THE CONTROL OF HETEROLOGOUS IFN-γ GENE EXPRESSION

IN RECOMBINANT CHO CELLS

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science

November 1995

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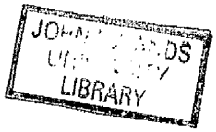

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ABSTRACT

Many factors control recombinant protein formation from vectors introduced into mammalian cells (transcription, transcript processing, translation and secretion) but few studies have been made of the importance of transcript processing and stability. Two CHO cell lines were generated to express human gamma interferon (IFN-γ), one containing the natural 3' untranslated region including an instability sequence (CHO 42) and the other with the instability sequence removed (CHO 43). After selecting and amplifying in media containing 1μM methotrexate, I found IFN-γ production was 20 times greater in CHO 43 cells than in CHO 42 cells.

IFN-γ messenger RNA (mRNA) levels were four-fold greater in CHO 43 cells than in CHO 42 cells and two IFN-γ mRNA species were found in CHO 42 cells (compared to only one in CHO 43 cells), one of the IFN-γ mRNA forms in CHO 42 cells was of the predicted size whereas the second form was 200 bases shorter than expected. To examine the importance of mRNA stability to IFN-γ production, RNA transcription was blocked and RNA degradation was measured. However, mRNA was stable in both cell lines (with half-life greater than 8 hours). Comparing nuclear/cytoplasmic distribution of IFN-γ pre/messenger RNA, I have shown that the smaller IFN-γ mRNA was a processed product which lacked the instability sequence. The sequence of DNA and mRNA showed that the smaller IFN-γ mRNA of CHO 42 cells composed two alternative splicing products.

Immunoblotting with an IFN-γ monoclonal antibody (20D7), exhibited that three forms of IFN-γ (with two forms being glycosylated) were produced by CHO 43 cells. However CHO 42 cells produced 5 types of IFN-γ. The majority of these forms appear to be abnormally sized. Immunoprecipitation of IFN-γ showed that the IFN-γ translation rate was 4.5-fold greater in CHO 43 cells than in CHO 42 cells. However, IFN-γ secretion are 20-fold greater in CHO 43 cells. The two high-molecular weight abnormal IFN-γ isoforms in CHO 42 cells were retained in cells for more than 4 hr.

From my data, the processing of the 3' untranslated region of IFN-γ mRNA in CHO cells can influence the amount of protein production either at the level of mRNA transcript stability or at translational level. The possiblity of IFN-γ pre-mRNA alternative splicing, abnormal IFN-γ isoform formation and subsequent retention within the secretory pathway is also discussed.

DECLARATION: 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.

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Acknowledgements:

I am very grateful to my Supervisor, Dr. Alan Dickson for his patient guidance and encouragement throughout the PhD program.

I also pay my acknowledgement to the Ministry of Education, Taiwan, ROC., who gave me the financial support so that I have the opportunity to study abroad, here in UK.

Every member of the laboratory has helped me somewhere along the way, but I would in particular like to thank Alison Bate, Dr. Rasida Anwar and Shen-Hsi Yang for giving practical help.

I would also like to thank the following colleagues for their generosity in providing various essential resources for the research: Fei-Ling Lim, Yuan Chen, Dr. Jia-Ching Shieh and Dr. Wen-Wu Zhai.

Finally, I must acknowledge the support of my family for their continued support, especially my wife, Pei-Fang. She has borne patiently my frustrations and depression, and her encouragement and understanding have supported me through these four years studying.

LIST OF ABBREVIATION

The standard abbreviations of the Biochemical Journal have been used throughout this thesis. All other abbreviations are defined in the text as appropriate; abbreviations encountered in more than one section of text are listed below:

Abbreviation	Item
ARE	AUUUA rich sequence
Asn	asparagine
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP
CAP	cap-binding protein
cDNA	complementary DNA
CHO	chinese hamster ovary cell
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEAE	diethylaminoethyl dextran
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DHFR	dihydrofolate reductase
DMEM	Dubecco's modified Eagle medium
DMSO	dimethyl sulphoxide
DRE	direct repeat element
dTTP	deoxythymidine triphosphate
EDTA	etylenediaminetetra-acetic acid

eIF2	protein synthesis initiation factor 2
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FCS	foetal calf serum
FDA	Food and Drug Administration
Fuc	fucose
Gal	galactose
gDNA	genomic DNA
GlnNac	N-acetylglucosamine
GM-CSF	granulocyte-macrophage colony stimulating factor
HIV	human immunodeficiency virus
hnRNA	heterogenous nuclear RNA
hnRNP	heterogenous ribonuclear protein
hr	hour
huIFN-γ	human interferon gamma
IFN-γ	interferon-gamma
IL	interleukin
IPTG	isopropyl-β-D-thiogalactopyranoside
IRE	iron response element
JAK	Janus kinase
kb	kilobase
kDa	kilodalton
KGB	potasium glutamate buffer
LB	Luria Bertani medium
LBA	LB agar plate
Man	mannose

MCS	multiple cloning site
mg	milligram
min	minute
ml	millilitre
mM	millimole
MOPs	3-(Morpholino)propanesulfonic acid
mRNA	messenger RNA
Mtx	methotrexate
N	any bases
ng	nanogram
NK cell	natural killer cell
NKSF	natural killer cell stimulatory factor
NP-40	Nonidet P-40 nonionic detergent
NPC	nuclear pore complex
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDI	isosulphide isomerase
pg	picogram
PHA	phytohaemaglutinin
PMSF	phenylmethylsulfonyl fluoride
Poly(A)	polyadenylation
$q_{IFN-\gamma}$	IFN-γ production rate
R	purine
rRNA	ribosomal RNA

SB	sodium bicarbonate
SCM	serum containing media
SDS	sodium dodecyl sulphate
Ser	serine
SF	splicing factor
SFM	serum free media
snRNA	small nuclear RNA
SNU	SDS-NP-40-urea buffer
SP	sodium pyruvate
SSC	sodium chloride/sodium citrate buffer
STAT	signal transducer and activator of transcription
SV40	simian virus 40
CETP	cholesteryl ester transfer protein
TBE	tris-borate-EDTA buffer
TCA	trichloroacetic acid
t_d	cell doubling time
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetrametylethylenediamine
TENN	tris-EDTA-sodium chloride-NP-40 buffer
TF IID	transcription factor IID
TLCK	tosyllysine chloromethyl ketone
TNF	tumour necrosis factor
tRNA	transfer RNA
3'UTR	3' untranslated region
x-gal	5-bromo-4-chloro-3-indolyl-6-D-galactoside

Y	pyrimidine
µg	microgram
µl	microlitre
μ_m	maximum specific growth rate
µm	micrometer

CHAPTER ONE : INTRODUCTION

There is a great interest in cloning cellular genes that code for polypeptides that have therapeutic use. Once the intact gene is isolated, it can be engineered into an expression vector which can transcribe when introduced into a host cell. After transfecting the vector into host cells, the expression of the gene is greatly affected by sequences in the vector and by the metabolism of host cell. In this thesis, I have compared γ-interferon (IFN-γ) protein production by two chinese hamster ovary cell (CHO) lines. These two CHO cell lines were generated from two vectors which share similar promoters, protein coding regions, poly (A) tail signals but which have different 3'untranslated regions (3' UTR). The p1042 vector (which was used to generate the CHO42 cell line) has a sequence rich in the ATTTA pentamer which can accelerate messenger RNA (mRNA) export and can make the mRNA unstable in some cell types whilst the p1043 vector (which was used to generate the CHO43 cell line) had the 3'UTR pentamer removed.

These two CHO cells produce hugely different amounts of IFN-γ (more than 20 times different). I have compared IFN-γ mRNA stability, IFN-γ mRNA and IFN-γ protein species, plus IFN-γ protein translation, secretion and retention in cells to define the mechanisms which cause the difference in productivity.

In this chapter, I wish to review the relevant work and background to this research area. There will be six sections

in this chapter. Firstly, I will introduce heterologous protein expression systems and explain why expression systems based on mammalian cells are important for many recombinant proteins in order to maintain their bioactivity. In the second section, I will describe the factors in vectors which are important for recombinant gene expression. In the third section, I will describe how metabolism may influence gene expression in host cells. Fourthly, I will describe mRNA structure and its function on gene expression. Fifthly, I will describe the characteristics of IFN-γ which is the recombinant product studied in this thesis. Lastly, I will outline the aims of the thesis.

1.1 Heterologous protein expression systems

Many proteins expressed by their natural producing cells are expressed at low level or need drugs or stress (eg. heat) to induce their expression. It is easier to express these kinds of protein using heterologous gene expression systems. Many heterologous expression systems have been defined in recent years. However, each system has its advantages and disadvantages. The choice of expression system largely depends on the properties of protein (Page, 1988; Goeddel, 1990) or the amount of protein (Hodgson, 1993) required for expression. Goeddel (1990) classified proteins into four groups:

1. Small peptides (less than 80 amino acids): these are easily expressed as fusion proteins in *Escherichia coli (E.*

coli).

2. Proteins which are normally secreted products (enzymes, cytokines, hormones) and which range in size from about 80-500 amino acids. This class of proteins is the most straightforward to express in all prokaryotic or eukaryotic expression system.

3. Very large secreted proteins (> 500 amino acids) and cell surface receptor proteins. This group generally has been expressed in insect or mammalian cell expression system.

4. Non-secreted proteins larger than 80 amino acids. The selection of an appropriate expression system should be based on the intended use of these proteins.

1.1.1 Recombinant protein expression systems

The choice of expression system, at its most extended, encompasses five kingdoms of organisms: bacteria (*E. coli*, Hockney, 1994; *Bacillus subtilis*, Henner, 1990), fungi (Raue, 1994; Peberdy, 1994), insect (Taticek et al., 1994), animal (mammalian cells, Reiter and Blüml, 1994; transgenic chickens, Sang, 1994) and plant (plants cell, Taticek et al., 1994; transgenic cereals, McElory and Brettell, 1994). However, only a few systems are in regular or large scale use. Hodgson (1993) and Liu (1992) have surveyed the expression systems used for the production of recombinant therapeutic proteins and have shown that *E. coli*, yeast and CHO cells are most widely used. In recent years, insect cells (O'Reilly et al., 1992) and transgenic organisms (McElroy and Brettel, 1994; Dorbrovolsky et al., 1993) have been

successfully used to produce recombinant proteins especially those which need post-translational glycosylation. Therefore, I will concentrate on *E. coli*, yeast, mammalian cells, insect cells and transgenic expression systems in the following section.

1.1.1.1 **Mammalian cells**

Mammalian cell cultures are widely used as expression systems for heterologous recombinant proteins. This is despite the fact that the medium used for mammalian cell culture (especially foetal calf serum supplements) is expensive, that cell density (up to 10^6 cells/ml) is lower than that obtained from bacteria or yeast culture (up to 10^8 cells/ml) and that stable cell expression can be more time-consuming than for bacteria and yeast. Mammalian cell expression systems have specific advantages for generation of therapeutic biopharmaceuticals (Page, 1988). Firstly, and most importantly, mammalian cells can correctly fold complex polypeptides and achieve the necessary tertiary conformational structure required for biological activity. Secondly, some polypeptides need post-translational modifications (eg., amidation, phosphorylation, carboxylation and glycosylation) for activity and mammalian cells express the enzyme systems for such modifications. Thirdly, if the gene product is naturally secreted, the cloned gene will contain information for a secretory signal sequence. This sequence is widely recognised by mammalian cells and results in the efficient secretion of gene product into culture

medium, thereby allowing for easier collection and purification.

At present, the most popular vehicle for expression of human recombinant glycoproteins are chinese hamster ovary (CHO) cell lines which have a set of glycosylation enzymes that are similar to those in human cells (James et al., 1995). The background of CHO cells is already well-defined (Liu, 1992).

1.1.1.1.1 Transient expression

For this type of expression, origin-defective SV40 mutant virus transformed monkey kidney CV-1 cells, called COS cells, are recipients for the expression of the cloned gene (Gluzman, 1981). These cells constitutively synthesise SV40 T antigen, which is required in trans to replicate DNA containing the SV40 origin, and therefore they are able to support the rapid replication of any recombinant plasmid with an SV40 origin. This generates a very high plasmid copy number in the cells and results in high levels of transient gene expression. However, the transfected cells die, possibly due to the high level of DNA replication.

1.1.1.1.2 Stable expression systems

Although the COS expression system (Section 1.1.1.1.1) can produce high levels of recombinant product, the cells die a few days after transfection and can no longer be used to produce recombinant protein. If we need continual production of protein, it is preferable to generate stable

expressing cell lines. To obtain recombinant gene products from stable cell lines, it is necessary to construct two separate expression cassettes. One cassette must produce an enzyme (eg. DHFR) to act as selectable marker, the other cassette produces the required recombinant product (Page, 1988). To express the required gene, the recipient cell for transfection must not produce the enzyme (eg. $dhfr^-$) so that it can be used to select those transfected cells. When the concentration of an inhibitor of that enzyme (eg methotrexate inhibits DHFR), the gene copy number of the plasmids in selected cells can be increased (Page, 1988).

1.1.1.2 **Nonmammlian cells**

1.1.1.2.1 *E. coli*

E. coli is good source for the synthesis of heterologous proteins of modest size with relatively simple structure from *E. coli* (Section 1.2; Hockney, 1994). A number of vectors are available for heterologous-protein expression, and *E. coli* can be grown to high cell density. These considerations have led to the development of inexpensive, high-yielding fermentation processes for production of recombinant proteins from *E. coli*. For small peptides, fusion protein expression strategies (Goeddel, 1990) ensure good translation. However, there are still some disadvantages to the use of *E. coli*. They include the fact that the reducing environment in *E. coli* does not permit cysteine-rich proteins to form the disulphide bonds essential for functional conformation, that unwanted N-terminal methionine can be

included on products, that proteolysis of heterologous proteins can be a problem and that recombinant proteins can accumulate intracellularly in the form of insoluble, biologically inactive inclusion bodies.

The high degree of certainty of expression with *E. coli* systems have made this a preferred method for generating immunogens (Watson et al., 1992). The anti-huIFN-γ monoclonal antibody, 20 D_7, used for western blotting (Section 3.1.2.3) and immunoprecipitation (Section 3.3.1) in this thesis was raised from recombinant IFN-γ which was a product of recombinant *E. coli* (Bulleid and Freedman, 1988).

1.1.1.2.2 Yeast (*Saccharomyces cerevisiae*)

Yeast offers many possible advantages as an expression system. Yeast grow rapidly (doubling time 2hr compared to 16 to 24 hr for mammalian cells; Watson et al., 1992), achieve high cell densities, propagate on simple defined media, can be transformed with a variety of either self-replicating or integrating plasmid vectors and are able to make post-translational modifications that *E. coli* is unable to perform (Goeddel, 1990; Emr, 1990). Although it easy to achieve high-level translation of heterologous proteins, yeast, like *E. coli*, (Section 1.1.1.2.1) degrades some protein products either during or shortly after synthesis (Emr, 1990). Some heterologous secreted polypeptides can be secreted from yeast (eg. interferon-γ; IFN-γ ; Hitzeman et al., 1983), but many "secreted" proteins are not secreted into medium using their natural signal

sequences (eg. human α_1-antitrypsin, Cabezon et al., 1984; calf prochymosin, Mellor et al., 1983). Though yeast can carry out post-translational glycosylation, as mentioned earlier, the glycoproteins are often hyper-glycosylated. This may affect the immunogenicity, activity and serum half-life of the product (Buckholz and Gleeson, 1991).

1.1.1.2.3 Insect cells

Some proteins are difficult to express in bacteria or yeast as described before (Section 1.1.1.2.1, 1.1.1.2.2; eg, some hormone receptors). For expression of certain proteins, the use of insect cells can be quicker and can give better protein yields than other systems (100-200 fold higher; Hodgson, 1993; Vlak and Keus, 1990). Another advantage of using the insect cell system is that it does not support the growth of viruses or expression of oncogenes affecting humans (Agathos et al., 1990). The baculovirus vector, the most frequently used vector for insect expression system, can accept large inserts up to 25 kbp in length (Van Lier et al., 1992).

The cost of culturing insect cells is more than that of culturing bacteria and yeast, but it is less than that for culturing mammalian cells. Most of the post-translational processing, proteolytic cleavage, phosphorylation, amidation and myristoylation in insect cells is similar to that of mammalian cells (Luckow and Summers, 1988), however, the oligosaccharides used in glycosylation are of the high mannose type (Kuroda et al., 1989).

1.1.1.2.4 Transgenics

There are many potential applications of transgenic technology in heterologous protein production. First, using transgenic plants or animals as a bioreactor can be cheaper to maintain than cell cultures (Medin et al., 1990). Secondly, they can be renewable and production is in substantial quantities (Glick and Pasternak, 1994). Lastly, because recombinant proteins can be produced in defined "compartments", eg. egg (Sang, 1994), seeds (McElory and Brettell, 1994) or milk (Dobrovolsky, 1993), they can be easy to harvest and purify (Glick and Pasternak, 1994).

For some proteins, especially for transmembrane proteins, yields are low in cell-based expression systems. This may arise from the accumulation of such proteins in the cell membrane of transfected cells. These kinds of protein can be produced through milk in transgenic animals because the fat from within the mammary gland cell is encapsulated by plasma membrane and together they are secreted into milk (Glick and Pasternak, 1994). There are other reasons for using mammary gland to produce pharmaceutical proteins in milk. Milk is a renewable, secreted body fluid that is produced in substantial quantities and which can be collected without harm to the animal. The secretion of protein has no side effects on the normal physiological processes of the transgenic animal and the protein should undergo post-translational modifications that match closely those in humans. Finally, purification of the desired protein from milk, which contains only a small number of protein types is

relatively easy.

Although transgenesis and generation of transgenic animals holds promise, there are some disadvantages (Old and Primrose, 1991; Glick and Pasternak, 1994). First, for domestic animals, relatively few eggs are produced, thus limiting material. Secondly, the efficiencies for the transgenesis process are low (less than 5%) so a significant number of animals may need to be used to generate a population for harvest. Thirdly, reimplantation of the manipulated fertilised eggs can be difficult. Lastly, it can take a long time to go from a fertilised egg to an adult animal, eg. 2 years for a calf, so producing large numbers of genetically-engineered animals is a slow process but one which can be ultimately very rewarding.

1.2 Host/vector systems for optimall heterologous gene expression

There are two general methods of transferring recombinant DNA into mammalian cells; those mediated by virus infection and those mediated by direct DNA transfer (Levinson, 1990; Kaufman, 1990). Virus infection is efficient and a wide range of host cells can be chosen. However, the relative difficulty of execution has limited recombinant virus utility (Levinson, 1990). As such, most attempts to express genes in mammalian cells depend on the construction of DNA vectors.

1.2.1 The host cell

The ideal mammalian cell for heterlogous gene expression

must be capable of being easily transfected by vector DNA and protein products should undergo correct post-translational modification. The mammalian cell line most widely used for commercial production of recombinant therapeutic proteins is the CHO cell (Hodgson, 1993; Liu, 1992). This is because CHO cells have been used for the isolation of mutants affecting intermediary metabolism, DNA, RNA and protein synthesis, membrane function and cell behaviour (Gottesman, 1987). From a commercial viewpoint, a mass of background data on CHO cells is already deposited with the Food and Drug Administration (FDA; Hodgson, 1993). The oligosaccharide structural components of recombinant glycoproteins produced in CHO cells are remarkably similar to those of natural proteins isolated from humans or produced using human diploid cells (Liu, 1992, Utsumi et al., 1989). However, CHO cells are not susceptible to infection by standard retroviruses which might be used as DNA vectors (Gottesman, 1987).

CHO cells were first isolated by Puck et al (1958). CHO-K1 was the original subline isolated and CHO-S was a further subline which was adapted to suspension growth (Gottesman, 1987). CHO cells grow optimally at 37°C and prefer a slightly alkaline pH. CHO-K1 cells were generated after mutagenesis using ethyl methanesulfonate, 6-thioguanine and γ-radiation. Mutants lacking a functional DHFR activity, CHO Duk cells, were selected and these CHO Duk cells require glycine, purine and thymidine for growth (Urlaub and Chasin, 1980). CHO Duk cells can be transfected with plasmids which contain the DHFR gene. The transfected cells can then be isolated in selective

media which lacks ribo- or deoxyribo-nucleosides. Cells selected on the basis of containing the DHFR sequences can be grown in progressively increased concentration of methotrexate, a DHFR enzyme inhibitor, to amplify their gene copy number (Butler, 1987).

1.2.2 Plasmid expression vectors for mammalian cells

Mammalian cells will express exogenously introduced genes provided that the genes possess the proper DNA and RNA regulatory elements recognized and used by the host cell. These elements include signals for transcription, eg. promoter and enhancer, and for RNA processing, eg. RNA splicing and poly(A) addition (Weymouth and Barsoum, 1987). In order to manipulate cloned genes (or cDNAs) for expression in mammalian cells a shuttle vector capable of replicating in both prokaryotic and mammalian cells is required. This kind of vector is normally a hybrid DNA molecule consisting of prokaryotic plasmid sequences combined with eukaryotic DNA and RNA regulatory elements (Sanders, 1990). In this section I will concentrate on the eukaryotic DNA and RNA regulatory elements.

1.2.2.1 Control of mRNA transcription: Promoter/5' cap and enhancer sequences

The sequences required for RNA polymerase binding to allow transcription of messenger RNA to be initiated are called promoter elements (Weymouth and Barsoum, 1987). Two kinds of promoter have been identified (Kaufman, 1990); one set is inducible and needs external stimuli to induce its

function and the other set is constitutive and expresses without other factors being required for stimulation.

1.2.2.1.1 Inducible promoters

In order to express a protein which is potentially cytotoxic to mammalian cells (or other host cells), it is advisable to use an inducible expression system (Kaufman, 1990). Such a system has also been used to examine stable mRNA decay without radioactive labelling of precursor pools or by use of transcription inhibitors (Helms and Rottman, 1990). Inducible promoters can be harvested from eukaryotic cells, eg. IFN-β promoter and heat-shock promoter (Kaufman, 1990), or from prokaryotic repressor/operator system which can regulate gene activity in eukaryotic cells, eg. *E. coli* lactose operon and tertracycline operon (Gossen et al., 1994).

1.2.2.1.2 Constitutive promoters

One critical feature of these promoter elements is that they all contain mRNA cap sites (mRNAs derive a m^7Gppp cap structure after transcription; Furuichi, 1975), the point at which the mRNA synthesis actually begins (Kriegler, 1990). Another frequently occurring region is the TATA box, which consists of the consensus sequence 5'-TATAAAT-3' and which is located about 25-35 base pairs just 5'- upstream of the mRNA cap site. The TATA box is required to position accurately the start site of transcription (Corden et al., 1980). Further upstream, the relative positioning of promoter regulatory

sequences becomes more variable. For simian virus (SV40) DNA, there are GC-rich regions (GC boxes) with the repeated sequence 5'-CCGCCC-3', located 35-107 nucleotides upstream of the mRNA cap site. For the human β-globin promoter, there is a CAAT box with 5'-GGCCAATC-3'sequence. All sequence elements are recognised by specific transcription factors, eg. the TATA box is recognised by TFIID, the CAAT box by CTF/NF1 and the GC box by SP1.

Enhancers are further cis-acting elements that can stimulate gene transcription by as much as 100-fold (Weymouth and Barsoum, 1987). The structural framework produced by DNA-protein interactions can result in the formation of higher order chromatin structures that may serve to regulate transcription of specific genes. Key properties of enhancers are that (1) they are relatively large elements and may contain repeated sequences that function independently, (2) they may act over considerable distances, up to several thousand base pairs, (3) they may function in either orientation, and (4) they may function in a cell-type or tissue-specific manner (reviewed by Kriegler, 1990). The SV40 enhancer, for example, is composed of three functional elements, A, B and C, each of which can cooperate with the others or with duplicates of itself to enhance transcription (Herr and Clarke, 1986). In addition, a single element is ineffective as an enhancer however, each element can act autonomously when present as multiple tandem copies. Studies with either B or C elements alone show that these elements

possess different cell-specific activities (Ondek et al., 1987).

Most vectors used for mammalian cell expression contain promoter and enhancer elements from efficient transcription elements such as the SV40 early gene, the Rous sarcoma virus, adenovirus major late gene and the human cytomegalovirus immediate early gene (Kaufuman, 1990).

1.2.2.2 RNA processing and translational control elements

1.2.2.2.1 RNA splicing signals

Although many genes do not require introns for mRNA formation when introduced into mammalian cells (eg. expression from cytomegalovirus or heat-shock promoter; Nueberger and Williams, 1988), there are several examples of genes that have strong requirements for the presence of an intron. For example, a 10- to 100-fold increase in β-globin gene expression is obtained when an intron sequence is included in the vector (Buchman and Berg, 1988). In order to maximize expression from transfected genes, a small intron with flanking splice donor (5'-GT...intron) and acceptor (intron...AG-3') sites is normally included in expression vectors. Apart from affecting mRNA level, some introns may also be involved in the 3' end poly(A) formation (Nesic et al., 1993). The intervening sequences from SV40 small t antigen mRNA or a hybrid intron from adenovirus and immunoglobulin sequences are frequently used (Kaufman, 1990).

1.2.2.2.2 mRNA 3' untranslated region (3'UTR)

The 3' UTR of mRNA has been found to affect the function of mRNAs in the cytoplasm in several ways (reviewed by Decker and Parker, 1995; Jackson, 1993). These influences include localisation of mRNAs, control of mRNA stability, regulation of translation efficiency and alteration of the coding capacity of mRNA. Kaufman (1990) suggested that it is better to remove as much as possible of 5' and 3' untranslated region when constructing expression vectors.

1.2.2.2.2.1 **AUUUA pentamer** (ARE)

Many cytokine mRNAs are unstable with half lifes of about 30 min (Stoecklin et al., 1994; Henics et al., 1994). These mRNA contain several ARE in their 3'UTR sequence. Shaw and Kamen (1986) introduced this sequence into the 3'UTR of the rabbit β-globin gene and caused the stable β-globin mRNA to become highly unstable in vivo. When some of the T residues of the pentamer which the cDNA were mutated, this reverted the stability of β-globin mRNA back to the original value. They concluded that these pentamers are the recognition signal for an mRNA processing pathway which specifically degrades the mRNA encoding for certain lymphokines, cytokines and proto-oncogenes. The mRNA degradation pathway is protein translation-dependent and target mRNA species can be stabilised by addition of translation inhibitors (eg. cycloheximide; Aharon and Shneider, 1993; Savant-Bhonsale and Cleveland, 1992; Shaw and Kamen, 1986). However, mRNA, containing ARE, can be

stabilized by factors in media, eg. IL-3 mRNA can be stabilised by inclusion of ionimycin in media (Stoecklin et al., 1994).

The presence of ARE-containing sequences can affect nucleocytoplasmic transport of mRNA (Katz et al., 1994; Muller et al., 1992) and translational efficiency in cytoplasm (Grafi et al., 1993; Aharon and Schneider, 1993; Koeller et al., 1991; Kruys et al., 1989; Kruys et al., 1988; Kruys et al., 1987) through interactions with many protein factors with different binding specificities and affinities in either the nucleus or cytoplasm. This binding with protein factors has also been linked to mRNA stability (Hamilton et al., 1993; Bohjanen et al., 1992; Gillis and Malter., 1991; Vakalopoulou et al., 1991; Malter, 1989).

1.2.2.2.2.2 Other factors

(A) Factors which influence mRNA stability: Some cis-factors in 3'UTR have been identified to stabilise mRNA by binding to protein factors. This RNA-protein interaction, which masks endonucleolytic cleavage sites, controls mRNA decay rate. Examples include the 8BP motif (GCYATCAY) in 3' UTR of transition protein 2 mRNA, (Schlüter et al., 1993), multiple repeats of the 17BP motif of X1hbox 2 mRNA (Brown et al., 1993) and five iron response elements (IRE) of the transferrin receptor mRNA (Binder et al., 1994).

(B) Factors which influence translational efficiency: In many cases, 3'UTR contains specific negative regulatory elements that interact with trans-acting

components to repress translation. For example, the sex-determining genes *tra-2* (Goodwin et al., 1993) and *fem3* (Ahringer and Kimble, 1991) of *Caenorhabditis elegans*, which transform hermaphrodites into females, contain copies of a direct repeat element (DRE). These DREs inhibit translation of a reporter RNA. Other factors which inhibit RNA translation include nanos responsiive elements (Dalby and Glover, 1993) in the hunchback and bicoid mRNAs of *Drosophila* (Wharton and Struhl, 1991) and AU-rich sequences in IFN-β (Whittemore and Miniatis, 1990).

(C) Factors which control cell growth and differentiation: Differentiated skeletal muscle cells cease dividing and sustain expression of tissue-specific genes, such as troponin I, tropomyosin and α-cardiac actin. The 3' UTR of these genes can augment the differentiation of wild-type muscle cells and suppress proliferation of 10T1/2 fibroblasts (Rastinejad and Blau, 1993).

(D) Factors which control coding capacity: A conserved RNA secondary structure within the 3'UTR of Type 1 deiodinase (Berry et al., 1991) and glutathione peroxidase (Shen et al., 1993) mRNAs is sufficient to direct the incorporation of selenocysteine at UGA codons that would otherwise specify translation termination.

1.2.2.2.3 3' End and poly(A) signals

For mRNA, creation of the 3' end is an essential step in mRNA synthesis (for tRNA and rRNA also). The mRNA needs to be free from the DNA template to function and the polymerase

needs to be released to transcribe again (Manley and Proudfoot, 1994). Most mRNAs have a homopolymer of 20-250 adenosine nucleotides at their 3' end. Such a poly(A) tail can stabilise mRNA and improve mRNA translation efficiency (Sachs and Wahles, 1993). However, histone mRNA does not have a poly(A) tail. The histone mRNA has a hairpin structure and highly conserved sequence (CAAGAAAGA) which are essential for the generation of a mature 3' end (Platt, 1986).

Some cis-factors have been identified which can influence poly(A) addition. These include the highly conserved sequence AAUAAA 10-30 nucleotides upstream of the cleavage site and a poorly defined GU- or U-rich sequence approximately the same distance downstream (Sachs and Wahles, 1993; Wickens, 1990a). These sequences bind to two protein factors, cleavage-polyadenylation-specific factor and cleavage-stimulatory factor to form a platform to which two additional cleavage factors (CF1 and CF2) then bind for cleavage to occur (Manley and Proudfoot, 1994). The poly(A) polymerase is also required for cleavage. Then, the addition of the poly(A) tail proceeds in two stages. At first, the addition of each of the first few adenosine nucleotides is dependent on AAUAAA. Once an oligo(A) primer of 10 or more nucleotides is synthesized, the requirement for AAUAAA is lost.

Poly(A) addition can activate translation, whereas poly(A) shortening may repress translation (Manley and Proudfoot, 1994). The same mRNA from different cell sources has different length of poly(A) tail (Wickens, 1990b). Thus

it is important to choose a good 3' end sequence for construction of an expression vector to maximise the length of the poly(A) addition. Efficient signals for polyadenylation can be derived from SV40 early transcription unit, the hepatitis B surface antigen transcription unit and the mouse β-globin gene and all are in common use (Kaufman, 1990).

1.2.2.3 Selective and amplifiable markers

One of the basic principles of heterologous gene expression is the use of biological markers to identify cells carrying recombinant DNA molecules. In bacteria, there are drug-resistance genes (ampicillin, tetracycline) and in yeast, there are nutrient auxotroph or temperature-sensitive mutants genes (Watson et al., 1992; Old and Primrose, 1989).

Many mutants of mammalian cells have been cloned (eg. CHO Duk, Section 1.1.1) and these established cell lines can be used as hosts. Plasmids which contain selectable marker genes (eg. dhfr) can be used to transfect these mutant cells. Successfully transfected cells can be isolated in selective media. DHFR is essential for purine and pyrimidine nucleotide synthesis (Benkovic, 1980). This enzyme can be inhibited by the folate analogue, methotrexate (Mtx) which is a potent competitive inhibitor the enzyme (Benkovic, 1980). Cells resistance to methotrexate correlates with increased copies of dhfr genes and expression of higher DHFR enzyme levels (Schimke, 1984). During the amplification process DNA that is amplified is much larger than dhfr gene alone, and there is often amplification of neighbouring DNA (Schimke, 1984).

Cells overproducing DHFR may contain over 1000 copies or more of the gene (Schimke et al., 1978) and highly resistant variants of the transfected cell line can be selected so as to give concomitant amplification of any co-transfected DNA (Wigler et al., 1980). Other selection (or amplification) markers are summarized in Table 1.1. These genes are used to transform cells to express particular drug resistance marker (as with DHFR resistance to Mtx), allowing transfected cells to be selected from heterogeneous cells. Some of the markers used in the selection can also be used to amplify gene copy number, eg. glutamine synthetase and aspartate transcarbamylase, whereas others are not. Drugs used in the selection procedures include G418, hygromycin-B, mycophenolate, N-phophonacetyl-L-aspartate and methionine sulphoximide.

