THE ROLE OF BASE AND DEOXYRIBOSE DAMAGE IN THE SENSITIVITY OF CELLS TO CYTOTOXIC AGENTS

A thesis submitted to the University of Manchester for the Degree of Doctor of Philosophy in the Faculty of Medicine

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by

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CONTENTS

ABSTRACT

nth and *nfo* encode endonuclease III and endonuclease IV, respectively. Endonuclease III removes 5,6 ring saturated and fragmented thymine derivatives from gamma irradiated DNA and has AP lyase activity, while endonuclease IV removes 3′ phosphate and 3′ phosphoglycolate termini from gamma irradiated DNA and has class II AP endonuclease activity. In order to assess the contribution of thymine modifications, apurinic/apyrimidinic (AP) sites and deoxyribose damage to the cytotoxic effects of ionising radiation and radiomimetic chemicals, the *nth* and *nfo* genes were expressed in mammalian cells and the effect of expression on cellular sensitivity to γ-rays, bleomycin sulphate and hydrogen peroxide was examined.

nth and *nfo* were ligated into pZipneoSV(X)1 and pLJ to generate pZipnth and pLJnfo, respectively. pZipnth was transfected into three types of Chinese hamster cell lines: RJKO (V79 lung fibroblasts), xrs7 (radiosensitive Chinese hamster ovary cells deficient in the repair of double strand breaks) and VG8 (radiosensitive Chinese hamster fibroblasts efficient in the repair of strand breaks). G418 resistant clones were examined for *nth* incorporation, transcription of the *nth* message, expression of endonuclease III and increase in apurinic/apyrimidinic (AP) endonuclease activity. Expression was achieved in two RJKO (1D and 7D) and two xrs7 (x7nth1 and 6) derived cell lines. By examining *nth* expression in these two cell lines, attempts were made to determine whether the effect of endonuclease III repair on cell survival was altered by the cells

inability to repair double strand breaks. Cytotoxicity studies showed that clones 1D and 7D were slightly sensitive to gamma radiation, while the radiosensitivity of x7nth1 and 6 was equivalent to the control cell lines. Only 7D, x7nth1 and x7nth6 showed increased resistance to hydrogen peroxide and increased sensitivity to bleomycin sulphate. The alterations in cell survival after treatment with bleomycin sulphate and hydrogen peroxide were much greater for x7nth1 and 6 compared to 7D.

An assay was not available to measure endonuclease III activity in cell-free extracts. Hence a rapid, simple endonuclease III assay was developed. The substrate consisted of a double stranded oligonucleotide containing thymine glycol. EIII-type activity cleaved the [^{32}P]-labelled substrate at thymine glycol and fragmentation of the substrate was detected using urea/ polyacrylamide gel electrophoresis and autoradiography.

pLJnfo was transfected into xrs7 cells and *nfo* incorporation, transcription of the *nfo* message and an increase in AP endonuclease activity was detected in extracts of one clone (x7nfo11). The sensitivity of x7nfo11 to gamma radiation or hydrogen peroxide was not significantly different from the control cell lines.

Attempts were made to express endonuclease III and endonuclease IV in the same cell line by transfecting pLJnfo into x7nth1 cells. However, functional expression of both enzymes was not achieved.

DECLARATION

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or other Institute of learning.

L. HARRISON

EDUCATION AND RESEARCH EXPERIENCE

July 1988	Graduated from the University of Sheffield with a B.Sc. Honours degree in Biochemistry.
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I dedicate this thesis to my parents and my family, who have provided support over the last three years, even when my problems were small in comparison to theirs.

ABBREVIATIONS

Ac	Acetate
AC	5-azacytidine
AMV	Avian myeloblastosis virus
AP	Apurinic/apyrimidinic
A-T	Ataxia-telangiectasia
ATP	Adenosine-5′ triphosphate
Bis	N,N′-Methylenebisacrylamide
BS	Bloom's syndrome
BSA	Bovine serum albumin
BUdR	Bromodeoxyuridine
Ci	Curie
CHO	Chinese hamster ovary
CFA	Colony forming ability
CS	Cockayne's syndrome
Da	Dalton
dATP	Deoxyadenosine-5′-triphosphate
dCTP	Deoxycytidine-5′-triphosphate
dGTP	Deoxyguanosine-5′-triphosphate
dTTP	Thymidine-5′-triphosphate
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DF	Degrees of freedom
DHT	Dihydrothymine
DMSO	Dimethyl-sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ds	Double stranded
dsb	Double strand break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
EIII	Endonuclease III
EIV	Endonuclease IV
EMS	Ethyl methanesulphonate
ENU	Ethylnitrosourea
ERCC	Excision repair cross-complementing
EVIII	Endonuclease VIII
FA	Fanconi's anaemia
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
Gy	Gray
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
$HO_2\cdot$	Hydroperoxyl radical
HPLC	High performance liquid chromatography
kb	Kilobase pairs

LET	Linear energy transfer
LMDS	Locally multiply damaged sites
LMP	Low melting point
LTR	Long terminal repeat
MEM	Minimal essential medium
MoMLV	Moloney murine leukaemia virus
MMC	Mitomycin C
MMS	Methyl methanesuphonate
MMTV	Mouse mammary tumour virus
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MPS	3-(N-Morpholino) propane-sulphonic acid
NAD	Nicotinamide
NCS	Neocarzinostatin
NFE	Neutral filter elution
NVS	Neutral velocity sedimentation
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenyl methylsulphonyl fluoride
RDDS	Randomly dispersed damaged sites
RE	Restriction endonuclease
RNA	Ribonucleic acid
RNaseA	Ribonuclease
SCE	Sister chromatid exchange
SDS	Sodium dodecylsulphate
SF2	Surviving fraction at 2 gray
SS	Sums of squares
ssb	Single strand break
TCA	Trichloroacetic acid
TdG	Thymidine glycol
TEAA	Triethylammonium acetate
TEMED	NNN'N'-tetramethylethylene-diamine
TFA	Trifluoroacetic acid
TG	Thymine glycol
Tm	"Melting" temperature
T4PNK	T4 polynucleotide kinase
Tris	Tris (hydroxymethyl) methylamine
UBA	beta ureidoisobutyric acid
UPAGE	Urea-polyacrylamide gel electrophoresis
U.V.	Ultra-violet
X-P	Xeroderma pigmentosum
XRCC	X-ray cross-complementing

CHAPTER 1: INTRODUCTION

1.1 Mechanism of action of radiation

1.1.1 Initial physical damage produced by ionising radiation

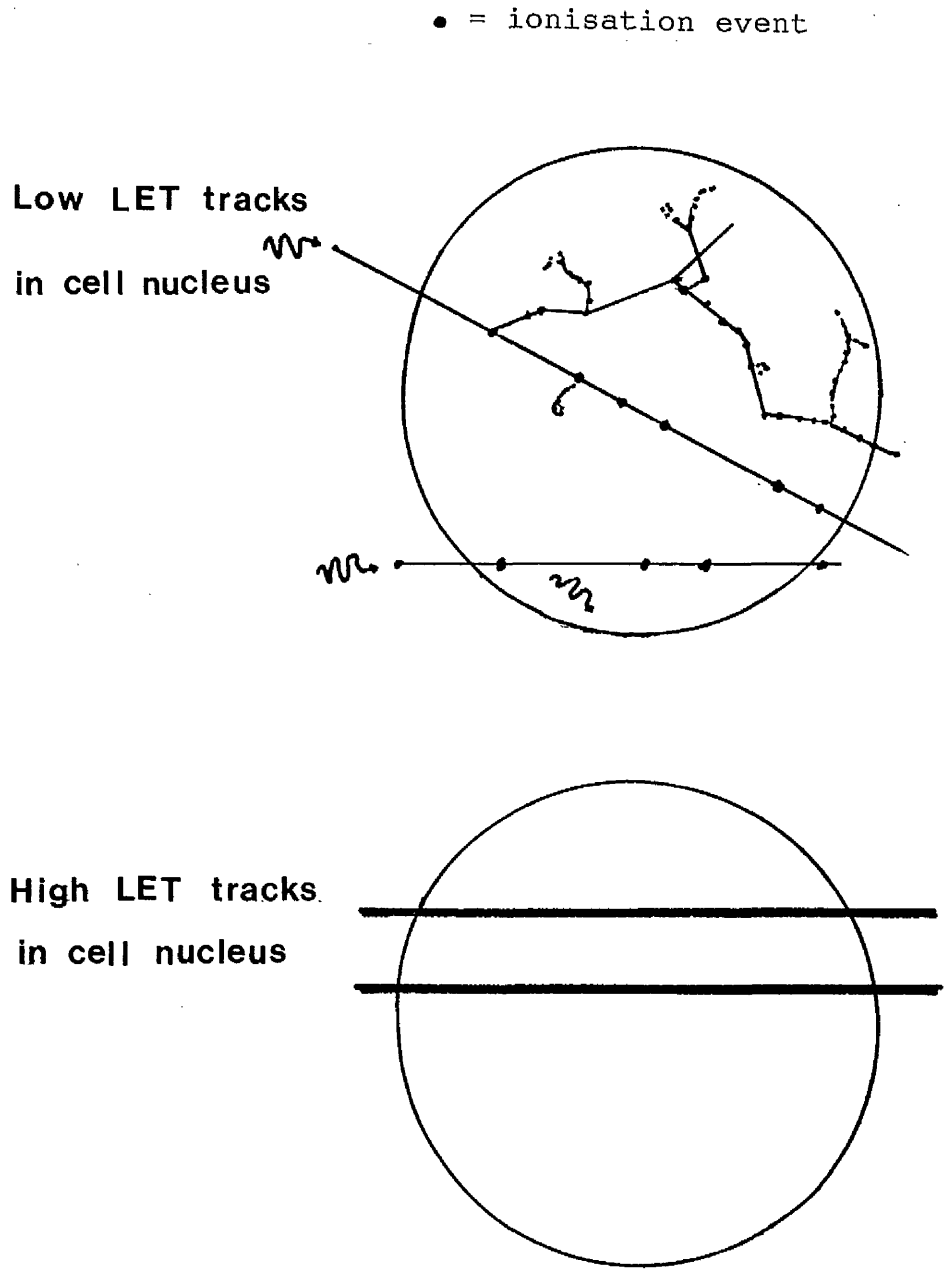
Damage produced by ionising radiation is due to the deposition of energy in cellular components, which causes initial physical damage in the form of ionisation or excitation of molecules at the site of interaction. This finally results in the formation of stable chemical lesions such as single strand breaks (ssb, see 1.2). Ionising radiation forms highly structured tracks of damage and this distinguishes it from other DNA damaging agents. Energy deposition is rapid (10^{-18}s, Ward 1990) and dependent on the quality of radiation, which is defined by the energy transfer/unit length of the track (linear energy transfer, LET). Gamma and X-rays are low LET radiations, while alpha particles are densely ionising and are classed as high LET radiation (Goodhead 1989).

For low LET radiations most of the energy is deposited in the form of sparse ionizations, but there is a substantial component that produces very localized clusters of ionisations. This is predominantly at the ionising track end and occurs due to low energy secondary electrons (see figure 1). High LET radiation produces larger and a higher number of clusters of ionisations (table 1, Goodhead 1989) and hence forms clusters of chemical lesions.

In DNA, due to the small target volume, the probability of overlapping ionising tracks is

FIGURE 1 - Schematic representation of tracks from low-LET or high LET radiation passing through a cell nucleus (reproduced from Goodhead 1989).

Most of the energy from low-LET radiation (e.g. gamma rays) is deposited in the form of sparse ionisations. However, localised clusters of ionisations occur at the track-ends of low-energy secondary electrons, which account for 30-50% of the local energy deposition of all low-LET radiations. High-LET radiation (e.g. alpha particles) is densely ionising and produces large clusters of ionisations at the site of interaction.



negligible, even at high doses (10-100Gy), and hence the interaction of chemical lesions, produced by different ionising tracks, to form stable complex lesions is unlikely. However, Goodhead (1989) and Ward (1981) have postulated that clusters of chemical lesions might be formed from a single ionising track: on interaction with the DNA, the DNA or those molecules close to it can receive small (resulting in a single ionisation or excitation) or large amounts of energy (tens of ionisations), producing damaged sites of different sizes.

Initial physical damage has been categorised into four classes (table 1).

TABLE 1 - Classes of initial physical damage (modified from Goodhead 1989)

CLASS	INITIAL PHYSICAL DAMAGE	COMMENT
1	sparse ionization/ excitation	little biological relevance? ssb?
2	moderate cluster	characteristic of low LET; particularly at ionising track ends, cause of DNA dsb?
3	large clusters	characteristic of high LET; complex damage, repairable?
4	very large clusters	unique to high LET; unrepairable; relevance?

The question has arisen whether the chemical DNA damage occurs at the site of energy deposition or whether the energy travels along the DNA and the damage is situated micrometer distances away from the initial excitation site (Baverstock 1989). It has been proposed that at electron track-ends the energy deposited in the molecule may result in DNA vibration as well as electronic excited states. If the vibrational energy is above a certain threshold, stable waves of energy (solitons) could be transmitted down the DNA by the vibration of covalent bonds. The solitons need only have a half-life of nanoseconds to transport the energy micrometer distances along the DNA (Baverstock 1989). It has also been suggested that energy may travel along the DNA by the transfer of ionisations through the hydrogen bonds of the water molecules in the water sheath (Pnevmatikos 1988).

1.1.2 Critical radiation target

It was originally proposed that ionising radiation resulted in two forms of damage (Alper 1963): type N, which contributed to cell death after irradiation under anoxic conditions and was the result of primary energy deposition in the DNA, and type O, which was responsible for the radiosensitization of cells in the presence of oxygen and was due to membrane damage. The cell membrane was known to be closely associated with the DNA: the bacterial membrane was found to be necessary for the replication of bacteriophage DNA (Puga & Tessman

1973) and transforming DNA required attachment to the cell membrane before replication and incorporation into the bacterial genome (Dooley & Nester 1973). Damage to the membrane was believed to result in cell death by the disruption of DNA structure and function (Alper 1979).

Experiments examining cell survival after alpha particle irradiation showed that radiosensitive sites were located in a thin peripheral region of the nucleus (Datta et al 1976) and nuclear transplantation experiments have demonstrated that the cytoplasm of the cell can withstand large doses of ionising radiation, without affecting cell viability. Although the latter two studies suggest that DNA may be the critical radiation target they do not eliminate the nuclear membrane as the target in eukaryote cells.

Early evidence in support of DNA as the major biological-important target was the radiosensitization of cells that had incorporated bromodeoxyuridine (BUdR) in their DNA (Djordevic & Szybalski 1960) and the log-linear relationship found between radiation lethality and the frequency of radiation induced mutations at the HGPRT (hypoxanthine-guanine phosphoribosyl transferase) locus in human, hamster and mouse cells (Thacker 1979, Thacker et al 1982). Further evidence is considered in section 1.7, where the biological consequences of specific types of DNA damage (e.g. double and single strand breaks, base damage) are discussed.

Although evidence indicates that DNA is the important biological target in cells, it is possible

that membrane damage may affect cell survival or even be involved in the generation of DNA damage, by the production of free radicals from the peroxidation of membrane associated lipids (Schaich & Borg 1984).

1.1.3 Mechanism for the production of chemical DNA damage

DNA damage is believed to be produced by two mechanisms: the direct effect, which involves the deposition of the energy of ionising radiation in the DNA molecule itself and the water sheath of the DNA, and the indirect effect in which radicals of oxygen, generated by the ionisation of water molecules not bound to DNA or other cell components, oxidize the DNA (von Sonntag 1987).

(i) Direct effect

Energy absorption leads initially to electron loss or electron capture by the bases of the DNA.

$$DNA \xrightarrow[\text{RADIATION}]{\text{IONISING}} DNA^{+} + e^{-} \qquad (1)$$

e^- can react with water and become hydrated.

$$DNA + e^{-} \longrightarrow DNA^{-} \qquad (2)$$

Electron spin resonance studies of DNA irradiated in frozen aqueous solution or in a dry state suggest that the guanosine cation radical and the thymidine anion radical are the primary intermediate species of base ionisation (Bernhard 1981). The radicals then degrade to produce chemical damage in the DNA: the major products of the thymidine and deoxycytidine anion

radicals are 5,6 saturated nucleosides, while the cation radical of these bases degrade to 5 hydroxymethyl-2' deoxyuridine and 2' deoxyuridine, respectively (Teebor et al 1984, Cadet et al 1987).

(ii) Indirect effect

Water comprises approximately 70% of the cell mass and upon irradiation water molecules are ionised.

$$H_2O \longrightarrow H_2O^+ + e^- \qquad (3)$$

e^- can become hydrated or react with DNA (equation 2). H_2O^+ is charged and has an unpaired electron and reacts rapidly with water molecules to form hydroxyl radicals (OH·)

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + OH\cdot \qquad (4)$$

OH· can diffuse a short distance and react with the DNA by addition or electron transfer (von Sonntag 1987).

$$DNA + OH\cdot \longrightarrow [DNA(OH)]\cdot \qquad (5)$$

$$DNA + OH\cdot \longrightarrow [DNA(-H)]\cdot + H_2O \qquad (6)$$

Genomic DNA is complexed with proteins to form chromatin and it has been suggested that protein radicals formed on irradiation can interact with and degrade the DNA (Schuessler 1987).

In cells there is a high concentration of organic material and hence it is likely that a high percentage of the radicals produced from the radiolysis of water will be scavenged and never react with DNA (von Sonntag 1987). Roots and Okada (1975) calculated that OH· were able to travel 6-9nm in mammalian cells and radicals produced by the indirect mechanism could only damage the

DNA if they were closely associated with the DNA (i.e. part of the water sheath). Water molecules in the water sheath have been found to have different properties compared to ordinary water, e.g. they do not rearrange into an ice-like structure even on cooling to -10°C (Ebert 1980, referenced in von Sonntag 1987) and they are not capable of hydrating electrons (van Lith et al 1986a,b). Therefore, electrons liberated in the radiolysis of the water sheath may interact directly with the DNA. The generation of OH· may be slower than in ordinary water, resulting in negative charge transferring from the DNA to H_2O^+, without the formation of OH· (Adams 1989). It has been suggested that the water molecules of the water sheath should be considered an integral part of the DNA and hence are involved in the production of DNA damage via the direct mechanism (von Sonntag 1987). The question then arises as to whether the indirect effect contributes to the production of chemical DNA damage. It has been found that irradiating cells in solutions containing high concentrations of OH· radical scavengers (DMSO and alcohol, Ashwood-Smith 1975, glycerol, Dewey 1963) increases cellular radioresistance and radical scavengers decrease OH· radical mediated production of thymine glycol (TG) in DNA *in vitro* and in intact HeLa cells (Remsen & Roti Roti 1977). However, at high concentrations, glycerol and DMSO are thought to alter the DNA structure (Raaphorst & Azzam 1981). Ethanol was also reported to protect the DNA from the production of

strand breaks by direct ionization (Schulte-Frohlinde 1986), which suggests that cellular radioresistance in the presence of ethanol was not necessarily due to the scavenging of radicals produced via the indirect mechanism.

DNA does not have a rigid structure and the degree of DNA packaging in the genome varies, e.g. packaging may not be as condensed at sites of replication and transcription compared to other regions of the DNA. Actively transcribed DNA regions are sensitive to nuclease digestion (Weintraub & Groudine 1976), which suggests that they may have a more "relaxed" structure: ssb (Chiu & Oleinick 1982) and TG (Patil et al 1985) have been found to be preferentially introduced into actively transcribed DNA and repaired at a faster rate than in non-transcribed DNA. The more "relaxed" structure may allow lesion production by the indirect mechanism as well as the direct mechanism, since radicals may be able to diffuse to the DNA more readily.

The damage produced by the two mechanisms is likely to be the same.

$$DNA^+ + H_2O \longrightarrow [DNA(OH)]\cdot + H^+ \quad (7)$$

$$DNA^+ \longrightarrow [DNA(-H)]\cdot + H^+ \quad (8)$$

If the DNA radical produced from the direct ionisation of DNA (reaction 1) reacted with the water of the water sheath to form a base-OH radical adduct (reaction 7) or release a proton (reaction 8), then the products of the indirect and direct effect would be the same (see reactions 5 and 6, von Sonntag 1987). It is

therefore impossible to distinguish between the products of the different mechanisms.

1.2 Types of chemical DNA damage produced by ionising radiation

A wide variety of chemical damage is produced following the decay of radicals in DNA. The lesions found in DNA include base modifications, ssbs and double strand breaks (dsbs), apurinic/apyrimidinic (AP) sites and deoxyribose modifications. AP sites and strand breakage are also produced during the repair of lesions, (see 1.6).

1.2.1 Base modifications

In order to determine the types of base modifications produced following irradiation, ionising radiation studies have been carried out on DNA, free bases and nucleosides and the radiation products analyzed by thin layer chromatography, high performance liquid chromatography (HPLC) and mass spectrometry. It has been found that the types and yields of modifications vary depending on the gaseous conditions under which irradiation is performed (i.e. DNA in aqueous solution saturated with air, nitrogen or oxygen) and on the conformation of the DNA: a higher yield of base damage is produced in heat denatured DNA compared to native DNA (Fuciarelli et al 1990).

(i) Thymine

The hydroxyl radical is the main reactive species with thymine in aerated solutions, while the superoxide

ion ($O_2 \cdot^-$) has been found to be inactive towards this base (Cadet & Teoule 1978). In the cell thymine base damage could be produced by the indirect effect, if such a mechanism is able to operate. The hydroxyl radical attacks preferentially at the C5 position in neutral solution (figure 2) and the C6 position in acidic solution and can also remove a hydrogen atom from the methyl group. C5 and C6 radicals are produced: the C5 radical is an oxidizing agent, while the C6 radical has a reducing nature (von Sonntag & Schuchmann 1986). Both radicals give rise to thymine hydroxyhydroperoxides.

In order to examine thymine products ^{14}C or ^{3}H labelled *E.coli* DNA was irradiated in solution and the stable modifications that remained attached to the DNA were identified by chromatography, after liberation from the DNA by formic acid hydrolysis at 90°C. The thymidine derivatives found on irradiation of the DNA in aerated solution included cis and trans TG, 5 hydroxy-5 methyl hydantoin, formyl urea, urea and N-formamido deoxyribose, which degraded to release carbonic oxide and pyruvamide (figure 3, Teoule et al 1977). The products formed when DNA was irradiated in deaerated conditions include 5,6 dihydrothymine, 5 hydroxy-5,6 dihydrothymine, cis and trans TG and 6 hydroxy-5,6 dihydrothymine (figure 4, Teoule 1987).

Cis and trans TG have also been found in U.V. irradiated DNA. Cyclobutane pyrimidine dimers were the major lesion following irradiation with far U.V. light (<300nm), but other products were formed with 254nm

FIGURE 2 - Attack of thymidine by hydroxyl radicals

The hydroxyl radical attacks preferentially at the C5 position in neutral solution and at the C6 position in acidic solution and can also remove a hydrogen atom from the methyl group.

dR = DEOXYRIBOSE

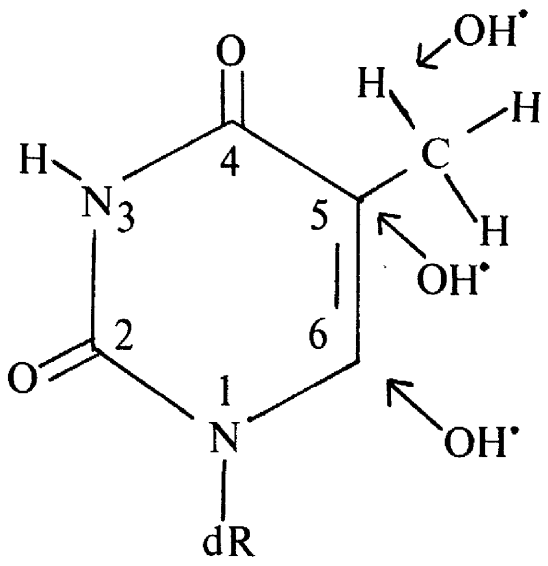


FIGURE 3 - Products of thymine following irradiation of DNA in aerated solution

I - 5-hydroperoxy-6-hydroxy-5,6-dihydrothymine
II - 6-hydroperoxy-5-hydroxy-5,6-dihydrothymine
III - cis thymine glycol
IV - trans thymine glycol
V - 5 hydroxy-5-methylhydantoin
VI - formamide
VII - N-formamido urea
VIII - urea

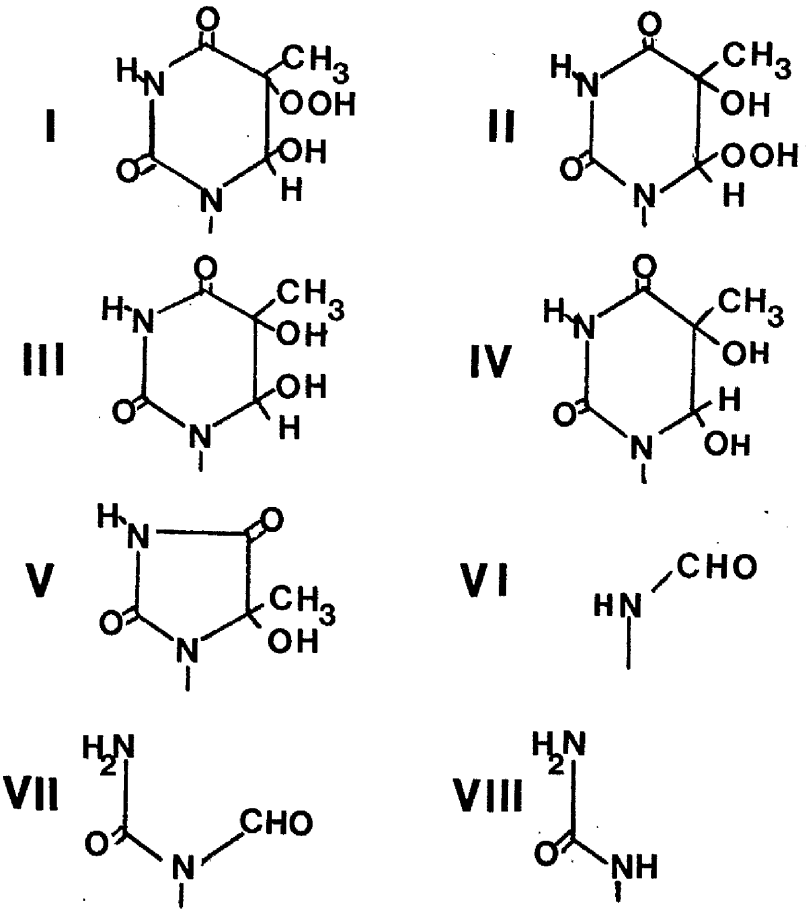
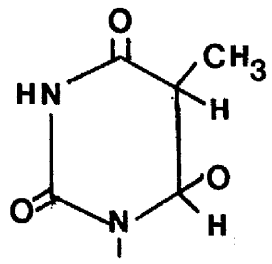
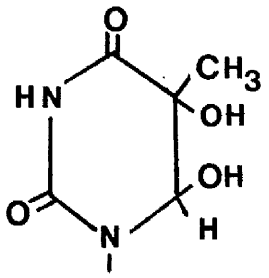


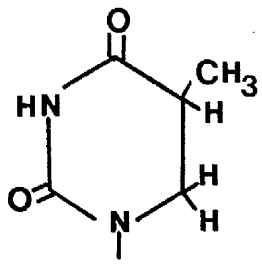
FIGURE 4 - Products of thymine following irradiation of DNA in deaerated solution



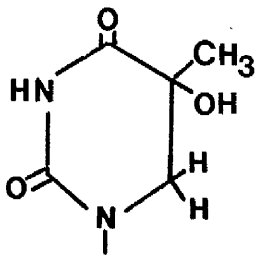
6-hydroxy-5,6-dihydrothymine



cis & trans thymine glycol



5,6 dihydrothymine



5-hydroxy-5,6-dihydrothymine

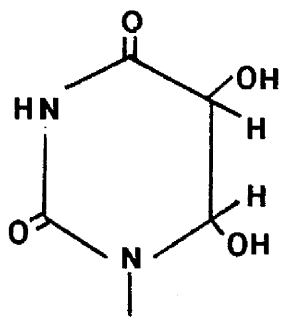
light (Wacker 1963), one of which was later identified as TG (Yamane et al 1967). Small amounts of 6 hydroxy-5,6- dihydrothymine were also produced on irradiation of DNA with 265nm light (Fisher & Johns 1973). Hariharan & Cerutti (1977) examined the formation of thymine derivatives using near U.V. light (300-380nm) irradiation and TG lesions were found to be major products. This is of particular interest as near U.V. light is part of the spectrum reaching the earth's surface (the ozone shield absorbs the far U.V. light) and so may be implicated in skin cancer formation. Further work (Leadon 1987), using a monoclonal antibody raised against TG in poly dT, has confirmed the production of TG by U.V. light and gamma irradiation.

Thymine cross-links can be produced in poly dT irradiated with gamma rays in deaerated solution by the formation of a single bond between two C6 radicals in either the same or separate polynucleotide chains (Dizdaroglu & Simic 1984a). Cross-links have also been found between thymine and amino acids of histone proteins (Simic & Dizdaroglu 1985). The positively charged aromatic amino acids (especially tyrosine) in close proximity to the DNA are involved in the cross-linking.

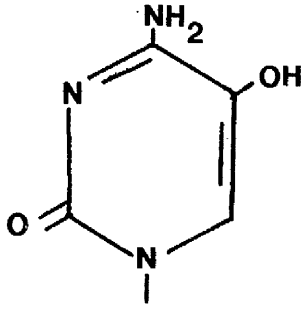
(ii) Cytosine

Uracil glycol and 5 hydroxycytosine are the major products formed in DNA following gamma-irradiation in aerated solution (figure 5) and cytosine, uracil glycol and 1-carbamoyl-5-hydroxy-hydantoin are spontaneously

FIGURE 5 - Products of cytosine following irradiation of DNA in aerated solution



cis & trans uracil glycol



5-hydroxycytosine

released from the DNA (Teoule 1987). In deaerated solution, ionising radiation has been found to cause the production of dimeric cytosine products in DNA, by the formation of a bond between the C5's of two cytosines (Dizdaroglu & Simic 1984b). Cytosine-tyrosine links, between C6 of cytosine and C3 of tyrosine, have also been detected in gamma irradiated acidic hydrolysates of calf thymus nucleohistones, although these DNA-protein bonds did not form in the presence of oxygen (Gajewski & Dizdaroglu 1990).

U.V. irradiation was found to produce pyrimidine dimers at cytosines located 3' to another pyrimidine (e.g. 5'-TC-3' or 5'-CC-3'). These dimers are called (6-4) photoproducts (Thacker 1986).

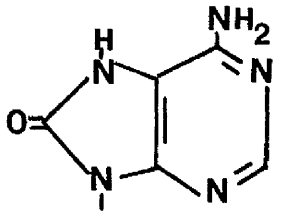
(iii) Adenine

Following gamma irradiation of DNA in aerated solution, 4,6 diamino-5-formamido pyrimidine and 7,8-dihydro-8-oxoadenine are released (Bonicel et al 1980, figure 6). Radiation-induced cyclization can also occur to form 8-5 cyclo-deoxyadenosine. This was detected in DNA irradiated in nitrogen-saturated solutions by Fuciarelli et al (1985), using a polyclonal antibody raised to the 8-5 cyclodeoxy-adenosine 5' mono-phosphate hapten. Hydroxyl radicals appear to initiate cyclization, as DMSO inhibits cyclization in irradiated polyadenylic acid (Fuciarelli et al 1985). Cyclization does not occur in aerated solutions.

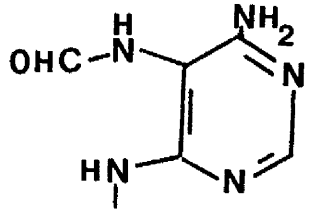
(iv) Guanine

In the presence of nitrous oxide (N_2O), in aerated

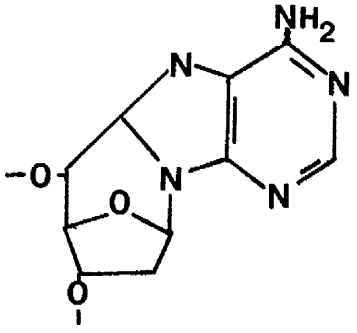
FIGURE 6 - Products of adenine following irradiation of DNA



7,8 dihydro-8-oxo-adenine



4,6 diamino-5-formamido-pyrimidine



8,5-cycloadenine

and deaerated solutions, 8 hydroxy-2'-deoxyguanosine is formed after gamma irradiation. The major product in deaerated solution is 2,4-diamino-5-formamido-pyrimidine -6-one, although 8 hydroxyguanine and 8,5'-cyclo-2'-deoxyguanosine have been detected (figure 7, Teoule 1987).

1.2.2 Alkali-labile sites

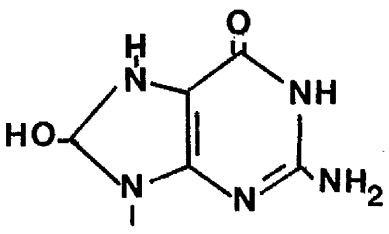
The damage is believed to occur by the abstraction of H-atoms from the deoxyribose moiety by hydroxyl radicals (section 1.1.3 equation 6, von Sonntag 1987). Oxidation occurs at positions 1, 2 and 4 of the deoxyribose moiety, but damage at position 2 only occurs in aerated solutions (Teoule 1987). Reaction of a DNA base radical with the deoxyribose can result in alkali-labile sites (Lemaire et al 1984, Deeble & von Sonntag 1984). This reaction may be at a neighbouring or more distant nucleotide, although it is possible that the base radical could react with the ribose to which it is attached (von Sonntag 1987).

There are four types of damage involving deoxyribose that are produced by irradiation of DNA (figure 8, von Sonntag 1987):

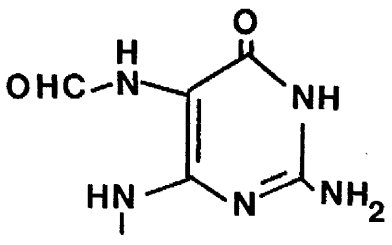
A) Altered ribose moieties bound to the DNA by one phosphate linkage. In this case the unaltered base is nearly always lost (1-5).

B) Liberation of free deoxyribose compounds: both phosphate bonds have been broken and the base lost (6-9).

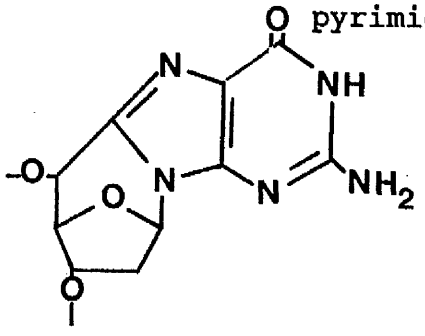
FIGURE 7 - Products of guanine following irradiation of DNA



8-hydroxyguanine



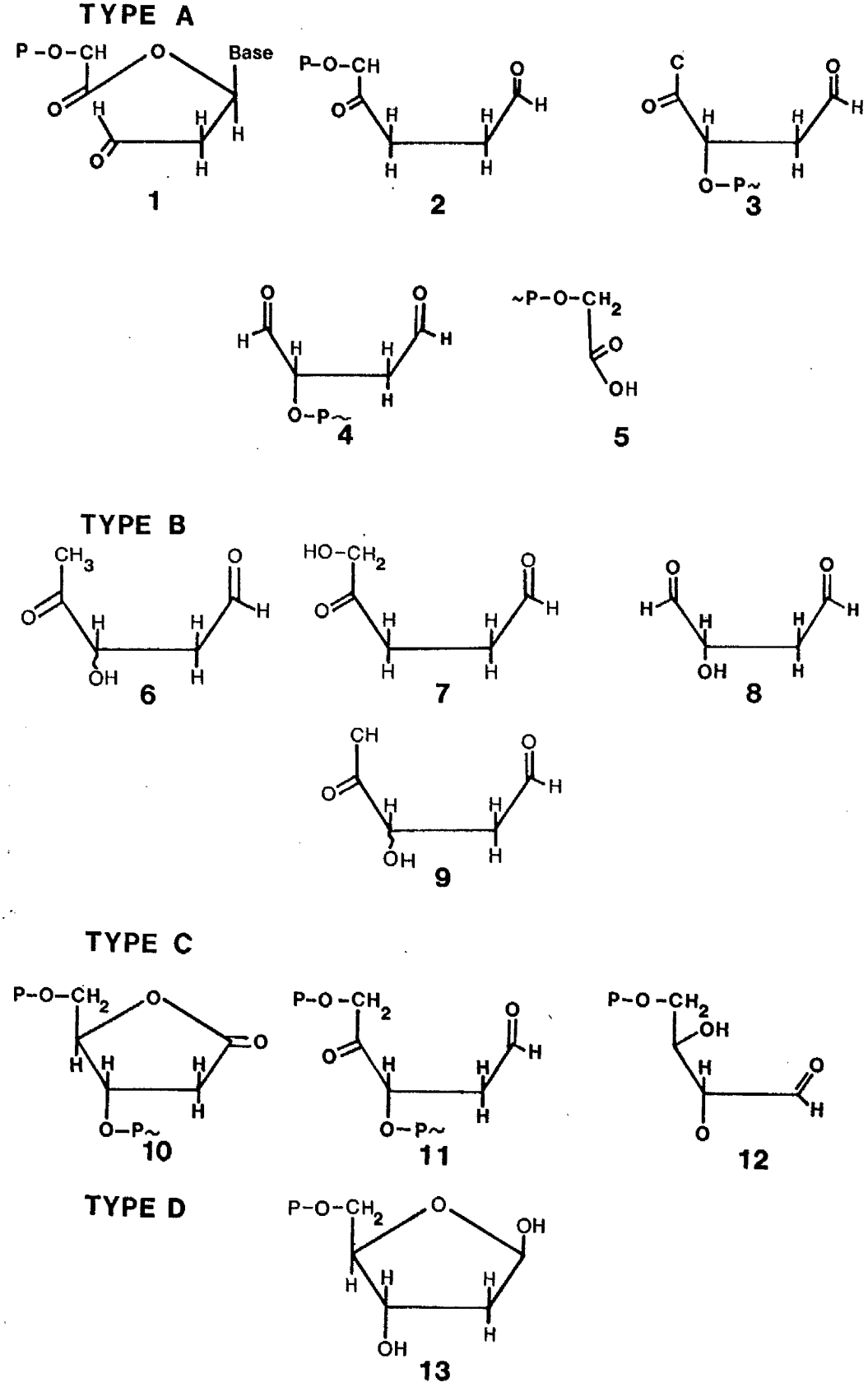
2,4-diamino-5-formamido-pyrimidin-6-one



8,5-cycloguanine

FIGURE 8 - Radiation-induced deoxyribose damage

Four types of damage are produced after irradiation of DNA. For details see text.



C) The unaltered base is lost and the sugar is altered either by oxidation, or fragmentation then oxidation (10-12).

D) The base is altered and the glycosyl linkage is no longer stable, the altered base is eliminated and an AP site is formed (13).

The products of A and B are strand breaks, while those of C and D will form strand breaks with 3′ phosphate termini if further treated with alkali (Teoule 1987).

1.2.3 DNA strand breakage

Ssbs and dsbs are produced by ionising radiation and occur following radical attack on the ribose moiety (see above). Analysis of gamma irradiated 5′ or 3′ [^{32}P]-labelled oligonucleotides, by Maxam and Gilbert sequencing methods, showed that one type of 5′ and two types of 3′ break termini are produced: 5′ phosphate, 3′ phosphate and 3′ phosphoglycolate ($OPO.OCH_2COOH$, Henner et al 1983). Seventy percent of breaks have 3′ phosphate termini and only thirty percent have 3′ phosphoglycolate termini (Ward 1987). Similar termini were detected at breaks in DNA isolated from X-irradiated monkey CV-1 cells (Feingold et al 1988). However, work by Jacobs et al (1972) indicated that at least 30% of ssb introduced by gamma irradiation of DNA could be directly rejoined by DNA ligase *in vitro*, suggesting that not all DNA breaks have the 3′ and 5′ termini mentioned above.

Two mechanisms have been suggested for dsb formation:

(i) Radical transfer mechanism

It was proposed by Siddiqi and Bothe (1987) that a radical is transferred to the DNA from a single hydroxyl radical, generated by ionising radiation. The DNA radical produces the first break in the DNA before transferring to a site on the complementary strand. The second break is then formed.

(ii) Multiple radical mechanism

Dsb are caused by two radical reactions (hydroxyl attack on or direct ionisation of the deoxyribose) which occur independently, to form two breaks in opposing strands of the DNA (Ward 1981).

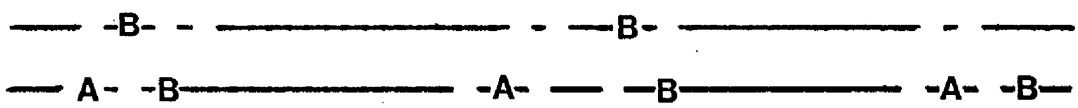
As discussed earlier (1.1.1), it is highly improbable that radicals produced from two separate energy depositions would be situated in close proximity in the DNA. However, single energy deposition can lead to the production of a cluster of radicals and hence damage a localized area of the DNA (Goodhead 1989). These localised areas (locally multiply damaged sites, LMDS, figure 9) would produce dsb by the multiple radical mechanism (hereafter called mechanism B, Ward 1981).

The two mechanisms are different in the way the ssb are generated: by the radical transfer mechanism (hereafter called mechanism A) production of the two ssb is sequential, while for mechanism B it is simultaneous (see table 2 and definition of mechanisms above).

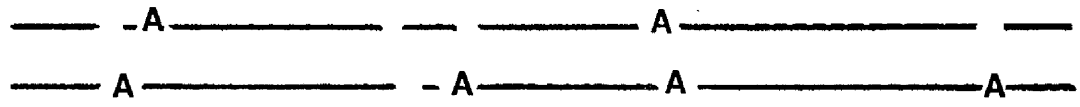
FIGURE 9 - Schematic representation of the distribution of lesions in the DNA after treatment with ionising radiation, bleomycin sulphate or hydrogen peroxide

It has been proposed that ionising radiation (1) and bleomycin sulphate (2) produce locally multiply damaged sites in DNA (Ward 1981, Povirk & Houlgrave 1988), while hydrogen peroxide produces randomly dispersed damaged sites (3).

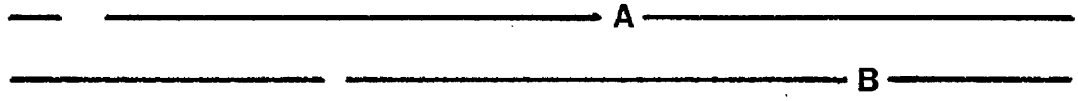
1 - Ionising radiation



2 - Bleomycin sulphate



3 - Hydrogen peroxide



A = AP site
B = Base modification
— — = single strand break

Theoretically, the dsb formed by mechanism B could be prevented by blocking the production of either break, but prevention of those formed by mechanism A could only occur by blocking the production of the first break. The two mechanisms would also produce different types of DNA dsb. Breaks produced by mechanism A would be expected to be within a fixed distance from one another, due to the radical of the first strand transferring to the second strand. Mechanism B would not necessarily produce breaks at a fixed distance (Ward 1987). The minimum distance required between two ssb, on opposite strands, to produce a dsb is not yet known. It is likely that the production of a dsb from two "staggered" ssb is dependent on the disruption of inter-strand hydrogen bonds in the sequence of DNA positioned between the ssb.

TABLE 2 - Comparison of the radical transfer and multiple radical mechanisms of dsb production (modified from Ward et al 1990)

FACET	MECHANISM	
	SINGLE RADICAL	MULTIPLE RADICAL
Formation of ssb	sequential	simultaneously
Damage structure	relatively simple	many types
Spacing of lesions	relatively constant	can vary from 0 - 20+bp
Lesion variation due to different LET	not easily explained	readily explained

LMDS in DNA are likely to contain a wide variety of lesions, i.e. base damage, deoxyribose damage, dsb and ssb. It has been proposed that in cells dsb might also be produced as a consequence of repair reactions, that generate ssb at or near an existing or repair generated ssb in the opposite strand (Ahnstrom & Bryant 1982).

The relationship of dose and dsb induction in cells has been found to vary depending on the method used to measure the breaks: neutral velocity sedimentation (NVS) has shown linear induction (Weibezahn & Coquerelle 1981), while neutral filter elution (NFE) showed non-linear induction (Blocher & Pohlit 1982). Repair of dsb measured using NVS is linear with time, but repair is non-linear when examined by NFE. However, it has been shown that the shape of the DNA elution dose response is dependent on the conditions of cell lysis and that linear elution versus dose can be achieved by lysing the cells with sodium-N-laurylsarcosine instead of SDS, or by carrying out lysis at 60°C. The non-linear induction found with NFE may be due to incomplete separation of DNA from other molecules (Okayasu & Iliakis 1989). Linear induction with dose suggests that dsb, like ssb, arise from a single energy deposition. Both mechanism A and B produce dsb from a single energy deposition. The same would be true of dsb production by repair endonucleases, since the damage (which is repaired, generating ssbs that interact to produce dsbs) within the LMDS would have originated from a cluster of ionisations generated by a single energy deposition.

1.3 Production of DNA damage by cellular metabolism

Active oxygen species (which can be produced in the cell by ionising radiation), such as superoxide ($O_2^{\cdot -}$), the hydroperoxyl radical ($HO_2^{\cdot}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$) are produced by the cells metabolic processes, an example of which is peroxisome mediated oxidation of fatty acids (Cerutti 1985). Evidence of continuous oxidative base damage and repair in mammalian systems was produced by Cathcart et al (1984), who detected TG and thymidine glycol in human and rat urine using HPLC. Prior exposure to ionising radiation had not occurred. The diet was ruled out as a source of these compounds by feeding rats a nucleic acid free diet. Tritiated thymidine glycol was administered to the rats by gastric intubation and only a small fraction was absorbed and excreted in the urine. This indicated that thymidine glycol produced by bacterial flora or the diet does not contribute significantly to urinary products.

To maintain steady-state concentrations of active oxygen species bacterial and mammalian cells have an elaborate defense system consisting of the antioxidant enzymes superoxide dismutase, glutathione peroxidase, glutathione transferases and catalase (Cerutti 1985). Superoxide dismutase and catalase degrade $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2), respectively. Strains of *E.coli* unable to produce active superoxide dismutase exhibited a 40-fold increase in spontaneous mutagenesis in the presence of oxygen (Denq et al 1989). Superoxide

dismutase and catalase prevented the accumulation of ssb and endonuclease III (EIII) sensitive lesions in $O_2^{\cdot -}$ and H_2O_2-treated pBR322 *in vitro* and were also found to protect against loss of infectivity of gamma irradiated ØX174 DNA (Denq et al 1989).

1.4 Production of DNA damage by radiomimetic chemicals

1.4.1 Bleomycin

Bleomycins are complex basic glycopeptide antibiotics, over 200 species of which have been found. They differ in structure only at the carboxy terminus, which consists of an amine or polyamine (Muller & Zahn 1977), and it is the removal of this terminal amine group by bleomycin hydrolase that inactivates the molecule (Sickic 1986).

Bleomycin reacts preferentially with double stranded DNA, but not RNA or poly ADP-ribose. The introduction of DNA damage inhibits the action of DNA polymerase and thus terminates DNA synthesis, while transcription and protein synthesis are not affected (Muller & Zahn 1977).

The damage introduced into the DNA is dependent on the conditions of the reaction. In the absence of oxygen or thiol compounds only base release occurs to produce AP sites (Burger et al 1982, Ross & Moses 1978). Bleomycin preferentially reacts with thymine residues (48% of the total reaction) and this may be due to the formation of relatively stable localised bonds on partial intercalation at thymine residues (Muller & Zahn 1977). Reaction also occurs with cytosine (27% of the

bases modified are cytosines), adenine (15%) and guanine (10%, Sausville & Horwitz 1979). In the presence of oxygen or thiol compounds strand breakage occurs. The mechanism of action is not fully understood, but one part of the molecule is thought to intercalate the DNA (Povirk et al 1979), while another section is capable of binding metal ions, e.g. iron (Sausville & Horwitz 1979). The primary attack is at the C4′ of the deoxyribose moiety (Wu et al 1983) and suggested mechanisms include:

1) $O_2 \cdot^-$ radicals, formed during the reaction of bleomycin(Fe^{2+}) with oxygen, could abstract a hydrogen atom from the C4′ (Takeshita & Grollman 1979). However, von Sonntag (1987) has suggested that this radical does not have the ability to remove a hydrogen atom from the deoxyribose moiety of DNA.

2) Damage might be produced by hydroxyl radicals which have been detected in bleomycin-O_2 and iron (II) systems (Oberley & Buettner 1979). However, scavengers do not affect the production of damage and it seems unlikely that these radicals are the dominant reactive species (von Sonntag 1987).

3) It is possible that on reaction of bleomycin(Fe^{2+}) with oxygen an "activated" complex is formed which then reacts with the DNA (von Sonntag 1987).

The DNA damage required to produce a lethal event in cells has been found to be equivalent to 40 dsb for ionising radiation (Elkind & Redpath 1977, Blocher 1982) and 30 dsb for bleomycin (Bradley & Kohn 1979). These

two agents may therefore produce similar lesion dispersion patterns, i.e. LMDS (figure 9, Ward 1987). It has recently been confirmed by Povirk & Houlgrave (1988) that even at low levels of DNA damage, complex lesions are produced that consist of AP sites close to strand breaks or AP sites in the opposite DNA strand. This was demonstrated by an increased production of dsb on incubating bleomycin-treated DNA *in vitro* with putrescine or EIII (both of which cleave at AP sites). It was suggested that these complex lesions were partially resistant to endonucleases, as high concentrations of EIII were required to produce dsb, and endonuclease IV (EIV) and exonuclease III did not increase the dsb formation. The DNA in these lesions may be single-stranded due to local denaturation after the formation of a ssb. This might explain the partial resistance to the endonucleases, since the enzymes mentioned above act preferentially on double stranded DNA (see 1.6). In chromatin, the double stranded structure may be maintained due to the presence of histone proteins and consequent DNA coiling, hence allowing repair of bleomycin-induced lesions by enzymes such as EIII.

Using the comet assay to examine DNA fragmentation in single cells, Ostling and Johanson (1987) showed that gamma radiation produced relatively uniform DNA breakage within a cell population after 200 or 400Gy. However, for a given dose (75 or 300ug/ml) bleomycin seemed to damage cells in a selective manner: the DNA of some cells within a population was heavily degraded, while

the damage to the DNA of other cells was moderate or not measurable. The amount of damage produced per cell by bleomycin may be dependent on the phase of the cell cycle that each cell is passing through : G1 is the most resistant and sensitivity of the phases to bleomycin increases in the order G1, late S, early S, G2 and mitosis (Sikic 1986). Since bleomycin requires thiol compounds to introduce breaks in DNA, an alteration in cellular sulphydryl concentration during the cell cycle could explain these changes in bleomycin sensitivity. Although Ohara and Terasima (1969) did detect such a variation, this was not confirmed in other work with HeLa cells (Klein & Robbins 1970).

1.4.2 Hydrogen peroxide

The variety of DNA lesions produced by H_2O_2 is similar to that of ionising radiation. Base damage and base release has been detected after H_2O_2 treatment of DNA (Rhaese & Freese 1968, Blakely *et al* 1990) and included cis and trans TG, cytosine glycol, 8 hydroxy and formamido pyrimidine derivatives of adenine and guanine. Bases are damaged by H_2O_2 in the order G (38% of the bases modified are guanines), A (32%), T (24%), C (5%, Blakeley *et al* 1990).

Hydroxyl radical attack at the deoxyribose moiety can result in strand breakage and significant levels of ssb were measured after treatment of DNA with 0.01mM H_2O_2 for 10 minutes at 0°C (Ward 1987). However, higher concentrations were required to detect dsb (1mM, 20

minutes, 0°C, Prise 1989). At equivalent cytotoxic doses H_2O_2 introduced five times more dsb into DNA than X-rays (Prise 1989) and, per lethal event, H_2O_2 produced 400,000 ssb (Ward 1987). It has been suggested that H_2O_2 produces randomly dispersed damaged sites (RDDS, previously known as singly damaged site, Ward 1987, see figure 9) and interaction of RDDS to produce lethal lesions (dsb or LMDS) may be necessary to cause cell death.

Hydrogen peroxide kills cells by two methods, as demonstrated in bacteria by Imlay & Linn (1986). Method 1 occurs at low concentrations (< 1mM in bacteria) and requires active cellular metabolism. Bacteria deficient in exonuclease III are more sensitive to killing by method 1, which appears to be due to DNA damage. Method 2 occurs at higher doses of hydrogen peroxide, does not require active metabolism, and the primary target for damage is not known, although it is possible that the cell membrane may be involved (Imlay & Linn 1986).

The amount of cell death by method 1 does not increase with increasing H_2O_2 concentration. This is consistent with the saturation of reactions converting H_2O_2 to a toxic radical species (suggested to be the hydroxyl radical) at some step in the damage producing pathway. Imlay and Linn (1986) proposed two possible explanations for the saturation:

1) The hydroxyl radical may be quenched directly by H_2O_2, producing a superoxide anion (or hydrated superoxide anion, equation 10), which may be less toxic

due to greater chemical stability, or vulnerability to degradation by superoxide dismutase.

2) The partial suppression of univalent H_2O_2 reduction by diversion of electrons to another process, e.g. divalent reduction of H_2O_2 by a peroxidase function.

The mechanism for the production of toxic radicals, from H_2O_2 is believed to involve a Fenton reaction. A metal ion, M (which could be iron or copper as these bind to DNA), may be required for the reaction as the addition of metal chelators to *E.coli* was found to block cell death by method 1 (Imlay et al 1988).

$$M^{n+} + H_2O_2 + H^+ \longrightarrow M^{(n+1)+} + OH\cdot + H_2O \qquad (9)$$

$$OH\cdot + H_2O_2 \longrightarrow HO_2\cdot + H_2O \qquad (10)$$

$$HO_2\cdot + M^{(n+1)+} \longrightarrow O_2 + M^{n+} + H^+ \qquad (11)$$

In summary:-

$$2H_2O_2 \xrightarrow{M} O_2 + 2H_2O \qquad (12)$$

If OH· is the toxic radical causing killing by method 1, equation 10 explains the suppression of method 1 toxicity at high H_2O_2 concentrations (i.e. H_2O_2 scavenges OH·) and the lack of dependence of cell killing with respect to H_2O_2 concentration. M^{n+} would be the limiting reactant at high H_2O_2 concentrations. *In vitro*, nanomolar amounts of iron mixed with H_2O_2 and DNA produced detectable amounts of DNA strand breakage and the extent of cleavage was proportional to the iron concentration (Imlay et al 1988). Increasing the H_2O_2 concentration decreased strand breakage, which is further evidence for the radical quenching at high concentrations. The addition of ethanol also decreased

strand breakage, but did not totally prevent it. Free hydroxyl radicals would be scavenged within a cell and so it was suggested that a metal hydroxide radical [$M^{(n+1)+}$-HO·] was the damaging species (equation 13, Imlay et al 1988).

$$M^{n+} + H_2O_2 + H^+ \longrightarrow M^{(n+1)+}\text{-}OH\cdot + H_2O \quad (13)$$

If the metal was bound to the DNA the radical would not be scavenged (Imlay et al 1988).

To maintain the reaction $M^{(n+1)+}$ would have to be reduced to M^{n+} and since the ion is probably chelated to the phosphodiester backbone the reducing agent would have to be a small diffusable molecule, such as NAD(P)H (Imlay & Linn 1988). This was supported by the finding that *E.coli* deficient in active NADH dehydrogenase, resulting in an accumulation of NADH, were highly sensitive to killing by method 1. *In vitro* systems, using Fe^{3+} chelated to EDTA, showed that H_2O_2 enhanced the conversion of Fe^{3+} to Fe^{2+} in the presence of NADH and that NADH stimulated H_2O_2 decomposition. On addition of DNA to the *in vitro* system, strand breaks were produced in the DNA (Imlay & Linn 1988). *In vivo*, one possible mechanism of damage production may be:

$$H^+ + (DNA)M^{n+} + H_2O_2 \longrightarrow (DNA)M^{(n+1)+}\text{-}OH\cdot + H_2O \quad (14)$$

$$(DNA)M^{(n+1)+}\text{-}OH\cdot \longrightarrow (DNA\cdot\text{-}H)M^{(n+1)+} + H_2O \quad (15)$$

In equation 15 the OH· abstracts a hydrogen atom from the DNA, resulting in transfer of the radical to the DNA and hence the formation of DNA damage, such as a ssb or base damage. For further damage to occur $M^{(n+1)+}$ would have to be reduced to M^{n+}:

$$2M^{(n+1)+} + NADH \longrightarrow 2M^{n+} + NAD^{+} + H^{+} \quad (16)$$

Unless metal ions were situated in close proximity in the DNA reaction 16 would have to occur before a dsb could be formed, which would explain why RDDS and not LMDS are produced in DNA by H_2O_2.

1.4.3 Neocarzinostatin (NCS)

NCS is a polypeptide, containing 109 amino acids and a bound chromophore, which is responsible for single strand break production. Activation by oxygen and thiol compounds is required for the production of ssb and alkali-labile sites at thymine residues (Hatayama & Goldberg 1980).

NCS has been found to introduce "complex" lesions that consist of more than one form of damage situated in close proximity in opposite strands of the DNA (Povirk & Houlgrave 1988), as found with bleomycin (see 1.4.1). NCS is therefore radiomimetic in two ways: firstly it produces strand breaks (some of which have been found to contain phosphate and glycolate termini, Deadon & Goldberg 1990) and secondly the lesions are complex and possibly similar to LMDS.

1.4.4 OsO_4, $KMnO_4$

These agents oxidise DNA and produce 5,6 ring saturated derivatives of thymidine (Beer et al 1966, Hubbard et al 1989, Iida & Hayatsu 1970). OsO_4 is more specific for TG production, although it appears to react to a small extent with cytosine (Beer et al 1966).

1.4.5 N-hydroxy-2-naphthylamine (NOH-2-NA)

NOH-2-NA is an active metabolite of the bladder carcinogen 2-naphthylamine and generates active oxygen species. This chemical is known to produce single strand breaks and TG in DNA. Direct action of NOH-2-NA with the DNA also leads to adducts of guanine and adenine (Leadon 1987).

1.5 Biological consequences of ionising radiation

Ionising radiation produces a complex pattern of damage which can lead to:

i) death of the irradiated parent cell

ii) death of the daughter cells produced from residual divisions of the "dying" parent cell (Hendry & Scott 1987).

iii) mutations in parent and daughter cells, some of which may lead to cell death (Seymour et al 1986) or carcinogenesis (UNSCEAR 1988) in subsequent cell generations.

The Gardner report (1990) has recently highlighted the importance of the latter consequence (iii). This report has proposed that the increased incidence of childhood leukaemias in the population living near the Sellafield nuclear power installation may be due to occupational radiation doses received by the father before conception of the child. Such low doses were originally thought not to affect the genetic integrity of the cell. Hence further research is now being carried out to determine whether the occupational doses

of radiation could have caused the leukaemias.

Lack of repair of the various types of DNA damage may cause the killing and mutations mentioned above. It is therefore important to understand the biological consequences of unrepaired and inaccurately repaired damage and how lesions may interact to cause lethality.

1.5.1 Biological consequences of base damage

AP sites, TG and urea residues have been the lesions most frequently studied, because they can be introduced into DNA *in vitro* specifically: AP sites by heat or acid treatment (Wallace 1987), TG by OsO4 (Beer et al 1966) and urea by alkali hydrolysis of DNA containing TG (Kow & Wallace 1985). Recently, it was found that dihydrothymine (DHT) could be incorporated into newly synthesized DNA *in vitro* ,using DNA polymerase I (Ide & Wallace 1988), and this has allowed the DHT lesion and its alkali cleavage product β-ureidoisobutyric acid (UBA) to be studied.

TG and DHT are structurally very similar (see figure 4) and have been used as models for the 5,6 ring saturated thymine lesions produced by ionising radiation, while urea and UBA are models for fragmented base damage.

i) Effect of TG and DHT on the DNA structure

TG and DHT maintain base pairing ability, however, they interact within the DNA structure to alter the base stacking. Using energy minimization routines, it was

found that there was very little difference between the two lesions with respect to the perturbation of the DNA molecule (Wallace & Ide 1990).

Clark et al (1987) used computer modelling to examine the effect of the presence of TG in DNA. The methyl group was found to be in axial configuration with respect to the non-planar pyrimidine ring, while the two hydroxyl groups project out into the major groove of the DNA. The disruption of structure is localized and depends upon the sequence surrounding the lesion. If guanine is 3′ to TG, there is the possibility of hydrogen bond formation between the hydroxyl group attached to the C6 of the glycol and N7 of the guanine. This causes the base moiety to move from an anti to a syn conformation. When adenine is 3′ to TG no hydrogen bond formation occurs and the TG remains in the anti conformation, but when it is 5′ to TG there is unfavourable steric overlap between the adenine and the methyl group of TG. The base pairing ability of bases 3′ and 5′ to the lesion are not altered, but the energetics of the stacking interactions are affected: the base pair 3′ to TG is stabilized, while the base pair 5′ to the lesion is destabilized.

ii) Effect of base damage on the transforming activity of pSV2 plasmids in human cells

TG and AP lesions did not affect the cell transforming activity of pSV2, but U.V. irradiation (254nm) of the plasmid increased the activity. This was

attributed to cyclobutane pyrimidine dimers in the plasmid DNA. These results were found with all the cell lines tested and were independent of the repair capacity of the recipient cells (Spivak et al 1988).

iii) Effects of base damage on replication *in vitro*

The base damage was introduced into single stranded bacteriophage M13 DNA and after annealing this to a primer, it was used as a template for DNA synthesis, by DNA polymerase I *in vitro* (Wallace 1987).

Replication was not inhibited by DHT, but halted at the residue 5′ to urea, UBA and AP sites and after the insertion of an adenine opposite TG. Termination also occurred opposite the UBA lesion. From these studies it was concluded that TG and DHT have base pairing ability and are non-mutagenic, while urea, UBA and AP lesions do not have base-pairing ability and are potentially mutagenic. Termination of DNA synthesis opposite UBA may be explained by the size or "bulkiness" of the lesion compared to urea, which could allow stabilization of base stacking by hydrogen bond formation (Wallace & Ide 1990). From these results it would be predicted that TG, UBA, urea and AP lesions would be lethal. Similar results were found for TG by Clark and Beardsley (1986) and Hayes and Le Clerc (1986).

DNA polymerase has been found to preferentially insert an adenine residue opposite non-instructive lesions (Loeb & Preston 1986). This should decrease the potential mutagenicity of urea and UBA, if DNA

polymerase is able to replicate the sequence containing the lesions. Using DNA polymerase I it was found that 3-4% of TG, 90% of DHT, but very few urea, UBA and AP lesions were by-passed *in vitro* (Wallace & Ide 1990). The by-pass of TG *in vitro* by DNA polymerase I, T4 DNA polymerase, polymerase α_2 and AMV reverse transcriptase was also examined by Clark and Beardsley (1987, 1989), using 18mer and 22mer templates with TG at position 16 from the 3′ end. Three parameters affecting synthesis past TG were identified:

a) Length of single-stranded template

TG was by-passed at a greater frequency when the 22mer template was used, which was explained by the availability of more nucleotides beyond the lesion site increasing the affinity of the polymerase for the substrate.

b) Identity of the base immediately 5' to TG

The presence of cytosine at this position results in a greater frequency of by-pass than is found with either adenine or guanine in this position. This agreed with molecular modelling studies of TG, which showed that the least distortion of the DNA structure occurred if cytosine was 5′ to TG (Clark et al 1987).

c) Presence (or absence) of 3'->5' exonuclease activity

The 3′->5′ exonuclease activity of the synthesizing enzyme inhibits by-pass of TG. DNA polymerase I, polymerase α_2 and T4 DNA polymerase have this activity and by-pass the lesion at a lower frequency than AMV reverse transcriptase, which lacks this "editing"

function. Nucleotide turnover at, or beyond, the site of this lesion may play an important role in the blocking of DNA synthesis by TG.

iv) Effects of base damage on replication *in vivo*.

This was examined by the introduction of the lesions into single-stranded or double-stranded phage DNA and the inhibition of DNA replication was measured as a function of the survival of the damaged phage following transfection into excision repair deficient host cells (Moran & Wallace 1988, Laspia & Wallace 1988a). DHT did not inactivate either single-stranded or double-stranded phage DNA. However, approximately one TG, urea, UBA or AP site per molecule effectively inactivated single-stranded phage DNA, while four lesions per molecule were required to inactivate double-stranded DNA. These results confirmed that TG, urea, UBA and AP lesions are able to inhibit DNA replication.

The induction of the SOS response in bacteria has been found to result in the by-pass of lesions during DNA synthesis. Although this was originally thought to occur even if mutations were introduced during the replication of the damaged DNA sequences (Radman 1975), Laspia and Wallace (1988b) showed that urea and AP lesions were not by-passed in SOS induced bacteria. However, 60-70% TG lesions, which are non-mutagenic, were by-passed.

1.5.2 Biological consequences of strand breakage.

Evidence indicates that ssb are relatively non-lethal: inactivation studies of double-stranded plasmid and viral DNA have shown that ssb only account for 5% of the inactivation events (Schulte-Frohlinde 1987) and as previously mentioned 400,000 ssb were measured at the H_2O_2 dose required to produce a lethal event in mammalian cells (Ward 1987).

Dsb appear to be the most important lesions with respect to cell-killing. Bacteria (Krasin & Hutchinson 1977), yeast (Ho 1975) and mammalian cells deficient in the repair of dsb show extreme sensitivity to ionising radiation and a 1:1 ratio of dsb:lethal event has been found in haploid yeast (Frankenberg 1983, referenced in Schulte-Frohlinde 1987) and exponentially growing *E.coli* (Kaplan 1966, Bonura et al 1975).

i) Use of restriction enzymes to examine the biological consequences of double strand breaks

Restriction endonucleases (RE) have been used to mimic the effects of radiation in Chinese hamster V79 cells (Bryant 1984) and examine dsb in relation to biological end-points, such as chromosomal aberrations, cell killing, sister-chromatid exchange and mutation induction. Two types of dsb termini structure can be introduced into DNA with RE: "blunt-ended" (e.g. by PvuII) and cohesive-ended (e.g. by BamHI). The majority of dsb produced by ionising radiation have cohesive ends, although it is likely that they do not have the "clean-ended" structure of the RE induced break, which

have 3'OH and 5'phosphate termini that can be rejoined by DNA ligase activity.

a) Chromosomal aberrations induced by RE.