Table 1.1 Selected drug resistance and gene amplification markers for mammalian cells

	Enzyme	Selection drug	Selection mechanism
1	Aminoglycoside phosphotransferase	G418	Inactivate G418
2	Hygromycin-B phosphotransferase	Hygromycin-B	Inactivate hygromycin-B
3	Thymidine kinase (TK)	Aminopterin	TK synthesise thymidylate
4	Xanthine-guanine phosphoribosyl-transferase (XGPRT)	Mycophenolic acid	XGPRT synthesise GMP from xanthine
5	Glutamine synthetase (GS)	Methionine sulphoximide	Overproduction GS
6	Aspartate transcar-bamylase (AT)	N-Phosphonacetyl-L-aspartate	Overproduction AT

(Reconstructed from Bebbington et al., 1992; Sanders, 1990; Watson et al, 1992; Old and Primrose, 1989)

1.2.2.4 Transfer of vectors into cells

Many methods have been developed to transfer vectors into mammalian cells, including calcium phosphate transfection (Chen and Okayama, 1988), DEAE-dextran (diethylaminoethyl-dextran) transfection (Sussman and Milman, 1984), electroporation (Potter, 1988) and liposome-mediated transfection (Felgner et al., 1987). The first two procedures produce a chemical environment which results in DNA attaching to the cell surface and DNA is then endocytosed by the cell. Electroporation uses an electric field to open up pores in cells and DNA diffuses into the cell through the pore. In liposome-mediated transfection, liposomes containing cationic and neutral lipids are used to mediate DNA transfection. All four methods can be used for transient transfection but only three, excluding DEAE-dextran transfection, produce cell lines containing stably integrated DNA.

1.3 Regulation of gene expression

1.3.1 Schemes of gene regulation

After successful transfection of a gene into mammalian cells, there are four key stages where gene expression may be regulated (Fig 1.1). These are transcriptional, post-transcriptional, translational, and post-translational regulation. In this section, I will concentrate mainly on post-transcriptional and post-translational regulation.

1.3.2 Gene regulation occurs at multiple levels

Regulation of specific gene expression is dynamic and

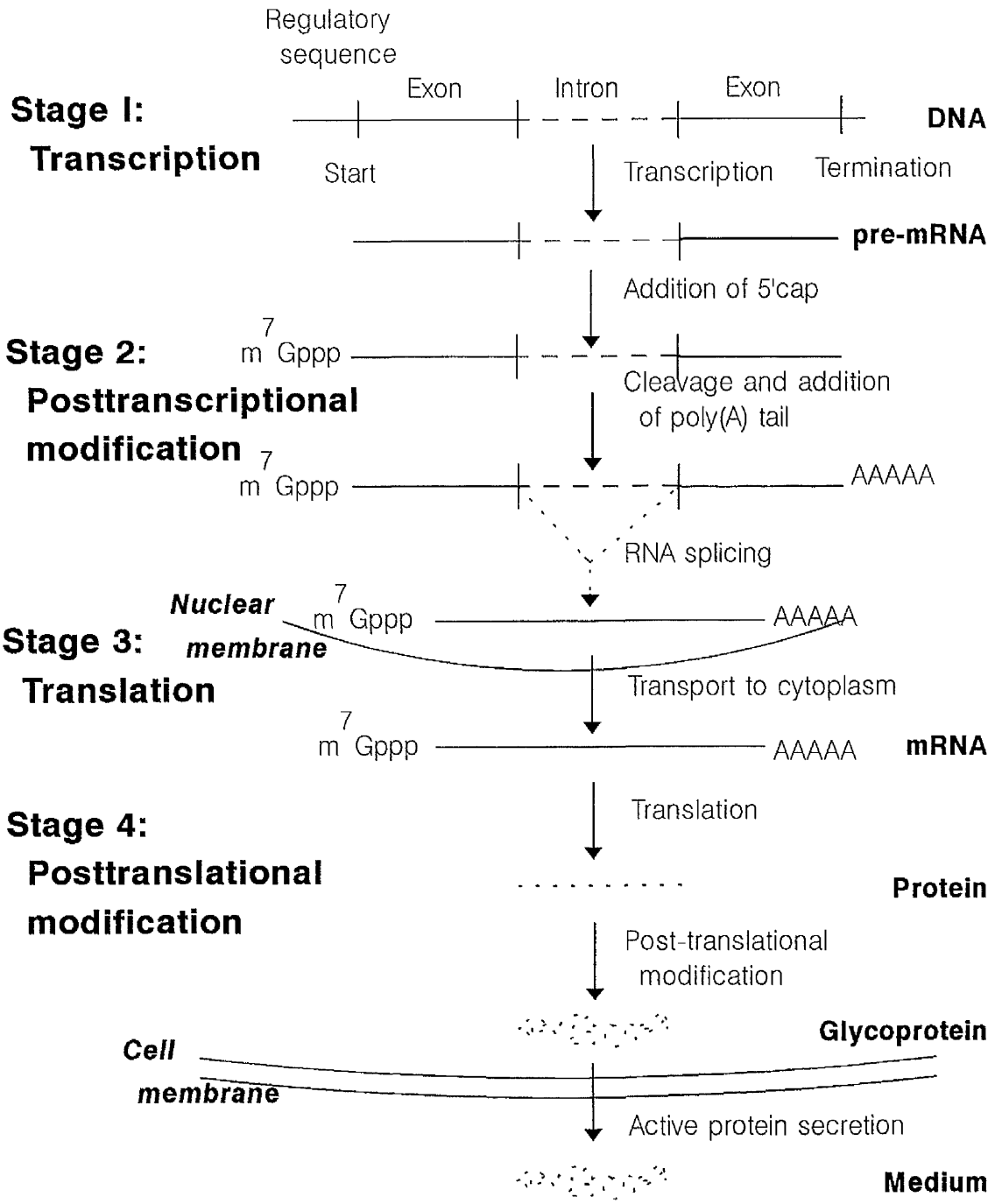


Fig 1.1 Stages in eukaryotic gene expression

integrated by contol at DNA, RNA and protein stages in the cell (Kyrpides and Quzounis, 1993). For any given gene several mechanisms may interact to produce a coordinated regulation at one point or/and regulate more than one point. For example, IFN-β expression in naturally-producing cells is regulated both at mRNA stability and at translational level (Kruys et al., 1987). Transcription and splicing are coordinately regulated in HeLa cells in response to viral or plasmid (with SV40 promoter) infection (Jimenez-Garcia and Spector, 1993), and when macrophages are exposed to bacterial endotoxin, it activates both transcription and translation of tumour necrosis factor (TNF; Han et al., 1991).

1.3.3 Regulation of mRNA expression

1.3.3.1 **Transcriptional regulation**

The control of transcription involves many transcriptional regulatory proteins and their binding to DNA cis-elements, eg transcription factor IID (TFIID) binds to the TATA box (Section 1.2.2.1.2; Latchman, 1991). Transcription factors from different families can interact with each other when bound to DNA regulatory cis-elements. This interaction of protein factors can result in cell specificity of transcriptional control and distinct regulatory patterns (Miner and Yamamoto, 1991). Transcriptional regulation may also involve alternative promoter selection (Stehle et al., 1993) and alternative selection of transcriptional termination sites (Greenblatt et al., 1993), either of these processes could modify gene

function.

In Section 1.2.2.3, I described how gene copy number can be amplified. Generally mRNA transcription correlates to gene copy number and to protein production (Kang and Perry, 1994; Cote et al., 1994). However, in some cases gene copy number has no correlation with the amount of mRNA (Miller et al., 1994), or high gene copy number may result in accelerated mRNA degradation (Jain and Belasco., 1995).

1.3.3.2 Post-transcriptional regulation

1.3.3.2.1 Splicing

In eukaryotic cells, genes are split into pieces by introns (Roger and Doolittle, 1993). Intron are removed from the RNA transcript to make a functional mRNA. The transcripts of protein-coding genes in the nucleus of eukaryotic cells are known as heterogenous nuclear RNA (hnRNA) or pre-mRNAs and contain introns. Removal of introns from hnRNA can produce alternative mRNAs (alternative or regulated splicing; Dibb, 1993).

1.3.3.2.2 Mechanism of splicing

Pre-mRNA together with some small nuclear RNAs (snRNA) form a complex of 60S which was designated to be a spliceosome (Grabowski et al., 1985). Within spliceosomes pre-mRNAs are spliced in a two-step pathway (Green, 1991). In the first step, the pre-mRNA is cleaved at the 5' splice site and generates two splicing intermediates, a linear exon-containing RNA and an intron-second exon RNA in a lariat

structure. In the second step, the 3' splice site is cleaved and the two exons are ligated to form a spliced mRNA. The intron is, then, degraded (Ruskin and Green, 1985).

The recognition of splicing sites is mediated by the pre-mRNA sequence, protein factors and snRNA (Sharp, 1994). The 5' splice-site consensus is AG/GURAGU (/: the boundary of the intron/exon; R: purine) and the 3' splice-site consensus sequence is YAG/G (Y: pyrimidine). In these sequences, the most highly conserved bases are the first and last two bases of the intron. Additional conserved sequence blocks include a branch point sequence, YNYURAY (N: any base), usually located about 30 nucleotide upstream from the 3' splice-site and a stretch of pyrimidines typically found immediately adjacent to the 3' splice site.

1.3.3.2.3 Regulated splicing (Alternative splicing)

Alternative splicing regulates the production of a number of important biological molecules. This regulation can involve on/off regulation of the products of particular genes (Bingham et al., 1988), or it can involve the production of alternative products with clearly separate functions (Mattox et al., 1992). It can also regulate the cytoplasmic accumulation of mRNA (Dix and Leppard, 1993).

Many factors are required for alternative splicing, these include sequences in pre-mRNA (Noble et al., 1988; Amrein et al.,1994), hnRNP (Pinol-Roma and Dreyfuss, 1992) and non-sn RNP protein splicing factors (Krainer et al., 1990; Lamm and Lamond, 1993). These factors interact with

each other to mediate alternative splicing (Smith et al., 1989; McKeown, 1992). For example, branch point sequence selection affects the large T antigen or t protein production of adenovirus which is mediated by a protein factor, alternative splicing factor SF2 (ASF/SF2; Noble et al., 1988). The concentration of ASF also affects the splice site selection (Fu et al., 1992; Mayeda and Krainer, 1992).

1.3.3.3 Nucleus/cytoplasmic transport

The pre-mRNAs are extensively processed and targeted to the cytoplasm to become functional, translatable mRNAs. mRNA transport from the nucleus to cytoplasm can be divided into two general phases. The first involves hnRNA movement from the transcription site and hnRNA assembly at the nuclear pore complex (NPC). NPCs are gate channels through which proteins and hnRNA enter and exit the nucleus (Izaurralde and Mattaj, 1995). Studies of gene regulation in human immunodeficiency virus (HIV) have suggested that gene expression may be regulated at the level of mRNA nuclear export (Rosen and Pavlakis, 1990). RNAs encoding structural polypeptides are retained in the nucleus and viral protein, Rev, modulates the redistribution of mRNA to the cytoplasm to allow protein synthesis.

The shuttling of hnRNA across the nuclear envelope is independent of ongoing transcription and translation, and it is temperature-dependent (Pinol-Roma and Dreyfuss, 1993). As the export is temperature-dependent, it has been suggested that it is energy-dependent and, therefore, result from an

active transport (Newmeyer, 1993). hnRNA sequence and structures can also affect its export (Terns et al., 1993; Izaurralde and Mattaj, 1995). Introns can form spliceosomes and serve as nuclear retention signals for unspliced pre-mRNA (Chang and Sharp, 1989). Sequences in 3'UTR also modulate mRNA export, eg. ARE binds to many protein factors, and one such protein called AU-A has been found to shuttle between the cytoplasm and nucleus, and modulate mRNA export (Katz et al., 1994). Others include Cap structure (Dargmont and Kuhn, 1992) which is mediated by cap-binding protein 20 (CBP20; Izaurralde et al., 1995) and mRNA 3'end cleavage and polyadenylation can stimulate mRNA export (Eckner et al., 1991).

1.3.3.4 **Messenger RNA stability**

In general, the rate of protein translation is directly proportional to the cytoplasmic level of the mRNA which encodes for it. Therefore, mRNA turnover is important for biological regulation (Hentze, 1991). This was confirmed by differences in degradation rates amongst mRNA molecules (Decker and Parker, 1994) and for specific mRNA under different nutritional states (Bridges and Cudkowicz, 1989), cell cycle (Morris et al., 1986) or different biological status. For example, induction of granulocyte-macrophage colony stimulating factor (GM-CSF) expression with different inducers results in 4-fold change to GM-CSF mRNA half-life (Schuler and Cole, 1988). The addition of an antibody to CD28, a T cell surface protein, to stimulate T cells,

stabilises by 5- to 20-fold four cytokine mRNAs, interleukin-2 (IL-2), TNF-α, GM-CSF, and IFN-γ (Lindsten et al., 1989).

Eukaryotic mRNA in the cytoplasm are usually associated with RNA binding proteins (Larson and Sells, 1987). The structure of mRNA molecules and their interactions with associated protein(s) are the primary determinants of mRNA stability (Saini et al., 1990). These interactions not only regulate mRNA stability but also have a strong influence on the translational efficiency of the message (Saini et al., 1990). For example, cap-binding protein and poly(A) binding protein can either stabilize mRNA or can increase translational efficiency (Peltz et al., 1987; Brawerman, 1981).

1.3.3.4.1 Mechanism of mRNA degradation

One major mRNA decay pathway is initiated by shortening of the poly(A) tail followed by decapping and 5' to 3' exonucleolytic degradation of the transcript (Beelman and Parker, 1995; Decker and Parker, 1994). This degradation can be either translational-dependent or -independent (Hentze, 1991). Other decay pathways are initiated, independent of deadenylation, by sequence- specific cleavage of mRNA. However, deadenylation does not occur at a uniform rate, it occurs in several phases. Some of the poly(A) tail is removed at an mRNA-specific rate, leaving an 25-60 adenylated residue tail (Shyu et al., 1991; Chen et al., 1994) which could result in loss of the poly(A)-binding protein associated with the transcript (Decker and Parker,

1993). The mRNA which loses the poly(A)-binding protein can become a substrate for the decapping reaction, thereby exposing the transcript to 5' to 3' exonucleolytic decay (Larimer and Stevens, 1990; Muhlrad et al., 1995). Eukaryotic mRNAs can also be degraded in a 3' to 5' direction following deadenylation (Higgs and Colbert, 1994). It is not known whether exonucleases, endonucleases or both are involved in 3' to 5' decay pathway. An individual mRNA can simultaneously be a substrate for more than one mechanism of decay. For example, the yeast *PGK*1 transcript undergoes a deadenylation-dependent 3' to 5' degradation in addition to a 5' to 3' decay mechanism (Muhlrad et al., 1995). Other mechanisms, including deadenylation-independent decapping (eg. degradation of mRNAs with nonsense stop codons; Hagan et al., 1995) and decay of mRNA via endonucleolytic cleavage (eg. in the coding region of the *c-myc*; Bernstein et al., 1992), tend to be mRNA-specific (Beelman and Parker, 1995).

1.3.3.4.2 3'UTR and mRNA stability (AU-rich sequence)

3'UTR of mRNA is well known to control mRNA half-life (Peltz et al., 1991). However, the mechanism of this modulation is still unknown. For example, β-globin mRNA is four- to sixfold more stable than δ-globin mRNA in human bone marrow cells (Ross and Pizarro, 1983). The mRNAs are approximately 92% homologous in the 5' UTR and coding region but differ by more than 50% in their 3' UTR. Examination of the sequences in the 3' UTR does not highlight any specific sequence which could modulate the difference in stability.

Lymphokine and serum response early gene mRNAs are unstable in vivo (Gillis and Malter, 1991). All these mRNAs contain a AU-rich sequence, with multicopies of ARE, which modulate rapid mRNA degradation (Gillis and Malter, 1991). The degradation of these mRNAs is through deadenylation, translation-dependent, 5' decapping pathway (Beelman and Parker, 1995; Aharon et al., 1993; Savant-Bhonsale and Cleveland, 1992). The AUUUA-mediated rapid degradation is modulated by trans-acting factors (Koeller et al., 1991), cooperating with adjacent sequences (Alberta et al., 1994) and can be stabilized by changing cell physiology (Lindsten et al., 1989).

1.3.4 Regulation of protein expression

Protein is the final product of gene expression. However, the proteins themselves are often further sites for regulation. For example, some proteins need addition of carbohydrate (glycosylation) or phosphate (phosphorylation) to gain their full activity.

1.3.4.1 **Translational efficiency**

The formation of the translational machinery is similar to the formation of spliceosome but utilises different components and is more complicated (Lodish et al., 1995). Firstly, an initiation factor (eIF2) binds a molecule of GTP and a molecule of Met-tRNA (transfer RNA) to form a ternary complex. Then, it binds to other initiation factors, to mRNA and small ribosomal subunit to make a 40S complex. Secondly,

once the Met-tRNA is positioned correctly at the AUG initiation codon, a large ribosomal subunit joins to complete the 80S initiation complex. The activity (or strength) of mRNA is determined first by the accessibility of the capped 5'-terminus to initiation factors (Rhoads, 1988) and by the involvement of the poly(A) tail (Galli and Tanguay, 1994), then by the ability of the 40S ribosome subunit to bind the mRNA and finally by the frequency of recognition of the initiation codon and the surrounding context (Hershey, 1991). After formation of the 80S initiation complex has been completed, the peptide is elongated along the mRNA until a stop codon is reached and translation terminates.

Many cis-acting sequences in 3' UTR of specific mRNA, together with other trans-acting factors, can regulate mRNA translation efficiency. For example, AU-rich sequence in lymphokine and serum-response early genes associated with poly(A) tails can regulate translational efficiency (Grafi et al., 1993). When poly(A)-rich, hu-IFN-β mRNA is an inefficient template for protein synthesis (Grafi et al., 1993). However, translational efficiency was improved when the poly(A) tail was shortened, or when the 3' AU-rich sequence was deleted. Binding of a phosphoprotein to the 3' UTR can repress protamine 2 mRNA translation (Kwon and Hecht, 1993) and some mRNAs in growing oocytes are dormant when they contain short poly(A) tails but are activated by cytoplasmic polyadenylation (Bachvarova, 1992; Huarte et al., 1992). Translational regulation can, also, occur through phosphorylation of factors of the protein synthetic machinery

(Barber et al., 1993). Phosphorylation of specific proteins involved in translation has been described to regulate translation rates at mitosis, nutrient starvation and stress (Hershey, 1989). For example, IFN-γ can induce expression of a 68kD protein kinase activity when cells are exposed to double-stranded RNA. Activation of this protein kinase, by binding double-stranded RNA to its N-terminal binding site, catalyses phosphorylation of the α subunit of eIF2 and inhibits translation at the stage of initiation (Barber et al., 1993).

1.3.4.2 Post-translational regulation

Once protein synthesis is complete, polypeptides introduced into the membrane and lumen of the ER must be matured, sorted, and transported. Many secretory or membrane proteins undergo four principal modifications as they mature to the cell surface (Freedman, 1984; Roth and Pierce, 1987): (1) formation of disulphide bonds, (2) proper folding of the protein, including formation of multichain complexes, (3) specific proteolytic cleavage, and (4) addition and modification of carbohydrate groups. Each modification takes place in a specific part of the membranous organelles through which proteins pass (Rothman and Orci, 1992). These modification help the protein achieve its functional form and in several cases, direct the protein to its ultimate destination in the cell. In this Section, I will concentrate on protein folding, secretion and glycosylation.

1.3.4.2.1 Protein folding

In eukaryotic cells, formation of all disulphide bonds occurs in the lumen of the rough ER (Freedman, 1989) which provides an oxidizing environment that favors formation of disulfide bonds (Bergeron et al., 1994). Many of these bonds are formed while the polypeptide is still growing on the ribosome (Freedman, 1989). Disulphide bonds stabilize the domains in proteins but if the proper bonds do not form, the protein may not achieve a functional conformation (Freedman, 1989).

The folding process is accelerated by the disulphide isomerase (PDI), peptidylprolyl isomerase and molecular chaperones of the Hsp70 and Hsp 90 families which are found in abundance in the ER of secretory tissues (Freedman, 1989; Helenius et al., 1992). The interaction of chaperones with the nascent chain forms a high molecular mass complex that allows the cotranslational formation of protein domains and the completion of folding once the chain is released from the ribosome (Frydaman et al., 1994).

1.3.4.2.2 The significance of protein glycosylation

For glycoproteins, the carbohydrate groups confer important physical properties such as conformational stability, protease resistance, charge and water binding capacity (Roth, 1987). These carbohydrate groups may also be involved in biological recognition, signals for protein targeting, cell-cell interaction (Paulson, 1989), enzyme activity (Parekh et al., 1989) and pharmacology clearance (Varki, 1993).

1.3.4.2.3 Overall pathway of secretory protein formation

The overall pathway of secretory protein formation is summarised in Fig 1.2. Protein is translated into rough endoplasmic reticulum (ER) and then this newly made protein is directed by the signal peptide to the lumen of the rough ER (Gilmore, 1993). The signal peptide is cleaved by signal peptidase (Dalbey and von Heijne, 1992). Protein transport from ER, through the cis-Golgi complex and then to the trans-Golgi complex is mediated in vesicles which are surrounded by a protein coat (Rothman, 1994). From there, the protein is sorted to secretory vesicles which fuse with the plasma membrane and release the protein to the cell exterior (Rothman, 1994).

There are two pathways of secretion: a regulated pathway and a constitutive pathway (Brion et al., 1992). Peptide hormones are stored in granules which undergo regulated release whereas other secreted proteins are externalized constitutively via a distinct set of vesicles. The constitutive secretory pathway is not affected by cycloheximide suggesting that operation of this pathway does not require components supplied by new protein synthesis (Wieland et al., 1987). However, cycloheximide treatment severely impairs the ability of cells to secrete protein by the regulated secretory pathway (Brion et al., 1992).

1.3.4.2.4 Quality control in the secretory pathway

The organelles (ER and Golgi) of the secretory pathway are responsible for the biosynthesis, folding, assembly and

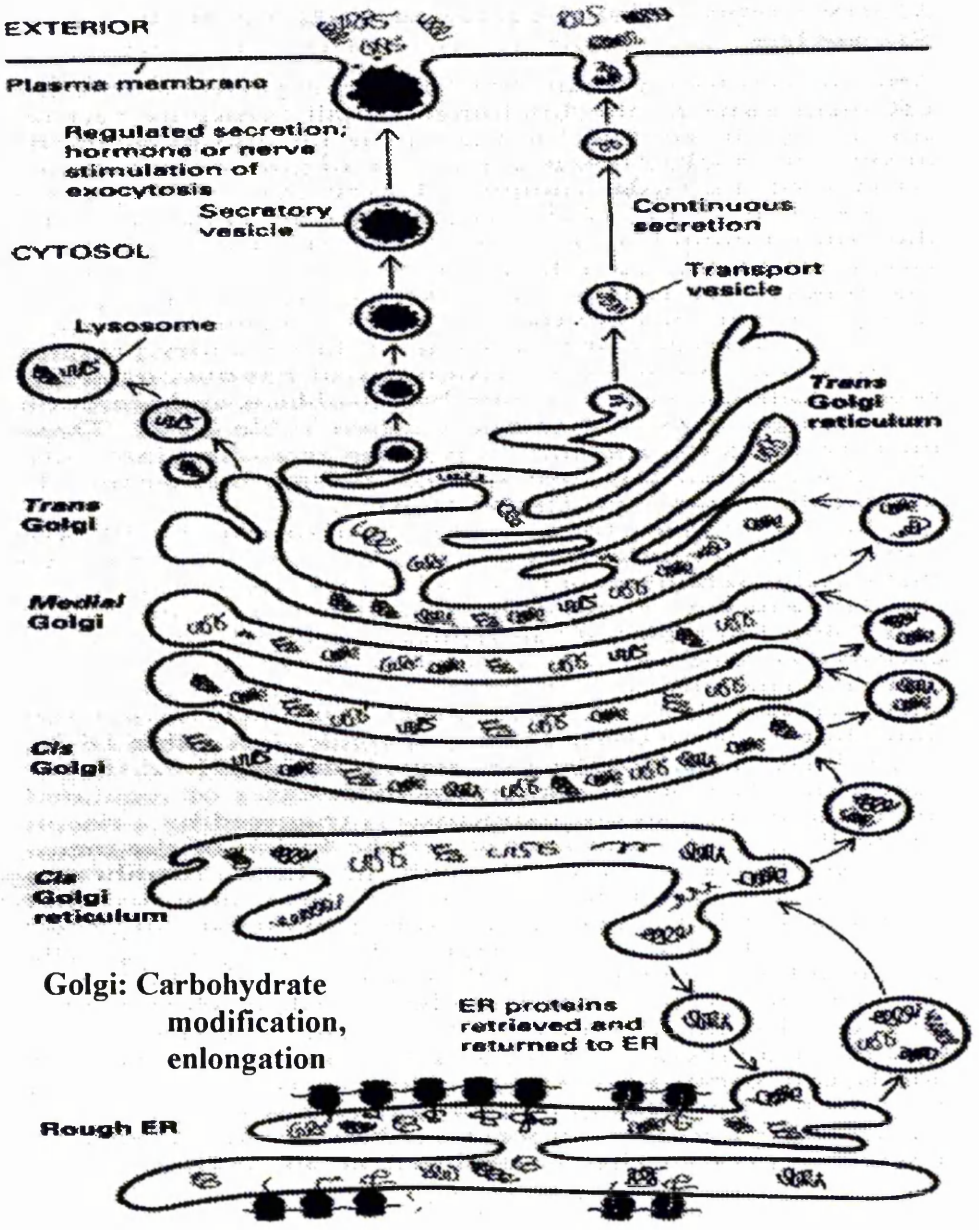


Fig 1.2 Glycoprotein secretion pathway (modified from Lodish et al., 1995)

modification of secreted protein. This capacity requires the ability to recognize, retain and degrade misfolded, incompletely-folded or partially- assembled proteins so that cell can ensure the structural and functional integrity of protein products. This conformation-based sorting phenomenon is called "quality control" (Hammond and Helenius, 1995).

For proper folding of oligomeric and glycosylated proteins, they are concentrated in shuttle vehicles (Quinn et al., 1984) and selectively transported to and through the Golgi (Pelham, 1995). Some transport of lumen-resident ER proteins occur, but these proteins carry a carboxyl-terminal KDEL signal which allows their receptor-mediated retrieval from post-ER compartments (Pelham, 1989).

Glycoprotein maturation is a pathway devoted to quality control in the ER and this control is mediated by calnexin (Bergeron et al., 1994), a phosphorylated transmembrane protein of 64.5 kDa, and calrediculin, a 45 kDa soluble homologue of calnexin present in the lumen of the ER (Peterson et al., 1995). The role of these chaperones is to assist the folding and oligomeric assembly of glycoproteins, to mediate ER retention of incompletely-folded and oligomerized proteins and prevent premature degradation (Hebert et al., 1995; Jackson et al., 1994). The period of chaperone binding is extended when proteins are permanently misfolded and usually lasts until they are degraded (Hammond et al., 1994).

Proteins that fail to fold are selectively degraded (Klausner and Sitia., 1990). This degradation can happen by

one of two general mechanisms. In the first, soon after the protein synthesized, it is degraded in a mechanism which requires ATP (Lippincott-Schwartz et al., 1988). In the second the protein is retained in ER by binding to the heavy chain binding protein, Bip (Hendershot et al., 1987), and route the protein to lysosomes where they are destroyed (Minami et al., 1987). The recognition of signals for degradation relies on the conformation and general properties expressed by incompletely-folded molecules rather than on structure or sequences (Hammond et al., 1994; Hebert et al., 1995). These include hydrophobic peptide elements exposed on the surface, the presence of free exposed sulhydryl groups, partially glucose-trimmed oligosaccharides, and the tendency to form large aggregates.

1.3.4.2.5 Protein glycosylation

Most plasma membrane and secretory proteins contain one or more carbohydrate chains. The structure of N- and O-linked oligosaccharide are very different and there are different sugars on each type (Stanley, 1992). For example, in O-linked sugars, N-acetylgalactosamine is linked to serine or threonine whereas in N-linked oligosaccharides, N-acetylglucosamine (GlcNAc) is linked to asparagine. O-linked oligosaccharides are generally short, often containing one to four sugars. The N-linked oligo-saccharides, in contrast, have a minimum of five sugars and always contain mannose as well as N-acetylglucosamine (Paulson, 1989). The N-linked oligosaccharides fall into several structural classes

(Kornfeld and Kornfeld, 1985):

1. Complex oligosaccharides containing mannose (Man), fucose (Fuc), galactose (Gal), GlcNAc and sialic acid (N-acetylneuraminic acids). These oligosaccharides are found in many serum and viral glycoproteins.
2. High-mannose oligosaccharides contain only GlcNAc and Man.
3. Hybrid N-linked oligosaccharides in which one of the branches has a typical complex structure while the other two contain only Man.

Glycosylation is the principle chemical modification to plasma and secretory proteins. Some glycosylation reactions occur in the lumen of the ER, others in the lumina of the cis-, medial- and trans-Golgi vesicles (Lodish et al., 1995). The biosynthesis of all N-linked oligosaccharides begins in the ER with a large precursor oligosaccharide, $(Glucose)_3(Man)_9(GlcNAc)_2$ (Kornfeld and Kornfeld, 1985). This precursor oligosaccharide is linked by a pyrophosphoryl residue to dolichol which acts as a carrier for the oligosaccharide. The oligosaccharide is transferred en bloc from the dolicol carrier to an asparagine residue on the nascent polypeptide with the sequence, Asn-X-Ser or Asn-X-Thr (X is any amino acid except proline). While the nascent polypeptide is still in the rough ER, immediately after oligosaccharide transfer, all three glucose residues and one mannose residue are removed and the glycoprotein is ready to move to the Golgi where further modifications occur. Different enzymes are located to the cis-, the medial, and the trans-Golgi vesicles and act sequentially as the protein

moves through these organelles en route to the cell exterior (Kornfeld and Kornfeld, 1985). The production of complex N-linked oligosaccharides is the result of a stepwise, coordinated set of reactions in which five more mannose residues are removed. Then, three GlcNAc, three Gal, one to three sialic acid and one Fuc are added, one at a time, to each oligosaccharide chain. High-mannose and hybrid oligosaccharides are produced if some of the enzymes cannot work on the N-linked oligosaccharide.

The conformation of the protein and its primary amino acid sequence determines whether an oligosaccharide is attached to a particular Asn-X-Ser/Thr sequence in the ER, since not all such possible sequences are utilized (Abeijon and Hirschberg, 1992). For example, the rapid folding of a segment of a protein containing an Asn-X-Ser/Thr sequence may prevent the oligosaccharide transferase enzyme from transferring an oligosaccharide to it. Similarly, the conformation of a segment of a protein may determine whether, in the Golgi, a particular N-linked oligosaccharide becomes complex, hybrid or high-mannose (Kornfeld and Kornfeld, 1985). Each specific cell type in an organism contains its own specific processing enzymes, thus the same protein produced by individual cell types may have differentially processed carbohydrate (Paulson and Colley, 1989).

1.4 mRNA secondary structure prediction

mRNA primary structure is important for coding sequences

and for provision of consensus sequences for the regulation of processing and translation (Konings et al., 1987; Section 1.3.3 and 1.3.4). In addition, secondary structures in mRNA species have been proposed to affect pre-mRNA 3' cleavage and polyadenylation (Birchmeir et al., 1983), splicing (Reed and Maniatis, 1986) and translation (Spena et al., 1985). mRNA structure is achieved by the molecule bending back on itself and forming helical regions stabilized by hydrogen bonds between complementary bases (Zuker, 1989). Base pairing can be three types: G with C, A with U and the weaker G with U. A basic feature of the secondary structure of RNA is the stem-loop structure (Pleij and Bosch, 1989). Pseudoknots arise on base pairing of the single-stranded loops enclosed by these stems, with complementary unpaired regions elsewhere in the mRNA chain. mRNAs in the cytosol are folded into a pseudoknot tertiary organisation (Pyle and Green, 1995). mRNAs in the cytosol often bind metal ions, eg. magnesium, and with proteins. Such interactions may change mRNA folded structure (Konings et al., 1987). Thus, it is difficult to predict the real secondary structure of mRNA in cells. However, by computation of the minimal free energy of folded mRNA, it is possible to obtain a first level prediction of mRNA secondary structure. Konings et al. (1987) used the minimal energy folding method to compare 38 mature mRNAs and fully or partly randomised sequences and concluded that the secondary structure surrounding the initiation site of translation conforms to a consensus structure. Loops have destabilizing energies and the overall free energy of a

folded mRNA is the sum of the energies of the stacked base pairs and the loops (Zuker, 1989). Comparison of the folding energy with the free energy of random sequences (or known structure) can also give some significant information about the folded structure (Grubskov and Devereux, 1991). If the energy of a folded mRNA is 50% higher than the random sequences, the mRNA structure may be unstable.

1.5 The characteristics of human IFN-γ

The interferons are a group of proteins and glycoproteins which can inhibit the growth of a wide range of virus in cells (Taylor-Papadimitriou, 1984). Interferons are classified into types on the basis of antigenic specificities (Stewart et al., 1980), alpha- (α), beta- (β) and gamma-interferon (γ) which have been categorised as leucocyte, fibroblast and type II (immune) interferons, respectively.

1.5.1 Function and cellular actions of IFN-γ

In addition to its antiviral activity, IFN-γ also exerts antitumour and antimicrobial activities (Kurzrock, 1992; Tyring 1995). All these effects are modulated by the actions of IFN-γ on regulation of the immune system. IFN-γ is a powerful stimulant of phagocytic macrophages (Murray, 1988) and induces expression of major histocompatibility complex antigens on various cells which may then be recognized by cytotoxic T lymphocytes (Kurzrock et al., 1986). IFN-γ also augments natural killer cell (NK cell) antibody-dependent

cellular cytotoxicity (Gidlund et al., 1987) and stimulates the maturation of B cells to active immunoglobulin secretion (Sidman et al., 1984).

In target cells IFN-γ induces the expression of many polypeptides (Weil et al., 1983). This results from the binding of IFN-γ to cell surface receptors which stimulate intracellular signalling which increases the expression of many genes (Lengyel, 1993). The IFN-γ receptor is a member of cytokine receptor family which activates tyrosine phosphorylation (Silva et al., 1994). The Janus kinase (JAK) family of tyrosine kinases play an integral role in intracellular signalling by the cytokine receptors (Ihle et al., 1994). Through JAK activation IFN-γ induces tyrosine phosphorylation of STAT1 (signal transducer and activator of transcription-1) and other transcription factors (Harroch et al., 1994; Shuai, 1994). When phosphorylated, these factors translocate to the nucleus and there they bind to enhancer elements with the consensus sequence TTnCnnnAA. Thus genes are activated that encode proteins that mediate various actions of the IFN-γ (Horroch et al., 1994; Shuai, 1994).

1.5.2 IFN-γ production

IFN-γ can be produced by T cells (Alzona et al., 1995), B cells (Mizrahi et al., 1990) and tonsil cells (Lebendiker et al., 1987) in response to interaction with a suitable mitogen, bacterial endotoxin, ionophore and bacteria or viral antigen (Mizrahi et al., 1990). IFN-γ has been expressed in various recombinant systems including *E. coli* (Gray et al.,

1982), Yeast *S. cerevisiae* (Derynck et al., 1983), *Xenopus laevis* oocytes (Devos et al., 1982), tobacco protoplasts (Mori et al., 1993), CHO cells (Nadler et al., 1994) and in transgenic mice (Dobrovolsky et al., 1993). IFN-γ mRNA has been translated in cell free systems, heterologous cells and *X. laevis* oocytes, but only oocyte translate IFN-γ mRNA efficiently (Sloma et al., 1981).

1.5.3. Molecular characteristics of human IFN-γ

In humans, the gene for IFN-γ is located on chromosome 12 and contains 3 introns and 4 exons (Farrar and Schreiber, 1993). An enhancer is found in a 220 bp sequence within the first intron and cis-acting promoter elements are located 700 bp 5' to the transcription start site. The gene codes for a single polypeptide made up of a signal peptide of 23 amino acids and a mature protein of 143 amino acids .

In the 3' UTR of IFN-γ mRNA, there are 5 copies of a ARE and mRNA stability is regulated by post transcriptional events which are sensitive, in naturally-producing cells, to external conditions (Kaldy and Schmitt-Verhulst, 1995). When transcription of the IFN-γ gene is induced by the ionophore ionomycin in the presence of agents which can increase cyclic AMP (cAMP), eg. prostaglandin E_2, IFN-γ mRNA has a half-life of more than 12 hr. However, inducing IFN-γ transcription with ionomycin in the presence of cyclosporine (an inhibitor of protein phosphatase 2B), the mRNA is rapidly degraded with half-life of 30 min (Kaldy and Schmitt-Verhulst, 1995). The half-life of IFN-γ mRNA can be increased in the presence of

the protein translation inhibitor, cycloheximide (Chan et al.,1992).

Two polypeptides of human IFN-γ (huIFN-γ) self-associate to form a homodimer with an apparent molecular weight of 34kDa (Farrar and Schreiber, 1993). Only in the dimerised form can IFN-γ display its biological activity and it has been shown that IFN-γ is secreted as a dimer (Ealick et al., 1991). IFN-γ has two potential N-linked glycosylation sites at Asn-25 and Asn-97 both of which are on the surface of the dimer in the natural conformation (Sareneva et al., 1995). The extent of glycosylation at these sites is influenced by cell culture conditions and cell type (James et al., 1995).

1.6 Aims of this project

Abundance of mRNA has been proposed to be the key point for determination of the extent of recombinant protein production (Atwater et al., 1990), the more mRNA then the greater the extent of protein produced. In most cases, this may be true. However, in some cases the quality of the mRNA may decide the level of protein production and the quality of the protein (Quinet et al., 1993). Although the design of recombinant vectors has been examined in depth and recombinant cells are placed in highly selective pressure in order to achieve the maximal protein production, the sequences of the mRNA may affect the gene regulation and result in alteration to protein production.

To understand the influences of abundance and quality (which may be affected by sequences in the 3' UTR) of

recombinant mRNA towards recombinant protein production, two recombinant CHO cells, were transfected with huIFN-γ expressing vectors with different 3' UTR. Several steps involved in the control of gene expression were examined and these included: (A) abundance of IFN-γ and DHFR mRNA, (B) IFN-γ and DHFR pre-mRNA processing, (C) IFN-γ mRNA sequences, (D) IFN-γ translation, (E) IFN-γ protein glycosylation, (F) IFN-γ protein secretion, (G) IFN-γ protein retention and (H) the quality of recombinant IFN-γ mRNA (secondary structure) and recombinant IFN-γ (secondary structure, surface probility and antigenic index) were also examined by computer programs.

CHAPTER TWO : MATERIALS AND METHODS

All concentrations given in this chapter as % are w/v unless stated otherwise.

2.1 Materials

Sources of materials used are given in Appendix 1. Further details about plasmids, oligonucleotides, antibodies and cells are given in Sections 2.1.1 to 2.1.2, respectively.

2.1.1 Plasmids, oligonucleotides and antibodies

2.1.1.1 Plasmids

Plasmid p1042

This plasmid was used to generate CHO 42 cells (Section 2.1.2.2), as a template for PCR (Section 2.6.3.1) and was used to probe AU-rich sequenceS in CHO 42 cell transcripts (Section 2.6.2.3). The restriction map of plasmid p1042 is given in Fig 2.1 and the size of IFN-γ transcript is given in Fig 2.2. The nucleotide sequence of p1042 is given in Appendix II.

Plasmid p1043

This plasmid was used to generate CHO 43 cells (Section 2.1.2.2) and was used as a PCR template (Section 2.6.3.1). Plasmid p1043 is identical to plasmid p1042 (Fig. 2.1) except for the lack of the AU-rich instability sequence in the IFN-γ cDNA insert. The sizes of IFN-γ transcripts is given in Fig 2.2.

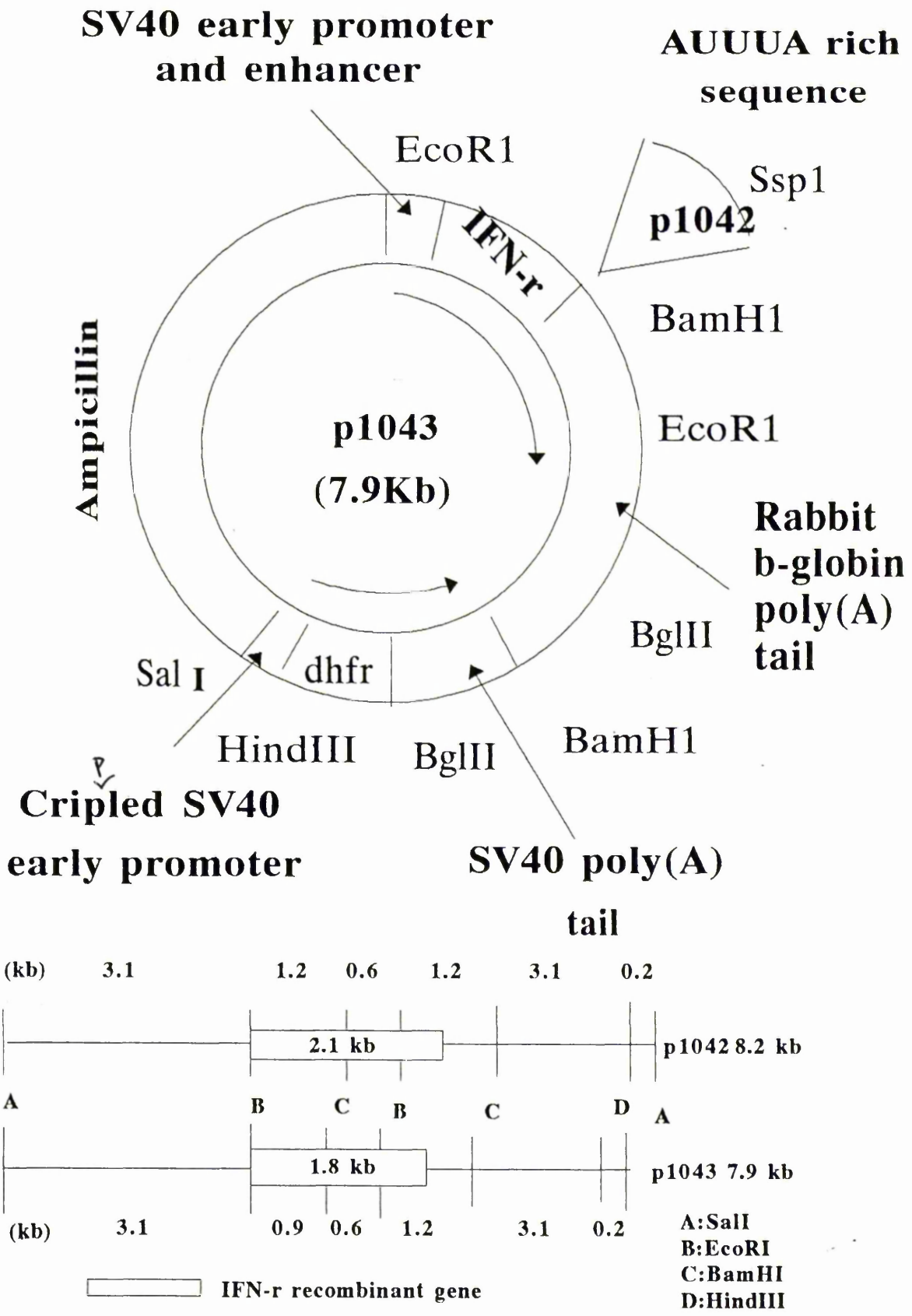


Fig 2.1 Plasmid p1042 and p1043

Plasmid pHIFN-γ

Plasmid pHIFN-γ was constructed by cloning the human IFN-γ cDNA into the plasmid pBR322. The map of this plasmid is given in Fig 2.3.

Plasmid pSV2-dhfr

The plasmid carries the dihydrofolate reductase gene and the map of this plasmid is given in Fig 2.4.

Plasmid p100-D9

This plasmid is derived from pBR322 and carries 200 base pairs of 18S ribosomal RNA cDNA (Fig 2.5).

Plasmid pGem-T

The plasmid pGem-T was used for subcloning PCR products (Section 2.6.3.3.2). It also contains a multiple cloning site (MCS) and SP6 and T7 RNA polymerase promoters (Fig 2.6).

Plasmid pBluescript-IISK

This plasmid was derived from pUC19 and contains a multiple cloning site and SK primer and KS primer site (Fig 2.7).

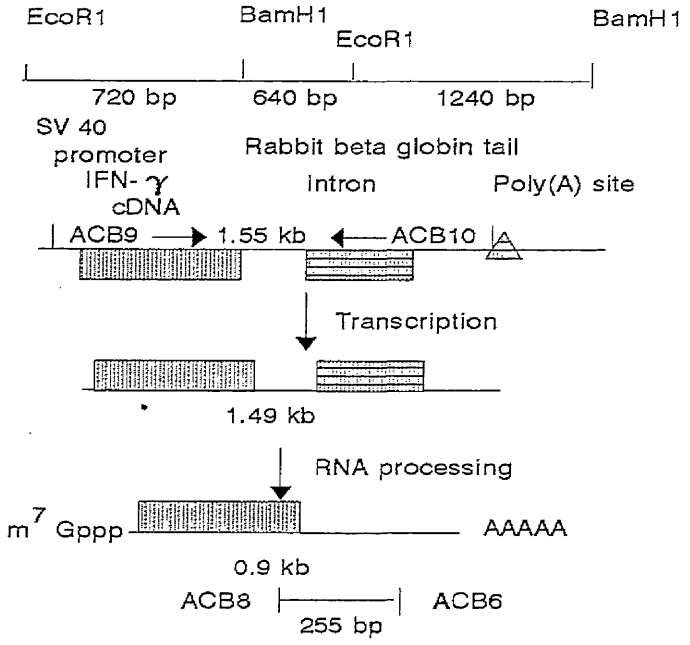
2.1.1.2 Oligonucleotides

The nucleotide sequence of the oligomers used is given below. Where applicable, the location of the primer in the relevant plasmid is also quoted.

ACB1

5'-GAA GCT TGA CTC GAG TCG ACA TCG ATT TTT TTT TTT TTT TTT-3'

This 24 mer contains 17 Ts and was used as the primer for first strand synthesis of cDNA (Section 2.6.3.2.1).

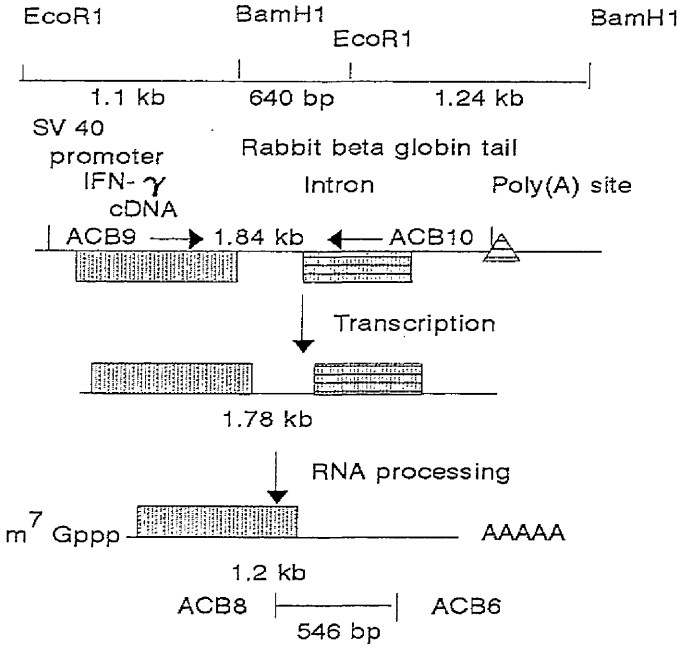


IFN-γ cDNA

Rabbit beta globin intron

ploy(A) tail

Fig 2.2A IFN-γ transcript expected from plasmid p1043

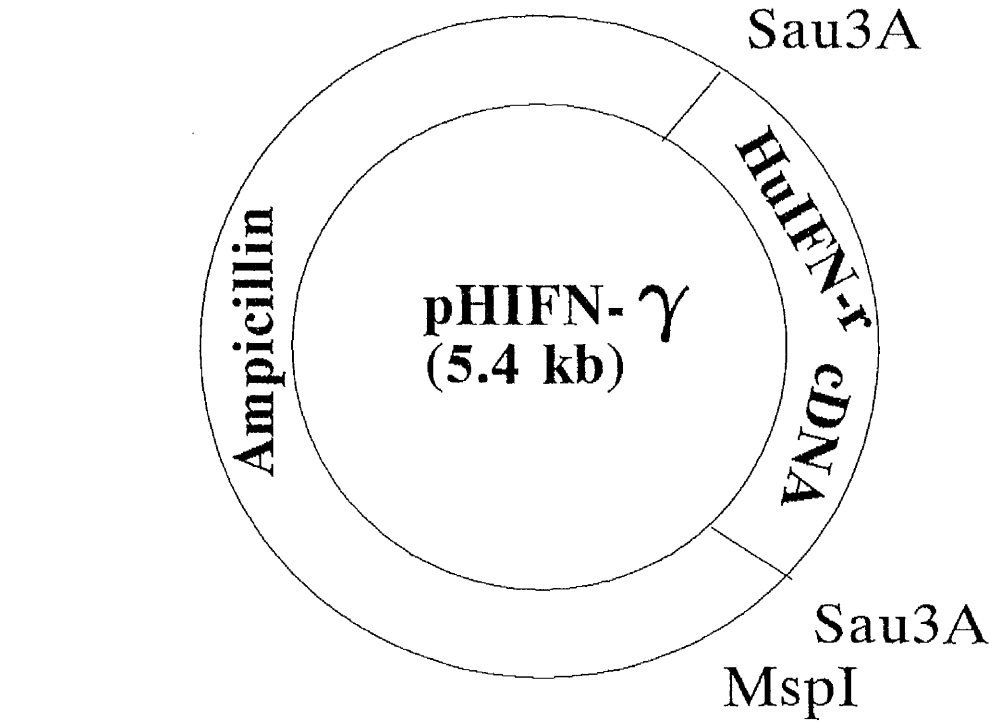


IFN-γ cDNA

Rabbit beta globin intron

poly(A) site

Fig 2.2B IFN-γ transcript expected from plasmid p1042



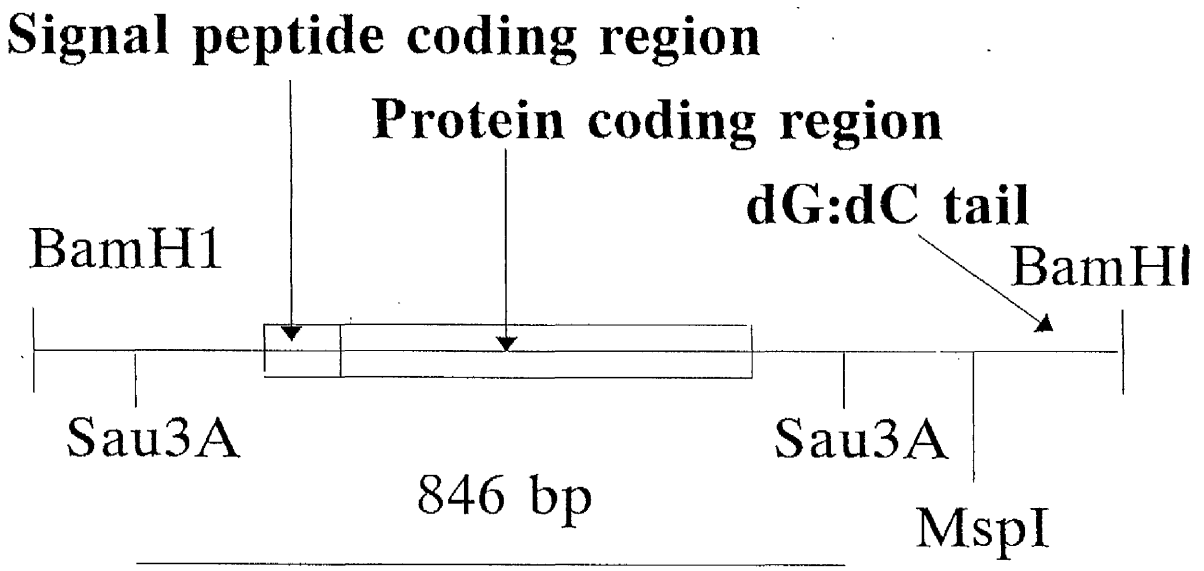
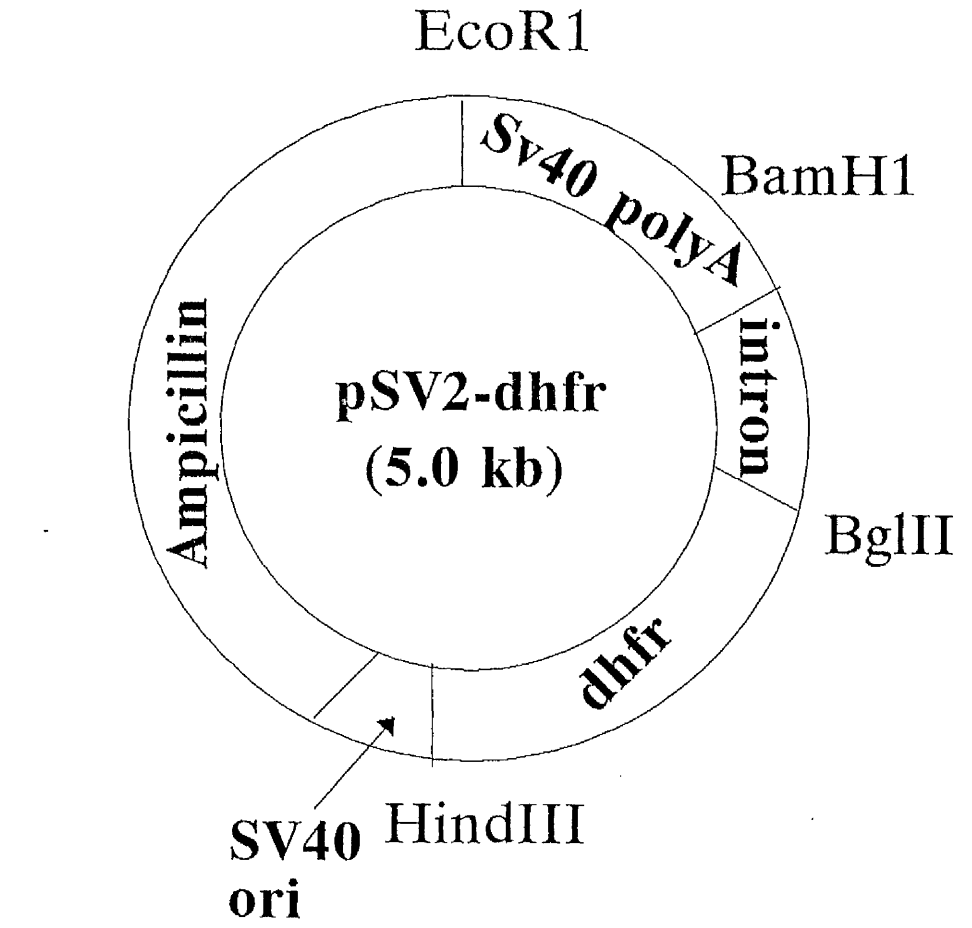


Fig 2.3 Plasmid pHIFN-γ



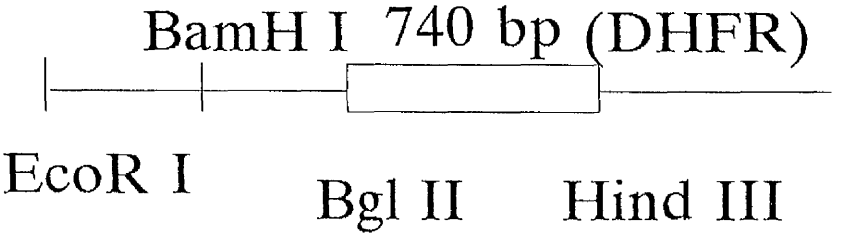
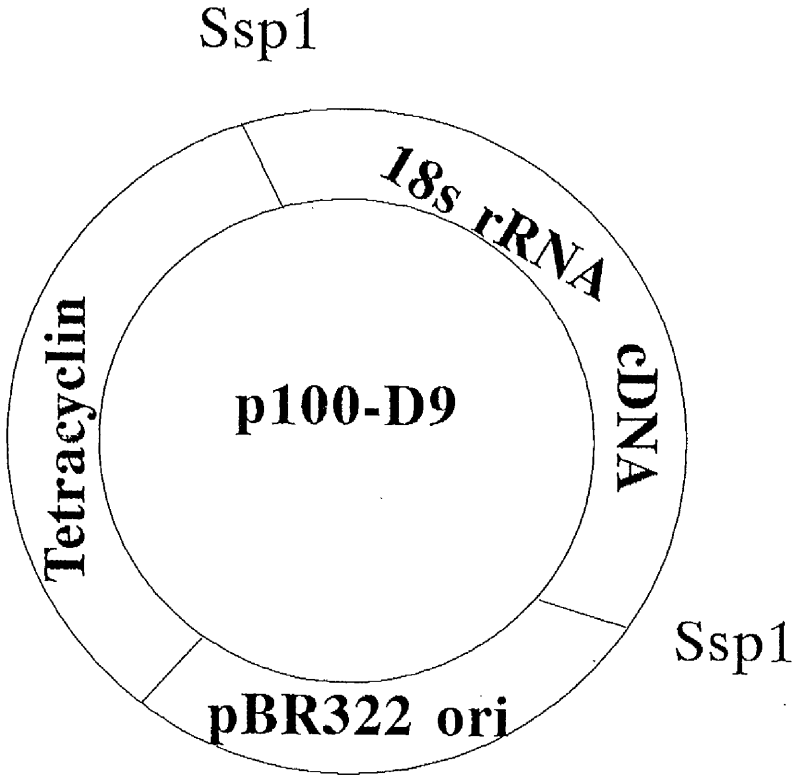


Fig 2.4 Plasmid pSV-dhfr



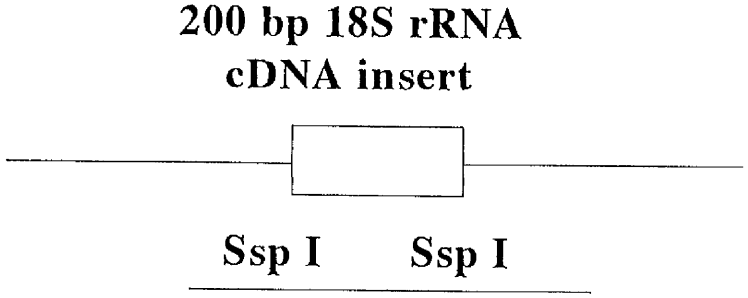
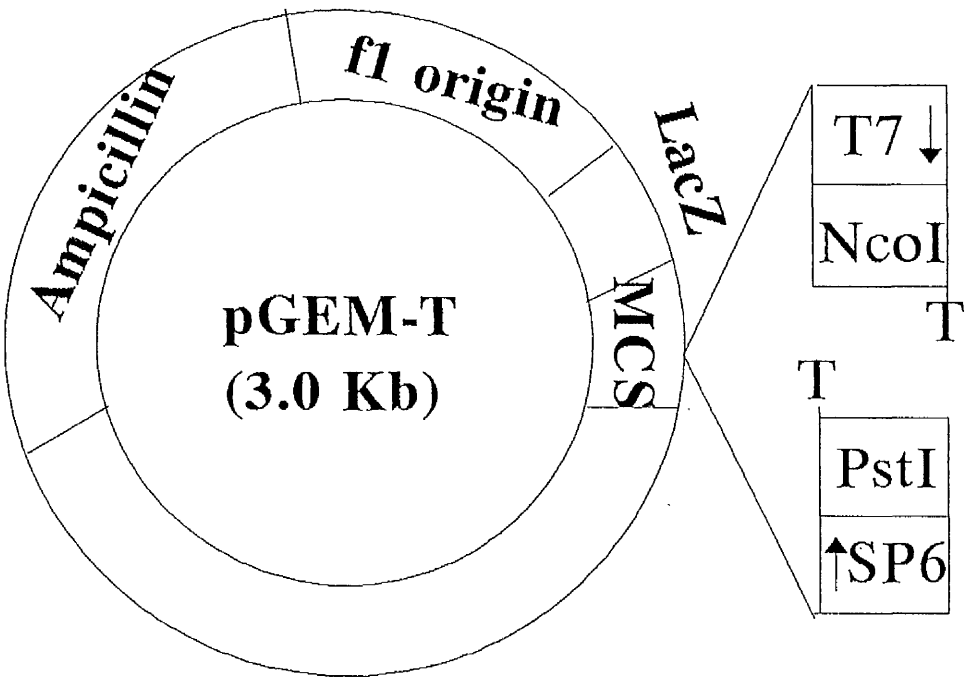
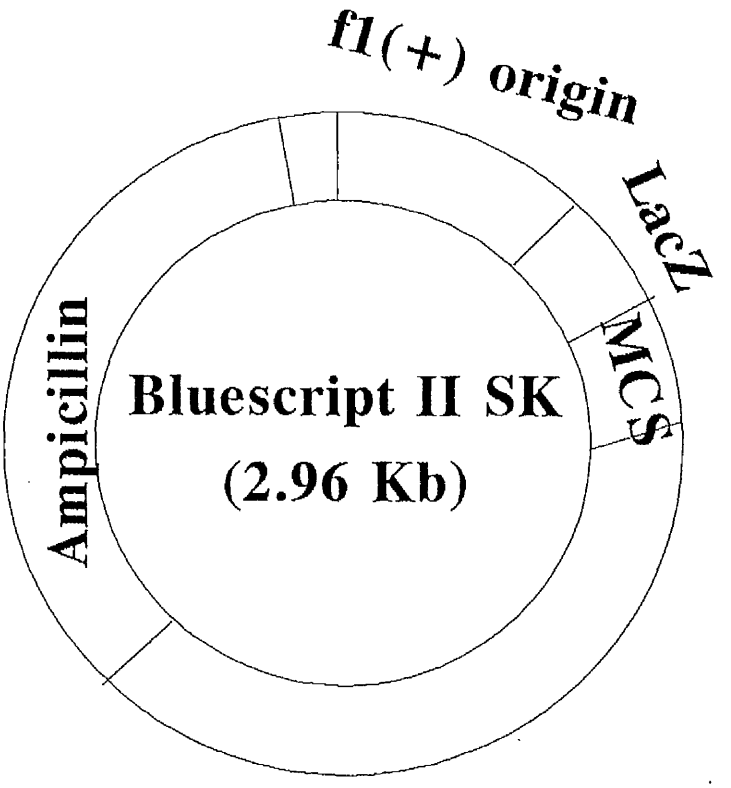


Fig 2.5 Plasmid p100-D9



MCS: multiple cloning site

Fig 2.6 Plasmid pGEM-T



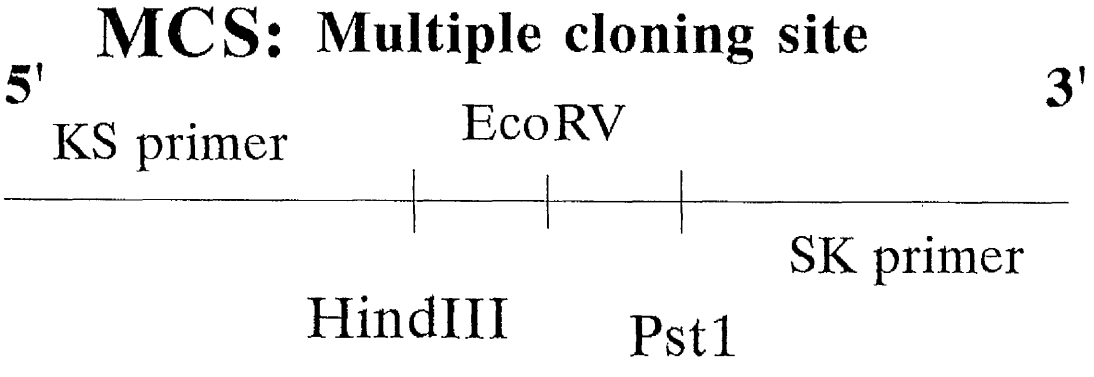


Fig 2.7 Plasmid pBluescript IISK

ACB6

5'-ATT TGT GAG CCA GGG CAT TG-3'

This sequence is complementary to bases 4980 to 4999 of plasmid p1042 (Appendix II) and to bases 1634 to 1653 of plasmid p1043 (Appendix III).

ACB8

5'-CAT CCA AGT GAT GGC TGA AC-3'

This sequence is complementary to bases 3896 to 3915 of plasmids p1042 (Appendix II) and to bases 825 to 844 of plasmid p1043 (Appendix III).

ACB9

5'-AGT AGG ATC CCC ATG GCT GAC TAA T-3'

The last 15 bases at 3' end of this 25 oligomer are complementary to bases 3307 to 3321 of plasmids p1042 (Appendix II) and to bases 236 to 250 of plasmid p1043 (Appendix III). The residual bases generate an EcoR I restriction site.

ACB10

5'-CCT CGA ATT CGA GTG AGA GAC ACA A-3'

The first 15 bases at the 5' end of this 25 oligomer are complementary to bases 5119 to 5133 of plasmid p1042 (Appendix II) and bases 1773 to 1787 of plasmid p1043 (Appendix III). The residual bases generate a BamH I restriction site.

KS primer

5'-TCG AGG TCG ACG GTA TC-3'

This sequence is located in the Multiple Cloning site (MCS) base 669 to 686 of pBluescript II SK (Fig 2.7).

SK primer

5'-CGC TCA AGA ACT AGT GGA TC-3'

This sequence is complementary to bases 772 to 791 located in the MCS of pBluescript II SK (Fig 2.7).

SP6 primer

5'-TTT AGG TGA CAC TAT AGA ATA CT-3'

This sequence is located in the MCS of the SP6 RNA polymerase promoter of pGem-T (Fig 2.6).

T7 primer

5'-TAA TAC GAC TCA CTA TAG GGC GA -3'

This sequence is located in the MCS of the T7 RNA polymerase promoter of pGem-T (Fig 2.6).

2.1.1.3 Antibodies

2.1.1.3.1 20B8, 20D7, R1PA and S1DM anti-huIFN-γ antibodies

The monoclonal antibodies, 20B8, 20D7 and 20G7 were raised in mouse from recombinant huIFN-γ, derived from recombinant *E. coli*, and they bind all forms (including proteolytically cleaved forms of natural, CHO- and *E. coli*-derived human IFN-γ. The polyclonal antibody R1PA was raised in rabbit and S1DM was raised in sheep.

2.1.1.3.2 Antimouse immunoglobulin polyclonal antibody

This antibody was directed against mouse immunoglobulins and was developed in goat. This antibody is conjugated with horseradish peroxidase.

2.1.2 Bacteria and CHO cells

2.1.2.1 Bacteria strains

Details of all characteristics of bacteria used are listed in Table 2.1. Both bacterial strains used are ampicillin- and tetracycline-sensitive strains and β-galactosidase-negative mutants.

Table 2.1 Characteristics of Bacteria used

Bacteria	Genotype
E. coli DH5α	F^-, ϕ80d*lacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17,* (r_K^-, m_K^+), *supE44, relA1, deoR,Δ(lacZYA-argF)U169*
E. coli XL-1 BlueMRF'	*Δ(mcrA)183,Δ(mcrCB-hsdSMR-mrr)173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac.*

2.1.2.2 CHO cell lines

The properties of all cell lines used are summarised in Table 2.2. All cell lines are adherent cell lines. The CHO Duk X cell line was derived from CHO K1 cells as mutants lacking DHFR activity (Urlaub and Chasin, 1980) and CHO 42 and CHO 43 cell lines were derived by transfection of DUK X cells.