Bryant (1984) found that RE-induced chromosomal aberrations in Chinese hamster V79 cells were of the same types as those produced by X-rays. The end structure of the dsb was found to be important in determining the frequency of chromosomal aberrations: "blunt-ended" dsb were very effective at inducing aberrations, whereas cohesive-ended dsb induced few aberrations. A similar result was obtained by Natarajan et al (1980), using Neurospora endonuclease. The distance between the two incisions of the RE has also been found to affect the aberration frequency: enzymes which introduce breaks separated by two residues (MspI) are more effective at producing aberrations than those which produce breaks separated by four residues (BamHI, Ward et al 1987). The importance of the end structure may be related to the ease of repair of the break. North et al (1990) have demonstrated that cohesive-ended dsb (which may be regarded as two ssb by repair enzymes) are repaired more efficiently than "blunt-ended" dsb. Repair of cohesive breaks separated by two bases may be slower than those separated by four bases, increasing the possibility of aberration formation (Bryant 1988).

b) Cell death induced by RE

Bryant (1985) showed that PvuII caused a dose dependent decrease in clonogenic survival, while BamHI had no effect. This is consistent with the findings of

the aberration studies (see above), in which PvuII, and not BamHI, produced chromosomal aberrations. These results support the idea that X-irradiated mammalian cells undergo a mode of death that involves dsbs in DNA resulting in chromosomal aberrations, which are lethal due to a loss of genetic material in the form of chromosome fragments or micronuclei (Joshi et al 1982).

c) Mutation induction by RE

Obe et al (1986), using AluI (generates dsb with blunt-ends), found an enzyme dose dependent increase in mutations in V79 hamster cells at the HGPRT locus, but not at the Na^+/K^+ ATPase locus. Similar results have also been reported by Zajac-Kaye & Ts'o (1984) using DNAase I and cultured Syrian hamster embryo cells, and by Thacker et al (1978) using ionising radiation and Chinese hamster cells. Mutations detected at the Na^+/K^+ ATPase locus tend to be point mutations (Obe et al 1986), since a large change in the DNA sequence at this locus is lethal (Thacker & Cox 1983). The lack of detection of mutation induction at this locus could be explained by the inability of AluI, DNase I and ionising radiation to induce point mutations (Obe et al 1986).

d) Sister-chromatid exchange (SCE) induction by RE

Natarajan et al (1985) showed that RE, producing cohesive and blunt-ended dsbs, induced SCE's. The highest frequencies were induced when cells were treated in early or mid S phase and it was concluded that the dsbs induced by RE led directly to the formation of SCE.

Alpha particles (3.2MeV) and neutrons (42MeV) have

been found to induce SCE (Aghamahammadi et al 1988, Savage & Holloway 1988), but although Nagasawa et al (1990) detected X-ray induced SCE, other studies indicated that low-LET radiation does not produce SCE (Perry & Evans 1975, Savage & Holloway 1988). This suggests that the complexity of DNA lesions varies with LET (Hendry 1991) and that RE produce damage similar to high LET radiation.

ii) Double strand breaks induced by ionising radiation

In normal mammalian cells it has been shown that 40 dsb are required to produce a lethal event after gamma or X-irradiation (Elkind & Redpath 1977, Blocher 1982), which suggests that not all dsb result in cell death. However, survival curves have been calculated (each taking into consideration a different repair time) and compared to experimentally determined survival curves. Blocher and Pohlit (1982) found that if the curve was calculated on the basis of 11 hours of repair and using the assumption that 1 dsb was lethal, the experimental and calculated curves were similar. This suggested that survival curves could be interpreted as 1 unrepaired dsb resulting in a lethal event. It is possible that only 1 of the 40 dsb measured at the radiation dose required to produce a lethal event could not be repaired.

If a dsb is situated within an LMDS other lesions present in the cluster of damage could prevent enzyme action at the dsb (Ward 1985). H_2O_2 (which is believed to produce RDDS, see earlier) requires 5 times more dsb than X-rays to produce the same lethal effect (Prise et

al 1989) and low LET-induced dsb can be repaired more easily than high LET-induced dsb (Coquerelle et al 1987). The latter finding may explain the 2 fold higher frequency of mutations (at the HGPRT locus) found to be induced by alpha particles compared to X-rays in mammalian cells (Thacker et al 1982). The lethality of dsb produced by these agents is therefore in the order of high LET radiation > low LET radiation > H_2O_2. This supports the suggestion that the lethality of a dsb may be dependent on the complexity of the lesions situated near it (Ward 1985), as well as the structure of the termini (i.e. cohesive or blunt). Therefore the radiation-induced "lethal" lesion may actually be an LMDS rather than a simple dsb.

1.5.3 Inhibition of DNA synthesis by ionising radiation

The dose response curve for the inhibition of DNA synthesis in mammalian cells consists of two components: an initial steep component at low doses (Do = 5Gy, where Do is the inverse of the gradient of the portion of the curve under consideration) and a shallow component at high doses (Do > 100Gy, Painter 1986). Okada (1970) suggested that the steep component was due to the inhibition of the initiation of DNA replication, while the shallow component reflected a decreased rate of elongation of the DNA chain. In support of this Watanabe (1974) showed, using fibre autoradiography, that ionising radiation reduced the length of the DNA chains and that the dose response (Do = 150Gy) was

equivalent to that of the shallow component for the inhibition of DNA synthesis. Also, a decrease in the number of short fragments was found and it was suggested that this may be due to inhibition of replicon initiation (see below). Makino and Okada (1975), using alkaline sucrose gradients, measured a large decrease in the number of low molecular weight fragments of nascent DNA, shortly after exposure of mouse L5187Y cells to low doses (<5Gy) of ionising radiation. This has also been demonstrated with Chinese hamster and HeLa cells (Painter & Young 1975) and supports the concept of low dose inhibition of initiation of DNA replication. Inhibition of DNA synthesis in normal cells could be part of a control process, whereby DNA replication is slowed down to enable more time for cells to repair the radiation damage (Painter 1986).

i) Mechanism of inhibition of replicon initiation and chain elongation

The mechanism of inhibition of replicon initiation is not well understood. Painter and Young (1976) suggested that production of DNA damage by radiation blocks the initiation of entire clusters of replicons, which is consistent with the idea that control of DNA synthesis initiation is at the level of replicon clusters (Hand 1978). Povirk and Painter (1976) proposed that the blockage of initiation at clusters was by "nick-induced" relaxation of the supercoiled cluster structure, which is approximately 1000kb of DNA (Painter 1978). However, relaxation by a ssb is unlikely as

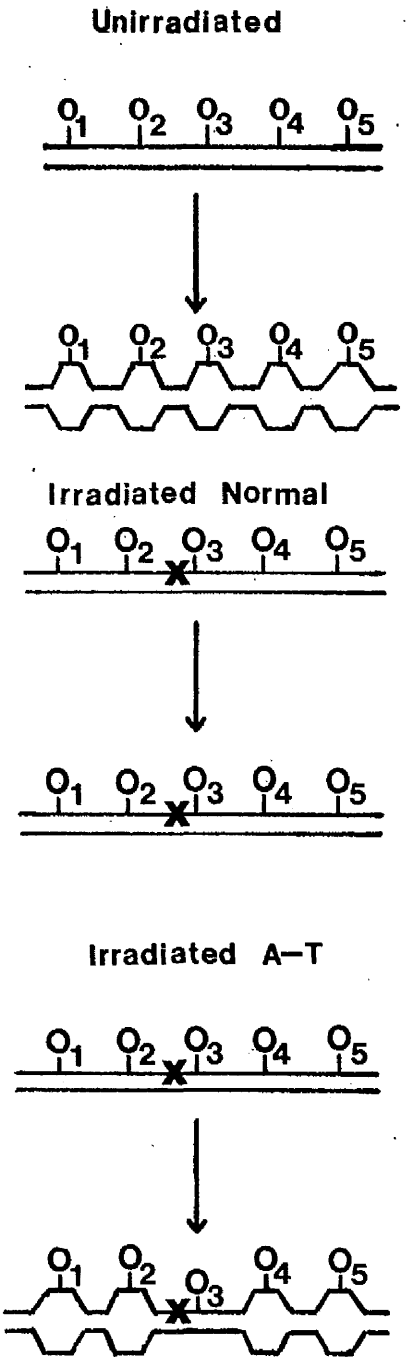
supercoiling tension within mammalian cells is taken up by nucleosomal interaction (Sinden et al 1980).

Ataxia telangiectasia (A-T) cells are hypersensitive to the cytotoxic effects of ionising radiation, but resistant to radiation-induced inhibition of DNA synthesis, as shown by alkaline sucrose gradients (Painter & Young 1980) and fibre autoradiography (Ockey 1983). A-T cells do not show the steep component in the dose response of DNA synthesis inhibition and also have been shown to be resistant to inhibition of chain elongation (Painter & Young 1980). DNA synthesis in A-T cells can only be blocked at replicon initiation and it has been suggested that these cells can initiate replication at each individual replicon, but not at a cluster. A-T cells may therefore lack a factor which in normal cells shuts down replicon initiation in a cluster of replicons and stops the advancing replication fork when damage occurs anywhere in the cluster (figure 10, Painter 1986).

Lamb et al (1989) has provided evidence to suggest that the factor is trans-acting. Following irradiation of mammalian cells containing extrachromosomal plasmid DNA, Lamb et al (1989) found that DNA synthesis was inhibited in plasmid DNA as well as in genomic DNA. Over the dose-range examined, no significant breakage of the plasmid occurred and by assuming that base damage was produced at less than 3 x frequency of strand breaks, they concluded that no significant base damage would have been produced in the plasmid. However, it is

FIGURE 10 - Inhibition of DNA synthesis

Painter (1986) proposed that in normal cells one lesion (X) inhibits the replication process at a cluster of replicons (O = replicon origin). The missing factor in A-T cells causes one lesion to inhibit at only one replicon. (Figure reproduced from Painter 1986).



now known that EIII-sensitive sites are produced in DNA at the same frequency as ssb (Bases et al 1990), but if measurement of strand breakage was accurate, there should still not have been a significant number of EIII-sensitive sites (and hence base damage) induced. Lamb et al (1989) concluded that the radiation-induced damage in the chromosomal DNA was inhibiting DNA synthesis of the plasmid DNA by producing or depleting a "trans-acting" factor.

ii) Effect of inhibition of DNA synthesis on cell killing by ionising radiation.

A number of workers have established A-T cell lines that have been transfected with human DNA and acquired increased radioresistance (Lehmann et al 1986, Kapp & Painter 1989, Ejima et al 1990). Although Lehmann et al (1986) and Kapp and Painter (1989) produced A-T cell lines with increased radioresistance, the cell lines continued to exhibit radioresistant DNA synthesis. Lehmann et al (1986) concluded that this demonstrated that the radiosensitivity of A-T cells was not due to the failure of the cells to inhibit DNA synthesis. It was proposed that the A-T phenotype may be due to deletions in two separate but closely linked genes, or a mutation in a regulator gene that affected cellular radiosensitivity and the ability to inhibit DNA synthesis. Kapp and Painter (1989) suggested that they and possibly Lehmann's group had produced cell lines that did not completely attain the radioresistant state of normal cells and that if transfection and/or

expression of the complete gene(s) had been achieved, both of the A-T characteristics (i.e. radiosensitivity and radioresistant DNA synthesis) would have been absent from the recipient A-T cell line.

1.6 Repair of radiation damage

Radiation-induced DNA damage can be repaired by two mechanisms: reversal of damage, which involves repair of breaks by the direct rejoining of ends, and excision repair, which involves the removal of modified residues from the DNA (Friedberg 1985). Most of the information presented below has been obtained from bacterial systems.

1.6.1 Reversal of damage

A fraction of ssb produced in DNA by ionising radiation are believed to be repaired by the simple rejoining of the ends (Jacobs et al 1972) and this is considered to be a direct reversal of DNA damage. The joining of the ends requires a polynucleotide ligase enzyme and has been measured *in vitro*, using gamma irradiated DNA, by the decrease in the fraction of total strand breaks as determined by sedimentation velocity in alkaline sucrose gradients. Approximately 30% of the ssbs examined under these experimental conditions were rejoined by polynucleotide ligase (Jacobs et al 1972). The *E.coli* enzyme requires nicotinamide, Mg^{2+} and "free" ends in the duplex DNA, which have 3′ hydroxyl and 5′ phosphate termini and no missing nucleotides at the break site (Friedberg1985).

1.6.2 Excision repair

Repair of oxidative DNA base damage and breaks that are more complex than phosphodiester bond scission, occurs by excision repair mechanisms in which the damaged residue is removed by either an endonucleolytic or glycosylytic reaction, as discussed below (figure 11). Following the excision of the damaged residue, the insertion of the correct nucleotides and the joining of the DNA termini are carried out by DNA polymerase and DNA ligase, respectively (Friedberg 1985). Certain enzymes (EIV and exonuclease III, see 1.6.3iii, iv) are able to remove 3′ phosphate and phosphoglycolate termini to allow completion of repair by DNA polymerase and DNA ligase at strand breaks.

(i) Glycosylytic reaction

This occurs in three steps (see figure 11):-

a) Removal of the modified base

Removal requires a DNA glycosylase (see later), which cleaves the N-glycosylic bond of the oxidized base producing an AP site in the DNA (Teoule 1987).

b) Introduction of a ssb next to the AP site.

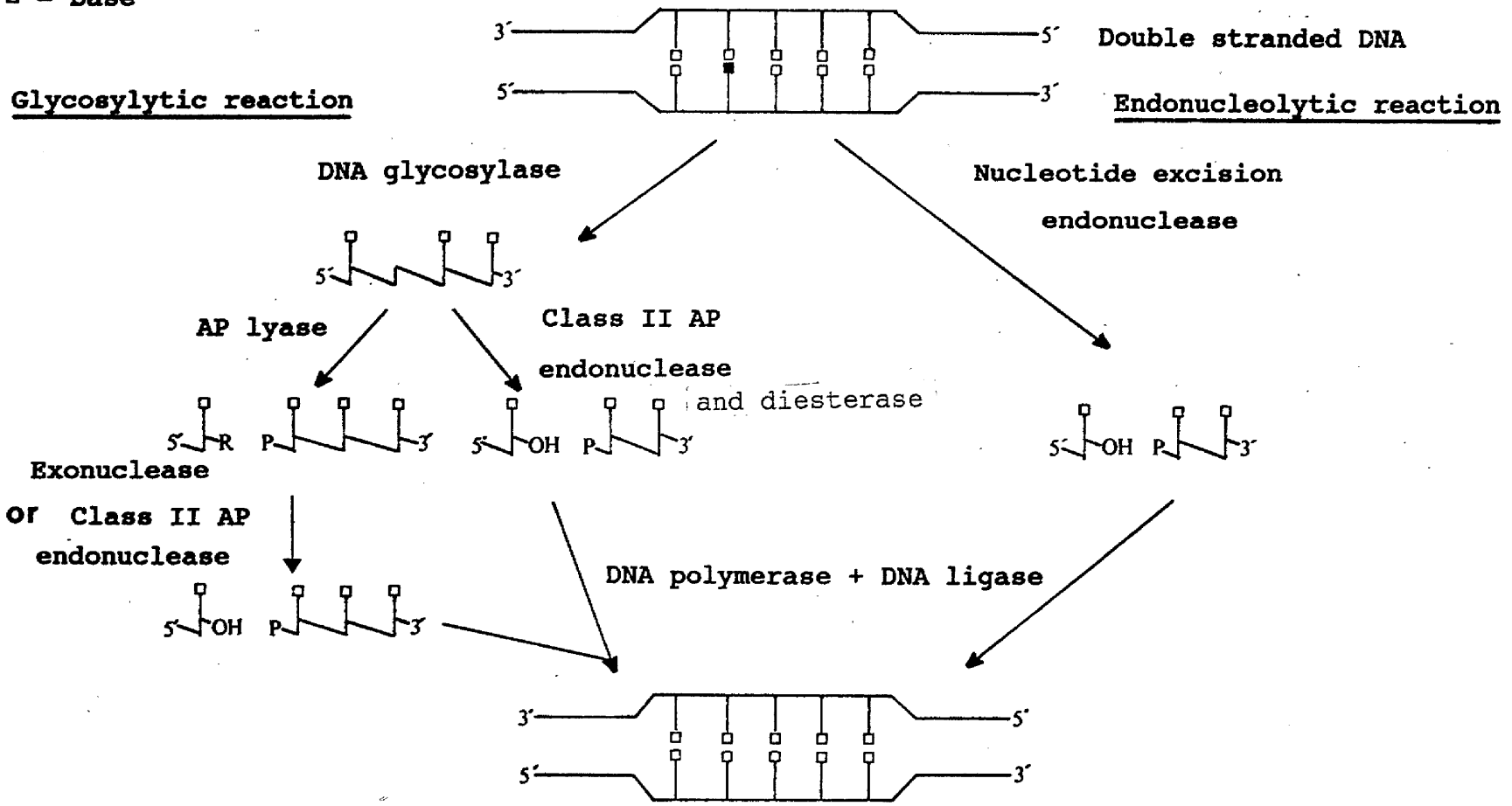
The break is produced by an AP endonuclease (see later). Two classes of the enzyme exist: those that cleave DNA at the 3′ side of the AP site (class I or AP lyase, Levin & Demple 1990, Bricteux-Gregoire & Verly 1991) and those that cleave at the 5′ side of the AP site (class II, Levin & Demple 1990).

c) Removal of the deoxyribose phosphate moiety.

The sequential action of a class I and class II AP

FIGURE 11 - Excision repair mechanism

Excision of a modified base can occur by a glycosylytic or endonucleolytic reaction. See text for details.
R = 2,3-didehydro-dideoxyribose
■ = modified base
□ = base



endonuclease could excise this moiety and this has been demonstrated *in vitro*. However, no evidence has been found for this occurring in the cell (Franklin 1987). Removal of the deoxyribose moiety may occur via an exonuclease, which degrades DNA in either the 5'->3' or 3'->5' direction at the break created by the endonuclease in reaction (b) (Lindahl 1982).

(ii) Endonucleolytic reaction

The endonucleolytic reaction results in the release of the modified base attached to the sugar phosphate without cleavage of the N-glycosylic bond. This class of endonuclease (see later) recognizes conformational distortions of the DNA structure, produced by a wide variety of base damage (Friedberg 1985). It was originally thought that the endonucleolytic reaction was carried out by an enzyme that incised at the 5' side of the damage and the excision of the damaged residue occurred by exonucleolytic degradation of the DNA in the 5'->3' direction (Hanawalt et al 1979). However, Sancar and Rupp (1983) have reported that there is an *E.coli* enzyme (UVRABC endonuclease, see 1.6.3iii) that incises on both sides of the base damage: it is not known whether this mechanism occurs in mammalian cells.

1.6.3 Prokaryote DNA repair enzymes

Table 3 summarises the types of enzymes involved in DNA repair and a more detailed discussion is presented below.

TABLE 3 - PROKARYOTE DNA REPAIR ENZYMES

ENZYME	ACTIVITY	DAMAGE RECOGNISED	SOURCE	REFERENCE
Uracil DNA glycosylase	DNA glycosylase	uracil	*E.coli* *M.luteus*	Delort et al 1985
Formamido-pyrimidine DNA glycosylase	DNA glycosylase AP lyase	2,6 diamino-4-hydroxy-5-(N-methyl formamido)-pyrimidine	*E.coli*	Chetsanga et al 1981
Pyrimidine dimer DNA glycosylase	DNA glycosylase AP endonuclease	thymine dimers	*T4 phage* *M.luteus*	Haseltine 1983
Endonuclease III	DNA glycosylase AP lyase	urea, cis & trans TG, 5-hydroxy-5-methylhydantoin, 6 hydroxy-5,6 dihydrothymine cytosine photo-product	*E.coli*	Katcher & Wallace 1983 Breimer & Lindahl 1984, 1985 Doetsch et al 1986
Endonuclease VIII	DNA glycosylase AP endonuclease	TG, urea, beta uriedoisobutylic acid, AP sites	*E.coli*	Melamede et al 1987
Endonuclease IV	3′ phosphatase 3′ phospho-glycolatase AP endonuclease	3′ phosphate, 3′ phosphoglycolate, AP sites, 2,3 di-dehydrodeoxyribose	*E.coli*	Saporito & Cunningham 1988 Demple et al 1986 Levin et al 1988
UVRABC endonuclease	damage specific nuclease	thymine dimers, cytosine (6-4) photoproducts, TG, AP sites	*E.coli*	Sancar & Rupp 1983 Lin & Sancar 1989
Exonuclease III	3′-5′exonuclease 3′ phosphatase 3′ phospho-glycolatase AP endonuclease	AP sites, fragmented bases, 3′ phosphate 3′ phosphoglycolate	*E.coli*	Cunningham & Weiss 1985 Kow et al 1987

(i) DNA glycosylases

Generally these enzymes have a low molecular weight, act at modified bases in double stranded DNA (Teoule 1987). An example is uracil DNA glycosylase (Delort et al 1985).

(ii) DNA glycosylases with associated AP endonuclease or AP lyase activity.

a) Pyrimidine dimer DNA glycosylase

Pyrimidine dimer DNA glycosylase is coded by the *denV* gene in T4 bacteriophage and is a 16,000 Da protein possessing two distinct enzymatic activities, which act to initiate repair of thymine dimers (Haseltine 1983). The glycosylase activity first cleaves one of the two bonds attaching the dimer to the DNA, leaving an AP site, and the endonuclease activity incises the DNA at the AP site. A similar enzyme is present in *M.luteus*

b) Endonuclease III

EIII is encoded by the *E.coli nth* gene and has a molecular weight 26,300 Da in the native state (Asahara et al 1989). It has both DNA glycosylase and AP lyase activities and does not require divalent metal ions for activity. EIII repairs double stranded DNA oxidized by hydroxyl radicals (see 1.2.1), but not singlet-oxygen (Muller et al 1990). Although this enzyme is not involved in the repair of pyrimidine dimers, it has been found to release an unidentified cytosine photoproduct from U.V. irradiated DNA (Doetsch et al 1986).

EIII DNA glysosylase activity releases a wide variety of damaged residues from damaged DNA (cis and

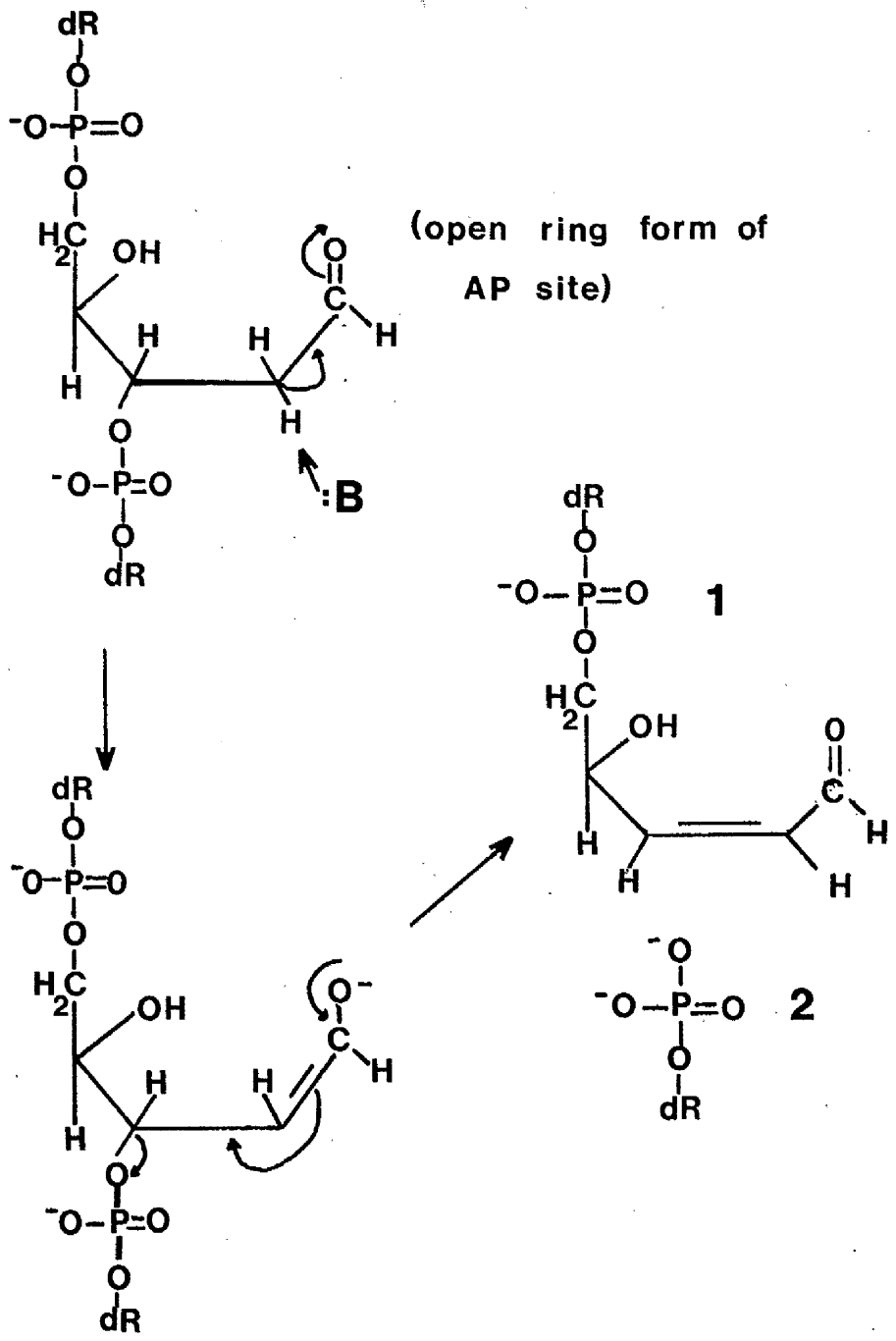
trans TG, 5 hydroxy-5 methylhydantoin, 6 hydroxy-5,6-dihydrothymine and urea, Breimer & Lindahl 1980, 1984, 1985, Katcher & Wallace 1983, Demple & Linn 1980, see figure 3 for structures of damage), and it is likely that a single active site recognises a structural feature common to all the pyrimidine derivatives. Recognition may be by the planar structure created when the 5,6 double bond is absent (Breimer & Lindahl 1984). It has been proposed that one of the intermediate steps of the EIII glycosylytic reaction is the formation of a Schiff's base between an enzyme amino group (possibly a lysine residue) and the aldehyde group of a ring open form of the AP deoxyribose residue, or the imine group of the ring open form of TG or urea residues (Kow & Wallace 1987).

After release of the damaged base, EIII catalyses a beta elimination reaction, which results in the cleavage of the phosphodiester bond 3′ to the AP site and catalyses the production of a 2,3-didehydro-dideoxyribose terminus (figure 12, Bailly & Verly 1987, Mazumder et al 1991). This break is a poor substrate for repair by DNA polymerase I (Kow & Wallace 1987), unlike the products of AP endonuclease reactions, which involve the hydrolysis of the phosphodiester bond leaving a deoxyribose moiety (Warner et al 1980).

Unlike other DNA binding proteins EIII is an iron-sulphur protein and contains a 4Fe-4S cluster in the 2+ oxidation state, a characteristic of proteins involved in electron transport. The iron-sulphur cluster in EIII

FIGURE 12 - Beta elimination reaction

After cleavage of the N-glycosylic bond of the modified base, endonuclease III catalyses a beta elimination reaction. A hydrogen is abstracted from the ribose moiety by B: and the products of the reaction are a 3′ 2,3-didehydro-dideoxyribose moiety (1) and a 5′ phosphate (2).



may function as a substrate binding site (Cunningham et al 1989). It does not appear to be involved in the beta elimination reaction, since other enzymes catalysing this type of reaction do not contain iron-sulphur centres, e.g. T4 bacteriophage pyrimidine dimer DNA glycosylase (Mazumder et al 1991).

Bacteria deficient in EIII (*nth* mutants) do not show increased sensitivity to H_2O_2 or gamma rays (Cunningham & Weiss 1985), in fact the mutants are slightly more resistant to H_2O_2. Bacteria deficient in endonuclease III and IV and exonuclease III (see later for description of the latter two enzymes) show a slight resistance to MMS, mitomycin C, U.V. and gamma radiation in comparison to the those deficient in endonuclease IV and exonuclease III. These results suggest that the AP lyase activity of EIII can be deleterious to cell survival. It is possible that another enzyme is able to remove the 5,6 ring saturated thymine damage produced by ionising radiation and H_2O_2 in *nth* mutants and it is the DNA glycosylase function of EIII that has caused this enzyme activity to be conserved through evolution: activities similar to EIII have been detected in bovine (Doetsch et al 1986), human (Higgins et al 1987) and yeast cells (Gossett et al 1988, see 1.6.4).

c) Endonuclease VIII

The substrates recognised by endonuclease VIII are TG, DHT, urea, AP sites and UBA (a product of DHT alkali cleavage). It is an *E.coli* enzyme that may substitute for EIII in the excision repair of TG, urea and AP sites

(Melamede et al 1987).

(iii) AP endonucleases

For a review of AP endonucleases see Weiss et al (1988).

a) Endonuclease IV

EIV is encoded by the *E.coli nfo* gene and has a molecular weight of 31,500 Da (Saporito & Cunningham 1988). It is a monomeric protein and does not require divalent metal ions for activity. However, pure protein is destabilized by EDTA in the absence of a DNA substrate, which suggests that the enzyme is a metalloprotein (Levin et al 1988).

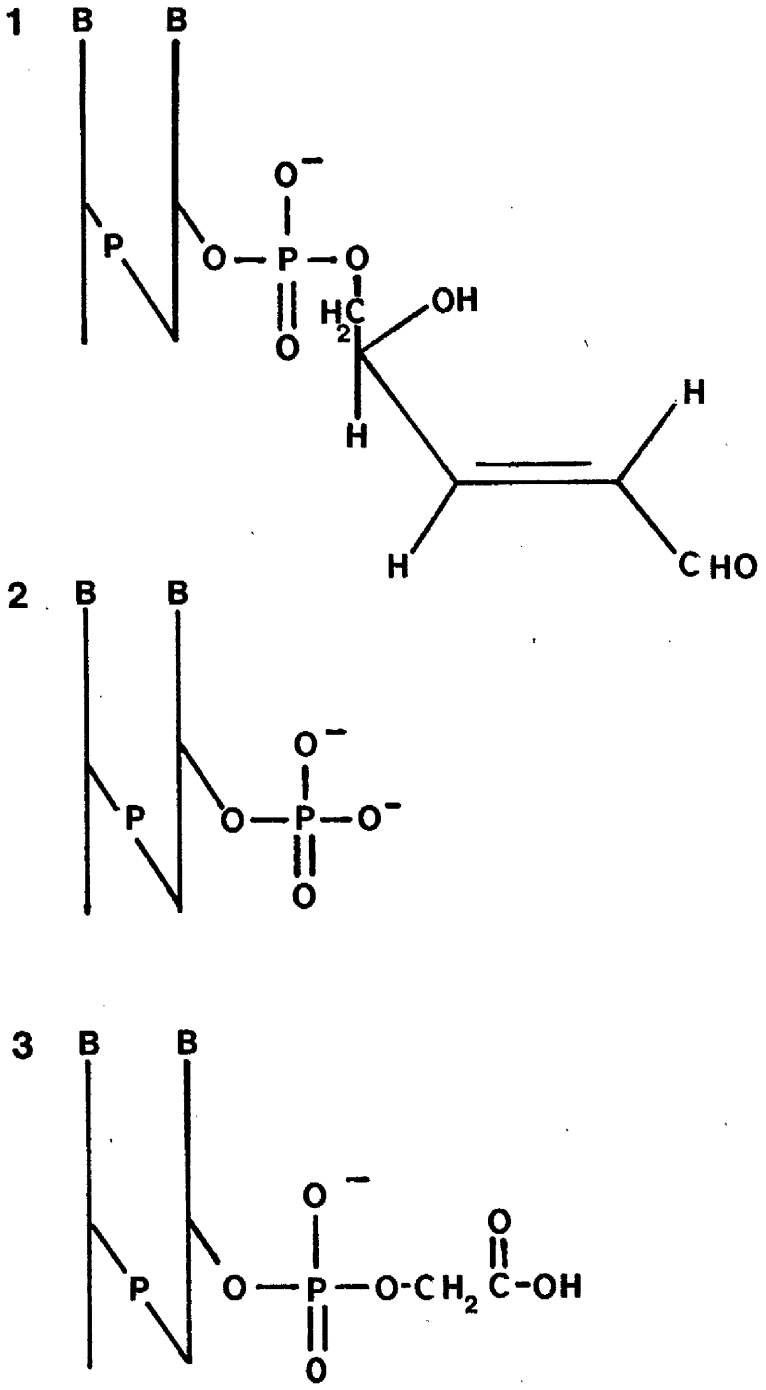
EIV is a class II AP endonuclease and accounts for approximately 10% of the AP endonuclease activity in *E.coli* (Siwek et al 1988). Action at AP sites results in a 3'OH group and a 5' terminal deoxyribose phosphate, which is "lost" spontaneously from the DNA by a beta elimination reaction. The 3' OH can then be used by DNA polymerase I to complete repair (Bailly & Verly 1989).

Other activities of EIV include 3' phosphatase (Levin et al 1988), 3'phosphoglycolatase (Demple et al 1986) and hydrolysis of the phosphodiester bond 5' to a 3' terminal base-free 2,3-didehydro-dideoxyribose moiety (Bailly & Verly 1989, see figure 13 for EIV substrates). Although radiation produces DNA strand breaks with 3' phosphate and 3' phosphoglycolate termini (Henner et al 1983) and EIV is known to remove 3' blocking termini and promote DNA synthesis in H_2O_2-damaged DNA (Demple et al 1986), EIV deficient bacteria (*nfo* mutants) do not show

FIGURE 13 - DNA Lesions recognised by endonuclease IV

Endonuclease IV removes 3′ 2,3-didehydro-dideoxyribose (1), 3′phosphate (2) and 3′ phosphoglycolate (3) termini from DNA to produce 3′ hydroxyl termini.

B = Base



increased sensitivity to ionising radiation or H_2O_2. However, bacteria deficient in both EIV and exonuclease III (see 1.6.3iv) are more sensitive to these cytotoxic agents than bacteria deficient in only exonuclease III (*xth* mutants). Exonuclease III can therefore substitute for EIV in *nfo* mutants, in the repair of ionising radiation and H_2O_2 DNA damage (Cunningham et al 1986). *nfo* mutants are more sensitive to bleomycin and t-butyl hydroperoxide than *xth* mutants, which indicates that the DNA damage produced by these agents is repaired more effectively by EIV than exonuclease III (Cunningham et al 1986).

EIV activity is induced 10-20 fold, in growing bacterial cultures, by paraquat and to a lesser extent by menadione, plumbagin, phenaxine methosulphate and H_2O_2, which produce superoxide ions (Chan & Weiss 1987). After treatment with paraquat, the AP endonuclease activity of EIV is equivalent to that of exonuclease III and the phosphoglycolatase activity constitutes approximately 20% of the cells activity, compared to 2% in uninduced bacteria (Siwek et al 1988). Bacteria deficient in superoxide dismutase are very sensitive to EIV induction, indicating that this process is mediated by superoxide ions (Chan & Weiss 1987).

b) UVRABC endonuclease

This *E.coli* multisubunit enzyme is the product of the *uvra*, *uvrb* and *uvrc* genes and acts to repair thymine cyclobutane dimers, cytosine (6-4) photoproducts, TG and AP sites (Lin & Sancar 1989).

The UVRABC endonuclease acts by the cleavage of phosphodiester bonds: it hydrolyses the 8th phosphodiester bond on the 5′ side of the damage and the 4th or 5th phosphodiester bond on the 3′ side and results in the removal of 12 nucleotides (Sancar & Rupp 1983).

(iv) Exonucleases

a) Exonuclease III

Exonuclease III is a 28kDa protein and is encoded by the *E.coli xth* gene. *xth* mutant bacteria show increased sensitivity to H_2O_2 (Cunningham & Weiss 1985) and long wavelength U.V. radiation (Kow et al 1987), but not ionising radiation (Cunningham & Weiss 1985). This may be explained by the overlapping activities of EIV and exonuclease III, i.e. AP endonuclease, 3′phosphatase and 3′ phosphoglycolatase activities (see 1.6.3 iiia). Exonuclease III accounts for 95% of 3′ phosphoglycolatase (Bernelot-Moens & Demple 1989) and 90% of AP endonuclease activity (Siwek et al 1988) in bacteria and can promote DNA synthesis in DNA isolated from H_2O_2-treated, *xth* mutant bacteria (Demple et al 1986). Unlike EIV, exonuclease III is not inducible and requires Mg^{2+} for activity (Siwek et al 1988). It also has 3′->5′ exonuclease and RNase H activities (Kow et al 1987).

b) DNA polymerase

E.coli DNA polymerases I, II and III have 3′->5′ exonuclease activity and I and III also have 5′->3′ exonuclease activity. It is possible that these

activities may remove AP or damaged nucleotides following the action of an endonuclease. DNA polymerase I was found to catalyse the removal of pyrimidine dimers from U.V. irradiated DNA *in vitro* (Friedberg 1985).

1.6.4 Eukaryote DNA repair enzymes

(i) EIII-like activities

Enzymes have been purified from bovine (Helland et al 1986) and yeast cells (Gossett et al 1988) and these have similar substrate specificities and mechanisms of action to EIII. This type of enzyme has been called a redoxyendonuclease and does not require divalent metal ions for activity. The bovine enzyme is 30,000Da, while the yeast enzyme is 38-42,000Da. Both enzymes are able to remove cytosine, thymine and guanine photoproducts in heavily U.V. irradiated DNA and cleave at thymine lesions in OsO_4 treated DNA (Helland et al 1986, Gossett et al 1988). Redoxyendonuclease activities have been detected in A-T (Remsen & Cerutti 1977), normal human and Xeroderma pigmentosum (X-P) fibroblasts (Doetsch et al 1987) and HeLa cells were found to contain an enzyme with TG DNA glycosylase activity (Higgins et al 1987).

Kim et al (1991) have purified enzymes from mouse MPC-11 cells and normal human fibroblasts, which have the same funtion as EIII, but preliminary experiments suggest that the mouse and human enzymes also cleave at the AP site generated by the breakage of one glycosyl linkage of a pyrimidine dimer. This type of enzyme has been called UV endonuclease III and appears to restore

U.V. DNA repair synthesis to X-P cells of complementation group D (Kim et al 1991).

(ii) EIV and exonuclease III-like activities

The cDNA of the major AP endonuclease of calf thymus has recently been cloned (Robson et al 1991) and it encodes a 38,000Da protein, which has been called BAP1. Incubation of H_2O_2 or bleomycin treated DNA with this enzyme *in vitro* removes damage that inhibits DNA synthesis, but although the predicted amino acid sequence of BAP1 has 50% homology with the peptide sequence of exonuclease III, BAP1 does not have exonuclease activity. Only 68 residues at the N-terminus are unique to BAP1. The function of the N-terminus is not known, but it has been suggested that it may interact with other enzymes in a multienzyme complex or contain nuclear localisation signals (Robson et al 1991).

Popoff et al (1990) have purified a 40,000Da yeast enzyme (APN1) that accounts for 97% of the AP endonuclease, 3′ phosphatase and 3′ phosphoglycolatase activities in yeast cells. The gene (*apn1*) encoding this enzyme has recently been cloned and the nucleotide and amino acid sequences of *apn1* and APN1 show high homology with the *nfo* and EIV sequences, respectively. Expression of the *apn1* gene in *nfo*, *xth* double mutant bacteria has been found to increase resistance to H_2O_2, t-butyl hydroperoxide, MMS, menadione, bleomycin and mitomycin C (Ramotar et al 1991), which confirms that EIV and APN1 have similar functions. The major difference between these proteins is at the C-terminus: APN1 has a highly

basic C-terminus which may interact with chromatin to allow the enzyme access to damaged DNA (Popoff et al 1990). Although the activities of APN1 are also similar to exonuclease III and BAP1, no significant amino acid homology was found between the sequences of APN1 and these two enzymes (Popoff et al 1990, Robson et al 1991).

Two 3' phosphoglycolatase activites have been separated by phosphocellulose chromatography from extracts of HeLa cells (Demple et al 1988), one of which corresponds to an enzyme detected in HeLa cells by Kane and Linn (1981). This enzyme (45,000Da) requires Mg^{2+} for activity and also has AP endonuclease activity. The other enzyme has yet to be characterised.

(iii) Enzymes encoded by the human *ERCC* genes

Functional complementation of U.V sensitive Chinese hamster ovary (CHO) mutant cells (defective in nucleotide excision repair) by HeLa cell genomic DNA has led to the isolation of five ERCC (excision repair cross-complementing) genes: *ERCC-1* (Westervald et al 1984), *ERCC-2* (Weber et al 1988), *ERCC-3* (Weeda et al 1990a), *ERCC-5* (Mudgett & MacInnes 1990) and *ERCC-6* (Troelstra et al 1990). The CHO mutants used have been assigned to different complementation groups (eight groups of U.V. sensitive Chinese hamster mutants exist, Collins & Johnson 1987, Busch et al 1989) and each ERCC gene corrects the defect of a cell line in a different complementation group. The characteristics of the genes are summarised in table 4.

TABLE 4 - CHARACTERISTICS OF THE ERCC GENES

GENE	SIZE	CHO MUTANT USED IN CLONING	DEFICIENCIES OF MUTANT CORRECTED BY GENE	CHARACTERISTICS OF PROTEIN	REFERENCES
ERCC1	15kb	43-3B (complementation group 1)	U.V. and MMC sensitivity	Homologus to RAD10 and regions of the UVRA and UVRC proteins of bacteria	Westerveld 1984 van Duin et al 1986
ERCC2	15-20kb	UV-5 (complementation group 2)	U.V sensitivity	High homology (73%) with RAD3, which is a single-stranded DNA dependent ATPase and an ATP dependent helicase	Weber et al 1988, 1990
ERCC3	35-45kb	27-1 (complementation group 3)	U.V. sensitivity Also corrects X-PB	Predicted to be 90kDa (782 amino acids) with putative nucleotide, chromatin and helix - turn - helix DNA binding domains and 7 consecutive motifs that are conserved in DNA and RNA helicases.	Weeda et al 1990a, b
ERCC5	32kb	UV-135 (complementation group 5)	U.V. sensitivity	----------	Mudgett & MacInnes 1990
ERCC6	~100kb	UV-61 (complementation group 6)	U.V. sensitivity, increases repair of U.V.-induced dimers	----------	Troelstra et al 1990

(iv) Enzyme encoded by *XRCC-1*

XRCC-1 (X-ray repair cross-complementing) is the only isolated gene that affects cellular radiosensitivity and it was cloned by techniques similar to those employed to isolate the ERCC genes. This gene is 33kb and complements EM9, which has reduced ssb repair capacity, increased production of sister chromatid exchange on incorporation of bromodeoxyuridine and is sensitive to EMS (ethyl methanesulphonate) and ionising radiation. The gene is predicted to encode a 69,500Da protein and has been assigned to human chromosome 19. The *XRCC-1* gene product may be involved in ssb repair (Thompson et al 1990).

1.7 Biological consequences of inaccurate DNA repair.

The absence of an intact complementary strand for use as a template for repair-synthesis can result in the introduction of mutations into the DNA sequence. When lesions, such as base damage or AP sites, are opposite one another the coding information for the DNA sequence is lost, which increases the possibility of point mutations (Ward 1987). In haploid cells, closely opposed lesions are more likely to be lethal or mutagenic, but in diploid cells the lost information is still available in the sister chromosome and the lesions can be repaired by recombination (Resnick 1976). A point mutation, induced by gamma radiation, activated the c-K-ras oncogene in mouse lymphomas (Guerro et al

1984). The mutation was a single base change (G -> A) in an exon sequence, which resulted in the insertion of an aspartic acid in the protein instead of a glycine, and was not found in non-malignant tissue DNA.

An assay has recently been developed which allows the fidelity, as well as the efficiency, of repair of dsbs (produced by RE) to be measured (North et al 1990). A dsb was introduced within the cloning region in the *lacZ* gene of pUC18 using a restriction endonuclease and the plasmid incubated with nuclear cell extract. The rejoining of the break was examined by Southern analysis, after gel electrophoresis, while the accuracy of repair was assessed by expression of the *lacZ* gene after bacterial transformation with the treated plasmid. The repair capabilities of nuclear extracts from immortalised A-T and normal human cells were examined and although the A-T cells were able to repair dsb at the same efficiency (or capacity) as the normal cells, the fidelity of repair was lower. It is not known how this is implicated in the A-T defect. It has been suggested that nucleases may degrade the sequence at the site of the break, resulting in a decrease in the fidelity of repair and that this may be due to the lack of a component(s) in A-T cells which protects the break termini from degradation (North et al 1990).

1.8 Cellular sensitivity to cytotoxic agents

The radiosensitivity of normal individuals varies: patients have exhibited abnormal skin reactions

following radiotherapy and fibroblasts from these patients showed marked *in vitro* radiosensitivity (Smith et al 1980, Woods et al 1988). Tumour cells (derived from individuals not suffering from the diseases mentioned in 1.8.1) also differ in their inherent radiosensitivity (Deacon et al 1984, Fertil & malaise 1985) and it is thought that these differences can affect the success of radiotherapy (Fertil & Malaise 1981). West et al (1991a) and other workers (Brock, Ramsey, Malaise, for review of work see West et al 1991b) are carrying out studies on the radiosensitivity of cell lines derived from human tumours, to determine whether the control of the disease by radiotherapy is related to the radiosensitivity of the tumour cells. West et al (1991a) have used the surviving fraction of cells at 2 Gy (SF2) as a measure of the radiosensitivity of human cervical carcinoma cell lines. Results from patients, at least 2 years after treatment, indicate that there is a good correlation between intrinsic radiosensitivity of cells and tumour control: radiosensitive (SF2 < 0.55) and radioresistant (SF2 > 0.55) tumours showed a 10 fold difference in tumour control. The SF2 value therefore appears to be a good prognostic factor for cervical carcinoma (West et al 1991a).

1.8.1 Human diseases which can result in sensitivity to ionising or U.V. radiation

Studies to try to determine the factors affecting cellular sensitivity to cytotoxic agents have used laboratory derived mutant cell lines (1.8.2) and cells

derived from patients with diseases found to be sensitive to ionizing or U.V. radiation. These include A-T (Bridges & Harnden 1982, Lehmann 1982a), X-P (Bootsma 1979), Huntington's chorea (Arlett 1979), Nijmegen breakage syndrome (Taalman et al 1983), inherited retinoblastoma (Arlett 1979), homocystinuria (Sinelshichikova et al 1987), Fanconi's anaemia (FA, Arlett 1979), Cockayne's syndrome (CS, Deschavanne et al 1984, reviewed by Lehmann 1987), Gardener's syndrome (Little et al 1980) and Bloom's syndrome (BS, Arlett 1979). The most studied of these are A-T, X-P, CS, FA and BS and the major characteristics and possible defects of these diseases are summarised in table 5 (for review see Setlow 1978, Lehmann 1982b).

Seven complementation groups (A-G) of excision repair defective X-P patients have been established and a connection has been found between X-P, CS and another disease trichothiodystrophy (TC). TC results in brittle, sulphur-deficient hair, mental and physical retardation, abnormal nail growth and scaly skin (Wood 1991). Approximately half of the 21 TC patients are photosensitive and have repair defectscharacteristic of X-PD patients. Patients with CS have also been found to show X-P characterisitcs: 3 with X-PB, 3 with X-PG and 1 with X-PD. It is now thought that there are seven genes, which if mutated at different alleles can result in one or more of these diseases (Wood 1991).

ERCC-3 has been found to correct the defect in X-PB cells. An X-PB patient was found to express one ERRC-3

TABLE 5 - CHARACTERISITICS OF HUMAN DISEASES RESULTING IN SENSITIVITY TO IONIZING OR U.V. RADIATION

DISEASE	CLINICAL SYMPTOMS	CHARACTERISITICS OF CELLS	REFERENCES
Ataxia telangiectasia	Occulocutaneous telangiectasia (chronic dilation of small blood cells) Progressive cerebellar ataxia (loss of muscular coordination) Impaired cell-mediated immunity Autosomal recessive disease	Sensitive to gamma and X-rays and bleomycin. Able to repair ssb and dsb. Low fidelity of break repair. Radioresistant DNA synthesis. Able to remove TG-type lesions from DNA.	Bridges & Harnden 1982 Remsen & Cerutti 1977 North et al 1990
Cockaynes Syndrome	Dermatosis. Physical and neurological defects. No increase in cancer frequency. Sensitive to sunlight	Sensitive to U.V. light. Subtle defect in excision repair (actively transcribed regions only).	Bootsma 1979 Venema et al 1990a Lehmann 1987
Fanconi's anaemia	Congenital anatomical defects. Hyperpigmentation. Mild mental defects. Hypoplasia of marrow cell lineages. Chromosome instability	Slightly sensitive to high doses of U.V. light. Able to repair strand breaks. Defective in repair of thymine dimers. 2 out of 4 cell lines tested were deficient in TG-type lesion removal.	German 1979 Poon et al 1974 Remsen & Cerutti 1976
Bloom's Syndrome	Growth deficiency. Sensitive to sunlight. Defective immunity. Increase risk of cancer. Recessive inherited disease	Slightly U.V. sensitive. Increase in SCE after EMS treatment. Increase in chromosome breakage after irradiation. Normal DNA ligase II activity. Defective DNA ligase I	German 1979 Setlow 1978 Lindahl et al 1989
Xeroderma Pigmentosum Variant	U.V. sensitive. Increased frequency of skin cancer	Defective in post replication repair. Slight increase in U.V. sensitivity. Increased U.V. mutagenesis.	Arlett 1979
Xeroderma Pigmentosum (A-G)	U.V. sensitive. Increased frequency of skin cancer	Defective in excision repair at the incision step of dimer removal. Increased U.V. mutagenesis. Very sensitive to U.V. radiation. X-PC able to repair dimers in actively transcribed chromatin.	Arlett 1979 Kim et al 1991 Venema et al 1990b

allele, which had a C->A mutation in the splice acceptor sequence of the last intron of the DNA sequence. This resulted in a 4bp insertion in the mRNA and inactivation of the C-terminus of the protein (Weeda et al 1990b).

1.8.2 Laboratory derived mutant cell lines

Many ionising radiation sensitive mutants have been derived from Chinese hamster cell lines by treatment with mutagens, such as ENU (ethylnitrosourea). Table 6 summarises the characteristics of these cell lines.

By the formation of hybrids of the various mutant cell lines it has been found that at least eight complementation groups of radiosensitive cell lines exist (Thacker & Wilkinson 1991). Table 7 summarises the results of the complementation studies and seven of the complementation groups. All of the mutations were found to be recessive, except for that of irs1SF, which was semi-dominant.

It is possible to reverse the xrs mutant phenotype by treatment with 5-azacytidine (AC, Jeggo & Holliday 1986). AC is a DNA demethylating agent and has been known to reactivate (i.e. promote transcription) non-transcribed genes on the inactive X chromosome (Graves 1982, Mohandas et al 1981). Two theories have been proposed to explain AC reversion of the xrs mutant phenotype (figure 14):

1) The xrs gene is hemizygous and EMS treatment of the CHO cells to isolate the mutant cell lines (xrs 1-7) resulted in hypermethylation and prevention of transcription of the xrs gene. AC reactivates the xrs gene.

TABLE 6 - CHARACTERISTICS OF RADIOSENSITIVE CHINESE HAMSTER MUTANT CELL LINES

CELL LINE	PARENTAL LINE	ISOLATING MUTAGEN	MUTANT PHENOTYPE	DEFECT	REFERENCES
xrs 1	CHOK1	EMS	sensitive to X-rays, bleomycin, EMS, slightly sensitive to MMS	dsb repair	Jeggo & Kemp 1983 Kemp et al 1984
2			sensitive to X-rays, bleomycin, slightly sensitive to U.V.	dsb repair	
3			slightly sensitive to X-rays + U.V.	?	
4			sensitive to X-rays, bleomycin, EMS, slightly sensitive to U.V.	dsb repair	
5			sensitive to X-rays, bleomycin, EMS MMS, U.V., slightly sensitive to MNNG	dsb repair	
6					
7			sensitive to X-rays, bleomycin, EMS, MMS, U.V., MNNG	dsb repair	
EM9 EM7	AA8	EMS	sensitive to gamma rays + EMS	ssb repair	Thompson et al 1980, 1982
VC4	V79	ENU	very sensitive to U.V., bleomycin, X-rays, MMS + MMC	Radio-resistant DNA synthesis	Zdzienicka & Simons 1987 Zdzienicka et al 1988, 1989
VG8			sensitive to X-rays, bleomycin, MMS slightly sensitive to U.V.		
VE5			sensitive to bleomycin, MMS, MMC + X-rays, slightly sensitive to U.V.		
irs 1 2 3	V79-4	EMS & gamma rays	sensitive to MMC, EMS, U.V. + X-rays	irs1 radio-resistant DNA synthesis, all decreased fidelity in dsb repair	Thacker & Ganesh 1990 Jones et al 1987
XR-1	CHOK1	EMS & gamma rays	sensitive to ionising radiation + bleomycin	dsb repair	Stamato et al 1983
irs-1SF	AA8	ICR-191 & X-rays	sensitive to ionising radiation, EMS + U.V.	slow ssb repair deficient in X-ray induced repair replication	Fuller & Painter 1988
BLM-2	CHOK1	Bleomycin	very sensitive to bleomycin, slightly sensitive to ionising radiation, EMS + MMC	strand break repair	Robson et al 1985, 1988

TABLE 7 - GENETIC ANALYSIS OF CHINESE HAMSTER MUTANTS

a) Complementation studies (modified from Thacker & Wilkinson 1991)

	EM7	VE5	irs1	irs2	irs3	XR-1	irs1SF	BLM2
xrs1	+	+	+	+	+	+		+
EM7		+	+	+	+	+	+	+
VE5			+	-	+	+		
irs1				+	+	+	+	+
irs2					+	+	+	+
irs3						+	+	+
XR-1							+	

+ = complementing
- = non-complementing
If + or - is not shown the result was not stated in the literature

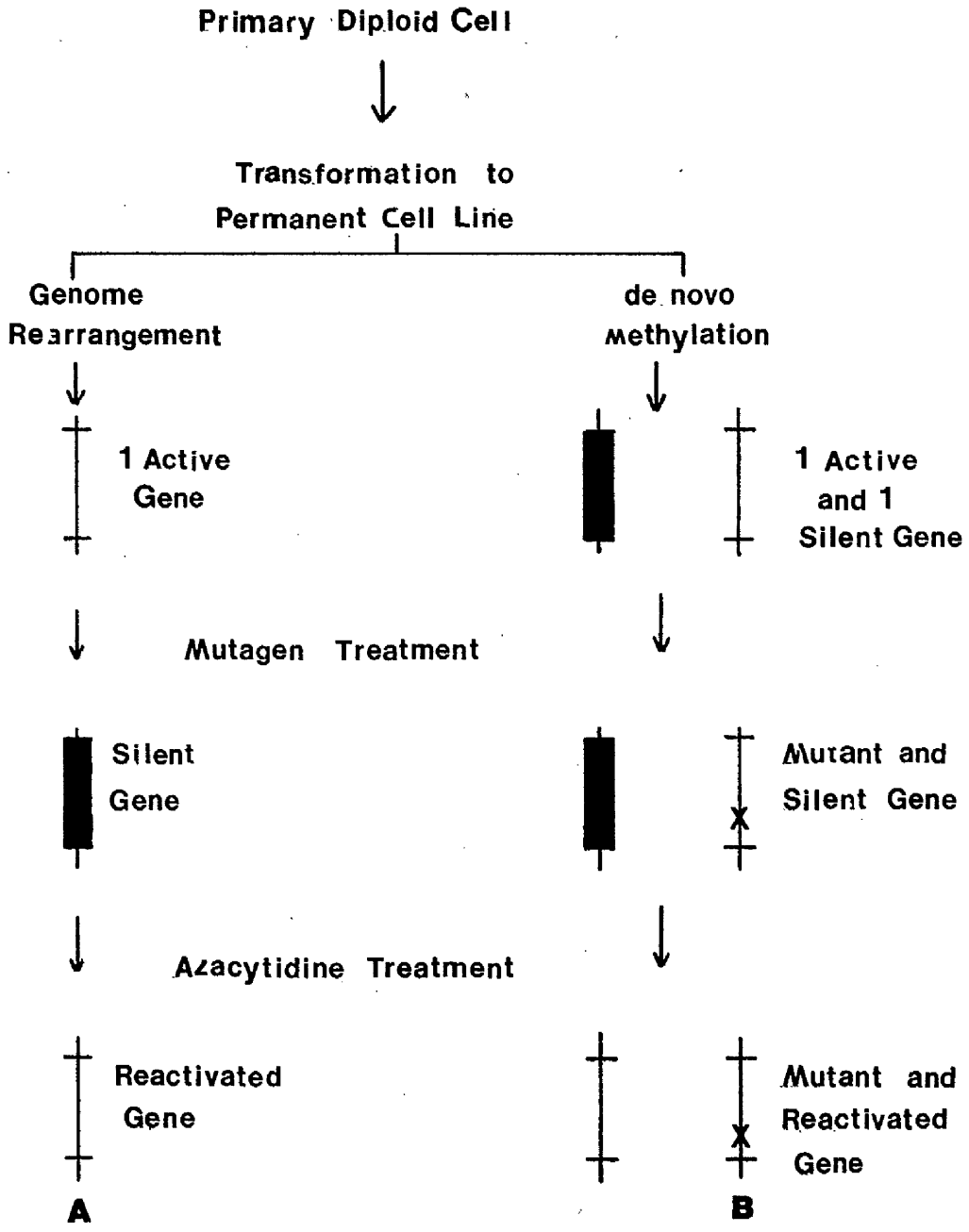
b) Summary of complementation studies

COMPLEMENTATION GROUP	CELL LINES
1	xrs1-7
2	irs1
3	irs2, VE5, VC4, VG8
4	irs3
5	EM7, EM9
6	BLM2
7	irs1SF

From the documented complementation studies the cell lines mentioned in table 6 form 7 complementation groups.

FIGURE 14 - Model for 5-azacytidine xrs gene activation

Possible mechanisms for the reacivation of the silent hypermethylated autosomal xrs gene by 5-azacytidine, assuming that the genome contains one (A) or two (B) copies of the gene (reproduced from Jeggo & Holliday 1986).



2) Two copies of the xrs gene are present in the CHO genome but only one is actively transcribed, the other is not transcribed due to methylation of the gene. The EMS treatment caused a mutation in the actively transcribed copy of the gene, which resulted in the xrs mutant phenotype. AC reactivates the non-mutated copy of the xrs gene.

The second theory is favoured since the phenotypes of the xrs mutants (which are all mutated in the same gene, Jeggo 1985) are not identical: they vary in their cross-sensitivity to DNA damaging agents (Jeggo & Kemp 1983) and in the rate at which they repair dsb (Kemp et al 1984).

1.9 Aims of the work of this thesis

Since ionising radiation produces a wide variety of lesions in DNA, it is difficult to determine whether specific types of lesions are toxic to cells. Studies *in vitro* have demonstrated that radiation-induced base damage (e.g TG, AP sites) can inhibit DNA synthesis and that certain lesions are mutagenic, but the contribution of base damage to radiation-induced cell death is not known. However, dsbs (possibly containing 3′ phosphate or phosphoglycolate termini) have been implicated as radiation-induced lethal lesions. To try to understand the role of base damage and breaks in cell death, genes encoding EIII or EIV were transfected into mammalian cells. By determining the effect of EIII expression on cell survival in a dsb repair deficient cell line and in

a cell line that was not particularly radiosensitive, after treatment with cytotoxic agents that produce thymine and AP modifications in different spatial distribution patterns in the DNA, the significance of base damage repair in relation to dsb repair was examined.

Since an assay was not available for the measurement of EIII-like activity in cell-free extracts, a further aim of this work was to develop a rapid, simple, specific assay for EIII.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Enzymes

Enzymes were stored at -20°C and used under the conditions stated by the manufacturers. Restriction endonucleases, DNA ligase and DNA polymerase I (Klenow fragment) were supplied by BRL-GIBCO and uracil DNA glycosylase was obtained from Boehringer Mannheim. T4 PNK and proteinase K were purchased from New England Biolabs and BDH, respectively and Taq DNA polymerase was supplied by Amersham International. RNase A (Sigma) was prepared at 10mg/ml in ddH_2O and heated to 100°C for 15 minutes before use.

2.1.2 Radiochemicals

All radiochemicals were obtained from NEN Dupont and stored at -20°C.

2.1.3 Chemicals for molecular biology

The formamide was obtained from BDH, while the nick-translation kit (stored at -20°C), TEMED, ammonium persulphate and the normal and LMP agarose were supplied by BRL-GIBCO. Agarose was prepared to 1% in TBE containing 0.5ug/ml ethidium bromide. Acrylamide solutions (30%, Protogel, and 40%, Acugel 40) were obtained from National Diagnostics and Hybond N and C were supplied by Amersham International. Work involving

TEAA (Applied Biosystems), acetonitrile (Rathburn Chemicals Ltd) or TFA (Sigma) was carried out in a fume cupboard, while wearing latex gloves. Protective clothing, i.e. gloves and safety glasses, were also worn when handling phenol (supplied by BDH), which after redistillation was equilibrated with water and stored at 4°C.

2.1.4 Separation products for molecular biology

Sephadex G50 and G25 (Pharmacia-LKB) was suspended in TE (see 2.1.12 viii), autoclaved and stored at 4°C. Double stranded (ds) DNA cellulose and ultragel A2 were obtained from Sigma and Pharmacia-LKB, respectively, and NENSORB^{TM} prep cartridges were supplied by NEN Dupont.

2.1.5 Oligonucleotides

Oligonucleotides were synthesized by Dr M Mackett on a Dupont Coder 3000 using standard phosphoramidite chemistry, which produced the oligonucleotide covalently bound to controlled porous glass beads. The sequences of the oligonucleotides used can be seen in figure 15.

2.1.6 Materials for OsO_4 oxidation of oligonucleotides

When preparing or using OsO_4 latex gloves and safety glasses were worn and all work was carried out in a fume cupboard. OsO_4 (BDH) was dissolved, by heating to 50°C, in deionized ddH_2O to produce a 10% (w/v) solution, which was stored in a sealed container at 4°C and used within three weeks of preparation. Piperidine was supplied by

FIGURE 15 - Oligonucleotide sequences

1 - Oligonucleotides complementary to *nth*

SENSE

5′ G G A T C C G A A T T C C T G A T G 3′

ANTISENSE

5′ C A C A A A G A T C T T C A A T A A T A C 3′

2 - Oligonucleotides complementary to *nfo*

SENSE

5′ C C A T G A A A T A C A T T G G A G C G C 3′

ANTISENSE

5′ G C G A A T G T T T T C T C G C A T T C G 3′

3 - Oligonucleotides complementary to *neo*

SENSE

5′ G G A G A G G C T A T T C G G C T A T G 3′

ANTISENSE

5′ G C C A A C G C T A T G T C C T G A T A 3′

4 - Oligonucleotides used to produce EIII substrate

A

5′ C A C C C G A A C A A C T T C A A C A C C C C 3′

B

5′ G G G G T G T T G A A G T T G T T C G G G T G 3′

BDH and pyridine by Hopkins and Williams Ltd.

2.1.7 Vectors

(i) Mammalian cell expression vectors

These vectors were designed to integrate into mammalian genomic DNA and express the selectable marker gene, e.g. *neo* or *hygromycin B*, and/or DNA sequences inserted at the unique cloning site (BamHI) situated within the transcriptional unit of pZipneoSV(X)1 and pLJ. pZipnth and pLJnfo were used to express EIII and EIV, respectively, in Chinese hamster cells (see chapters 3 and 5).

a) pZipneoSV(X)1

pZipneoSV(X)1 (10.2kb, Cepko et al, 1984) consists of the transcriptional unit of the Moloney murine leukaemia virus (Hoffman et al, 1982) ligated into pBR322 (see figure 16). The 5′ and 3′ LTR's of the transcriptional unit are required for the initiation of transcription and polyadenylation of transcripts, respectively, and for the integration of vector sequences into mammalian genomic DNA. Expression of the truncated *neo* gene results in cellular resistance to Geneticin (G418), allowing the selection and isolation of cells that had incorporated pZipneoSV(X)1 into their genome.

b) pZipnth

pZipnth (11.2kb) consists of the *E.coli nth* protein coding sequence (1kb) ligated into the unique BamHI site of pZipneoSV(X)1. Figure 16 shows the method of construction of pZipnth (produced by M.Skorvaga, PICR).