Table 2.2 Sources of CHO cell lines and growth media used

Cell line	Vector	Amplification MTX level(μM)	Growth medium	Parent cell line
CHO 42	p1042	1.0	DMEM+FCS	CHO Duk
CHO 43	p1043	1.0	DMEM+FCS	CHO Duk

Abbreviation: DMEM, Dulbecoo Modified Eagle Medium; FCS, Foetal calf serum; MTX, Methotrexate

2.2 Bacterial cell culture

2.2.1 Growth media

The medium used for bacterial cell culture was Luria-Bertani medium (LB medium). The contents of media used are shown in Table 2.3.

Table 2.3 Growth medium for bacteria *E. coli*

Medium	Bacto-tryptone (%)	bacto-yeast (%)	sodium chloride (%)	bacto-agar (%)
LB	1	1	0.5	0
LBA	1	1	0.5	1.5

LBA: LB plus 1.5% agar medium

All media was autoclaved at 15Ib/in^2 on liquid cycle for 20 min and antibiotics were added, if appropriate, after media was cooled to 45°C. Antibiotics were used a final concentrations of, ampicillin, 100 µg/ml (from 50 mg/ml stocks) and, tetracycline, 40 µg/ml (from 20 mg/ml stocks). sterilised by filtration through 0.2 µm filters. For LBA plates, the media was allowed to cool to around 45°C then 20 ml was poured into each plate. After the media solidified, the surface of LBA plates was dried by placing in a 37°C oven for 30 min.

2.2.2 Cell culture

E. coli was streaked directly from a frozen stock onto the surface of LBA plates. Plates were incubated overnight at 37°C. Then, a single colony was transferred into 200 ml LB in a one litre flask. After incubation overnight at 37°C, cells were harvested.

2.2.3 Cryo-preservation cell stocks

A single colony was transferred into a culture flask containing 5 - 10 ml of LB media and this was incubated at 37°C overnight. The culture was transferred into sterile vials, 0.85 ml per vial, each containing 0.15 ml sterile glycerol. The contents were mixed thoroughly by vortexing and the cultures were stored at -70°C.

2.3 CHO cell culture

2.3.1 Growth media

All growth medium for mammalian cell culture was sterilised by filtration through 0.2 µm filters. The media prepared below is based on final volume of 500 ml and pH value was adjust to pH 7.4 with 1 M sodium hydroxide before it was filtered.

Table 2.4 Composition of CHO cell lines growth medium

Cell line	DMEM	FCS		Glutamine	MTX	SB	SP	Water	
	10X	SCM	SFM	(ml)				SCM	SFM
CHO 42	50	50	0	10	0.5	24.5	5	360	410
CHO 43	50	50	0	10	0.5	24.5	5	360	410

Abbreviations: DMEM 10X, ten times concentrated Dulbecco's modification of Eagles's medium; FCS, foetal calf serum (10% final); SCM, serum-containing medium; SFM, serum-free medium; Glutamine (4 mM final); MTX, methotrexate (1 µM final); SB, sodium bicarbonate (43.8 mM final); SP, sodium pyruvate (1 µM final).

2.3.2 Routine cell culture

The anchorage-dependent CHO cells were routinely cultured in plastic flasks at 37°C and with the pH of the medium maintained at pH 7.4 by growth in Heraeus air/CO_2 incubators containing sterile 5% CO_2 in air. CHO cells were maintained by routine subculture. The old medium was removed and the cell sheet was washed twice with PBS. The monolayer of cells was then trypsinized with 1% trypsin containing 2% EDTA for 1 min. Cells detachment was completed with the aid a physical tapping of the flask onto the bench and then trypsin action was inhibited by the addition of 10 ml fresh growth medium. Finally the cells were harvested by centrifugation (130g for 3 min) and the cell pellet was suspended in fresh medium for subsequent inoculation. For CHO 43 cells the final inoculation density was 1 X 10^5 cells/ml and for CHO 42 cells it was 2 X 10^5 cells/ml.

2.3.3 Cell viability determination

The Neuhauer haemocytometer slide was used to determine cell concentrations (Patterson, 1979). Cell viability was determined by adding trypan blue (0.2% in PBS) to cell suspensions. Viable cells, with an intact cell membrane, are able to exclude the dye while non-viable cells stain blue.

2.3.4 Cryo-preservation of cell stocks

Cells were trypsinised as described before (Section 2.3.2). Following harvest of cells, cells were suspended in

FCS (100%) to a density of 10^6 cells/ml for CHO 43 cells and 2 X 10^6 cells/ml for CHO 42 cells. DMSO was added dropwise to cells to give a final concentration of 10% (v/v) while mixing the cell suspension gently. Cells (1 ml aliquots) were immediately dispensed into sterile polypropylene cryotubes which were then placed in a polystyrene box at -80°C to cool slowly overnight. Cells were then transferred for storage in liquid nitrogen. When initiating new cultures from frozen stocks, cell were rapidly thawed at 37°C, added to prewarmed medium and centrifuged (130g for 3 min) to remove the DMSO. Cells were then inoculated into a T_{25} flask and subsequently subcultured as described in Section 2.3.2.

2.4 Bacterial cell transformation and plasmid isolation

2.4.1 Generation of competent bacteria

After overnight growth at 37°C, a colony was picked from LBA plates and transferred into 100 ml LB broth in a 1 litre flask. The culture was incubated for about 3 hours at 37°C with vigorous shaking. Cells were collected into 50 ml polypropylene tubes when the OD_{600} was about 0.3-0.4 and cells were chilled to 0°C by storing the tubes on ice for 10 min. Then, cells were collected by centrifugation at 4°C and resuspended in 10 ml of ice-cold 0.1M calcium chloride. After storing the suspension on ice for 10 min, the cells were collected by centrifugation at 4°C. Cells were resuspended in 2 ml of ice-cold 0.1 M calcium chloride for each 50 ml of original culture, then 200 µl portions of the suspension were transferred into sterile microfuge tubes. The cells were

ready for immediate use or could be stored frozen at -70°C for more than 6 month. The frozen competent cells were thawed just before use in transformations by gentle warming and were used immediately.

2.4.2 Transformation procedures

Fifty ng of plasmid in 10 µl water was used for transformation of each 200 µl aliquot of competent cells. The plasmid was added into a tube containing an aliquot of competent bacterial cells and this was mixed well by gently swirling and the mixture was placed on ice for 30 min. Then, the tube was transferred to a 42°C water bath. The tube was left in the bath for 90 seconds and transferred to an ice bath for 2 min. Then 800 µl of LB was added into each tube and tubes were incubated for 1 hr in a 37°C water bath. Up to 200 µl was then spread on LBA medium plate containing the appropriate antibiotic. Plates were inverted and incubated at 37°C, colonies should appear after overnight incubation.

Table 2.5 Plasmids used in transformation and their selection marker

Plasmid	Competent bacterial cell	Selection marker
p1042	*E. coli* DH5α	Ampicillin
p1043	*E. coli* DH5α	Ampicillin
pHIFN-γ	*E. coli* DH5α	Ampicillin
pSV-dhfr	*E. coli* DH5α	Ampicillin
p100-D9	*E. coli* DH5α	Tetracyclin
pGem-T constructs	*E. coli* XL-1 blue MRF^-	Ampicillin; Blue/white
pBluescript II SK constructs	*E. coli* XL-1 blue MRF^-	Ampicillin; Blue/white

Plasmid construction and blue/white selection were described in Section 2.6.3.3.

2.4.3 Plasmid isolation

Bacteria cells were lysed by an alkali method (Sambrook et al, 1989). The solutions used for lysis of bacteria cells are listed below.

Table 2.6 Composition of plasmid preparation solution

Solution I	Solution II	Solution III
50mM Glucose 25mM Tris-HCl 10mM EDTA	0.2 M NaOH 1% SDS	3M $K(CH_3COO)$ 2M Acetic acid

Solution I was autoclaved and stored at 4°C. The pH value of solution I was adjusted to pH 8.0.
Solution II was freshly prepared before use.
Solution III was autoclaved and stored at 4°C.

2.4.3.1 Mini-prep procedure

A single bacterial colony was transferred into 10 ml LB medium containing the appropriate antibiotic in a loosely sealed 25ml universal tube. The bacterium soups were incubated overnight at 37°C with vigorous shaking. After this incubation, 1.5 ml of the culture was transferred into microfuge tubes which were centrifuged at 15,000g for 1 min at 4°C. The medium was removed and pellets were resuspended in 100 µl of ice-cold solution I. Then 200 µl solution II was added to lyse the bacteria and 150 µl solution III was then added to precipitate protein. Tubes were stored on ice for 5 min and centrifuged at 14,900g for 5 min at 4°C. The supernatants were transferred into fresh tubes and each solution was extracted with an equal volume of phenol : chloroform (Section 2.5.1.1.2). After centrifugation at 14,900g for 2 min at 4°C, supernatants were transferred to fresh tubes and the double-stranded DNA was precipitated by addition of 2 volumes of ethanol.

2.4.3.2 Large-scale procedure

A single colony of bacteria was transferred into 10 ml LB medium (containing the appropriate antibiotic) in a 25 ml universal tube for overnight incubation at 37°C. The culture was then poured into 200 ml LB medium (containing the appropriate antibiotic) in a 1 litre flask and incubation was continued overnight at 37°C. Bacteria were harvested by centrifugation at 2,830g for 15 min at 4°C. After draining the supernatant, cells were resuspended in 20 ml solution I and lysed by addition of 40 ml solution II. Then 30 ml of solution III was added and the mixture was centrifuged for 10 min at 4°C. Next, double-stranded DNA was precipitated by addition of an equal volume of isopropanol. Then the precipitate was dissolved in 4.3 ml TE buffer and 4.8 g of caesium chloride and 0.1 ml of ethidium bromide (10 mg/ml) were added. The mixture was then transferred into a 6 ml Quick-seal tube (total weight should be 9.6 g and final density was 1.55 g/ml) and this was centrifuged at 290,000g for 16 hr in VTi65 rotor. Two bands were found in the preparation. The lower (major) band was collected by puncturing the tube using a 1 ml syringe with a 21-gauge hypodermic needle in as low a volume as possible. The ethidium bromide was removed by butanol extraction (repeated four times) and the caesium chloride was removed by the addition of 3 volumes of water and the plasmid DNA was precipitated with the addition of 2 volumes of ethanol. This was left for 15 min at 4°C and then centrifuged at 12,000g for 15 min at 4°C to harvest DNA.

2.5 Isolation of CHO cell DNA and RNA

2.5.1 General method for DNA and RNA preparation

2.5.1.1 Phenol extraction of nucleic acids

2.5.1.1.1 Phenol preparation

Phenol was melted at 65°C and was extracted with an equal volume of 1 M Tris-HCl, pH 8.0, followed by two further extractions with 0.1 M Tris-HCl, pH 8.0. The phenol solution was then equilibrated with 0.1 M Tris-HCl and 0.1 % of 8-hydroxquinoline was added. The phenol solution was put into a dark container and stored at 4°C.

2.5.1.1.2 Phenol:chloroform extraction

For nucleic acid samples, equal volumes of phenol-chloroform solution (phenol:chloroform:isoamyl alcohol, 25:24:1) was added and this was vortexed for 1 min. The tube was centrifuged at 2,830g and the aqueous (upper) layer was transferred to new microfuge tube. This aqueous layer was then extracted with phenol-chloroform and centrifuged again. The nucleic acid in the aqueous layer was ethanol precipitated.

2.5.1.2 Ethanol precipitation

To the aqueous phase obtained from nucleic acid samples (Section 2.5.1.1), 0.1 volume 3 M sodium acetate, pH 5.8, and two volumes of ethanol were added and this mixture was mixed well by inverting the tube a few times. After mixing well, 2 volume of ethanol was added and the precipitated mixtures were stored in either -20°C for 2 hr or

-70°C for 30 min. The nucleic acid samples were precipitated by centrifugation at 14,900g at 4°C for 10 min. The nucleic acid was washed with ice-cold 70% ethanol in water and centrifuged each time as before to collect nucleic acids. For DNA samples, the precipitate was dissolved in TE buffer (Section 2.4.3) and RNA samples were dissolved in DEPC-treated water (0.1% diethyl pyrocarbonate in distilled water overnight and autoclaved before use).

2.5.1.3 UV spectrophotometric determination of the amount of DNA and RNA

For nucleic acid samples, absorption values at 260nm and 280nm were measured. The readings (after subtraction of solvent blanks) at 260nm were used to calculate the concentration of nucleic acid in each sample. An OD of 1 corresponds to approximately 50 µg/ml for DNA and 40 µg/ml RNA (Sambrook et al., 1989). The ratio between the reading at 260 and 280 nm was used to estimate the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively (Sambrook et al., 1989).

2.5.2 DNA extraction

The media of CHO cells was removed and cell monolayers were washed twice with ice-cold PBS. The CHO cells were scraped, using a rubber policeman, from culture flasks. Cells were collected by centrifugation and suspended in TE buffer (containing 10 mM Tris-HCl and 0.1 mM EDTA, pH 8.0) at a concentration of 5×10^7 cells/ml in 50 ml centrifuge tube. Then 10 ml of extraction buffer (containing 10 mM Tris-HCl,

0.1 mM EDTA, 0.5% SDS, pH 8.0 and 20 µg/ml RNAse) was added into the cell suspension. Samples were incubated at 37°C for 1 hr and then proteinase K was added into each tube (mixed well using a glass rod) to a final concentration of 100 µg/ml. The tubes were transferred to a 50°C water bath for 3 hr and they were swirled periodically. After incubation, the tubes were cooled to room temperature and the mixtures were extracted three times with phenol-chloroform (Section 2.5.1.1). During extraction mixtures were extracted by rotary mixing and not by vortexing. The viscous aqueous phase was transferred to a clean centrifuge tube with a wide-bore pipette and DNA was precipitated by ethanol (Section 2.5.1.2). The DNA pellets were dissolved in TE buffer and DNA concentration was estimated using UV spectrophotometer (Section 2.5.1.3).

2.5.3 RNA extraction

All solutions used were prepared using DEPC-treated water. DEPC (1.8 ml) was added to Duran bottles containing 1.8 litre Milli-Q water and the solution was shaken vigorously . After shaking, the solution was stored (leaving the cap loose) at room temperature overnight and this solution was then autoclaved.

2.5.3.1 Total RNA extraction

The single step RNA isolation method of Chomczynski and Sacchi (1987) was used with some modifications. Cells were lysed in RNA extraction solution (25 mM Na-citrate, pH 7.0,

containing 4 M guanidinium thiocyanate, 0.5% sarkosyl and 0.1 M 2-mercaptoethanol). Sequentially, 0.1 volumes of 2 M sodium acetate, pH 4.0, an equal volume of phenol (Section 2.5.1.1.1) and 0.2 volume of chloroform : isoamylalcohol (24:1) were added to the lysate and the suspension was shaken vigorously. Samples were cooled on ice for 15 min, centrifuged at 2,830g for 15 min and the aqueous phase was removed and re-extracted with phenol:chloroform (Section 2.5.1.1.2). RNA in the extracted aqueous phase was precipitated by addition of an equal volume of isopropanol. Following sedimentation (-20°C for 2 hr) the mixture was centrifuged at 2,830g for 10 min. The resulting RNA pellet was dissolved in 0.4 ml RNA extraction solution and this was ethanol precipitated (Section 2.5.1.2). The final RNA pellet was resuspended in DEPC- treated water and the RNA was quantified by spectrophotometry (Section 2.5.1.3).

2.5.3.2 Isolation of nuclear and cytoplasm RNA

For cells growing in T_{75} culture flasks, the adherent cell sheets were washed twice with 5 ml of ice-cold PBS and cells were scraped into 0.5 ml of ice-cold PBS using a rubber policeman. Cells were collected by centrifugation at 2500g for 5 min at 4°C and then they were further washed with ice-cold PBS. The cell pellet was loosened by gentle agitation and 5 ml NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, containing 100 mM sodium chloride, 5 mM magnesium chloride, 0.5% nonidet P-40, 20 mM vanadyl ribonucleoside complex) was

added to cells which were then vortexed briefly. The samples were left on ice for 5 min and then centrifuged at 400g for 5 min at 4°C. The supernatant was saved for preparation of cytoplasmic RNA and the pellet (containing nuclei) was resuspended in 3 ml NP-40 lysis buffer, vortexed briefly and harvested as before. The residual pellet was resuspended in 1 ml ice-cold NP-40 lysis buffer and 5 ml of RNA extraction solution was then added to the tube. As a result of nuclear lysis, nuclear RNA could then be recovered and purified with phenol:chloroform as before (Section 2.5.1.1).

The supernatant from nuclear RNA extraction (above) was added to a 5 ml of RNA extraction solution and cytoplasmic RNA was recovered following phenol:chloroform extraction (Section 2.5.1.1.2) as described before.

2.5.4 Isolation of poly(A)$^+$ RNA

The method described in Ausubel et al (1991) was used to isolate poly (A)$^+$ RNA. Oligo(dT)-cellulose was suspended in 0.1 M sodium hydroxide, and 0.5 ml (packed volume, sufficient for use with 2-5 mg of RNA) columns were prepared (in 1ml syringes, syringes prewashed with 10 ml of 5M sodium hydroxide and rinsed with DEPC-treated water). The oligo(dT)-cellulose was then washed with poly(A) loading buffer (10 mM Tris-HCl buffer, pH 7.5, containing 0.5 M lithium chloride, 1 mM EDTA and 0.1% SDS) until the pH of the eluant was pH 7.5. The RNA, for purification, was heated at 70°C for 10 min

and lithium chloride was added (from 10 M lithium chloride stock solution) to produce a final concentration of 0.5 M. The RNA solution was passed through the oligo(dT) column and the column was washed with 1 ml poly(A) loading buffer. The eluant was collected and passed through the column twice more. Then, the column was rinsed with 2 ml of middle wash buffer (same as poly(A) loading buffer except that the lithium chloride concentration was 0.15 M). Then, the poly(A)$^+$-containing RNA was eluted from the column into a fresh tube by passage of 2 ml of 2 mM EDTA/0.1% SDS solution through the resin. The resultant RNA was precipitated from this solution by ethanol precipitation (Section 2.5.1.2).

2.6 Characterization of DNA and RNA

2.6.1 Agarose gel analysis

2.6.1.1 DNA gels and electrophoresis

One gram of agarose powder was transferred into a 500 ml flask, then 10 ml of 10X TBE buffer (containing 0.89 M Tris-borate and 25 mM EDTA, pH 8.3) and 90 ml of distilled water were added. The flask was heated by microwave to melt the agarose and the flask was left at room temperature until the agarose solution had cooled to around 60°C. Then 5 µl of ethidium bromide (10 mg/ml water) was added. The appropriate amount of agarose solution was poured into a horizontal electrophoresis gel tank and left for 30 min to allow the agarose to set completely. The gel tank was filled with 1X

TBE buffer sufficient to cover the gel to a depth of about 1 mm.

DNA samples were mixed with DNA gel loading buffer (1 mM EDTA containing 20% Ficoll 400, 50% glycerol, 0.05% bromophenol blue and 0.05% xylene cyanol) in a ratio of 10:1. The mixture was loaded into sample wells and a voltage of 5 V/cm was applied to separate the DNA species. The electric current was turned off when the front of dye reached the end of the gel. The DNA species were visualized and photographs were taken if necessary, by examination of agarose gel on a UV (302 nm) transilluminator. The molecular sizes of DNA species were calculated by reference to a 1 kb DNA Ladder (containing 1 to 12 repeats of 1018 bp DNA fragment and 12 bands of vector DNA; Hartley and Donelson, 1980).

2.6.1.2 RNA gels and electrophoresis

RNA (15 µg/10µl/lane of total, nuclear and cytoplasmic RNA; 0.2 µg poly $(A)^+$ RNA) was resolved in 1.5% agarose gels containing 6.7% formaldehyde. For 100 ml of gel mixture, 1.5 g of agarose was dissolved in 75 ml DEPC-heated water by microwave. This was cooled to 55°C and 10 ml of 10X MOPS buffer (0.2 M MOPS containing 100 µM sodium acetate and 10 mM EDTA, pH 7.0 with acetic acid. The buffer was prepared by using DEPC-treated water and by autoclaving) and 18 ml of 37% formaldehyde was added and the gel was poured immediately into a horizontal electrophoresis tank.

Denaturing buffer (10 µl; 52.4% formamide, 10.6%

formaldehyde and 1.9X MOPS buffer, made freshly before use) was added to RNA samples 10 µl and samples were incubated at 65°C for 15 min. Samples were cooled on ice, and 2 µl of agarose gel loading buffer was added (1 µm EDTA containing 20% Ficoll, 50% glycerol, and 0.04% bromophenol blue) and the samples were applied to the gel.

Electrophoresis was conducted in 1X MOPS buffer. The electric current was turned off when the dye front reached the end of the gel. Then gels were stained for 30 min in water containing ethidium bromide (2 µg/ml) and destained for at least 45 min in 500 ml water. The integrity of the resolved RNA was assessed by visualising the ribosomal RNA bands with a UV transilluminator. The molecular sizes of RNA transcripts were calculated with 18S and 28S rRNA (1.87 and 4.7 kb, respectively).

2.6.2 DNA restriction enzyme digestion

2.6.2.1 Digest procedures

DNA samples (2.5 µg plasmid DNA or 10 µg genomic DNA, gDNA) were transferred to microfuge tubes containing 2.5 µl of the restriction buffer appropriate to the enzyme under study. The volume was made up to 24 µl with water and 5 units of restriction enzyme was added. The tube was incubated at 37°C water bath for one hour. After incubation, samples were ready to be resolved in DNA separating gels (Section 2.6.1.1). DNA samples and restriction enzymes used are listed below.

Table 2.7 DNA restriction enzyme digestion

DNA	Restriction enzyme	Buffer	Fragment (Kb)	Purpose
CHO 42 gDNA	Hind III	B	8.2	PCR ampl.
CHO 43 gDNA	Hind III	B	7.9	PCR ampl.
p1042	Hind III	B	8.2	PCR ampl.
p1042	BamH I, Ssp I	KGB	0.225	Probe
p1043	Hind III	B	7.9	PCR ampl.
pHIFN-γ	Msp I, Sau 3A	B	0.85	Probe
pSV-dhfr	Bgl II, Hind III	C	0.75	Probe
pGem-Tcon	Nco I, Pst I	KGB	3.0	Size
pBluescript SK II	EcoR V	D	2.9	T vector
pBluescript SK II-Tcon	Hind III, Pst I	B	2.9	Size

Note: Buffer used was supplied by the restriction enzyme supplier. KGB containing 100 mM potassium glutamate, 25 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate, 50 μg/ml BSA and 0.5 mM β-mercaptoethanol.
Fragments listed here refer to the fragment isolated for the purpose specified.
gDNA refers to genomic DNA and this was extracted and purified as described in Section 2.5.2.
PCR ampl: Used as PCR amplification template.
pGem-Tcon and pBluescript IISK-Tcon are constructs listed in Section 2.6.3.3.2.

2.6.2.2 Harvest of DNA restriction fragments

DNA species were separated by electrophoresis through a 1% agarose gel (Section 2.6.1.1). A slit was then cut in the gel immediately ahead of the DNA fragments of interest and a sliver of DEAE-cellulose membrane (2 mm larger than the width of well, prewashed with a solution containing 10 mM EDTA and 0.5 M sodium hydroxide solution, then rinsed six times with sterile water prior to use) was inserted into the slit. Electrophoresis was continued until all of the DNA in the band of interest had been collected onto the membrane (visualised with a hand-held UV lamp). The membrane was removed from the slit and washed free of contaminants in a buffer of low ionic strength (50 mM Tris-HCl and 10mM EDTA,

pH 8.0 containing 0.15 M sodium chloride). The DNA was eluted from the membrane in a buffer of high ionic strength (50 mM Tris-HCl and 10 mM EDTA, pH 8.0 containing 1 M sodium chloride) at 65°C for 30 min. The DNA elute was then extracted with phenol-chloroform (Section 2.5.1.1.2) and DNA was precipitated by ethanol (Section 2.5.1.2).

2.6.2.3 AU-rich sequence preparation

Plasmid p1042 was digested with restriction enzymes and the fragment corresponding to the AU-rich sequence was collected. Plasmid p1042 was, first, digested with EcoR I and BamH I (Fig 2.8A), then the 1.2 kb fragment (Section 2.1.1.1.1), corresponding to IFN-γ cDNA and the SV40 promoter (shown in Fig 2.1), were collected as described in Section 2.6.2.2. This 1.2 kb fragment was digested, again, with Ssp 1 and a 232 bp product corresponding to the AU-rich sequence (Fig 2.8B) was harvested. This 232 bp fragment was used to prepare AU-rich hybridisation probe.

2.6.3 PCR amplification procedures

2.6.3.1 Plasmid and genomic DNA amplification

One µg of genomic DNA (digested with HindIII and purified with three phenol extractions and one chloroform extraction; Section 2.5.1.1.1) was added to 50 pmole of each of the appropriate oligonucleotides (Section 2.1.1.2), 10 µl of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, containing 15 mM magnesium chloride, 500 mM potassium chloride and 0.1% gelatin) and 20 pmole dNTPs (dATP, dCTP, dGTP and dTTP). The

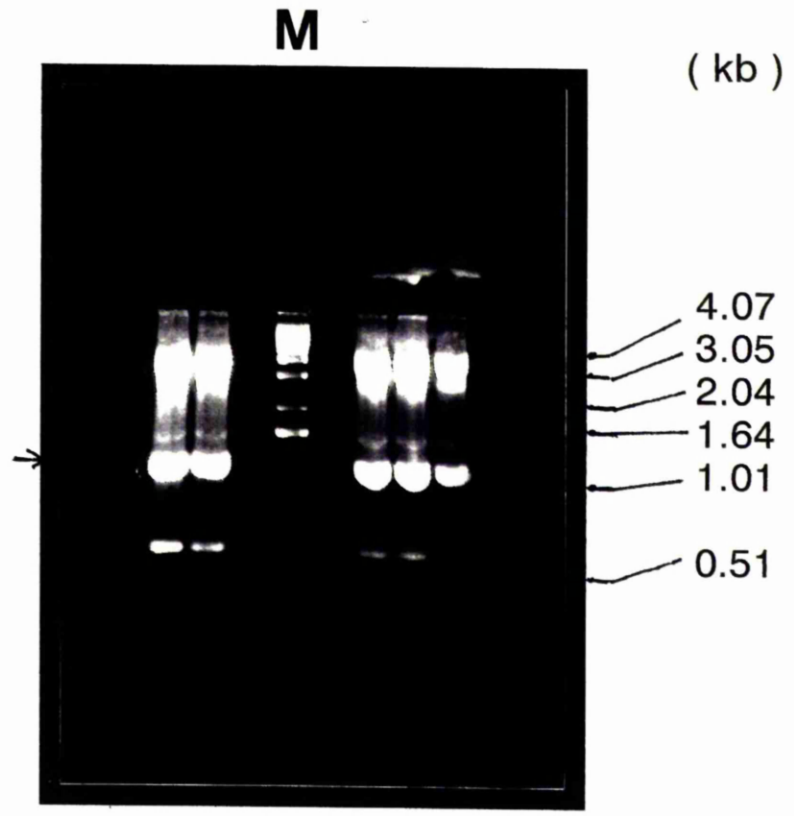
Figure 2.8 Source of AU-rich sequence: two stage digestion of plasmid p1042

(A) Plasmid p1042 was digested with restriction enzymes, BamH I and EcoR I as described in Section 2.6.2.1. The 1.18 kb IFN-γ cDNA product was collected, purified (Section 2.6.2.2) for a further digestion. Lane M is 1 kb ladder marker and other five lanes are from plasmid p1042.

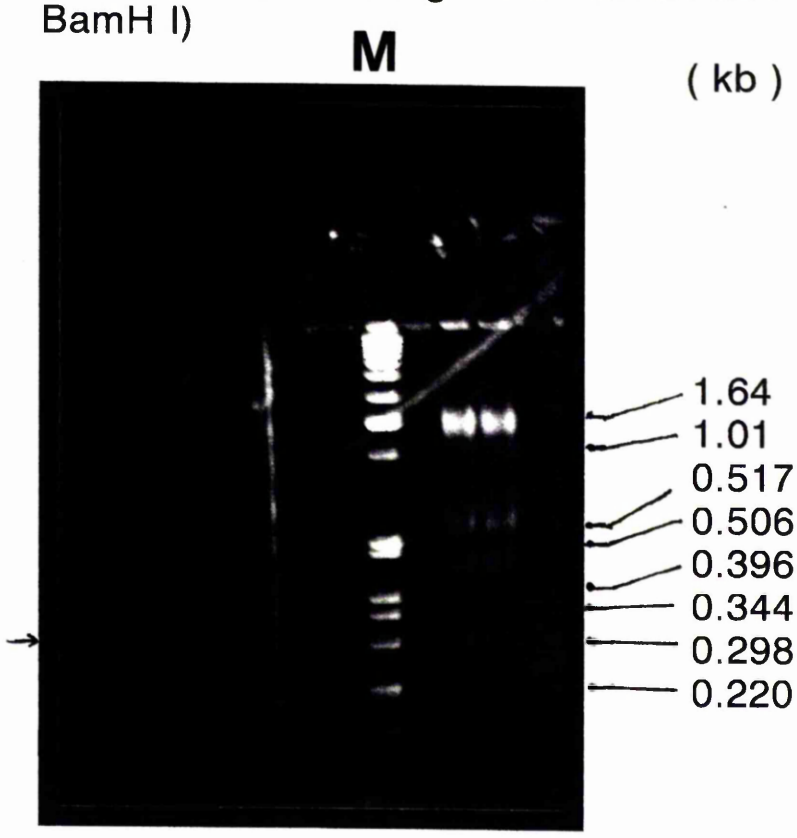
(B) The purified 1.18 kb fragment was digested with Ssp 1, the 232 bp fragment corresponding to part of AU-rich sequence was collected and purified for ^{32}P-labelling to probe AU-rich sequences in IFN-γ RNAs. Lane M is 1 kb ladder marker and other two lanes are from 1.18 kb fragment .

The 1.2 kb and 232 bp fragments are marked with arrows.

Photo	Enzyme	Expected fragments (kb)			
A	BamH I/EcoR I	4.95	1.25	**1.18**	0.65
B	Ssp 1	1.25	0.51	0.42	**0.23**



(A:Plasmid p1042 digested with EcoR I and BamH I)



(B: AU-rich sequence of plasmid p1042)

volume was made up to 100µl with sterile water and the solution was overlaid with 100 µl of light mineral oil.

The tubes were placed in a Techne PHC-3 thermal cycler, and the DNA was denatured at 94°C for 10 min. Then 0.5 unit of Taq DNA polymerase was added to each reaction. Thirty cycles of incubation were performed (94°C for 2 min, 50°C for 2 min and 72°C for 3 min) and the reaction ended by incubation at 72°C for 10 min. Reaction products (5 µl) were analyzed by agarose gel electrophoresis.

2.6.3.2 **RNA amplification**

2.6.3.2.1 RNA reverse transcription

Two µg of Poly $(A)^+$ RNA (Section 2.5.4) were transferred into a 1.5 ml microfuge tube and Pharmacia First-strand cDNA synthesis kit was used make the first strand cDNA. The ACB1 oligonucleotide (0.2 µg/µl; Section 2.1.1.2) was used as a primer and the reactions followed the supplier's instructions. The reaction was monitored by incorporation of ^{32}P-dCTP during polymerisation.

2.6.3.2.2 cDNA amplification

Two µl of cDNA product (Section 2.6.3.2.1) was added to 50 pmole of the ACB6 and ACB8 oligonucleotides, 10 µl of 10X PCR buffer (Section 2.6.3.1) and 20 pmole dNTPs (Section 2.6.3.1). The volume was made up to 100 µl with sterile water and the solution was overlaid with 100 µl of light mineral oil. The temperature and time of DNA melting, primer annealing and template extension in PCR reaction were set as described in genomic DNA amplification (Section 2.6.3.1).

2.6.3.3 DNA ligation

2.6.3.3.1 Plasmid pBluesript IISK T-vector construction

Plasmid pBluescript IISK was digested with EcoR V (Section 2.6.3.3.1) and incubated with Taq polymerase (1 unit/µg plasmid/20µl) using PCR buffer (Section 2.6.3.1) in the presence of 2 mM dTTP for 2 hr at 70°C. After phenol-chloroform extraction (Section 2.5.1.1.2) and ethanol precipitation (Section 2.5.1.2), the T-vector is ready for cloning (Marchuk et al, 1990).

2.6.3.3.2 DNA ligation

PCR products were resolved from DNA agarose electrophoresis (Section 2.6.1.1) and fragments of interest were harvested (Section 2.6.2.2). The concentration of these fragments was estimated using UV spectrophotometry (Section 2.5.1.3). Using equation 2.1 the amount of DNA fragments required for reaction with 50 ng of T-vector in a ratio of 3 to 1 can be calculated.

$$\text{ng of insert} = \left\{\frac{50\text{ng of vector} \times \text{Kb size of insert}}{\text{Kb size of vector}}\right\} \times \left\{\text{molar ratio}\ \frac{\text{insert}}{\text{vector}}\right\} \quad \text{------Equation 2.1}$$

T-vectors and DNA insert were mixed and the volume was made up to 8 µl with water. One µl of 10X ligation buffer (0.5 M Tris-HCl, pH 7.6 buffer containing 100 mM magnesium chloride, 100 mM dithiothreitol, 500 µg/ml BSA and 10 mM ATP) and 1 unit of T_4 DNA ligase were added and this solution was mixed by brief vortexing. Tubes were incubated at 14°C overnight. Constructed vectors (5 µl of reaction mixture)

were then used to transform *E. coli* XL-blue bacteria (Section 2.4.2). Transformants were selected by ampicillin resistance and blue/white selection. 20 µl X-gal (40 mg/ml in dimethylformamide) and 100 µl IPTG (100 µg/ml, filter sterilised) were spread on the surface of LBA plate containing ampicillin (Section 2.2.1). The plates were placed in a 37°C oven for 30 min. Bacterial cells were then spread on these plates and they were incubated at 37°C for 16 hr. After incubation, only those containing inserts transformants produced white colonies. Vector self-ligation transformants produced blue colonies (Ullmann et al., 1967). Inserts, vectors and bacteria used in this Section is listed below.