FIGURE 16 - Construction of pZipnth

Site directed mutagenesis was used to remove an out-of-frame translation initiation codon, which was situated 7 base pairs upstream of the *nth* translation initiation codon in pRPC53 (a), and to conveniently introduce an EcoRI restriction endonuclease site. Following EcoRI digestion the *nth* gene was ligated into pUCDUB, after which the construct was digested with BamHI to allow ligation of the *nth* gene into the BamHI site of pZipneoSV(X)1 (b). (Not all the restriction sites for the enzymes mentioned are shown on the diagram.)

a)

OUT-OF-FRAME TRANSLATION INITIATION CODON

nth TRANSLATION INITIATION CODON

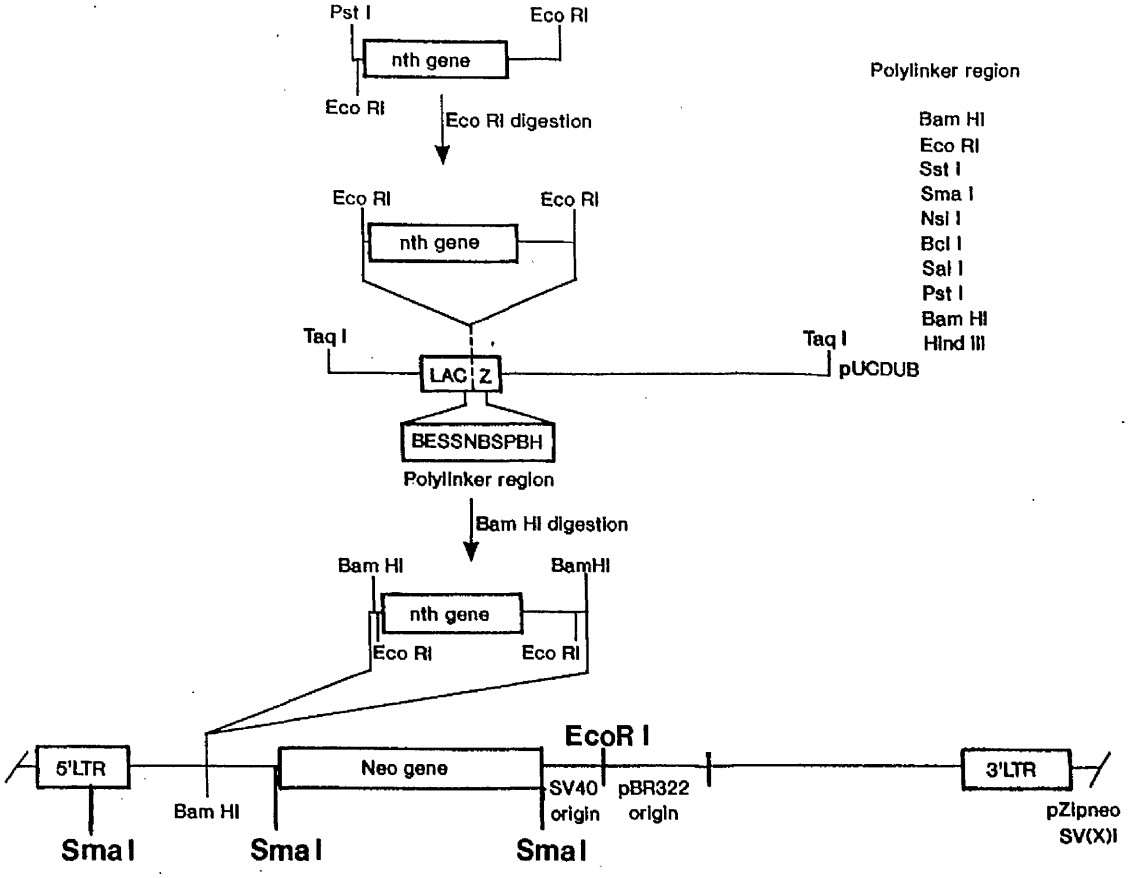
5′ A A C A G G G A A T G T C T G A T G A A T A A A 3′

SITE DIRECTED MUTAGENESIS

5′ A A C A G G G A A T T C C T G A T G A A T A A A 3′

EcoRI SITE CREATED

b)



c) pLJ

pLJ (9.2kb, obtained from Dr. Whitehead, MIT) contains the transcriptional unit of the Moloney murine leukaemia virus (see pZipneoSV(X)1 for details) ligated to sequences from the early region of the polyoma virus (figure 17).

d) pLJnfo

pLJnfo (10.1kb) consists of the *E.coli nfo* protein coding sequence (0.9kb) ligated into the unique BamHI site of pLJ (figure 17, for method of construction see chapter 5).

e)pMSGneo

pMSGneo (Analects 1987) was the source of the *neo* probe used in DNA analyses, but was designed for stable integration and inducible expression of genes in mammalian cells. The inserted gene is operated by the dexamethasone inducible promoter, which is part of the LTR of the Mouse Mammary Tumour Virus (MMTV), and SV40 polyadenylation signals. *Neo* is operated from an SV40 promoter and has a separate SV40 polyadenylation signal (figure 18).

f)pSV2hyg

This vector was cotransfected with pLJnfo into x7nth1 cells, which were already resistant to G418 (see chapter 3). pSV2hyg (5.3kb, figure 19) consists of the hygromycin B gene ligated into pSV2 and confers resistance to hygromycin in mammalian cells (Gritz & Davies 1983, Blochlinger & Diggelmann 1984). Co-transfection therefore allowed the selection of cells

FIGURE 17 - pLJ and pLJnfo

nfo was ligated into the BamHI cloning site of pLJ to produce pLJnfo. pLJnfo contains the 5′ and 3′ LTR of the Moloney murine leukemia virus (MoMLV), which provides the promoter and polyadenylation signals for *nfo*. SV40 sequences provide the promoter for *neo*, which confers resistance to G418 sulphate in mammalian cells. Sequences other than the transcriptional unit are from the early region of the polyoma virus. For details of pLJnfo construction and endonuclease restriction sites see figure 61.

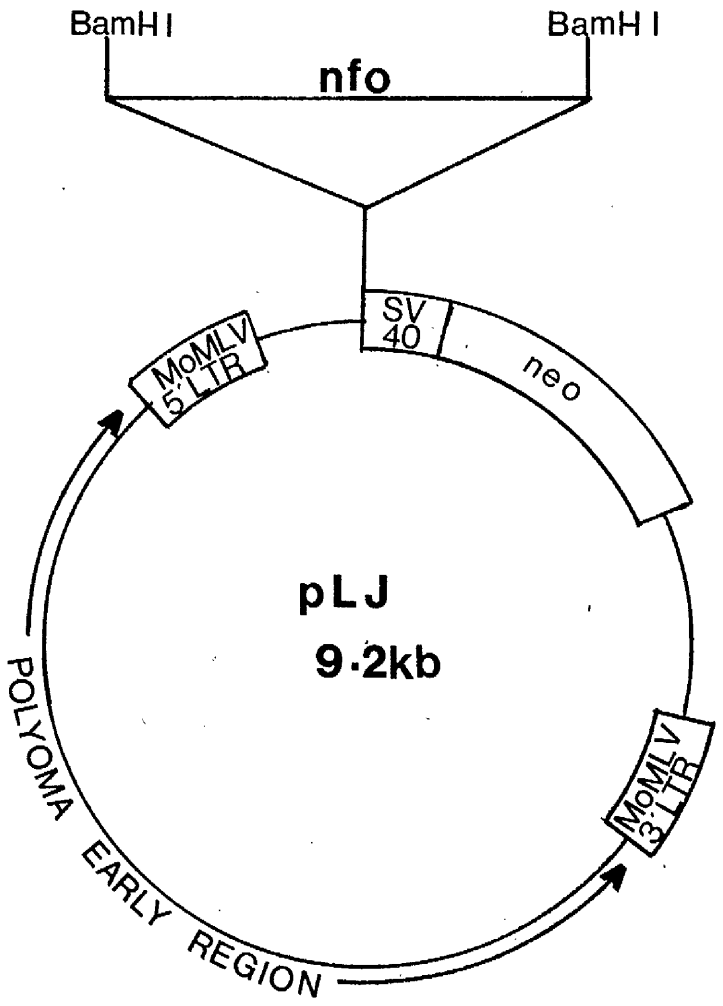


FIGURE 18 - pMSGneo

pMSGneo contains the gene encoding resistance to ampicillin (amp) and *neo*, which confers resistance to G418 in mammalian cells and is operated by SV40 promoter and polyadenylation signals. A unique EcoRI site allows the insertion of gene sequences into pMSGneo and subsequent expression of these sequences in mammalian cells. (Not all the sites are shown for the restriction endonucleases mentioned.)

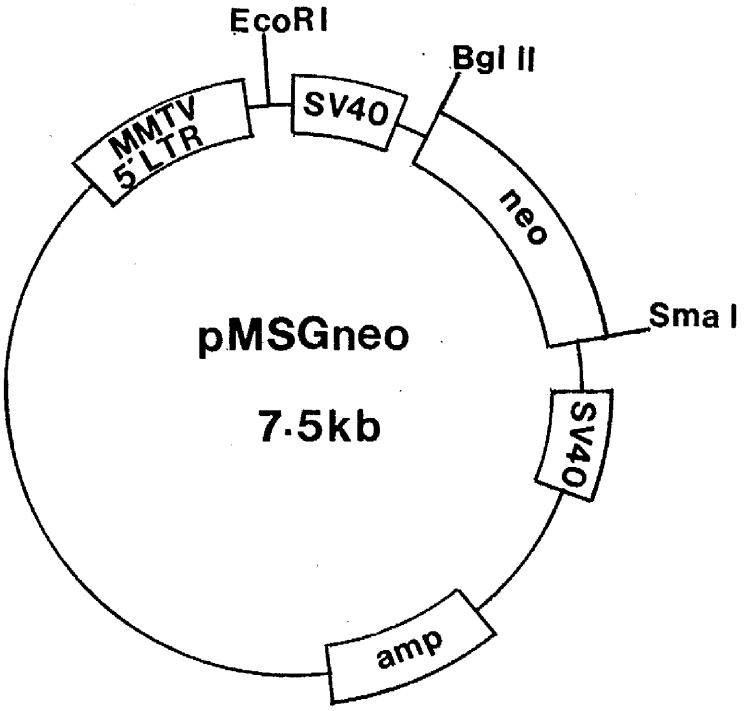
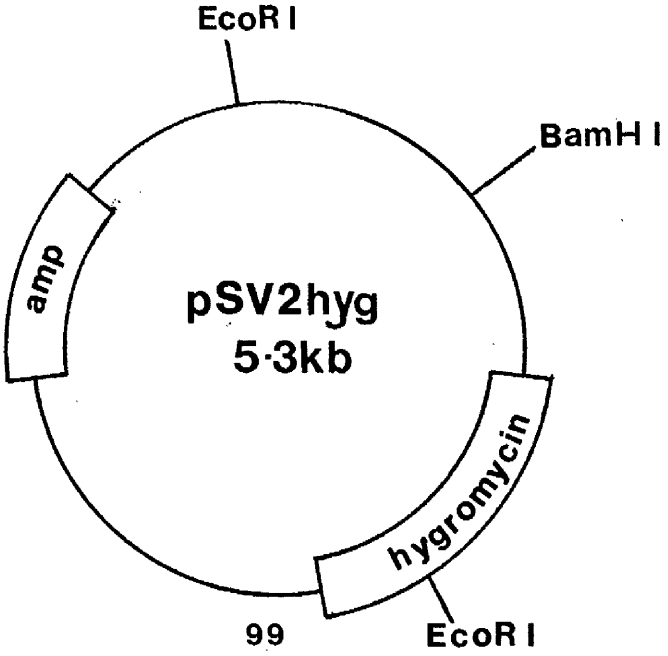


FIGURE 19 - pSV2hyg

pSV2hyg contains the gene encoding resistance to ampicillin (amp) and the gene conferring resistance to hygromycin B (hygromycin) in mammalian cells.



that had incorporated pSV2hyg and possibly pLJnfo.

(ii) Bacterial expression vectors

a) pUC8.1nth

A 1kb DNA fragment containing the *nth* protein coding sequence was ligated into the EcoRI site of the multiple cloning region (polylinker) of pUC8.1 (figure 20), which is a derivative of pUC8. These vectors contain a truncated *lac* gene, that encodes beta galactosidase, and the polylinker is situated downstream of the *lac* translation initiation codon. Insertion of DNA fragments into a restriction site within the polylinker prevents expression of a functionally active beta galactosidase. When host bacteria are grown in the presence of IPTG (an inducer) and X-gal (an indicator dye) only those containing recombinant plasmid produce white colonies, bacteria actively expressing the *lac* gene form blue colonies.

pUC8.1nth was used to isolate the *nth* probe, which was used in dot blot, Southern and northern analyses (see later).

b)pUCDUB

This vector (2.5kb) is a derivative of pUC19 and was provided by Dr G. Clark. The polylinker was removed from pUC19 and replaced with a ds oligonucleotide, containing the restriction sites shown in figure 21, to generate a different multiple cloning region. pUCDUB and pUC19 are similar to pUC8, although these vectors differ at the polylinker region. The truncated *nfo* gene was ligated into

FIGURE 20 - pUC8.1nth

EcoRI digestion of pUC8.1nth was used to isolate the *nth* probe, which was used in the DNA analyses of pZipneoSV(X)1, pZipnth, pLJ or pLJnfo transfected cell lines.

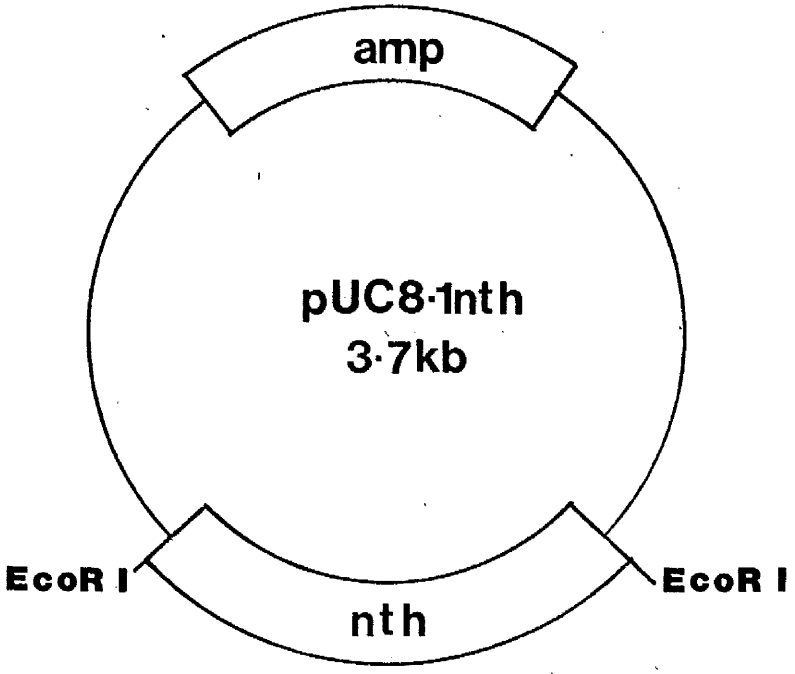
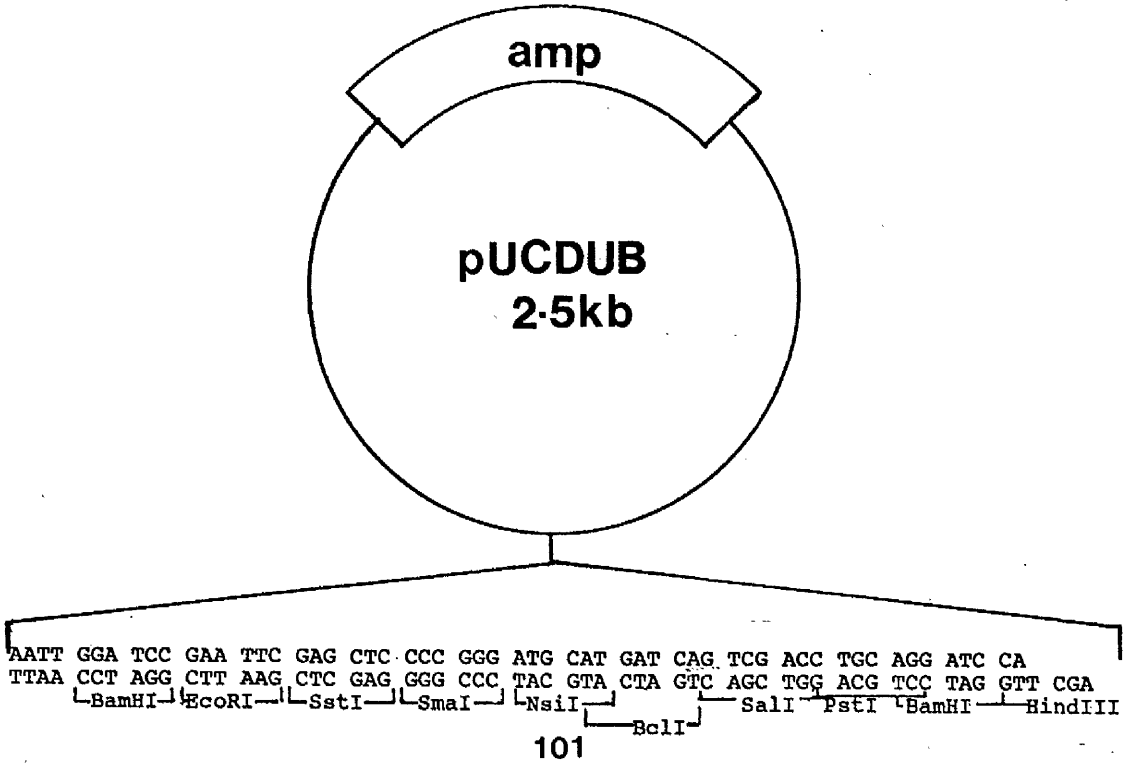


FIGURE 21 - pUCDUB

pUCDUB is a derivative of pUC19 and contains the gene encoding resistance to ampicillin (amp). The sequence of the multiple cloning site is shown.



pUCDUB (see chapter 5), from which *nfo* with BamHI termini was isolated. This allowed *nfo* to be ligated into the BamHI site of pLJ.

c) pRPC53

A 2kb fragment of *E.coli* DNA containing the *nth* gene (Cunningham & Weiss 1985) was cloned into pSP65 to produce pRPC53 (provided by Dr R.P.Cunningham, SUNY Albany, USA).

d) pRPC124

The *nfo* gene was subcloned from a ColE1 hybrid plasmid (pLC38-27) from the Clarke-Carbon collection and localized to a 3.9kb fragment on a plasmid, pWB21. After removal of the BamHI-HindIII fragment from pBR322, a 1.2kb fragment of pWB21 was ligated into the BamHI-HindIII sites of pBR322 to produce pRPC124 (Saporito & Cunningham 1988).

2.1.8 Cell culture media and materials

Plastic cell culture dishes and flasks were supplied by Falcon. All solutions were filter-sterilized using Falcon 0.22um filters. G418 (BRL-GIBCO) and L-glutamine (Sigma) were prepared with ddH_2O as 100mg/ml and 200mM stock solutions, respectively, and were stored at -20°C. 0.05% (w/v) trypsin (Sigma) and 0.02% (w/v) EDTA (Sigma) were dissolved in PBS (phosphate buffered saline) and stored at -20°C. Sterile PBS was supplied by central washing services.

Chinese hamster V79 lung fibroblast and Chinese hamster ovary cells were grown in MEM (minimal essential

medium) containing 10% FCS (foetal calf serum).

MEM + 10% FCS: 767ml ddH_2O

100ml FCS (Sigma)

10ml 200mM L-glutamine

27ml 7.5% (w/v) sodium bicarbonate (BRL-GIBCO)

100ml 10 x concentration MEM (BRL-GIBCO)

This was stored at 4°C.

2.1.9 Bacterial Culture media and materials

(i) Media

Bactotryptone and yeast extract were obtained from Difco. LB and TYM were autoclaved and stored at room temperature.

LB : 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract 0.17M NaCl, pH 7.5

TYM : 2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract 0.1M NaCl, 10mM $MgSO_4$

M9 : 0.8% (w/v) glucose, 90mM Na_2HPO_4, 22mM KH_2PO_4, 8.5mM NaCl, 19mM NH_4Cl

A 20% glucose solution was used to prepare M9 and was filter sterilized with a 0.2um acrodisc filter (Gelman), while a solution containing the salts in M9 was prepared as a 5 x stock solution and was autoclaved.

Supplements of 40ug/ml thymidine, 50ug/ml thymine, 1ug/ml thiamine, 20ug/ml tryptophan and 0.001M $MgSO_4$ were used. The 20mg/ml stock solutions of thiamine and tryptophan, the 3mg/ml stock solution of thymine and

0.01M $MgSO_4$ were filter sterilised.

Top agar : 0.7% (w/v) bacto-agar

Bottom agar: 1.5% (w/v) bacto-agar

The bacto-agar was dissolved in LB and autoclaved. LB agar plates, required for bacterial growth, were prepared using bottom agar and were stored at 4°C.

(ii) Antibiotics

Ampicillin (5mg/ml (w/v) in 50% methanol), chloramphenicol (34mg/ml (w/v) in ethanol) and kanamycin (10mg/ml (w/v) in ddH_2O) were filter-sterilised as above and stored at -20°C.

(iii) Bacterial strains

E.coli strains HB101 and DH5α (supplied by Dept. Carcinogenesis, PICR) were used in the preparation of competent bacteria (see 2.2.1vii), while XA103 was used to prepare stock solutions of T4U phage particles, and BW313 (uracil DNA glycosylase deficient) was used in the production [^{3}H] uracil-containing phage DNA. XA103, BW313, T4U phage and MM294 (transformed with pRPC124 or pRPC53) were provided by Dr R.P. Cunningham. MM294 required 40ug/ml thymidine in LB for growth.

2.1.10 Cytotoxic agents

Bleomycin sulphate (Lundbeck Ltd) was obtained from the NHS pharmacy and dissolved at 5mg/ml in ddH_2O: aliquots were stored at -20°C. Hydrogen peroxide (BDH) was obtained as a 30% solution and diluted to the appropriate concentration immediately before use. The 30% solution was stored at 4°C.

2.1.11 Anti-thymidine glycol antibody

The anti-thymidine glycol (anti-TdG) monoclonal antibody was provided by Dr. Osamu Mikaido and was raised against OsO_4 treated poly(dT). The antibody was stored at 4°C.

2.1.12 Buffers and reagents

The buffers described are 1 x "working" concentration, and were prepared as 10 x stock solutions and stored at room temperature unless otherwise stated.

(i) Electrophoresis

MOPS : 20mM 3-(N-morpholino) propane-sulphonic acid (MPS), 5mM NaAc, 1mM EDTA, pH 8

TBE : 90mM boric acid, 1mM EDTA, 90mM Tris-HCl, pH 8.3

SDS PAGE : 50mM Tris, 192mM glycine, 0.1% (w/v) SDS

Electroblot: 25mM Tris, 192mM glycine, 20% methanol (The Tris-glycine 10 x stock solution was diluted to 1 x with methanol and ddH_20).

(ii) Loading Buffers

Agarose electrophoresis : 4% (w/v) sucrose, 10mM EDTA, 0.025% (w/v) bromophenol blue.

SDS PAGE : 63mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue (Prepared as a 5 x stock solution).

UPAGE : 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue, 20mM EDTA in 96% formamide.
(Prepared as a 2 x stock solution).

(iii) Chromatography buffers

Buffers were prepared as 1 x stock solutions.

Buffer I : 50mM Tris-HCl, pH 8.3, 1mM EDTA, 3mM DTT
This was stored at 4°C.

TES : 10mM Tris-HCl, pH 8, 1mM EDTA, 0.1% (w/v) SDS.
The Tris-EDTA solution was autoclaved before the addition of SDS.

(iv) Plasmid extraction solutions

Buffers were prepared as 1 x stock solutions.

CA : 75mM NaCl, 50mM NaAc, pH4 (autoclaved)

P1 : 50mM glucose, 25mM Tris-HCl, 25mM EDTA, 5mg/ml lysozyme (Sigma, added immediately before use)

P2 : 0.2M NaOH, 1% (w/v) SDS
(Prepared immediately before use)

P3 : 3mM NaAc, pH 4.8 (autoclaved)

P4 : 3M KAc, pH 4.5 (autoclaved)

(The appropriate pH of P3 and P4 was obtained by the addition of glacial acetic acid).

(v) Phage preparation solutions

Phage particles were prepared using the QIAGEN midi

Lambda DNA preparation kit.

SM : 50mM Tris-HCl, pH 7.5, 0.01% (w/v) gelatin 0.1M NaCl, 8mM $MgSO_4$ (Autoclaved)

L1 : 20mg / ml RNase A, 6mg /ml DNase I, 0.2mg/ml BSA, 10mM EDTA, 100mM Tris-HCl, pH 7.5, 300mM NaCl (stored at 4°C)

L2 : 30% (v/v) polyethylene glycol (PEG 6000), 3M NaCl (stored at 4°C)

L3 : 100mM Tris-HCl, pH 7.5, 100mM NaCl, 25mM EDTA

L4 : 4% (w/v) SDS

L5 : 2.55M KAc, pH 4.8

QBT : 750mM NaCl, 50mM MPS, 15% (v/v) ethanol, 0.15% (v/v) Triton X-100, pH 7

QC : 1M NaCl, 50mM MPS, 15% (v/v) ethanol, pH 7

QF : 1.25M NaCl, 50mM MPS, 15% (v/v) ethanol, pH 8.2

(vi) Solutions for the preparation of competent bacteria

Buffers prepared as 1 x stock solutions and autoclaved.

TFB1 : 30mM KAc, 50mM $MnCl_2$, 100mM KCl, 10mM $CaCl_2$, 15% (v/v) glycerol

TFB2 : 10mM NaMPS, 75mM $CaCl_2$, 10mM KCl, 15% (v/v) glycerol, pH 7

(vii) Reaction buffers

Unless stated otherwise 10 x buffer solutions were

stored at -20°C.

Ligation buffer	: 50mM Tris-HCl, pH 7.6, 10mM $MgCl_2$, 10mM DTT, 50ug/ml BSA
T4 PNK	: 70mM Tris-HCl, pH7.6, 10mM $MgCl_2$, 1mM DTT
EIII reaction buffer	: 50mM Tris-HCl, pH 7.6, 100mM KCl, 1mM EDTA, 1mM DTT, 0.1mg/ml BSA (Prepared immediately before use. Stock solutions of 1M DTT and 10mg/ml BSA were stored at -20°C).
EIV reaction buffer	: 50mM Hepes-KOH, pH 8.2, 1mM EDTA, 1mM DTT, 0.1mg/ml BSA (Prepared immediately before use. Stock solutions of 1M DTT and 10mg/ml BSA were stored at -20°C).
Uracil DNA Glycosylase Reaction buffer	: 60mM Tris-HCl, pH 8, 1mM EDTA, 1mM DTT 0.1mg/ml BSA
PCR Reaction buffer	: 50mM KCl, 10mM Tris-HCl, pH 8.3, 2.5mM $MgCl_2$, 0.1mg/ml (w/v) gelatin, 0.45% (v/v) NP40, 0.45% (v/v) Tween 20

One times buffer containing 0.06mg/ml proteinase K was used to prepare the cell extracts for PCR analysis. The cell extract buffer was prepared immediately before use.

(viii) Buffers for the preparation of mammalian genomic DNA

Buffers were autoclaved.

Homogenization buffer	: 0.05M EDTA, 0.05M Tris-HCl, pH 8 (Prepared as 1 x solution)

TE : 10mM Tris-HCl, pH 8, 1mM EDTA

(ix) Hybridization buffers

Blotto : 0.25% (w/v) Cadbury's Marvel (Gateway, Withington, prepared as 1 x solution)

SSC : 0.15M NaCl, 15mM Na citrate, pH 7 (Prepared as a 20 x stock solution).

FBS : 50% (v/v) formamide, 6 x SSC, 0.25% (w/v) Cadbury's Marvel, 0.1% (w/v) SDS (Prepared as 1 x solution)

(x) RNA isolation buffer

All solutions were prepared as 1 x solutions, filtered through nitrocellulose and stored in plastic containers at room temperature, unless stated otherwise.

Tris-saline : 25mM Tris-HCl, pH 7.4, 130mM NaCl, 5mM KCl

NDD buffer : 1% (v/v) Nonidet P40 (Sigma), 0.5% (w/v) sodium deoxycholate (Sigma), 0.01% (w/v) dextran sulphate (Prepared using Tris-saline. The Nonidet P40 was added after filtration).

DEPC H_2O : 1ml DEPC, 999ml ddH_2O After 30 minutes this was autoclaved.

(xi) Stains

Colony-forming assay : 1% (w/v) Gentian violet 2B (BDH), 70% ethanol, 5% formaldehyde

Chromosome preparations	: 2% (v/v) Giemsa, 16mM NaH_2PO_4, 50mM KH_2PO_4. pH 6.4
Protein gels	: 0.1% (w/v) coomassie blue G250 (Sigma) 30% (v/v) methanol, 10% (v/v) acetic acid

2.2 METHODS

2.2.1 Molecular Biology

(i) Small scale plasmid preparation from bacteria

The method of Zasloff et al (1978) was used. The appropriate antibiotic (e.g. ampicillin 100 ug/ml or kanamycin 50 ug/ml) was added to 5ml LB in a sterile universal tube. This was innoculated with 10ul of the bacterial frozen stock and incubated overnight at 37°C in an orbital shaker (275 rpm). Addition of the antibiotic ensured that the bacteria retained the plasmid during growth.

A 1.5ml aliquot of the culture was centrifuged for 15 seconds at 13,000 rpm. The cells were resuspended in 100ul of solution P1 (2.1.12iv) and incubated at room temperature for 10 minutes. To this 200ul of P2 (2.1.12iv) were added and the solution mixed thoroughly. After 5 minutes at 0°C, 150ul P3 (2.1.12iv) were added, the solution mixed by inversion and placed at 0°C for 10 minutes. The sample was centrifuged at 12,000 rpm and 0°C for 5 minutes and the supernatant transferred to a new eppendorf tube. To this 2 volumes of cold ethanol were added and the sample placed at -20°C for 1 hour. Following centrifugation (at 12,000 rpm and 0°C for 15

minutes), the plasmid DNA was washed with 70% (v/v) ethanol, dried and resuspended in 30ul TE. Plasmid DNA was stored at -20°C.

(ii) Large scale plasmid preparation from bacteria

An overnight culture was grown (see 2.2.1.i) and used to innoculate 500ml LB, containing 100ug/ml ampicillin. This was placed at 37°C in an orbital shaker (275 rpm) and when the absorbance of the culture at 550nm reached 0.8, chloramphenicol was added to a final concentration of 170ug/ml, to amplify the plasmid. The culture was grown overnight and harvested by centrifugation (5000 rpm, 10 minutes). The bacteria were resuspended in 10ml of P1 (2.1.12iv) and after the addition of 20ml of P2 (2.1.12iv), the suspension was placed at 0°C for 5 minutes. To this 15ml of P4 (2.1.12iv) were added, the sample mixed thoroughly and placed at 0°C for 15 minutes. After centrifugation (10,000 rpm, 14°C for 30minutes) the supernatant was transferred to a new tube and the nucleic acids precipitated by the addition of 0.8 volumes of isopropanol. After 5 minutes at room temperature the solution was centrifuged at 5000rpm for 20 minutes and the pellet resuspended in 1.4ml TE. To this 100ul ethidium bromide (10mg/ml) and 13.5ml caesium chloride (1g/ml) were added and the solution transferred to a bell-top quick seal centrifuge tube (Beckman). Centrifugation at 40,000 rpm, 20°C for 16 hours separated the RNA and DNA.

The plasmid DNA formed a band in the caesium chloride

gradient, which appeared red due to the ethidium bromide bound to the DNA. This was collected using a syringe and needle, after the top of the tube had been pierced using a separate needle (see Sambrook et al 1989). To extract the ethidium bromide from the DNA, the solution was transferred to a 15ml tube, 3 volumes of ddH_2O were added and the DNA precipitated (-20°C, 30 minutes) using 2 volumes of cold ethanol. The DNA was isolated by centrifugation (10,000 rpm, 30 minutes) and resuspended in 500ul of ddH_2O. Reprecipitation (-20°C, 20 minutes), by the addition of 0.02 volumes of 5M NaCl and 2 volumes of ethanol, was carried out twice. After centrifugation (10,000 rpm, 20 minutes) the DNA was washed with 70% (v/v) ethanol, dried and resuspended in 200ul ddH_2O. Plasmid DNA was stored at -20°C.

(iii) Restriction endonuclease analysis

Digestion of DNA (2ug) was carried out in a total volume of 30ul containing the appropriate buffer (supplied by the manufacturers) and restriction endonuclease (approximately 10 units). The reaction was incubated at 37°C for 2 hours, after which 0.1 volumes of 10 x loading buffer (2.1.12 ii) were added and 15ul were subjected to electrophoresis in a 1% agarose gel. Electrophoresis was initially at 100V and this was decreased to 30V after 5 minutes. When the bromophenol blue had moved 3/4 of the length of the gel, the gel was examined on a U.V. transilluminator (302nm).

(iv) Isolation of a DNA fragment from LMP agarose

The DNA was digested, as described in 2.2.1 iii, and subjected to electrophoresis in a 1% LMP agarose gel. Following electrophoresis, the region of the gel containing the required fragment was cut out using a scalpel and placed in an eppendorf tube. To this 0.1 volumes of 1M NaCl were added and the gel heated to 65°C, until molten. Phenol was equilibrated with 0.1M NaCl in TBE (LMP phenol) and 0.75 volumes LMP phenol were added to the molten gel. After mixing vigorously the sample was centrifuged at 13,000 rpm for 5 minutes and 0.75 volumes of LMP phenol were added to the aqueous phase and the extraction repeated. The aqueous phase was then extracted with 2 volumes of butanol until the volume was reduced to 50ul. To this 2 volumes of cold ethanol were added and precipitation of DNA was carried out at -20°C for 1 hour. After centrifugation (18,000 rpm, 0°C for 15 minutes) the DNA was dissolved in 50ul H_2O and reprecipitated with 0.3M NaAc and 2 volumes of cold ethanol. This was centrifuged (18,000 rpm, 0°C for 15 minutes) and the DNA washed extensively with 70% (v/v) ethanol, dried and redissolved in 20ul TE. The DNA was stored at -20°C.

(v) Production of a "blunt-ended" DNA fragment

The reaction was carried out in 40ul and contained 34ul of the XhoII-PleI digest of the *nfo* 0.9kb fragment (approximately 100ng), 1 unit of DNA polymerase I (Klenow fragment), 5mM $MgCl_2$ and 0.05mM deoxynucleotide

triphosphates (dGTP, dTTP, dATP and dCTP). This was incubated at room temperature for 15 minutes and the DNA polymerase I was then inactivated by heating the reaction to 75°C for 10 minutes.

(vi) Ligation of a DNA fragment and linearised plasmid DNA

A ratio of 3:1 of fragment termini: plasmid termini was required to ensure that the ligation reaction was efficient. Appropriate amounts of the plasmid and DNA fragment (e.g. 28ng pUCDUB {2.5kb} and 28ng DNA {0.9kb} containing the truncated *nfo* gene) were mixed in a 15ul volume of 1 x ligation buffer (see 2.1.12 viii) with 1 unit of T4 DNA ligase. A 15ul control reaction, containing the plasmid DNA, ligation buffer and T4 DNA ligase, was also carried out. The reactions were incubated at room temperature overnight and then stored at -20°C.

(vii) Preparation of competent bacteria

A sterile loop was used to innoculate an LB agar plate with approximately 10ul of the frozen bacterial stock, which was incubated overnight at 37°C. One colony from the plate was used to innoculate 5ml of TYM and this was grown overnight at 37°C in an orbital shaker (275 rpm). The 5ml culture was then used to innoculate 500ml of TYM, which was placed at 37°C in an orbital shaker until the OD_{600} reached 0.6 (i.e. 10^8 bacteria/ml). The culture was cooled, by placing the flask on ice, and centrifuged in sterile tubes at 4000 rpm for 15 minutes

at 4°C. The bacteria were resuspended gently in 33ml of cold TFB1 (2.1.12 vi), centrifuged at 3000 rpm for 10 minutes at 4°C and resuspended in 20ml of cold TFB2 (2.1.12vi). Aliquots (500ul) of the bacterial suspension were pipetted into chilled eppendorf tubes, rapidly frozen by immersing the tubes in methanol containing solid carbon dioxide and stored at -80°C.

(viii) Transformation of competent bacteria

An aliquot of frozen competent cells (prepared as in 2.2.1 vii) was defrosted and 200ul pipetted into a transformation tube, which had been placed on ice. To this 2ul of the ligation reaction (approximately 4ng of the construct, see 2.2.1 vi) were added and the bacteria kept on ice for 30 minutes. Control transformations were carried out using 4ng of circular parent plasmid and 2ul of the control ligation reaction (see 2.2.1 vi). The samples were incubated at 37°C for 5 minutes, diluted 1:5 with LB and allowed to grow for 90 minutes at 37°C in an orbital shaker (275 rpm). Aliquots of each culture (between 50 and 200ul) were then spread over the surface of LB agar plates, containing the appropriate antibiotic for the selection of transformed bacteria. The plates were dried and placed at 37°C.

(ix) Isolation of genomic DNA from mammalian cells

Approximately 10^8 cells were trypsinized (2.2.2 ii) and harvested. After washing with PBS the cells were resuspended in 4mls of homogenization buffer (2.1.12

viii) and placed in a Teflon/glass Potter Elvehjem homogeniser. After five passes, the suspension was transferred to a Sarstedt tube and 40ul 10% (w/v) SDS and 40ul RNase A (10mg/ml) were added. The solution was incubated at 37°C for 30 minutes and 20ul proteinase K (20mg/ml) were added before the solution was incubated at 37°C overnight.

The samples were extracted with 4mls of phenol (equilibrated to pH 8 with 1M Tris-HCl) and centrifuged at 10,000 rpm for 15 minutes at room temperature. The aqueous layer was extracted with an equal volume of chloroform: butanol (25:1 v/v). Following centrifugation (10,000 rpm, 15 minutes), the aqueous layer was removed and 2 volumes of cold ethanol added. Gentle mixing precipitated the DNA, which was transferred to an eppendorf tube using a sterile, disposable innoculating loop. The DNA was washed exten-sively with 70% (v/v) ethanol, dried and resuspended in 1ml TE. The DNA concentration was determined by absorbance at 260nm (1 E_{260} = 50ug/ml). DNA in TE was stored at 4°C.

(x) Isolation of RNA from mammalian cells

RNA was isolated from mammalian cells as described by Wilkinson (1988). Approximately 4 x 10^6 cells were trypsinized (see 2.2.2ii) and harvested. After washing with PBS the cells were resuspended in 1ml cold Tris-saline (2.1.12 x) and transferred to a 2ml glass homogeniser, which had previously been treated with Decon 90 to remove ribonucleases and rinsed with DEPC water.

Following homogenization, the suspension was transferred to a 1.5ml ependorf tube and centrifuged at 6000 rpm for 30 seconds at 4°C. The pellet was resuspended in 400ul cold Tris-saline, to which 100ul cold NDD buffer were added. The tube was inverted 10 times and centrifuged at 6000 rpm for 30 seconds at 4°C. The supernatant was transferred to a tube containing 25ul 20% (w/v) SDS and 15ul 5M NaCl, which had been filtered through nitrocellulose, and 500ul of (1:1 v/v) phenol:chloroform were added, (the phenol had previously been equilibrated to pH8 with 1M Tris-HCl). After mixing vigorously, the sample was centrifuged (13,000 rpm, room temperature, 2 minutes) and extracted twice with phenol-chloroform (1:1, v/v). The final aqueous layer containing the RNA was extracted once with chloroform: isoamylalchol (24:1, v/v) and transferred to a new tube. The RNA was precipitated with 2 volumes of ethanol at -20°C for 30 minutes. After centrifugation (16,000 rpm, 20 minute, 0°C), the RNA was washed with 80% (v/v) ethanol and dried. It was then redissolved in 50ul DEPC water and used immediately. To store the RNA, it was reprecipitated using 0.3M NaAc and 2 volumes of cold ethanol and kept at -70°C.

(xi) Preparation of cell extracts for PCR analysis

Extracts to be used in polymerase chain reactions (PCR) were prepared as described in Higuchi (1989). Cells were harvested by trypsinization (see 2.2.2 ii), washed twice with 10ml PBS and counted using a Coulter counter. After centrifugation (1,500 rpm, 5 minutes)

they were resuspended in PCR buffer containing 0.06mg/ml proteinase K (2.1.12 vii) at a cell density of 6 x 10^6/ml and incubated at 55°C for 1 hour. Cell extracts were then heated to 90°C for 5 minutes to inactivate the proteinase K and were stored at -20°C.

(xii) PCR analysis

PCR was used for the detection of specific DNA sequences (*nth* or *nfo*) in cell extracts and/or genomic DNA and required two oligonucleotide primers derived from the 5′ and 3′ regions of the DNA of interest (Saiki et al 1985, 1988).

Reactions were carried out in 50ul of PCR buffer (2.1.12 vii) and contained 1ug mammalian DNA (or 25ul of cell extract, prepared as described in 2.2.1 xi), 100ng of each of the appropriate sense and antisense oligonucleotides (see figure 15), 100uM of dGTP, dATP, dCTP and dTTP and 1 unit of Taq DNA polymerase. After vortexing, 50ul of mineral oil was added and the reactions centrifuged for 30 seconds at 13,000 rpm. They were then transferred to a PCR machine (M.J. Research Inc.) and a programme incorporating the following steps was used to amplify and hence detect *nth* or *nfo* DNA sequences:

1 93°C for 1.5 minutes

2 49°C for *nth*, or 57°C for *nfo* oligonucleotides, for 2 minutes

3 72°C for 3 minutes

The temperature of step 2 (during which the oligo-

nucleotides anneal to the target sequence) is 5°C below the Tm [2°C(A + T) + 4°C(G + C)] of the oligonucleotides.

After 30 cycles, 400ul of chloroform were added to each reaction to remove the mineral oil from the PCR products and samples were vortexed before centrifugation for 1 minute at 13,000 rpm. The aqueous layer was transferred to a new eppendorf tube and extracted with an equal volume of water-saturated ether to remove any remaining chloroform. A 5ul aliquot of the aqueous layer was subjected to electrophoresis in a 1% agarose gel and the PCR products examined using a U.V. transilluminator (302nm).

(xiii) ^{32}P labelling of DNA

a)"Nick-translation"

The reaction was usually carried out in a total volume of 50ul and consisted of DNA polymerase I (1.2 units), the DNA to be labelled (usually 50ng), 5ul (50uCi) [^{32}P] dCTP (3000 Ci/mmole) and 5ul of buffer, which contained 0.2mM of each of dGTP, dTTP and dATP. It was incubated at 15°C for 1 hour, after which time 100ul of TE were added. A G50 sephadex column ("spun column", volume 1cm^3) was prepared (see Sambrook et al 1989) and approximately 20ug herring sperm DNA dissolved in TE were added to block non-specific DNA binding sites. After centrifugation at 400rpm (MSE coolspin) for 10 minutes, the reaction mix was applied to the column and the [^{32}P] labelled DNA was recovered by centrifugation at 800 rpm for 6 minutes. This step

removed unincorporated [^{32}P] dCTP.

The labelled DNA was denatured by heating to 95°C for 10 minutes.

b) 5' labelling of oligonucleotides

The reaction (10ul) contained 50ng of oligonucleotide, T4 PNK (10 units), 1ul 10 x T4 PNK buffer (2.1.12vii) and 1ul (10uCi) [^{32}P] ATP (6000 Ci/mmole) and was incubated at 37°C for 1 hour. Unincorporated [^{32}P] ATP was removed by the use of a G25 sephadex 1cm^3 "spun column" (see above).

(xiv) Preparation of filters for DNA and RNA analyses

a) DNA dot blot analysis

The required size of Hybond N (nylon filter) was soaked in 2 x SSC (2.1.12ix) and placed in position in the dot blot apparatus. DNA solutions were diluted with 2 x SSC to obtain samples containing 1ug of DNA in 100ul. These were pipetted into the wells of the dot blot apparatus and the vacuum applied. The vacuum was disconnected when the DNA solution had been "pulled" onto the filter. The filter was then layed on the surface of denaturing solution (0.5M NaOH, 1.5M NaCl) for 1 minute and neutralising solution (0.5M Tris-HCl, 3M NaCl pH 7.5) for 1 minute. After drying, it was wrapped in Saran wrap and irradiated with U.V. (302nm) light for 5 minutes, using a U.V. trans-illuminator. The side of the nylon to which the DNA or RNA had been applied was placed against the transilluminator. The filter was stored at 4°C and hybridization with [^{32}P] labelled DNA was carried out as

described in 2.2.1xva.

b) RNA dot blot analysis

The negative (RNA-free) controls (produced by incubating 2ug RNA with 1 ul of RNase A at room temperature for 10 minutes) and the positive samples (2ug RNA) were made up to a volume of 50ul with 20 x SSC and heated to 65°C to denature any 2° structure. These were pipetted into the wells of the dot blot apparatus (assembled as above) and the RNA solution "pulled" onto the filter. The RNA was bound to the nylon and the filter was stored as described above. Hybridization of [^{32}P] labelled DNA was carried out as described in 2.2.1xvb.

(c) Southern analysis

BamHI and SmaI (when analysing *nth* containing clones) or BamHI and EcoRI (when analysing *nfo* containing clones) digests of 10ug DNA from each parental and transfected cell line were carried out, as described in 2.2.1 iii, and subjected to electrophoresis in a 0.8% agarose gel, at 28V overnight.

The gel was immersed in denaturing solution for 2 hours and then neutralising solution for 2 hours, after which the DNA was transferred to a nylon membrane by capillary action (Southern 1975). Transfer occurred over a 24 hour period, using 6 x SSC as the transfer buffer. The DNA was bound to the nylon and the filter stored as described above. Hybridization with [^{32}P] labelled DNA

was carried out as described in 2.2.1xva.

(d) Northern analysis

RNA (10ug) from each parental and transfected cell line (dissolved in 6% formaldehyde, 1 x MOPS, 50% formamide) was heated to 65°C to denature any secondary structure present and cooled at 0°C. Agarose was dissolved in 1 x MOPS (2.1.12 i) and 6% formaldehyde (in a fume cupboard) and used to prepare a 1.5% agarose gel. The RNA samples were then subjected to electrophoresis, using 1 x MOPS as the electrophoresis buffer.

Following electrophoresis the RNA was transferred to a nylon membrane (see above), using 20 x SSC as the transfer buffer. The RNA was bound to the nylon and the filter stored as described above. Hybridization with [^{32}P] labelled DNA was carried out as described in 2.2.1xvb.

(xv) Hybridization of ^{32}P labelled DNA with DNA/RNA attached to nylon

a) DNA attached to nylon

Filters (prepared as in 2.2.1xiv) were prehybridized in 6 x SSC, blotto at 65°C for 4 hours, in a sealed bag. Hybridization with [^{32}P] labelled DNA (prepared as in 2.2.1xiiia) was carried out at 65°C overnight in 15mls of hybridization mixture, which contained 10% (w/v) dextran sulphate, 6 x SSC, blotto and the ^{32}P labelled DNA. The membrane was then rinsed in 6 x SSC and washed at low (2 x SSC, 0.1% SDS for 1 hour at 65°C) or high stringency (0.1 x SSC, 0.1% SDS for 1 hour at 65°C), depending on the amount of radioactivity bound to the membrane, which

was assessed using a Geiger counter. The nylon membrane was dried, wrapped in Saran wrap and exposed to X-ray film, with an intensifying screen, at -80°C. Time of exposure varied depending on the signal intensity.

b)RNA attached to nylon

The procedure used was similar to that described above, except 6 x SSC, blotto was replaced with FBS (2.1.12ix), pre-hybridizations and hybridizations were carried out at 42°C and the membranes were washed at low stringency.

2.2.2 Culture of mammalian cells

(i) Maintenance of cell lines

Cells were grown as monolayers in MEM (Eagle 1959) containing 10% (v/v) FCS, at 37°C, 5% CO_2 in a humidified atmosphere. Transfected Chinese hamster V79 lung fibroblasts and transfected Chinese hamster ovary cells were grown in medium containing 1mg/ml and 300ug/ml G418, respectively.

(ii) Routine subculture

On reaching confluence (approximately every 4 days) the cells were subcultured. The medium was removed and the cells were washed with 5ml PBS. To obtain a cell suspension the cells were treated with 1ml 0.02% EDTA for 1 minute at room temperature and 2ml 0.05% trypsin for 5 minutes at 37°C. After this time 5ml MEM containing 10% (v/v) FCS were added to the flask and the cell suspension centrifuged at 1000rpm for 5 minutes. The cell pellet

was resuspended in 5ml of medium (MEM, 10% v/v FCS), 0.5ml of which was used to determine the cell density using a Coulter counter. A new flask containing 10ml medium was innoculated with 2 x 10^5 cells. The cells were then incubated as described above.

(iii) Freezing, storage and thawing

Trypsinization was carried out as described above. The cells obtained from each flask (75cm^2 in area) were resuspended in 4ml 10% (v/v) DMSO, which was prepared by the addition of 400ul DMSO to 3.6ml MEM containing 10% (v/v) FCS. Aliquots (1ml) of the cell suspension were slowly frozen in sterile plastic ampoules, in liquid nitrogen vapour. Ampoules were stored in liquid nitrogen in a Jencons VHC35 liquid freezer.

(iv) Transfection of mammalian cells by lipofection

Lipofectin reagent (BRL) is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3 dioleyoxy)-propyl]-N,N,N-trimethyl ammonium chloride (DOTMA) and dioleoyl phosphatidyl ethanolamine (DOPE) in filtered ddH_2O. It interacts with DNA spontaneously to form lipid-DNA complexs such that the DNA is enclosed within the liposomes (Felgner et al 1987).

To form the lipid-DNA complex 50ul of lipofectin reagent (BRL) was mixed with 50ul sterile ddH_2O containing 10ug vector DNA in a polystyrene tube and incubated at room temperature for 15 minutes. Co-transfection of pSV2hyg and pLJnfo (see chapter 5)

required the mixing of the reagent with 50ul sterile ddH_2O containing 2ug pSV2hyg and 20ug pLJnfo.

Cells (at approximately 60% confluency) were washed twice with 3ml OPTIMEM 1 (serum-free medium) and the lipofectin-DNA complex added dropwise to the flask, which contained 4ml OPTIMEM 1. After a 24 hour incubation at 37°C, 4ml MEM containing 20% (v/v) FCS were added to the flask and the cells were incubated for a further 48 hours. When confluent, the cells were trypsinised (see 2.2.2ii) and 2 flasks, containing 10ml medium (MEM,10% v/v FCS) and the appropriate concentration of G418 (e.g. 500 ug/ml for xrs7 cells)or hygromycin (100 ug/ml for x7nth1 cells), were innoculated with 2 x 10^5 cells. The remaining cells were frozen and stored at -195°C (2.2.2 iii). Cells in selective medium were incubated at 37°C, 5% CO_2 in a humidified atmosphere. The medium was changed every 4 days until colonies were visible. The colonies were then isolated, by placing a sterile 0.5cm diameter cloning ring around each one, harvested by trypsinization (2.2.2 ii) and the cells of each colony transferred to a separate tissue culture flask (25cm^2 in area) containing 5ml selective medium. When confluent the cells were further expanded and the cell lines characterised (see chapters 3 and 5).

(v) Cell cycle analysis

Exponentially growing cells were trypsinized as above and 10^6 cells fixed in 1ml of 70% (v/v) cold ethanol at 4°C for a minimum of 30 minutes (Vindelov 1977). The

cells were washed in PBS and resuspended in 1ml RNase A (1mg/ml in PBS), which had been heated to 95°C to destroy contaminating DNase. After 40 minutes at 37°C the cells were resuspended in 1ml propidium iodide (Sigma, 50ug/ml in PBS) and placed on ice for a minimum of 15 minutes. The percentage of cells in each phase of the cell cycle was determined by fluorescent activated cell sorting, using an EPICS 5 FACS machine. The fluorescence of the cells was measured at 520nm after excitation at 488nm, which enabled the cells to be "sorted" according to DNA content.

(vi) Determination of growth rate

Exponentially growing cells were trypsinized (as above), counted using a Coulter counter and 2 x 10^4 cells were added to 60mm plastic tissue culture dishes containing 4ml medium (MEM, 10% FCS), which were then incubated as described in 2.2.2i. Increase in cell number was measured as a function of incubation time. Every 12 hours cells were harvested, by trypsinization, from 3 dishes and the number of cells/dish was determined. Linear regression was used to analyse the exponential portion of growth, to obtain the cell doubling time.

(vii) Chromosome preparation.

Two days after subculturing 100ul colcemid (10ug/ml in PBS, GIBCO) was added to two flasks of cells of each cell line and the flasks incubated (see 2.2.2i) for 2

hours. The cells were trypsinized (2.2.2ii) and the cell suspension centrifuged (1,500 rpm, 5 minutes) in a 15 ml Falcon tube. After decanting the medium, the cell pellet was resuspended in the 0.5ml medium remaining in the tube and 5ml of 0.05M KCl were added slowly to the cells, with intermittant shaking. The cells were then incubated at 37°C for 20 minutes. After centrifugation (1,500 rpm, 5 minutes), 5ml cold fixative (3:1, v/v, methanol: glacial acetic acid) were added slowly and the cells centrifuged (1,500rpm, 5 minutes). The solution was removed and the cells were washed twice in fixative and then placed at 4°C, overnight. The fixative wash was repeated three times before the cells were finally resuspended in 0.3ml of fixative.

Microscope slides were washed in methanol and cooled on solid CO_2. Approximately 50ul of the cell suspension was dropped onto a frosted slide, which was tilted at an angle of 45° to the horizontal. The slide was then passed over a low flame, which evaporated the fixative and the contents of the cells were distributed over the area of the slide. After the slides were dry, they were stained with 2% giemsa (see 2.1.12 xiv) and cover slips were fixed over the chromosome preparations.

(viii) Clonogenic survival assay

This was based on the method by Puck and Marcus (1955, 1956). Subconfluent cells were trypsinized (2.2.2ii) and resuspended in 5mls medium. A Coulter counter was used to determine the cell density of the

suspension, as this ensured reproducible cell density determination in the independent survival experiments.

a) Radiation survival

For each radiation dose point 8 x 10^5 cells were suspended in 2ml medium in a 20ml sterile plastic universal tube and "gassed" with air for 20 seconds before irradiation, to prevent hypoxia. The suspension of cells was irradiated at room temperature for various times with gamma radiation, using a ^{137}Cs sealed source (dose rate = 3.75 Gy/minute).

Following irradiation the cells were serially diluted. The cell densities, prepared for each dose point, were chosen to obtain an optimal number of colonies (approximately 70) for counting, e.g. 100 and 200 cells/ml were prepared for RJKO cells irradiated at 0 and 4 gray. A 1ml aliquot of each dilution was added to a 60mm plastic dish containing 4ml medium (MEM, 10% FCS). Dishes were prepared in triplicate for each cell density and two cell densities were examined for each dose point.

b) Bleomycin and hydrogen peroxide survival

After trypsinization, cells were serially diluted to obtain the appropriate cell densities for each dose (see 2.2.2viiia) and 1ml of each dilution was added to a 60mm plastic dish containing 3ml medium (MEM, 10% FCS). Dishes were prepared in triplicate and two cell densities were examined for each dose point. Cells were incubated (2.2.2i) for 4 hours to allow attachment. Immediately before treatment an aliquot of bleomycin sulphate (the same batch of bleomycin was used for the parental and

transfected clones of a particular cell line) or the hydrogen peroxide was diluted. The appropriate amounts of bleomycin sulphate or hydrogen peroxide and MEM + 10% FCS were added to the dishes to obtain the correct dose of the cytotoxic agent in 5mls. Treatment times at 37°C were 1 hour for hydrogen peroxide and 24 hours for bleomycin sulphate.

After treatment the medium containing the cytotoxic agent was removed from the dishes and replaced with fresh medium.

RJKO and xrs7 cells were incubated (2.2.2i) for 7 and 8 days, respectively, after which time the medium was removed from the dishes and the colonies stained by addition of 1% (w/v) gentian violet (2.1.12 xiv). This was removed after 2 minutes and the dishes rinsed with water.

Cell toxicity was measured by the reduction in colony-forming ability (CFA).

2.2.3 Protein Biochemistry

(i) Preparation of cell-free extracts

Approximately 4 x 10^6 cells were trypsinized, harvested and resuspended in 1ml of sonication buffer (buffer I or EIII or EIV reaction buffer when extracts were to be used for assay). The suspension was sonicated at 10u peak to peak distance for 10 seconds and cooled at 0°C. Resonication was carried out at 12u peak to peak distance for 10 seconds and 0.01 volumes of phenyl

methylsulphonyl fluoride (PMSF, 8.7mg/ml in ethanol) was added to inhibit proteases. Centrifugation at 4°C and 16,000 rpm for 5 minutes separated the cell debris from the cell-free extract, which was transferred to a new ependorf tube and kept at 0°C until required.

(ii) Protein concentration estimation

The protein assay is based on the "shift" in absorbance from 465nm to 595nm of coomassie brillant blue G250 which occurs when it is bound to protein (Bradford 1976).

A standard curve was set up in duplicate, using BSA concentrations ranging from 0-1mg/ml. The procedure involved the addition of 2mls of filtered Biorad protein reagent to 40ul of each protein solution. After 15 minutes at room temperature the absorbance at 595nm was determined.

Estimation of protein in the cell-free extracts was carried out in duplicate. Samples were diluted to obtain an absorbance approximately equal to that of the 0.3mg/ml standard solution. The concentration of protein in the samples was determined by extrapolation from the standard curve.

(iii) SDS PAGE of proteins

SDS PAGE was performed using vertical slab gels in a BioRAD Protean II (16 x 16cm) or a BioRAD Mini Protean II system (8 x 7cm) depending on the volume or concentration of the protein samples to be analysed.

The 30% Protogel stock solution was diluted to 12.5% for the resolving gel and 3% for the stacking gel, using 0.375M Tris-HCl, pH 8.5, and 0.125M Tris-HCl, pH 6.8, respectively.

The 12.5% resolving gel solution (40mls for the Protean II system and 3mls for the mini-system) was prepared and vacuum degassed, before addition of SDS to a final concentration of 0.1%. After the addition of 0.001 volumes TEMED the solution was mixed thoroughly and 0.004 volumes of 10% (w/v) ammonium persulphate were added. The gel solution was pipetted between the two glass plates (1.5mm apart in the Protean II system and 0.75mm apart in the mini-system) of the vertical gel slab apparatus. Butanol was layered on to the surface of the gel, which was then allowed to polymerise.

The stacking gel (10mls for the Protean II system and 1ml for the mini-system) was prepared in a similar manner to the resolving gel using a 3% acrylamide solution. After the removal of the butanol from the polymerised resolving gel, a well-forming comb was placed in position and the stacking gel pipetted onto the resolving gel. After polymerisation the well-forming comb was removed and the well slots washed thoroughly with SDS-PAGE electrophoresis buffer (2.1.12i).

Samples were heated to 100°C in SDS-PAGE loading buffer (2.1.12ii) for 10 minutes and then pipetted into the well slots. Denatured prestained molecular weight protein markers were also applied to the gel. Electrophoresis was at 200V until the bromophenol blue

reached the end of the gel. The proteins in the resolving gel were transferred to nitrocellulose (see 2.2.3v) or stained by immersing the gel for 1 hour in 0.1% w/v coomassie blue, 30% v/v methanol, 10% v/v acetic acid. The gel was then destained in 30% v/v methanol, 10% v/v acetic acid for 10 hours to visualise the proteins.

(iv) Partial purification of cell-free extracts and preparation of samples for SDS PAGE

The cell-free extract (see 2.2.3 i) was applied to a 1cm x 1cm ds DNA cellulose column, equilibrated with buffer I (2.1.12iii). The elutant was monitored by a U.V. (280nm) flow cell, which was connected to a chart recorder. A flow rate of 20ml/hour was achieved by use of a peristaltic pump and 3ml fractions were collected. The column was washed with buffer I after addition of the cell-free extract and almost immediately a large peak was recorded. This was the "breakthrough" protein peak. When the absorbance returned to base-line level, 0.75M NaCl in buffer I was used to elute the DNA binding proteins. The relevant fractions were pooled for the "breakthrough" proteins and DNA binding proteins.

Dialysis tubing was boiled in 10mM EDTA and the protein binding sites blocked with BSA. The "breakthrough" and DNA binding protein fractions were dialysed overnight against 1 litre of ddH_2O, to remove NaCl, and freeze-dried. The "breakthrough" proteins and the DNA binding proteins were dissolved in 200ul and 80ul ddH_2O, respectively. A 4ul aliquot of each sample was

subjected to SDS PAGE (see 2.2.2 xiv) in a BioRAD Mini-Protean II gel apparatus. After staining the proteins in the gel (see 2.2.3iii), it was possible to compare the amount of protein in each sample. This enabled SDS-PAGE to be repeated with equal quantities of protein for each sample. The proteins in this gel were transferred to nitrocellulose (2.2.3v) and used in Western analysis (incubation of filters containing proteins with anti-EIII antibody was carried out by Mrs H.Ahern, SUNY Albany, USA).

(v) Electrotransfer of proteins

Two pieces of porous matting, two pieces of Whatman 3MM paper and a nitrocellulose membrane, the same size as the gel, were pre-soaked in electrotransfer buffer (2.1.12 i).

The gel was placed against the nitrocellulose membrane and assembled in a sandwich in between two pieces of Whatman filter paper and the porous matting.

The sandwich was placed in an electrotransfer tank with the nitrocellulose membrane next to the anode and electro-transfer was carried out overnight at 0.1mA. The membrane was then dried, wrapped in Saran wrap and stored at 4°C.

The residual gel was stained with coomassie blue (see 2.2.3 iii) after transfer to confirm that protein transfer had been efficient.

(vi) Development of an endonuclease III assay

a) Purification of oligonucleotides

The oligonucleotide, which was covalently attached to controlled porous glass beads, was placed in a 2ml plastic ampoule and this was then filled with 35% ammonia and sealed. The ampoule was incubated at 55°C overnight to detach the oligonucleotide from the glass beads. To prevent hydrolysis of the trityl group on the oligonucleotide 0.5M NaOH was added to a final concentration of 5mM before ammonia was evaporated.

A NENSORB^{Tm} prep column was activated by washing with 10ml methanol and equilibrated with 5ml TEAA. The flow rate did not exceed 10ml/minute during the purification, which was carried out in a fume cupboard. The sample, dissolved in 4ml TEAA, was applied to the column and the column washed with 10ml aceto-nitrile/TEAA (1:9 v/v), which removed failure sequences, salts and synthetic by-products. The trityl group on the oligonucleotide was hydrolysed by washing the column with 25ml 0.5% (v/v) trifluoroacetic acid, and 10ml TEAA were used to remove the acid. The oligonucleotide was finally eluted in 5ml 35% (v/v) methanol and 1ml fractions were collected. The absorbance of each fraction at 260nm was measured and those containing the oligonucleotide were dried and pooled after resuspension in 1ml ddH_2O. The concentration of the solution was determined by measuring the absorbance at 260nm (1 E_{260} = 33ug/ml). The oligonucleotide was stored at -20°C.

b) OsO_4 oxidation of strand A oligonucleotide

Oxidation of 1ug ^{32}P labelled strand A oligonucleotide (see figure 15) was carried out in an 80ul reaction, using 2% OsO_4 and 5% pyridine, at room temperature for 30 minutes (Clark & Beardsley 1986). A control reaction, omitting OsO_4, was also performed. Addition of pyridine accelerates the rate of formation of cis glycols by OsO_4 by several orders of magnitude (Schroder 1980), which allowed the reaction to be carried out at room temperature. To remove excess OsO_4 the reaction mix was applied to a Sephadex G25 column (volume $1cm^3$, see 2.2.1xiii), which had been pretreated with 1ug unlabelled oligonucleotide to block non-specific DNA binding sites and equilibrated with ddH_2O. After centrifugation at 800rpm for 6 minutes, the eluant (approximately 350ul) contained the ^{32}P labelled oligonucleotide, which was dried using a vacuum centrifuge (Gyro Vap) and resuspended in 40ul ddH_2O. Oxidized and control oligonucleotides were stored at $-20^{o}C$.

c) Piperidine treatment of oligonucleotides

Piperidine was added to 50ng oxidized (or control) oligonucleotide dissolved in 30ul ddH_2O, to a final concentration of 0.5M. This was heated to $90^{o}C$ for 30 minutes, after which time the sample was dried (using the Gyro Vap) and washed with 10ul ddH_2O. The sample was re-dried and the oligonucleotide dissolved in 10ul 0.1M NaOH, 1mM EDTA. After addition of 10ul 0.1M HCl to

neutralize the solution, it was subjected to UPAGE to determine the size of the piperidine treated oligonucleotide, (see 2.2.3 vig).

d) Annealing complementary oligonucleotides

Quantitation of the strand B oligonucleotide (see figure 15, complementary to strand A) was achieved by measuring the absorbance of the oligonucleotide solution at 260nm (1 E_{260} = 33ug/ml). However, it was necessary to assume that 100% of the oxidized (or control) strand A oligonucleotide was recovered from the G25 sephadex "spun" column (see above), since the low concentration of the oligonucleotide solution prevented E_{260} determination.

Equal amounts of strand B and oxidized (or control) oligonucleotides were mixed and the solution was heated to 70°C for 5 minutes to denature any illegitimate secondary structure that had formed between or within the oligonucleotides. The Tm [2°C(A + T) + 4°C(G + C)] of the oligonucleotides was 62°C and so the solution was incubated at 55°C for 1 hour to allow annealing and cooled over a period of 5 hours to maintain the double strand structure. The ds oligonucleotide was stored at -20°C.

e) Endonuclease III assay

See chapter 4, section 4.3. and 4.5, for the assay procedure.

f) Separation of oligonucleotide fragments following EIII or piperidine treatment

UPAGE (using BioRAD mini Protean II system) was used to achieve separation of oligonucleotides produced from the treatment of oxidized (or control) oligonucleotide solutions with EIII or piperidine. A 0.75mm, 20% polyacrylamide/7M urea gel was prepared from a solution of 5ml 40% Acugel 40 stock acrylamide solution, 1ml 10 x TBE and 5g urea, which was made up to a volume of 10ml. After addition of 12ul TEMED the solution was mixed and 60ul 10% ammonium persulphate were added. The solution was poured into the vertical gel slab apparatus, a well-forming comb was placed in the top of the gel and it was allowed to polymerise.

The oligonucleotide samples and 13mer and 23mer size markers were heated to 100°C for 3 minutes in 1 x UPAGE loading buffer, after which they were subjected to UPAGE for 45 minutes at 400V, with TBE as the electrophoresis buffer. The gel was wrapped in Saran wrap and exposed to X-ray film, without the use of an intensifying screen, at room temperature for 1-4 hours, depending on the signal intensity.

g) Analysis of autoradiographs by scanning densitometry

Autoradiographs were analysed using a Shimadzu CS930 dual wavelength scanning densitometer. The absorbance at 595nm of each lane of the autoradiograph was measured and the absorbance versus distance in cm was plotted by the machine. The area under each peak (i.e. signal intensity) was expressed as a percentage of the area of

all the peaks (i.e. of the total signal intensity of the lane).

h) Immunoprecipitation of oxidized (or control) oligonucleotides with an anti-TdG monoclonal antibody.

After mixing anti-TdG monoclonal antibody with the [^{32}P] labelled oxidized (or control) oligonucleotide (for quantities see chapter 4) and allowing time for hybridization (for conditions see chapter 4), ddH_2O was added to the reaction to increase the volume to 100ul. An equal volume of cold, saturated ammonium sulphate solution was then added and after mixing thoroughly the reaction was incubated at 0°C for 1 hour or 24 hours. Centrifugation at 15,000 rpm at 4°C for 15 minutes was used to separate insoluble oligonucleotide-antibody complexes from the solution. The radioactivity in 150ul of the supernatant was measured to determine the amount of oligonucleotide remaining in solution.

j) Immobilization of the anti-TdG monoclonal antibody.

A range of dilutions (3 to 3000 x) of the anti-TdG monoclonal antibody were prepared using 50mM sodium borate buffer, pH 9.5, and 90ul of each dilution was pipetted in duplicate into the wells of a microtitre plate (Costar). This was wrapped in foil to prevent light from inhibiting antibody binding and incubated at room temperature overnight. The liquid in the wells was then removed and each well washed five times with PBS containing 0.05% Tween 20 (using a microtitre plate

washer). Various attempts were made to bind oxidized oligonucleotide to the antibody attached to the microtitre plate (see chapter 4).

(vii) AP endonuclease assay

a) Determination of the titre of phage particles

A single colony of XA103 or BW313 was used to innoculate 50ml LB containing 0.5ml 20% (w/v) maltose, which was then incubated overnight at 37°C in an orbital shaker (275rpm). After centrifugation (3000rpm, 20°C for 15 minutes) the bacteria were resuspended in 5ml of 0.01M $MgSO_4$ and the concentration of the suspension determined by measuring the absorbance of the solution at 600nm (1 OD = 0.8 x 10^9 bacteria/ml). The solution was diluted to 1.6 x 10^9 bacteria/ml using 0.01M $MgSO_4$ and stored at 4°C until required.