Table 2.8 PCR constructs for sequencing

Insert	Vector	Bacteria
42 gDNA	pGem-T	*E. coli* DH5α
42 RNAU	pBluescript IISK-T	*E. coli* XL-blue
42 RNAL	pBLuescript IISK-T	*E. coli* XL-blue
43 gDNA	pGem-T	*E. coli* DH5α
43 RNA	PGem-T	*E. coli* DH5α

gDNA: IFN-γ genomic DNA from CHO cells (Section 2.6.3.1)
RNA: IFN-γ cDNA from CHO cells (Section 2.6.3.2.2)

2.6.4 Hybridisation procedures

2.6.4.1 Generation and purification of cDNA probes

The cDNAs were incorporated with α^{32}P dCTP and α^{32}P dATP using different methods to make probes.

Table 2.9 Methods and isotope used for probe preparation

Probe	Method	Isotope
IFN-γ	Random priming	α^{32}P dCTP
ARS	Random priming	α^{32}P dATP
DHFR	Random priming	α^{32}P dCTP
18S rRNA	Nick translation	α^{32}P dCTP

ARS: AU-rich sequence of IFN-γ cDNA 3'UTR

2.6.4.1.1 Random priming

Sixty ng of DNA fragment (in 14 µl water) was incubated in boiling water for 2 min and immediately chilled on ice and this DNA solution was labelled by klenow incorporation of α^{32}P dCTP (or dATP; 3000 µCi/mmole) by the random priming technique (Feinberg and Vogelstein, 1983 and 1984). Unincorporated nucleotides were removed by passage through a Sephadex G-50 column (dry Sephadex G-50 was suspended in 10 mM Tris-Hcl pH 7.4 buffer containing 0.1 mM EDTA, and autoclaved). Spun columns were prepared using 1 ml disposable syringes plugged with sterile glass wool, as described by Sambrook et al (1989). The specific activity of the labelled probe was determined by Cerenkov counting. In general, labelled probe DNA was used at 1 X 10^7 cpm/10 ml hybridisation buffer.

2.6.4.1.2 Nick translation

Plasmid DNA (100 µg/µl) was transferred to 1.5 ml microfuge tubes and 5 µl of α^{32}P dCTP (3000 µCi/mmole) was added. The Boehringer Mannheim Nick Translation kit (containing dATP, dGTP, and dTTP solution; enzyme and buffer) was used to make probe. The reaction solutions were added sequentially following the supplier's instruction (made up to final volume of 15 µl with water). This reaction solution was incubated at 15°C for 1 hr. Unincorporated nucleotides were removed by passage through a Sephadex G-50 column as described above (Section 2.6.4.1).

2.6.4.2 Southern analysis of DNA

DNA samples (having been digested with restriction enzymes) were resolved in agarose gel electrophoresis (Section 2.6.1.1). The gel was soaked in 0.05 M sodium hydroxide solution for 20 min and neutralized in neutralizing solution (1 M Tris-HCl buffer pH 8, containing 1.5 M sodium chloride) twice, 30 min each time. The DNA was transferred to Hybond-N membrane using 10X SSC (containing 1.5 M sodium chloride and 0.15 M tri-sodium citrate, pH 7.4) using capillary elution method as described by Sambrook (1989). After DNA transferance, the Hybond-N membrane was washed in 4X SSC solution. The membrane was, then baked in an 80°C vacuum oven for 2 hr.

The membrane was rinsed with 4X SSC and was prehybridised in sealed plastic bag which was filled with prehybridisation solution (0.5 M disodium hydrogenphosphate containing 0.1% BSA, 1 mM EDTA and 7% SDS) at 65°C for 5 min. The prehybridisation solution was, then substituted with hybridisation buffer (1X SSC containing 6% polyethylene glycerol 6000) and the cDNA probe (heated in boiling water for 2 min and chilled on ice immediately) was added. The DNAs were hybridised with cDNA probes at 65°C overnight. The membrane was washed once with washing buffer (40 mM disodium hydrophosphate containing 1% SDS) at 65°C for 15 min and sequentially washed twice with different concentrations of SSC washing solution (0.5X SSC and 0.2X SSC solution containing 0.01% SDS). The washed membrane was, then ready for autoradiography.

2.6.4.3 Autoradiography

The excess water was drained from the membrane and the membrane was covered with Saran Wrap. The wrapped membrane was placed on a X-ray film (pre-flashed with flash unit which was covered with an orange filter 50 cm high above the film and an intensifying screen was placed behind the X-ray film. This cassette was put in a -70°C freezer until the OD_{545} of the image on X-ray film was 0.1 to 1.0.

2.6.4.4 DNA and RNA (cDNA) sequencing

Plasmid DNA (4 µg, containing PCR insert; Section 2.6.3) was transferred into A 1.5 ml microfuge tube and 1 µl of 2 M sodium hydroxide was added, then the solution was made up to a final volume of 10 µl with sterile water. The tube was then incubated at 37°C for 15 min. After incubation, 3 µl 3 M sodium acetate, 1 µl 1 mM primer and 75 µl ethanol was added to each tube and mixed. Tubes were stored at -20°C overnight and annealed DNAs were precipitated by centrifugation. Sequencing reactions were carried out using the Amersham Sequenase2 Kit following the suppliers instructions.

The sequencing products were resolved in 7 M urea, 6% polyacrylamide denaturing gels using 1X TBE electrophoresis buffer (Section 2.6.1.1) at a constant power of 55 watts, sufficient to maintain the gel at 55°C. Following electrophoresis the gels were dried directly onto a piece of 3 mm filter paper by placing onto a vacuum gel drier at 80°C. Autoradiography (Section 2.6.4.3) was performed at room temperature.

2.6.4.5 **Northern blotting**

RNA samples (15 µl/lane) were separated by 1.5% agarose electrophoresis (Section 2.6.1.2) and gels were soaked in 500 ml of 0.05 M sodium hydroxide solution for 20 min. The agarose gel was, then washed with DEPC water and soaked in neutralization buffer (Section 2.6.4.2) for 40 min. The gel was placed in contact with nylon membrane and RNA was transferred onto membrane in an ascending flow of 10X SSC as described by Sambrook et al (1989). After transferring the RNA, the membrane was soaked in 4X SSC for 5 min and baked for 2 hr at 80°C in a vacuum oven.

The filters were sealed in plastic bags containing the prehybridisation solution (50% formamide containing 6X SSPE, 5X Denhardt's solution, 0.5% SDS and 100 µg/ml sheared salmon sperm DNA; 20X SSPE contains 3.6 M sodium chloride, 0.2 M sodium phosphate, 0.02 M EDTA pH 7.4; 100X Denhardt's solution contains 2% each of BSA, Ficoll and polyvinylpyrolidine) and the bags were submerged in a 42°C shaking water bath for 1 hr. The radio-labelled DNA probe (Section 2.6.4.1), denatured in boiling water for 5 min, was then added to the bag and hybridisation was allowed to proceed overnight at 42°C.

Following hybridisation the filters were removed from the bags and they were washed by sequential incubation at 55°C (except as mentioned) for 15 min periods (all the washing solutions contained 0.1% SDS): once in 2X SSC, twice in 1X SSC, twice in 0.5X SSC and twice at 65°C in 0.1X SSC solution. The localisation of hybridisation on the filter was

defined by autoradiography (Section 2.6.4.3) and, if needed, the membrane could be stripped for further probing.

Boiling strip solution (500 ml; 0.1% SDS) was poured into sandwich boxes containing those membranes which required stripping of the radio-labelling probe. This was left until the strip solution had cooled to room temperature. The strip efficiency was monitored by autoradiography (Section 2.6.4.3). If the radio-labelled probe was stripped off, membranes were incubated in 80°C vacuum oven. These membranes were ready to be reprobed.

2.7 Analysis of macromolecular turnover

2.7.1 Total RNA content (Orcinol method, Dawson et al., 1986)

CHO 42 and CHO 43 cells were cultured in T_{75} flask to mid-log phase and harvested, trypsinized as described in Section 2.3.2. Cells were collected in 1.5 ml microfuge tubes and extracted with a series of solvent before used for RNA estimation. Firstly, cells were extracted twice with 0.5 ml methanol by vortexing and following a centrifugation to recollect cells. Further extractions were done in the following order: 2 times with 0.05 M formic acid in methanol, 2 times with 5% TCA in methanol, once with ethanol, twice with ethanol : chloroform (3 : 1, v/v), once with ethanol : ether (3 : 1, v/v) and finally with ether. After the ether was evaporated, the dry pellet was digested with 1 ml 0.3 M potassium hydroxide at 37° C for 1 hr, the suspension was centrifuged and the resultant pellet was discarded. To the

supernatant was added 400 µl of 5% perchloric acid and samples were left on ice for 15 min. After centrifugation, the supernatant was ready for RNA estimation.

To 200 µl sample solution from above, 300 µl of distilled water was added followed by the addition of 1 ml of orcinol acid reagent (10% ferric chloride in conc. HCl, w/v) and 66 µl of orcinol alcoholic reagent (6% orcinol in ethanol). The mixture was mixed well and heated at 90° C for 20 min in a water bath. After cooling, the OD of the solution was measured at 660 nm against a reagent blank and with nucleotide standards (0 - 132 µg; containing 1 X 10^{-4} M of each AMP, GMP, CMP and UMP).

2.7.2 RNA pulse label analysis

CHO cells were seeded in 24-well culture dishes for overnight incubation. Then medium was removed and cells were washed twice with warmed PBS. One ml of serum-free media (SFM; Section 2.3.1) was added to each well then sequentially actinomycin D (up to 20 µg/ml; 1 mg/ml dissolved in ethanol) and [^{3}H]-uridine (30 µCi/ml; 1 mCi/mmole) were added. CHO cells were then incubated at 37°C for 6 hr. The cells were washed twice with PBS and cells were lysed with SNU solution (6 M urea containing 0.5% SDS and 1% Nonidet P-40). RNAs were precipitated from cell lysates by the addition of an equal volume of 20% TCA as described by Lewis (1987). RNA precipitates were dissolved in 0.5 ml of 0.1 M sodium hydroxide and 0.3 ml extracts were transferred to scintillation vials. Then, 3 ml of Ecoscint A scintillation

cocktail was added and the solutions were mixed thoroughly. Radioactivity of samples was counted using the Packard 2000CA liquid scintillation analyzer.

2.7.3 Total protein content (Bradford Method; Bradford, 1976)

CHO 42 and CHO 43 cells were cultured in T_{75} flasks to mid-log phase and harvested, trypsinized and counted as described in Section 2.3.2. Cells were suspended in PBS to give 1 X 10^4 cells/ml. 1ml of Bradford solution (containing 0.01% Coomasie Brilliant Blue G250 in 4.7% ethanol and 85% (w/v) phosphoric acid) was added to 100 µl of cell suspension and this was mixed well with vortexing. After 10 min incubation at room temperature the absorbance of the mixture was read at 595 nm against a reagent blank and with a BSA standard curve (1 to 50 µg/ml).

2.7.4 ELISA analysis of interferon expression

A three layer sandwich ELISA, as described by Gould (1992), was used to determine amounts of IFN-γ. 96 well Microtire plates were coated, overnight at 4°C, with 50 µl antibody R1PA/well (polyclonal anti-human IFN-γ raised from rabbit, dissolved in 100 mM citric acid) and the wells were washed twice with 100 µl wash buffer/well (0.05% casein and 0.1% Tween 20 in PBS). Non-specific binding in plates was minimised by the addition of 100 µl block buffer (0.5% casein and 0.1% Tween 20 in PBS) per well. This was left for two

hours at room temperature and then the wells were washed twice with wash buffer. Samples (50 µl, around 1-10 units IFN-γ) or standards (0, 1, 2, 5, 10, 15 and 20 units) were added to each well and the reaction was allowed to proceed for 1 hr. The plates were then washed twice in wash buffer. Then, 50 µl monoclonal antibody 20B8 (10 µg/ml in wash buffer) was added to each well and the plates were incubated for a further 1 hour at room temperature. After this, the plates were washed twice with wash buffer. Then peroxidase conjugated anti-mouse polyvalent antibody (50 µl/well; 10 µg/ml in washing buffer) was added to each well for an hour and the plates were washed twice with wash buffer. Finally, 100 µl of substrate solution [one tablet of o-phenyldiamine dihydrochloride dissolved in 25 ml substrate buffer (containing 51.2 mM disodium hydrophosphate and 24.4 mM tri-sodium citrate, pH 5.0) just prior to use; after the tablet had dissolved, 3 µl 30% hydrogen peroxide was added] was added into each well and incubated at room temperature. The reaction was stopped by addition of 10 µl 20% sulphuric acid to each well and the absorbance at 490 nm was measured. The concentration of IFN-γ was calculated by comparison to a standard curve (OD_{490} 0.3 - 1.0).

2.7.5 SDS-PAGE analysis of protein

2.7.5.1 Protein sample preparation

CHO cells were cultured and media was transferred to 15 ml centrifuge tubes. The cell sheets were washed twice with

PBS and trypsinized as described in Section 2.3.2. Cells were suspended in PBS to give a concentration of 4 X 10^6 cells/100µl (CHO 42 cells) or 1 X 10^6 cells/100µl (CHO 43 cells). Equal volumes of cells and protein loading buffer (125 mM Tris-HCl buffer, pH 6.8 containing 20% glycerol, 4% SDS and 0.01% bromophenol blue and 18 µl 2-mercaptoethanol/ml, added just before use) were mixed and this was boiled for 5 min and cooled to room temperature prior to loading on gels.

The medium (5 ml for CHO 42, 1 ml for CHO 43) was mixed with an equal volume of 10% TCA and was incubated on ice for 15 min. This was centrifuged at 2500g for 15 min at 4°C. The supernatant was aspirated and pellets were washed twice with 5% TCA. The protein pellets were then dissolved in 50 µl PBS (CHO 42) or 100 µl PBS (CHO 43). Equal amounts of medium extracts and protein loading buffer were mixed. This was boiled for 5 min and cooled to room temperature prior to loading on gels.

2.7.5.2 SDS-PAGE

The gel system was based on a 12% separating gel overlaid by a stacking gel containing 4% polyacrylamide as described by Dickson (1991). The separation gel was made by mixing 4 ml 30% acrylamide solution (30% acrylamide containing 0.8% N,N-methylene bisacrylamide, stored in dark at 4°C), 2.5 ml separating buffer (1.5 M Tris-HCl, pH 8.8 containing 0.4% SDS and stored at room temperature) and 3.5 ml water. The mixture was mixed with 100 µl ammonium

persulphate (10% solution, fresh preparation) and 10 µl TEMED. This was immediately poured into the gel sandwich of a Biorad Mini Protein 2 slab gel system. The top was then covered with 0.1 ml isobutanol until the gel had set. The isobutanol was removed and the top of the gel was rinsed with water. The gel was then overlaid with 4% stacking gel [mixing 1.32 ml 30% acrylamide solution, 2.5 ml stacking buffer (0.5 M Tris-Hcl, pH 6.8 containing 0.4% SDS), 6.18 ml water; this was added with 100 µl ammonium persulphate and 10 µl TEMED prior to use]. The 10 well Teflon comb was placed before the gel had set.

Protein samples (from Section 2.7.5.1) were loaded into wells. Electrophoresis was run at 60V until the bromophenol blue marker reached the interface between stacking and separating gels then the voltage was increased and maintained at 150V until the marker dye was near the end of the separating gel.

2.7.6 Western blotting

At the end of the SDS-PAGE, the gel was soaked in blotting buffer (25 mM Tris base, pH 7.4, containing 100 mM glycine and 20% methanol) for 30 min and the fractionated proteins were transferred onto nitrocellulose membranes by electroblotting using an LKB multiphor II electrotransfer apparatus.

Nitrocellulose membranes were covered with blocking buffer (10 mM tris base pH 7.4, containing 140 mM sodium

chloride, 0.1% V/V Tween 20 and 5% casein milk powder) and they were agitated for 30 min. The buffer was removed, replaced with fresh blocking buffer containing the 20D7 monoclonal antibody or S1DM polyclonal antibody directed against IFN-γ (1 μg/ml) and the blots were gently agitated for 60 min. Blots were then washed five times (10 min each time) in blocking buffer and the antimouse IgG antibody (conjugated with peroxidase and diluted 1:1000) was added in blocking buffer. The 60 min incubation was repeated and the filters were washed in fresh blocking buffer as before. The membranes were finally washed twice with TBS/Tween (blocking buffer minus casein milk powder). The antigen/antibody complexes were detected using an enhanced chemiluminescence (ECL) kit following the suppliers instructions.

2.7.7 Protein sample preparation and pulse and chase labelling

2.7.7.1 **Cell labelling procedures**

2.7.7.1.1 [^{3}H]-leucine pulse labelling

CHO cells were seeded in 24-well culture dishes for overnight incubation. Then medium was removed and cells were washed twice with warmed PBS. One ml of serum-free media (SFM; 2.3.1) was added to each well then cycloheximide (up to 20 μg/ml; 1 mg/ml in water) and L-[4,5- ^{3}H]-leucine (30 μCi/ml; 1 mCi/mmole) were added sequentially. CHO cells were then incubated at 37°C for 6 hr. Medium was transferred to 1.5 ml microfuge tubes and tubes were centrifuged at 14,900g for 1 min. 0.5 ml of medium was then transferred to new tubes

and 500 µl of 10% TCA was added. Proteins were precipitated leaving tubes on ice for 15 min and were redissolved in 500 µl 5% TCA (containing 10 mM leucine). CHO cells were washed twice with washing buffer (25 mM Tris, pH 7.5 containing 20 mM sodium chloride) and 500 µl 5% TCA (containing 10 mM leucine) was added to each well. CHO cells, then were scraped off and cell suspensions were transferred to microfuge tubes.

Both medium and cellular samples were heated at 90°C for 15 min to hydrolyse any charged tRNA, then samples were centrifuged at 14,900g for 2 min. The protein precipitates were washed three times with 500 µl 5% TCA (containing 10mM leucine) and tubes were re-centrifuged. 30 µl of NCS tissue solubilizer were added to each tube and protein pellets were dissolved by stirring. The radioactivity was counted in the presence of 2 ml of Ecoscint plus 10 µl 10 M hydrochloric acid.

2.7.7.1.2 [^{35}S]-methionine/cysteine labelling

CHO cells were seeded in 6-well culture dishes for 48 hr incubation. Then medium was removed and cells were washed twice with 1ml warmed labelling medium minus methionine and cysteine (DMEM metabolite medium supplemented with L-arginine, D-glucose, i-inositol, L-leucine and sodium dihydrophosphate). After washing, 2 ml warmed labelling medium minus methionine and cysteine were added to each well and CHO cells were then incubated at 37°C for 15 min. The medium was aspirated and 1ml labelling medium supplemented with 100 µCi/ml of Tran ^{35}S-label (containing 70% [^{35}S]-

methionine and 20% [^{35}S]-cysteine; 1 mCi/mmole) was added. For identifying the primary translation IFN-γ products, the labellings were set for a short period of 5-15 min. For chasing IFN-γ metabolites, the labellings were set for 1 hr. After labelling, medium was aspirated and cell sheets were washed twice with warmed labelling medium (for chasing) and PBS (for pulsing). Following a pulse with radio-labelled Tran^{35}S-label, chases with the SCM culture medium (Section 2.3.1) were performed for up to 4 hr. Cell sheets were washed with PBS and cells were lysed with NP-40 lysis buffer [(50 mM Tris-HCl, pH 7.5, containing 150 mM sodium chloride, 0.05% SDS, 1% NP-40; Dorner and Kaufman (1990) and protease inhibitor (including 200 μM PMSF, 1 μM leupeptin, 1 μM pepstatin and 100 μM TLCK; Dickson (1991)]. The cellular and medium samples were analysed by immuno-precipitation.

A 5 μl aliquot of cell lysate was spotted on a Whatman 3MM filter paper and this was air dried. Filters were then washed on ice three times with 10% TCA, once with 100% ethanol and then they were air dried. The radioactivity left on the filter was measured by liquid scintillation counting (Section 2.7.7.1.1).

2.7.7.2 **Immuno-precipitation analysis**

Protein A-Sepharose was prepared as follow: Protein A-Sephrose beads were prepared by suspending 0.1 g of protein A-Sepharose in 20 ml TENN buffer (50 mM Tris-HCl, pH 5.5 containing 5 mM EDTA, 0.15 M sodium chloride and 0.5% NP-40) and protein A-Sepharose beads were allowed to settle. This

was washed once more with TENN buffer and was allowed to settle. The final volume was adjusted with TENN buffer to 2.5 ml and stored at 4°C until used.

IFN-γ in cell extracts underwent reaction with antibody by addition of 2.5 µl 20D7 antibody (10 mg/ml; supplemented with protease inhibitor as defined in Section 2.7.7.1.2) as described by Doner and Kaufman (1990). This was incubated overnight in a rotating platform at 4°C. Then 30 µl of protein A-Sepharose solution supplemented with protease inhibitors (Section 2.7.7.1.2) was added and this was incubated for a further 1 hr at 4°C. The suspension was centrifuged (3 min at 14,900g, 4°C) and the supernatant was aspirated and sequential washings and centrifugation at 4°C were performed, twice with PBS containing 1% Triton X-100, once with PBS with 0.5% Triton X-100 and once with PBS containing 0.05% Triton X-100. An aliquot of 50 µl protein loading buffer was added to the precipitates and these were mixed. This was boiled for 5 min and samples were ready for SDS-PAGE separation.

The immuno-precipitate was resolved by 12% SDS-PAGE (Section 2.7.5) and gels were fixed in 10% acetic acid for 30 min. They were then washed with water for 30 min. After washing, gels were soaked in 1M sodium salicylate for 30 min. Then, the gel was placed onto a piece of Whatman 3MM filter paper and a piece Saran film was covered on top of the gel. This was dried at 80°C for 2 hr using a vacuum gel dryer. A sheet of X-ray film was put on dried gels to detect signals (Section 2.6.4.3).

2.8 Data anylysis

2.8.1 Parameters of growth and productivity

The CHO 42 and CHO 43 cell growth and IFN-γ production analysis were performed as described by Rasida Anwar (1994).

(A) Cell growth

(a) Maximum specific growth rate (μ_m)

During a time interval (dt), the increase in cell number (dx) is proportional to the amount (x) present and to the time interval:

$$dx = \mu x \times dt \qquad (1)$$

$$\mu x = dx/dt \qquad (2)$$

When μ is constant, integration of equation (2) results:

$$\ln x = \ln x_0 + \mu t \qquad (3)$$

At mid-log phase

$$\mu_m = \ln (x - x_0)/t \qquad (4)$$

where x_0 is the cell number at time t_0.

(b) Cell doubling time (t_d)

By putting equation (3) in the form

$$\ln(x/x_0) = \mu t \qquad (5)$$

When $x = 2x_0$ and $t = t_d$ equation (4) becomes:

$$t_d = \ln 2/\mu = 0.693/\mu \qquad (6)$$

(B) IFN-γ production in batch culture ($q_{IFN-\gamma}$)

The IFN-γ formed in an small time interval (dt) is given by :

$$d(IFN-\gamma) = q_{IFN-\gamma} \times (x) \times dt \qquad (7)$$

when $q_{IFN-\gamma}$ is constant, intergration of equation (7) gives:

$$\ln (IFN\text{-}\gamma_t) = \ln (IFN\text{-}\gamma_0) \times q_{IFN\text{-}\gamma} \times (x)$$

$$q_{IFN\text{-}\gamma} = \ln (IFN\text{-}\gamma_t - IFN\text{-}\gamma_0) / (x)$$

where x = cell number

$IFN\text{-}\gamma_t$ and $IFN\text{-}\gamma_0$ are IFN-γ production at time t and t_0

$q_{IFN\text{-}\gamma}$ = specific rate of IFN-γ formation

2.8.2 Statistical Methods (Harper, 1988)

The standard deviation (SD) from the mean of samples and the standard error of the mean of n experiments (SEM) were calculated from the formulae :

$$SD = \sqrt{\Sigma (\bar{x}-x)^2 / (n-1)}$$

$$SEM = \sqrt{\Sigma (x-m)^2 / n(n-1)}$$

$$= SD / \sqrt{n}$$

where m = arithmetic mean of n experiments

x = an observed value

$\bar{x}$ = arithmetic mean of n times of observations

n = number of independent observations

n-1 = numbers of degrees of freedom

3.1 CHO 42 and CHO 43 characteristics

In this section, the characteristics of CHO 42 and CHO 43 cells will be described in terms of growth (including cell morphology), DHFR mRNA and IFN-γ mRNA species and IFN-γ protein species.

3.1.1 CHO cell physiology

3.1.1.1 CHO cell growth and morphology

The growth curves for CHO 42 and CHO 43 cells are shown in Fig 3.1. CHO 43 cells were seeded at a concentration of 1 X 10^5 cells/ml and reached their maximum concentration of 1 X 10^6 cells/ml by 4 days of culture. CHO 42 cells were seeded at a concentration of 2 X 10^5 cells/ml and grew to a maximum concentration of 2 X 10^6 cells/ml by 5 days of culture. Under these conditions, CHO 42 cells grew in a manner similar to their progenitor cell, CHO Duk cells, which attained a maximum concentration of 2.2 X 10^6 cells/ml by 5 days of culture (Anwar, 1994).

Both recombinant CHO cells grew slower than CHO Duk cells. CHO 42 and CHO 43 cells had doubling times of 28 hr and 24 hr, respectively, and CHO Duk cells had a doubling time of 17.5 hr (Anwar, 1994). All three dhfr-deficient cell lines grew slower than their predecessor CHO cells which have been reported to have a doubling time of 12 hr (Gottesman, 1987).

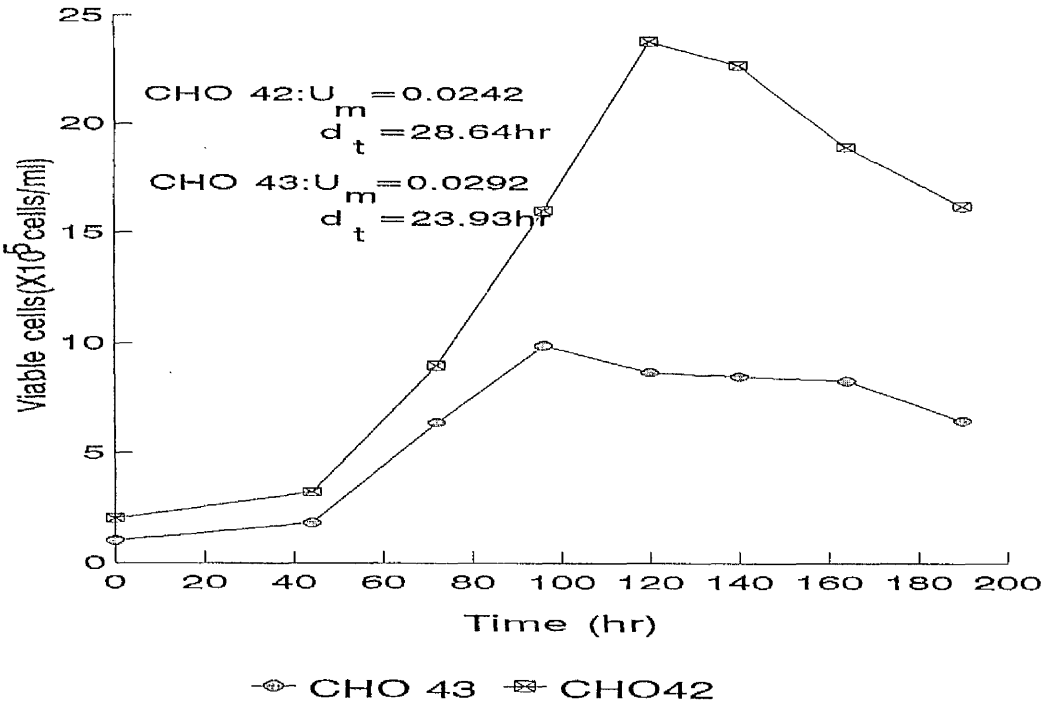
Figure 3.1 CHO 42 and CHO 43 cell growth through batch culture

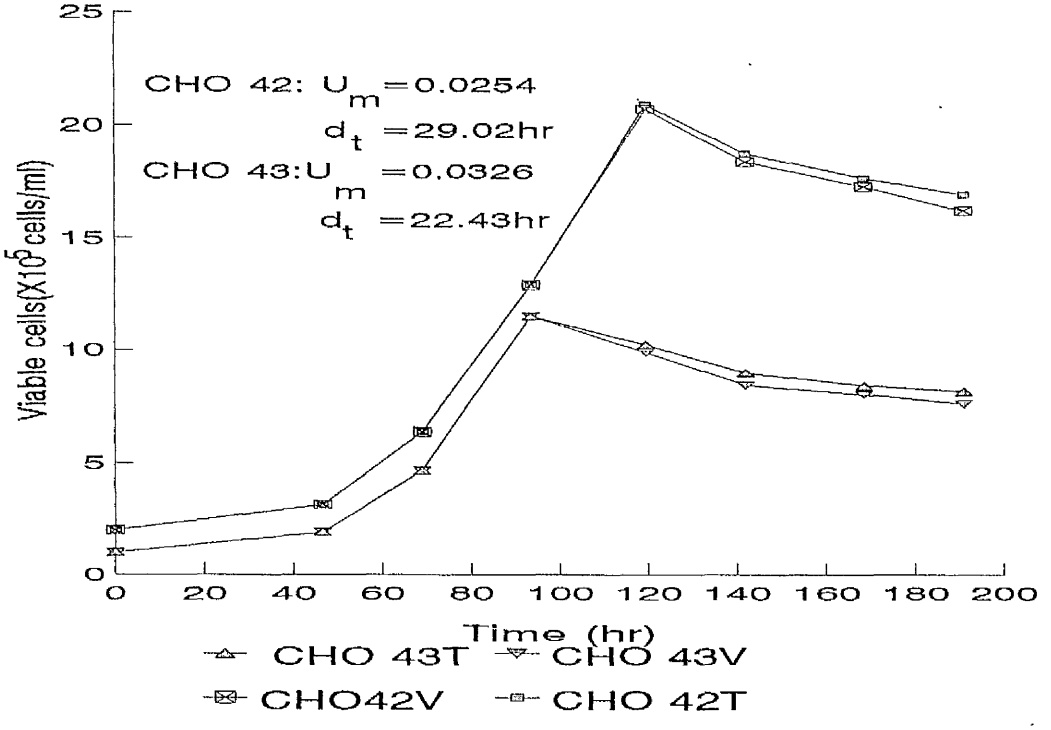
CHO 42 and CHO 43 cells from early stationary phase of culture were trypsinised, harvested, suspended in fresh medium to cell density of 2 X 10^5 cells/ml CHO 42, 1 X 10^5 cells/ml CHO 43 (Section 2.3.2). 6 ml was aliquoted per T_{25} flask and cells were cultured as described in Section 2.3.2. Cells were counted on a haemocytometer slide and cell viability was determined by prior incubation with trypan blue.

U_m: Maximum specific growth rate was calculated as shown in Section 2.8.

d_t: Cell doubling time was calculated as shown in Section 2.8.

CHO 42T and CHO 43T in Fig 3.1B represent total CHO 42 and CHO 43 cells in culture. CHO 42V and CHO 43V are viable CHO 42 and CHO 43 cells in culture.


CHO 42:U m =0.0242
d t =28.64hr
CHO 43:U m =0.0292
d t =23.93hr
Viable cells(X10^5 cells/ml)
Time (hr)
CHO 43
CHO42


CHO 42: U m =0.0254
d t =29.02hr
CHO 43:U m =0.0326
d t =22.43hr
Viable cells(X10^5 cells/ml)
Time (hr)
CHO 43T
CHO 43V
CHO42V
CHO 42T

CHO 42 cells were smaller than CHO 43 cells. The average diameter of CHO 42 and CHO 43 cells were 13.9±0.9µm (data from 40 cells, mean±SD; average volume 0.01125 µl) and 20.09±5µm (data from 40 cells, mean±SD; average volume 0.03396 µl), respectively. When initially cultured, both cell types exhibited an irregular shape.

CHO 42 cells, when initially attached, had a shape that was thin and long by comparison to the wide and broad shape of CHO 43 cells (Fig 3.2). After 7 days of continuous culture, both cells types had changed to a round or oval shape.