The stock solution of phage particles was serially diluted (10-10^6 times) and 0.1ml of bacterial solution was added to 0.1ml of each dilution of the phage solution. The cultures were mixed and incubated at 37°C for 20 minutes to allow phage infection. To each phage-bacteria culture 3ml of 0.7% LB agar (42°C) was added before it was poured onto an LB agar plate. After 5 minutes at room temperature the plates were incubated at 37°C overnight. Phage plaques produced from the dilutions of the stock solution were counted to determine the concentration of phage particles in the stock solution.

b) Production of a stock solution of phage particles from a single phage plaque

A single plaque (produced as in 2.2.3viia) was removed using a pasteur pipette and placed in 1ml SM (2.1.12v) containing a drop of chloroform. After incubating the solution at room temperature for 1 hour the phage particles had diffused into the SM. A 10^4 dilution of this solution was used to prepare an LB agar plate containing phage-infected XA103 bacteria (as described above), which was incubated at 37°C for approximately 12 hours. When the phage particles had lysed all the bacteria 5ml SM was pipetted onto the plate, which was incubated at 4°C for 7 hours with intermittant shaking. The SM was then pipetted into a 15ml polypropylene tube and an extra 1ml SM was added to the plate for 15 minutes to collect the remaining phage particles. After pooling the SM containing the phage particles, the solution was centrifuged at 6000rpm for 10 minutes at 4°C to remove bacterial debris. The supernatant was transferred to a new tube, 1 drop of chloroform added and the phage solution stored at 4°C.

c) Production and isolation of [^{3}H]-labelled partially depyrimidinated phage DNA

This was prepared as described by Cunningham & Weiss (1985). A single colony of BW313 bacteria was used to innoculate 50ml of M9 (2.1.9 i) containing 1mM $MgSO_4$, thymine (50ug/ml) and thiamine (1ug/ml). The culture was incubated at 37°C in an orbital shaker (275rpm) until the OD_{600} = 0.25 (i.e. 2 x 10^8 bacteria/ml). The solution was centrifuged at 3000rpm for 10 minutes at 20°C and the

bacteria resuspended in M9 containing 1mM $MgSO_4$, thiamine (1ug/ml) and tryptophan (20ug/ml). After the addition of tritiated thymidine (5uCi/ml, 76Ci/mmole) and deoxy-adenosine (400ug/ml) 0.4 x 10^9 phage particles were added and the culture allowed to grow for 2 hours at 37°C in an orbital shaker. Chloroform (0.3ml) was then added and the culture re-incubated for 15 minutes to complete the lysis of the bacteria. The solution was centrifuged at 3000rpm for 10 minutes at 20°C and the supernatant transferred to a new tube.

Purification of the phage DNA was carried out using the midi QIAGEN lambda kit. To degrade bacterial RNA and DNA 100ul buffer L1 (20mg/ml RNase, 6mg/ml DNase I, 0.2mg/ml BSA, 10mM EDTA, 300mM NaCl, 100mM Tris-HCl pH 7.5) was added to the supernatant and after a 30 minute incubation at 37°C 10ml of buffer L2 (30% v/v PEG, 3M NaCl) were added. The solution was mixed gently and incubated on ice for 1 hour to precipitate the phage particles. After centrifugation at 10,000 rpm for 10 minutes, the pellet was resuspended in 3ml buffer L3 (100mM NaCl, 25mM EDTA, 100mM Tris-HCl pH 7.5) and 4ml buffer L4 (4% w/v SDS) were then added. This was incubated at 70°C for 20 minutes and cooled on ice, before the addition of 3ml buffer L5 (2.55M KAc). The solution was mixed gently and centrifugation at 18,000 rpm at 4°C for 10 minutes removed contaminating protein, leaving the phage DNA in solution. The supernatant was re-centrifuged (18,000 rpm, 4°C, 10 minutes) to obtain a clear lysate.

A QIAGEN-tip 100 was equilibrated with 3ml buffer QBT (750mM NaCl, 50mM MPS, 15% v/v ethanol, 0.15% v/v Triton X-100, pH 7) and the clear lysate was applied to the column. After washing the column with 10ml buffer QC (1M NaCl, 50mM MPS, 15% v/v ethanol, pH 7), the phage DNA was eluted with 5ml buffer QF (1.25M NaCl, 50mM MPS, 15% v/v ethanol, pH 8.2) and precipitated using 0.7 volumes of isopropanol. The phage DNA was collected by centrifugation (10,000 rpm, 4°C, 30minutes), washed with 70% ethanol and dissolved in 100ul ddH_2O. Phage DNA was stored at 4°C.

To produce the [^{3}H]-labelled AP DNA, the [^{3}H]-labelled uracil containing phage DNA (approximately 10ug) was incubated with 5 units of uracil DNA glycosylase in 500ul of 1 x uracil DNA glycosylase buffer (see 2.1.12 vii), at 37°C for 4 hours and the product stored at 4°C.

d) AP endonuclease assay procedure

The reaction mixture (200ul) was prepared using 1 x EIII or EIV reaction buffer (2.1.12 vii) and contained 2000 cpm [^{3}H] AP DNA and 20ug of cell-free extract (prepared as described in 2.2.3i). Assays were incubated at 37°C for 30 minutes.

To determine the level of AP sites in the substrate 50ul 0.3M NaOH were added to 50ul [^{3}H] AP DNA (2000cpm) and the solution incubated for 30 minutes at 37°C. The reaction was then neutralised by the addition of 100ul 0.15M HCl.

To precipitate the high molecular weight DNA

remaining after treatment with alkali or cell-free extract, 100ul of salmon sperm DNA (2.5mg/ml) was added, the solutions mixed and 300ul cold 10% (w/v) TCA added. After 10 minutes on ice the precipitated DNA and acid soluble fraction were separated by centrifugation at 15,000 rpm for 5 minutes at 0°C. The radioactivity was measured in 500ul of the supernatant by a Berkard scintillation counter.

viii) "Plasmid-nicking" assay

pBR322 DNA (0.1ug/ul) was irradiated with 3000J/m^2 254nm light, on a plastic tray at 4°C and used as the substrate for this assay.

Assays containing 0.5ug irradiated or unirradiated DNA and 12ug cell-free extract (prepared as described in 2.2.3i) in 20ul EIII reaction buffer (2.1.12vii) were incubated at 37°C for 30 minutes. Addition of 1ul proteinase K (20mg/ml) and incubation at 4°C, overnight degraded the protein in the samples. SDS was added to a final concentration of 0.05% and the samples placed at 60°C for 5 minutes to ensure the remaining protein was soluble. After chilling, 2ul of loading buffer (see 2.1.12 ii) were added to the samples and they were subjected to 0.7% agarose gel electrophoresis, using TBE containing 0.5ug/ml ethidium bromide as the electrophoresis buffer.

Quantitation of the conversion of circular to linear plasmid DNA, and hence endonuclease activity, was by the intensity of the ethidium bromide stained plasmid bands

on examination of the gel using a U.V. transilluminator (302nm).

ix) Assay to detect the enzyme cleavage pattern.

The substrate for this assay was ^{32}P-labelled, U.V. irradiated pBR322 DNA. The plasmid DNA (10ug) was digested with 16 units of AvaI in a volume of 20ul, at 37°C for 2 hours and labelled by the addition of 20uCi alpha ^{32}P dTTP and 2 units of Klenow fragment. After 15 minutes at room temperature, the reaction was extracted twice with phenol (equilibrated to pH 8 with TE) and precipitated using 0.1 volume of 3M NaAc and 2 volumes of cold ethanol. The DNA was washed twice with 70% ethanol, dried and resuspended in 50ul of 1 x EIII reaction buffer (2.1.12 vii). The DNA was irradiated on a plastic tray at 4°C, using 254nm U.V. light. The total dose was 10,000 J/m^2.

The reaction was carried out in 10ul 1 x EIII reaction buffer and contained 6ug cell-free extract (prepared as in 2.2.3i) and 0.8ug of irradiated DNA. After 30 minutes at 37°C, 4ul of loading buffer were added and the samples heated to 90°C for 5 minutes. A 5ul aliquot of each sample was then subjected to UPAGE (2.2.3 vif), using an 8% polyacrylamide gel, at 100W for three hours. The gel was then autoradiographed at -80°C overnight.

2.2.4 Toxicity analysis

Model equations were used to fit the survival data,

using a computer program called DRFIT (Roberts 1990). This uses maximum likelihood techniques and analysis of variance to test for significant differences between data sets. Empirical equations were used, which adequately fitted the shapes of the survival curves as follows:

Alpha, beta model (Chadwick & Leenhouts 1973)

$$S = A \exp(-\alpha D - \beta D^2) \qquad (17)$$

where S = CFA at a given dose

A = CFA at zero dose

D = dose

Alpha is the number of lethal events produced/ unit dose (at low doses), presumed to result from the direct production of dsb in DNA by single charged particle tracks.

Beta is related to the number of lethal events generated by the accumulation of sublethal injury, which may be due to the interaction of two ssb. It can also be accounted for by the time dependent production of dsb possibly in the course of repair of a ssb opposite base (or sugar) damage or two closely opposed base (or sugar) lesions (Ahnstrom & Bryant 1982, Ward 1987).

Alpha, beta model adapted to incorporate a quenched-dose parameter

$$S = A \exp\{-\alpha Deff - \beta Deff^2\} \qquad (18)$$

where $Deff = Q_D [1 - \exp(D/Q_D)]$

and D = dose

S = CFA at a given dose

A = CFA at zero dose

Deff = effective dose

Q_D = maximum value of Deff, above which further doses are quenched

α = number of lethal events produced/unit dose (at low doses)

β = relates to the number of lethal events generated by accumulation of sublethal damage

Twin-exponential model

$$S = A [B \exp(-D/{}_1Do) + (1-B)\exp(-D/{}_2Do)] \quad (19)$$

where D = dose

S = CFA at a given dose

A = CFA at zero dose

B = sensitive portion of the total population

$_1Do$ = Do of sensitive population

$_2Do$ = Do of resistant population

Do is the inverse of the gradient of the exponential portion of the curve.

Single-hit, multitarget model, adapted to incorporate a quenched-dose parameter

$$S = A \{1 - [1- \exp(-Deff/Do)]^N \} \quad (20)$$

where $Deff = Q_D \{1 - \exp(-D/Q_D) \}$

and D = dose

S = CFA at a given dose

A = CFA at zero dose

Do = Do at high dose if no quenching occurs

Deff = effective dose

Q_D = maximum value of Deff, above which further doses are quenched

N = extrapolation number

Data analysed by these models are used by the computer in the above equations to determine the best estimates of the "fitted" parameters and hence the shape of the curve.

From the "fitting" of the curve the sums of squares, which relates to the amount of "scatter" of the points about the "fitted" curve, and the degrees of freedom, which is the number of observations minus the number of fitted parameters, are calculated.

In order to compare survival curves produced from different data sets, the sums of squares (SS) and the degress of freedom (DF) were required for the individual curves and for the one curve which was produced by "pooling" the data sets under consideration (all data sets were given common parameters, except for the CFA at zero dose, to achieve this). Consider the example of data sets 1 and 2:-

	SS	DF
Data set 1	SS_a	DF_a
Data set 2	SS_b	DF_b
Pooled set	SS_2	DF_2

$$SS_a + SS_b = SS_1 \qquad (21)$$

$$DF_a + DF_b = DF_1 \qquad (22)$$

By plotting the 2 separate survival curves for data set 1 and data set 2 during the same programme "run", the

computer automatically calculated the values of SS_1 and DF_1. The values were then used in the following equations:-

$$N_1 = \frac{SS_1}{DF_1} \qquad (23)$$

$$N_2 = \frac{SS_2 - SS_1}{DF_2 - DF_1} \qquad (24)$$

$$F = \frac{N_2}{N_1} \qquad (25)$$

The F value was then used by the programme to determine the p value. If $p < 0.05$ the curves were considered significantly different.

To determine which portion of the curves was different, the data sets were analysed again by the programme with only one common parameter. The sums of squares and degrees of freedom obtained were used in equation (24) instead of DF_2 and SS_2. The N value was then used in equation (25) instead of N_2 to obtain an F value and hence a p value. This was repeated for each parameter.

There is convincing evidence that dsb are the principal lethal lesions produced by ionising radiation (Blocher & Pohlit 1982, Bryant 1984, 1985, see 1.5.2), but the involvement of specific types of base damage in cell killing is difficult to assess due to the complex spectrum of radiation-induced DNA damage. In order to determine whether the TG-type lesion was toxic to cells the effects of EIII expression on cell survival was examined by transfecting pZipnth into cells of differing repair capacity. The ideal cell line would have been one deficient in the EIII-type activity, however, no such cell line was available at the start of this work. Three cell lines were chosen: RJKO, Chinese hamster V79 lung fibroblasts, xrs7, radiation sensitive Chinese hamster ovary cells deficient in double strand break (dsb) repair, and VG8, radiation sensitive Chinese hamster fibroblasts efficient in dsb repair. Since these cell lines differ in their ability to repair dsb, the contribution of thymine modifications to cell death could be assessed with respect to the repair of dsb.

Three cytotoxic agents were used in cell survival studies: ionising radiation, hydrogen peroxide and bleomycin sulphate. These agents produce thymine modifications and/or AP sites and it has been calculated that they produce different levels of DNA strand breakage per lethal event per cell (Ward et al 1987, Ross & Moses 1978, Bradley & Kohn 1979). The spatial distribution of

lesions produced in the DNA by these agents is also different (see figure 9): hydrogen peroxide is thought to produce randomly dispersed damaged sites (RDDS), while ionising radiation and bleomycin sulphate are believed to produce lesions in clusters known as local multiply damaged sites (LMDS). Survival studies using these cytotoxic agents allowed the relevance of the repair of thymine modifications to be assessed with respect to different lesion dispersion patterns.

3.1 Expression of *nth* in RJKO cells.

Expression of the bacterial *ogt* and *ada* genes had previously been achieved in RJKO cells following transfection of Moloney murine leukaemia retrovirus based vectors (pZipneoSV(X)1) containing *ada* or *ogt* (Brennand & Margison 1986, Harris 1990). This suggested that the expression of EIII should also be possible in RJKO cells using a similar mammalian cell expression system.

Transfection of pZipnth and pZipneoSV(X)1 into RJKO cells was achieved using calcium phosphate precipitation. Following selection in medium containing 1.5mg/ml G418, three clones (1D, 7D and 9D) and the vector control cell line (6E) were isolated from pZipnth and pZipneoSV(X)1 transfected cells, respectively. Transfected cell lines were maintained in medium containing 1mg/ml G418.

3.1.1 Molecular analysis of G418 resistant clones.

(i) DNA analysis

Genomic DNA was isolated (2.2.1ix) from the parental (RJKO) and the transfected cell lines (1D, 7D, 9D and 6E) and dot blot (2.2.1xiva) and Southern analyses (2.2.1xivc) were carried out using the 1kb [^{32}P]-labelled *nth* probe, which was isolated from an EcoRI digest of pUC8.1nth, using LMP agarose (2.2.1iv).

a) DNA dot blot analysis

Unlabelled probe (3.1.1.i) was used as the positive control. Strong hybridization signals were found with the positive control, 1D and 7D (figure 22A), but the signal was more intense for 7D compared to 1D, indicating a higher *nth* copy number in 7D. Although no signal was seen with RJKO DNA, the probe hybridized to the DNA of the vector control cell line (6E) and 9D (a pZipnth transfected cell line) to produce a faint signal, suggesting the presence of sequences complementary to the probe. This may have been due to pUC8.1 sequences contaminating the probe, since pUC8.1 and pZipneoSV(X)1 do consist of approximately 80% and 40% pBR322 sequences, respectively. Re-purification of the probe by LMP agarose might reduce this in future experiments.

b) Southern analysis

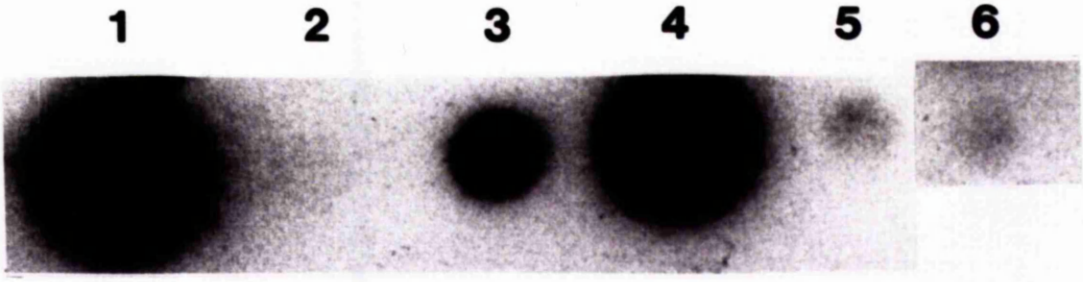
Genomic DNA from 1D, 7D, 9D and 6E cells was digested (2.2.1iii) with BamHI or SmaI for Southern analysis (2.2.1xivc).

FIGURE 22 - DNA analysis of RJKO and RJKO-transfected cell lines

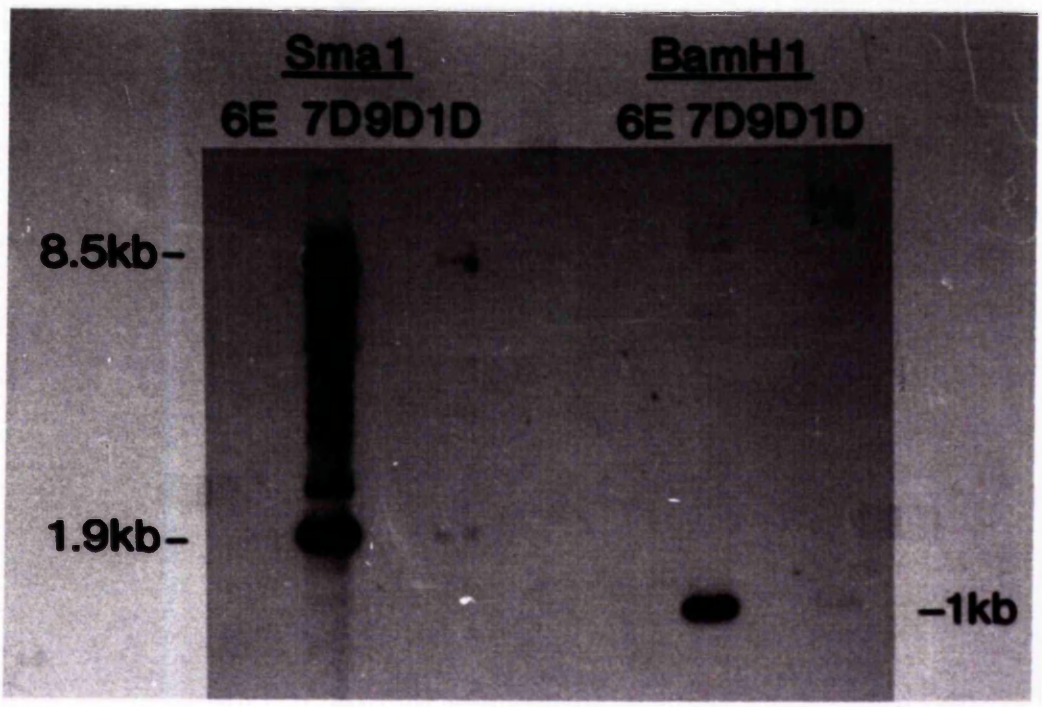
Filters were probed with the 1kb *nth* probe.

A - DNA dot blot analysis

1 positive control
2 RJKO
3 1D
4 7D
5 9D
6 6E



B - Southern analysis



BamHI digests of DNA from 1D and 7D produced a hybridization signal of approximately 1kb, confirming the presence of *nth* (figure 22B). The signal intensity was greater for 7D compared to 1D, as found in the DNA dot blot analysis. Gene dosage analysis would be necessary to obtain an accurate estimate of the *nth* copy number. Less intense signals between 5.6kb and 11kb and signals of 11kb and 12kb were also present in the 1D and 7D lanes, respectively. This may be due to incomplete BamHI digestion of the DNA or may indicate that only part of the *nth* sequence has been retained within the genome or a small fragment of pZipnth incorporated.

SmaI digestion of clone 1D DNA produced hybridization signals of approximately 1.9kb and 8.5kb, while clone 7D showed signals ranging in size from 1.9kb to 8.5kb. pZipnth contains 4 SmaI sites (figure 16). Independent of the insertion site a 1.9kb signal would be expected on SmaI digestion. Other signals resulting from SmaI digestion may be due to:

1) Partial SmaI digestion

SmaI will not cleave the DNA if a 5 methylcytosine is present at the third C in the recognition sequence (CCCGGG). It is possible that a fraction of pZipnth may have been methylated by a Chinese hamster DNA-cytosine methyltransferase, following insertion into the genome and DNA replication. A range of hybridization signals would also be produced if incomplete digestion of the unmethylated SmaI sites had occurred.

a) 2.3kb if the SmaI site in the pUCDUB polylinker

remained intact.

b) 3.7kb if the SmaI site in the pUCDUB polylinker and at the 5′ end of the *neo* gene remained intact.

c) 9.5kb if the SmaI site in the 5′LTR remained intact.

2) Site of linearization of pZipnth

Circular DNA was used in the transfection, hence linearization of the construct must have occurred before pZipnth inserted into the genomic DNA. A variation in the point of linearization from the 5′ LTR would produce a range of signal sizes if the SmaI site in the 5′LTR remained intact. The minimum size would be 2.1kb, which is the distance from the SmaI site within the pUCDUB polylinker to the end of the 5′ LTR. The size seen would depend on the position of the next SmaI site, either in the genomic DNA or in pZipnth sequences in the case of tandem insertions. The presence of copies inserted in tandem repeats could be examined by digesting the genomic DNA with a restriction endonuclease that cleaves once within *nth* but not within pZipneoSV(X)1, e.g. MaeIII or EcoRII. On Southern analysis a signal of 11.2kb would be seen, if no vector sequence was lost on incorporation.

3) Degradation of vector DNA before insertion into the genome

Insertion occurs via recombination of the LTR's with the genomic DNA. It is possible that sequences outside the LTR's may have degraded before incorporation of the vector DNA in the genome, producing a variation in the signal sizes detected.

(ii) RNA analysis

Total RNA was isolated from 1D, 7D, 9D and 6E (see 2.2.1x) and dot blot (2.2.1xivb) and northern analyses (2.2.1xivd) were carried out using the *nth* probe (3.1.1i).

a) RNA dot blot analysis

Because of the possibility of contamination of the RNA with cellular DNA, RNase A degradation of an aliquot of the RNA from each sample was carried out to obtain the negative (RNA-free) controls for this analysis (see 2.2.1vixb).

The probe hybridized to sequences in each of the positive and negative samples (figure 23A). Since the negative controls did not contain RNA, the signals were due to non-specific hybridization with contaminating genomic DNA. Clones 1D and 7D were found to have greater hybridization signals for the positive samples compared to the corresponding negative (RNA-free) controls. This suggested that these two clones expressed the *nth* mRNA.

b) Northern analysis

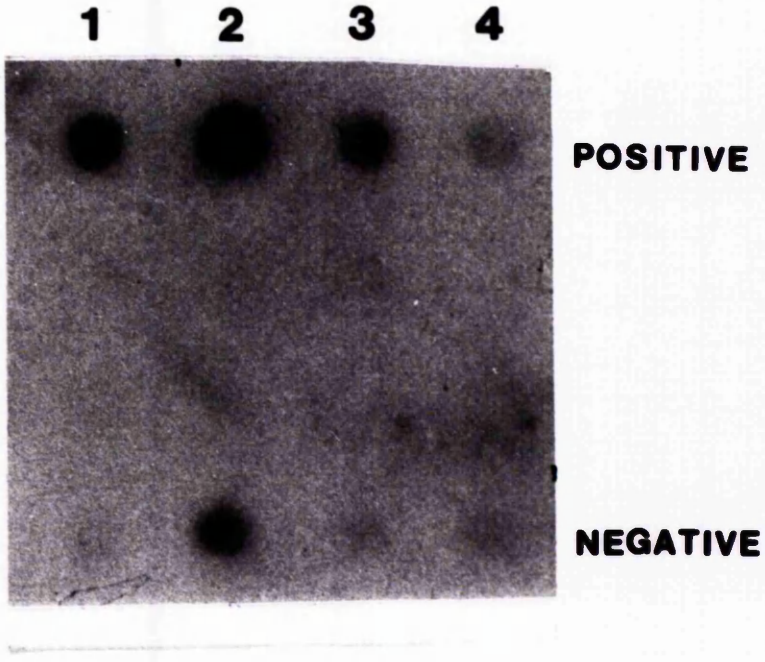
RNA from clones 1D, 7D, 9D and 6E showed three identical hybridization signals, which may have been due to hybridization with ribosomal RNA or with other mammalian message sequences (figure 23B). In addition to these common signals, RNA from clones 1D and 7D hybridized with the probe to produce an extra signal, which was not seen in RNA of clones 9D and 6E even after longer exposure. It was not possible to estimate the

FIGURE 23 - RNA analysis of RJKO and RJKO-transfected cell lines

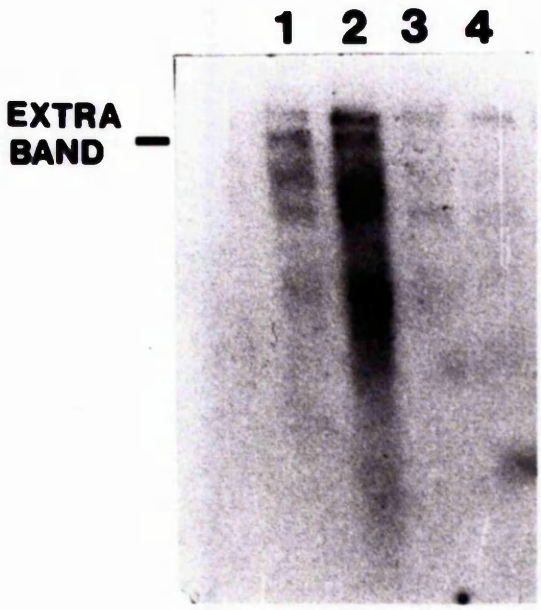
Filters were probed with the 1kb *nth* probe.

1 1D
2 7D
3 9D
4 6E

A - RNA dot blot analysis



B - Northern analysis



size of these signals due to the underloading of the RNA markers. However, taking into consideration the results of the DNA analysis (3.1.1i) and since the additional signal was not seen in clones 9D and 6E, it is likely that 1D and 7D transcribe the *nth* message. Without an estimation of the size of the additional signal it is not possible to determine whether this corresponds to the full length (4.4kb) or spliced transcript (2.2kb) of pZipnth.

(iii) Western analysis

Cell-free extracts (prepared from 1D, 7D, 6E and RJKO, 2.2.3i) and partially purified extracts (prepared from 7D and bacteria, 2.2.3iv) were subjected to SDS PAGE (2.2.3iii) and electrotransfer (2.2.3v) to a nitro-cellulose membrane. The filters were hybridized with an anti-EIII polyclonal antibody or pre-immune serum by the collaborating group.

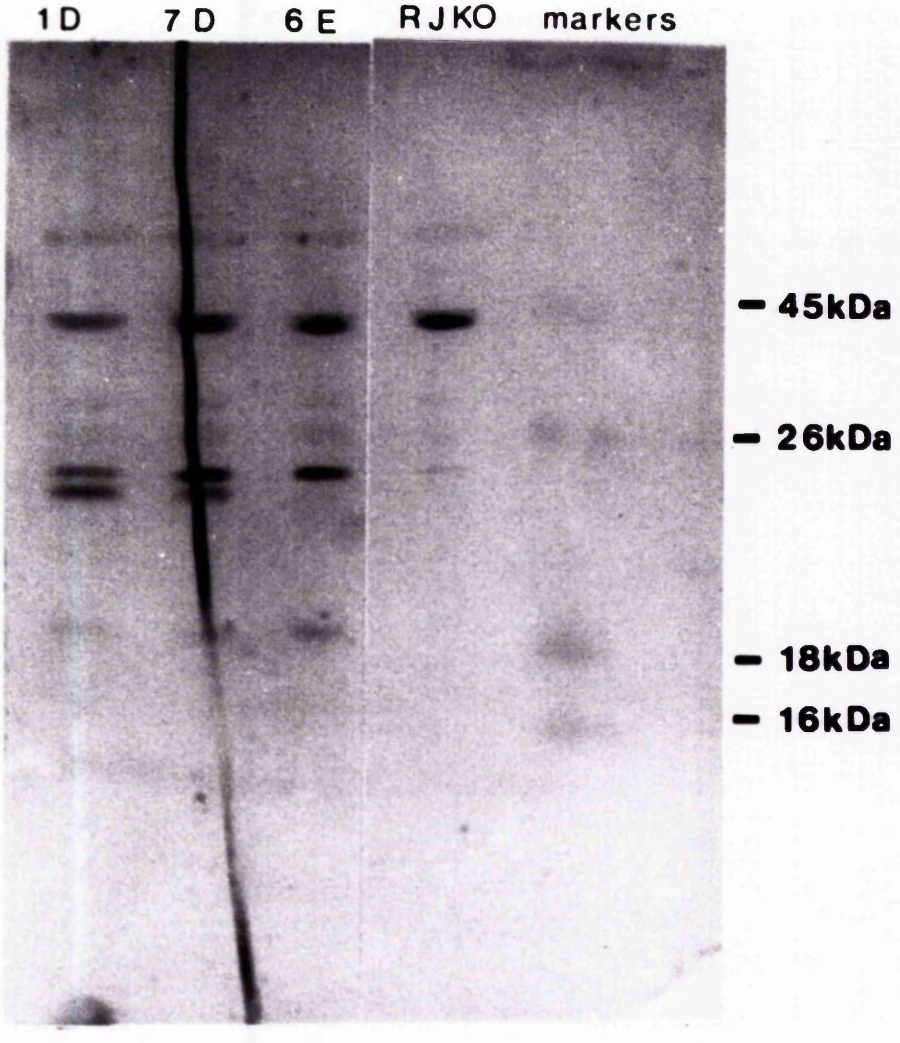
a) Analysis of cell-free extracts

After hybridization with the anti-EIII antibody, bands corresponding to molecular weights of approximately 18kDa, 26kDa, 30kDa, 45kDa and 60kDa were seen in all lanes (figure 24). An extra band was detected at approximately 25kDa in lanes containing extracts of clones 1D and 7D but not RJKO and 6E. This band corresponds to the size of the EIII protein and indicates that the extracts contain the bacterial EIII protein.

To ensure that the bands seen in the original

FIGURE 24 - Western analysis of cell-free extracts of RJKO and RJKO-transfected cell lines

The nitrocelluose filter containing cell-free extracts of RJKO and RJKO-transfected cells was probed with an anti-EIII polyclonal antibody.



analysis were due to the anti-EIII antibody and not other antibodies in the serum, western analysis was repeated using pre-immune serum (figure 25A). Bands were not detected in the 1D and 7D lanes. Therefore, the cross-reactions ranging in size of 18kDa-45kDa in all the Chinese hamster extracts and the 25kDa band in extracts of 1D and 7D were the result of hybridization with proteins containing epitopes recognised by the anti-EIII antibody. Enzymes with N-glycosylase and AP endonuclease activity analogous to EIII have been purified from bovine, mouse and human cells and these proteins have molecular weights of approximately 30kDa (Doetsch et al, 1987, Kim et al, 1991). It is possible that the cross-reactions seen in all the extracts (18-45kDa) may have been due to Chinese hamster enzyme(s) equivalent to EIII.

b) Analysis of DNA cellulose purified samples

Cell-free extracts of clone 7D and *E.coli* harbouring pRPC53 were subjected to DNA cellulose chromatography (2.2.3iv) and the load, breakthrough and 0.75M NaCl eluant subjected to western analysis.

Multiple bands ranging from 25kDa to 70kDa were seen in the *E.coli* samples (figure 25B). The most prominant band corresponded to a 25kDa protein. The 0.75M NaCl eluant contained more of the 25kDa protein than the other bacterial samples and it is not unreasonable to conclude that this represents the EIII protein. The other cross-reactions of the anti-EIII antibody may have been due to other bacterial proteins contaminating the EIII used to

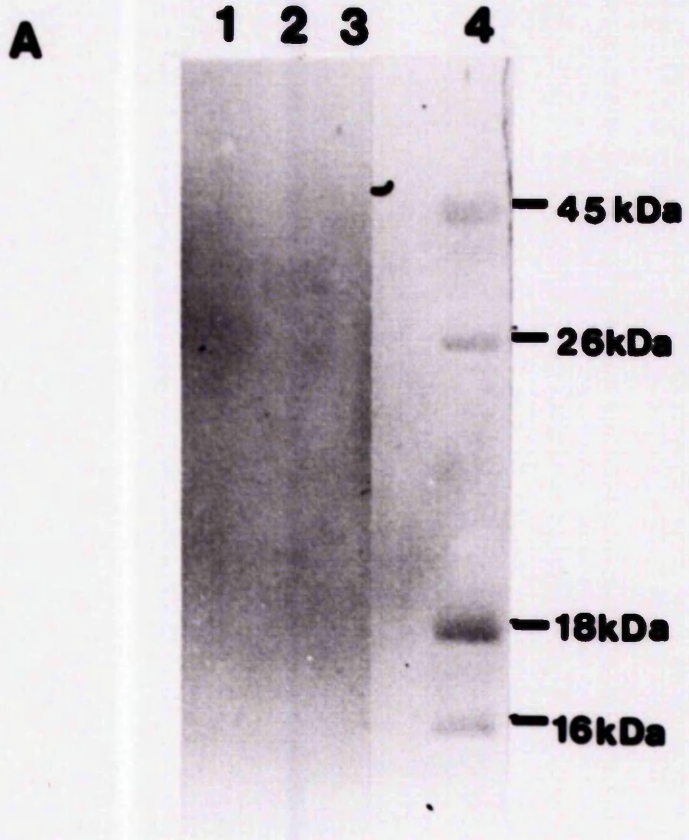
FIGURE 25 - Western analysis of RJKO and RJKO-transfected cell lines using:

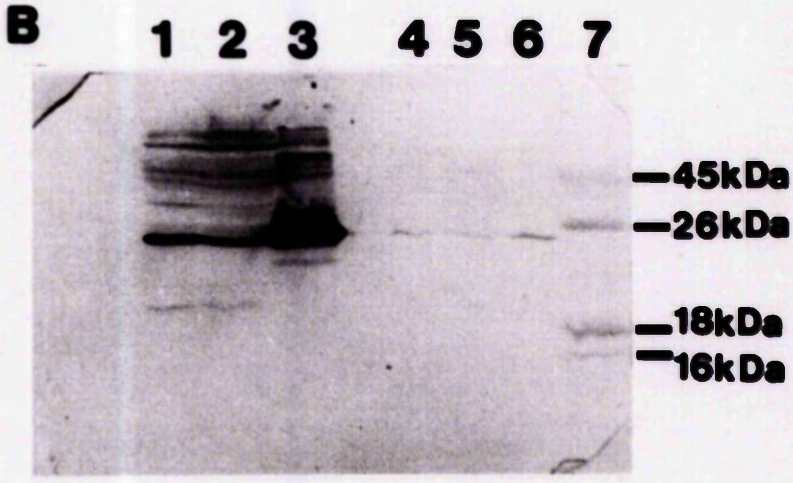
A - Pre-immune serum and cell-free extracts

1 1D
2 7D
3 RJKO
4 Markers

B - Anti-EIII polyclonal antibody and partially purified extracts

1 Total extract of pRPC53 containing bacteria
2 Breakthrough of pRPC53 containing bacteria
3 0.75M NaCl sample of pRPC53 containing bacteria
4 Total extract of 7D
5 Breakthrough of 7D
6 0.75M NaCl sample of 7D
7 Markers


A
1 2 3 4
45kDa
26kDa
18kDa
16kDa


B
1 2 3 4 5 6 7
45kDa
26kDa
18kDa
16kDa

produce the antibody. It is also possible that the polyclonal antibody was raised against EIII epitopes that are common in bacterial proteins.

Although less intense, a band at 25kDa was seen in all three 7D samples. This was more intense in the 0.75M NaCl eluant compared to the load and the break-through, which showed the faintest band. These results confirmed that EIII was present in 7D extracts.

Since EIII was expected in only the cell-free extracts and the 0.75M NaCl eluant, the band in the break-through may be explained by an overloading of the DNA cellulose column, the loss of DNA binding capacity during the extraction or the production of a non-DNA binding form of the enzyme in the cells. The high intensity of the bands seen with the bacterial samples indicates that the DNA cellulose column may have been overloaded as it is unlikely that bacteria would produce an inactive form of the enzyme. Since protein with DNA binding capacity was detected in the 7D extract, the most likely explanation of EIII in the break-through is that a proportion of the EIII lost DNA binding activity during the extraction.

3.1.2 Assay of AP endonuclease activity

An assay that would only measure EIII-type activity was not available (see chapter 4). Hence an AP endonuclease assay was used to try to determine whether the EIII expressed in clones 1D and 7D was active.

Cell-free extracts of RJKO, 1D, 7D and 6E were

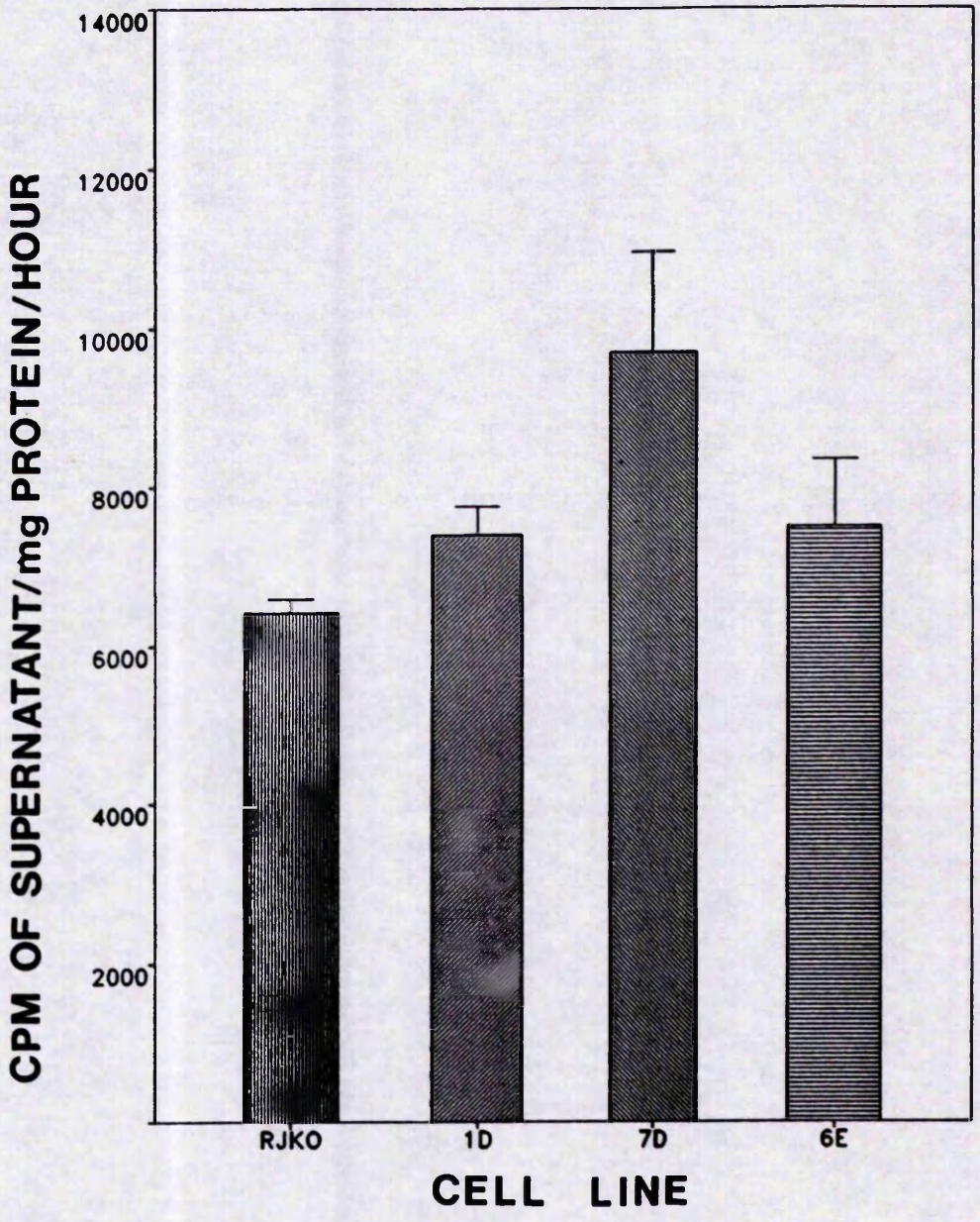
prepared as described in 2.2.3i, using the EIII reaction buffer (2.1.12vii), and assayed in duplicate for their ability to release radioactivity from tritiated, partially depyrimidinated DNA (see 2.2.3viid).

An increase in AP endonuclease activity was not detected in the extract of clone 1D compared to RJKO and 6E (figure 26). However, the extract of clone 7D did show a slight increase in activity compared to RJKO and 6E cells.

Mammalian AP endonucleases also fragmented the partially depyrimidinated substrate and detection of a relatively low level of EIII in a cell-free extract with high endogenous AP endonuclease activity would be difficult: the endogenous AP endonuclease activities of RJKO and 6E were high (6420 cpm released/mg protein/hour) in comparison to another Chinese hamster cell line, xrs7 (3990 cpm released/mg protein/hour). This may explain why an increase in activity was not found in the 1D extract compared to RJKO or 6E. The small increase in AP endonuclease activity in the 7D extract indicates that EIII may be active and it would therefore not be unreasonable to suggest that the EIII in clone 1D could also be active. However, before it can be concluded that active expression of *nth* has been achieved in these cells, it will be necessary to measure activity in cell-free extracts with an assay that can distinguish between EIII and endogenous AP endonucleases (see chapter 4).

FIGURE 26-AP endonuclease activity of RJKO, 1D, 7D and 6E cell-free extracts

Cell-free extracts were assayed in duplicate for the fragmentation of [^{3}H]-labelled partially depyrimidinated T4 phage DNA. The height of the bar represents the average of the two assays and the error is the difference between the duplicates.



3.1.3 Cell cycle and growth analysis

Radiosensitivity is cell cycle dependent (Sinclair & Morton, 1966) and changes in growth have been found to alter the sensitivity of cells to cytotoxic agents such as nitrogen mustard (Wilkinson & Nias 1971). Hence changes in cellular parameters could have caused an alteration in radiosensitivity that was not directly due to the action of EIII, but a consequence of incorporation of bacterial DNA, expression of bacterial message and or protein and even the subcloning procedure. It was therefore necessary to carry out growth and cell cycle analyses on RJKO cells and clones 1D, 7D and 6E.

Growth rate was measured as an increase in cell number with incubation time (see 2.2.2vi). The exponential portion of growth was analysed by linear regression to obtain the doubling time for RJKO, 1D, 7D and 6E. No significant alteration in the doubling time was detected for any of the transfected cell lines compared to the parental cells (table 8).

The proportion of cells in each phase of the cell cycle was determined by FACS of propidium iodide stained cells (see 2.2.2v). A minimum of four samples from different cell passages of exponentially growing cultures were analysed. No change was detected in transfected cell lines compared to the parental cell line (table 8). In clone 7D the apparent higher proportion of cells in G2/M and lower proportion in G1 compared to RJKO cells was not statistically significant.

TABLE 8 - Analysis of cell cycle and growth of RJKO, 1D, 7D and 6E

CELL LINE	% G1	% S	% G2/M	DOUBLING TIME (HOURS)
RJKO	45 ± 2	38 ± 2	16 ± 2	10.9 ± 0.7
1D	47 ± 1	38 ± 3	16 ± 1	12.1 ± 0.3
7D	40 ± 6	36 ± 9	24 ± 3	13.0 ± 1.7
6E	47 ± 5	37 ± 6	17 ± 2	12.5 ± 0.5

A minimum of four samples from different cell passages of exponentially growing cells were stained with propidium iodide and analysed by FACS to obtain the proportion of cells in each phase of the cell cycle (±SE). The doubling time (±SE) was obtained by analysing the exponential portion of the growth curve by linear regression.

3.1.4 Chromosome number

Kasid et al (1989) found that the radiosensitivity of four out of six transfected and two out of four non-transfected clonal cell lines differed significantly from parental NIH/3T3 cells ($p < 0.05$). Detailed karyotype analysis of two nontransfected clonal cell lines with different radiosensitivities showed variation in chromosomal composition. A minute chromosome was found in one of these cell lines but not in the other and this chromosome was only present in approximately 10% of metaphases of the parental cells.

In view of the influence of chromosomal composition on radiosensitivity (for further discussion see 3.4), the average number of chromosomes for clones 1D, 7D and 6E and RJKO cells was determined as described in 2.2.2vii (table 9).

TABLE 9 - Average chromosome number of RJKO and RJKO-transfected cell lines

CELL LINE	AVERAGE CHROMOSOME NUMBER	% TETRAPLOID	PASSAGE NUMBER
RJKO	21 ± 1	0%	25
1D	22 ± 1	0%	11
7D	22 ± 1	0%	6
6E	21 ± 1	0%	11

No statistically significant difference was detected in the chromosome number of clones 1D, 7D and 6E compared to RJKO.

3.1.5 Results of cytotoxicity studies

A colony-forming assay was used to assess the sensitivity of RJKO and the transfected clones 1D, 7D and

6E to killing by radiation, hydrogen peroxide and bleomycin sulphate (see 2.2.2viii). At least three independent survival experiments, using cells of different passage number, were carried out for each cell line. Data were analysed using selected mathematical models in the DRFIT computer programme. The radiation and bleomycin sulphate results were analysed by the alpha, beta model (equation 17, see 2.2.4), which provides the simplest mathematical description of a "shouldered" curve, and attempts were made to analyse the hydrogen peroxide data using the alpha, beta and single-hit multitarget models, adapted to incorporate a quenched dose parameter (section 2.2.4, equations 18 and 20).

For clarity the effects of EIII expression on cell survival will be discussed in 3.4.

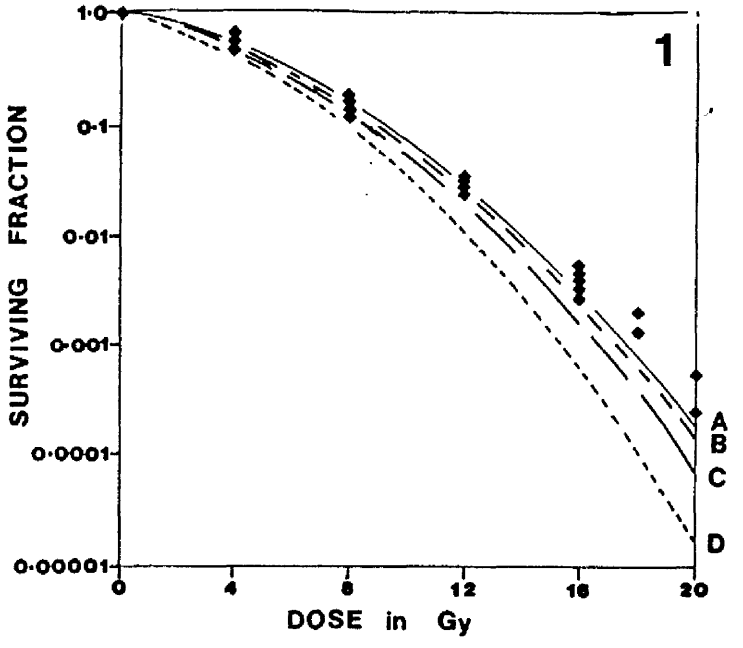
(i) Ionising Radiation

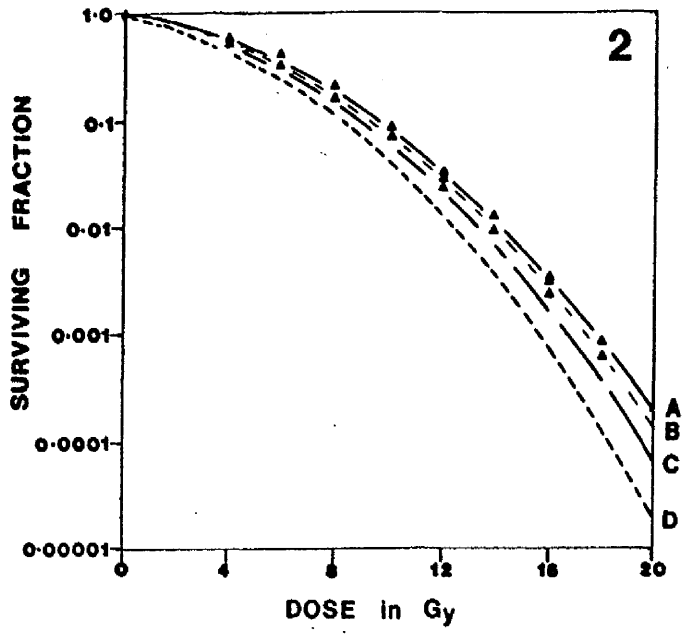
Each of the survival curves had a shoulder and a "near-exponential" terminal portion (figure 27). Analysis of the individual experiments for each cell line (table 10a) showed there was no statistically significant difference between the experiments, except for those of 7D which differed in the beta component.

Data were pooled to compare the radiosensitivities of the cell lines (table 10b). No difference was found between RJKO and 6E, indicating that the transfection process, incorporation of vector sequences in the genome or expression of *neo* did not affect the radiosensitivity of the cells. However, clones 1D and 7D were slightly

FIGURE 27-Survival of RJKO, 1D, 7D and 6E after gamma irradiation

Three independent experiments were carried out for RJKO (A), 1D (C) and 7D (D) and two for 6E (B), using cells of different passage number. The data were analysed by the alpha, beta model of the DRFIT computer programme. The points from all the experiments performed are shown for each cell line individually in graphs 1 (RJKO), 2 (6E), 3 (1D) and 4 (7D).

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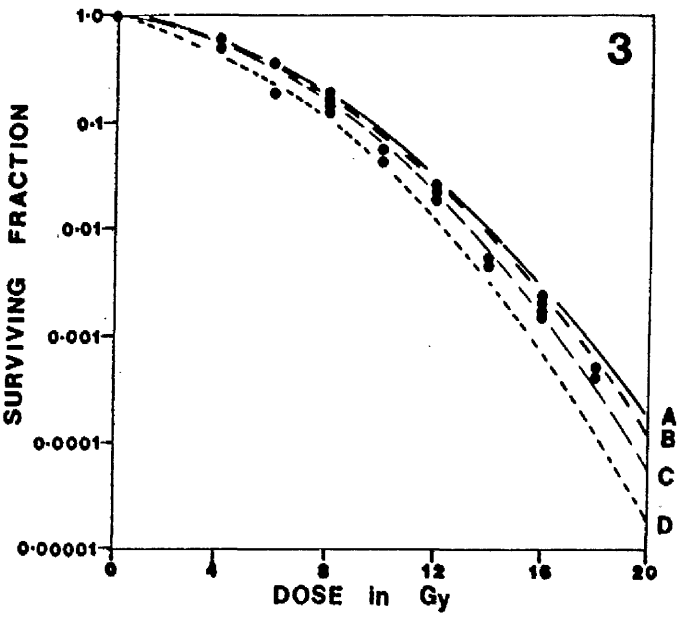
SURVIVING FRACTION
1·0
0·1
0·01
0·001
0·0001
0·00001
0
4
8
12
16
20
DOSE in Gy
A
B
C
D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SURVIVING FRACTION
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0·01
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0·0001
0·00001
0
4
8
12
16
20
DOSE in Gy
A
B
C
D

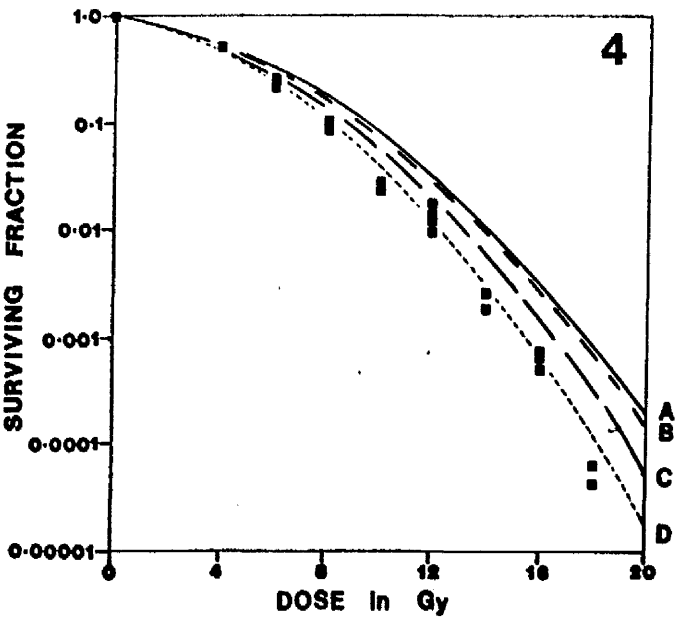

4
SURVIVING FRACTION
1·0
0·1
0·01
0·001
0·0001
0·00001
0
4
8
12
16
20
DOSE in Gy
A
B
C
D

TABLE 10 - Analysis of the Radiosensitivity of RJKO, 1D, 7D and 6E (± SE)

a) Analysis of Individual Experiments

CELL LINE	EXPERIMENT N°	ALPHA (Gy^{-1})	BETA (Gy^{-2})
RJKO	1	0.10 ± 0.02	0.016 ± 0.002
	2	0.10 ± 0.02	0.017 ± 0.002
	3	0.08 ± 0.02	0.018 ± 0.001
1D	1	0.06 ± 0.02	0.021 ± 0.001
	2	0.10 ± 0.03	0.020 ± 0.003
	3	0.05 ± 0.02	0.023 ± 0.001
7D	1	0.07 ± 0.03	0.029 ± 0.003
beta*	2	0.08 ± 0.02	0.024 ± 0.002
	3	0.14 ± 0.02	0.019 ± 0.002
6E	1	0.07 ± 0.02	0.018 ± 0.001
	2	0.09 ± 0.02	0.019 ± 0.002

* - $p < 0.05$ for this parameter

b) Analysis of pooled data

CELL LINE	ALPHA (Gy^{-1})	ALPHA RATIO	BETA (Gy^{-2})	BETA RATIO
RJKO	0.083 ± 0.01	1.00	0.017 ± 0.001	1.00
1D	0.066 ± 0.01	1.26 ± 0.32	0.021 ± 0.001*	0.81 ± 0.06
7D	0.11 ± 0.02	0.76 ± 0.16	0.022 ± 0.002*	0.77 ± 0.07
6E	0.083 ± 0.01	1.00 ± 0.22	0.018 ± 0.001	0.94 ± 0.07

* - $p < 0.05$ compared to RJKO

Data were analysed by the alpha, beta model of the DRFIT computer programme.

more radiosensitive than 6E and RJKO. Differences in the beta component of 1D and 7D compared to the control cell lines indicated an increase in the production of lethal lesions/unit dose at high doses.

(ii) Bleomycin sulphate

The RJKO and 6E data produced curves (figure 28) that consisted of an exponential portion, while 1D produced a slightly shouldered curve and 7D produced a slightly concave curve. Analysis of individual experiments for each cell line showed there was no difference between the experiments, except those of 1D which differed in both the alpha and beta components (table 11a). The value of the alpha component for 6E, experiment 4, also appears to differ from that of the other 6E experiments.

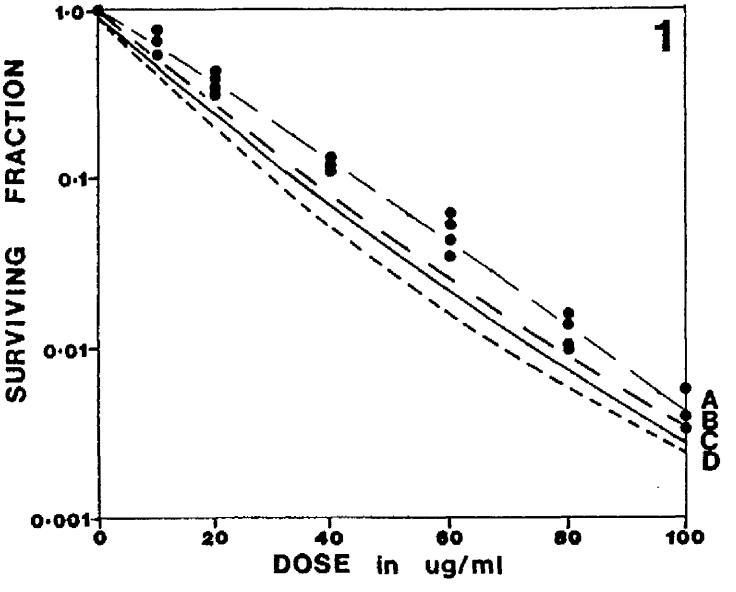
After pooling the data for each cell line it was found that 6E had equivalent clonal survival to RJKO (table 11b). However, clone 1D showed a slight resistance and clone 7D a slight sensitization to bleomycin sulphate, as seen from the significantly different alpha and beta values of these clones compared to the control cells. The alteration is more pronounced in the beta component, suggesting a greater change in sensitivity at high doses.

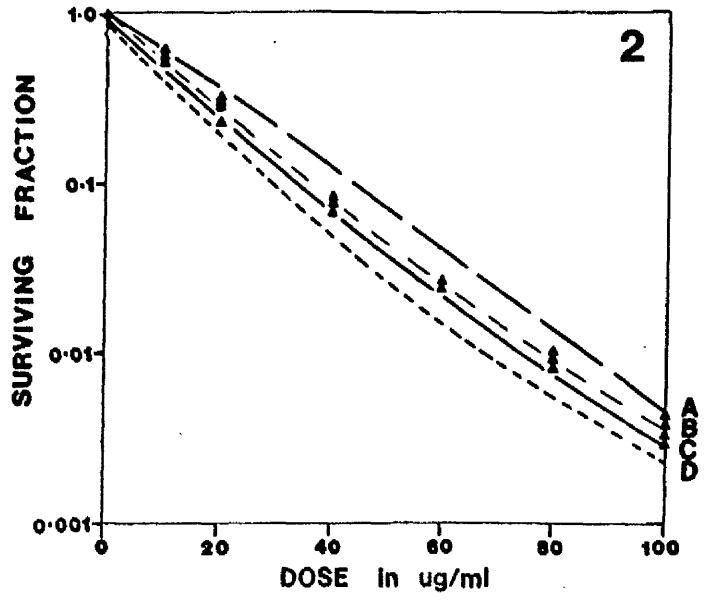
(iii) Hydrogen peroxide

Each of the survival curves had a shoulder, an exponential portion and a plateau region (figure 29). Comparison of the individual experiments for RJKO, 1D, 7D

FIGURE 28- Survival of RJKO, 1D, 7D and 6E after treatment with bleomycin sulphate

At least three independent experiments were carried out using cells of different passage number for 1D (A), 6E (B), RJKO (C) and 7D (D). Data were analysed by the twin exponential model. The points from all the experiments performed are shown for each cell line individually in graphs 1 (1D), 2 (6E), 3 (RJKO) and 4 (7D).

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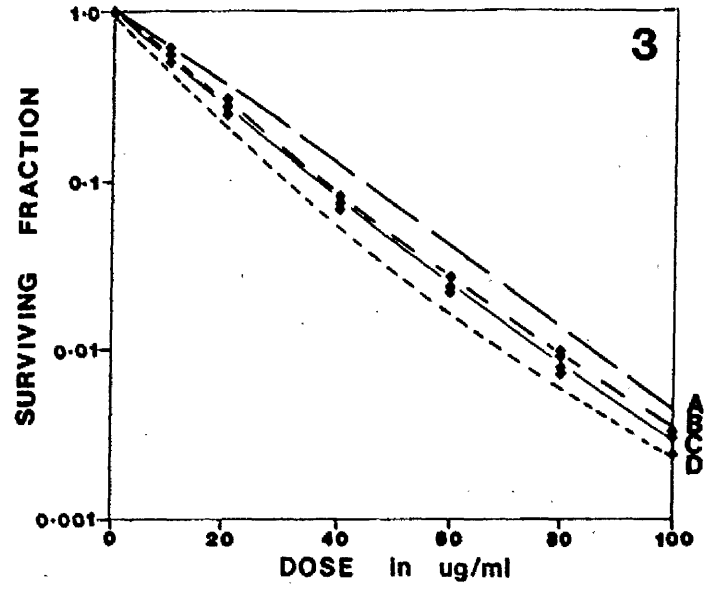
SURVIVING FRACTION
1·0
0·1
0·01
0·001
0
20
40
60
80
100
DOSE in ug/ml
A
B
C
D


3
SURVIVING FRACTION
1·0
0·1
0·01
0·001
0
20
40
60
80
100
DOSE in ug/ml
A
B
C
D

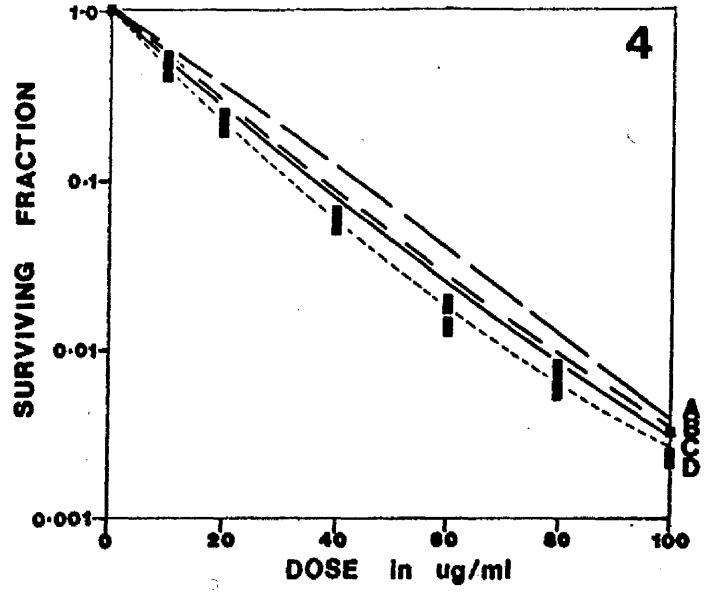

4
SURVIVING FRACTION
1·0
0·1
0·01
0·001
0
20
40
60
80
100
DOSE in ug/ml
A
B
C
D

TABLE 11 - Analysis of the Cytotoxicity Data of RJKO, 1D, 7D and 6E After Bleomycin Sulphate Treatment (± SE)

a) Analysis of Individual Experiments

CELL LINE	EXPERIMENT N°	ALPHA $(ug/ml)^{-1}$	BETA $(ug/ml)^{-2}$
RJKO	1	0.068 ± 0.004	$-0.1x10^{-3} \pm 0.4x10^{-4}$
	2	0.064 ± 0.005	$-0.5x10^{-4} \pm 0.5x10^{-4}$
	3	0.069 ± 0.003	$-0.1x10^{-3} \pm 0.4x10^{-4}$
1D	1	0.075 ± 0.006	$-0.4x10^{-3} \pm 0.1x10^{-3}$
alpha*	2	0.057 ± 0.004	$-0.4x10^{-4} \pm 0.4x10^{-4}$
beta*	3	0.042 ± 0.003	$0.7x10^{-4} \pm 0.3x10^{-4}$
	4	0.062 ± 0.004	$-0.3x10^{-4} \pm 0.5x10^{-4}$
7D	1	0.077 ± 0.004	$-0.2x10^{-3} \pm 0.5x10^{-4}$
	2	0.079 ± 0.005	$-0.2x10^{-3} \pm 0.6x10^{-4}$
	3	0.084 ± 0.005	$-0.3x10^{-3} \pm 0.5x10^{-4}$
	4	0.079 ± 0.003	$-0.2x10^{-3} \pm 0.4x10^{-4}$
6E	1	0.054 ± 0.007	$-0.5x10^{-5} \pm 0.7x10^{-4}$
	2	0.066 ± 0.007	$-0.1x10^{-3} \pm 0.7x10^{-4}$
	3	0.066 ± 0.005	$-0.7x10^{-4} \pm 0.5x10^{-4}$
	4	0.082 ± 0.006	$-0.3x10^{-3} \pm 0.8x10^{-4}$

* - $p < 0.05$ for this parameter

b) Analysis of Pooled Data

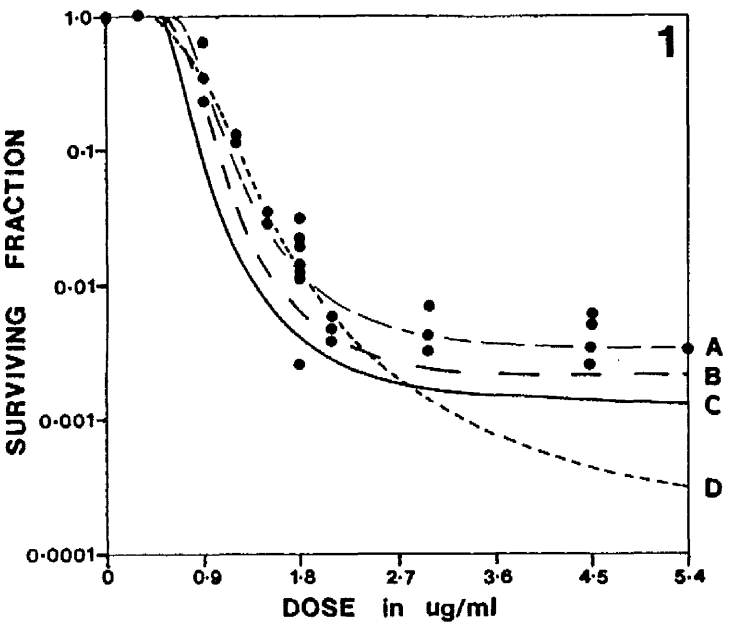
CELL LINE	ALPHA $(ug/ml)^{-1}$	ALPHA RATIO	BETA $(ug/ml)^{-2}$	BETA RATIO
RJKO	0.068 ± 0.003	1.00	-0.00009 ± 0.000037	1.00
1D	0.051 ± 0.003*	1.33 ± 0.10	0.00004 ± 0.00003*	-2.22 ± 1.87
7D	0.08 ± 0.003*	0.85 ± 0.05	-0.00020 ± 0.00003*	0.46 ± 0.20
6E	0.068 ± 0.003	1.00 ± 0.06	-0.00010 ± 0.00003	0.83 ± 0.41

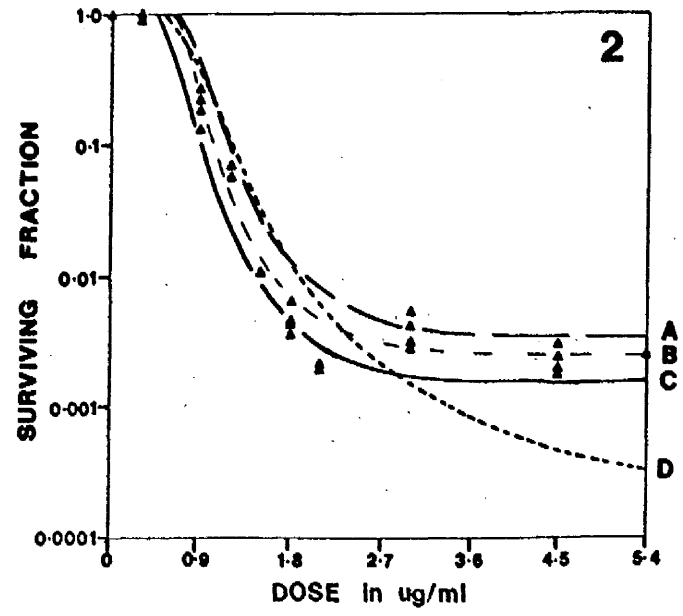
* - $p < 0.05$ compared to RJKO

Data were analysed using the alpha, beta model of the DRFIT computer programme.

FIGURE 29-Survival of RJKO, 1D, 7D and 6E after treatment with hydrogen peroxide

At least three independent experiments were caried out using cells of different passage number for 1D (A), 6E (B), RJKO (C) and 7D (D). Data were analysed by the single-hit, multitarget model, incorporating a quenched dose parameter. The points from all the experiments performed are shown individually for each cell line in graphs 1 (1D), 2 (6E), 3 (RJKO) and 4 (7D).

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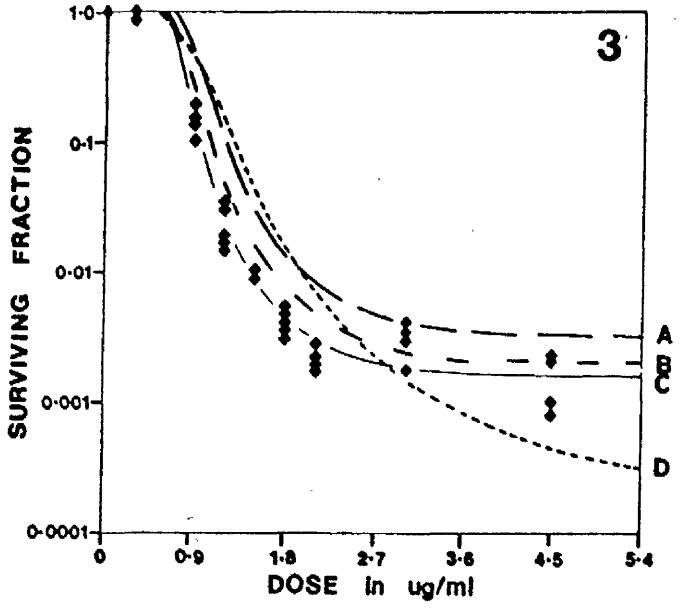
1·0
0·1
0·01
0·001
0·0001
A
B
C
D
0
0·9
1·8
2·7
3·6
4·5
5·4
DOSE in ug/ml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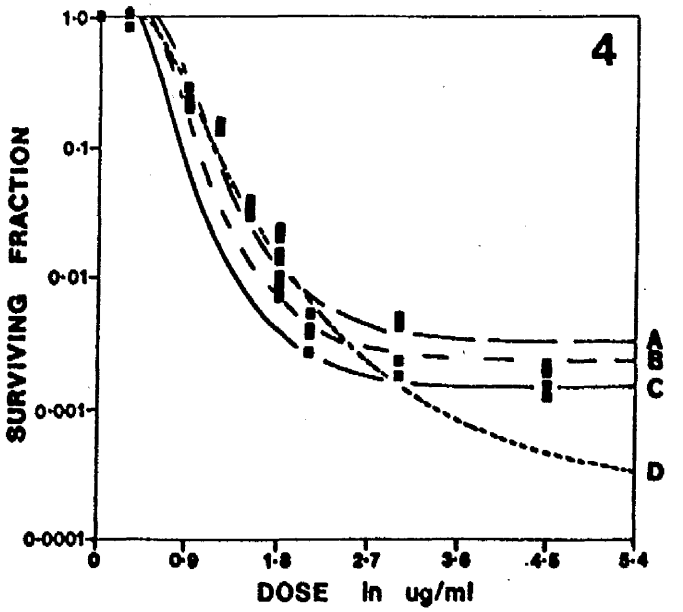
C
D
0
0·9
1·8
2·7
3·6
4·5
5·4
DOSE in ug/ml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1·0
0·1
0·01
0·001
0·0001
A
B
C
D
0
0·9
1·8
2·7
3·6
4·5
5·4
DOSE in ug/ml

and 6E was not possible since surviving fractions at doses between 0.3ug/ml-5.4ug/ml were required to accurately determine the shape of the curve and each experiment only consisted of four different dose points.

Data were pooled to compare the sensitivities of the cell lines (table 12). The standard errors on the fitted parameters were very large (approximately 50%), especially when the alpha, beta model, incorporating a quenched dose, was used. This prevented meaningful statistical analysis. However, 7D was found to be more resistant to the cytotoxic effects of hydrogen peroxide than RJKO and 6E (table 12b) on analysis of the data using the adapted single-hit multitarget model.

3.2 Expression of *nth* in xrs7 cells.

Transfection of pZipnth into RJKO cells showed that it was possible to express EIII in Chinese hamster cells. These cells were not particularly radiosensitive (α = 0.08 Gy^{-1}) and only small alterations in sensitivity to cytotoxic agents were found between 1D and 7D and control cell lines. It was not possible to conclude from these results that the repair of thymine and AP modifications was necessary for cell survival after treatment with the agents mentioned above. On the contrary the results suggested that expression of EIII caused a slight sensitization to radiation and bleomycin sulphate. This may be explained by the introduction of ssb (during EIII repair) opposite other ssb or base damage, increasing the level of dsb, some of which may remain unrepaired (see

TABLE 12 - Analysis of Cytotoxicity Data of RJKO, 1D, 7D and 6E After Treatment with Hydrogen Peroxide (± SE)

a) Using the alpha, beta model incorporating a quenched dose parameter

CELL LINE	ALPHA $(ug/ml)^{-1}$	BETA $(ug/ml)^{-2}$	Q_D (ug/ml)
RJKO	$0.71 \times 10^{-6} \pm 2.5$	7.4 ± 4.0	1.0 ± 0.07
1D	$0.27 \times 10^{-5} \pm 2.1$	6.4 ± 2.7	1.0 ± 0.03
7D	$0.31 \times 10^{-3} \pm 1.7$	4.0 ± 2.3	1.4 ± 0.15
6E	$0.89 \times 10^{-7} \pm 2.3$	6.6 ± 3.2	1.0 ± 0.03

b) Using the single hit, multitarget model, incorporating a quenched dose parameter

CELL LINE	N	Do (ug/ml)	Do RATIO	QD (ug/ml)	RATIO QD
RJKO	$0.9 \times 10^6 \pm 0.4 \times 10^7$	0.03 ± 0.01	1.00	0.61 ± 0.08	1.00
1D	$0.4 \times 10^6 + 0.2 \times 10^7$	0.04 + 0.02	1.23 + 0.8	0.68 + 0.17	1.11 ± 0.32
7D	50 + 77**	0.14 + 0.05**	4.67 + 2.3	1.80 + 0.87**	2.95 ± 1.48
6E	$0.9 \times 10^6 + 0.5 \times 10^7$	0.03 + 0.01	1.00 + 0.6	0.62 + 0.12	1.02 ± 0.24

** $p < 0.05$ compared to RJKO

At least three independent experiments were carried out using cells of different passage number. Data were pooled and analysed using the alpha, beta or single hit multitarget models adapted to incorporate a quenched dose parameter.

3.4 for discussion). Xrs7 cells are sensitive to radiation and bleomycin sulphate and are deficient in the repair of dsb. It was thought that pZipnth-transfected xrs7 cells would be unable to repair the additional dsb introduced by EIII and would be hypersensitive to these cytotoxic agents. Xrs7 cells were therefore transfected with pZipnth to examine if EIII repair could increase cell death after radiation and bleomycin sulphate treatment and to examine further whether EIII-repairable lesions were cytotoxic after H_2O_2 treatment.

3.2.1 Transfection of pZipnth into xrs7 cells.

Lipofection was used to transfer pZipnth and pZipneoSV(X)1 into xrs7 cells, as described in 2.2.2iv. Clones were selected in medium containing 500ug/ml G418 and the transfection frequency (G418 resistant colonies/ cells used to innoculate the culture) was approximately 10^{-5} and 2 x 10^{-4} for pZipnth and pZipneoSV(X)1, respectively. Nine clones were isolated following the transfection of pZipnth and six of these survived: x7nth1, 3, 5, 6, 7 and 9. The pZipneoSV(X)1-transfected control cell line, x7neo1, was also isolated. All clones were maintained in culture medium containing 300ug/ml G418.

3.2.2 Molecular analysis of G418 resistant clones

(i) PCR of "cell-free" extracts

The presence of *nth* in the G418 resistant clones was assessed by PCR (2.2.1xii) amplification of cell extracts

(2.2.1xi), which was predicted to produce a 600bp fragment. The positive and negative controls were cell extracts of clone 7D (RJKO derived cell line) and xrs7, respectively. After gel electrophoresis, the PCR products of six of the seven pZipnth-transfected clones (including the 7D sample) were found to contain a 600bp fragment. The amplified DNA was transferred to a nylon membrane (2.2.1xiv) and hybridized with the [^{32}P]-labelled *nth* probe (see 3.1.1i). Signals of approximately 600bp were found in the PCR products of 7D, x7nth1, 3, 5, 6 and 7, confirming that they contained *nth* (figure 30). The *nth* probe did not hybridize to the negative control, but a very faint 600bp signal was seen in the x7nth9 sample. This indicated that x7nth9 had also incorporated *nth*, even though the 600bp amplified fragment was not apparent after gel electrophoresis.

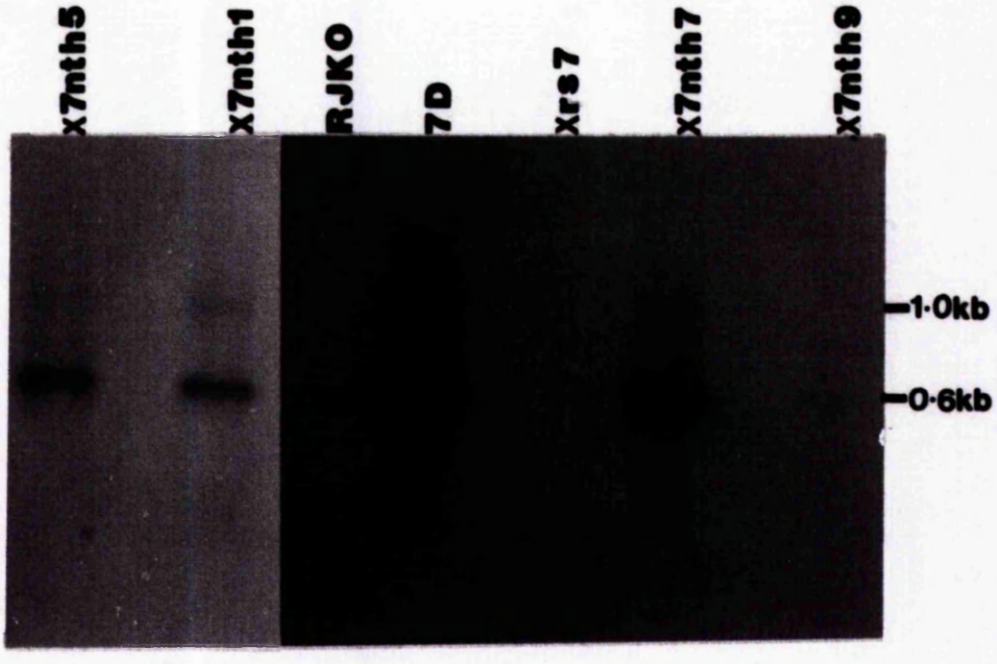
A less intense signal (approximately 1kb) was detected in all the PCR products, except those of x7nth9 and xrs7, which may have been due to amplification of sequences complementary to the *nth* probe in pZipnth.

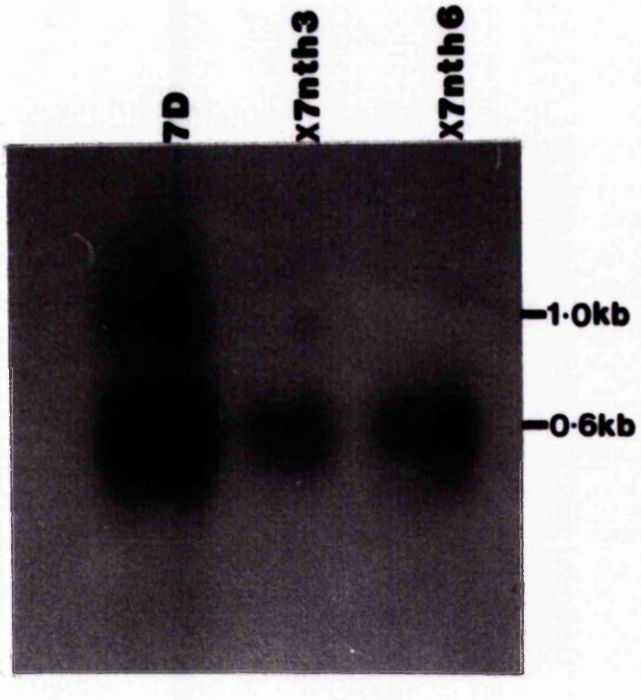
(ii) DNA analysis

Genomic DNA was isolated from xrs7 cells and clones x7nth1, x7nth6 and x7neo1 (2.2.1ix) and dot blot (2.2.1xiva) and Southern analyses (2.2.2xivc) were carried out using the 1kb, [^{32}P] labelled *neo* probe (isolated from pMSGneo) and the *nth* probe (see 3.1.1i), respectively.

FIGURE 30 - PCR analysis of cell extracts of xrs7 and pZipnth or pZipneoSV(X)1 transfected xrs7 cell lines

Attempts were made to amplify *nth* by PCR using oligonucleotides complementary to the nth sequence.





a) DNA dot blot analysis

The negative and positive controls were xrs7 and 6E (RJKO derived cell line) genomic DNA, respectively.

A hybridization signal was detected in each of the transfected clones (figure 31A), but not the negative control, confirming the incorporation of vector sequences into the genomic DNA of transfected cell lines. x7nth1 DNA hybridized to the *neo* probe to produce a more intense signal than DNA from the other clones and hence x7nth1 appears to contain a higher copy number of *neo* than x7neo1, x7nth6 or 6E.

b) Southern analysis

Genomic DNA of xrs7 and clones x7nth1, 5, 6, and 7 and x7neo1 was digested with BamHI or SmaI and subjected to Southern analysis. Analysis of x7nth5 and 7 DNA was carried out in a separate experiment.

A 1kb signal was detected in the BamHI digest of x7nth1 DNA (figure 31C) after an overnight exposure of the filter to X-ray film, but it was necessary to autoradiograph the filters for 3 days in order to detect the 1kb signal in the x7nth5 (figure 31B), 6 (figure 31D) and 7 samples. Southern analysis therefore confirmed that *nth* had incorporated into the genomes of x7nth1, 5, 6 and 7. Signals were not seen in the xrs7 and x7neo1 lanes, even after the 3 day exposure.

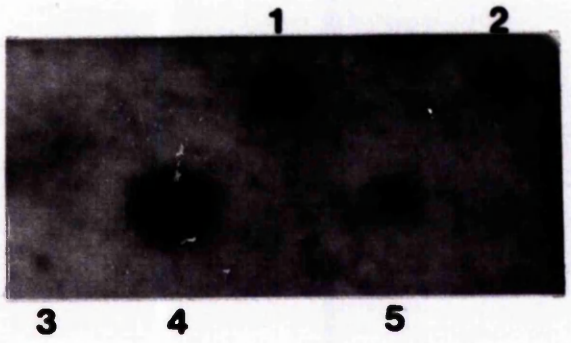
Six other signals (ranging in size from 1.9kb-10kb) were also detected in the BamHI digest of x7nth1 DNA and a 2.5kb signal was seen with BamHI digested x7nth5 DNA.

FIGURE 31 - DNA analysis of xrs7 and pZipnth or pZipneoSV(X)1 transfected xrs7 cells.

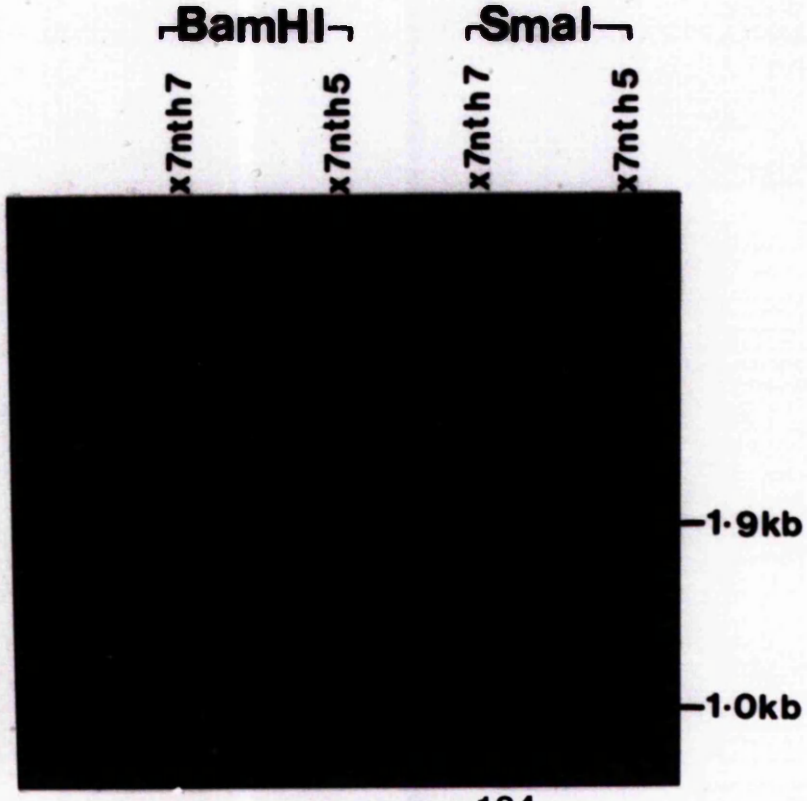
The dot blot and was probed with the *neo* probe and filters for Southern analysis were probed with the *nth* probe.

A - DNA dot blot analysis

1 6E
2 x7neo1
3 xrs7
4 x7nth1
5 x7nth6

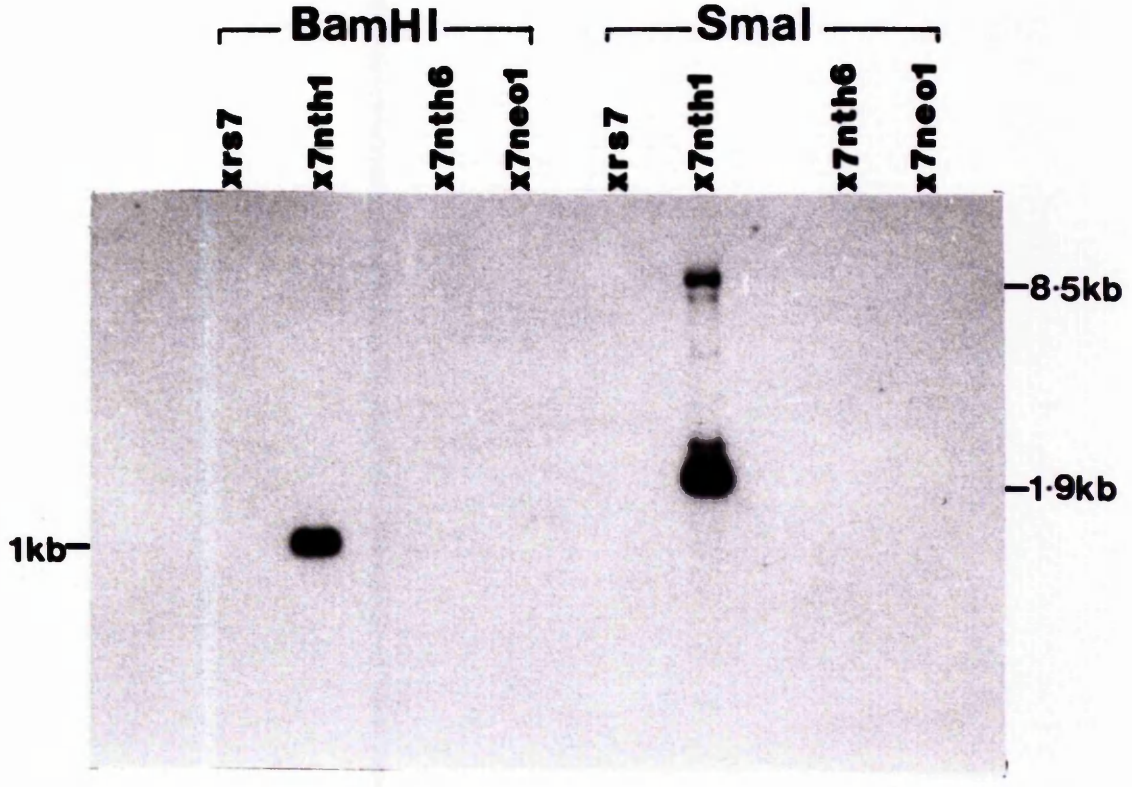


B - Southern analysis of x7nth5 and 7

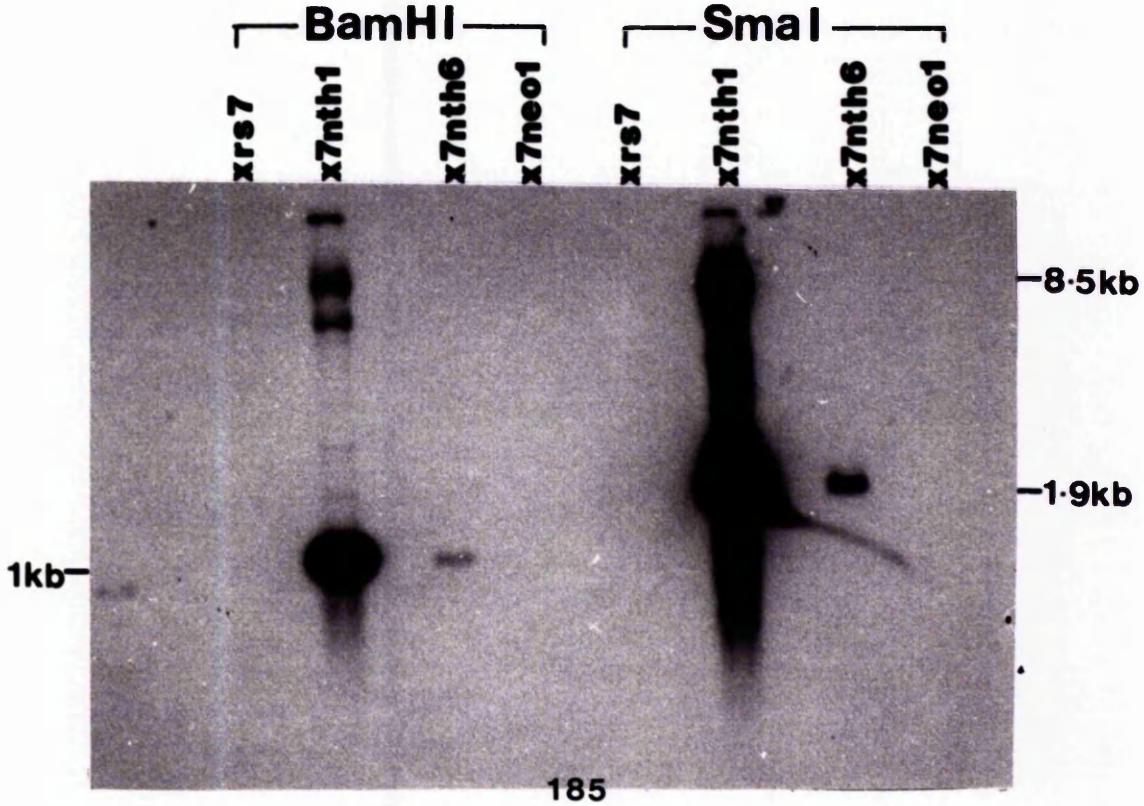


C - Southern analysis of xrs7, x7nth1, x7nth6 and x7neo1

1 - Overnight exposure



2 - Three day exposure



These signals may be due to incomplete BamHI digestion or may indicate that only part of *nth* has been retained within the genome or a small fragment of the pZipnth incorporated.

A 1.9kb signal was present in the SmaI digests of clones x7nth5, 6 and 7, as expected, but one other signal (2.2kb), was also found in the x7nth5 lane. SmaI digestion of x7nth1 DNA produced a range of signals from 1.9kb - 8.5kb, but the 1.9kb signal was the most intense. A range of signals of similar size were found on Southern analysis of RJKO transfected clones and may be explained by partial SmaI digestion, variation in the site of linearization of the vector DNA or degradation of the vector DNA before incorporation (see 3.1.1i b).

By examining the intensity of the signals of the two autoradiographs it is clear that clone x7nth1 has incorporated more copies of *nth* compared to x7nth6, while clone x7nth5 contains a higher copy number than clone x7nth7.

(ii) Western analysis

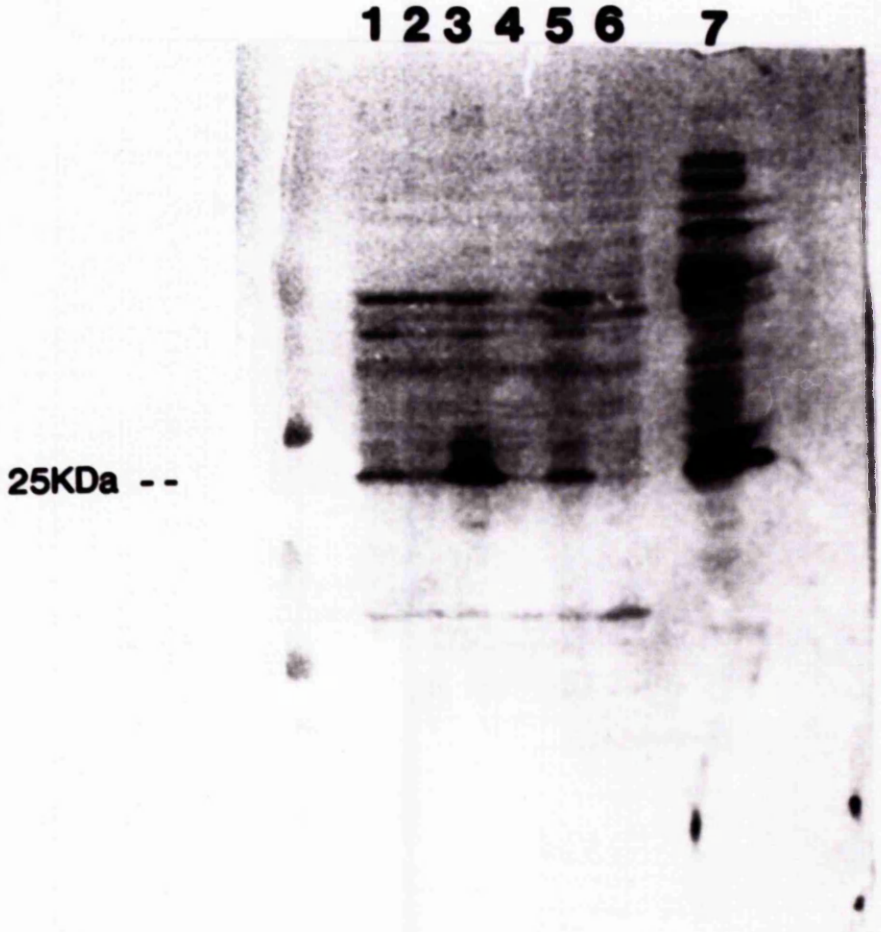
Cell-free extracts of clones x7nth1, 3, 5, 6 and 7 were subjected to western analysis (2.2.1iii) using an anti-EIII polyclonal antibody. The positive control was an extract of *E.coli* harbouring pRPC53, while the negative control was an extract of xrs7 cells.

As previously found (3.1.1iii) the polyclonal antibody cross-reacted extensively with proteins from the bacteria and the Chinese hamster ovary cells (figure 32).

FIGURE 32 - Western analysis of cell-free extracts of xrs7, pZipnth or pZippeSV(X)1 transfected xrs7 cell lines and bacteria harbouring pRPC53.

The filter was probed with the anti-EIII polyclonal antibody.

1	x7nth3	5	x7nth6
2	x7nth5	6	xrs7
3	x7nth1	7	bacteria
4	x7nth7		



The intensity of the cross-reacting bands did confirm that equal quantities of protein from each extract were analysed.

A prominent 25kDa band corresponding to EIII was present in the bacterial extract, as found on analysis of partially purified bacterial extract (see 3.1.1iiib). Bands of similar size were present in extracts of x7nth1, 3, and 6 and not in the xrs7, x7nth5 and 7 extracts. It is therefore reasonable to conclude that transcription and translation of *nth* has been achieved in clones x7nth1, 3 and 6. The intensity of the bands in these three clones indicated that the x7nth1 extract contains more EIII protein than x7nth6 and x7nth3.

(iv) Southern analysis using HpaII and MspI digestion

Clones x7nth1, 5, 6, and 7 have incorporated *nth* (see 3.2.1ib), but only cell-free extracts (3.2.1.iii) of clones x7nth1 and x7nth6 contained EIII protein.

It is possible that methylation of the *nth* sequence by Chinese hamster DNA-cytosine methyltransferases may have occurred after incorporation of pZipnth into the genome. Methylation of *nth* or the promoter from which it is operated (provided by the 5'LTR of the MoMLV) could prevent transcription of the sequence. An alteration in the methylation pattern of the *ogt* gene (bacterial gene encoding alkytransferase) was previously detected in genomic DNA of an *ogt* transgenic mouse, which did not express *ogt* (Harris 1990). It is unlikely that methylation of the promoter prevented *nth* expression since

neo is operated from the same promoter as *nth* in pZipnth and x7nth5 and 7 are G418 resistant.

Certain RE are methylation sensitive, i.e. will not cleave the DNA if the recog-nition sequence is methylated in a certain position. Isoschizomeric RE are also available that cleave the DNA at similar recognition sequences, even if the DNA is methylated. Examples of these are HpaII and MspI. Both recognise the following sequence:

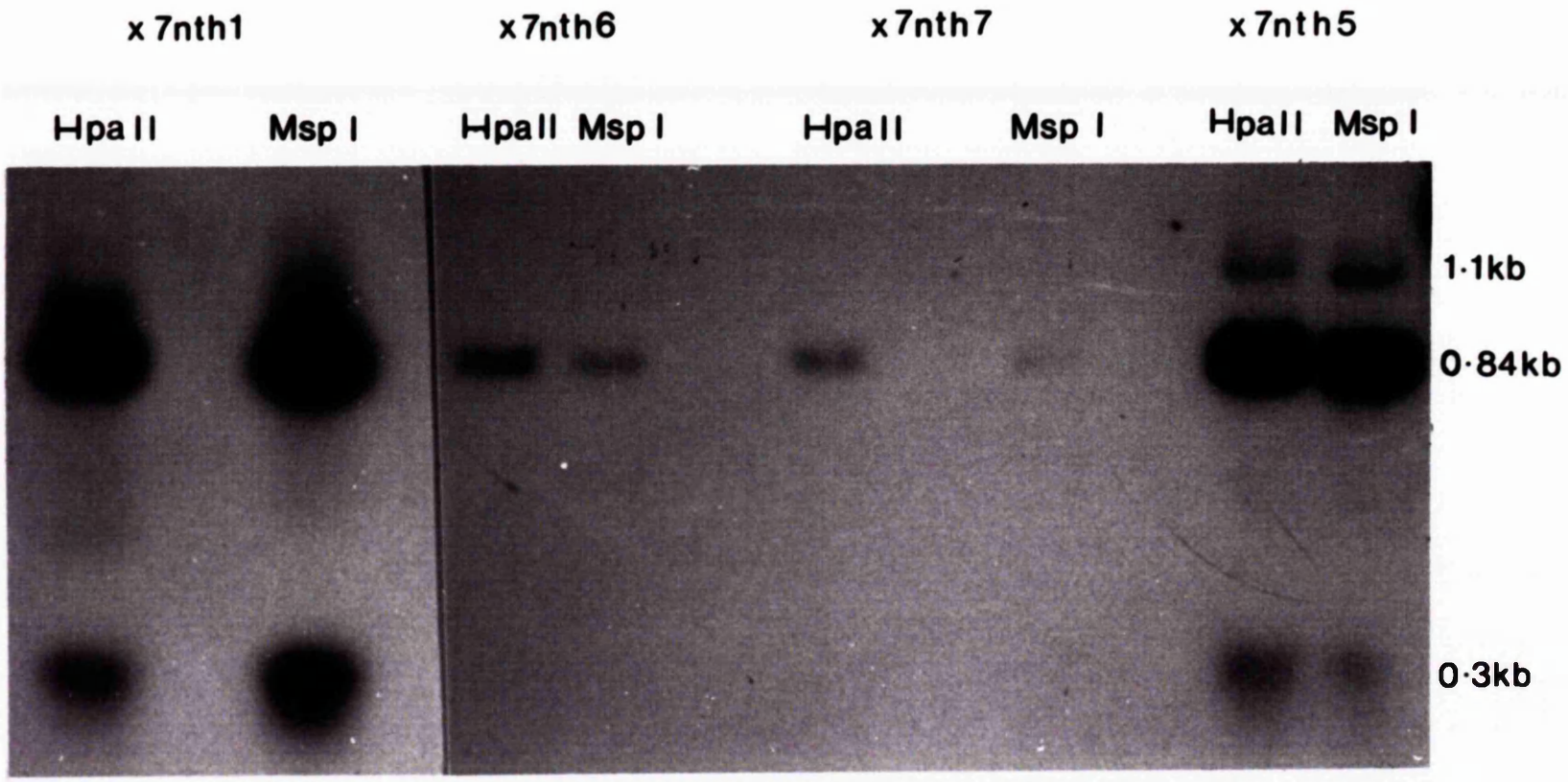
5′ C C G G 3′

If 5 methyl-cytosine is situated at C in the recognition sequence HpaII will not cleave the DNA. Five HpaII sites are known to exist in pZipnth: *nth* contains 4 (170, 317, 348 and 458bp downstream of the 5′ end of *nth*) and 1 site is situated at approximately the 5′ end of *neo*. If no other recognition sites are present in the remaining sequences between *nth* and *neo*, four bands of sizes 147, 31, 110 and 0.9kb should be seen on Southern analysis using the *nth* probe. The size of the fragment resulting from the cleavage of the HpaII site at the 5′ end of the *nth* sequence will be determined by the next site upstream of *nth*.

Genomic DNA of x7nth1, 5, 6 and 7 was digested with HpaII or MspI (2.2.1iii) and subjected to Southern analysis (2.2.1xivc), using the *nth* probe (see 3.1.1i). A 0.84kb signal was detected in all the digests (figure 33) and the probe also hybridized to DNA of 1.1kb and 0.3kb in the x7nth5 HpaII and MspI samples. Only x7nth1 DNA

FIGURE 33 - Southern analysis using HpaII and MspI digests of DNA from x7nth1, 5, 6 and 7

The filter was probed with the *nth* probe.



produced different signals in the HpaII and MspI lanes: 0.84kb and 0.3kb signals were produced by MspI digestion, while 1kb, 0.84kb, 0.54kb, 0.4kb and 0.3kb were detected in the HpaII lane. Since the "extra" signals in the HpaII digest were faint, it is possible that only a fraction of *nth* in the x7nth1 genome is methylated. There was no indication of methylation in x7nth5 and 7 DNA. However, it is possible that methylation may have occurred at sites that could not be detected by DNA digestion with these RE.

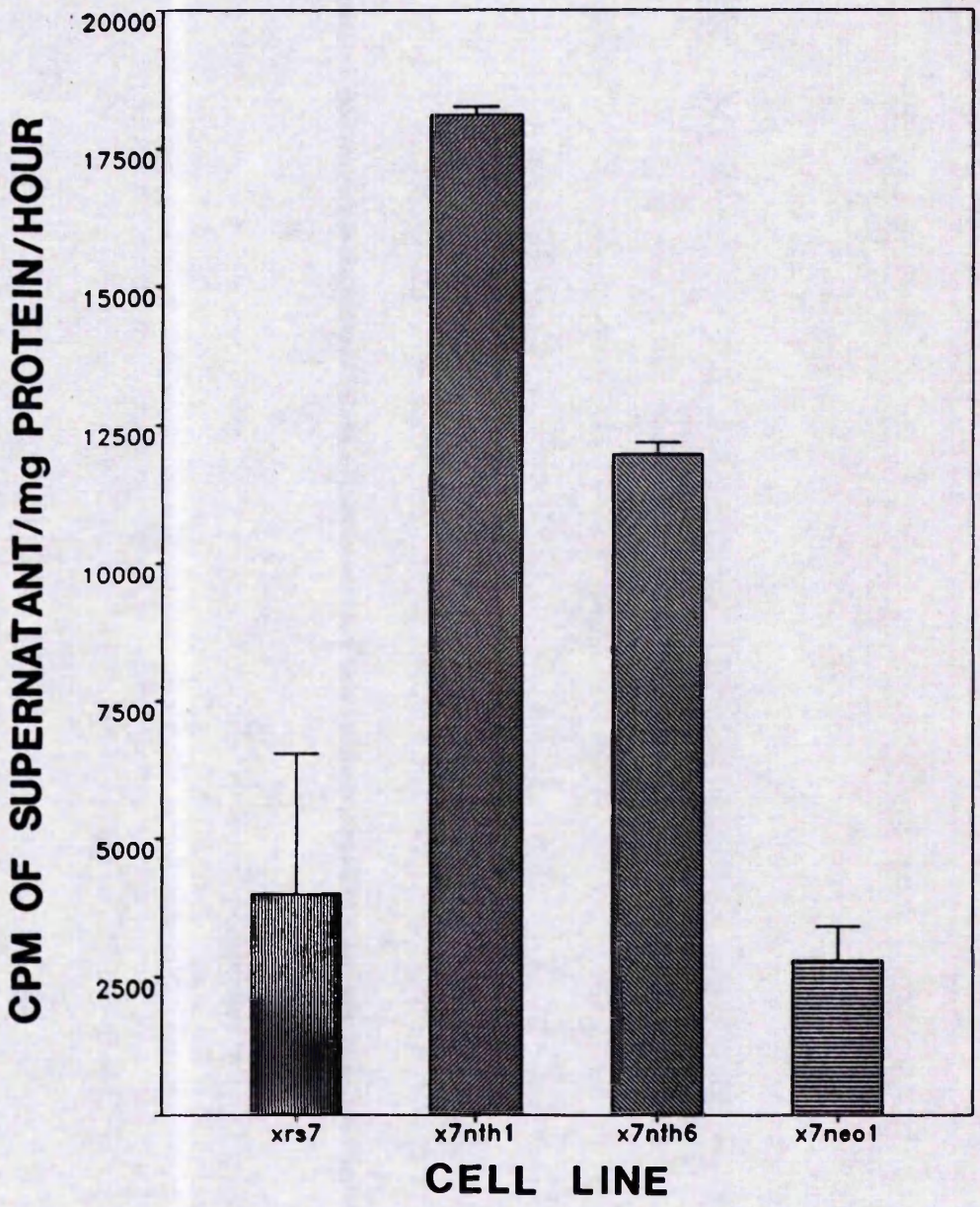
3.2.3 Assay of AP endonuclease activity

Cell-free extracts of xrs7 and clones x7nth1, x7nth6, x7neo1 were assayed in duplicate for AP endonuclease activity (see 2.2.3vii).

Clone x7neo1 had equivalent AP endonuclease activity to xrs7 (figure 34). Hence the insertion of vector sequences into the genome and expression of *neo* did not affect the endogenous AP endonuclease activity. However, AP endonuclease activity 3 and 4 times that of the control cell lines was detected in x7nth6 and x7nth1, respectively. These results confirmed that EIII expression was greater in clone x7nth1 compared to x7nth6. The intensity of the EIII protein signal on western analysis suggested that the x7nth1 extract contained at least 50% more EIII than x7nth6. X7nth1 does appear to express approximately twice the additional AP endonuclease activity of x7nth6, if the endogenous AP endonuclease activity (3990cpm/mg protein /hour) is

FIGURE 34-AP endonuclease activity of xrs7, x7nth1, x7nth6 and x7neo1 cell-free extracts

Cell-free extracts were assayed in duplicate for the fragmentation of [^{3}H]-labelled partially depyrimidinated T4 phage DNA. The height of the bar represents the average of the two assays and the error is the difference between the duplicates.



subtracted from the activities in extracts of x7nth1 (18100 cpm/mg protein /hour) and 6 (11970 cpm/mg protein /hour).

3.2.4 Cell cycle and growth analysis

It was found that clones x7nth6 grew slower and x7neo1 grew faster than xrs7 (table 13), while clone x7nth1, which expressed EIII to a greater extent than x7nth6 (see above), grew at approximately the same rate as the parental cells. This suggests that changes in the growth rate of pZipnth-transfected cells were not necessarily due to expression of EIII. Therefore it is possible that incorporation of vector DNA, expression of *neo* or subcloning the cell population may have been responsible.

The proportion of cells in each of the phases of the cell cycle was determined by FACS analysis of propidium iodide stained cells (see 2.2.2viii). No significant differences were found when comparing xrs7 and clone x7nth6. However, clone x7nth1 had an increased percentage of cells in G1 and a decreased percentage in G2/M compared with xrs7 (table 13). Clone x7neo1 was found to have a lower proportion of cells in G1 compared to xrs7, but the other phases had not been significantly altered by the transfection.

3.2.5 Chromosome number

The average number of chromosomes was determined for xrs7 and clones x7nth1, x7nth6 and x7neo1 (2.2.2vii).

TABLE 13 - Analysis of cell cycle and growth of xrs7, x7nth1, x7nth6 and x7neo1

CELL LINE	% G1	% S	% G2/M	DOUBLING TIME (HOURS)
xrs7	50 ± 3	13 ± 2	37 ± 2	17.4 ± 0.5
x7nth6	52 ± 3	16 ± 2	32 ± 2	21.2 ± 0.6*
x7nth1	64 ± 3*	9 ± 1	27 ± 2*	18.5 ± 0.2
x7neo1	42 ± 2*	23 ± 3*	36 ± 3	15.1 ± 0.5*

* $p < 0.05$ compared to xrs7 cells

The exponential portion of the growth curves of the parental and transfected cell lines were analysed by linear regression to obtain the doubling time for each cell line (± SE). The percentage of cells in each of the phases of the cell cycle (± SE) was determined by FACS analysis of propidium iodide stained cells. A minimum of four samples of different passage number were examined.

Only 50 metaphases were counted for the x7nth1, passage 4 sample.

TABLE 14 - Average chromosome number of xrs7 and xrs7-transfected cell lines

CELL LINE	AVERAGE CHROMOSOME NUMBER	% TETRAPLOID	PASSAGE NUMBER
xrs7	21 ± 1	0%	14
x7nth6	21 ± 2	6% (39 ± 3)	7
x7nth1	36 ± 4	91%	14
x7nth1	36 ± 3	92%	4
x7neo1	21 ± 1	11% (39 ± 5)	14

Although no statistical difference in chromosome number was found between x7nth6 or x7neo1 and xrs7, clone x7nth1 was found to be tetraploid. This was not due to subculturing the cell line, since cells of passage 4 were also tetraploid. Tetraploid metaphases (average number of chromosomes for these metaphases is shown in brackets in table 14) were present in x7neo1 and x7nth6 and also in x7pLJ9 samples (see chapter 5) and a small percentage of x7nth1 metaphases were found to be diploid in cells of passage 4 (8% had an average chromosome number = 21 ± 2) and 14 (9% had an average chromosome number = 22 ± 2). The effect of increase in ploidy on cellular sensitivity to cytotoxic agents will be discussed in 3.4.

3.2.6 Results of cytotoxicity studies

The twin-exponential model of the DRFIT computer programme (see 2.2.4) was used to analyse the radiation and bleomycin sulphate results, since this model provided the simplest mathematical description of a biphasic

curve. Attempts were made to analyse the hydrogen peroxide data using the alpha, beta model, incorporating a quenched dose parameter. However, from the large errors on the alpha and beta parameters (see 3.2.6iii) it was apparent that this model was unable to accurately analyse the data. Therefore hydrogen peroxide data were analysed by the single-hit multitarget model, adapted to incorporate a quenched dose parameter (see 2.2.4).

For clarity the effect of *nth* expression on cell survival will be discussed in 3.4.

(i) Ionising radiation

It was found that there were no significant differences between the individual experiments for x7neo1 or x7nth1 (table 15a), indicating that the experiments for these particular cell lines were reproducable. The errors of the fitted parameters are large and are probably due to difficulties in determining the shape of the curve at the point where the sensitive portion ends and the resistant portion begins. The inaccurate fitting of the first portion of the curve occurred if doses < 1 Gy were not examined in the experiment. The data of the x7nth6 individual experiments were only significantly different in the percentage of the resistant portion of the population. The xrs7 individual experiments were not different in the $_1$Do (Do of the sensitive portion of the population), but comparison of the $_2$Do (Do of the resistant portion of the population) parameter and the percentage of the resistant portion of the population was

TABLE 15 - Analysis of the Radiosensitivity of xrs7, x7nth1, x7nth6 and x7neo1 (± SE)

a) Analysis of individual experiments

CELL LINE	EXPERIMENT N°	$_1$Do Gy	$_2$Do Gy	% RADIO-RESISTANT POPULATION
xrs7	1	0.066 ± $3x10^6$	1.17 ± 0.17	25 ± 10
	2	0.092 ± 639	1.20 ± 0.15	14 ± 5
	3	0.270 ± 0.05	0.88 ± 0.09	30 ± 9
	4	0.370 ± 0.13	1.21 ± 0.45	20 ± 18
x7nth6	1	0.130 ± 11	1.23 ± 0.17	13 ± 5
% pop*	2	0.290 ± 0.06	1.01 ± 0.11	32 ± 8
	3	0.260 ± 0.17	1.00 ± 0.1	22 ± 8
x7nth1	1	0.089 ± 2723	1.36 ± 0.20	27 ± 10
	2	0.410 ± 0.17	1.26 ± 0.63	25 ± 26
	3	0.300 ± 0.40	1.18 ± 0.30	31 ± 22
	4	0.350 ± 0.11	3.95 ± 11	10 ± 15
x7neo1	1	0.480 ± 0.29	0.83 ± 0.57	32 ± 100
	2	0.230 ± 0.15	0.89 ± 0.08	54 ± 16
	3	0.042 ± $1x10^{11}$	0.84 ± 0.11	44 ± 22
	4	0.440 ± 0.20	0.86 ± 0.28	29 ± 42

* $p < 0.05$ for this parameter

b) Analysis of pooled data

CELL LINE	$_1$Do (Gy)	$_1$Do RATIO	$_2$Do (Gy)	$_2$Do RATIO
xrs7	0.31 ± 0.04	1.00	1.13 ± 0.07	1.00
x7nth6	0.29 ± 0.03	1.07 ± 0.17	1.12 ± 0.07	1.01 ± 0.09
x7nth1	0.34 ± 0.04	0.91 ± 0.16	1.25 ± 0.11	0.90 ± 0.10
x7neo1	0.39 ± 0.11	0.80 ± 0.24	0.85 ± 0.12	1.33 ± 0.21

% OF RADIORESISTANT PORTION (± SE)

xrs7	18 ± 7
x7nth6	18 ± 3
x7nth1	26 ± 4**
x7neo1	38 ± 21

** $p < 0.05$ compared to xrs7 cells

Data were analysed using the twin-exponential model of the DRFIT computer programme. Analysis of the individual experiments of xrs7 found that the experiments were significantly different. However, it was not possible to determine whether % of resistant population or $_2$Do parameters of the xrs7 experiments were statistically different, but $_1$Do was not significantly different for these experiments.

not possible since the iteration procedures in the analysis did not converge.

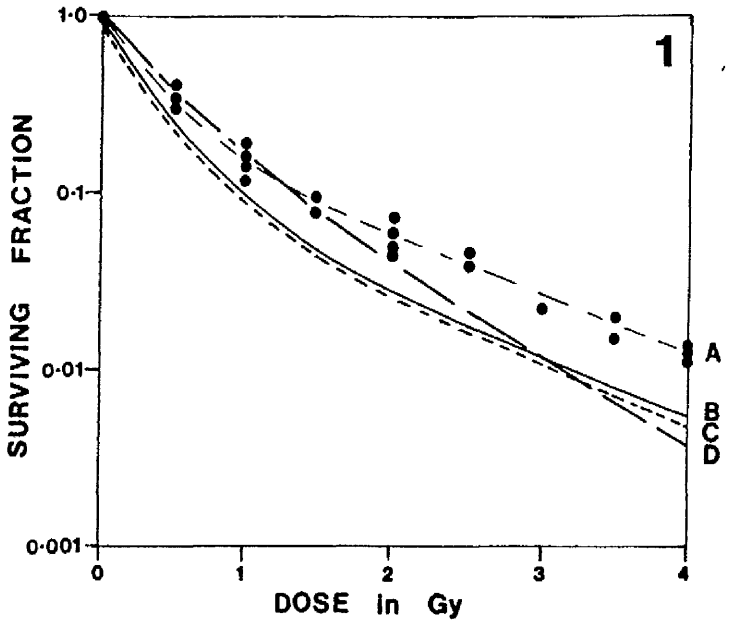
Data for the individual experiments were pooled for each cell line and the analysis to compare the sensitivities of the cell lines was carried out. The survival curves of xrs7, x7nth1, x7nth6 and x7neo1 are shown in figure 35 and the data is summarised in table 15b. The parental and transfected cell lines were not significantly different in the Do values of either the sensitive and resistant portions of the population. However, the x7nth1 population contained a higher resistant portion compared to xrs7, which may have been a result of the alterations detected in the cell cycle (see 3.2.4), since it has previously been reported that G2/M is the most sensitive and S the most resistant phase in Chinese hamster cells (Sinclair & Morton 1966). Other changes in cellular characteristics (see 3.2.4) and the increase in ploidy of x7nth1 cells (3.2.5, see 3.4 for discusson) did not significantly affect the radio-sensitivity parameters of the transfected clones compared to the parental cells.

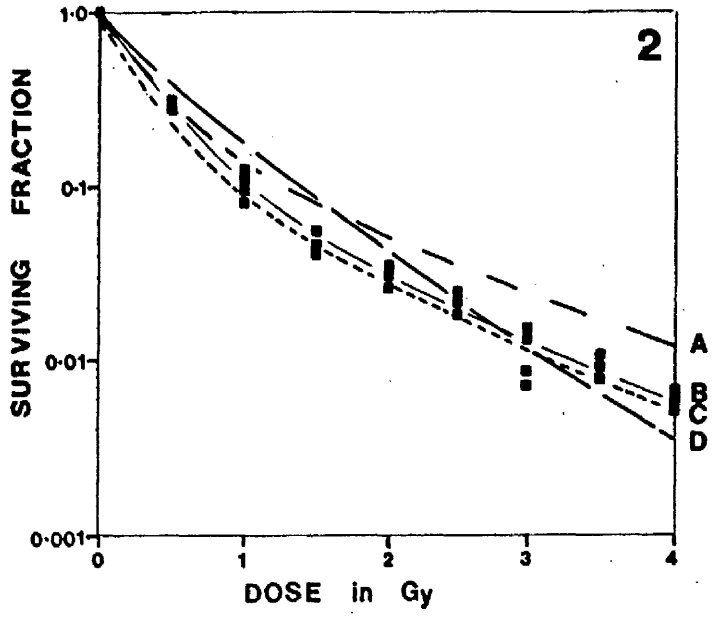
(ii) Bleomycin sulphate

Significant differences were not detected between the individual experiments of each cell line (table 16a). The standard errors of the parameters were large, probably due to the fitting of a biphasic curve. When the data of the individual experiments were pooled for each cell line (see table 16b) the analysis was more

FIGURE 35- Survival of xrs7, x7nth1, x7nth6 and x7neo1 after gamma irradiation

Four independent experiments were carried out using cells of different passage number for x7nth1 (A), xrs7 (B), x7nth6 (C) and x7neo1 (D). Data were analysed by the twin exponential model. The points from all the experiments performed are shown individually for each cell line in graphs 1 (x7nth1), 2 (xrs7), 3 (x7nth6) and 4 (x7neo1).

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4
DOSE in Gy
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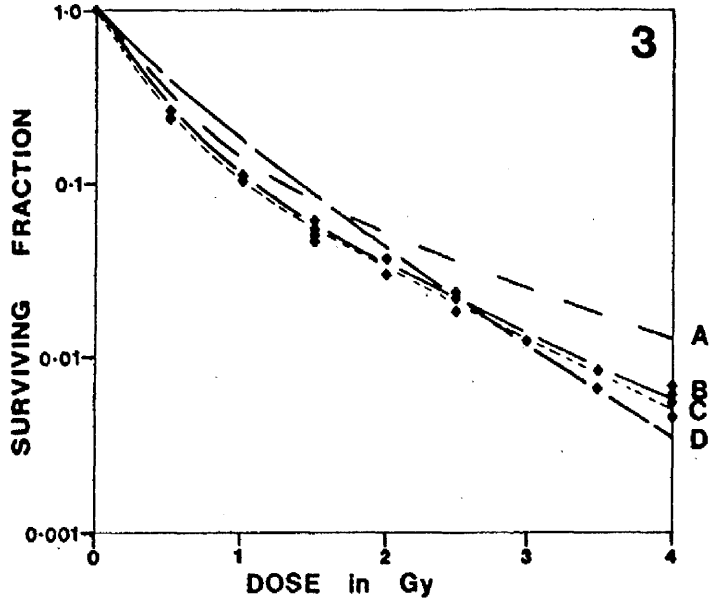
3
SURVIVING FRACTION
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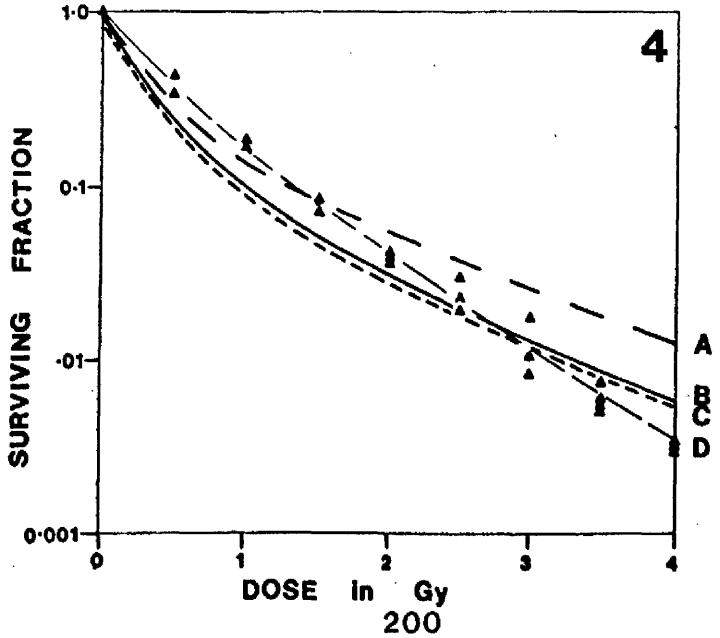
4
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2
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4
DOSE in Gy
A
B
C
D

TABLE 16 - Analysis of the Cytotoxicity of xrs7, x7nth1, x7nth6 and x7neo1 After Treatment with Bleomycin Sulphate (± SE)

a) Analysis of individual experiments

CELL LINE	EXPERIMENT No	$_1D^o$ (ug/ml)	$_2Do$ (ug/ml)	% RESISTANT POPULATION
xrs7	1	0.150 ± 0.050	0.60 ± 0.22	34 ± 20
	2	0.130 ± 0.030	0.50 ± 0.07	31 ± 9
	3	0.240 ± 0.040	1.75 ± ~3	5 ± 7
x7nth6	1	0.067 ± 0.005	0.46 ± 0.04	4 ± 1
	2	0.052 ± 0.003	0.54 ± 0.09	4 ± 1
	3	0.046 ± 0.003	0.42 ± 0.05	6 ± 1
x7nth1	1	0.140 ± 0.051	0.59 ± 0.50	8 ± 11
	2	0.024 ± ~9	0.26 ± 0.04	48 ± 20
	3	0.120 ± 0.027	0.39 ± 0.09	17 ± 10
x7neo1	1	0.059 ± 1678	0.45 ± 0.03	71 ± 10
	2	0.330 ± 8	0.43 ± 2	81 ± 33
	3	0.065 ± 1700	0.48 ± 0.04	86 ± 16

b) Analysis of pooled data

CELL LINE	$_1Do$ (ug/ml)	$_1Do$ RATIO	$_2Do$ (ug/ml)	$_2Do$ RATIO
xrs7	0.12 ± 0.03	1.00	0.48 ± 0.04	1.00
x7nth6	0.05 ± 0.01*	2.45 ± 0.55	0.41 ± 0.04	1.17 ± 0.14
x7nth1	0.12 ± 0.01	1.00 ± 0.23	0.35 ± 0.04*	1.37 ± 0.18
x7neo1	0.01 ± 2.60	~11	0.45 ± 0.02	1.07 ± 0.10

% BLEOMYCIN SULPHATE RESISTANT PORTION (± SE)

xrs7	39 ± 7
x7nth1	21 ± 6*
x7nth6	7 ± 1*
x7neo1	84 ± 7*

* $p < 0.05$ compared to parental cells

Data were analysed by the twin-exponential model of the DRFIT computer programme. Standard errors of the parameters of the analysis of the individual experiments were large. To obtain an accurate fitting survival data was required at the doses at the end of the first and beginning of the second exponential phase but each experiment consisted of only four different dose points. Hence accurate analysis of individual experiments was not possible.

accurate and it was possible to compare the sensitivities of the cell lines.

Both x7nth1 and x7nth6 were markedly more sensitive than xrs7 and x7neo1 (figure 36): the sensitive portion of x7nth6 shows a 2.5 fold decrease in Do, while it is the resistant portion of x7nth1 which has a 1.4 fold decrease in Do compared to xrs7 (table 16b). Each of the transfected cell lines also showed a change in the percentage of sensitive and resistant portions of the population compared to xrs7: x7neo1 had an increased resistant portion, while x7nth1 and x7nth6 had a decreased resistant portion.

Expression of EIII therefore greatly enhanced the sensitivity of xrs7 cells to bleomycin sulphate.

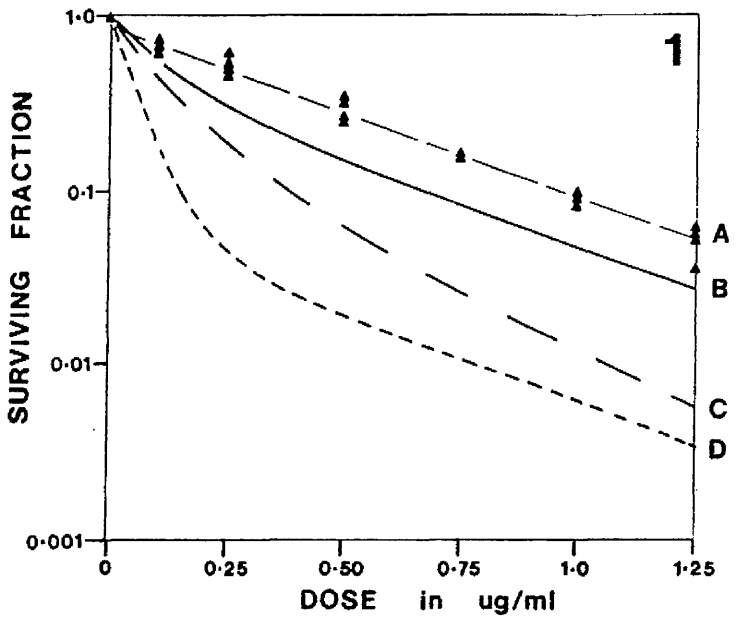
(iii) Hydrogen peroxide

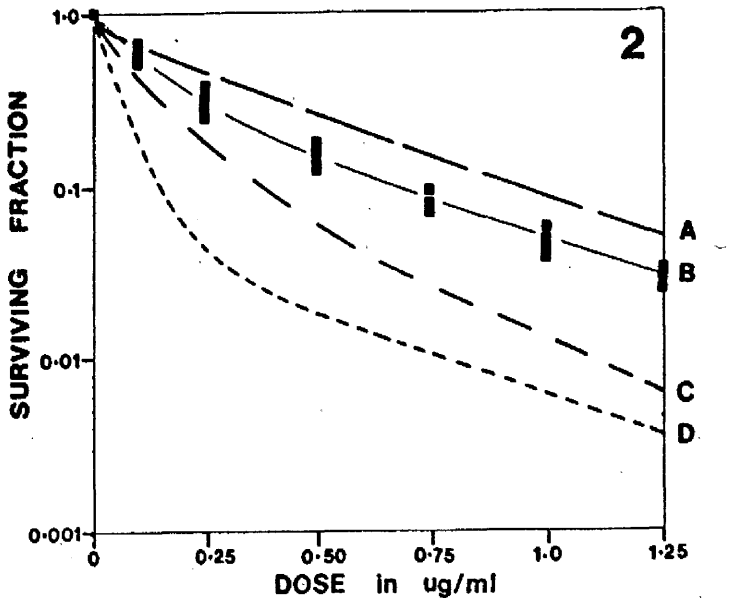
It was not possible to examine the reproducibility of the individual experiments for each cell line, as each experiment examined only four different doses. For an accurate fitting of the curve and hence an accurate analysis, survival data was required for doses between 0.09ug/ml-3ug/ml. This could only be achieved by pooling the data from the individual experiments for each cell line.

Each of the survival curves had a shoulder, an exponential portion and a plateau (figure 37). Clone x7nth1 is significantly more resistant to hydrogen peroxide than xrs7 and x7neo1, showing a two fold increase in Do and Q_D (table 17b), while x7nth6 is

FIGURE 36- Survival of xrs7, x7nth1, x7nth6 and x7neo1 after treatment with bleomycin sulphate

Three independent experimnts were carried out using cells of different passage number for x7neo1 (A), xrs7 (B), x7nth1 (C) and x7nth6 (D). Data were analysd by the twin exponential model. The points from all the experiments performed are shown individually for each cell line in graphs 1 (x7neo1), 2 (xrs7), 3 (x7nth1) and 4 (x7nth6).

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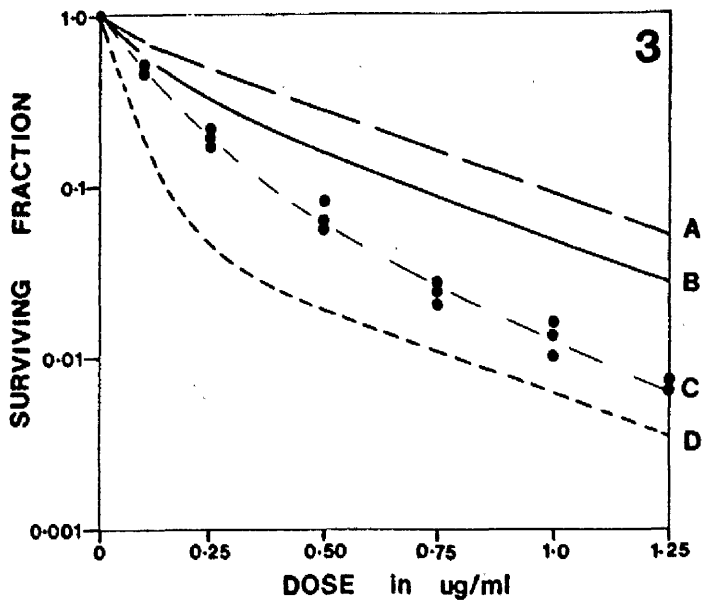
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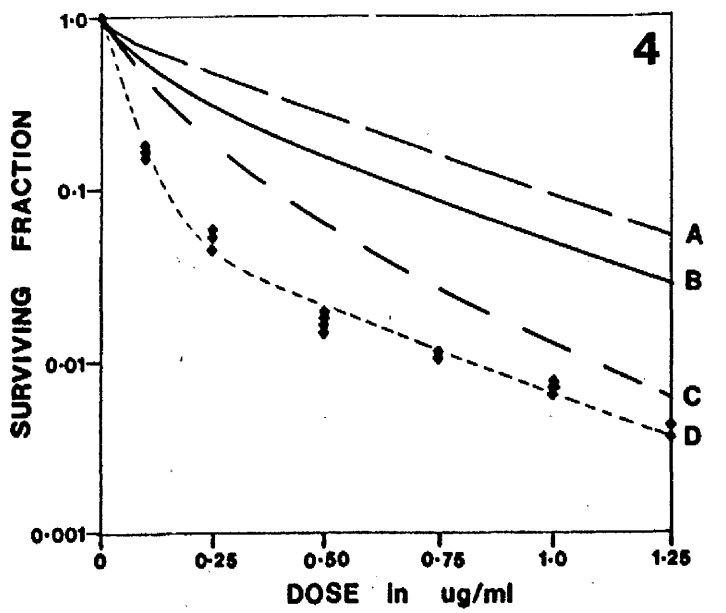
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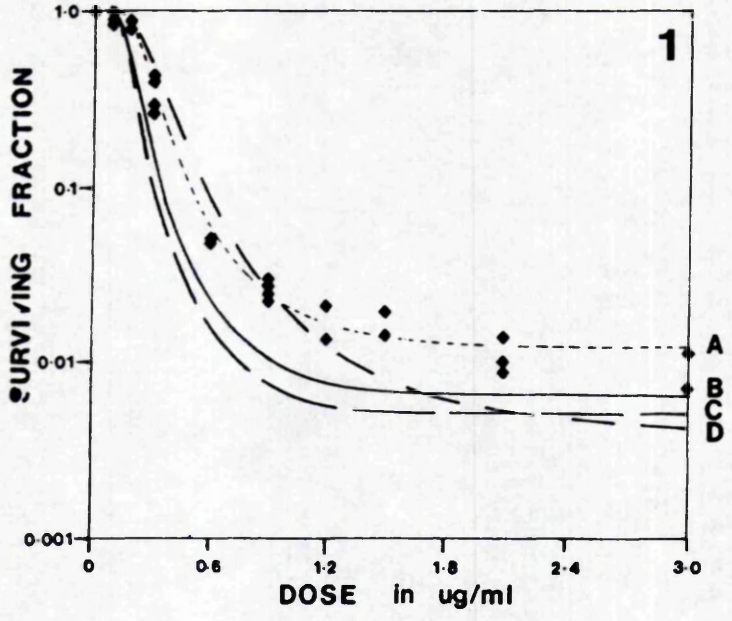
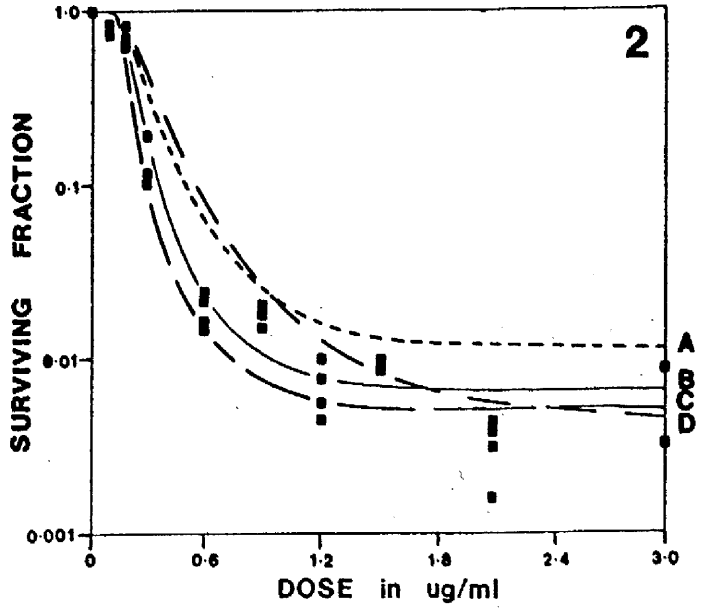
1·25
DOSE in ug/ml
A
B
C
D

FIGURE 37- Survival of xrs7, x7nth1, x7nth6 and x7neo1 after treatment with hydrogen peroxide

At least three independent experiments were carried out using cells of different passage number for x7nth6 (A), xrs7 (B), x7neo1 (C) and x7nth1 (D). Data were analysed by the single-hit multitarget model, incorporating a quenched dose parameter. The points from all the experiments performed are shown individually for each cell line in graphs 1 (x7nth6), 2 (xrs7), 3 (x7neo1) and 4 (x7nth1).