3.1.1.2 Cellular RNA and protein contents

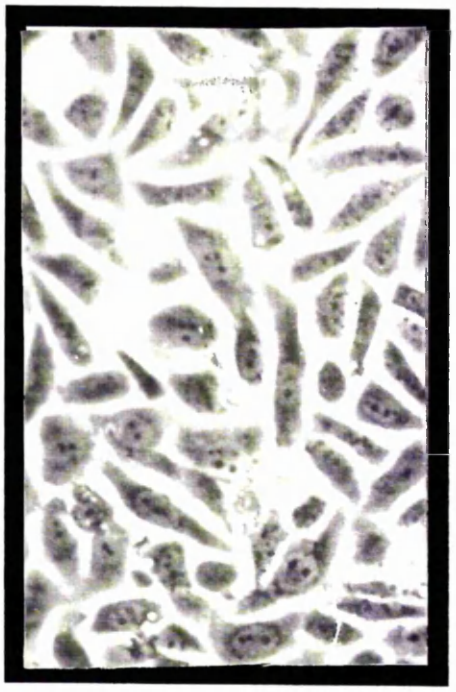
RNA and protein contents of CHO 42 and CHO 43 cells are summarized in Table 3.1. CHO 43 cells have three times the RNA (30 pg ± 4 per cell, CHO 43; 10.3 pg ± 3.7, CHO 42 cells) and twice the protein (0.59 ng ± 0.19 per cell, CHO 43; 0.34 ng ± 0.11 CHO 42 cells) content of CHO 42 cells.

3.1.1.3 Transcription and translation rates

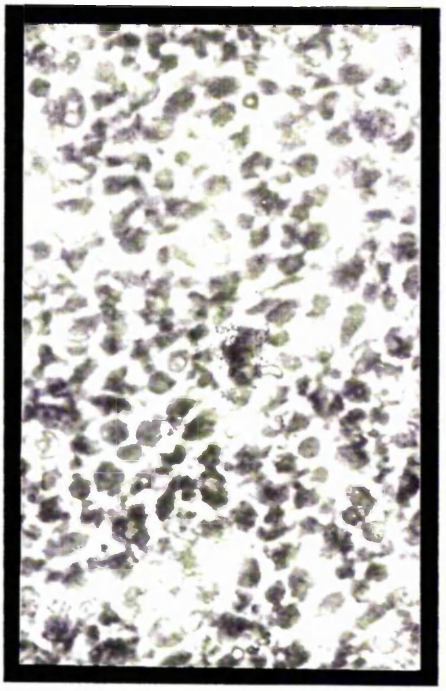
Cells, at mid-log phase, were examined for transcriptional and translational characteristics, and these results are summarized in Table 3.2. CHO 43 cells exhibited twice the RNA and protein productivities of CHO 42 cells. When cells were labelled with ^{3}H-uridine, CHO 43 cells incorporated 2.4 times more radio-labelled uridine into trichloroacetic acid-precipitable material (acid-precipitable; 2.11±0.34 dpm/cell/hr) than did CHO 42 cells

Figure 3.2 CHO 42 and CHO 43 cell culture morphology

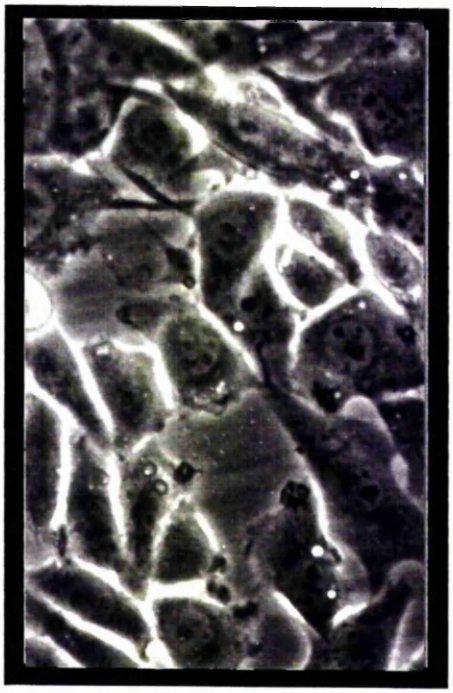
CHO 42 and CHO 43 cells were examined in exponential phase (2 day) and late stationary phase (7 day). The cells in T_{25} flasks were photographed under light microscopy. The bar shown under the photographs is 100μm.



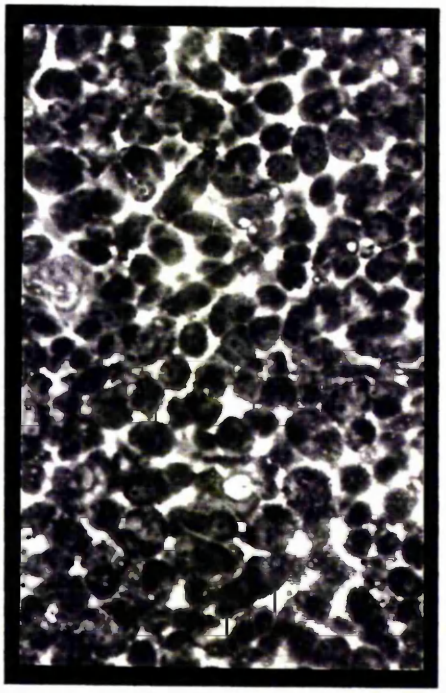
CHO 42 (2 day)



CHO 42 (7 day)



CHO 43 (2 day)



CHO 43 (7 day)

100 μm

Table 3.1 RNA and protein contents of CHO 42 and CHO 43 cells at mid-log phase

Cell	RNA (pg) per cell	Protein (ng) per cell
CHO 42	10.3 ± 3.7	0.34 ± 0.11
CHO 43	30.0 ± 4.0	0.59 ± 0.19

CHO cells were cultured in T_{75} culture flask, media were aspirated, cell sheet washed twice with PBS and cell were trypsinised. Cell number were counted by using haemocytometer (Section 2.3.3), total RNA was estimated by Orcinol method (Section 2.7.1) and total protein was evaluated by Bradford method (Section 2.7.3).

Table 3.2 RNA transcription and protein translation in CHO 42 and CHO 43 cells

Cells	Transcription	Translation (dpm/hr/cell)	Secretion
CHO 42	0.864±0.068(1.0)	0.0180±0.0021(1.0)	0.0025±0.0004(1.0)
CHO 43	2.105±0.342(2.4)	0.0397±0.0059(2.2)	0.0195±0.0025(7.8)

Transcription rate : Cells were cultured in 24 well plates overnight. The medium was aspirated and cells were cultured in SFM containing 30 μCi [^{3}H3,5]-uridine for 6 hr. Cells were lysed with SNU solution (Section 2.7.2) and RNA was precipitated with TCA. The radioactivity of RNA samples was counted in a liquid scintillation counter. Transcription rate was calculated as radioactivity per cell.

Translation rate: Cells were cultured in 24 well plates overnight, then cells were cultured in SFM containing [^{3}H]-leucine for 6 hr. Cells were lysed with NP-40 lysis solution (Section 2.7.7.1). The protein in both cellular and medium samples was precipitated with TCA. The radioactivity of protein samples was counted in a liquid scintillation counter. Translation and secretion rates were calculated from radioactivity in cellular and medium sample, respectively, on a cellular basis.

(0.86±0.07 dpm/cell/hr). Cells were also labelled with ^{3}H-leucine to examine translational rate. CHO 43 cells have a 2.2 times greater rate of incorporation into acid-precipitable material (0.40±0.06 dpm/cell/hr) than did CHO 42 cells (0.18±0.02 dpm/cell/hr). For both RNA and protein the cell differences could be a result of the greater volume of CHO 43 cells (Section 3.1.1.1). CHO 43 cells secreted protein (acid-precipitable; 0.195±0.025 dpm/cell/hr) at a rate 7.8 times greater than CHO 42 cells (0.025±0.004 dpm/cell/hr).

3.1.2 IFN-γ gene expression

CHO 42 and CHO 43 cells exhibit several differences (eg. cell volume, growth rate, cell density, Section 3.1.1). These differences could influence expression of recombinant IFN-γ. Here, I would like to compare IFN-γ mRNA content and IFN-γ protein expression in these two cell lines.

3.1.2.1 IFN-γ mRNA species

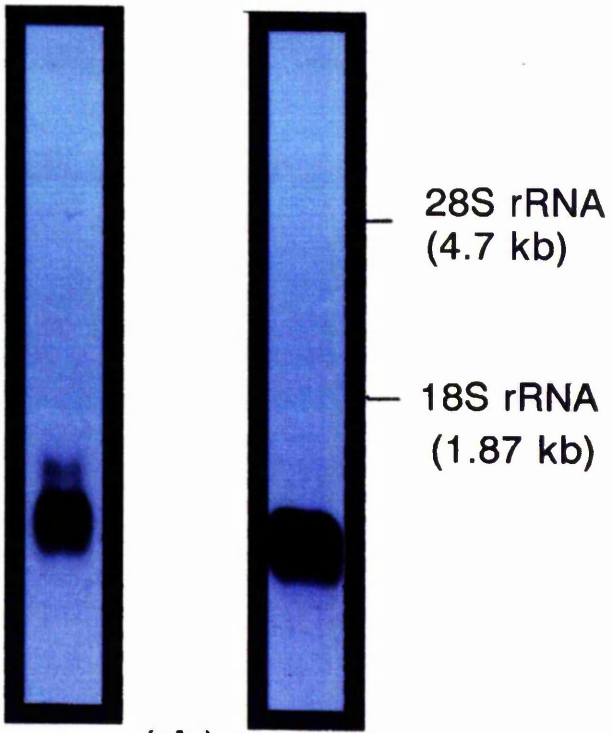
Northern hybridisation was performed on IFN-γ mRNA species isolated from CHO 42 and CHO 43 cells and the results are presented in Fig 3.3. The amount of IFN-γ mRNA in CHO 43 cells (1.14±0.45 arbitrary standardised densitometric units) is about 1.6 times greater than CHO 42 cells (0.71±0.07 arbitrary standardised densitometric units). There are two IFN-γ mRNA species in CHO 42 cells, but only one species in CHO 43 cells. In CHO 43 cells the single mRNA species had an estimated size of 0.93 kb and this is close to the predicted size for IFN-γ mRNA (Section 2.1.1.2). The major IFN-γ mRNA

Figure 3.3 IFN-γ mRNA species in CHO 42 and CHO 43 cells

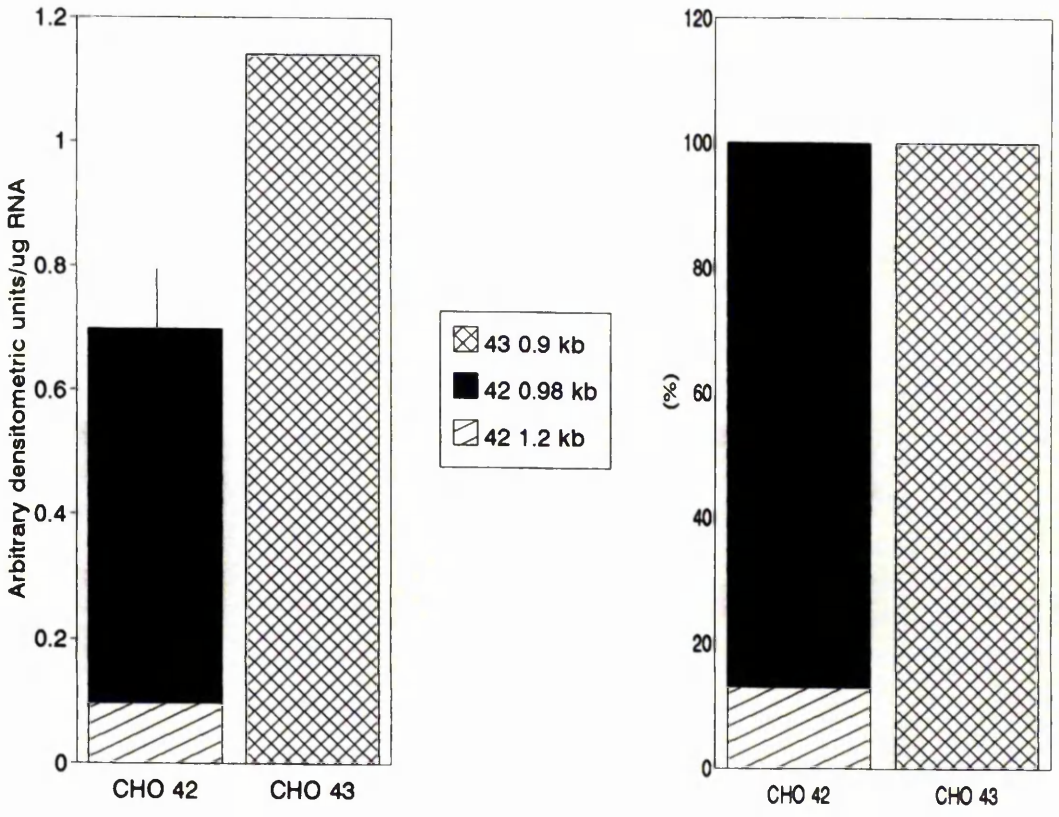
(A) CHO 42 and CHO 43 cells were cultured for two days (mid-log phase) and total RNA was extracted (Section 2.5.3.1). Fifteen µg of each RNA sample was resolved in 1.5% agarose gel (Section 2.6.1.2), Northern blotted (Section 2.6.4.5) and probed with ^{32}P-labelled IFN-γ cDNA as described in Section 2.6.4.1.

(B) The autoradiographs of IFN-γ MRNA were scanned as described in Section 2.6.4.3, values shown in Fig 3.4B and 3.4C are means of 5 separate experiments. The amount of mRNA was calculated from 15 µg of total RNA. Legend shows the mRNA species of CHO 42 and CHO 43 cells and its size (eg.: 43 0.9 kb means mRNA of CHO 43 and its size is 0.9 kb).

(C) The percentage of each IFN-γ mRNA species in total IFN-γ mRNA of CHO 42 and CHO 43 cells is presented as a % of total.



CHO 42 (A) CHO 43



(B) (C)

species (0.98 kb) in CHO 42 cells accounts for about 80% (78.5±7%, from 6 experiments) of the total amount of IFN-γ mRNA and was about 200 bases less than the predicted size (Section 2.1.1.1). The higher molecular weight (minor) species in CHO 42 cells was the predicted size for IFN-γ mRNA transcribed from plasmid p1042 (1.22 kb; Section 2.1.1.1).

3.1.2.2 IFN-γ protein production

IFN-γ secreted by CHO 42 and CHO 43 cells was collected in medium samples and the amount of IFN-γ was analyzed using ELISA (Fig 3.4). CHO 43 cells accumulated 270,000 units/ml after 8 days of incubation, this was 11 times greater than the amount of IFN-γ produced by CHO 42 cells (24,000units/ml) at the equivalent culture time. Production of these amounts of IFN-γ is equivalent to 2.7 mg and 0.24 mg IFN-γ protein produced per litre of culture medium, respectively (10^7 units equal 1 mg protein; Sareneva et al, 1994).

In batch culture, CHO 42 and CHO 43 cells produced greater amounts of IFN-γ than reported for cytolytic T cells superinduced with the phorbol ester PMA and ionomycin (1500 units/ml; Kaldy and Schmitt-Verhulst, 1991), CD30 T cells activated with IL-12 (1948 pg/ml; Alzona, 1995), human T lymphocytes stimulated with natural killer cell stimulatory factor (NKSF), IL-2 and phytohaemagglutinin (PHA; 447 units/ml; Chan et al, 1992) or tonsil cells superinduced with cycloheximide and low dose γ-irradiation (3 X 10^{-2} units/ml; Lebendiker et al, 1987). CHO 43 also produced higher amounts of recombinant IFN-γ than reported for other recombinant CHO

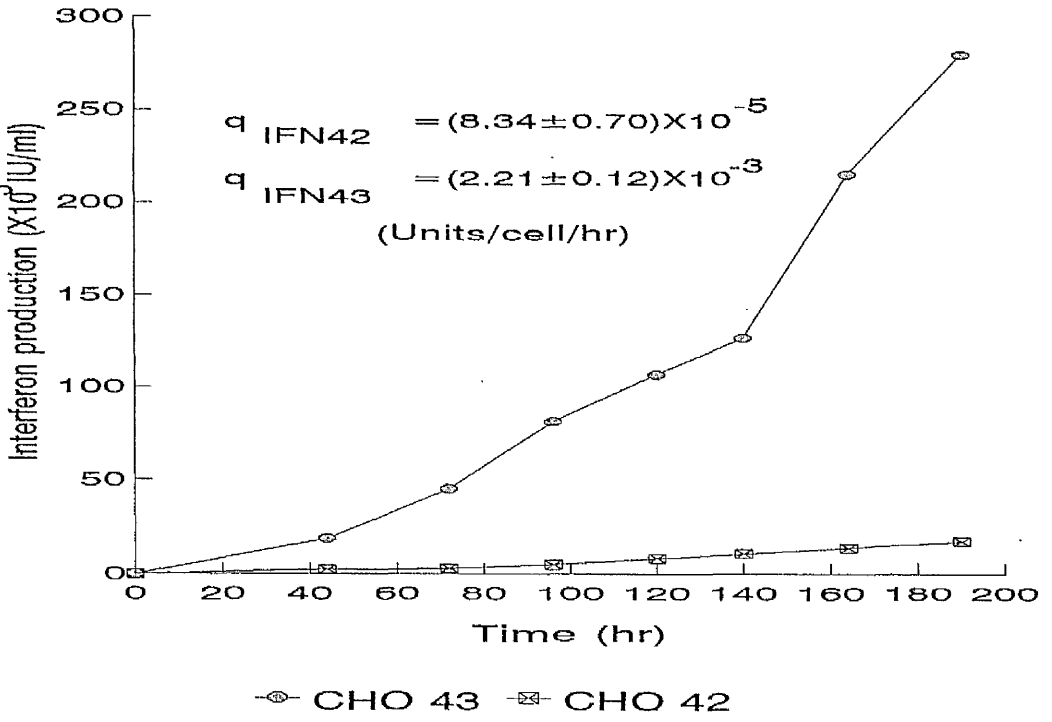
Figure 3.4 IFN-γ production by CHO 42 and CHO 43 cells through batch culture

CHO 42 (2 X 10^5 cells/ml) and CHO 43 (1 X 10^5 cells/ml) cells were cultured as described in Section 2.3.2. The medium of these batch culture was collected and amounts of IFN-γ were analyzed by ELISA (Section 2.7.4).

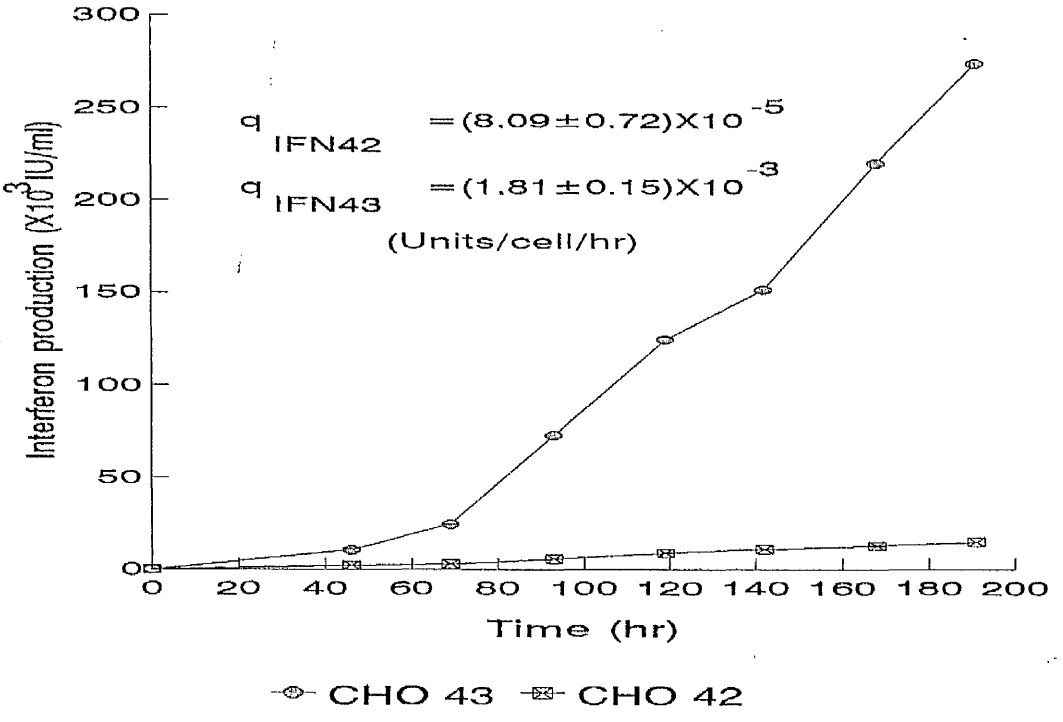
q_{IFN42} and q_{IFN43}: specific IFN-γ production rate of CHO 42 and CHO 43, respectively and were calculated as described in Section 2.8.1.

Fig 3.4A and Fig 3.4B are plotted from two separate experiments.

A


300
250
200
150
100
50
0
Interferon production (X10^{3}IU/ml)
q_{IFN42} = (8.34±0.70)X10^{-5}
q_{IFN43} = (2.21±0.12)X10^{-3}
(Units/cell/hr)
0 20 40 60 80 100 120 140 160 180 200
Time (hr)
CHO 43
CHO 42

B


300
250
200
150
100
50
0
Interferon production (X10^{3}IU/ml)
q_{IFN42} = (8.09±0.72)X10^{-5}
q_{IFN43} = (1.81±0.15)X10^{-3}
(Units/cell/hr)
0 20 40 60 80 100 120 140 160 180 200
Time (hr)
CHO 43
CHO 42

cells (CHO E10B and CHO E10C; Scahill et al, 1983) generated by the cotransformation of pSV2 IFN-γ (IFN-γ cDNA with SV40 poly (A) tail and SV40 early gene promoter) with pAdD26SV(A)-3 (DHFR expression vector) into CHO DHFR$^-$ cells. It was reported that these two cell lines produced 5 X 10^4 units/ml. IFN-γ CDNA has also been expressed in *E. coli* (25 units/ml cell extracts, after an overnight incubation; Gray et al, 1982), monkey COS-7 cells (100 units/ml; Gray et al, 1982), monkey AP8 cells (100 units/ml; Devos et al, 1982), yeast (3500 units/ml; Derynck et al, 1983) and tobacco protoplasts (5-10% of total protein, Mori et al, 1993). All these recombinant cells, except tobacco protoplasts, produced smaller amounts of IFN-γ than either CHO 42 and CHO 43 cells.

3.1.2.3 IFN-γ protein species

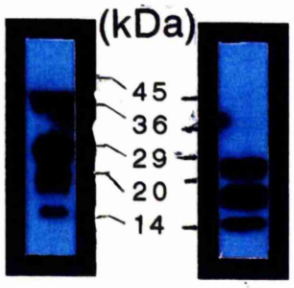
CHO 42 and CHO 43 cells secreted glycosylated forms of IFN-γ as shown by the pattern assessed by western blotting (Fig 3.5). CHO 43 cells produced three forms of recombinant IFN-γ with molecular weights of 17, 21 and 25 kDa. This is consistent with earlier findings from recombinant CHO cells (Anwar, 1994; Scahill et al., 1983; Mutsaers et al., 1986) suggesting that they correspond to non-glycosylated, singly-glycosylated and doubly-glycosylated forms of IFN-γ. CHO 42 cells also produced those three forms of IFN-γ but in medium from CHO 42 cells, an extra form of IFN-γ was detected. Thus CHO 42 cell medium contained 17, 21, 25 and 36 kDa IFN-γ products. The high molecular weight product was 25.9% of total

Figure 3.5 Secreted IFN-γ species of CHO 42 and CHO 43 cells

(A) CHO 42 cells were cultured, as described in Section 23, for 8 days and medium proteins were concentrated 10X with ice cold TCA. CHO 43 cells were cultured for 4 day and medium was collected. The protein samples were resolved in 12% SDS-PAGE (Section 2.7.5.1), Western blotted and detected by ECL (Section 2.7.6). The primary antibody used was monoclonal 20D7 (Section 2.1.1.3.1).

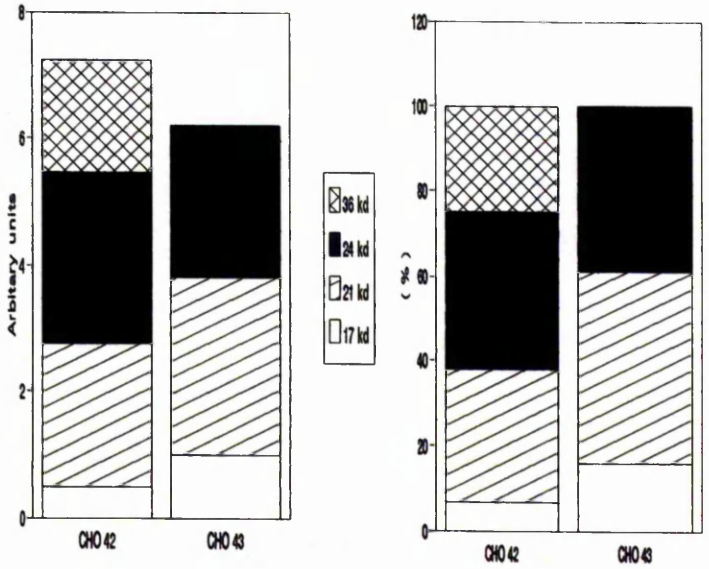
(B) The autoradiograph was scanned as described in Section 2.6.4.3, values are from one experiment. Legends show the molecular weight of each band (eg., 36kd means its molecular weight is 36 kDa).

(C) The percentage of each IFN-γ isoform is expressed as a % of all isoforms of IFN-γ detected.



CHO 42 CHO 43

(A)



(B) (C)

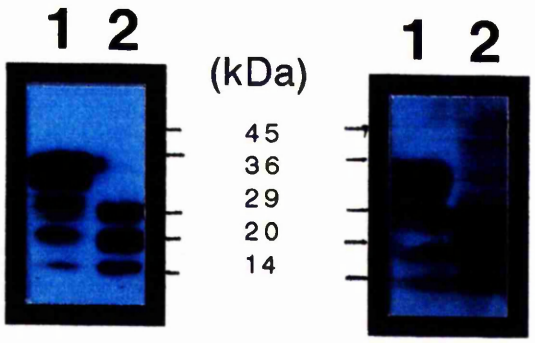
Figure 3.6 Isoform of IFN-γ in CHO 42 and CHO 43 cells

(A and B) CHO 42 and CHO 43 cells were cultured, as described in Section 2.3, for 2 days, cells were trypsinised, washed with PBS to generate a cell suspension of 4 X 10^5 cells/10µl PBS of CHO 42 and 1.5 X 10^5 cells/10µl PBS of CHO 43 cells. Equal amounts of protein sample buffer (Section 2.7.5) were added to the cell suspension and this mixture were resolved in 12% SDS-PAGE (Section 2.7.5.1), Western blotted and IFN-γ was detected by ECL (Section 2.7.6). The primary antibody used was monoclonal 20D7 (A; Section 2.1.1.3.1) and polyclonal, raised in sheep, against *E. coli* derived human IFN-γ (B; Section 2.1.1.3.1).

1 and 2 shown on fig A and Fig B are protein samples of CHO 42 and CHO 43 cells, respectively.

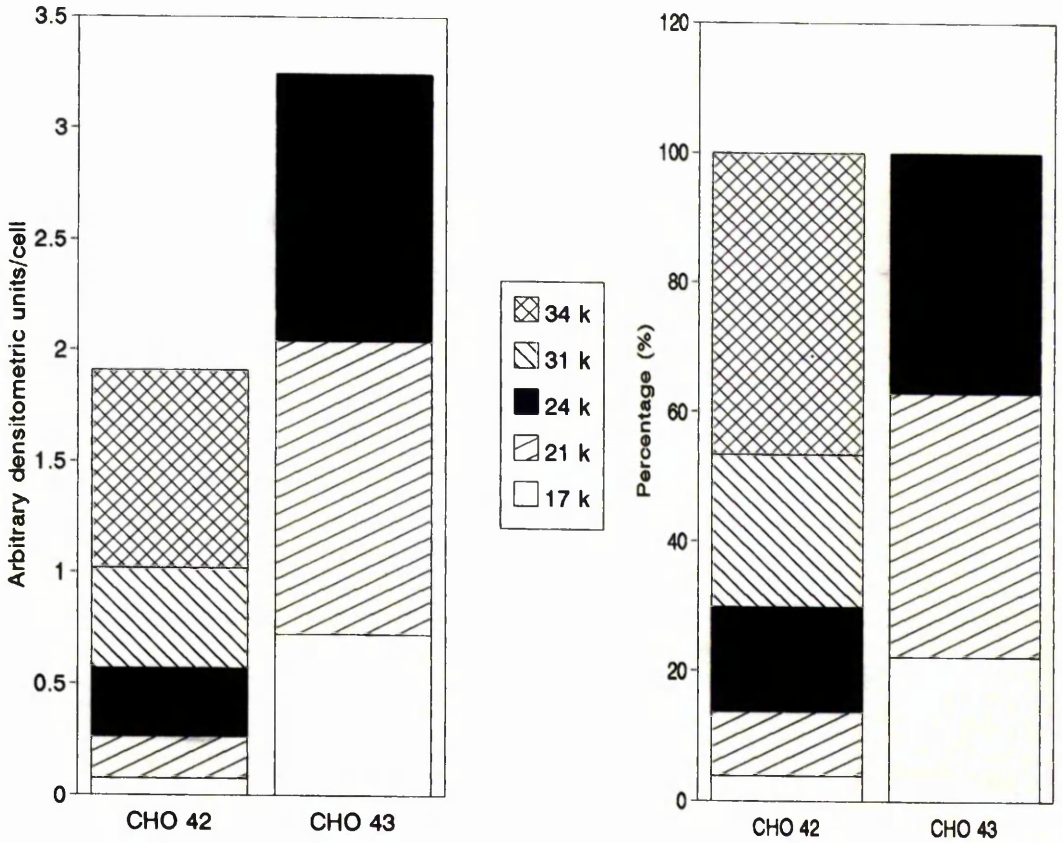
(C) The autoradiographs of IFN-γ were scanned as described in Section 2.6.4.3, values are means of 3 separate experiments. The amount of IFN-γ was calculated from each 1 X 10^5 cell. Legends shows the molecular weight of each band (eg., 34kd means its molecular weight is 34 kDa).

(D) The percentage of each isoform is expressed as a % of all forms of IFN-γ in CHO 42 and CHO 43 cells.


1 2
(kDa)
45
36
29
20
14
1 2

(A)

(B)


Arbitrary densitometric units/cell
3.5
3
2.5
2
1.5
1
0.5
0
CHO 42
CHO 43
34 k
31 k
24 k
21 k
17 k
Percentage (%)
120
100
80
60
40
20
0
CHO 42
CHO 43

(C)

(D)

immunoreactive IFN-γ secreted by CHO 42 cells. The singly-glycosylated form (45.7%) was the major IFN-γ secreted by CHO 43 cells but the doubly-glycosylated form (37.9%) was the major product of CHO 42 cells. Both cell lines secreted little of the non-glycosylated form of IFN-γ (6.9% for CHO 42 cell, 16.4% for CHO 43 cell).

IFN-γ expressed within cells is presented in Fig 3.6. The forms inside CHO 43 cell extracts are similar as those found in media. Based on cellular content CHO 43 cells contained 1.7 times more IFN-γ than CHO 42 cells (Fig 3.6C). Each cell type contains different kinds of isoform of IFN-γ. CHO 42 cells contain five isoforms but CHO 43 cells contain only three isoforms with molecular sizes related to those secreted. In addition to those 3 bands, CHO 42 cells produced two extra immunoactive forms of higher molecular weight, 31 and 34 kDa. These bands were detected with two distinct IFN-γ antibodies (Fig 3.6A and B). These two higher molecular weight products were the major IFN-γ products localised within CHO 42 cells (70.2% of total products). The doubly-glycosylated form (16.03%) was about twice as prevalent as the singly- glycosylated form (9.85%). However, the singly-glycosylated form (40.7%) was the major product within CHO 43 cells.

The three major (17, 21, 25 kDa) forms of recombinant IFN-γ produced by CHO cells have similar mobility to the natural forms of IFN-γ (Mutsaers et al., 1986). Although multiple forms of IFN-γ are produced by tobacco protoplasts (14.5, 18, 23 kDa; Mori et al., 1993) and insect cells (15,

20, 23 kDa; Sareneva et al., 1994) these are of smaller molecular weight than the forms secreted by CHO 42 and CHO 43 cells.

3.1.2.4 IFN-γ gene integrity

My data shows that the major IFN-γ mRNA in CHO 42 cells was 200 bases shorter than would be expected from the structure of p1042 (Section 3.1.2.1). IFN-γ gene integrity was checked in both CHO 42 and CHO 43 cell DNA. Plasmids (p1042 and p1043) and genomic DNA were subjected to restriction digests with the restriction enzyme Hind III and used as templates to amplify the whole IFN-γ gene by PCR using oligomer ACB9 and ACB10. The amplified fragment is shown Fig 3.7A. PCR products showed that the amplified IFN-γ genes from CHO 42 (1.84 kb) and CHO 43 (1.55 kb) cellular DNA had the sizes expected from the structure of p1042 and p1043. These PCR products of the IFN-γ gene were digested with BamH I and EcoR I restriction enzymes and the fragments obtained had the same sizes as predicted from digestion of plasmid p1042 and p1043 (Fig 3.7B). Thus the IFN-γ gene in CHO 42 and CHO 43 cells is intact at this gross level, and the IFN-γ mRNA species in CHO 42 cells are not the result of a gross rearrangement.