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3·0
DOSE in ug/ml
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B
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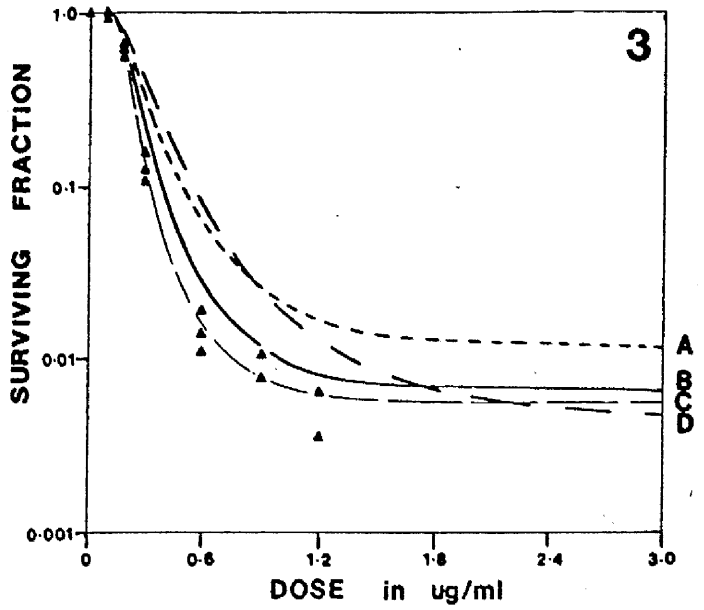
D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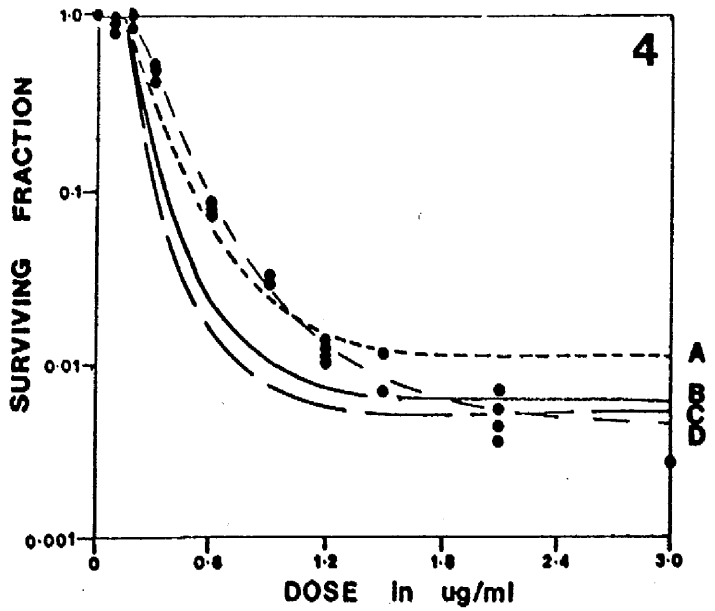
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0
0·6
1·2
1·8
2·4
3·0
DOSE in ug/ml
A
B
C
D

TABLE 17 - Analysis of the Cytotoxicity of xrs7, x7nth1, x7nth6 and x7neo1 After Treatment with Hydrogen Peroxide (± SE)

a) Using the alpha, beta model, incorporating a quenched dose parameter

CELL LINE	ALPHA $(ug/ml)^{-1}$	BETA $(ug/ml)^{-2}$	Q_D (ug/ml)
xrs7	0.17 ± 2.02	43.34 ± 6.83	0.35 ± 0.02
x7nth6	0.20 ± 1.57	22.19 ± 3.94*	0.49 ± 0.05
x7nth1	0.35×10^{-7} ± 0.98*	19.75 ± 2.44*	0.53 ± 0.05*
x7neo1	0.57×10^{-3} ± 2.69	36.66 ± 13.02	0.45 ± 0.04

b) Using the single-hit multitarget model, incorporating a quenched dose parameter

CELL LINE	N	Do (ug/ml)	Do RATIO v. xrs7	Do RATIO v. x7neo1	Q_D (ug/ml)	Q_D RATIO v. xrs7	Q_D RATIO v. x7neo1
xrs7	57 ± 43	0.034 ± 0.006	1.00	0.79 ± 0.20	0.31 ± 0.03	1.00	
x7nth6	39 ± 21	0.046 ± 0.007	0.74 ± 0.17	0.59 ± 0.24*	0.37 ± 0.03	0.84 ± 0.12	0.73 ± 0.12*
x7nth1	18 ± 6	0.072 ± 0.009	0.47 ± 0.10*	0.38 ± 0.22*	0.61 ± 0.06	0.51 ± 0.07*	0.44 ± 0.08*
x7neo1	126 ± 86	0.027 ± 0.005	1.26 ± 0.33	1.00	0.27 ± 0.04	1.15 ± 0.19	1.00

* $p < 0.05$ compared to xrs7

Data were analysed using the multitarget model, adapted to incorporate a quenching dose parameter (Q_D), of the DRFIT computer programme. At least three independent survival experiments were carried out for each cell line using cells of different passage number.

significantly more resistant than clone x7neo1 but not xrs7. However, clone x7neo1 contains the vector sequences involved in expression of the *neo* and *nth* genes and may be the more appropriate control cell line.

Therefore, expression of EIII in xrs7 cells caused an increase in resistance to hydrogen peroxide.

3.3 Expression of the *nth* gene in VG8 cells

VG8, VC4 and VE5 cells are radiation sensitive Chinese hamster V79 fibroblasts (Zdzienicka & Simons 1987). Since VG8 has a lower spontaneous mutation rate than VC4 and VE5, it was thought that this cell line would be able to survive the subcloning procedure following transfection without "loss" of genetic integrity. VG8 cells were not assayed to determine the level of endogenous AP endonuclease activity before they were transfected with pZipnth, since the non-specific AP endonuclease assay was not available at this time. However, the literature stated that VG8 cells were proficient in the repair of strand breakage (double and single) and showed similar characteristics to A-T cells, i.e. were unable to inhibit DNA synthesis after irradiation (Zdzienicka et al, 1989). VG8 cells also demonstrated a cross-sensitivity to a number of cytotoxic agents, which included bleomycin and X-rays. It was thought interesting to examine the effects of EIII expression on the radiosensitivity of these cells and to compare the effects with the normal Chinese hamster *nth*-expressing clones and those that were unable to repair

dsb. In this way the biological relevance of thymine modifications could be assessed in relation to dsb repair.

3.3.1 Transfection of pZipnth into VG8 cells.

Lipofection was used to transfer pZipnth and pZipneoSV(X)1 into VG8 cells, as described in 2.2.2iv. Initially clones were selected in medium containing 1mg/ml G418. Three out of 49 colonies were isolated from the pZipneoSV(X)1 transfection (the transfection frequency was 4 x 10^{-4}) and maintained for use as vector control cell lines (VG8neo1, 2, 3). Four G418 resistant clones were isolated from the pZipnth transfection, three of which survived: VG8nth2, 3 and 4. Gel electrophoresis analysis of PCR products from the amplification of cell extracts (see 3.3.2i) showed that *nth* was not present in these clones. Therefore the pZipnth transfection was repeated. The clones were selected in medium containing 500ug/ml G418, since it was possible that only three clones survived from the previous transfection due to the high (1mg/ml) G418 concentration used in the selection. Medium containing 500ug/ml G418 did kill the parental cells, although cell death was not detected until 2 weeks after the start of selection. Again the transfection frequency of pZipnth was low (approximately 3 x 10^{-5}) and only four clones survived (VG8nth5, 8, 9 and 16) out of the twelve colonies isolated. The transfected cell lines were maintained in medium containing 300ug/ml G418.

3.3.2 Molecular analysis of G418 resistant clones

(i) PCR of cell extracts

PCR amplification of *nth* sequences in cell-free extracts of VG8 and clones VG8nth2, 3, 4, 5, 8, 9 and 16 and 7D (RJKO derived cell line) was carried out (2.2.1xii) and products analysed by gel electrophoresis. Clone 7D was the positive control and VG8 was the negative control. Only two extracts of VG8 derived clones contained a DNA fragment of approximately 600bp: VG8nth5 and VG8nth8. This was confirmed by autoradiography of the *nth* probed filter containing the PCR products (figure 38).

(ii) DNA analysis

Genomic DNA was isolated from VG8 and clones VG8neo1, 2 and 3 and VG8nth2, 3, 4, 5 and 8, as described in 2.2.1ix, and used in dot blot and Southern analyses. The dot blot and Southern membranes were hybridized with the [^{32}P]-labelled *neo* (3.2.2ii) and *nth* (3.1.1i) probes, respectively.

a) DNA dot blot analysis

Clone 6E (RJKO derived vector control cell line) DNA was the positive control and VG8 DNA the negative control. Only the transfected cell lines hybridized to the *neo* probe (figure 39A), confirming that G418 resistance was due to the incorporation of pZipnth or pZipneoSV(X)1 and expression of *neo*. Therefore pZipnth-transfected clones that did not contain *nth* (VG8nth2, 3

FIGURE 38 - PCR analysis of cell extracts of VG8 and VG8-transfected cell lines

The filter was probed with the *nth* probe.

1	VG8	6	VG8nth3
2	7D	7	VG8nth4
3	VG8nth5	8	VG8nth9
4	VG8nth8	9	VG8nth16
5	VG8nth2		

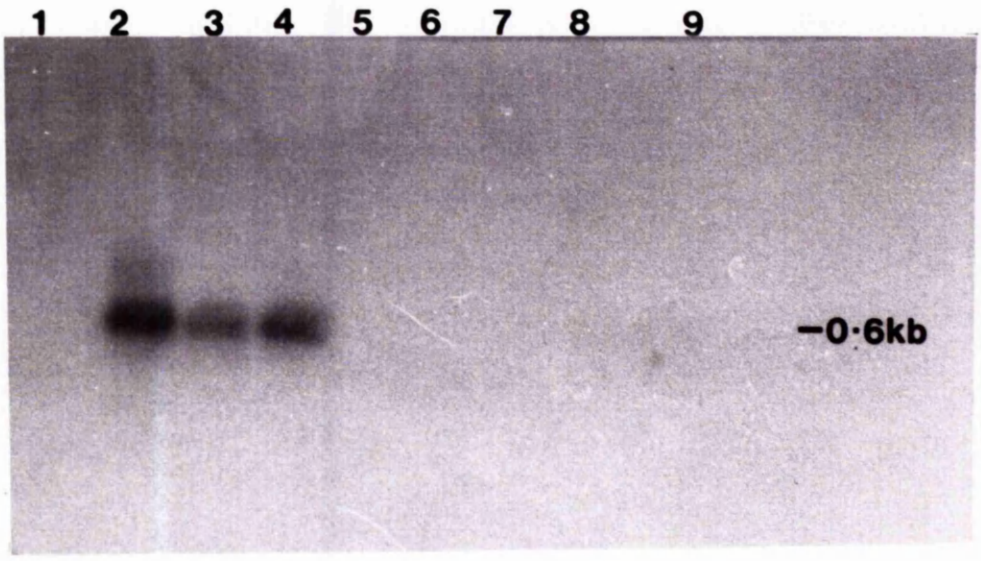
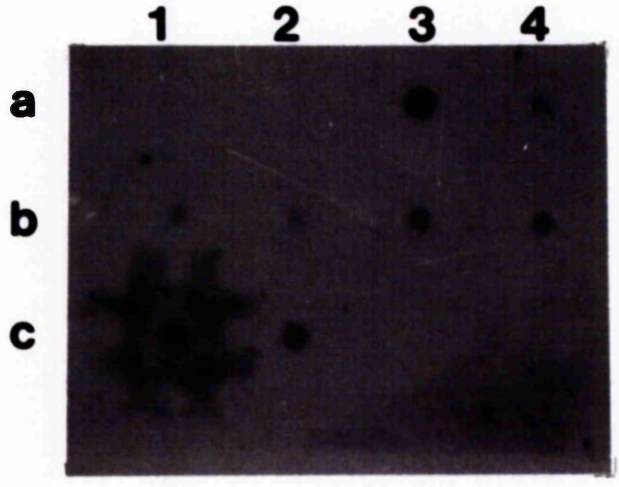


FIGURE 39 - DNA analysis of VG8 and VG8-transfected cell lines

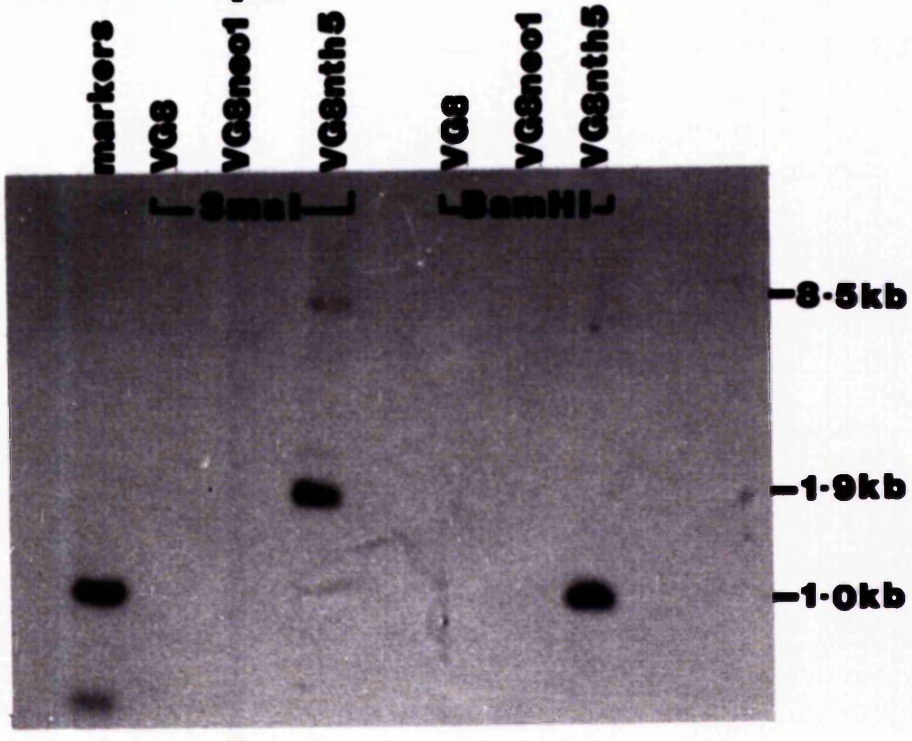
The dot blot was probed with the *neo* probe and the filter for Southern analysis was probed with the *nth* probe. A faint signal corresponding to the VG8neo1 sample is present on the autoradiograph of the dot blot. Unfortunately this signal cannot be seen on photograph A.

A - DNA dot blot analysis

a	1	VG8	b	1	VG8nth3	c	1 VG8nth4
	2	VG8neo1		2	VG8nth8		2 6E
	3	VG8neo2		3	VG8nth2		
	4	VG8neo3		4	VG8nth5		



B - Southern analysis



and 4) had maintained the vector sequences and had not achieved resistance to G418 by increased expression of endogenous resistance factors.

b) Southern analysis

Genomic DNA of VG8, VG8neo1 and VG8nth5 was digested with BamHI or SmaI and subjected to Southern analysis (2.2.1xivc) using the *nth* probe. No hybridization signals were detected in VG8 and VG8neo1 (figure 39B), but 1kb and 1.9kb signals were seen in the BamHI and SmaI digests, respectively, of VG8nth5, confirming the results of the PCR analysis.

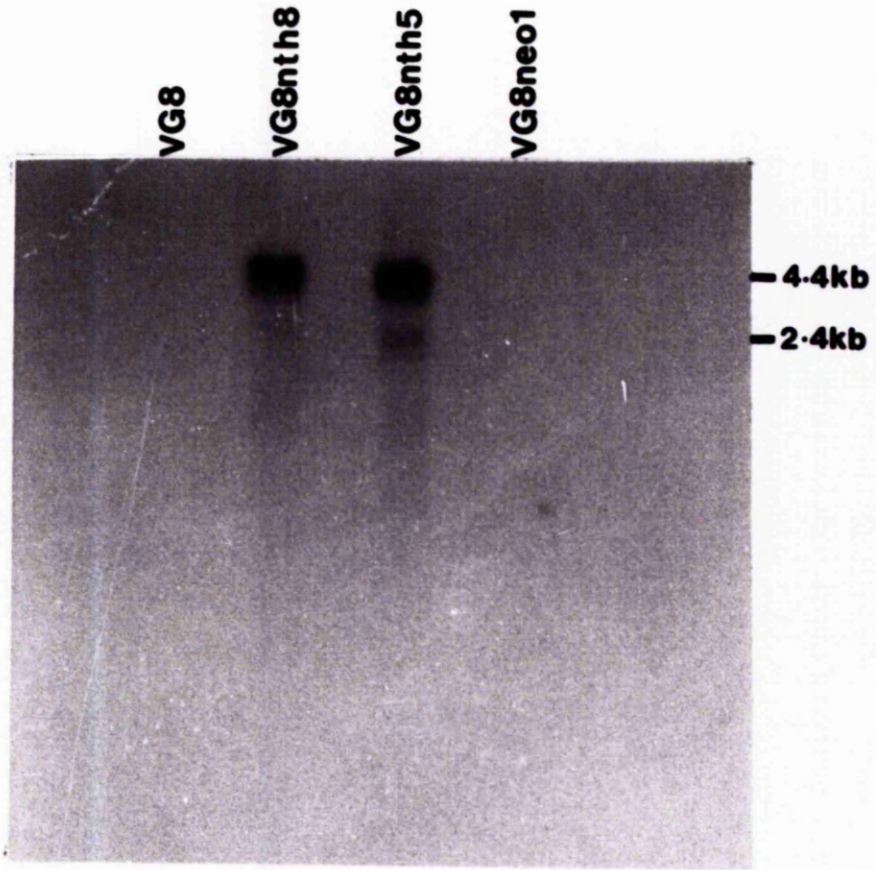
(iii) Northern analysis

Total RNA was isolated from VG8 and clones VG8neo1, VG8nth5 and VG8nth8 (see 2.2.1x) and subjected to northern analysis (2.2.1xivd).

VG8 and VG8neo1 RNA did not hybridize to the *nth* probe (figure 40). However, two hybridization signals at sizes of 4.4kb and 2.4kb were detected in the VG8nth5 and VG8nth8 samples. The 4.4kb message corresponds to the size of the full length message expected to be produced from the transcription of pZipnth. Cleavage of the full length transcript at the 3′ splice site (situated in the sequence between the 3′ end of *nth* and 5′ end of *neo*) would produce a 2.2kb message containing *nth* sequences and it is therefore likely that the 2.4kb hybridization signal corresponds to the spliced message.

FIGURE 40 - Northern analysis of VG8 and VG8-transfected cell lines

The filter was probed with the *nth* probe.



3.3.3 Assay of AP endonuclease activity

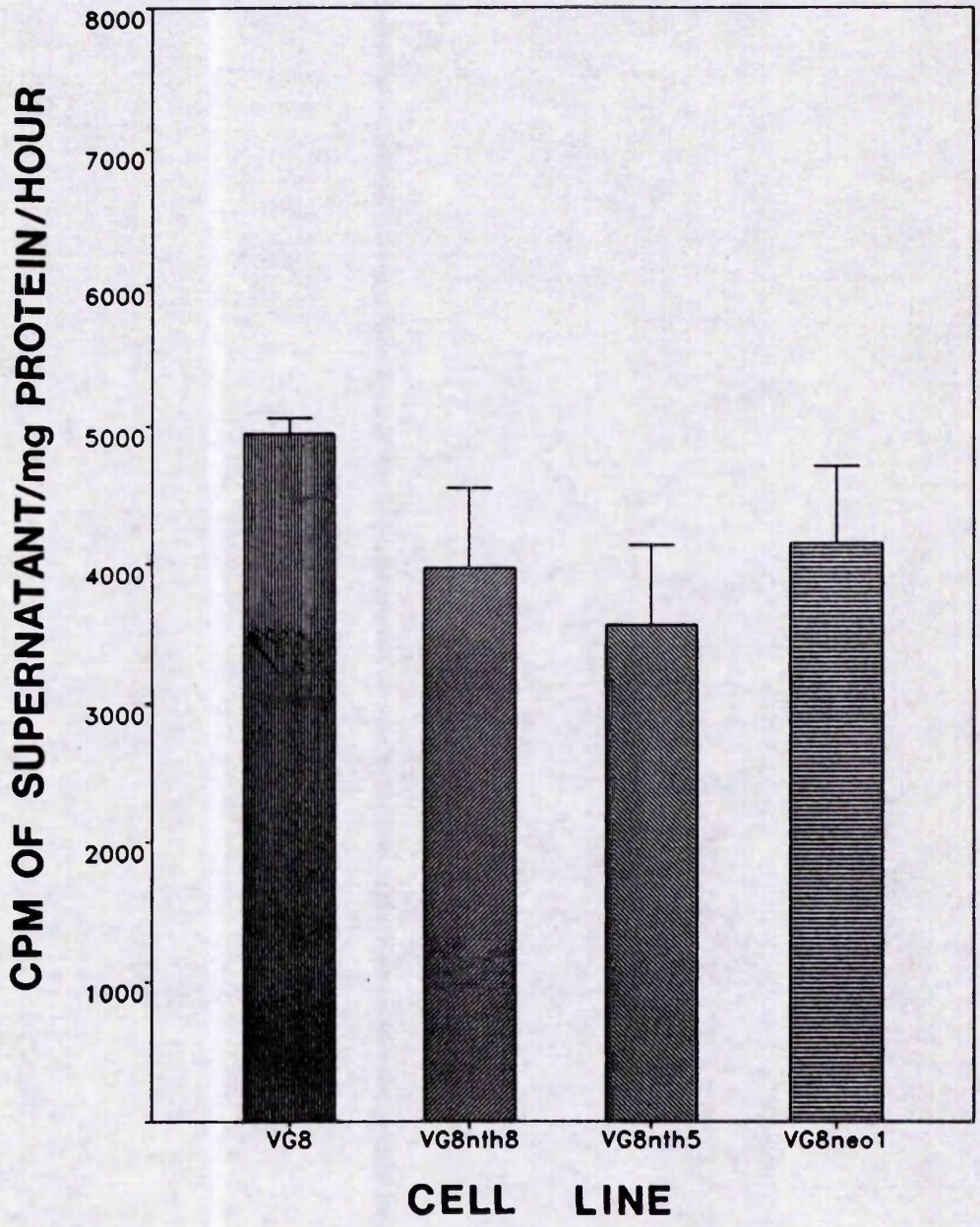
Cell-free extracts from parental and transfected cell lines were assayed in duplicate for AP endonuclease activity (see 2.2.3vii).

An increase in activity was not detected in clones VG8nth5 and VG8nth8 compared to VG8 and VG8neo1 (figure 41), even though the *nth* message was present in these two pZipnth-transfected clones. Work with RJKO-derived clones 1D and 7D showed that the presence of the *nth* message correlated with production of EIII protein and an increase in AP endonuclease activity was found in xrs7-derived clones x7nth1 and x7nth6, which were positive on western analysis using the anti-EIII antibody. It is therefore possible that clones VG8nth5 and VG8nth8 actively express EIII. Although detection of EIII using this non-specific assay has proved difficult, partial purification of the extracts by DNA cellulose chromatography (see 2.2.3iv) would concentrate the DNA binding proteins and may allow detection of low EIII expression. However, it is possible that the conditions required (0.75M NaCl) to release EIII from the DNA cellulose affinity column would also release endogenous AP endonucleases, which may continue to prevent detection of the EIII activity.

Due partly to the difficulties in detection of EIII-activity and partly to the shortage of time, survival experiments with VG8 and the VG8 transfected clones were not carried out.

FIGURE 41-AP endonuclease activity of VG8, VG8nth5, VG8nth8 and VG8neo1 cell-free extracts.

Cell-free extracts were assayed in duplicate for the fragmentation of [^{3}H]-labelled partially depyrimidinated T4 phage DNA. The height of the bar represents the average of the two assays and the error is the difference between the duplicates.



3.4 Discussion of the effect of EIII expression on cell survival of RJKO and xrs7-derived cell lines

(i) Cytotoxicity of ionising radiation

The radiosensitization of clones 1D and 7D cannot be accounted for by the alteration of cellular parameters (table 8) or chromosome number (table 9), since no such changes were detected. It is not unreasonable to suggest, therefore, that the repair of thymine lesions and AP sites by EIII may have caused the radiosensitization. EIII repair results in the production of a 3′ terminal 2-3 didehydro-dideoxyribose moiety at the site of the lesion (Bailly & Verly 1987) and this DNA terminus cannot readily be used by DNA polymerase I for repair synthesis (Warner et al 1980). It is therefore possible that the break introduced by EIII will persist in the DNA for longer than ssbs produced by other mechanisms. Although ssbs have not themselves been implicated in cell death (see 1.5.2), during irradiation the interaction of a single energy track with the DNA, or the water or protein closely associated with the DNA, can produce a high number of ionizations and excitations at the site of energy deposition (Goodhead 1989, see 1.1.1). This is believed to result in clusters of DNA damage called LMDS. Radicals have been found to produce a wide variety of DNA damage (see 1.2, Teoule 1987, Fuciarelli et al 1990) and it is therefore likely that thymine damage and AP sites will be present in the LMDS. It is possible that the

EIII-induced ssb could interact with other ssb (including other endogenous repair enzyme or EIII-induced ssb) within a cluster of damage, resulting in the production of lethal dsbs.

The difference in radiosensitivity between 1D and 7D (figure 27) may be explained by 7D expressing a higher level of EIII than 1D (figure 26): a slight increase in AP endonuclease activity was only detected in extracts of clone 7D and not 1D.

Xrs7 cells are dsb repair deficient and hence if EIII did cause increased production of dsbs a greater radiosensitization compared to control cell lines might have been expected with clones x7nth1 and 6 than with clones 1D and 7D. However, x7nth1 and 6 had equivalent radiosensitivites compared to xrs7 and x7neo1 (figure 35, table 15). The alteration of the ploidy of x7nth1 (table 14) does not appear to have affected the radiosensitivity of the cell line. A number of groups have found that an increase in ploidy can result in an increase in radioresistance of cell lines derived from human tumours of the uterus and cervix (De 1961, Puck 1960) and *in vivo* studies, using diploid (2n) and tetraploid (4n) lymphocytic leukaemic cell lines grown in mice, showed that 4n cells were more resistant to gamma and X-rays than 2n cells (Do = 1.6Gy for 2n and 1.8Gy for 4n cells under oxic conditions, Berry 1963). It was suggested that 4n cells had twice the number of targets of 2n cells and that this resulted in an increase in radioresistance. However, Revell (1983) found that 4n Syrian hamster cells

had approximately twice the chromosome aberration frequency and fragment loss/unit dose of 2n cells, which could result in equivalent radiosensitivites of 2n and 4n cells, but nonsynchronous populations of these cells showed that 4n cells were more radioresistant than 2n cells. To explain this Revell (1983) proposed that 4n cells may tolerate fragment loss better than 2n cells. However, Radford and Hodgson (1987) and Jones et al (1988) found no significant alteration in the radiosensitivity of tetraploid compared to diploid Chinese hamster cells. The reason(s) for the conflicting results are not known.

Possible explanations of the sensitization of clones 1D and 7D, and not x7nth1 and x7nth6 are:

1) Endogenous levels of repair enzymes

RJKO cells appear to contain a higher level of endogenous AP endonuclease activity. A small increase in EIII expression in RJKO cells may have resulted in an increase in breakage for which repair could not be completed. Other repair enzymes are required in the repair of radiation-induced lesions. If these enzymes, e.g. those that repair ssb, are not able to repair the damage before EIII introduces ssbs on the opposite DNA strand close to the damage, there will be an increase in the probability of lesion interaction and formation of dsbs.

2) No significant alterations in cellular parameters were detected for the RJKO-derived clones (table 8, 9).

If changes had occurred that were not detected by the

growth and cell cycle assays used (see 2.2.2v, vi), they could have altered the radiosensitivity of clones 1D and 7D. However, alterations in both growth and cell cycle were detected for the xrs7-derived clones, but the radiosensitivity of x7nth1, x7nth6 and x7neo1 was equivalent to xrs7.

(ii) Cytotoxicity of bleomycin sulphate

Bleomycin sulphate produces AP sites and DNA strand breakage (Ross & Moses 1978, Bradley & Kohn 1979, see 1.4.1) and by extrapolation from the level of lesions required to produce a lethal event, may have a similar lesion distribution to that of radiation: 40 dsb for radiation and 30 dsb for bleomycin (Ward et al 1987). It might therefore be expected that a sensitization of clones 1D and 7D and x7nth1 and x7nth6 would be seen following bleomycin sulphate treatment.

Only small changes in the mathematical parameters could be detected after bleomycin sulphate treatment of 1D and 7D (table 11): a slight sensitization was found with clone 7D and a slight resistance with 1D (figure 28). The sensitization of clone 7D may therefore be due to EIII action increasing the level of dsbs, as discussed above. Clone 1D appears to express a lower level of EIII than 7D (figure 26). Low EIII expression could confer a resistance to bleomycin sulphate if the repair of EIII-induced ssb occurred before the lesions interacted to produce lethal dsb. A sensitization to radiation and resistance to bleomycin sulphate might have been found if

radiation produced more EIII-repairable lesions, and hence more repair-induced ssb, than bleomycin sulphate. However, it has recently been reported that EIII sensitive sites are introduced into DNA by radiation at the same frequency as the ssb (Bases et al 1990), while 5-6 thymines are released (i.e. generating 5-6 AP sites) per ssb by bleomycin (Muller & Zahn 1977). On comparing the level of ssb introduced per cell per lethal event, Ward et al (1987) found that 1000 ssb were introduced by radiation compared to 150 ssb for bleomycin. This suggests that radiation and bleomycin produce almost equivalent amounts of EIII sensitive sites per lethal event and hence does not explain the resistance to bleomycin sulphate observed with clone 1D. However, ionising radiation introduces many types of base damage, which may be repaired by DNA glycosylase and/or AP endonuclease activities that could also produce ssbs in DNA. If bleomycin produces base damage (other than AP sites) at a much lower frequency than ionising radiation, EIII-induced ssb would have a lower probability of interacting with ssb introduced by other repair enzymes after treatment of the cells with bleomycin compared to ionising radiation. EIII activity could then cause 1D to be slightly radiosensitive yet slightly resistance to bleomycin.

In contrast to the small differences in survival found with clones 1D and 7D compared to 6E and RJKO, a marked sensitivity to bleomycin sulphate was observed with x7nth1 and 6 (figure 36, table 16). The enhanced

sensitivity of x7nth1 and 6 can be explained by EIII-induced ssbs resulting in an increase in dsbs, which cannot be repaired due to the decreased efficiency of dsb repair of x7nth1 and 6.

The sensitization of x7nth1 and x7nth6 to bleomycin sulphate and not radiation may be due to the different treatment times of the cytotoxic agents. During a 24 hour treatment at 37°C EIII repair would have continued while bleomycin sulphate damaged the DNA. Therefore the ssb, dsb and AP sites would have increased as the EIII-induced breaks increased. Cells were irradiated while in suspension at room temperature and it is likely that under these conditions repair of lesions would be slower than at 37°C. The time of irradiation would have allowed little repair to occur while the damage was being introduced: the longest irradiation for xrs7 cells was 66 seconds, while the half life of ssb and thymine glycol in DNA in mammalian cells is 4 and 7 minutes, respectively (Ward et al 1987). The probability of the interaction of AP sites or ssb with an EIII-induced break (which as mentioned earlier may have a longer half life than ssb produced by other mechanisms) would have been greater during the 24 hour bleomycin sulphate treatment.

The cause of the xrs7 biphasic survival curves is not yet understood. Suggestions of transient expression of repair genes during DNA synthesis, in the period before methylation has occurred, have been considered to explain the resistant portion of the population (Denekamp et al 1989). This would be dependent on the completion of

transcription of the non-mutated allele(s), that encode the protein(s) which are normally deficient in xrs7 cells, in the time available before methylation and on the spatial arrangement of the DNA synthesizing, transcription and methylation complexes at the gene(s) in question. Since the reason for the sensitive and resistant portions of the xrs7 cell population is not known it is not possible to explain why different portions of the x7nth1 and x7nth6 population were sensitized: $_1$Do and $_2$Do were lower for x7nth6 and x7nth1, respectively, compared to xrs7 and x7neo1 (table 16). Although the fact that the x7nth1 cells are tetraploid did not affect the radiosensitivity of the cells, it may explain why x7nth1 cells are more resistant to bleomycin sulphate than x7nth6. Diploid, tetraploid and octaploid cell lines of a RIF-1 mouse sarcoma were found not to differ in sensitivity to X-rays and adriamycin, but the increase in ploidy resulted in resistance to CCNU (cyclohexyl-chloroethylnitrosourea, a cross-linking alkylating agent, Reeve et al 1983). The effect of ploidy on survival may therefore vary with different cytotoxic agents.

(iii) Cytotoxicity of hydrogen peroxide

Clones 7D (table 12, figure 29), x7nth1 and x7nth6 (table 17, figure 37) showed a decrease in the susceptibility to the cytotoxic effects of hydrogen peroxide in comparison to control cells, while clone 1D had equivalent survival to 6E and RJKO. The difference

in survival of clones 1D and 7D, and x7nth1 and x7nth6 may be due to higher EIII expression in clone 7D compared to 1D (figure 26) and in x7nth1 compared to x7nth6 (figure 34). The difference in sensitivity of x7nth1 and 6 however, could also be due to the tetraploid nature of x7nth1 cells.

Hydrogen peroxide damages the DNA via hydroxyl radicals, which are produced following a Fenton reaction (Imlay et al 1988), and a wide variety of DNA lesions are introduced, as found with ionising radiation. Relative magnitudes of the yields of hydrogen peroxide DNA damage are in the order of base damage > ssb > dsb > cross-links (Massie et al 1972). The increased resistance of clones 7D, x7nth1 and x7nth6 to hydrogen peroxide compared to control cells indicates that EIII-repairable lesions are potentially toxic lesions. It has been suggested that hydrogen peroxide produces randomly dispersed damaged sites (RDDS, previously referred to as singly damaged sites by Ward et al 1985) in the DNA rather than LMDS and that the interaction of RDDS may be one cause of lethality (Ward et al 1985, see 1.4.2). In *nth*-expressing cells the interaction of EIII-induced breaks with other damage to form a lethal lesion would thus seem to be more probable in an LMDS rather than an RDDS situation and hence after treatment with bleomycin sulphate rather than hydrogen peroxide. This may explain why clones 7D, x7nth1 and x7nth6 were sensitive to bleomycin sulphate, yet resistant to hydrogen peroxide.

3.5 Summary

Differences detected in cell survival studies of 1D and 7D (RJKO-derived EIII expressing cells) were small. Both clones were slightly more radiosensitive than control cell lines and 7D had a small increase in sensitivity to bleomycin sulphate and resistance to hydrogen peroxide. The small differences may have been due to low EIII expression in comparison to endogenous enzymes.

Clones x7nth1 and x7nth6 (xrs7-derived cell lines) did not show increased sensitivity to radiation, but were markedly more sensitive to bleomycin sulphate and showed a resistance to hydrogen peroxide compared to control cell lines.

The slight radiosensitizations of 1D and 7D and the sensitization of 7D, x7nth1 and x7nth6 to bleomycin sulphate suggest that EIII repair can cause increased cell death. It is proposed that the single strand DNA scission produced by EIII during repair can increase the production of lethal lesions by interacting with other lesions or products of repair by other AP endonucleases. Since 7D, x7nth1 and x7nth6 had increased resistance to hydrogen peroxide it appears that the distribution of damage in the DNA is important. If the lesions are randomly dispersed (as is thought to occur after hydrogen peroxide damage) EIII-induced breaks have a lower probability of interacting with other lesions to produce a dsb or LMDS. In this situation increased repair capacity enhances cell survival, suggesting that the

EIII-repairable lesions can be potentially toxic, but when this form of lesion is present in a cluster of damage (LMDS) repair can cause an increase in the production of lethal lesions. Bleomycin and radiation produce equivalent amounts of EIII-repairable lesions at equivalent cytotoxic doses. The sensitization of x7nth1 and x7nth6 to bleomycin sulphate can probably be explained by the 24 hour bleomycin treatment time, during which EIII repair would have occurred while the DNA was being damaged. It is possible that hydrogen peroxide produces more EIII-repairable lesions than radiation as a radioresistance was not seen, even in a situation where repair and DNA damage were unlikely to interact.

To determine if the treatment time was critical in observing increased cell death of *nth*-expressing clones when using a cytotoxic agent producing LMDS DNA damage, the sensitivity of x7nth1 and x7nth6 could be examined using a shorter bleomycin sulphate treatment time, under conditions preventing repair, e.g. 4°C, or an increased time of irradiation, using a low dose-rate source (e.g. ^{60}Co). It would also be interesting to measure the induction of double and single strand breaks before and after repair, following the treatments mentioned above.

Different types of radiation are thought to produce different sizes of clusters of damage in the DNA (Goodhead 1989, see 1.1.1). Low LET radiation is thought to produce smaller clusters of lesions in comparison to high LET radiation. Therefore it would be interesting to compare the effect of EIII repair after alpha

irradiation, since alpha radiation produces larger lesion clusters.

The explanations of the changes in cytotoxicity observed with *nth*-expressing clones are dependent on the assumption that EIII can repair lesions in mammalian DNA. Previously, it has been shown that *E.coli* ada protein expressed in mammalian cells was able to repair damage in the host cell genome and increase cell survival after exposure to monofunctional alkylating agents, such as MNU (Brennand & Margison 1986). This suggests that other bacterial enzymes should also be able to carry out successful repair of DNA in chromatin. In the case of EIII, it should be possible to examine this by treating *nth*-expressing cells with hydrogen peroxide or radiation and measuring the removal of TG from the genome, using an anti-TdG monoclonal antibody.

EIII-repairable lesions appear to be toxic when cells are treated with hydrogen peroxide. It is therefore likely that these lesions are potentially toxic after treatment with other DNA damaging agents. Mutation and chromosome aberration studies of xrs7-derived clones using radiation, bleomycin sulphate, hydrogen peroxide and other agents that produce EIII-repairable damage (e.g. U.V.), will help to further explore the biological relevance of thymine and AP modifications and the possibility that EIII repair can increase cell killing when lesions form LMDS. An EIII deficient cell line is required for pZipnth transfection to confirm EIII-repairable lesions are potential toxic, radiation lesions.

CHAPTER 4 - DEVELOPEMENT OF AN ASSAY SPECIFIC FOR ENDONUCLEASE III

Assays available for the detection of EIII activity are either time consuming or utilize substrates containing DNA damage that is also recognised by other enzymes. Examples include:

I - AP endonuclease assay

The substrate for this assay (for preparation see 2.2.3 viic) is [^{3}H]-thymidine labelled, partially depyrimidinated T4 phage DNA ([^{3}H] AP DNA). Incubation with an AP endonuclease results in the introduction of single strand breaks at the AP lesions and fragmentation of the substrate. The high molecular weight DNA fragments are precipitated (see 2.2.3.viid) and the radioactivity remaining in solution is a measure of the fragmentation of the substrate and hence of the AP endonuclease activity (Cunningham & Weiss 1985).

Although the assay is rapid and simple, it is not specific for EIII, since other enzymes will introduce a ssb at AP sites and fragment the substrate, e.g. EIV, exonuclease III. In the absence of an assay specific for the measurement of EIII or EIV activity, the AP endonuclease assay was used to try to determine whether active expression of EIII or EIV had been achieved in Chinese hamster cells transfected with the *nth* or *nfo* genes (see chapters 3 and 5). An increase in AP endonuclease activity (approximately 3-4 fold) was detected in cell-free extracts of xrs7 cells transfected with pZipnth or pLJnfo, compared to parental and vector

control cell lines.

II - "Plasmid-nicking" assay

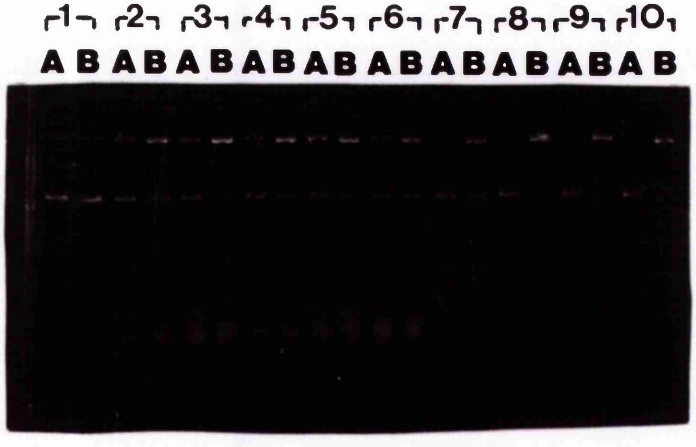
pBR322 DNA irradiated with 3000J/m^2 254nm U.V. light is the substrate for this assay (see 2.2.3viii, assay procedure provided by Dr R.P. Cunningham, pers. communication). After incubation with cell-free extract (bacterial or mammalian), the DNA is subjected to gel electrophoresis, as described in 2.2.3viii. The level of endonuclease activity is determined by the conversion of the plasmid DNA from the supercoiled to the open circle form, the conformations of which have different mobilities during electrophoresis. Studies have shown that although the TG-type damage is produced in DNA by 254nm U.V. light, cyclobutane pyrimidine dimers are the predominant lesions (Hariharan & Cerutti 1977). Therefore enzymes that have the ability to remove pyrimidine dimers and introduce a ssb will cleave the U.V. damaged plasmid DNA to a greater extent than EIII.

An attempt was made to compare the activities of the RJKO-transfected cell lines and xrs7 and x7nth1 (see figure 42). Before the reaction products were subjected to gel electrophoresis it was necessary to treat the samples with proteinase K (see 2.2.3viii), since protein affected the electrophoretic mobility of the plasmid DNA. Conversion of the supercoiled to the open circle form was evident when irradiated DNA was used as the substrate. However, an increase in activity was not detected in the pZipnth-transfected cell lines compared to the control cell lines, although increased AP endonuclease activity

FIGURE 42 - Attempt to detect increased endonuclease activity in cell-free extracts of 1D, 7D and x7nth1 compared to control cell lines using the "plasmid-nicking" assay

Each extract was incubated with unirradiated (A) and U.V. irradiated (B) plasmid DNA. Partially purified EIII was the positive control (final EIII concentrations in the reactions are shown).

1 - no extract
2 - 1D
3 - 7D
4 - 6E
5 - RJKO
6 - xrs7
7 - x7nth1
8 - 0.15ng/ul EIII
9 - 0.05ng/ul EIII
10 - 0.025ng/ul EIII



was found in the x7nth1 extract compared to xrs7, using the AP endonuclease assay (I).

The "plasmid-nicking" assay is simple and does not involve radioactivity, but it is not as sensitive as assay (I) and will not distinguish between EIII and other U.V.-DNA repair endonucleases in cell-free extracts.

III - Assay to detect the cleavage pattern of the enzyme.

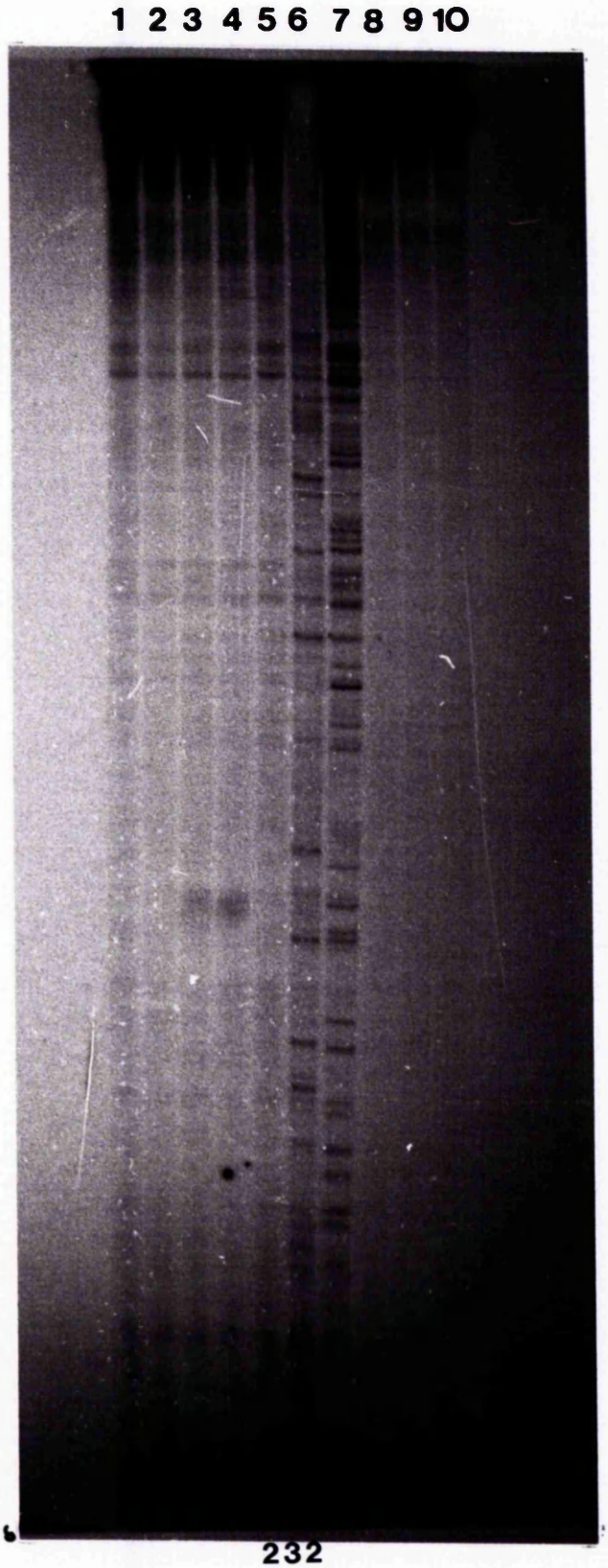
pBR322 DNA labelled with [^{32}P] and irradiated with 10,000J/m^2 254nm U.V. light (see 2.2.3ix) is the substrate for this assay. After incubation with cell-free extract the DNA is subjected to UPAGE, as described in 2.2.3ix. Autoradiography is used to detect the fragmentation pattern of the substrate (Gossett et al 1988).

Similar cleavage patterns were detected after UPAGE of the substrate treated with cell-free extracts of 1D, 7D, RJKO, 6E and xrs7 (see figure 43), while the x7nth1 extract produced a different fragmentation pattern compared to EIII and the other mammalian extracts. It is likely that the Chinese hamster cell-free extracts contained a number of enzymes that cleaved the U.V. damaged DNA. A combination of EIII and the endogenous Chinese hamster enzymes would therefore produce a different cleavage pattern from either EIII or the other Chinese hamster cell-free extracts.

Although it is possible to determine the site at which the enzyme cleaves the DNA, the assay is non-specific (due to the wide variety of modifications

FIGURE 43 - Attempt to detect endonuclease III activity in cell-free extracts of 1D, 7D and x7nth1 by examining the enzyme cleavage pattern of U.V. irradiated DNA.

1	1D	6	x7nth1
2	7D	7	EIII
3	6E	8	U.V. irradiated DNA
4	RJKO	9	EIII + unirradiated DNA
5	xrs7	10	unirradiated DNA



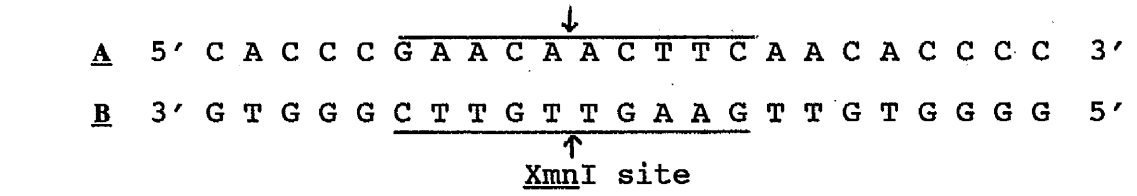
introduced by U.V. irradiation), time consuming and when examining cell-free extracts the results are difficult to interpret. Using this assay it was not possible to show that EIII was expressed in the Chinese hamster cells, although there was an indication of different U.V. DNA repair activities in x7nth1 cells compared to xrs7.

It was considered worthwhile to try to establish a more convenient assay, which was able to measure EIII activity in cell-free extracts and had the advantages of the assays mentioned above, i.e. a rapid, simple procedure, that would allow the determination of the enzyme cleavage sites of the substrate.

A double stranded (ds) oligonucleotide (see figure 44) was designed to contain a RE site (XmnI), which had two thymidine residues in the recognition sequence that could be oxidized to produce TG (see 4.1).

FIGURE 44 - Structure of the oligonucleotides used in the EIII assay

TG is generated in oligonucleotide A by OsO_4 oxidization and the product is annealed to oligonucleotide B to produce the EIII substrate. The TG (T′) residues are situated in the XmnI recognition site.



EIII substrate:

5′ C A C C C G A A C A A C T′T′C A A C A C C C C 3′

3′ G T G G G C T T G T T G A A G T T G T G G G G 5′

It was proposed that the TG lesions would prevent XmnI recognition and cleavage of the oxidized ds oligonucleotide, providing a simple test for the presence and extent of damage in oligonucleotide A. The assay would be specific for enzymes with TG glycosylase and endonuclease activities, e.g. EIII and EVIII. If EIII or EVIII removed the TG from the substrate and introduced a break between or at the 3′ side of the newly generated AP site(s), oligonucleotide A would be fragmented to a 13/14mer. The extent of fragmentation (visualised by UPAGE and autoradiography) would be a measure of enzyme activity.

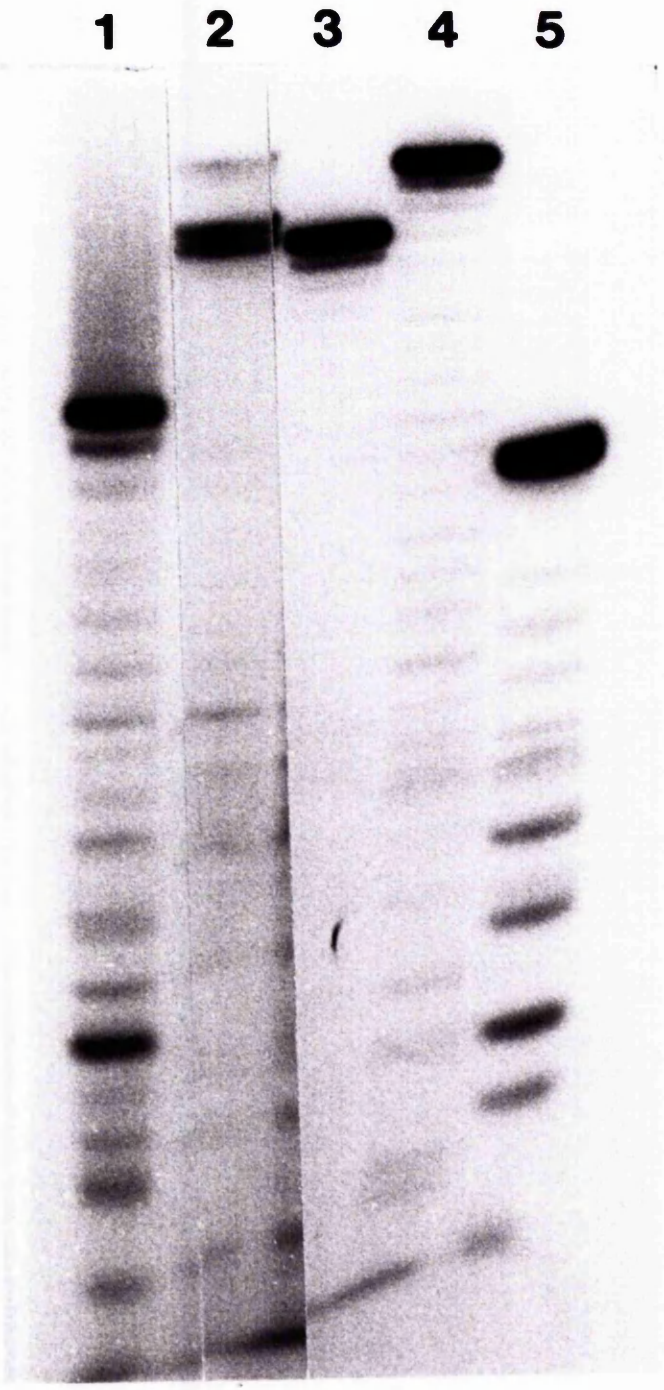
4.1 Production of the thymine glycol containing substrate

Oligonucleotides A and B (figure 44) contain 23 nucleotides and are complementary. Purification was achieved as described in 2.2.3via and analysis of the purity of the products was by UPAGE (2.2.3viif) using a DNA sequencing gel apparatus, after labelling the 5′ end of each oligonucleotide with ^{32}P (2.2.1xiii). A and B did show a slight contamination of smaller oligonucleotides (figure 45), but this was considered negligible.

OsO_4 was chosen to modify oligonucleotide A, as it oxidizes the thymine base readily with only a slight reaction with cytosine and no reaction with guanine or adenine (Beer et al 1966). Clark and Beardsley (1987) found that >90% of the oxidation products of thymidine in an 18mer oligonucleotide (under similar conditions as

FIGURE 45 - Examination of the purity of oligonucleotides A and B

1 Standard 17mer
2 Double stranded oligonucleotide (A and B annealed)
3 Single stranded A
4 Single stranded B
5 Standard 13mer



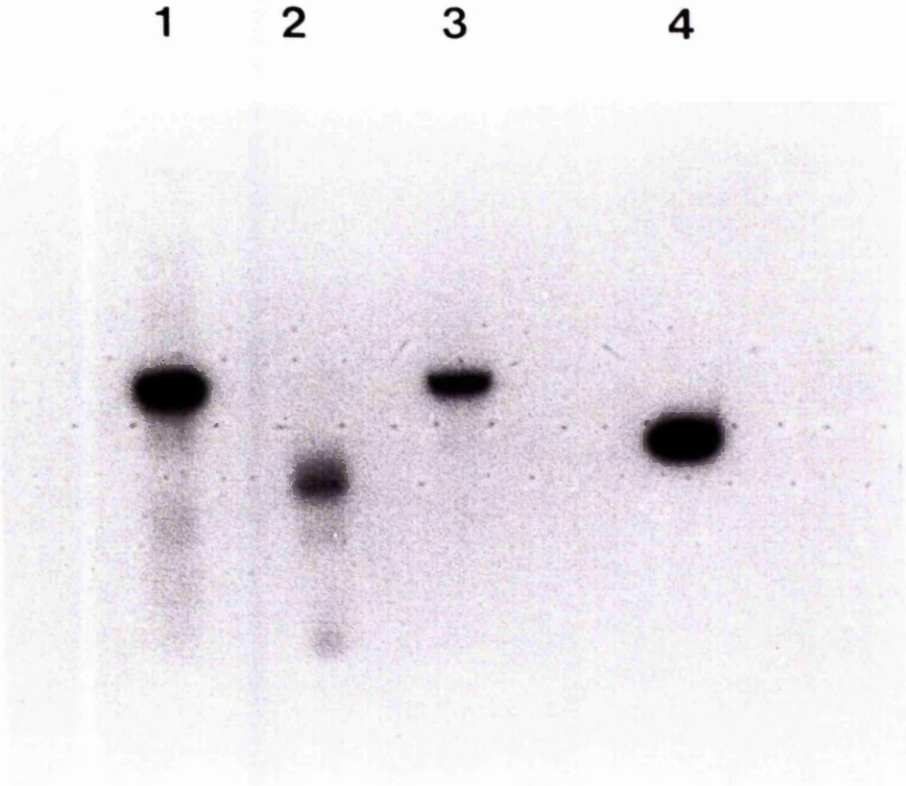
described in 2.2.3vib) were TG. In DNA the oxidation of cytosine, in comparison with thymine, is low: when 90% of thymine residues were oxidized in DNA only 10% of cytosines were modified (Beer et al 1966). The cytosine residues in oligonucleotide A should therefore only affect the TG substrate to a limited extent.

Oxidation of [^{32}P]-labelled oligonucleotide A is described in 2.2.3vib and a control oligonucleotide was produced by substituting water for OsO_4.

Initially the modification of oligonucleotide A was verified by treatment with piperidine and alkali (2.2.3vic). Piperidine removes modified base moieties from the oligonucleotide producing AP site(s), which are converted to ssb by alkali. Cytosine and thymine modifications would therefore be detected by this procedure. Following UPAGE (2.2.3vif) piperidine-treated, oxidized oligonucleotide A was expected to yield a 12/13 mer. The control oligonucleotide was not fragmented (figure 46), but signals corresponding to approximately the size of a 12mer and a 13mer (which was very faint) were detected with oxidized oligonucleotide A (hereafter referred to as the oxidized oligonucleotide). Analysis of the signal intensity using scanning densitometry (2.2.3vig) revealed that 97% of the oxidized sample was at the 12/13mer position, while 100% of the control sample was at the 23mer position. The signals corresponding to the 12 and 13mers in the oxidized sample were not completely resolved by scanning densitometry. However, the trace did show a shoulder on the 12mer peak,

FIGURE 46 - Analysis of piperidine and alkali treated control and oxidized oligonucleotides

1 Untreated oligonucleotide A
2 Treated oxidized oligonucleotide
3 Treated control oligonucleotide
4 Standard 13mer



confirming the presence of a second signal positioned close to the 12mer (figure 47). This indicated TG was present at position 13 and possibly 14 in the oxidized oligonucleotide.

Annealing the control or oxidized oligonucleotides to oligonucleotide B (2.2.3vid) produced the control substrate or oxidized substrate (refered to hereafter as EIII substrate).

4.2 Digestion of EIII substrate with XmnI

Oligonucleotide B was 5′ [^{32}P] labelled and annealed to 5′ [^{32}P] labelled oligonucleotide A, or oxidized or control oligonucleotide, to produce double stranded oligonucleotide (A and B annealed), EIII substrate or control substrate. XmnI digestion was carried out as described in 2.2.1iii.

The double stranded oligonucleotide digested to produce 10mer and 13mer fragments, as expected (figure 48), while the control substrate digested to produce a 13mer. Complete digestion of the ds oligonucleotide was not achieved, as bands were seen in the positions of oligonucleotides A and B. Oligonucleotide A was not seen in the control sample due to a lower level of labelling, which also explains the absence of a 10mer. The EIII substrate, however, did not digest. This is further evidence for the presence of modified residue(s) and indicates the damage was introduced within the recognition site of XmnI.

FIGURE 47 - Analysis of the piperidine-treated oxidized and control oligonucleotides by scanning densitometry.

The scanning densitometry traces obtained from the analysis of the autoradiograph are shown.

1 - 23mer
2 - piperidine-treated, oxidized oligonucleotide
3 - piperidine-treated, control oligonucleotide
4 - 13mer

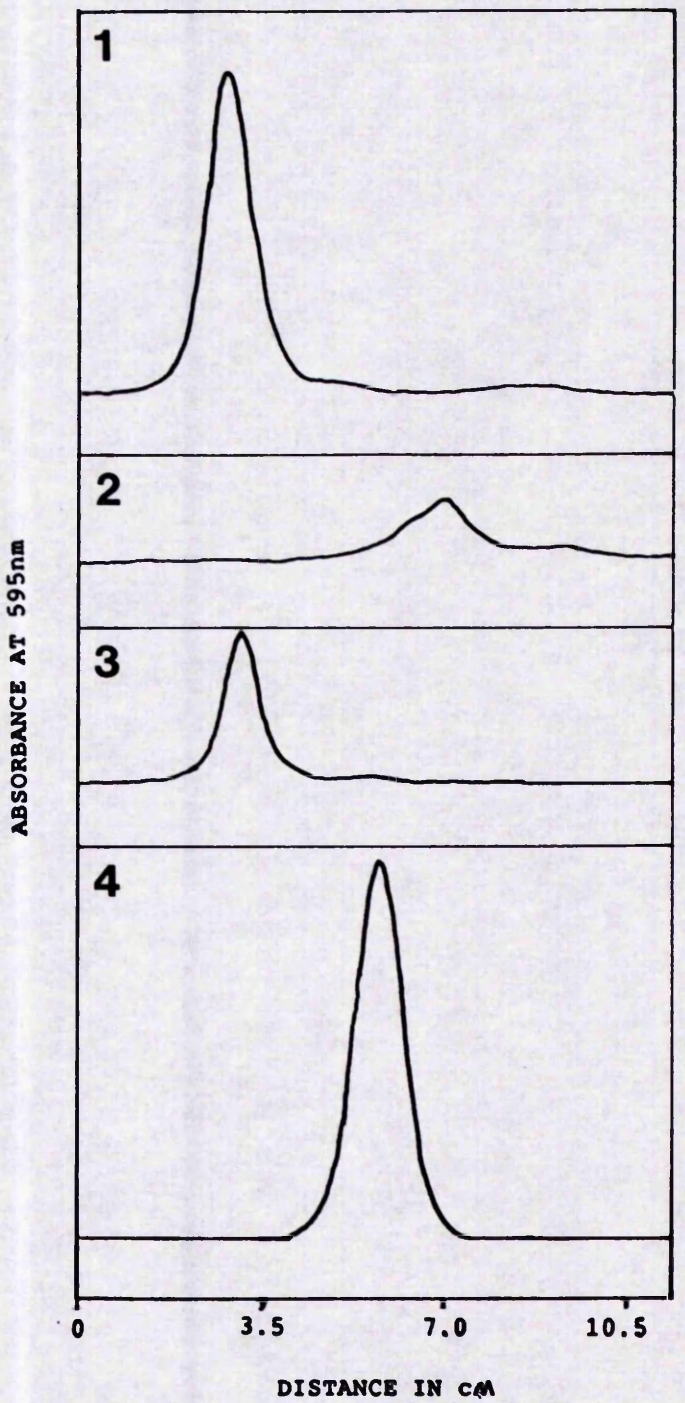
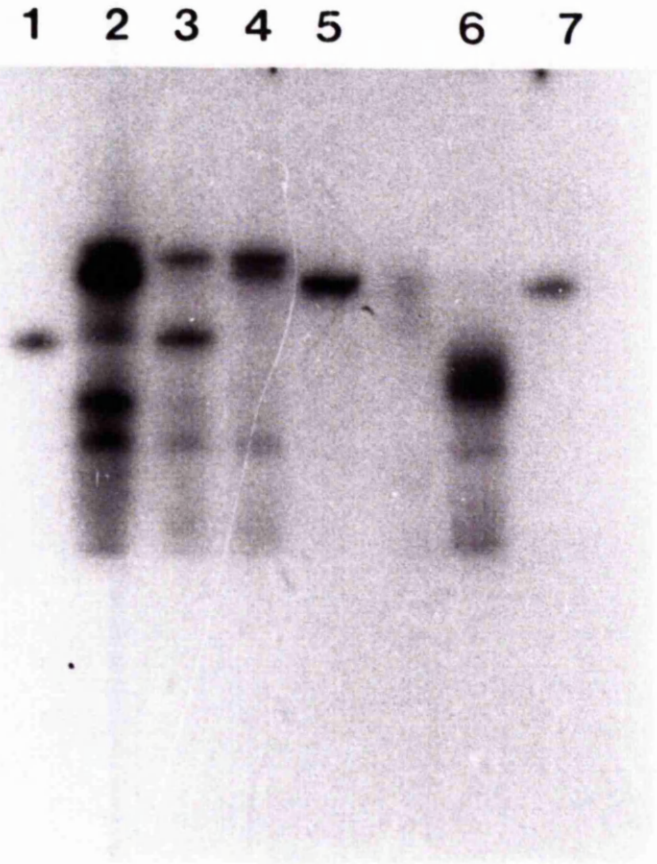


FIGURE 48 - XmnI digestion of double stranded oligonucleotide, control substrate and EIII substrate

1 Standard 13mer
2 Digestion of double stranded oligonucleotide
3 Digestion of control substrate
4 Digestion of EIII substrate
5 Piperidine treated control oligonucleotide
6 Piperidine treated oxidized oligonucleotide
7 Oligonucleotide A



4.3 Attempts to determine the sensitivity of the EIII assay

A range of EIII concentrations (0.5ug/ml-40ug/ml) were prepared in a total volume of 20ul containing 0.05M Tris-HCl pH7.6, 0.1M KCl, 0.001M EDTA, 0.001M DTT, 0.1mg/ml BSA (EIII reaction buffer) and 30ng of EIII substrate, while 30ug/ml of EIII and 30ng of control substrate were used in the control assay. After 30 minutes at 37°C reactions were stopped by the addition of 20ul of 2 X UPAGE loading buffer. Samples were denatured by heating at 90°C for 3 minutes and a 5ul aliquot was subjected to UPAGE (20% acrylamide). Electrophoresis was carried out at 150V for approximately 2 hours, using the mini-protean II BioRad apparatus. The gel was then placed against X-ray film for 50 minutes at room temperature.

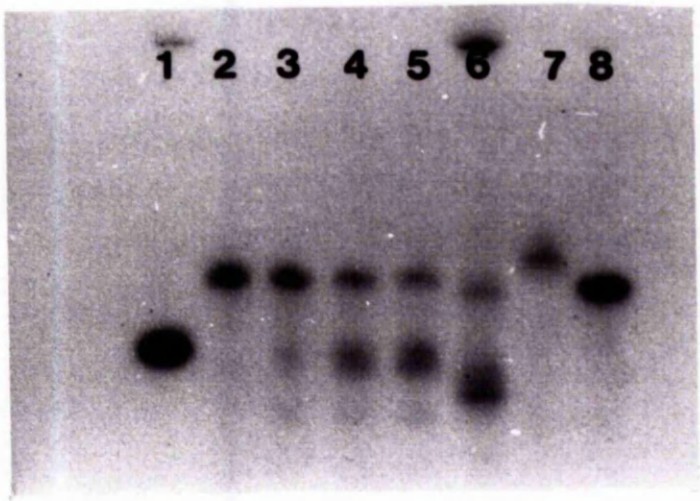
The intensity of the 13mer signal increased with EIII concentration (figure 49), while the 23mer decreased: the lowest concentration at which fragmentation was detected was 0.5ug/ml. This was confirmed from the analysis of the autoradiographs by scanning densitometry (2.2.3vig), which also showed that maximum conversion of 23mer to a 13mer occurred when the EIII substrate was incubated with 10ug/ml EIII (figure 50A).

The fragmentation pattern of the piperidine-treated, oxidized oligonucleotide suggested modifications at positions 13 and 14 and EIII did appear to produce two signals in the appropriate region: although scanning densitometry did not resolve the two signals, the trace of the 13mer peak did have a shoulder (figure 51), as

FIGURE 49 - Attempt to determine the sensitivity of the EIII assay using EIII

Final EIII concentrations are shown.

A
1. Standard 13mer
2. 0ug/ml EIII
3. 0.5ug/ml EIII
4. 5ug/ml EIII
5. 10ug/ml EIII
6. Piperidine treated oxidized oligonucleotide
7. Control assay
8. Oligonucleotide A



B
1. Control assay
2. Standard 13mer
3. 0ug/ml EIII
4. 20ug/ml EIII
5. 30ug/ml EIII
6. 40ug/ml EIII
7. Piperidine treated oxidized oligonucleotide
8. Oligonucleotide A

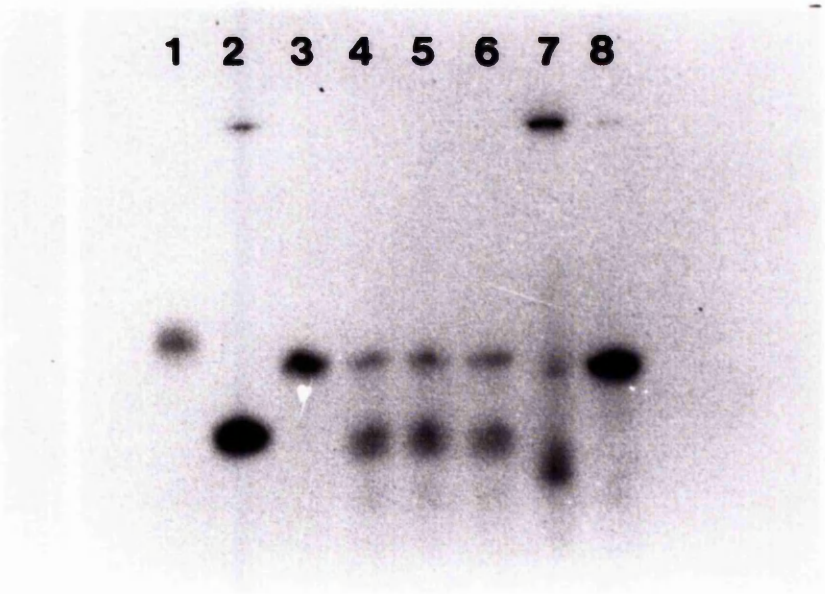
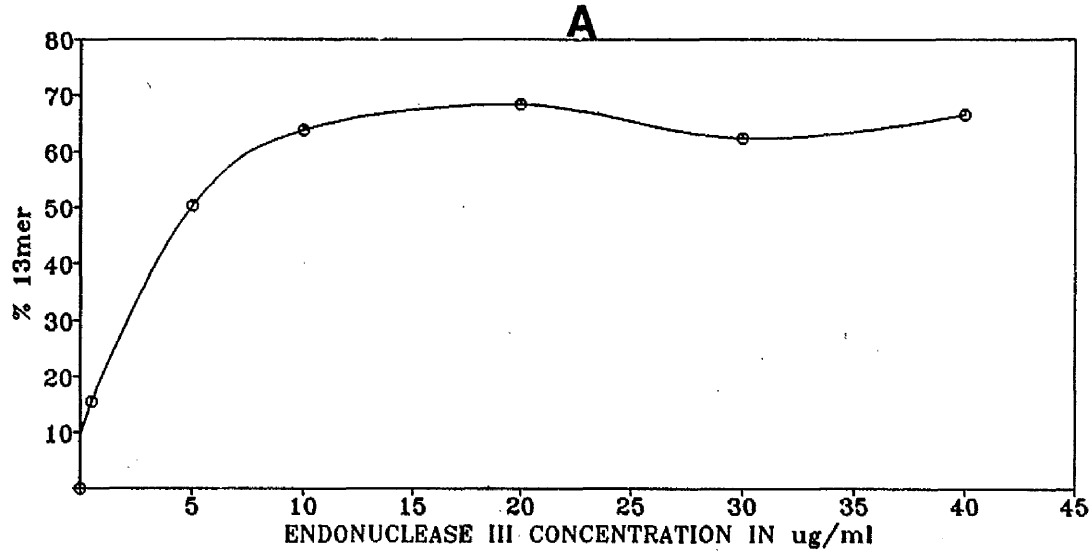
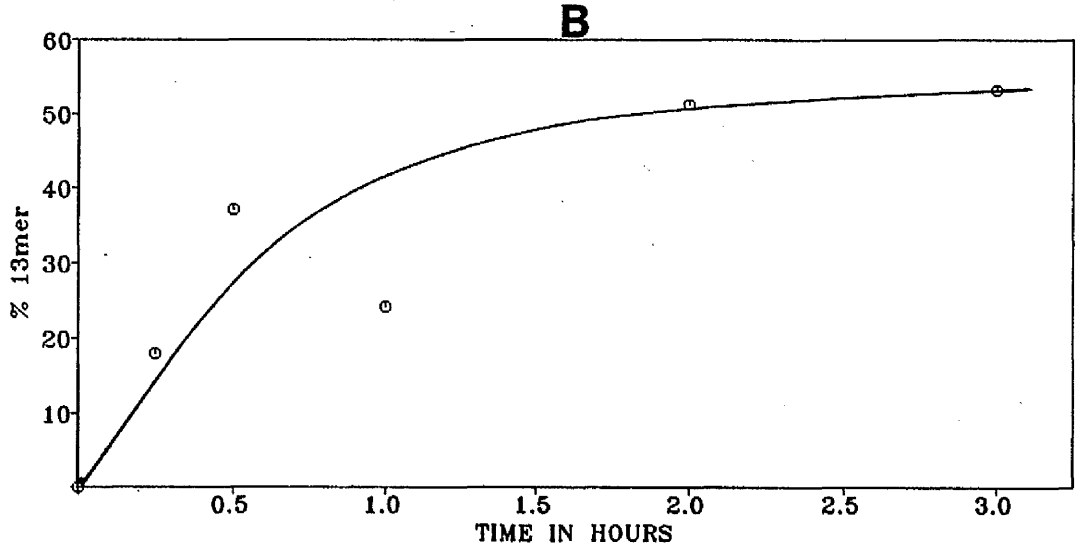


FIGURE 50 - Analysis of the autoradiographs from the determination of the sensitivity of the EIII assay using EIII (A), and bacterial cell-free extract (C) and the time course of the EIII assay (B), using scanning densitometry.


A
% 13mer
80
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40
45
ENDONUCLEASE III CONCENTRATION IN ug/ml


B
% 13mer
60
50
40
30
20
10
0.5
1.0
1.5
2.0
2.5
3.0
TIME IN HOURS

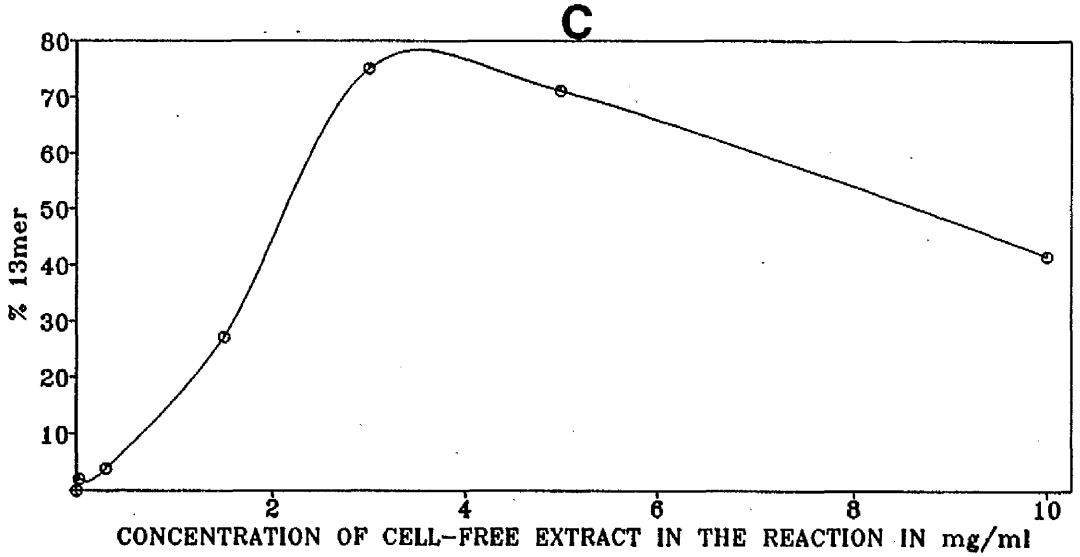
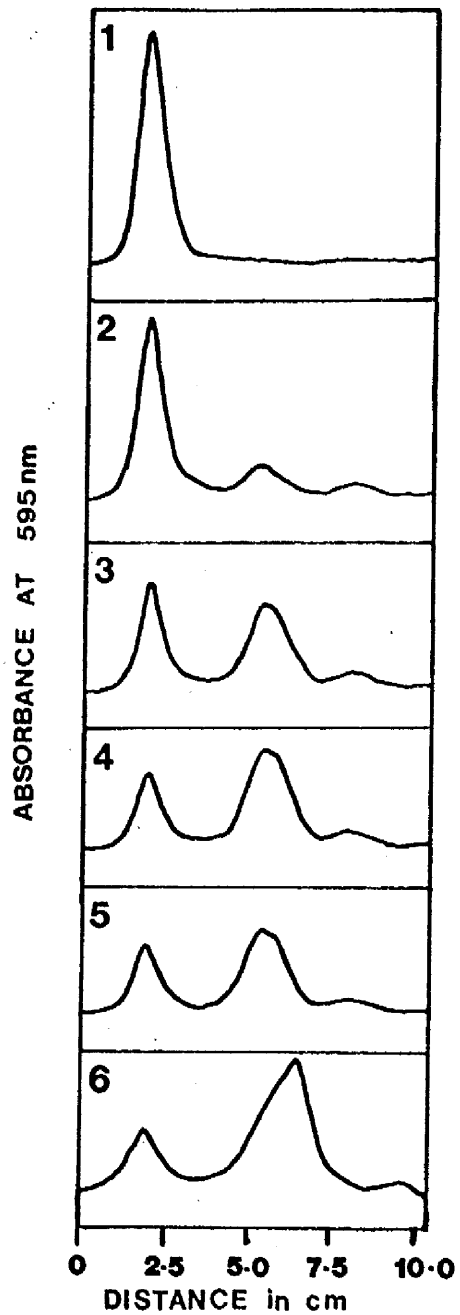

C
% 13mer
80
70
60
50
40
30
20
10
2
4
6
8
10
CONCENTRATION OF CELL-FREE EXTRACT IN THE REACTION IN mg/ml

FIGURE 51 - Analysis of the reaction products from the incubation of EIII substrate with EIII using scanning densitometry.

The scanning densitometry traces obtained from the analysis of the autoradiographs are shown.

1 - control sample
2 - incubation with 0.5ug/ml EIII
3 - incubation with 5 ug/ml EIII
4 - incubation with 10ug/ml EIII
5 - incubation with 40ug/ml EIII
6 - piperidine-treated, oxidized oligonucleotide



previously found in 4.1.

In each of the samples containing EIII (except when the control substrate was used) an extra signal (approximately a 10mer, figure 49) was detected, which was 5% of the total signal. It is possible that this was due to cleavage at a modified base in oligonucleotide A other than thymine, e.g. cytosine (present at positions 1, 3, 4, 5, 9, 12, 15, 18,20, 21, 22, 23), since EIII has been found to incise U.V. irradiated DNA at cytosine (Helland et al 1986).

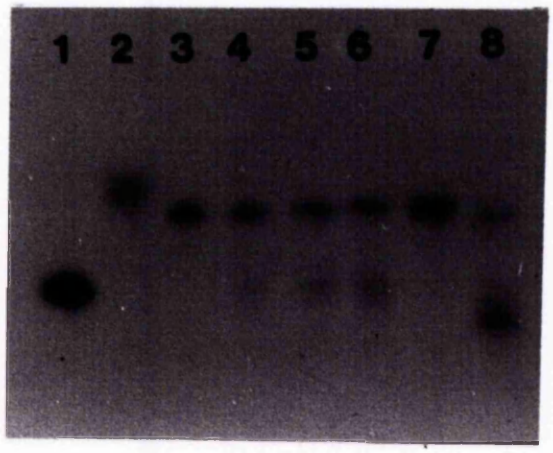
4.4 Time course of the EIII assay

Assays were prepared using the EIII buffer (2.1.12vii), 30ng of EIII substrate and a final concentration of 2.5ug/ml EIII in 20ul. Incubation times of 0, 0.25, 0.5, 1, 2 and 3 hours at 37°C were examined. The reactions were stopped by the addition of an equal volume of 2 X PAGE loading buffer and 5ul was subjected to UPAGE. Autoradiography of the gel detected an increase in the intensity of the 13mer with increasing incubation time (figure 52). This was confirmed by scanning densitometry, which showed that fragmentation of the substrate was linear with respect to time until approximately 0.5 hours and a plateau was reached after 2 hours (figure 50B). The optimum incubation time for the assay was therefore 0.5 hours. Examination of the autoradiograph and the pattern of signal distribution again suggests the presence of a doublet signal at the 13mer and additional base modification(s) in the EIII

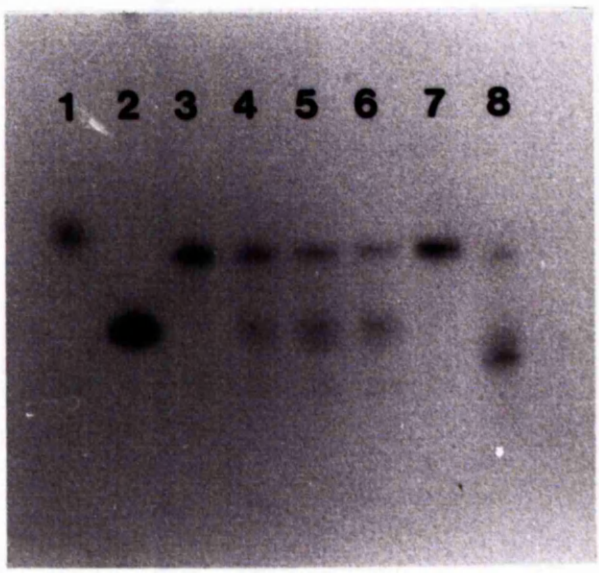
FIGURE 52 - Time course of the EIII assay

Incubation times of the assays are shown.

A
1 Standard 13mer
2 Control assay
3 0 hours
4 0.25 hours
5 0.5 hours
6 1 hour
7 Oligonucleotide A
8 Piperidine treated oxidized oligonucleotide



B
1 Control assay
2 Standard 13mer
3 0 hours
4 0.5 hours
5 2 hours
6 3 hours
7 Oligonucleotide A
8 Piperidine treated oxidized oligonucleotide



substrate at position(s) upstream of the thymidine residues. About 7% of the total signal in the EIII containing samples was due to a fragment approximately the size of a 10mer.

4.5 Detection of EIII-type activity in the cell-free extract of bacteria harbouring pRPC53

Bacteria containing pRPC53 (see 2.1.7) produce approximately 10 times the level of EIII of untransformed bacteria (pers. communication Dr R.P. Cunningham) and western analysis confirmed EIII was present in the cell-free extract of these bacteria (see 3.1.1iii). This extract was therefore chosen as a positive control to determine whether the assay developed could be used to measure EIII activity in cell-free extracts.

Bacterial cell-free extract was prepared as described in 2.2.3i, using EIII reaction buffer as the sonication buffer and 100ul of extract (equivalent to approximately 1mg of protein) was incubated with 100ng of EIII substrate (or control substrate) for 30 minutes at 37°C. Initial attempts to analyse the fragmentation pattern were thwarted by the presence of protein in the sample, which affected the resolution of the fragments. Phenol extraction of the samples removed the protein but was also found to fragment the substrate (control and EIII substrate) and precipitation (using 2M NH_4Ac and 2 volumes cold ethanol) removed the protein and also a high proportion of the oligonucleotide from solution.

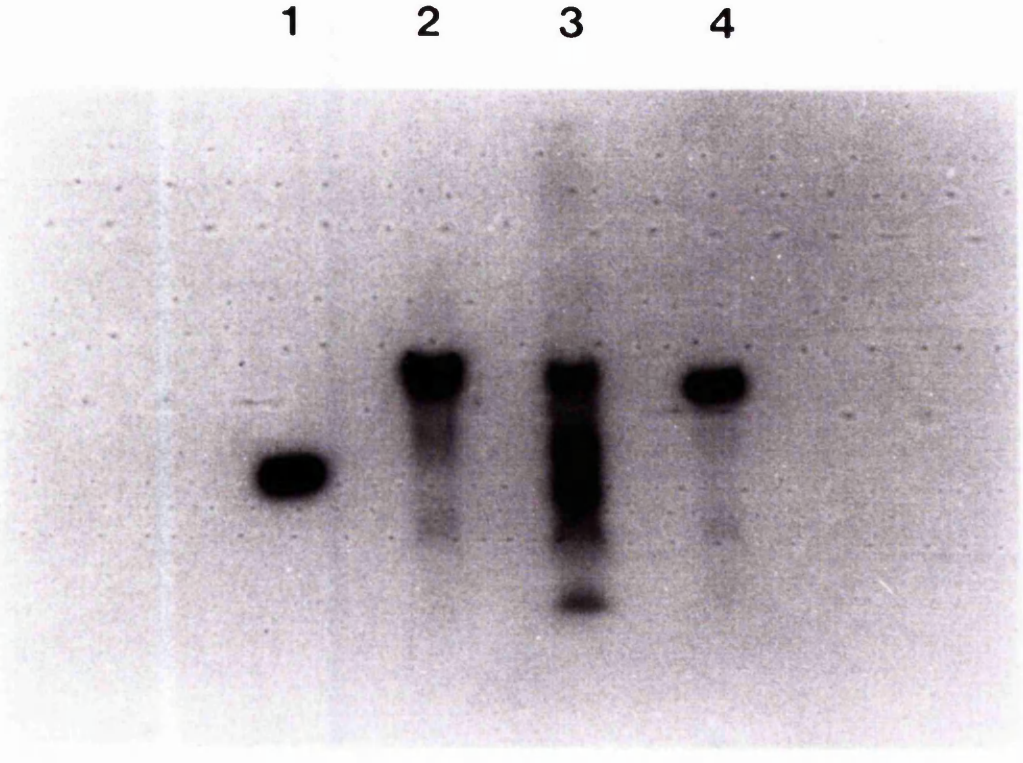
However, it was found that heating the sample precipitated most of the protein. Therefore, 50ul of each assay was heated to 100°C for 5 minutes and centrifuged at 13,000 rpm for 5 minutes at room temperature. The oligonucleotides in the supernatant were co-precipitated with 5ug of calf thymus DNA, using 2M NH_4Ac and 2 volumes of cold ethanol at -20°C for approximately 2 hours. After centrifugation at 16,000 rpm, 0°C for 15 minutes the pellet was washed with 70% (v/v) ethanol, dried and finally resuspended in 20ul of 1 X UPAGE loading buffer. The 20ul sample was heated to 100°C for 5 minutes again, as small amounts of contaminating protein prevented resolution of fragments if the sample was subjected to UPAGE at this point. After centrifugation (13,000 rpm, 2 minutes) the supernatant was subjected to UPAGE.

The control substrate was not fragmented by the bacterial cell-free extract (figure 53). However, two signals were seen at the 13 and 14mer positions in the sample containing the EIII substrate. The cell-free extract did not fragment 100% of the EIII substrate: 6.5% of the total signal was at the 23mer position, 29.4% was at the 14mer position and 54% corresponed to the 13mer signal. This again demonstrates that the EIII substrate was damaged at the two thymidines of oligonucleotide A. Since the oligonucleotide was ^{32}P labelled at the 5′ termimus the site of cleavage of the substrate nearest to the 5′ end would be detected by this assay. Therefore, it is not possible to determine the extent of

FIGURE 53 - Detection of EIII-type activity in the cell-free extract of bacteria harbouring pRPC53

Control or EIII substrate was incubated at 37°C with bacterial cell-free extract.

1 Standard 13mer
2 Control substrate
3 EIII substrate
4 Oligonucleotide A



modification at position 14, since is likely that cleavage at position 14 was not detected in the proportion of EIII substrate damaged at positions 13 and 14. However, these results suggest that 54% of the EIII substrate was modified at position 13.

These results showed that the assay could be used to quantitate EIII activity.

4.6 Attempts to determine the sensitivity of the EIII assay using cell-free extract of bacteria harbouring pRPC53

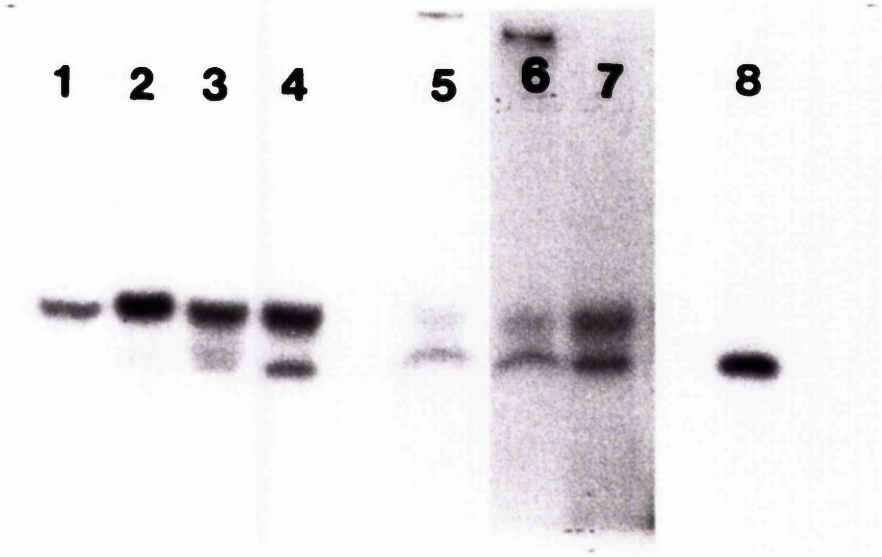
Bacterial cell-free extract was prepared as described in 2.2.3i and the protein concentration of the extract estimated (see 2.2.3ii). Assays were carried out in 100ul using 100ng of EIII substrate, with final concentrations of cell-free extract ranging from 0.03mg/ml-10mg/ml. After incubation at 37°C for 30 minutes the protein was removed from the samples (see 4.5) before they were subjected to UPAGE.

Fragmentation of the EIII substrate was detected at the lowest concentration of cell-free extract assayed (0.03mg/ml) and the intensity of the 13mer increased with bacterial protein concentration (figure 54). Maximum fragmentation occurred at 3mg/ml (figure 50C), but at concentrations greater than approximately 5mg/ml the reaction appeared to be inhibited. It is likely that EIII is only a small percentage of the total protein of the bacterial extract. A high protein concentration may therefore prevent interaction of the EIII with the substrate and result in an inhibition of the reaction.

Figure 54 - Attempts to determine the sensitivity of the EIII assay using cell-free extract of bacteria harbouring pRPC53

Final concentrations of bacterial extract are shown.

1	Oligonucleotide A	5	3.0mg/ml extract
2	0.03mg/ml extract	6	5.0mg/ml extract
3	0.3mg/ml extract	7	10mg/ml extract
4	1.5mg/ml extract	8	Standard 13mer



4.7 Attempts to detect EIII-type activity in cell-free extracts of RJKO-transfected cell lines.

Comparison of the intensity of signals corresponding to EIII on western analysis of partially purified cell-free extracts of 7D and bacteria transformed with pRPC53, showed that EIII expression was much higher (approximately 100-200 times) in the bacteria than in 7D (see 3.1.1iii). Therefore to try to detect EIII, assays using a final concentration of 7mg/ml of mammalian cell-free extract and 100ng substrate (control or EIII) in 100ul were attempted. The positive control was bacterial cell-free extract assayed at an equivalent protein concentration.

RJKO and 1D extracts did not fragment the EIII substrate (figure 55A). Therefore the protein concentration was increased to 13mg/ml, but fragmentation was still not seen using extracts of RJKO, 1D, 7D, or 6E (figure 55B, C). It was therefore not possible to detect the endogenous EIII-like activity in the Chinese hamster cell-free extracts or the EIII expressed in clones 1D and 7D using this assay. It is possible that the high protein concentration may have inhibited the reaction, as found in 4.4. The positive control (13mg/ml of bacterial cell-free extract), however, does appear to have fragmented the EIII substrate to produce a signal of approximately the size of a 10mer (figure 55B), which may be explained by modification(s) upstream of the thymidine residues in oligonucleotide A. Scanning densitometry of the signal obtained from piperidine-treated, oxidized

FIGURE 55 - Attempts to detect EIII-type activity in cell-free extracts of RJKO and RJKO-transfected cell lines

A - Reaction of RJKO and 1D extracts at a final concentration of 7mg/ml extract

1 Oligonucleotide A
2 Standard 13mer
3 Piperidine treated oxidized oligonucleotide
4 Bacterial extract incubated with control substrate
5 1D incubated with EIII substrate
6 RJKO incubated with EIII substrate
7 1D incubated with control substrate
8 Bacterial extract incubated with EIII substrate
9 RJKO incubated with control substrate

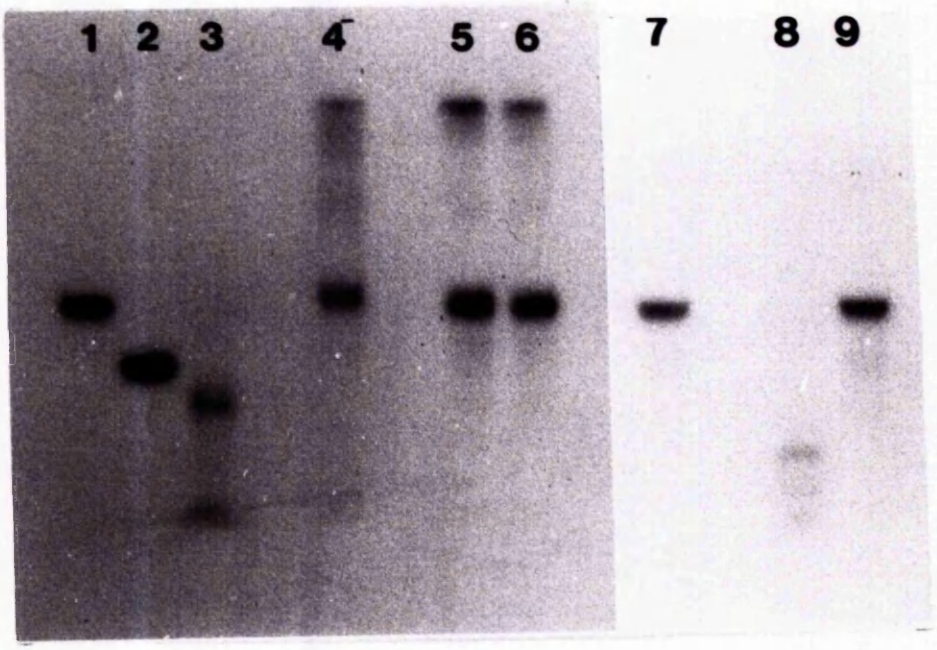
B - Reaction of RJKO, 1D, 7D and 6E extracts at a final concentration of 13mg/ml with EIII substrate

1 Bacterial extract
2 RJKO
3 7D
4 1D
5 6E
6 Piperidine treated oxidized oligonucleotide
7 Standard 13mer
8 Oligonucleotide A

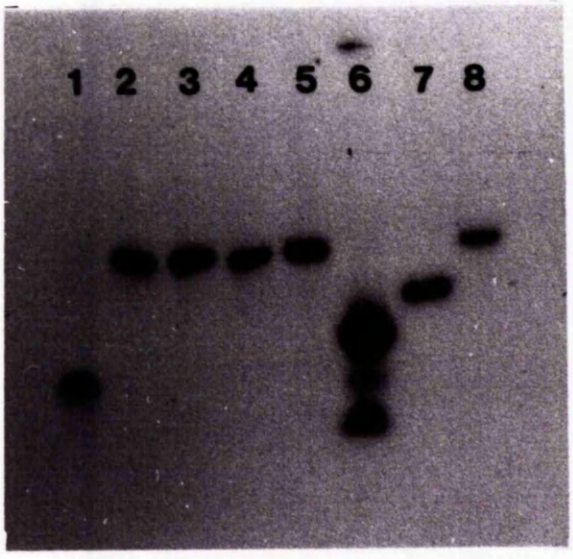
C - Reaction of RJKO, 1D, 7D and 6E extracts at a final concentration of 13mg.ml with control substrate

1 Standard 13mer
2 Oligonucleotide A
3 Piperidine treated control oligonucleotide
4 6E
5 1D
6 7D
7 RJKO

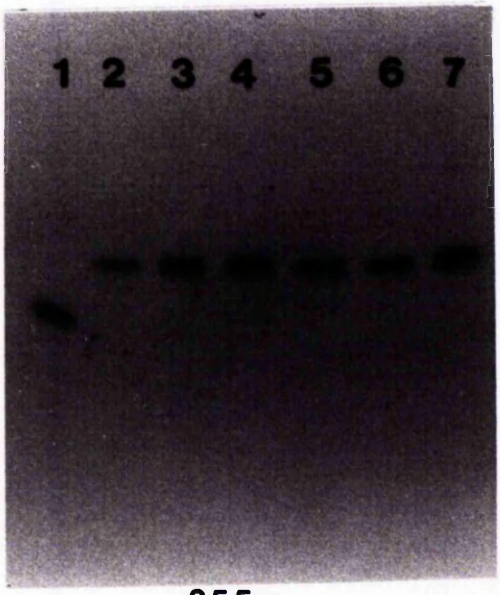
A



B



C



oligonucleotide showed that 4% of the oligonucleotide was damaged at approximately the 10mer position, but 95% of the EIII substrate was fragmented to approximately a 10mer by the bacterial extract. It is possible that in reactions using a final concentration of 13mg/ml of extract, non-specific endonucleases, present in the bacterial but not the mammalian extracts, degraded the substrate.

Without further modification the assay developed cannot be used to screen pZipnth transfected clones for EIII activity. Partial purification of cell-free extracts for example, using DNA cellulose chromatography (see 2.2.3iv) would concentrate the DNA binding proteins and allow lower total protein concentrations to be assayed. It would be interesting to try to measure EIII-activity in assays containing partially purified EIII and large ammounts of bacterial extract to examine which activity (non-specific endonucleases or EIII) would preferentially fragment the substrate. Increasing the EDTA concentration may inhibit other nucleases and resolve the problem of fragmentation by non-specific nucleases.

4.8 Attempts to immunoprecipitate the oxidized oligonucleotide with an anti-thymidine glycol antibody

The assay procedure was lengthened considerably by removal of the protein from the samples to allow UPAGE analysis, and by quantitation of EIII substrate fragmentation by scanning densitometry. It was proposed that

the assay would be easier and faster if the unrepaired oligonucleotide in the sample could be immunoprecipitated with an anti-thymidine glycol (TdG) monoclonal antibody (2.1.11). If the repaired, fragmented substrate remained in solution after centrifugation, the radioactivity in the supernatant would be a measure of EIII activity. It would also still be possible to visualise the substrate cleavage pattern by analysing a fraction of the supernatant by UPAGE.

The anti-TdG monoclonal antibody had been found to have a higher affinity for TG in single stranded rather than double stranded DNA (pers. communication Dr Osamu Nikaido). Therefore, a variety of hybridization and precipitation conditions were used in attempts to immunoprecipitate [^{32}P]-labelled oxidized oligonucleotide A and not the EIII substrate, which is double stranded. A portion of oxidized oligonucleotide had previously been treated with piperidine and alkali and analysed by UPAGE to confirm that the oligonucleotide was damaged. Non-specific binding of the anti-TdG antibody to [^{32}P]-labelled control oligonucleotide was also examined.

4.8.1 Attempts to bind the anti-TdG antibody to oxidized oligonucleotide

Hybridization reactions (30ul) containing 5ng of oxidized or control oligonucleotide (final concentration of 167pg/ul) and 0.3%-16.7% final percentage of antibody were prepared in duplicate. The samples were incubated at either 37^{o}C for 1 hour or at 0^{o}C overnight, and 70ul ddH_2O and 100ul cold saturated ammonium sulphate solution

were added. The samples were mixed and placed on ice for 1 hour to precipitate antibody-oligonucleotide complexes. After centrifugation (15,000 rpm, 15 minutes, 0°C) the radioactivity in 150ul of the supernatant was determined by scintillation counting. No precipitation of the control or oxidized oligonucleotide was detected with samples incubated at 37°C or 0°C (figure 56).

4.8.2 Effect of decreasing the oligonucleotide concentration

To explore if the ratio of oligonucleotide to antibody had prevented detection of a small amount of precipitation, hydridization reactions containing a range of oligo-nucleotide concentrations (0.17pg/ul - 333pg/ul) and a final percentage of antibody of 16.7% were prepared and incubated at 37°C for 1 hour. However, precipitation was not detected with the control or oxidized oligonucleotide (figure 57) at any of the oligonucleotide concentrations examined.

4.8.3 Effect of water and PBS on the binding of the antibody to the oligonucleotide at 37°C

The antibody had been stored in PBS, but previous hybridization reactions had been prepared using ddH_2O. To determine if this had hindered antibody binding, samples (30ul) containing 10ng (333pg/ul) of control or oxidized oligonucleotide and final percentages of 25%-75% antibody were prepared using PBS or ddH_2O. The samples were incubated at 37°C for 1.5 hours and any antibody-oligonucleotide complexes were precipitated, as described

FIGURE 56 - Attempt to bind the anti-TdG monoclonal antibody to control (——) or oxidized (- - -) oligonucleotide at 37°C for 1 hour (A) and 0°C overnight (B).

Incubations were carried out in duplicate and the results are represented by the upper and lower limits of the error bar, while the point is the average of the two assays.

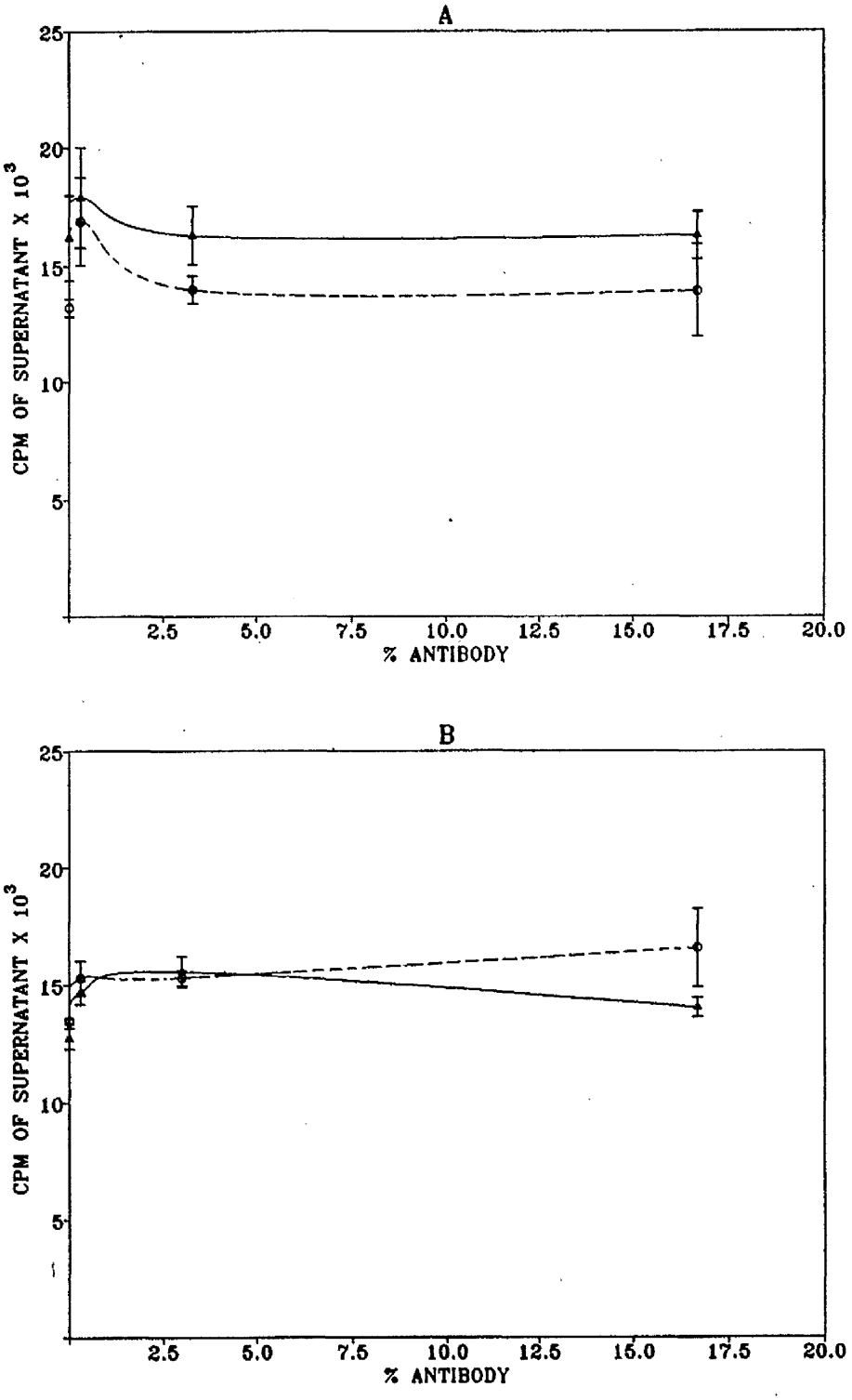
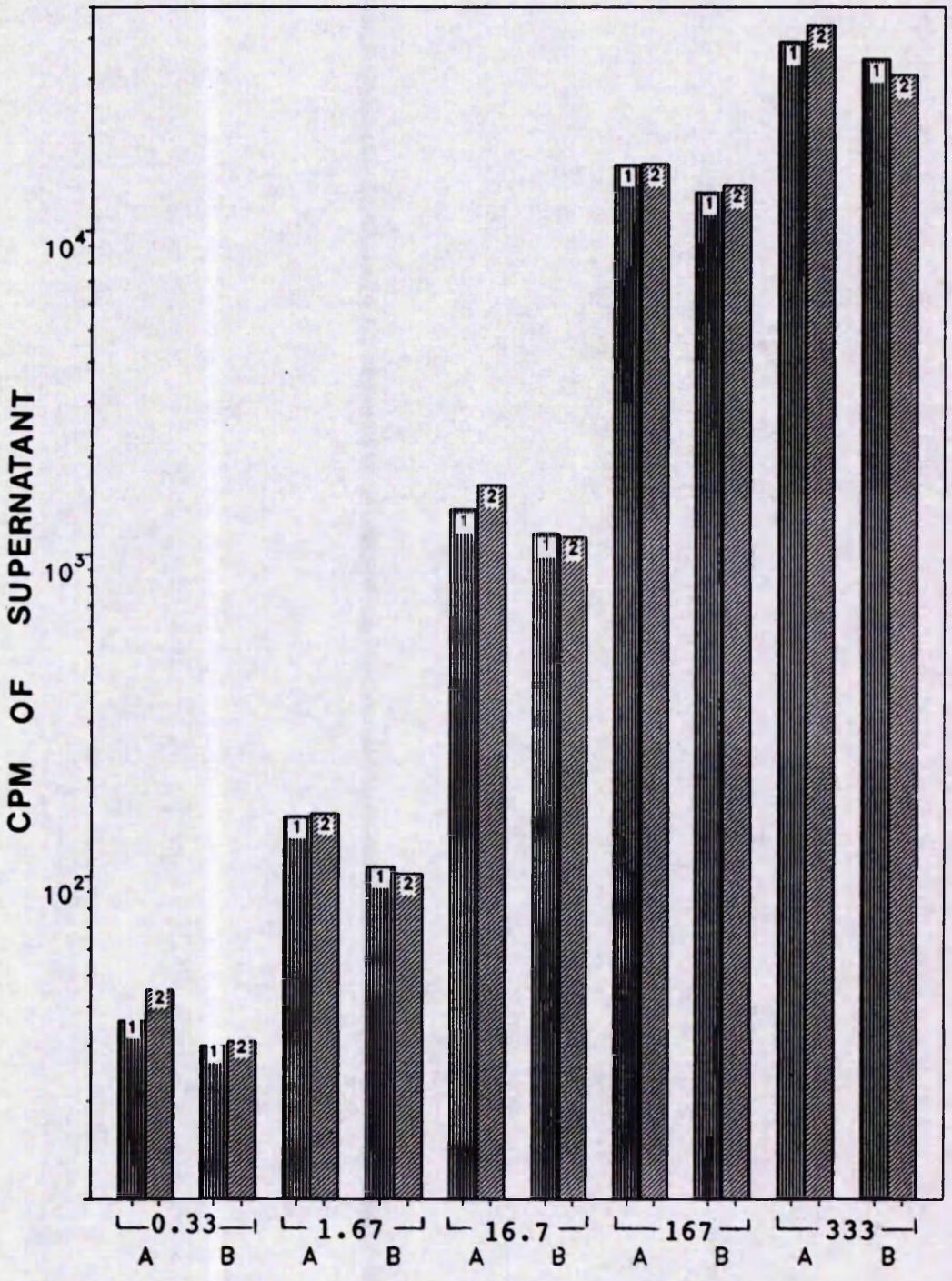


FIGURE 57 - Effect of decreasing the oligonucleotide concentration on the binding of the anti-TdG monoclonal antibody to control (A) or oxidized (B) oligonucleotides

Incubations without (1) and including (2) the anti-TdG monoclonal antibody were carried out at 37°C for 1 hour.



above. No precipitation was detected (figure 58) with either diluent, even though a higher percentage of antibody had been used in this experiment. However, it was interesting to find that when using unsiliconized eppendorf tubes (previously siliconized tubes had been used) oligonucleotide bound to the tube in the absence of antibody (figure 58A). This did not occur when PBS was used as the diluent (figure 58B). Therefore PBS and the anti-TdG antibody blocked the oligonucleotide binding sites on the eppendorf tubes.

4.8.4 Effect of increasing the time of precipitation

Samples containing 10ng of oligonucleotide (333pg/ul) and a range of antibody concentrations (37.5%-75%, final percentage), were incubated at 37°C for 1 hour and placed on ice for 24 hours. Increasing the precipitation time did not alter the amount of oligonucleotide in the supernatant (figure 59). Hence the 1 hour precipitation time previously used had not prevented the detection of oligonucleotide-antibody binding.

4.9. Attempts to bind the oligonucleotide to immobilized antibody

In view of the lack of immunoprecipitation of the oxidized oligonucleotide, it was considered worthwhile to try to bind the antibody to the oligonucleotide while the antibody was bound to the surface of the wells of a microtitre plate. This would ensure that the precipitation procedure had not resulted in the dissociation of oligonucleotide-antibody complexes.

FIGURE 58 - Effect of water (A) and PBS (B) on the binding of the anti-TdG monoclonal antibody to the control (——) or oxidized (- - -) oligonucleotides.

Duplicate samples were examined. The points represent the average of the two assays, while the upper and lower limits of the error bars are the duplicate results of each sample.

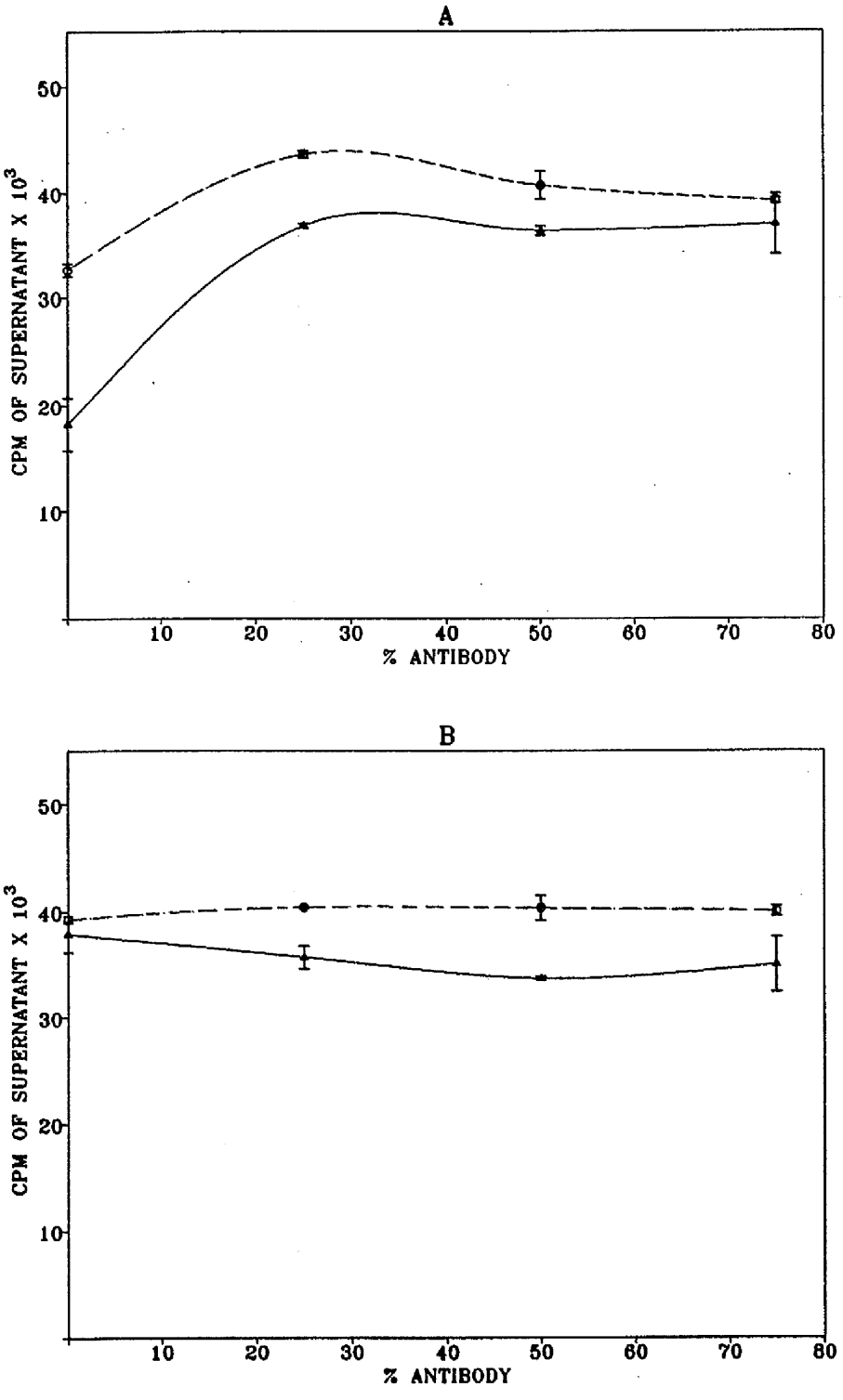
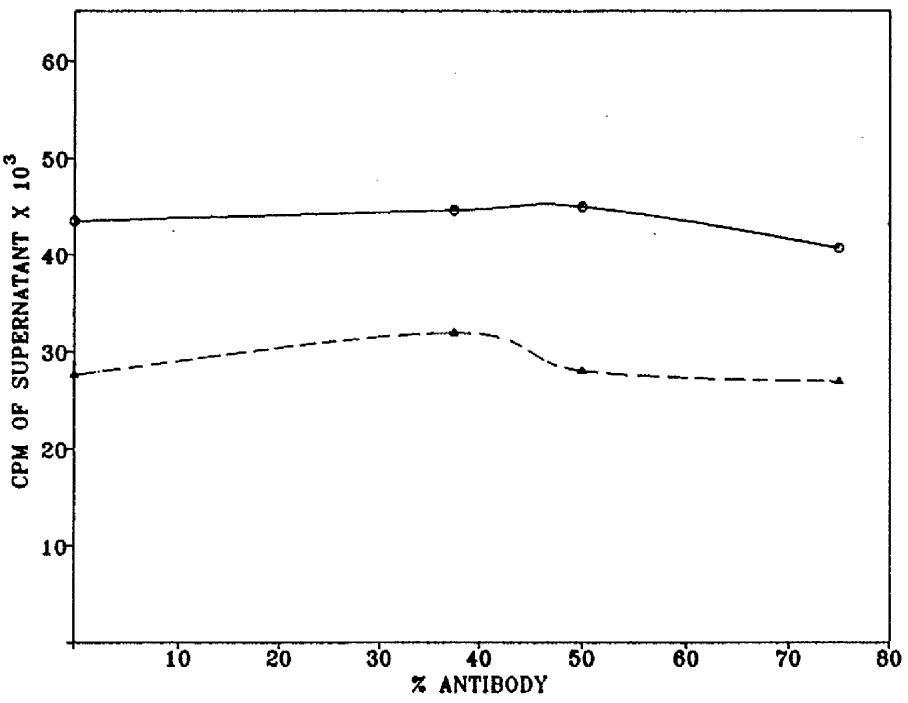


FIGURE 59 - Attempts to immunoprecipitate the control (——) or oxidized (- - -) oligonucleotides using the anti-TdG monoclonal antibody by increasing the time of precipitation to 24 hours.



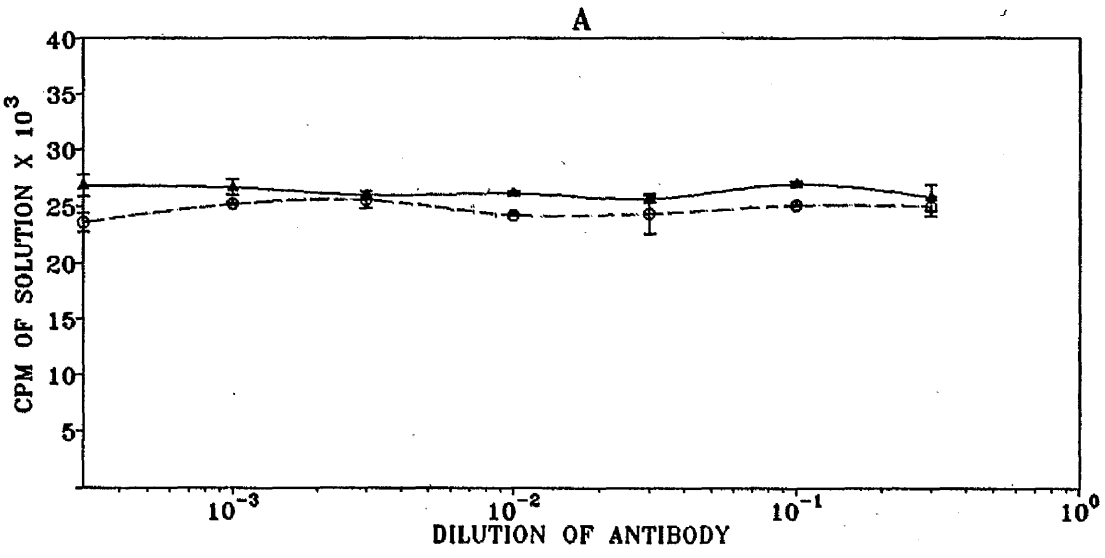
A range of dilutions of the antibody (3x to 3000x) were prepared and the antibody attached to the wells of a microtitre plate (see 2.2.3vij). A variety of hybridization conditions, using 5ng of control or oxidized oligo-nucleotide in 100ul PBS, were examined in duplicate: 37°C for 1.5 hours, room temperature for 6 hours and 4°C for 30 hours. The radioactivity was measured in 80ul of the solution in the wells of the microtitre plate, by scintillation counting. Binding of the control or oxidized oligonucleotide was not detected (figure 60).

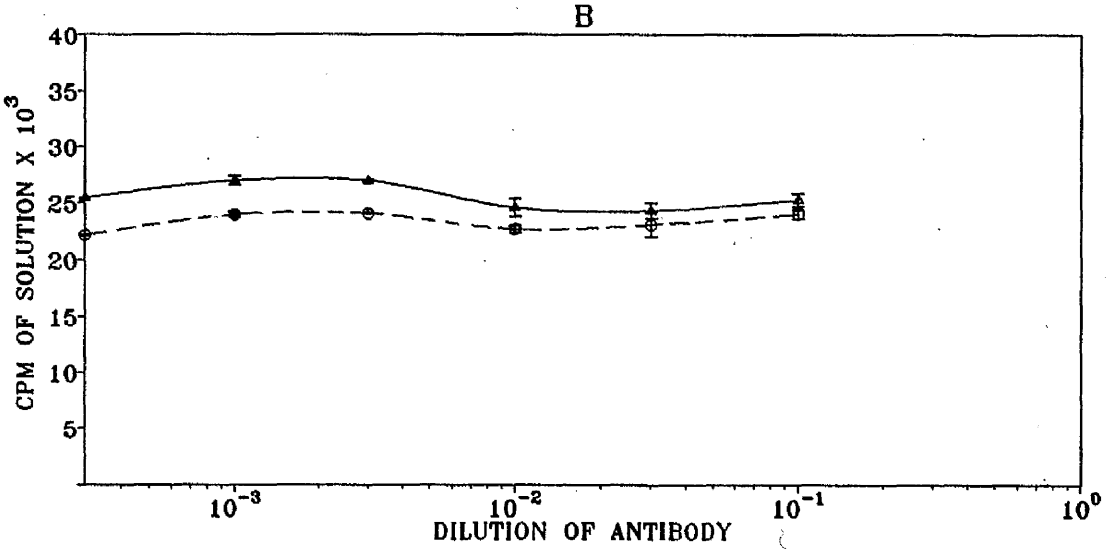
It is possible that the antibody did not bind to the microtitre plate or that on immobilization the antibody was not able to bind the TG antigen in the oligonucleotide.

Considering these results and those of the immunoprecipitation experiments, it is not unreasonable to suggest that this antibody will not bind the oxidized oligonucleotide, certainly under the variety of conditions assessed. The antibody was raised to OsO_4-generated TG in single stranded poly-dT. However, it is possible that the close proximity of two TG residues in the oligonucleotide prevented binding by masking the hapten recognised by the antibody or by distorting the DNA structure. Since a positive control for antibody binding was not available, it is also possible that the antibody no longer has the ability to bind TdG.

FIGURE 60 - Attempt to bind control (——) or oxidized (- -) oligonucleotide to immobilized anti-TdG monoclonal antibody

A variety of hybridization conditions were examined: 37°C for 1.5 hours (A), room temperature for 6 hours (B) and 4°C overnight (C). Each sample was carried out in duplicate. The points represent the average of the two assays, while the upper and lower limits of the error bars are the duplicate results of each sample.


A
CPM OF SOLUTION X 10^3
40
35
30
25
20
15
10
5
10^{-3}
10^{-2}
10^{-1}
10^{0}
DILUTION OF ANTIBODY


B
CPM OF SOLUTION X 10^3
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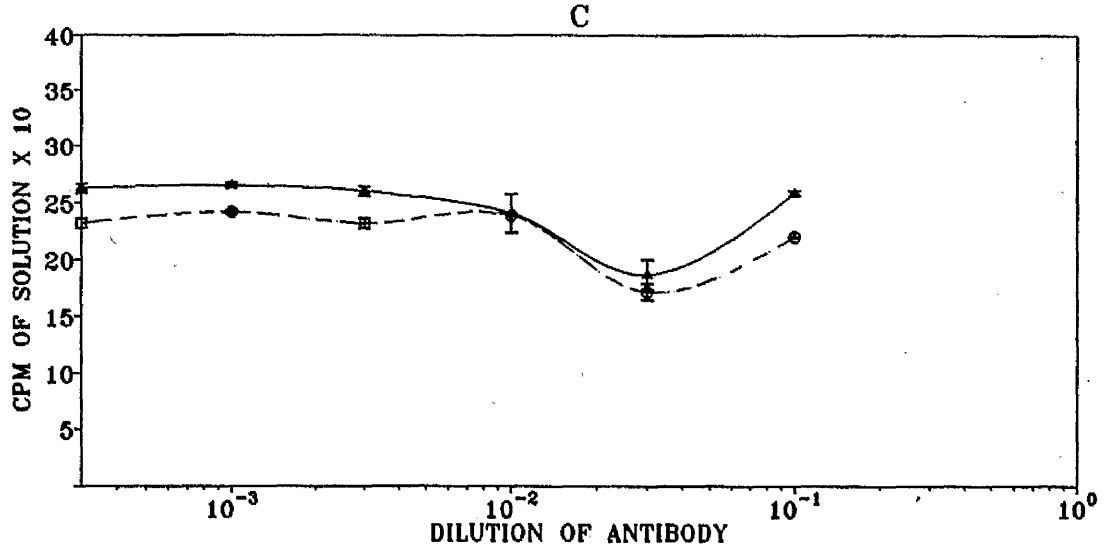
10^{-3}
10^{-2}
10^{-1}
10^{0}
DILUTION OF ANTIBODY


C
CPM OF SOLUTION X 10
40
35
30
25
20
15
10
5
10^{-3}
10^{-2}
10^{-1}
10^{0}
DILUTION OF ANTIBODY

4.10 Summary

The oligo-based EIII assay developed was able to detect 0.5ug/ml of EIII and EIII-type activity in 0.03mg/ml of cell-free extract of bacteria harbouring pRPC53. No activity could be detected in the cell-free extracts of RJKO and RJKO-transfected cell lines, although it is possible that endogenous activity was not seen because the high protein concentration (7 and 13mg/ml) inhibited the reaction. Time was not available to assay the xrs7-transfected clones (x7nth1 and x7nth6), which did have increased AP endonuclease activity compared to the parental cells.

The main advantage of this assay in comparison to those mentioned earlier (I, II, III) is that it is specific for EIII-type enzyme activity. The cleavage pattern of the substrate can be seen by resolution of fragments using a mini-protean II apparatus after 50 minutes of electro-phoresis. The non-specific assay that detects the EIII cleavage pattern (III) does not distinguish between EIII-type activity and U.V. DNA damage repair enzymes in cell-free extracts and requires separation of the reaction products using a large acrylamide gel, which is time consuming (approximately 4-5 hours to complete electrophoresis).

The main disadvantages with the assay developed are that the protein has to be removed from the reaction products before UPAGE analysis and that ^{32}P is used to detect the oligonucleotide. If oligonucleotide A was labelled with a fluorescent nucleotide, the assay would

be safer to carry out. The segments of the gel containing the 13/14mer fragments could be removed and the oligonucleotide extracted from the acrylamide (Maniatis et al 1989) into TE and the fluorescence of the oligonucleotide solution would be a measure of enzyme activity. Two out of the three assays discussed earlier also have the problem of the removal of the protein before analysis (II and III). Immunoprecipitation of unrepaired EIII substrate using the anti-TdG antibody and measurement of the radioactivity of repaired, fragmented substrate would have provided a method of quantitating the EIII activity without UPAGE analysis. However, the anti-TdG antibody available would not bind the oxidized oligonucleotide.

The assay could be modified by chemically attaching one end of oxidized oligonucleotide A, which had been labelled at the other end with ^{32}P (5′ end labelled) or a fluorescent label (3′ end), to commercially available magnetic beads (DYNABEADS, DYNAL Ltd). It would then be annealed to oligonucleotide B to produce EIII substrate. After incubation with the extract, the solution would be heated to denature the substrate and release the fragmented, oxidized oligonucleotide. By placing the eppendorf tubes containing the reaction products into a magnetic holder (MPC, DYNAL) the unfragmented/ fragment of the oxidized oligonucleotide attached to the beads would adhere to the side of the tube, leaving the labelled fragment of the oxidized oligonucleotide in solution. The radioactivity, assessed by scintillation

counting, or fluorescence of the solution would be a measure of EIII-type activity. This would be a more rapid, simple and possibly more sensitive assay.

CHAPTER 5: EXPRESSION OF THE *E.coli nfo* GENE in MAMMALIAN CELLS.

The *E.coli nfo* gene encodes EIV, which has AP endonuclease activity. Although EIII incises at the 3′ side of AP sites (class I activity, AP lyase), EIV catalyses cleavage at the 5′ side (class II activity) and produces a 3′OH termini that can be used by DNA polymerase to complete repair synthesis (see figure 11). Other activities of EIV include the removal of 3′ phosphate and phosphoglycolate DNA termini, cleavage of the C(3′)-O-P bond 5′ to a 3′ terminal AP site, and cleavage of the C(3′)-O-P 5′ to a 3′ terminal base-free 2′3′-unsaturated sugar (Ljungquist 1977, Saporito & Cunningham 1988, Levin et al 1988, Bernelot-Moens & Demple 1989).

Cytotoxicity studies using bacteria deficient in EIV and exonuclease III indicate that EIV is involved in the repair of lesions contributing to the cytotoxic effects of radiation, hydrogen peroxide and bleomycin (Cunningham et al 1986). It is likely that EIV is involved in the repair of radiation-induced DNA breaks, since 70% and 30% of these breaks have 3′ phosphate and 3′ phosphoglycolate termini, respectively (Henner et al 1983). Thus, it was considered worthwhile to examine the effects of EIV expression on survival of xrs7 cells (deficient in dsb repair) to determine whether the removal of 3′ phosphate and 3′ phosphoglycolate termini and AP sites was necessary for xrs7 cell survival after treatment with ionising radiation and hydrogen peroxide.

This would also allow a comparison of the effects of EIII and EIV expression on xrs7 cytotoxicity.

5.1 Production of the *nfo* mammalian cell expression vector

The method of vector construction is summarised in figure 61 .

The *nfo* nucleotide sequence was examined and it was found that the protein coding region of the gene could be isolated by digestion of pRPC124 with PleI and XhoII. Since XhoII digestion of pRPC124 produced 14 fragments, 6 of which ranged between 0.8kb-1.4kb, a preliminary BamHI and HindIII digest (2.2.1iii) was carried out. The 1.2kb fragment isolated (2.2.1iv) was digested with PleI and XhoII to obtain the *nfo* sequence (881bp).

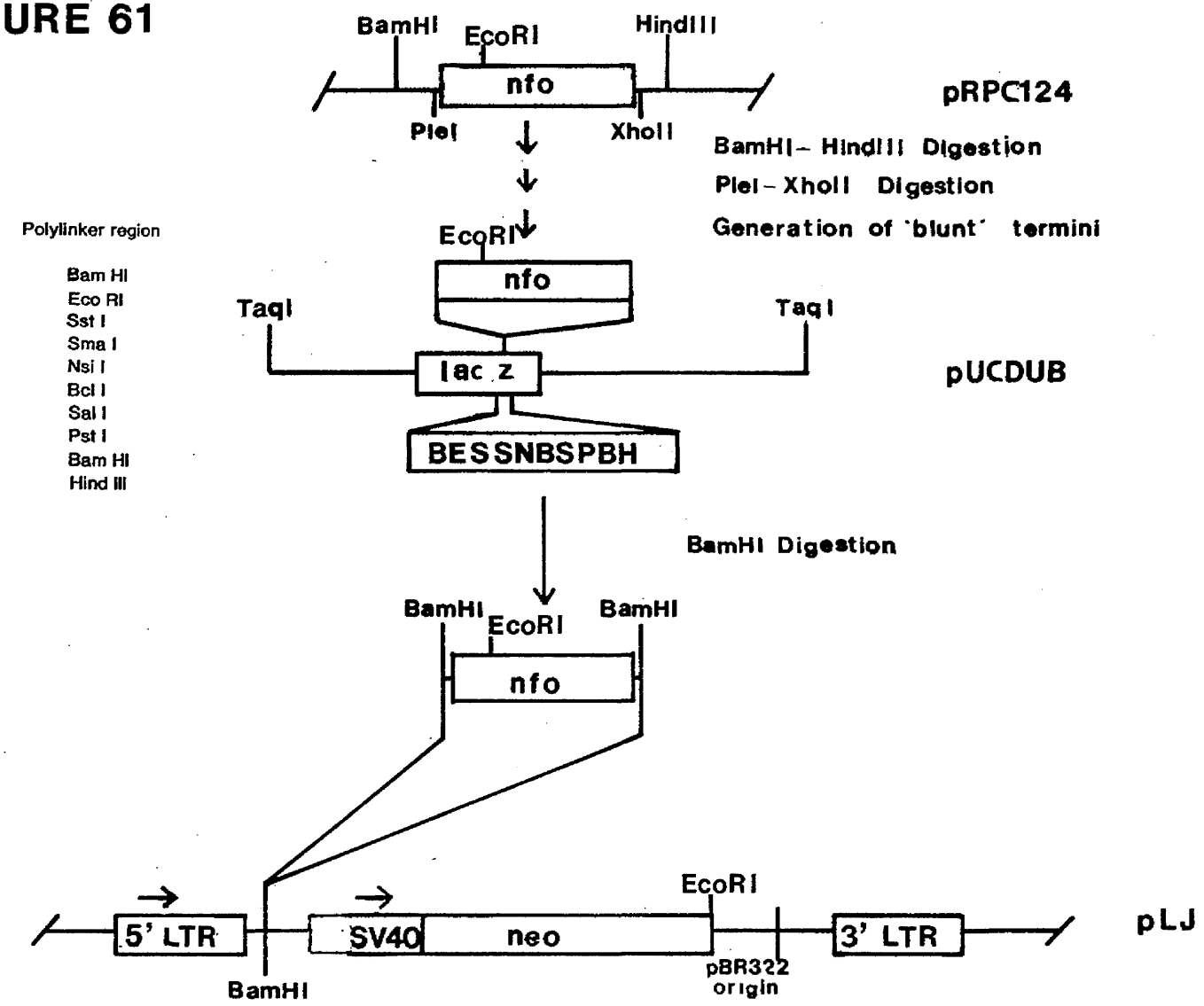
pUCDUB was digested with SmaI, treated with calf intestinal alkaline phosphatase to remove the phosphates from the termini of the plasmid and isolated using LMP agarose (2.2.1iv). Linearization of pUCDUB was confirmed by HaeII digestion, which produced fragments of 1.9kb, 0.5kb and two of 0.2kb, while digestion of circular plasmid produced 1.9kb, 0.5kb and 0.4kb fragments.