The IFN-γ gene in CHO 43 genomic DNA has been proven to be intact using Southern hybridisation (Anwar, 1994). Genomic DNA was also extracted from CHO 42 cells and it was digested with combinations of BamH I, EcoR I and Hind III. The result

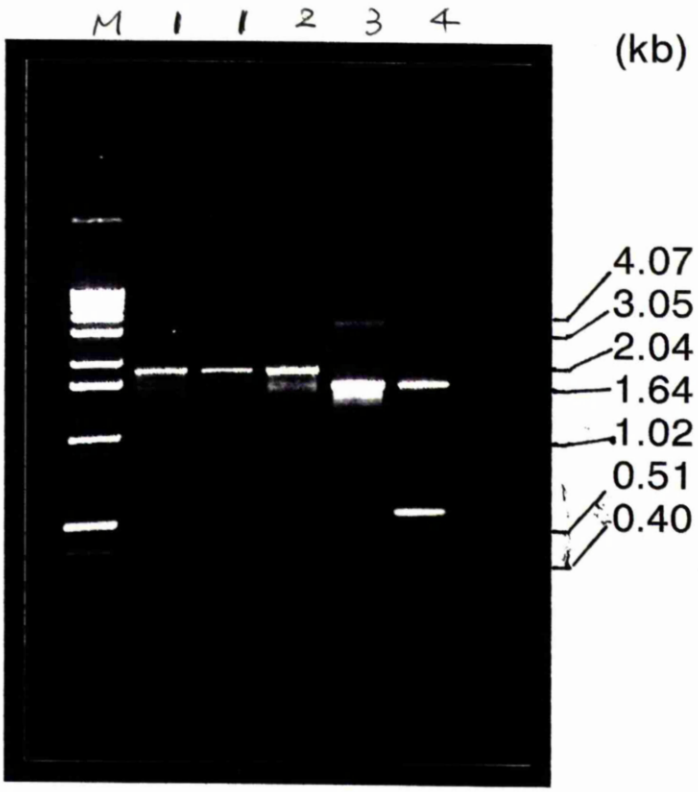
Figure 3.7 PCR amplification of IFN-γ in CHO 42 and CHO 43 cells

(A) Genomic DNA of CHO 42 and CHO 43 cells was extracted as described in Section 2.5.2, 2 µg and 1 µg of CHO 42 (Lane 2) and CHO 43 (Lane 4) genomic DNA and 1ng of plasmid p1042 (Lane 1) and p1043 (Lane 3). All plasmids and genomic DNAs were digested with Hind III before use (Section 2.6.2.1) and used for PCR amplification. Primers used in these amplifications were ACB9 and ACB10 (Section 2.1.1.2), and these cover all the 5' SV40 promoter, IFN-' coding region and 3' β-globin poly(A) tail as described in maps of plasmid p1042 and p1043 (Section 2.1.1.1). The reaction conditions were as described in Section 2.6.3.1. The PCR products in plasmid p1042 and CHO 42 genomic DNA should be 1.82 kb and in plasmid p1043 and CHO 43 genomic DNA should be 1.55 kb.

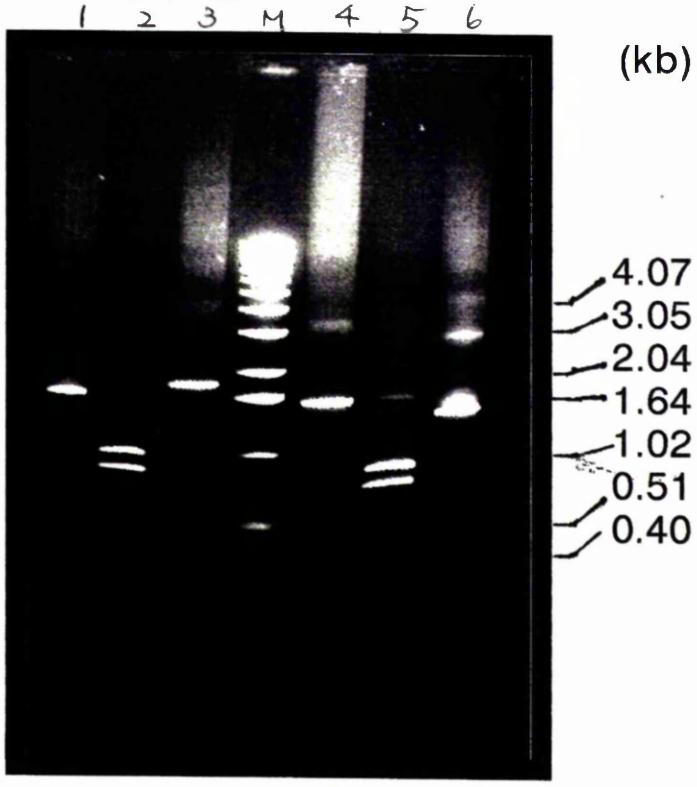
Lane	Source	Expected fragments (kb)	Amplified fragments (kb)
1	p1042	1.82	1.82
2	CHO 42	1.82	1.82
3	p1043	1.55	1.55
4	CHO 43	1.55	1.55, 0.57

(B) The amplified fragments of genomic DNA (Lane 3 and Lane 4; CHO 42 and CHO 43, respectively) were digested with EcorR I (Lane 1; should be 0.21 and 1.61 kb and Lane 6; should be 0.21 and 1.34 kb) and BamH I (Lane 2; should be 0.85 and 0.97kb and Lane 5; should be 0.74 and 0.85 kb).

Lane	Enzyme/source	Size of fragments (kb)	
1	EcoR I, CHO 42	1.61	
2	BamH I, CHO 42	0.97	0.85
3	PCR, CHO 42	1.82	
4	PCR, CHO 43	1.55	
5	BamH I, CHO 43	0.85	0.74
6	EcoR I, CHO 43	1.34	



A:PCR products of IFN-γ in CHO 42 and CHO 43 cells

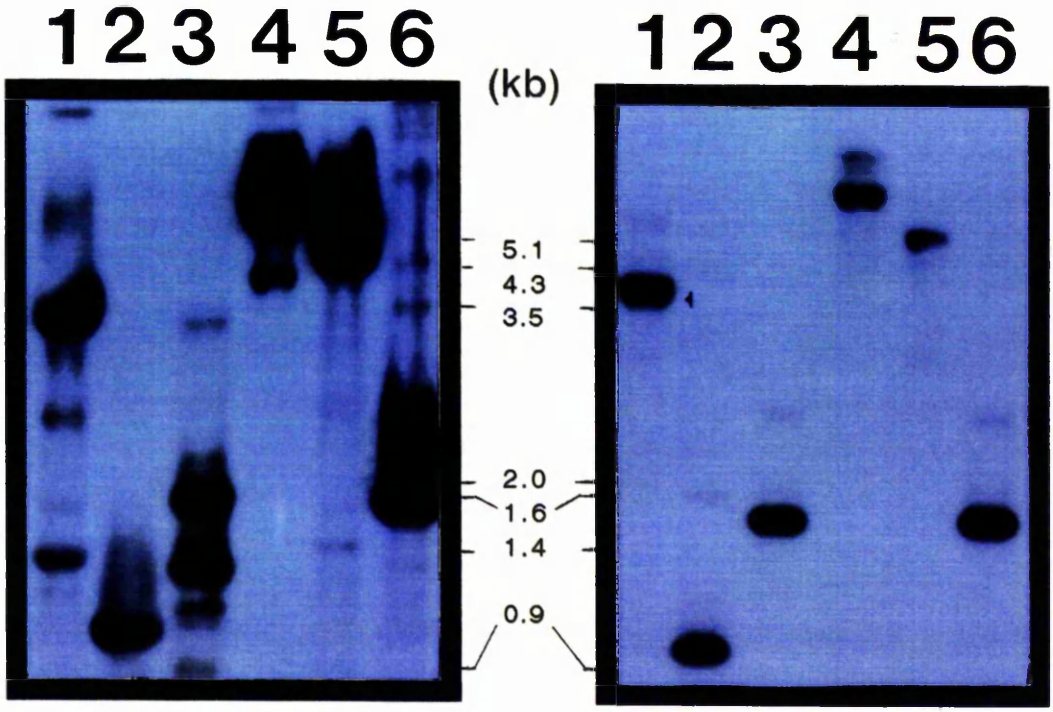


B:EcoR I and BamH I digestion

Figure 3.8 Southern blotting of IFN-γ of plasmid p1042 and CHO 42 cell DNA

Genomic DNA was extracted as described in Section 2.5.2, 10X restriction buffer was added to 10 µg genomic DNA and 1ng of plasmid p1042 samples and these reaction mixtures were incubated overnight with restriction enzyme (Section 2.6.2.1). Digested DNA samples were resolved in 1% agarose gels, Southern blotted, probed with ^{33}P-labelled IFN-γ cDNA. The enzyme used in each lane and expected fragments are listed below, those fragments corresponding to IFN-γ are shown in bold.

		Hybridisation (kb)	
Lane	Enzyme	Expected	Obtained
1	BamH I/Hind III	**4.53**	4.4
2	BamH I/EcoR I	**1.20**	1.1
3	EcoR I/Hind III	**1.85**	1.6
4	Hind III	7.93	7.93
5	BamH I	**6.03**	6.03
6	EcoR I	**1.85**	1.6



(A: Plasmid p1042)

(B: CHO 42 genomic DNA)

Southern hybridisation of CHO 42 (courtesy of Alison Bate)

of Southern hybridisation of IFN-γ is shown in Fig 3.8. The result indicated that the IFN-γ gene in CHO 42 cells is intact. The major hybridisation bands in genomic DNA extracts, digested with BamH I (6.03 kb), EcoR I (1.85 kb), Hind III (7.93 kb), BamH I and EcoR I (1.2 kb), BamH I and Hind III (4.53 kb), and EcoR I and Hind III (1.85 kb), are the same as those obtained from plasmid p1042 digested with equivalent enzymes.

3.1.3 DHFR mRNA expression

Northern hybridisation of DHFR mRNA of RNA samples from CHO 42 and CHO 43 cells is presented in Fig 3.9. There were three DHFR mRNA species in both CHO 42 and CHO 43 cells and they were shown to have molecular sizes of 1.1, 1.6 and 2.4 kb. The 1.6 kb transcript was the major DHFR mRNA in both CHO 42 and CHO 43 cells. In amount the 1.1 kb and 2.4 kb transcripts represented about 20% and 3%, respectively, of the 1.6 kb DHFR mRNA. The 1.6 kb mRNA was the size expected for a transcript from the DHFR cDNA in plasmid p1042 and p1043 (Section 2.2.3). As the 1.1 kb and 2.4 kb are transcripts from the endogenous DHFR gene in CHO cells (Alison Bate personal communication; Carothers et al., 1983), in this thesis I will only focus in 1.6 kb DHFR mRNA.

3.1.4 IFN-γ and DHFR mRNA through batch culture

3.1.4.1 IFN-γ mRNA

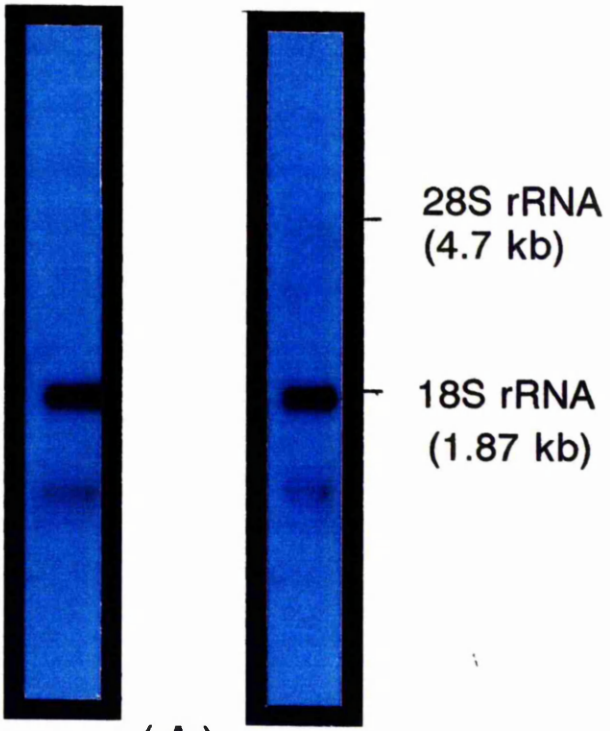
Northern hybridisation of the IFN-γ mRNA through growth is presented in Fig 3.10 and the results are summarised in Fig 3.11. At log-phase CHO 42 cells had two

Figure 3.9 DHFR mRNA expression in CHO 42 and CHO 43 cells

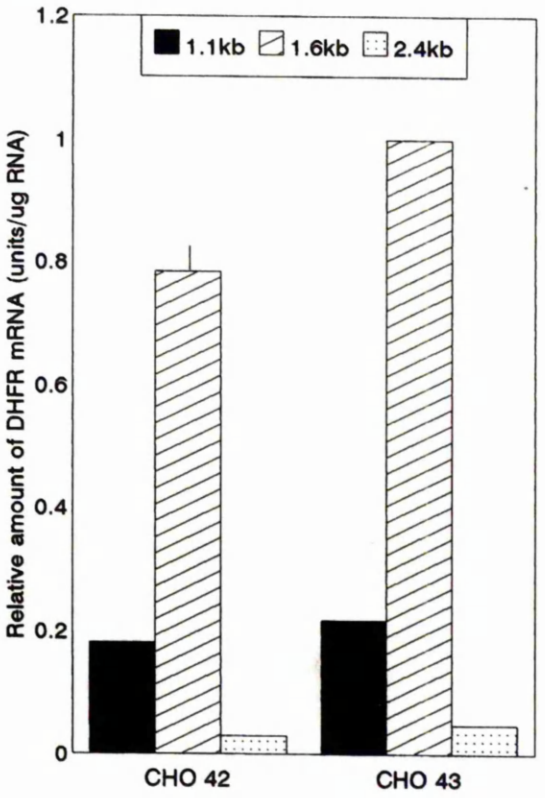
(A) CHO 42 and CHO 43 cells were cultured for two days (mid-log phase) and total RNA was extracted (Section 2.5.3.1). Fifteen µg of each RNA sample was resolved in 1.5% agarose gel (Section 2.6.1.2), Northern blotted (Section 2.6.4.5) and probed with ^{32}P-labelled DHFR cDNA as described in Section 2.6.4.

(B) The autoradiographs of DHFR mRNA were scanned as described in Section 2.6.4.3, values are means of 5 separate experiments. The amount of mRNA was calculated from 15 µg of total RNA. Legends shows the mRNA of CHO cells and its size (eg.: 1.6kb means DHFR mRNA of CHO 42 and CHO 43 and its size is 1.6 kb).

The paragraph shows here is average of 5 separate experiment and is not the data from 3.9A.



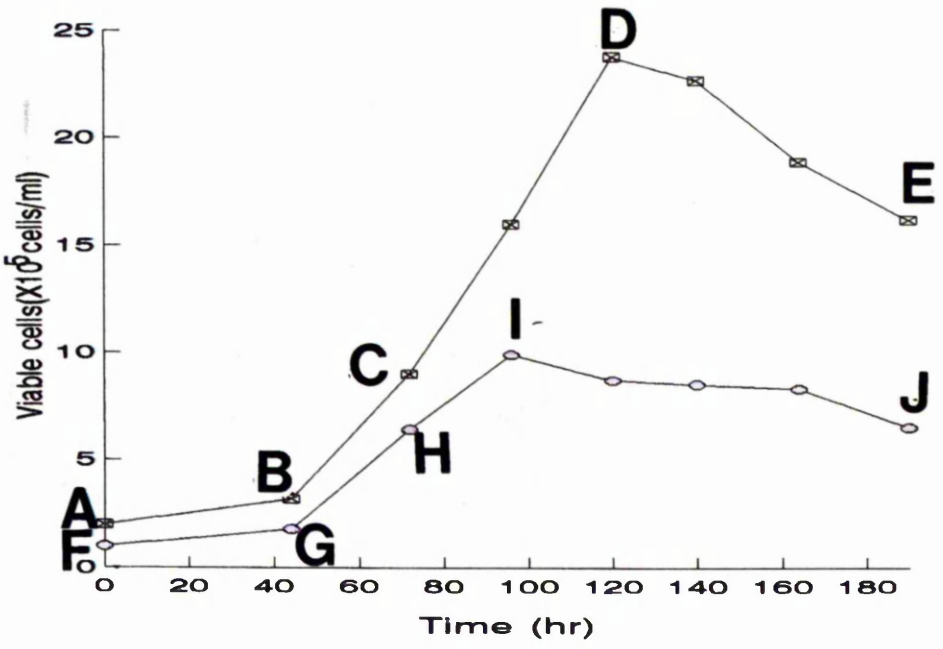
CHO 42 (A) CHO 43



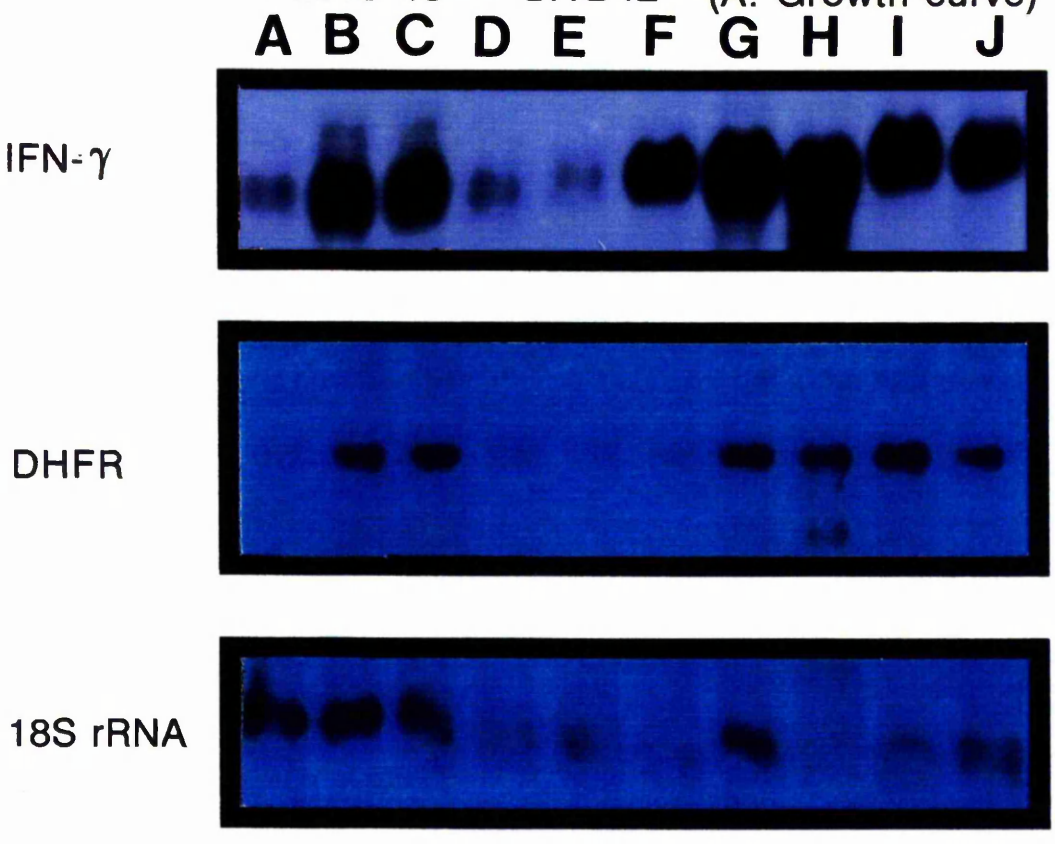
(B)

Figure 3.10 Northern blotting of IFN-γ, DHFR and 18S rRNA through batch culture

CHO 42 and CHO 43 cells were cultured for up to 8 days (decline phase) and total RNA was extracted (Section 2.5.3.1). Fifteen μg of RNA extracts were resolved in 1.5% agarose gels (Section 2.6.1.2), Northern blotted (Section 2.6.4.5) and probed with ^{32}P-labelled DHFR cDNA as described in Section 2.6.4. Samples were taken from each stage of growth curves, A to E from CHO 42 cells and F to J from CHO 43 cells as indicated. A and F were cells from freshly trysinized cells, B and G were cells at early log-phase, C and H were at late log-phase, D and I were early stationary phase and E and J were at death phase.



CHO 43 CHO42 (A: Growth curve)



(B: Autorad)

Figure 3.11 Relative amounts of IFN-γ mRNA through batch culture

(A) The autoradiographs of IFN-γ mRNA in Fig 3.10 were scanned as described in Section 2.6.4.3, values are means of 3 separate experiments. The amount of IFN-γ mRNA was calculated from 15 μg of total RNA and normalised by 18S rRNA. Legends shows the mRNA of CHO cells and its size (eg.: 42 0.98kb means IFN-γ mRNA of CHO 42 and its size is 0.98 kb). Each lane corresponds to those in growth curve, A to E from CHO 42 cells and F to J from CHO 43 cells (Fig 3.10); A and F were from freshly trysinized cells, B and G were cells at early log-phase, C and H were at late log-phase, D and I were early stationary phase and E and J were at death phase. The amount of IFN-γ mRNA in Lane G was set as 1 and others are relative to this sample.

(B) The percentage of each form is given as a % for all forms at each time point.

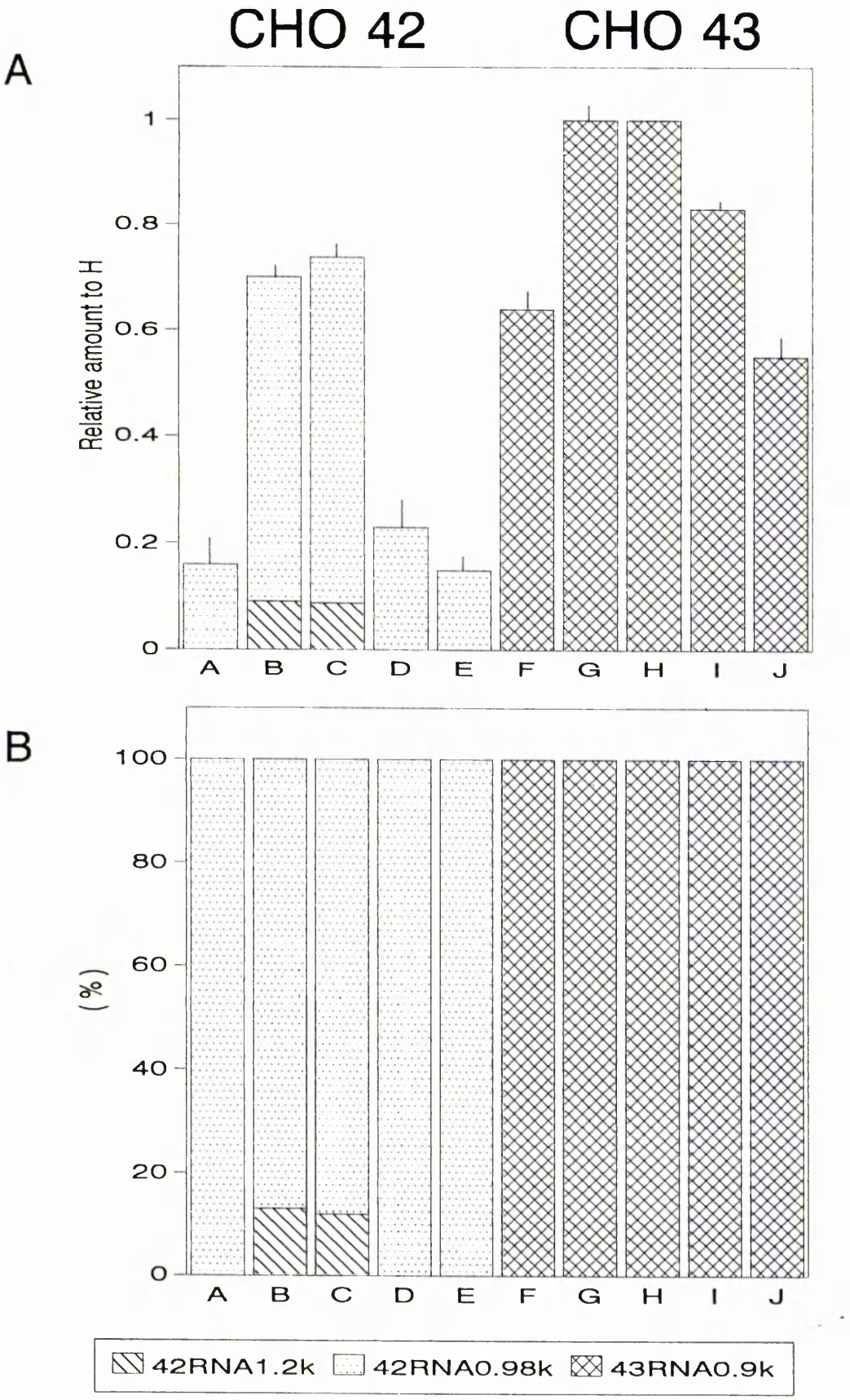

CHO 42
CHO 43
A
Relative amount to H
1
0.8
0.6
0.4
0.2
0
A B C D E F G H I J
B
(%)
100
80
60
40
20
0
A B C D E F G H I J
42RNA1.2k
42RNA0.98k
43RNA0.9k

Figure 3.12 Relative amounts of DHFR mRNA through batch culture

The autoradiographs of DHFR mRNA in Fig 3.10 were scanned as described in Section 2.6.4.3, values are from 1 experiment. The amount of mRNA was calculated from 15 µg of total RNA. Legends shows the 1.6 kb mRNA of CHO 42 and CHO 43 cells. Each lane of sample is shown in growth curve, A to E from CHO 42 cells and F to J from CHO 43 cells (Fig 3.11); A and F were from cells trysinized, B and G were cells at early log-phase, C and H were at late log-phase, D and I were early stationary phase and E and J were at death phase. The amount of DHFR mRNA in Lane G was set as 1 and others are relative to this sample.

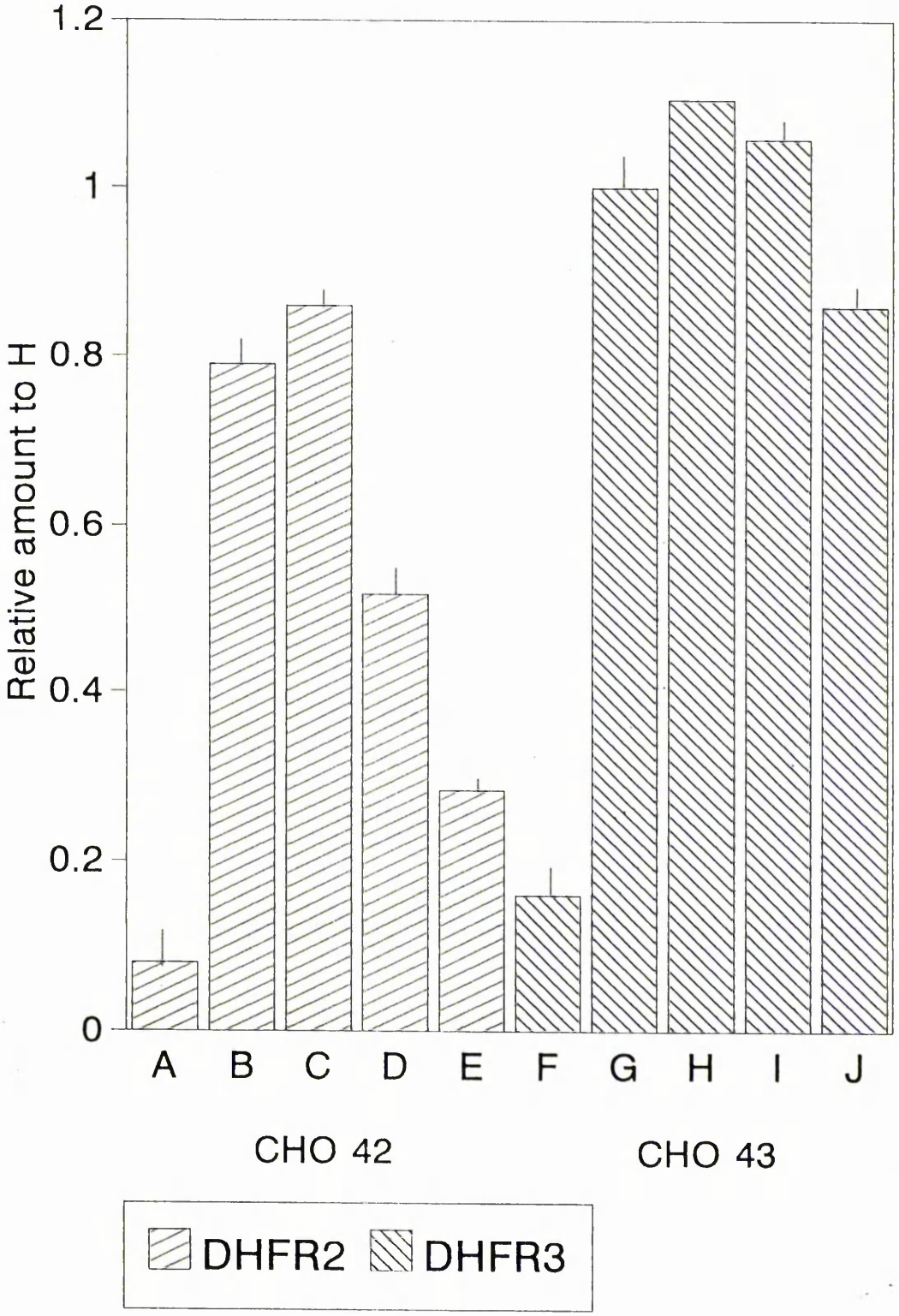
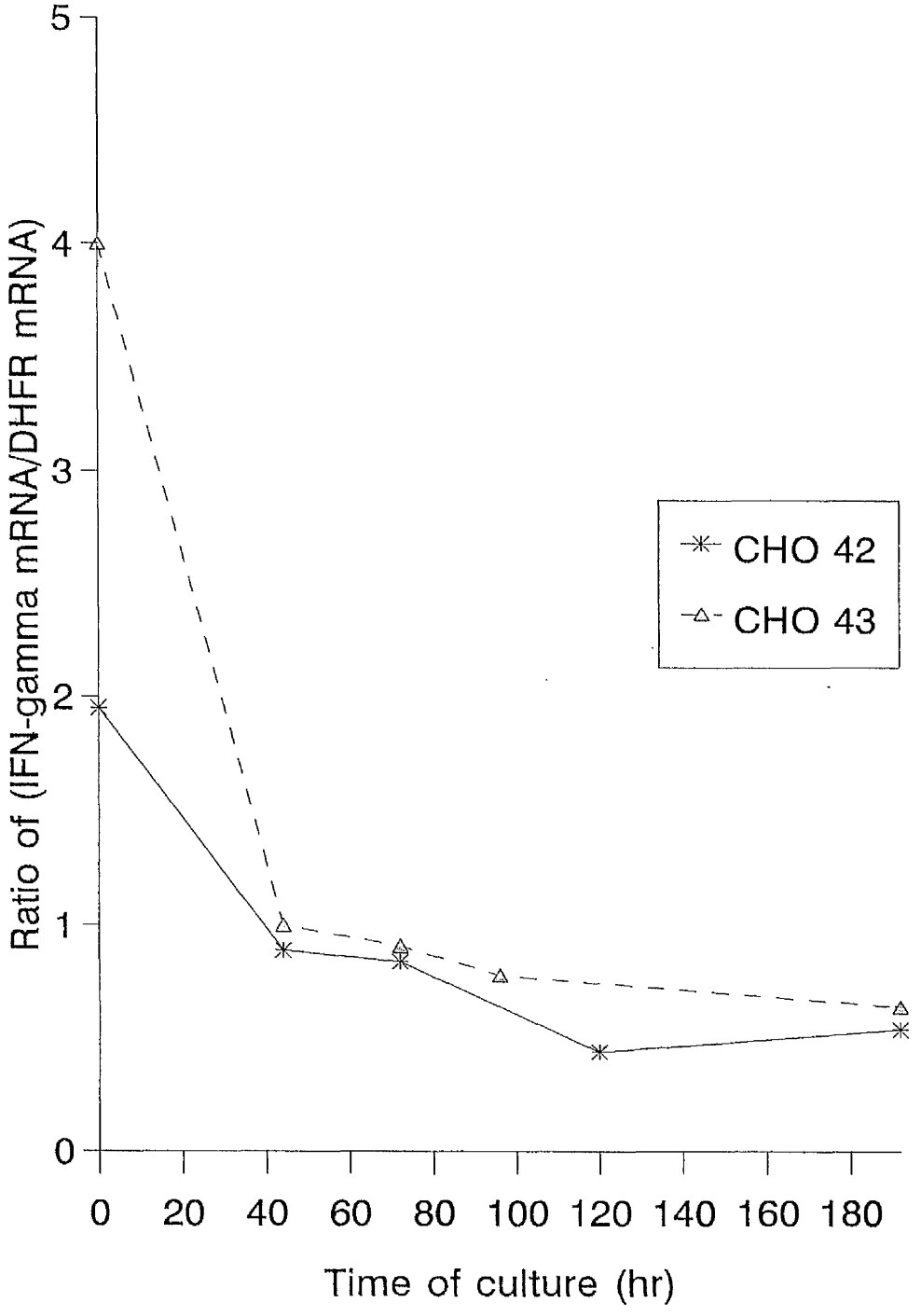

1.2
1
0.8
0.6
0.4
0.2
0
Relative amount to H
A
B
C
D
E
F
G
H
I
J
CHO 42
CHO 43
DHFR2
DHFR3

Figure 3.13 Relative expression of IFN-γ and DHFR mRNA through batch culture

The amounts of IFN-γ mRNA in each sample in Fig 3.11 was divided by amount of DHFR mRNA (Fig 3.12) and the ratio of each sample was plotted in this figure.