The 881bp fragment containing the *nfo* sequence was "blunted-ended" (2.2.1v) and ligated (2.2.1vi) into the SmaI site of pUCDUB. A 2ul aliquot of the ligation reaction was used to transform competent *E.coli*, strain DH5α (see 2.2.1viii), which were then grown on LB agar containing 100ug/ml ampicillin. Plasmid DNA was isolated (2.2.1i) from twenty-four ampicillin resistant clones and eight were found to contain a 3.4kb plasmid,

FIGURE 61 - Construction of pLJnfo

A 1.2kb fragment, isolated by BamHI and HindIII digestion of pRPC124, was digested with PleI and XhoII to produce an 881bp fragment containing the *nfo* protein coding sequence. This was ligated into pUCDUB and after BamHI digestion the 0.9kb fragment of *nfo* was isolated and ligated into the BamHI site of pLJ. The 5′ and 3′ LTR's of the Moloney Leukaemia virus provide the promoter and polyadenylation signals for the expression of EIV in mammalian cells. The truncated *neo* gene is operated from an SV40 promoter and expression of *neo* confers resistance to G418 in mammalian cells. (Not all the restriction sites are shown for the enzymes mentioned in the diagram).

FIGURE 61


BamHI
EcoRI
HindIII
nfo
PleI
XhoII
pRPC124
BamHI – HindIII Digestion
PleI – XhoII Digestion
Generation of 'blunt' termini
Polylinker region
Bam HI
Eco RI
Sst I
Sma I
Nsi I
Bcl I
Sal I
Pst I
Bam HI
Hind III
EcoRI
nfo
TaqI
TaqI
lac z
pUCDUB
BESSNBSPBH
BamHI Digestion
BamHI
EcoRI
BamHI
nfo
EcoRI
5' LTR
SV40
neo
3' LTR
pLJ
BamHI
pBR322
origin

which was digested with EcoRI to determine the orientation of the *nfo* sequence in pUCDUB. EcoRI cleaves the *nfo* sequence to produce 0.15kb and 0.73kb fragments and also linearizes pUCDUB. Fragments of approximately 0.16kb and 3.2kb or 0.73kb and 2.6kb were expected after electrophoresis if the *nfo* sequence was in the correct or incorrect orientation, respectively. Five clones contained pUCDUBnfo, i.e. with the *nfo* sequence in the correct orientation.

pUCDUBnfo was digested with BamHI to isolate a DNA fragment of approximately 0.9kb, containing the *nfo* sequence, and pLJ was linearized by BamHI digestion and treated with calf intestinal alkaline phosphatase. Since the size ratio of pLJ: *nfo* was 10:1 and a ratio of 1: 3 of pLJ termini: *nfo* termini was required for ligation, 600ng of pLJ and 200ng of 0.9kb fragment were used in the ligation reaction (see 2.2.1vi). Competent *E.coli*, strain HB101, were transformed (2.2.1viii) with 1ul of the ligation mixture and the bacteria grown on LB agar containing 50ug/ml kanamycin. Plasmid DNA was isolated from twelve kanamycin resistant clones (2.2.1i) and BamHI digestion showed that the plasmid DNA from ten of the clones contained the 0.9kb fragment. EcoRI digestion was used to determine the orientation of *nfo* in pLJ: sizes of 3.55kb, 3.96kb, 2.42kb and 0.15kb or 4.3kb, 3.96kb, 1.67kb and 0.15kb were expected if the fragment was in the correct or incorrect orientation, respectively. Five clones contained pLJnfo.

5.2 Expression of *nfo* in xrs7 cells

5.2.1 Transfection of pLJnfo into xrs7 cells

Lipofection was used to transfer pLJ and pLJnfo into xrs7 cells (2.2.2iv) and clones were selected at 500ug/ml G418. The transfection frequency (number of G418 resistant colonies/number of cells used to innoculate the flask) of pLJ and pLJnfo was found to be 2 x 10^{-4}. Twenty-three G418 resistant clones were isolated from the pLJnfo transfection and ten survived: x7nfo8, 11, 13-19, 22 and 23. The vector control cell line, x7pLJ9, was one of five G418 resistant clones isolated from the pLJ transfection. Once established the cell lines were maintained in medium containing 300ug/ml G418.

5.2.2 Molecular analysis of G418 resistant clones

(i) PCR analysis

Attempts were made to screen the pLJnfo transfected clones for *nfo* incorporation by PCR of cell extracts (see 2.2.1xii). The oligonucleotides designed for this purpose are shown in figure 15.

The presence of the truncated *nfo* gene would have been confirmed by the amplification of a 605bp DNA fragment. However, no fragments were amplified even when using the oligonucleotides and plasmid DNA (pLJnfo). Each oligonucleotide was therefore used in a PCR reaction with the appropriate *neo* sense or antisense oligonucleotide and pLJnfo in an attempt to determine whether, and which of, the *nfo* oligonucleotides were defective. Amplification using the *nfo* sense and *neo* antisense oligonucleotides

produced a 2.5kb DNA fragment and the *neo* sense and antisense oligonucleotides amplified a 624bp fragment, as expected (figure 62). However, PCR using the *nfo* antisense and *neo* sense oligonucleotides did not produce any fragments. This suggested that it was the antisense *nfo* oligonucleotide that was defective. PCR analysis (2.2.1xii) using DNA of the clones mentioned above was carried out using the *nfo* sense and *neo* antisense oligonucleotides. Although a 2.5kb fragment was amplified using x7nfo11 and 8 DNA, a 2kb fragment was amplified using DNA from x7nfo15, 19 and 23 (figure 62). Two fragments (approximately 0.6kb and 0.4kb) were also amplified using DNA from xrs7 and x7nfo15, 19 and 23. DNA dot blot and Southern analyses were therefore carried out to confirm that pLJnfo had incorporated into the genomes of x7nfo11 and 8.

(ii) DNA analysis

Genomic DNA was isolated from each of the ten pLJnfo-transfected clones and from the five pLJ-transfected clones and used in dot blot analysis (2.2.1xiva). DNA from xrs7, x7pLJ9 and x7nfo8, 11, 13 and 19 was subjected to Southern analysis (2.2.1xivc).

The probe was the ^{32}P-labelled 1.2kb DNA fragment, isolated from a BamHI and HindIII digest of pRPC124 (see 5.1).

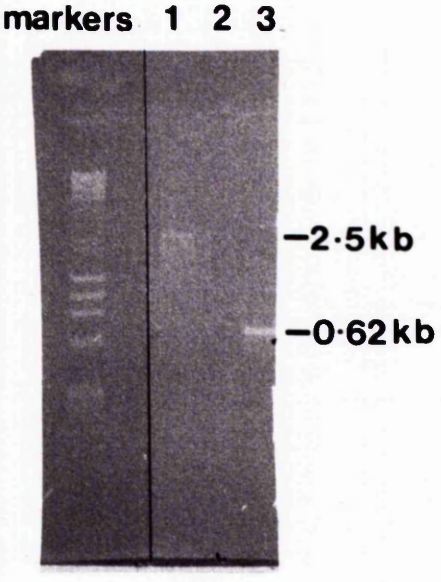
a) DNA dot blot analysis

The positive control was 50ng of pUCDUBnfo and 1ug xrs7 DNA was the negative control. Intense hybridization

FIGURE 62 - PCR analysis

A - Of pLJnfo using:

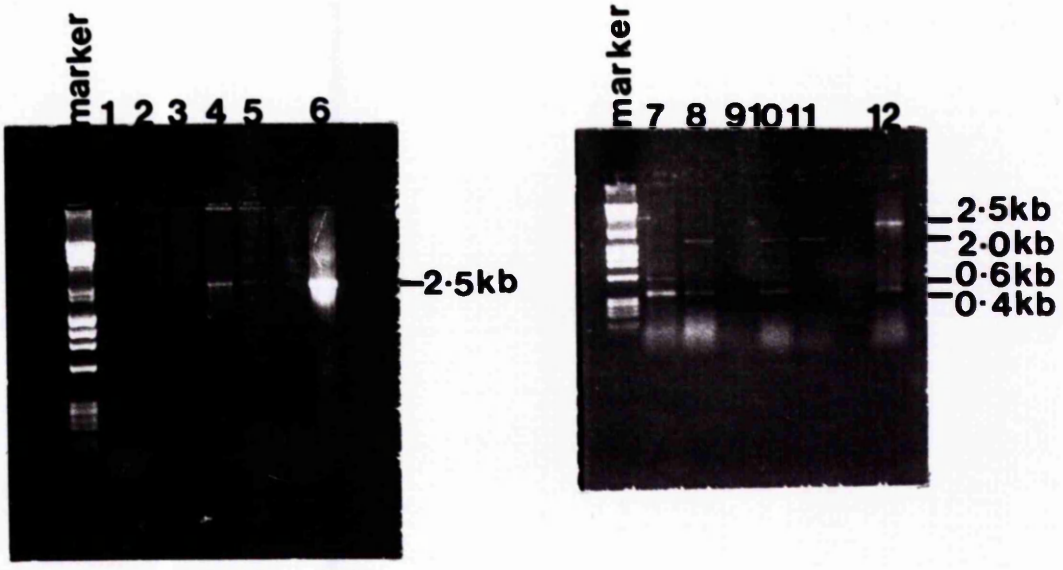
1 *nfo* sense and *neo* antisense oligonucleotides
2 *neo* sense and *nfo* antisense oligonucleotides
3 *neo* sense and antisense oligonucleotides



B - Of DNA from:

1	xrs7	7	xrs7
2	x7nfo14	8	x7nfo15
3	x7nfo16	9	x7nfo18
4	x7nfo11	10	x7nfo19
5	x7nfo8	11	x7nfo23
6	pLJnfo	12	x7nfo11

Attempts were made to detect *nfo* by PCR using the *nfo* sense and *neo* antisense oligonucleotides. Amplifications 1-6 and 7-12 were separate experiments.



signals, indicating the presence of the *nfo* sequence, were found with the positive control and the DNA of clones x7nfo11, 13 and 19, but not x7nfo8 (figure 63A). Faint signals were also detected in four of the vector control cell lines and four other pLJnfo-transfected clones. Similar signals were seen with DNA of 6E and 9D after probing the DNA dot blot with the 1kb *nth* probe (see 3.1.1i). The signals may be due to a cross-reaction of the probe with vector sequences or mammalian DNA. Xrs7 DNA did hybridize to the probe (lane c5), however, it is possible that this may have been due to a contamination of the xrs7 sample with *nfo* clone DNA or pUCDUBnfo, since the signal intensity was greater than that of the vector control cell lines.

b) Southern analysis

Genomic DNA of xrs7, x7pLJ9 and x7nfo8, 11, 13 and 19 was digested using BamHI or EcoRI, which on Southern analysis was expected to produce signals of 0.9kb or 0.15kb and 2.4kb, respectively, if pLJnfo had been incorporated into the genome of the cells.

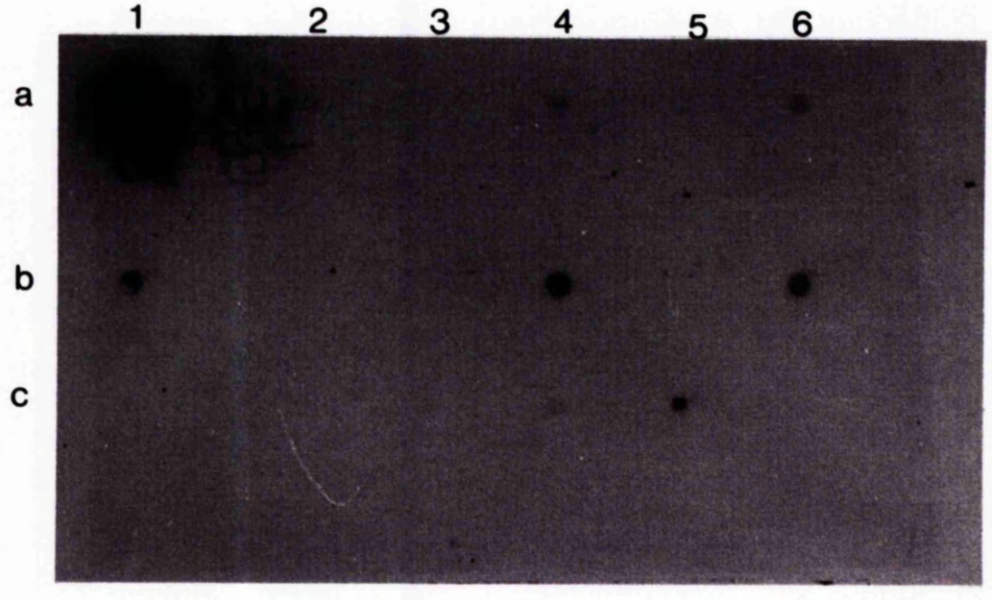
A 0.9kb signal was seen in the BamHI digests of x7nfo13 and 11 and a 2.4kb signal was found in the EcoRI digests of x7nfo11, 13 and 19 (figure 63B). The signal intensity was much greater for x7nfo11, suggesting the presence of a higher *nfo* copy number in this clone. Clone x7nfo19, although positive by dot blot analysis, did not produce the expected digestion patterns: signals of 9kb, 4.9kb, 2kb and 1.3kb were found after BamHI digestion and

FIGURE 63 - DNA analysis of xrs7 and pLJ or pLJnfo-transfected xrs7 cell lines

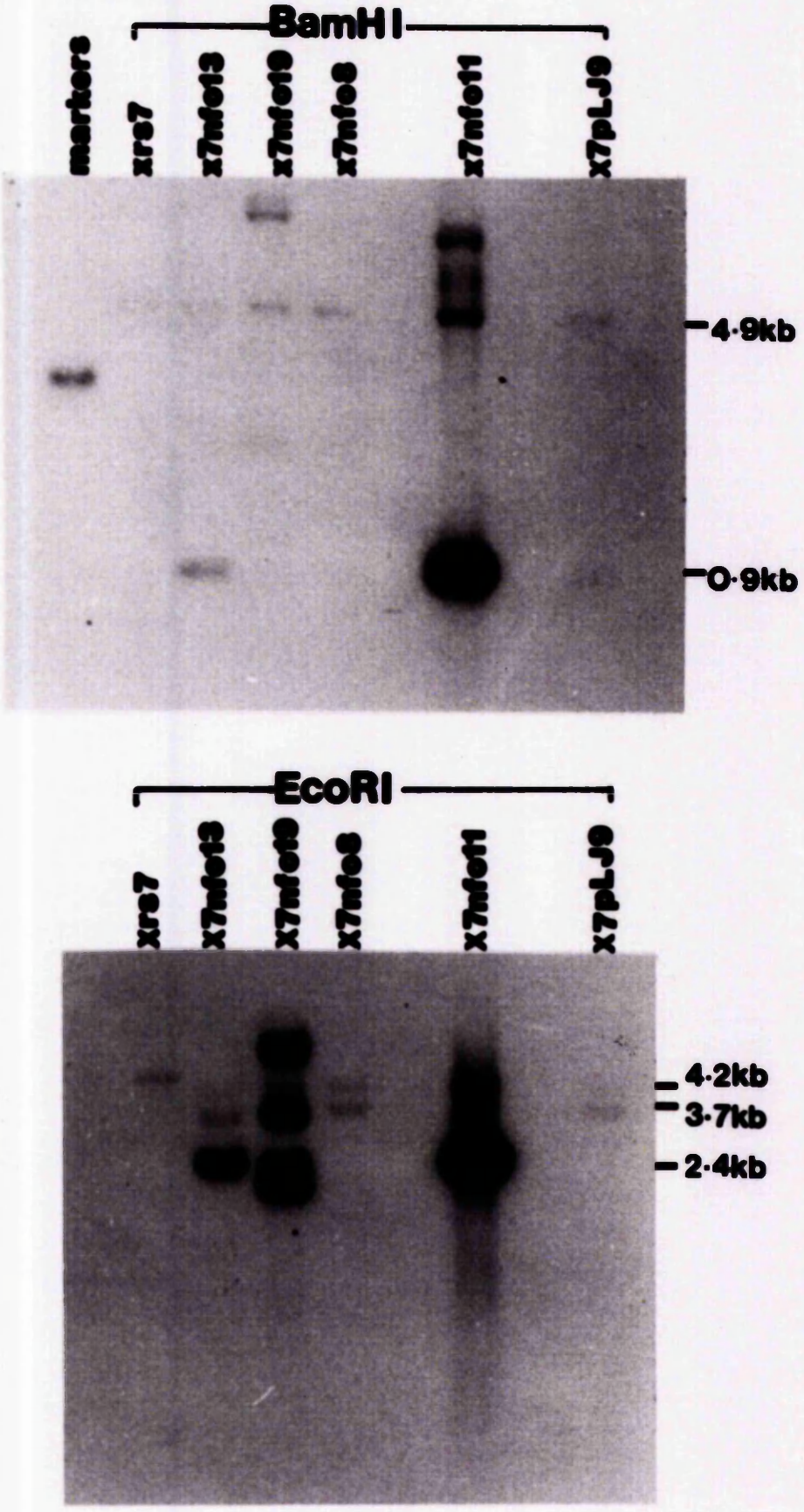
Filters were probed with the *nfo* probe.

A - DNA dot blot analysis

a	b	c
1 pUCDUBnfo	1 x7nfo13	1 x7nfo18
2 x7pLJ9	2 x7nfo15	2 x7nfo23
3 x7pLJ1	3 x7nfo22	3 x7nfo14
4 x7pLJ6	4 x7nfo11	4 x7nfo16
5 x7pLJ8	5 x7nfo8	[redacted]
6 x7pLJ5	6 x7nfo19	5 xrs7



B - Southern analysis



5.6kb, 4.2kb, 3.7kb, 2.4kb and 2.2kb were found after EcoRI digestion. It is possible that this banding pattern was due to partial insertion or rearrangement of pLJnfo on insertion into the genome.

A signal of 4.9kb was present in all of the BamHI digested samples, including the xrs7, x7pLJ9 and x7nfo8, and in the EcoRI digest of xrs7 DNA. If the signal had been due to contamination of DNA solutions or buffers with pLJnfo or pUCDUBnfo signals of 11kb or 3.4kb, respectively, would have been detected. Thus, the 4.9kb signal suggests that there are sequences in the Chinese hamster DNA that are complementary to *nfo*. It is possible that the probe cross-reacted with the DNA sequence that encodes a Chinese hamster EIV-type enzyme, since the DNA and protein sequences of *nfo* and EIV have been found to be highly complementary to the yeast *apn1* gene and APN1 protein sequences (Popoff et al 1990) and may show similar homology to other eukaryote genes encoding EIV-type enzymes.

In EcoRI digests of DNA from the transfected cell lines, signals corresponding to the sizes of 3.7kb and 4.2kb were also present. These may be due to a cross-reaction of the probe with pLJ vector sequences, which would also explain the faint hybridization signals detected on DNA dot blot analysis with DNA from four vector control cell lines and four pLJnfo-transfected cell lines that were resistant to G418, but had not incorporated *nfo*.

(ii) Northern analysis

Total RNA was isolated from xrs7 and clones x7pLJ9, x7nfo11 and 13 and subjected to northern analysis (2.2.1xivd).

A 4.4kb signal was only detected in the RNA of x7nfo11 (figure 64). This corresponded to the size of the message expected to be produced when transcription started at the 5′ MoMLV LTR and finished at the end of the 3′ MoMLV LTR. Therefore, it is probable that clone x7nfo11 produces EIV mRNA. Although x7nfo13 was positive for *nfo* on Southern analysis, the *nfo* message was not detected in the RNA isolated from this cell line. It is possible that the message was produced at a lower level in x7nfo13 than in x7nfo11 and could not be detected. However, since the *nfo* promoter is independent of the *neo* promoter in pLJnfo, x7nfo13 cells could be resistant to G418 and not express *nfo* due to methylation of the *nfo* promoter or DNA sequence.

5.2.3 AP endonuclease activity

Cellular protein extracts (prepared as in 2.2.3i) of xrs7, x7pLJ9 and x7nfo11 were assayed in duplicate for their ability to fragment [^{3}H]-labelled partially depyrimidinated T4 phage DNA (2.2.3viid).

Approximately 2.5 times the AP endonuclease activity of the parental and vector control cell lines was detected in the x7nfo11 extract (figure 65). An anti-EIV antibody was not available for western analysis, however, considering the results of the AP endonuclease assay and

FIGURE 64 - Northern analysis of xrs7, x7pLJ9, x7nfo11 and x7nfo13

The filter was probed with the *nfo* probe.

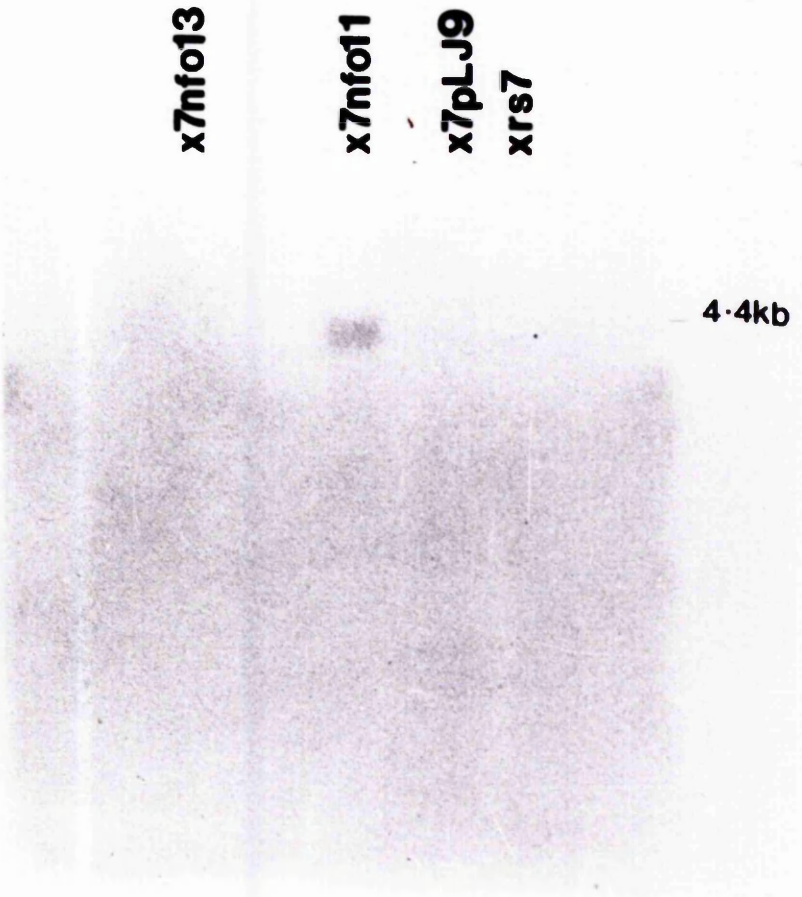
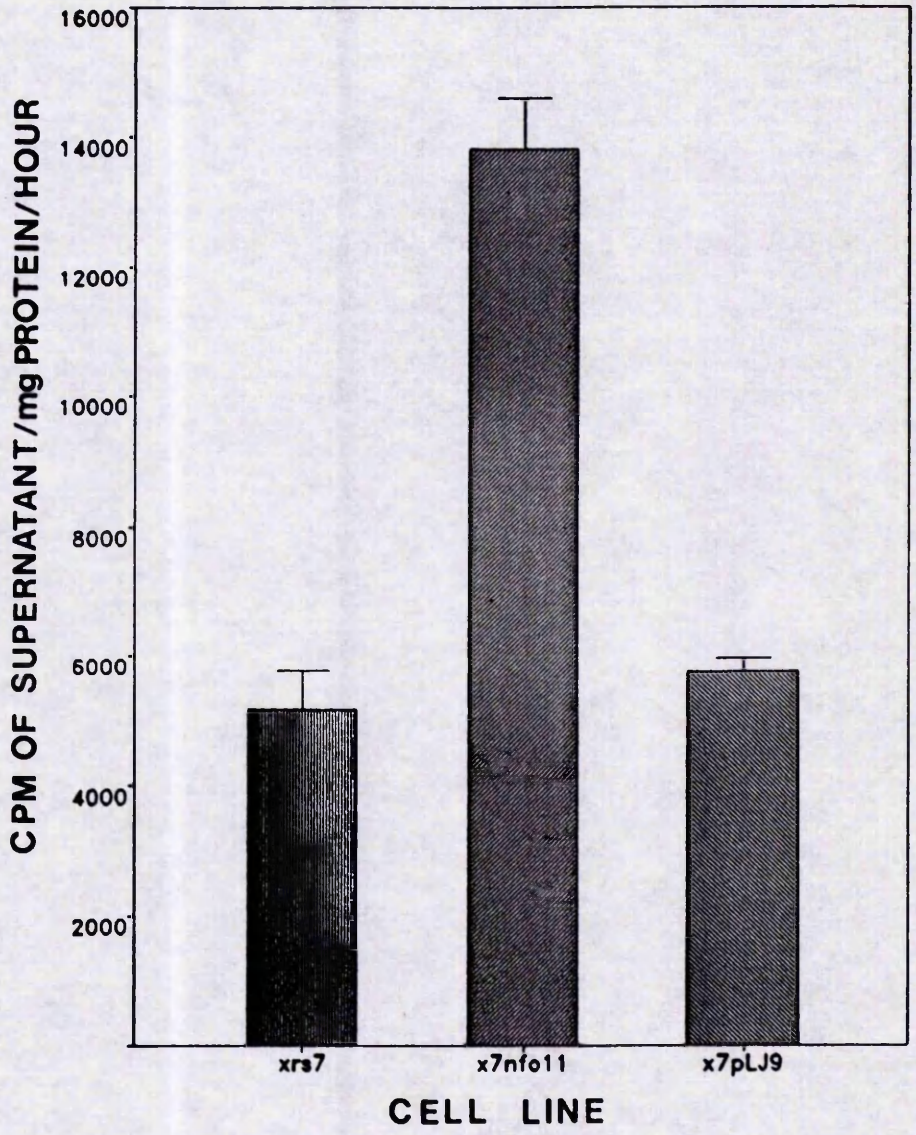


FIGURE 65-AP endonuclease activity of xrs7, x7nfo11 and x7pLJ9 cell-free extracts

Cell-free extracts were assayed in duplicate for the fragmentation of [3H]-labelled partially depyrimidinated T4 phage DNA. The height of the bar represents the average of the two assays and the error is the difference between the duplicates.



Southern and northern analyses, it is not unreasonable to suggest that this clone actively expresses EIV.

5.2.4 Cell cycle and growth analyses

The exponential portion of growth (determined as described in 2.2.2vi) was analysed by linear regression to obtain the doubling time for x7pLJ9 and x7nfo11. It was not possible to compare the doubling times of x7pLJ9 and x7nfo11 with that of xrs7, since the xrs7 growth analysis was carried out using medium containing a different batch of FCS.

From table 18 it can be seen that x7nfo11 grew significantly slower than x7pLJ9.

Examination of the proportion of cells in each of the phases of the cell cycle (see 2.2.2v) showed that there was no significant difference between the parental and transfected cell lines, except for the G2/M phases of x7pLJ9 and x7nfo11, which were significantly higher than that of xrs7 (table 18).

Alterations in growth rate or cell cycle were also detected in pZipnth and pZipneoS(V)X1 transfected cell lines (see chapter 3). Since changes were detected not only in cells expressing EIII or EIV but also in vector control cell lines, it is likely that vector incorporation, *neo* expression or subcloning the cells may have caused these alterations.

TABLE 18 - Cell cycle and growth analyses of xrs7, x7pLJ9 and x7nfo11

CELL LINE	% G1	% S	% G2/M	DOUBLING TIME (HOURS)
xrs7	46 ± 2	30 ± 2	24 ± 1	17.4 ± 0.5
x7pLJ9	39 ± 3	27 ± 2	34 ± 1*	19.4 ± 0.5
x7nfo11	43 ± 2	29 ± 2	29 ± 1*	22.5 ± 0.7**

* $p < 0.05$ compared to xrs7
** $p < 0.05$ compared to x7pLJ9

The exponential portion of growth was analysed by linear regression to obtain the doubling time (± SE) for each cell line. The doubling time of xrs7 was determined using medium containing a different batch of serum and hence the growth of x7nfo11 was compared to x7pLJ9. At least three samples of different passage were stained with propidium iodide and analysed by FACS to determine the proportion of cells in each phase of the cell cycle (± SE).

5.2.5 Chromosome number

As stated previously, it has been found that the transfection and subcloning of a population of cells can result in chromosome abnormalities (e.g. NIH3T3 cells, Kasid et al 1989) and clone x7nth1 was found to be tetraploid (see 3.2.5). Chromosomes were prepared from xrs7, x7pLJ9 and x7nfo11 cells (see 2.2.2vii) and one hundred metaphases were examined and the chromosomes counted.

TABLE 19 - Average chromosome number of xrs7, x7pLJ9 and x7nfo11

CELL LINE	AVERAGE CHROMOSOME NUMBER	% TETRAPLOID	PASSAGE NUMBER
xrs7	21 ± 1	0%	14
x7pLJ9	20 ± 1	12% (36 ± 3)	9
x7nfo11	21 ± 2	0%	10

No statistically significant difference was detected in the number of chromosomes of clones x7pLJ9 and x7nfo11 in comparison to xrs7 (table 19). Twelve percent of the x7pLJ9 metaphases examined were tetraploid and these had an average chromosome number of 36 ± 3.

5.2.6 Results of cytotoxicity studies

Survival after treatment with ionising radiation and hydrogen peroxide was assessed as described in 2.2.2viii. At least three independent experiments were carried out for each cell line, using cells of different passage number, except for the hydrogen peroxide experiments of xrs7 for which two experiments were carried out. Radiation survival data were analysed by the twin

exponential model. Although attempts were made to analyse the hydrogen peroxide data using the alpha, beta model incorporating a quenched dose parameter, the errors of the parameters were large. Hence the single hit multitarget model, incorporating a quenched dose parameter was used to analyse the hydrogen peroxide data. The results are presented below but for clarity the effect of EIV expression on cell survival after treatment with ionising radiation or hydrogen peroxide will be discussed in 5.4.

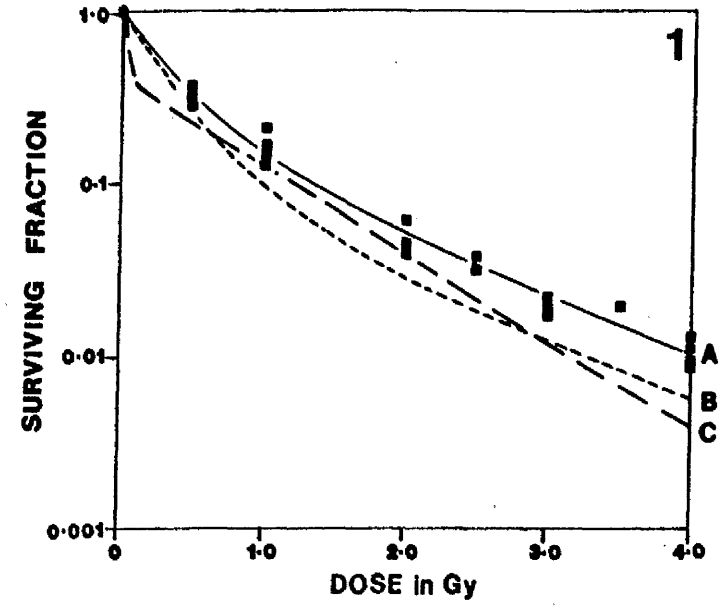
(i) Ionising Radiation

The survival curves were biphasic (figure 66) and significant differences were not detected between the independent experiments of x7pLJ9 and x7nfo11, except for the proportion of the radioresistant population in the x7nfo11 experiments (table 20a). Results of xrs7 survival experiments are repeated from chapter 3. Previous analysis of the independent experiments (see 3.2.6) showed that the $_1$Do parameter was not different for the four experiments and that it was not possible to analyse the proportion of the radioresisant population and the $_2$Do parameters.

No significant difference was detected between xrs7 and x7nfo11 (table 20b), which suggests that the decrease in growth rate of clone x7nfo11 and slight increase in the proportion of cells in the G2/M phase did not affect the radiosensitivity of the cells. However, x7pLJ9 showed alterations in each of the parameters. The

FIGURE 66 - Survival of xrs7, x7pLJ9 and x7nfo11 after gamma irradiation

At least three independent experiments were carried out, using cells of different passage number, for x7nfo11 (A), xrs7 (B) and x7pLJ9 (C). The data were analysed using the twin exponential model of the DRFIT computer programme. The points from all the experiments performed are shown for each of the cell lines individually in graphs 1 (x7nfo11), 2 (xrs7) and 3 (x7pLJ9).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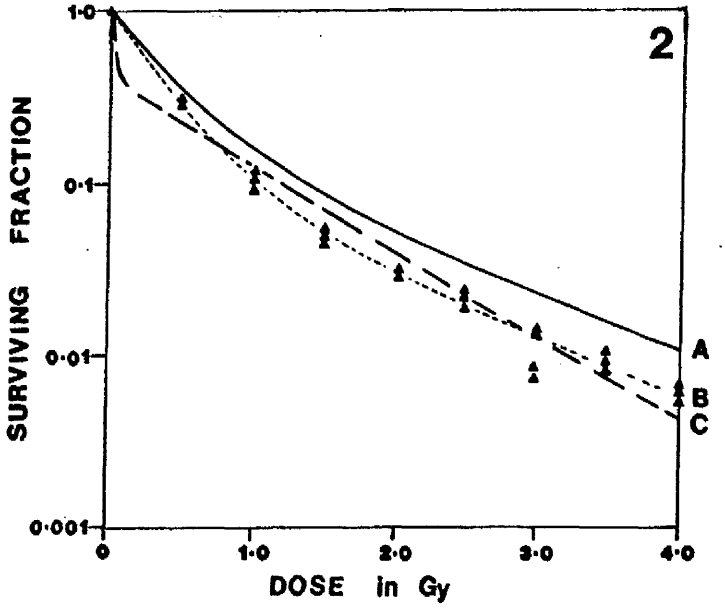
0
1·0
2·0
3·0
4·0
DOSE in Gy
A
B
C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0
1·0
2·0
3·0
4·0
DOSE in Gy
A
B
C

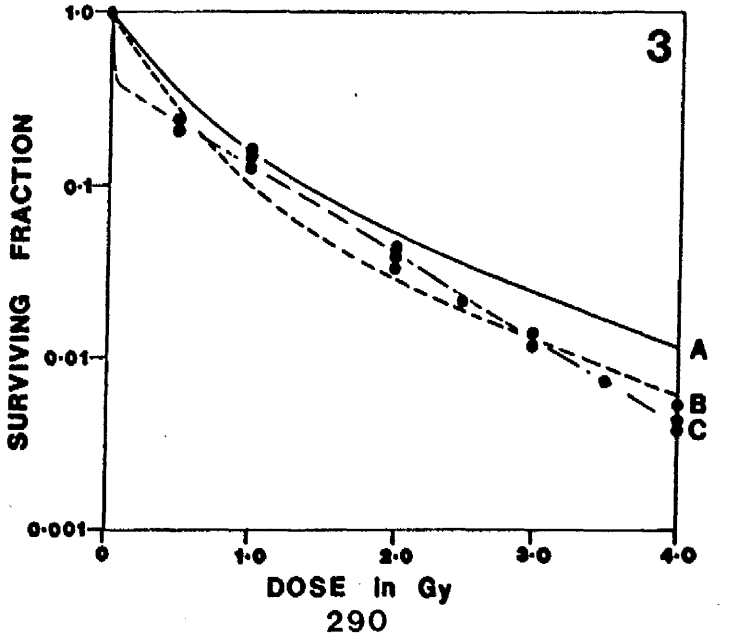

3
SURVIVING FRACTION
1·0
0·1
0·01
0·001
0
1·0
2·0
3·0
4·0
DOSE in Gy
A
B
C

TABLE 20 - Analysis of the Radiosensitivty of xrs7, x7pLJ9 and x7nfo11 (± SE)

a) Analysis of individual experiments

CELL LINE	EXPERIMENT N°	$_1$Do (Gy)	$_2$Do (Gy)	% RADIORESISTANT POPULATION
xrs7	1	0.07 ± $3x10^6$	1.17 ± 0.17	25 ± 10
	2	0.09 ± 639	1.20 ± 0.15	14 ± 5
	3	0.27 ± 0.05	0.88 ± 0.09	30 ± 9
	4	0.37 ± 0.13	1.21 ± 0.45	20 ± 18
x7nfo11	1	0.38 ± 0.05	1.43 ± 0.24	13 ± 5
% population*	2	0.32 ± 0.03	1.44 ± 0.13	19 ± 4
	3	0.25 ± 0.07	1.02 ± 0.09	41 ± 9
x7pLJ9	1	0.32 ± 0.05	0.95 ± 0.11	24 ± 8
	2	0.16 ± 0.05	0.93 ± 0.07	27 ± 7
	3	0.44 ± 0.09	1.04 ± 0.12	31 ± 13

b) Analysis of pooled data

CELL LINE	$_1$Do (Gy)	$_1$Do RATIO	$_2$Do (Gy)	$_2$Do RATIO
xrs7	0.33 ± 0.04	1.00	1.24 ± 0.13	1.00
x7nfo11	0.33 ± 0.04	1.00 ± 0.17	1.25 ± 0.11	1.01 ± 0.14
x7pLJ9	0.01 ± $5x10^9$**	----	0.84 ± 0.04**	0.72 ± 0.12

% OF RADIORESISTANT PORTION

xrs7	15 ± 4
x7nfo11	25 ± 7
x7pLJ9	39 ± 6**

* $p < 0.05$ for the independent experiments
** $p < 0.05$ compared to xrs7

Data were analysed using the twin exponential model of the DRFIT computer programme. It was not possible to determine whether the percentage of radioresistant population or the $_2$Do parameter for the individual xrs7 experiments were statistically different (repeated from table 15), since the iteration procedure would not converge during the analysis. However, the $_1$Do parameter of the individual experiments did not significantly alter.

sensitive portion of the x7pLJ9 population was relatively small and survival below 0.5Gy would have to have been examined to achieve an accurate assessment of this population. This explains the large standard error on the $_1$Do parameter of x7pLJ9 and the inaccurate fit of the first exponential of this curve. A similar problem was found on examination of the radiosensitivity of clone x7neo1 (pZipneoSV(X)1 vector control cell line, see chapter 3), which suggests that incorporation and expression of vector sequences in xrs7 cells may affect the proportion of the radiosensitive population. The $_2$Do parameter of x7pLJ9 was significantly smaller than that of xrs7 and x7nfo11, which indicates that the x7pLJ9 radioresistant population is more radiosensitive compared to xrs7 and x7nfo11. This may be explained by the increase in the proportion of cells in the G2/M phase of x7pLJ9 (see 5.2.4) in comparison to xrs7 and x7nfo11.

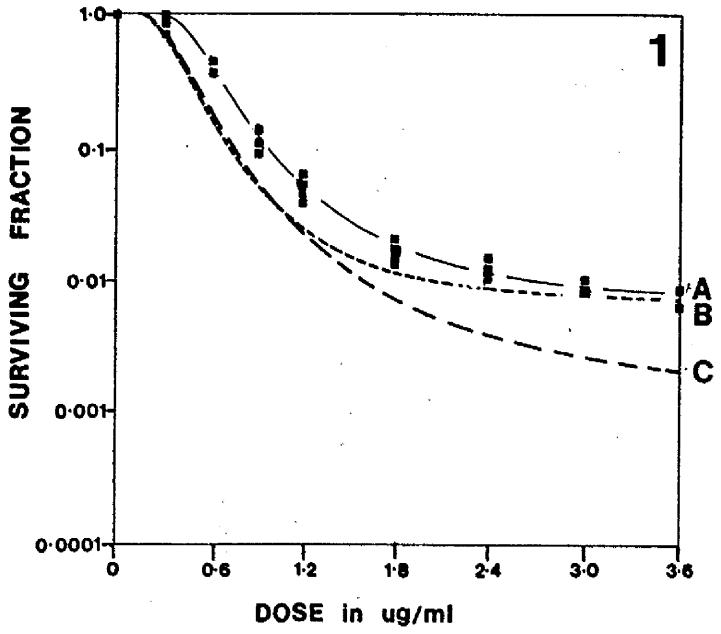
(ii) Hydrogen Peroxide

Analysis of the independent experiments for each cell line was not carried out as an accurate assessment of the shape of the curve could not be obtained without data for doses between 0.3ug/ml and 3.6ug/ml and each experiment examined only four different doses.

No difference in the hydrogen peroxide cytotoxicity was detected between xrs7 and x7nfo11 (figure 67). Although the QD for x7pLJ9 was not significantly different from that of x7nfo11 (table 21b), x7pLJ9 had a significantly higher QD than xrs7.

FIGURE 67 - Survival of xrs7, x7pLJ9 and x7nfo11 after treatment with hydrogen peroxide

Four independent experiments were carried out for x7nfo11 (A) and x7pLJ9 (C) and two for xrs7 (B), using cells of different passage number. The data were analysed using the single hit multitarget model of the DRFIT computer programme adapted to incorporate a quenched dose parameter. The points from all the experiments performed are shown for each cell line individually in graphs 1 (x7nfo11), 2 (xrs7) and 3 (x7pLJ9).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0·01
0·001
0·0001
0
0·6
1·2
1·8
2·4
3·0
3·6
DOSE in ug/ml
A
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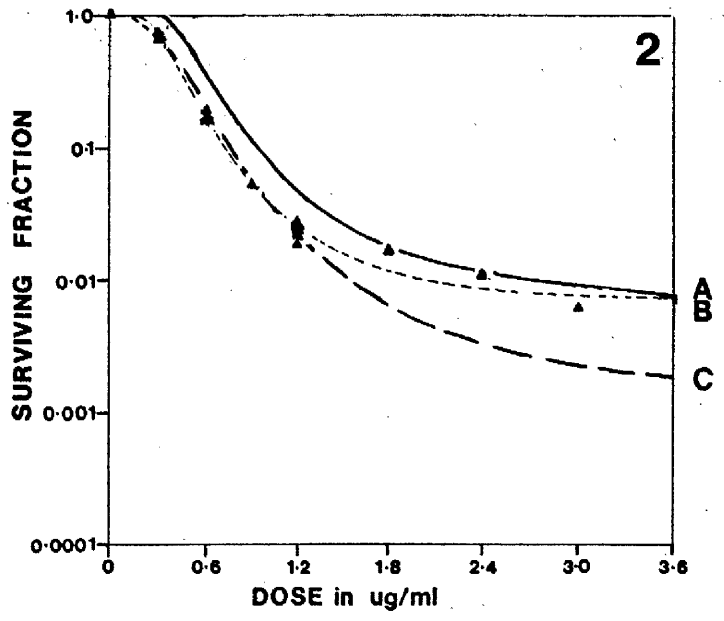
C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DOSE in ug/ml
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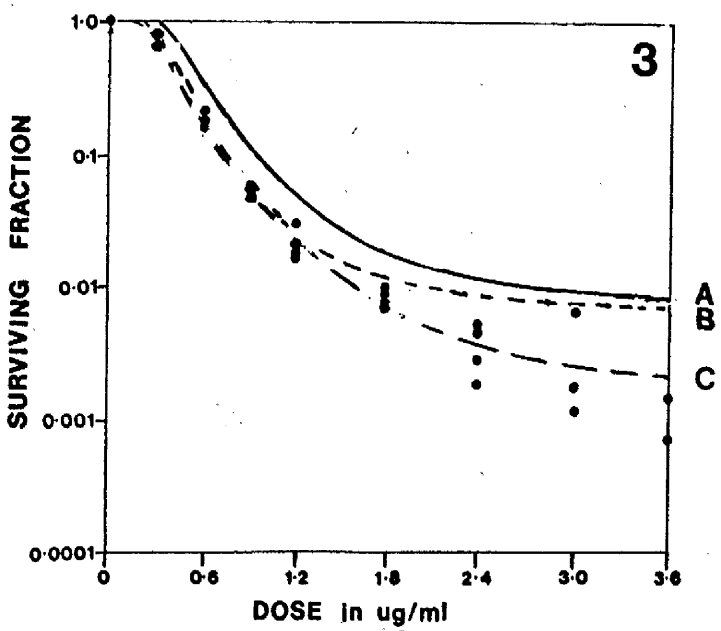
3
SURVIVING FRACTION
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0·1
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0·0001
0
0·6
1·2
1·8
2·4
3·0
3·6
DOSE in ug/ml
A
B
C

TABLE 21 - Analysis of the Cytotoxicity Data of xrs7, x7pLJ9 and x7nfo11 After Treatment with Hydrogen Peroxide (± SE)

a) Using the alpha, beta model, incorporating a quenched dose parameter

CELL LINE	ALPHA $(ug/ml)^{-1}$	BETA $(ug/ml)^{-2}$	Q_D (ug/ml)
xrs7	4.40 ± 1.73	0.28 ± 2.93	1.39 ± 0.47
x7pLJ9	2.12 ± 0.82	2.60 ± 1.55	1.60 ± 0.32
x7nfo11	0.72 ± 1.15*	2.19 ± 2.62	3.83 ± 7.43

b) Using the single-hit, multitarget model, incorporating a quenched dose parameter

CELL LINE	N	Do(ug/ml)	RATIO Do	Q_D(ug/ml)	RATIO QD
xrs7	27 ± 15	0.078 ± 0.010	1.00	0.650 ± 0.054	1.00
x7pLJ9	12 ± 5	0.111 ± 0.014	1.42 ± 0.25	0.980 ± 0.114*	1.51 ± 0.22
x7nfo11	49 ± 30	0.092 ± 0.014	1.18 ± 0.23	0.810 ± 0.085	1.25 ± 0.17

* $p < 0.05$ compared to xrs7

Data were analysed using the alpha, beta or single-hit multitarget models, incorporating a quenched dose parameter. Four independent experiments were carried out for x7pLJ9 and x7nfo11, while two were carried out for xrs7.

5.3 Expression of *nfo* in x7nth1 cells

Clones x7nth1 and x7nth6 were found to be resistant to hydrogen peroxide, but markedly more sensitive to bleomycin sulphate (see chapter 3). As discussed in 3.4 the sensitization to bleomycin sulphate may have been due to EIII-induced ssb interacting with other breaks and increasing the number of lethal lesions. The AP lyase activity of EIII results in a 3′ 2,3-didehydro-dideoxyribose moiety which can be removed by EIV, allowing repair to be completed. It was therefore considered worthwhile to try to express EIV in x7nth1 cells and hence test the idea that the x7nth1 sensitization was due to EIII repair.

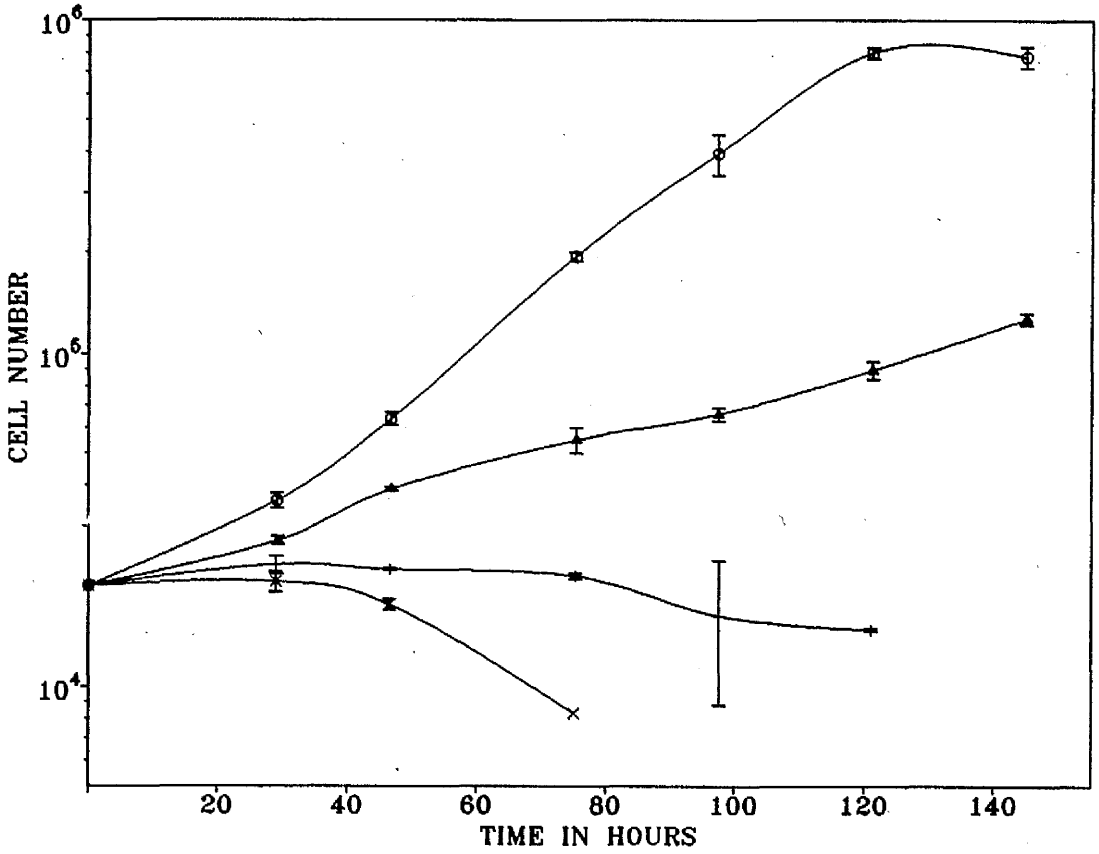
5.3.1 Co-transfection of pLJnfo and pSV2hyg

pLJnfo or pLJ was co-transfected by lipofection into x7nth1 with pSV2hyg (see 2.2.2iv), which contains the gene that confers hygromycin resistance in mammalian cells. To determine the level of hygromycin required to kill x7nth1 cells, the growth of the cells was examined (2.2.2vi) in medium containing 0, 50, 100 and 150ug/ml hygromycin. x7nth1 cells were able to grow in medium containing 50ug/ml, but not 100ug/ml and 150ug/ml of hygromycin (figure 68). The co-transfected cells were therefore selected in medium containing 100ug/ml hygromycin.

Ten hygromycin resistant clones were isolated from the co-transfection of pLJnfo and pSV2hyg, seven of which survived (x71nfo3-7, 9 and 10), and four vector control

FIGURE 68-Effect of hygromycin on the growth of x7nth1 cells

The increase in cell number with incubation time was examined in duplicate, using medium containing 0ug/ml (o), 50ug/ml(Δ), 100ug/ml (+) and 150ug/ml (x) hygromycin. Each point represents the average of the duplicate samples and the error is the difference between the two samples.



cell lines were isolated (x71pLJ3-6) from the pLJ and pSV2hyg transfection. After the cell lines had been subcultured twice, they were no longer maintained in medium containing hygromycin, but were grown in medium containing 300ug/ml G418 to retain pLJnfo (or pLJ) and pZipnth in the genome.

5.3.2 Molecular analysis of hygromycin resistant clones

DNA was isolated (2.2.1ix) from each of the hygromycin resistant clones mentioned above and used in DNA dot blot (2.2.1xiva) and Southern analyses (2.2.1xivc). The probe was the ^{32}P-labelled *nfo* sequence (5.2.2).

(i) DNA dot blot analysis

The positive control was 10ng pUCDUBnfo and the negative controls were 1ug x7nth1 DNA and 10ng pSV2hyg.

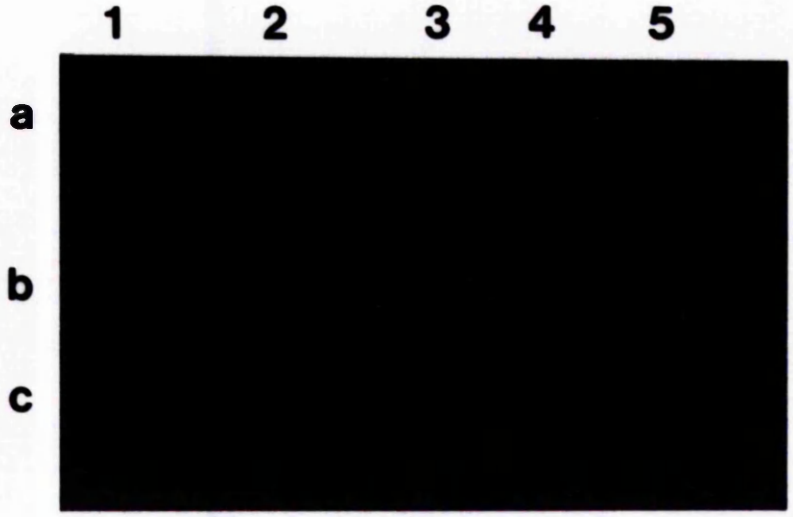
Intense hybridization signals were detected in the positive control, x71pLJ3 and pSV2hyg (figure 69A), which suggested that the *nfo* sequence cross-reacted extensively with pSV2hyg or that the pSV2hyg DNA had been contaminated with pLJnfo or pUCDUBnfo. A faint hybridization signal was also seen in x7nth1. This may have been due to complementary *nfo* sequences in pZipnth or contamination of the *nfo* probe with vector sequences. Signals of similar intensity were also found with x71pLJ4, x71nfo7, 9 and 10. Two other clones (x71nfo3 and 4) did show stronger signals than x7nth1, although these were not as intense as x71pLJ3. Results from the

FIGURE 69 - DNA dot blot (A) and Southern (B) analyses of x7nth1 and x7nth1-transfected cell lines

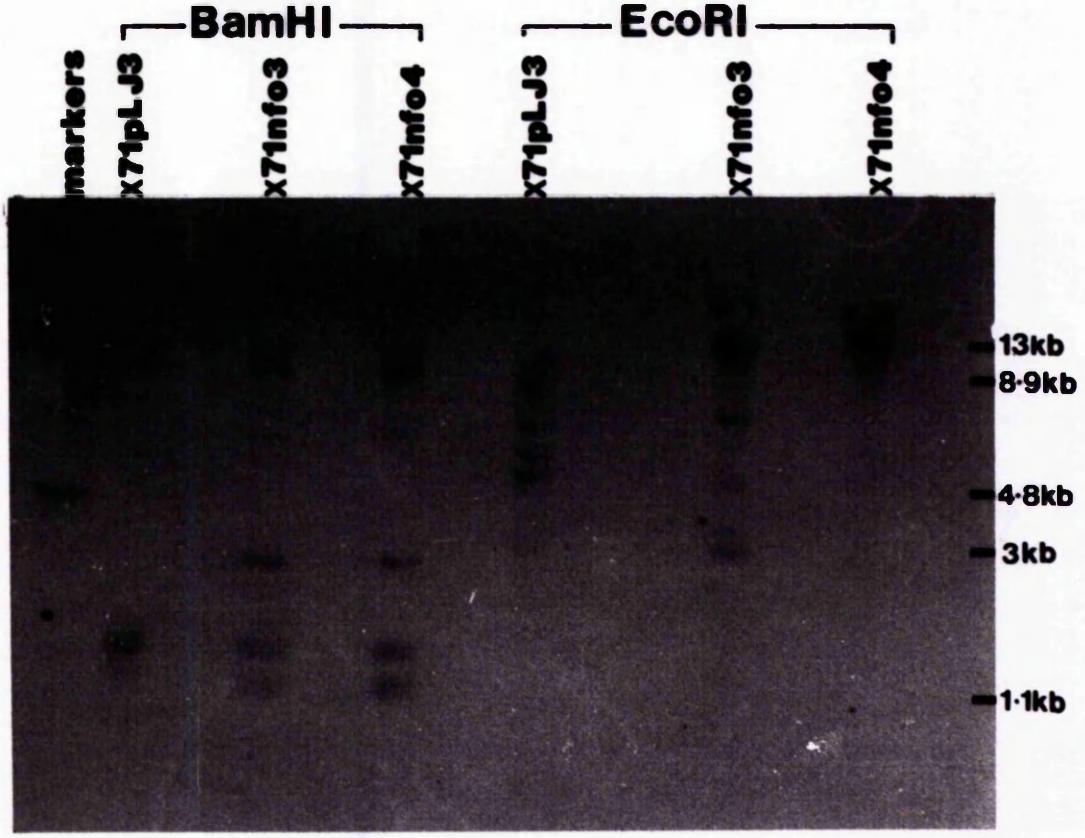
Filters were probed with the *nfo* probe.

A - DNA dot blot analysis

a 1 pUCDUBnfo	b 1 x71pLJ3	c 1 x71nfo5
2 pSV2hyg	2 x71pLJ6	2 x71nfo10
3 x7nth1	3 x71nfo9	3 x71nfo4
4 x71pLJ4	4 x71nfo3	4 x71nfo6
5 x71pLJ5	5 x71nfo7	



B - Southern analysis



DNA dot blot analysis suggested that pLJnfo may have incorporated into the genome of x71nfo3 and 4, but no definite conclusions could be reached due to the intense hybridization signals obtained with pSV2hyg and x71pLJ3.

(ii) Southern analysis

DNA from x71pLJ3 (negative control) and x71nfo3 and 4 was digested by BamHI or EcoRI and subjected to Southern analysis (2.2.) Many hybridization signals were detected in each of the DNA digests (figure 69B). BamHI digestion of x7pLJ9, x71nfo3 and 4 DNA produced signals of 8.9kb, 1.4kb and 1.1kb and signals of sizes 5.6kb and 2.7kb were also seen in the BamHI digests of x71nfo3 and 4 DNA. EcoRI digestion of x71pLJ3 and x71nfo3 DNA produced signals of 13kb, 4.8kb and 3kb and approximately 5 faint signals were seen ranging in size from 13-4.8kb in the EcoRI digest of x71pLJ3 DNA. The 13kb and the 4.8kb signals were also found in the EcoRI digest of x71nfo4 DNA. The 13kb signal had the greatest intensity, suggesting that the EcoRI digest was not complete. Two other signals of 7.6kb and 3.5kb were also present in the EcoRI digest of x71nfo3 DNA.

The presence of the *nfo* sequence was expected to result in 0.9kb and 2.4kb signals after BamHI and EcoRI digests, respectively. The hybridization signals that were detected may have been due to:

1) Rearrangement of pLJnfo on incorporation into the genome

pLJnfo (10.1kb), pLJ (9.2kb) and pZipnth (11.2kb)

contain the transcription unit of the Moloney leukaemia virus (4.9kb). Since there is approximately 50% homology between the vectors, homologous recombination between pLJnfo (or pLJ) and pZipnth could have occurred, which would have resulted in partial incorporation of pLJnfo (or pLJ) and rearrangement of incorporated vector sequences.

2) A cross-reaction of the *nfo* probe with pSV2hyg

pSV2hyg is linearized by BamHI and has a size of approximately 5.2kb, while EcoRI cuts twice within this vector to produce signals of 2.3kb and 3kb (figure 19).

3) A cross-reaction of the *nfo* probe with the *nth* sequence

BamHI or EcoRI digestion of pZipnth would produce signals of approximatly 1kb. Partial EcoRI digestion could produce signals of 3kb, 4kb, 5.5kb or 6.5kb, as EcoRI cuts twice in pZipneoSV(X)1 and cleaves immediately 5′ and 3′ to the truncated *nth* gene (see figure 16).

4) A cross-reaction of the *nfo* probe with mammalian or vector sequences

BamHI digestion of xrs7, x7pLJ9 and x7nfo11, 8, 13 and 19 produced a 4.9kb signal on Southern analysis (5.2.2ib) and signals of 4.2kb and 3.7kb were seen on EcoRI digestion of transfected cell lines.

(iii) PCR of DNA samples

Since the results of the DNA dot blot and Southern analyses were difficult to interpret, DNA from the seven pLJnfo-pSV2hyg co-transfected clones and x71pLJ3 and x71pLJ4 was analysed by PCR (2.2.1xii), using the sense

nfo and the antisense *neo* oligonucleotides (see figure 15). The presence of *nfo* would have resulted in the amplification of a 2.5kb fragment, as found after PCR of x7nfo11 DNA (the positive control, figure 70). Amplification of DNA sequences of the appropriate size was not detected in any of the other samples, demonstrating that *nfo* had not been incorporated in the genome of these cell lines in a form that might be expected to result in active EIV expression. Amplification of a DNA fragment of approximately 200-300bp was seen in the x71nfo3 and 4 samples, which suggests that rearrangement of pLJnfo (as discussed above) may have occurred.

5.4 Discussion of the effect of EIV expression on xrs7 cytotoxicity

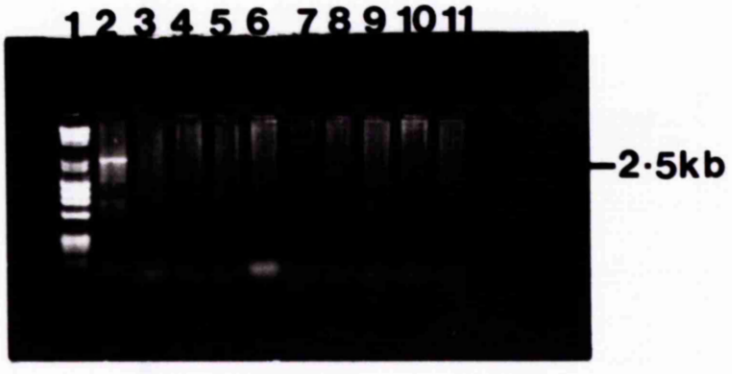
Although cytotoxicity studies using *nfo* and *xth* mutant bacteria indicated that EIV is involved in the repair of toxic lesions induced by ionising radiation and hydrogen peroxide, expression of EIV did not alter the sensitivity of xrs7 cells to these two DNA damaging agents (figures 66, 67). This suggests that EIV-repairable lesions are not toxic to xrs7 cells. However, it is possible that the parental cells contain an EIV-type enzyme that is repair efficient and hence expression of EIV activity did not significantly increase the cells repair capacity.

The defect in xrs7 cells that results in cellular radiosensitivity and inability to repair dsb, is not yet understood. If the deficient enzyme(s) was involved in the repair pathway at a rate-limiting step, e.g. in the

FIGURE 70 - PCR analysis of x7nth1-transfected cell lines

Attempts were made to detect *nfo* in DNA of x7nth1-transfected cell lines, by PCR using the *nfo* sense and *neo* antisense oligonucleotides.

1 Markers
2 x7nfo11
3 x71pLJ3
4 x71pLJ4
5 x71nfo4
6 x71nfo3
7 x71nfo5
8 x71nfo10
9 x71nfo7
10 x71nfo9
11 x71nfo6



unfolding of the chromatin structure or the recognition of dsb damage, an increase in EIV activity would not alter survival. This, however, would not explain the increase in resistance to hydrogen peroxide of EIII expressing clones (figures 29, 37).

The EIV survival results could also be explained by the inability of EIV to be transported into the nucleus or to repair DNA damage in the host cell genome. EIV has similar activities and extensive homology to the yeast APN1 enzyme, but APN1 has been found to have a highly basic 10kDa C-terminus that is not present in EIV (Popoff et al 1990). The function of the C-terminus is not known, although it has been suggested that it may be a nuclear localisation signal or involved in DNA binding, possibly in competition with the histone proteins, to allow the enzyme access to the DNA in chromatin (Popoff et al 1990). However, the human EIV-type enzyme does not appear to have the highly basic C-terminus of APN1 and EIV is able to repair DNA damage in yeast cells (B. Demple, personal communication). This suggest that EIV might be able to repair lesions in Chinese hamster chromatin.

The major EIV activity involved in the repair of hydrogen peroxide and radiation-induced lesions is probably the 3′ diesterase repair function (i.e. removal of 3′ phosphate and 3′ phosphoglycolate termini), but it was only possible to measure the AP endonuclease activity of cell-free extracts of transfected cells. If the EIV expressed in the Chinese hamster cells did not have 3′

diesterase activity (i.e. if the appropriate metal ions had not bound to the the three dimensional protein [EIV is a metalloprotein] to produce active enzyme) it is possible that increased AP endonuclease activity alone would not enhance cell survival after treatment with radiation or hydrogen peroxide. As mentioned earlier, the TG DNA glycosylase may be the EIII repair function that increases the resistance of x7nth1 and 6 to hydrogen peroxide.

The level of EIII or EIV expressed in the cells may also account for EIII, but not EIV, expression increasing the resistance of xrs7 cells to hydrogen peroxide: in comparison to parental cells x7nfo11 has approximately 2-2.5 times more AP endonuclease activity (figure 65), while x7nth1 and x7nth6 have 4 and 3 times the activity (figure 34), respectively. The increase in survival of x7nth6 was less than that of x7nth1 and the Do of x7nth6 (0.046ug/ml) is only 1.35 times that of xrs7 (0.034ug/ml). Therefore the increase in AP endonuclease activity, and possibly other EIV activites, in x7nfo11 cell-free extracts may not have been sufficient to enhance cell survival compared to control cell lines.

To try to further understand the action of EIV and EIII in these cells, studies using bleomycin, mitomycin C and t-butyl hydroperoxide could be carried out, examining the effects expression on cell survival, mutation and chromosome abberation induction. In addition, *in vitro* studies have shown that EIV increases the number of dsb in NCS but not bleomycin treated DNA, whereas EIII was

found to produce dsb in both: a higher concentration of EIII was required to produce a similar level of dsb as EIV with the NCS treated DNA (Povirk & Houlgrave, 1988). Therefore had time allowed it would have been interesting to examine survival, mutation and chromosome aberration frequency of x7nfo11, x7nth1 and x7nth6 after NCS treatment.

Attempts were made to express EIV and EIII in the same cell to examine how simultaneous repair by EIII and EIV affected cell survival. However, Southern analysis of DNA from hygromycin resistant clones suggested that recombination of pZipnth and pLJnfo may have caused rearrangement of the truncated *nfo* gene. To achieve expression of both enzymes in the same cell line it may be necessary to construct a vector that contains and expresses both *nth* and *nfo*. It would be possible to remove the truncated *neo* gene from pZipnth by XhoI digestion, "blunt-end" the termini of the vector and insert the 0.9kb fragment containing *nfo* (isolated from SmaI digestion of pUCDUBnfo) into pZipnthΔneo. pZipnthnfoΔneo could be co-transfected into mammalian cells with pSV2hyg or pMSGneo. x7nth6 would probably be used as the recipient cell line for this transfection, since these cells showed the greatest sensitization to bleomycin sulphate and were diploid, unlike x7nth1 cells.

It would be of interest to progress from expression of EIII, and possibly EIV, in cellular systems to expression in whole animals, by the generation of

transgenic mice. This might allow the effect of EIII or EIV-type repair activities on tissue sensitivity and tumour induction to be assessed after irradiation or treatment with cytotoxic agents, such as bleomycin. However, with the recent cloning of mammalian DNA repair genes, e.g. the human (pers. communication B. Demple) and bovine AP endonucleases (Robson et al 1991), it may be more appropriate to use the mammalian genes to generate the transgenic mice. This would eliminate the questions concerning bacterial protein transport into the nucleus and its ability to repair DNA in chromatin.

CHAPTER 6: REFERENCES

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