Ratio of (IFN-gamma mRNA/DHFR mRNA)
5
4
3
2
1
0
0
20
40
60
80
100
120
140
160
180
Time of culture (hr)
CHO 42
CHO 43

IFN-γ mRNA (1.22 and 0.98 kb) but only one (0.98 kb) at other stages of culture. The 0.98 kb IFN-γ mRNA was the major form detected accounting for more than 80% of the total IFN-γ mRNA and it should be stressed that this was 200 bases less than the size expected (Section 3.1.2.1). In CHO 43 cells, only one IFN-γ mRNA (0.9 kb) was detected. The relationship between the amount of IFN-γ mRNA and growth exhibited a similar pattern in both CHO 42 and CHO 43 cells; the highest content was found at log phase and gradually diminished through stationary phase, and into death phase. In CHO 42 cells the rate of change was more dramatic than for CHO 43 cells.

3.1.4.2 DHFR mRNA

Northern hybridisation of DHFR mRNA through growth is presented in Fig 3.10 and standardised results are presented in Fig 3.12. Patterns of DHFR mRNA expression throughout growth are similar to those determined for IFN-γ (Section 3.1.4.1). At log-phase, for both CHO 42 and CHO 43 cells, the content of DHFR mRNA was greatest and the amount decreased in stationary and death phases.

3.1.4.3 Relative amounts of DHFR and IFN-γ mRNA in CHO 42 and CHO 43 cells

DHFR mRNA was 1.2-3.7 times greater in CHO 43 cells than in CHO 42 cells. The relationship between DHFR mRNA and IFN-γ mRNA is examined in Fig 3.13. There was a similar trend for CHO 42 and CHO 43 cells for expression of DHFR and IFN-γ mRNA. However, this ratio was slightly higher in CHO 43

cells. The ratio of IFN-γ to DHFR mRNA was high at the beginning of cell culture (1.95 in CHO 42, 4 in CHO 43) and this ratio decreased sharply to under 1 after 44 hr of culture (0.89 in CHO 42 cells, 1 in CHO 43 cells). The ratio slowly decreased until 8 days culture (0.54 in CHO 42 cells, 0.64 in CHO 43 cells).

3.2 Messenger RNA stability

The AU-rich sequence contained within natural IFN-γ mRNA (and within the IFN-γ gene in p1042) generates a short half-life to IFN-γ mRNA in natural IFN-γ-producing cells (Chan et al., 1992). However, IFN-γ mRNA can be stabilised by blocking protein translation (Chan et al., 1992; Kaldy and Schmitt-Verhulst, 1991). IFN-γ protein and mRNA expression (Sections 3.1.2.2 and 3.1.2.1) are lower in CHO 42 cells than in CHO 43 cells. This may be a consequence of the differences in IFN-γ mRNA stability. To compare mRNA stability, RNA transcription was inhibited using actinomycin D. Protein translation was blocked using cycloheximide to examine the IFN-γ mRNA stabilisation and possible superinduction in CHO 42 and CHO 43 cells.

3.2.1 Effect of actinomycin D

3.2.1.1 Effect of actinomycin D on CHO 42 and CHO 43 cells

To determine RNA stability, RNA transcription in CHO 42 and CHO 43 cells was inhibited using actinomycin D. Actinomycin D has been reported to change the morphology of

Table 3.3 Effect of actinomycin D on ^{3}H-uridine incorporated into CHO cell lines

Act.D (ug/ml)	CHO42		CHO43	
	DPM	Relative ratio(%)	DPM	Relative ratio(%)
0	43193	100	52635	100
2	19557	25.3	15674	29.8
5	10039	23.2	8777	16.7
10	5171	12.0	7331	14.0
25	1821	4.2	1252	2.4
50	1391	3.2	-150	0

CHO cells were cultured in 24 well plates. After 24 hours incubation, actinomycin D and ^{3}H-uridine were added and incubated for 6 more hours. Then cells were harvested and lysed with SNU solution. RNA was precipitated by adding equal volumes of 20% trichloroacetic acid to lysates. Then RNA precipitates were counted by liquid scintillation counting (Section 2.7.2).

Figure 3.14 Effect of actinomycin D on CHO 42 and CHO 43 cell attachment

CHO 42 and CHO 43 cells were cultured in T_{25} flasks for two days, media were aspirated and cell sheets were washed two times with PBS. One set of cells were replenished with fresh media containing 10 µg/ml actinomycin D, and a control set of cells were replenished with fresh media containing 1 µl/ml ethanol (actinomycin D was dissolved in ethanol to a concentration of 10 mg/ml). Cells were then incubated for up to 7 hr. At each time of sampling cells were washed with PBS, trypsinised, counted as described in Section 2.3.3. Those cells treated with actinomycin D were compared to the control set to determine the relative amount of attached cells.

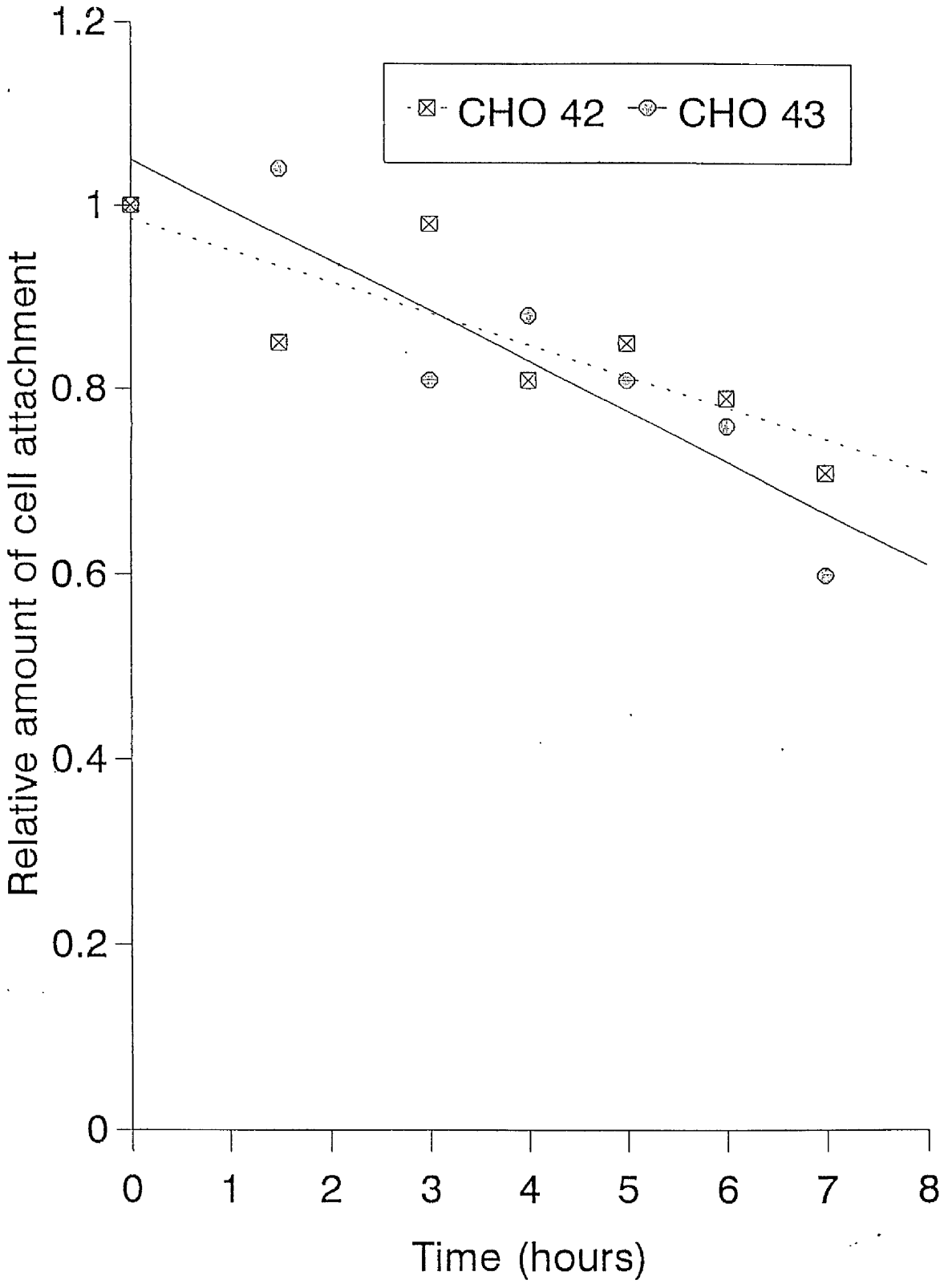
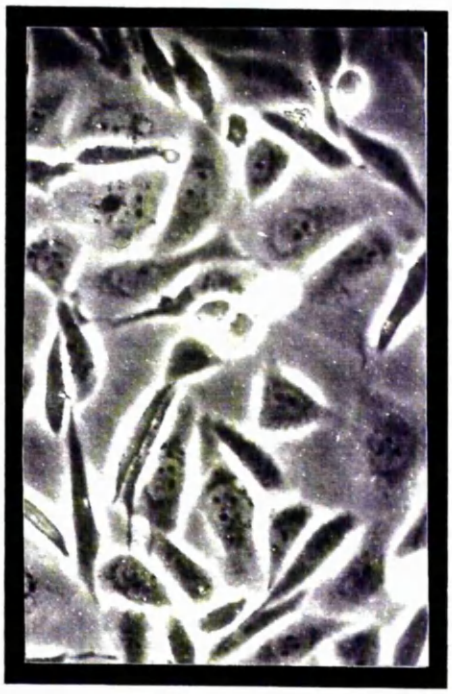
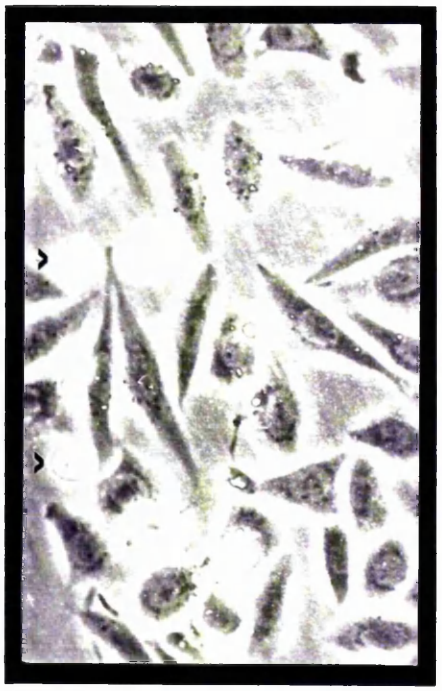

CHO 42
CHO 43
Relative amount of cell attachment
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
Time (hours)

Figure 3.15 Effect of actinomycin D on CHO 42 and CHO 43 cell morphology

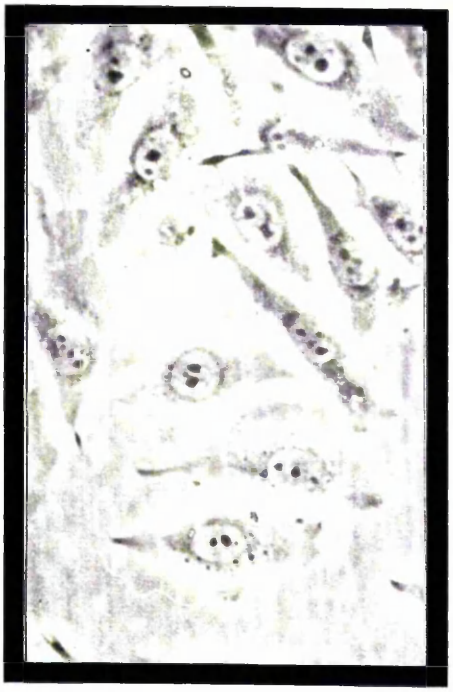
CHO 42 and CHO 43 cells were cultured in T_{25} flasks for two days, medium was aspirated and cell sheets were washed two times with PBS. One set of cells were replenished with fresh media containing 10 µg/ml actinomycin D, and the control set of cells were replenished with fresh media containing 1µl/ml ethanol (actinomycin D was dissolved in ethanol to a concentration of 10 mg/ml). Cells were then incubated for up to 7 hr. At the end of 7 hr incubation cells were photographed under light microscopy.



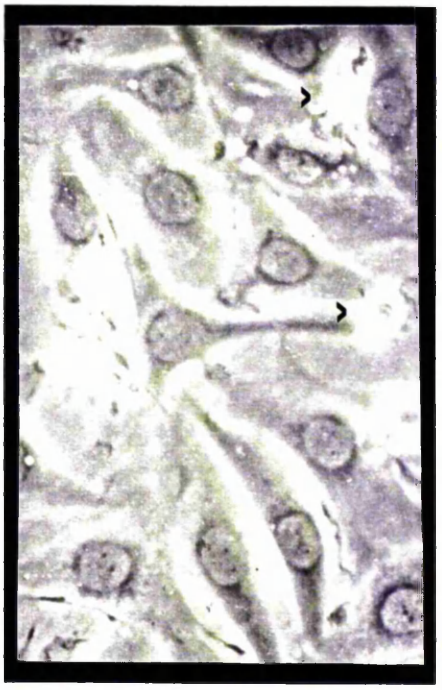
CHO 42 (control)



CHO 42 (actinomycin D)



CHO 43 (control)



CHO 43 (actinomycin D)

100 μm ——————

nuclei (Bernhard, 1971) and to change the expression pattern of hnRNA (Levis and Penman, 1977). Before I started to inspect RNA stability, the effects of actinomycin D on cell morphology, cell attachment and inhibition of total RNA transcription was assessed. The effects on cell attachment are shown in Fig 3.14. After 7 hr of treatment, more than 80% of CHO 42 cells and 70% of CHO 43 cells were still attached to flasks and the morphology of the attached cells was not changed (Fig 3.15). The effects of actinomycin D on transcription was also investigated (Table 3.3). As little as 10 μg actinomycin D/ml was sufficient to inhibit 90% of transcription in both CHO 42 and CHO 43 cells.

3.2.1.2 IFN-γ mRNA stability

Northern hybridization of IFN-γ mRNA in the presence of actinomycin D was used to assess mRNA stability in CHO 42 and CHO 43 cells and this is shown in Fig 3.16 and the standardised results are summarised in Fig 3.17. IFN-γ mRNA in both CHO 42 and CHO 43 cells was relatively stable with a half-life greater than 8 hr. As CHO 42 cells contained two species of IFN-γ mRNA (Section 3.1.2.1), the half-lives of these two mRNA were analyzed separately (Fig 3.17). The 1.22 kb IFN-γ mRNA had a half-life of about 5 hr (5.2 and 5.3 hr in two separate experiments), but the shorter form of IFN-γ mRNA was more stable with a half-life greater than 8 hr. All these three IFN-γ mRNA in CHO 42 and CHO 43 cells exhibit a longer half-life than IFN-γ mRNA in PHA-activated T cell blasts where IFN-γ mRNA had a half-life of less than 2 hr (Chan et al., 1992).

Figure 3.16 Northern blotting of IFN-γ, DHFR and 18S rRNA of actinomycin D-treated cells

CHO 42 and CHO 43 cells were cultured in T_{75} flasks for two days, medium was aspirated and cell sheets were washed two times with PBS. One set of cells were replenished with fresh media containing 10 µg/ml actinomycin D, and a control set of cells were replenished with fresh media containing 1 µl/ml ethanol (actinomycin D was dissolved in ethanol to a concentration of 10 mg/ml). Cells were then incubated for up to 8 hr.

Total RNA was extracted (Section 2.5.3.1) from both actinomycin D-treated and control cells. Fifteen µg of RNA extracts were resolved in 1.5% agarose gel (Section 2.6.1.2), Northern blotted (Section 2.6.4.5) and probed with ^{32}P-labelled cDNA (IFN-γ, DHFR and 18S rRNA) as described in Section 2.6.4. Lane A to G were from control cells and H to N were from actinomycin D-treated cells. A and H were cells from trysinization, B and I, C and J, D and K, E and L, F and M, G and N were cells incubated for 1, 2, 3, 4, 6, 8 hr respectively.

Fig 3.16A and Fig 3.16B show autograph from CHO42 and CHO43, respectively

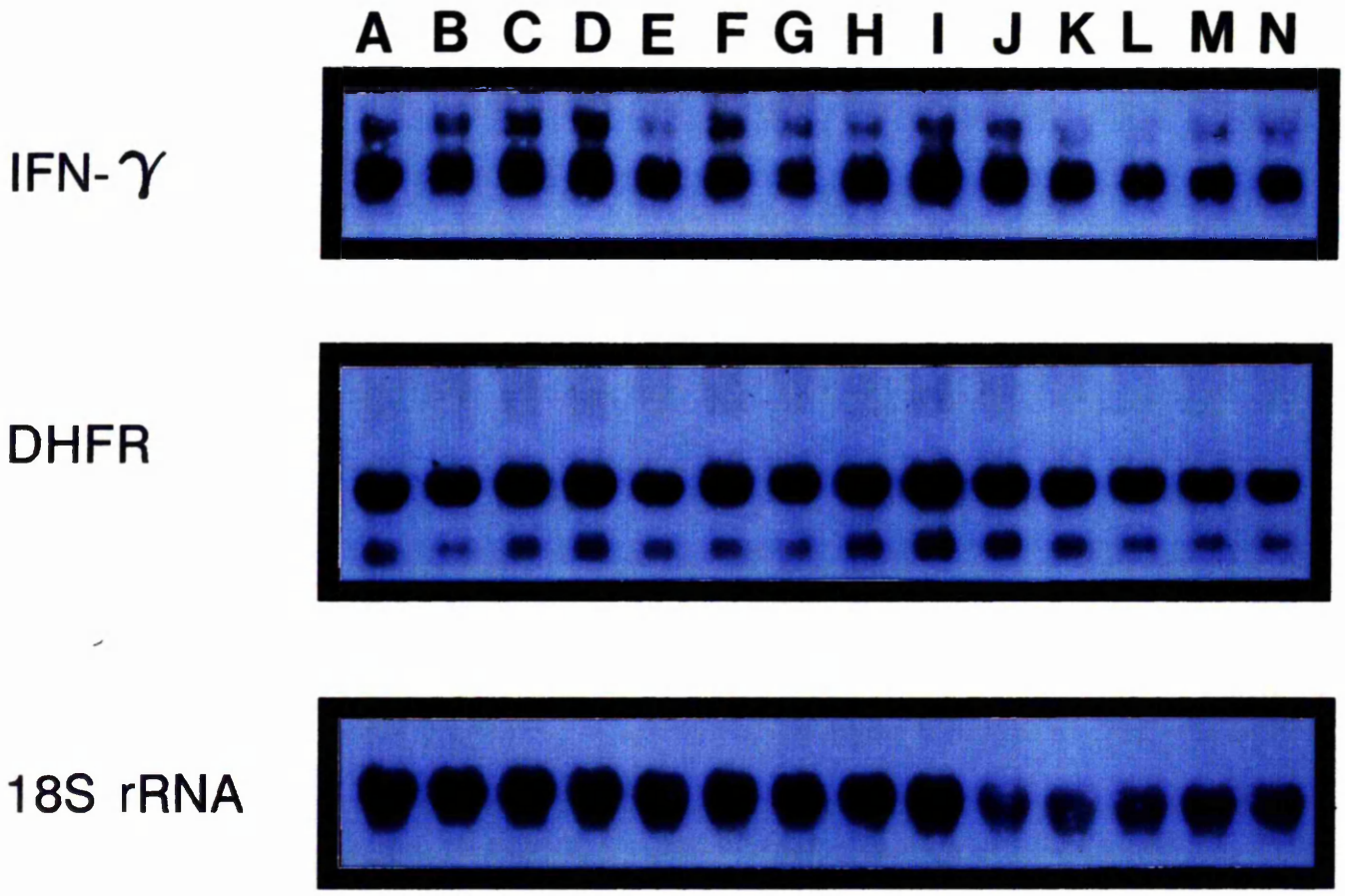


Fig 3.16A IFN-γ and DHFR mRNA stability (CHO 42)

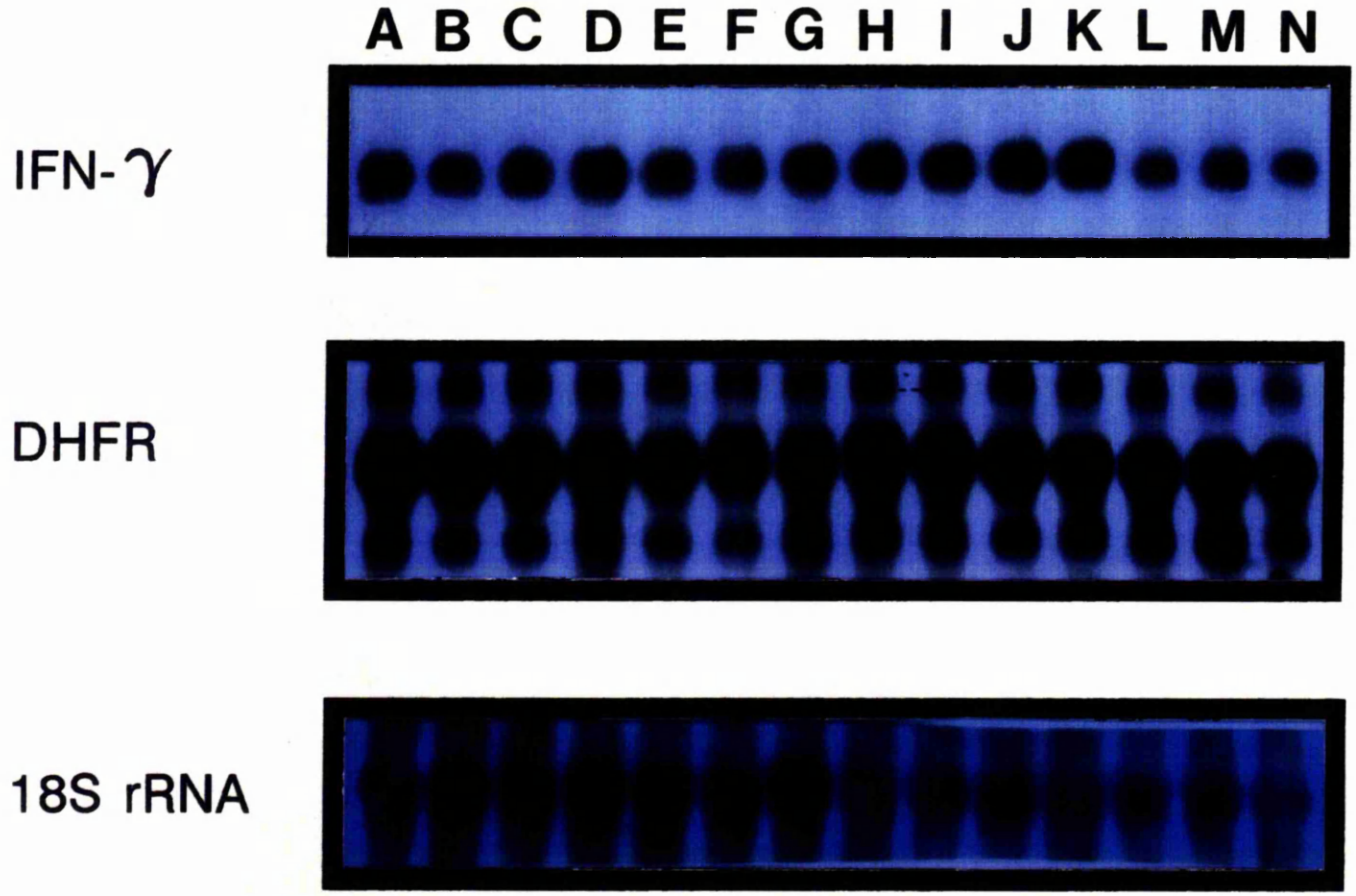
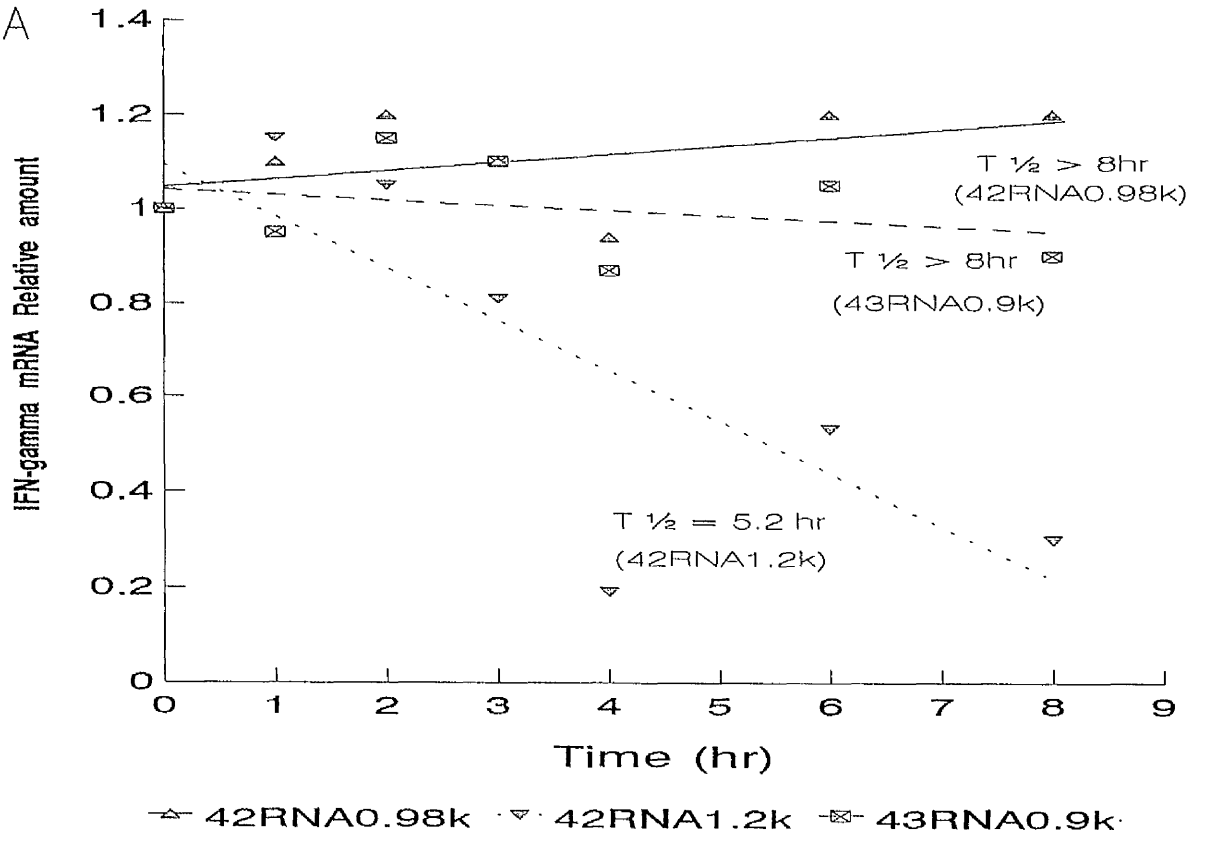


Fig 3.16B IFN-γ and DHFR mRNA stability (CHO 43)

Figure 3.17 IFN-γ mRNA stability in CHO 42 and CHO 43 cells

Autoradiographs of IFN-γ mRNA in Fig 3.16 were scanned as described in Section 2.6.4.3, two separate experiments are shown in this figure. The amount of mRNA was calculated from 15 µg of total RNA and normalised with 18S rRNA. Legends shows the mRNA of CHO cells and its size (eg.: 42 0.98kb means IFN-γ mRNA of CHO 42 and its size is 0.98 kb).

A


IFN-gamma mRNA Relative amount
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9
Time (hr)
T ½ > 8hr
(42RNA0.98k)
T ½ > 8hr
(43RNA0.9k)
T ½ = 5.2 hr
(42RNA1.2k)
42RNA0.98k
42RNA1.2k
43RNA0.9k

B

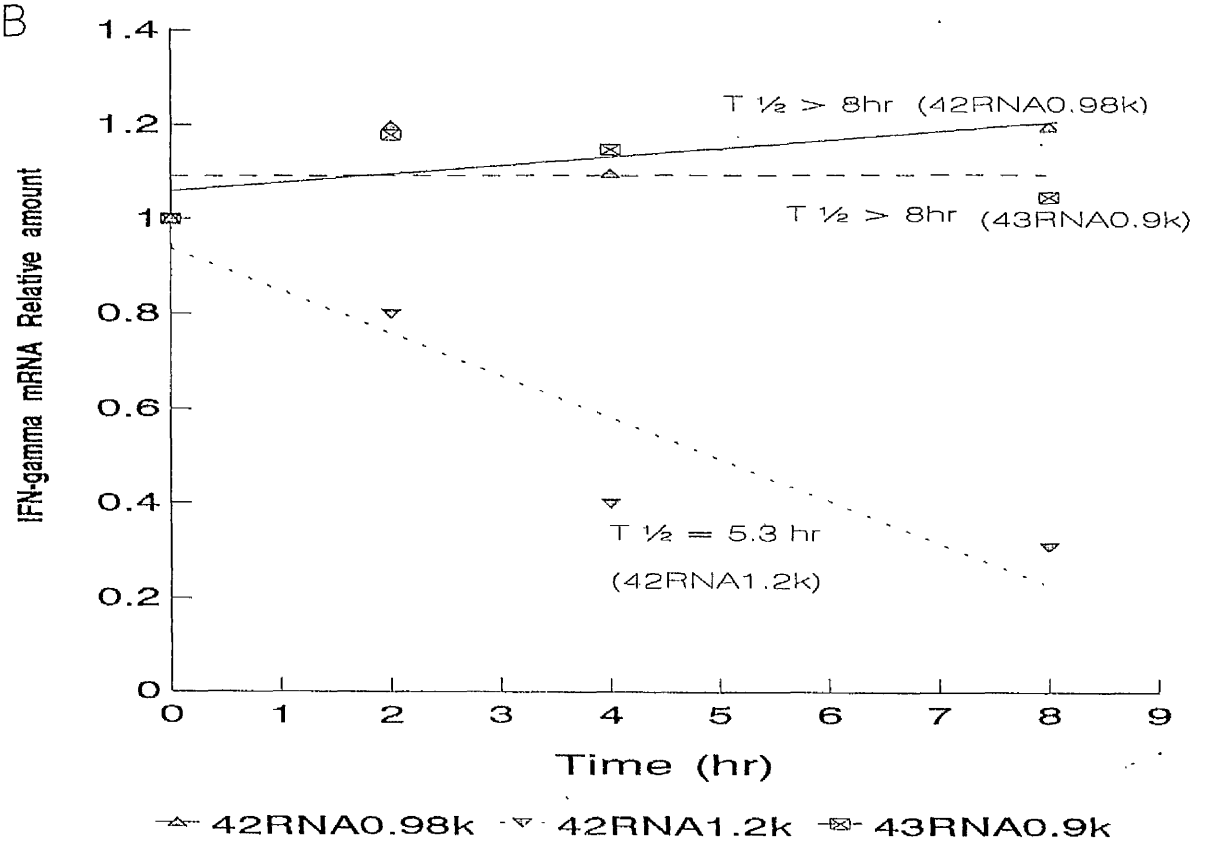
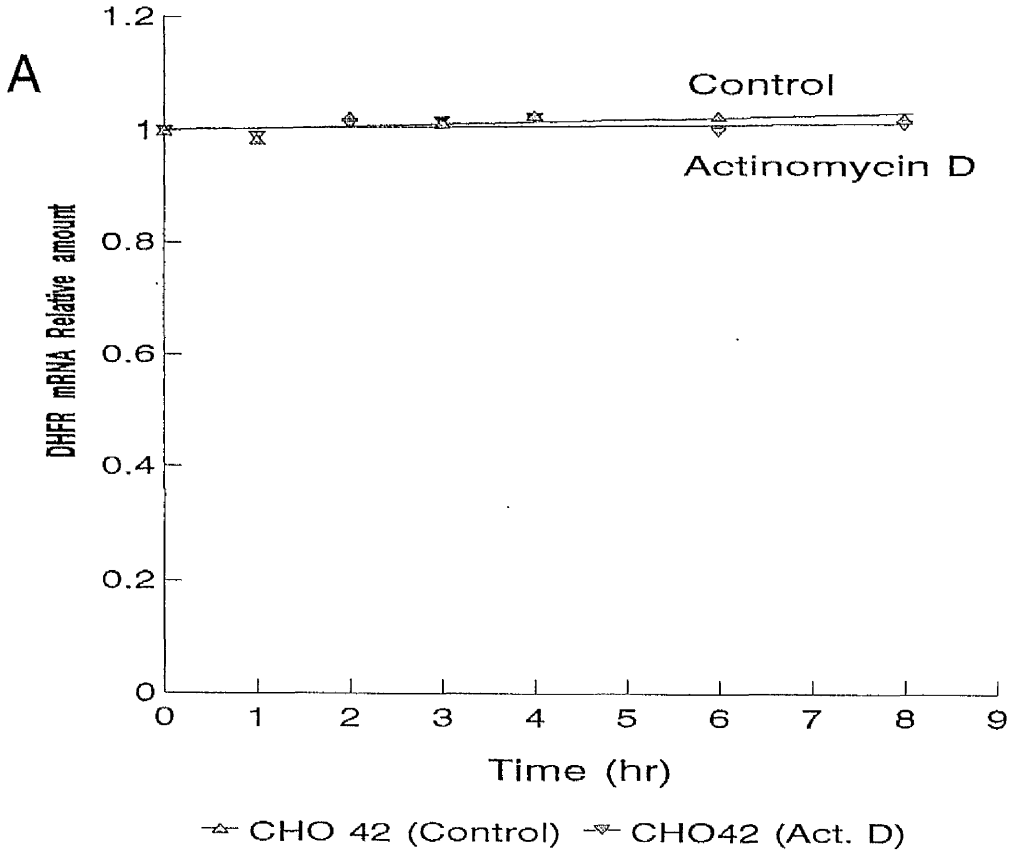
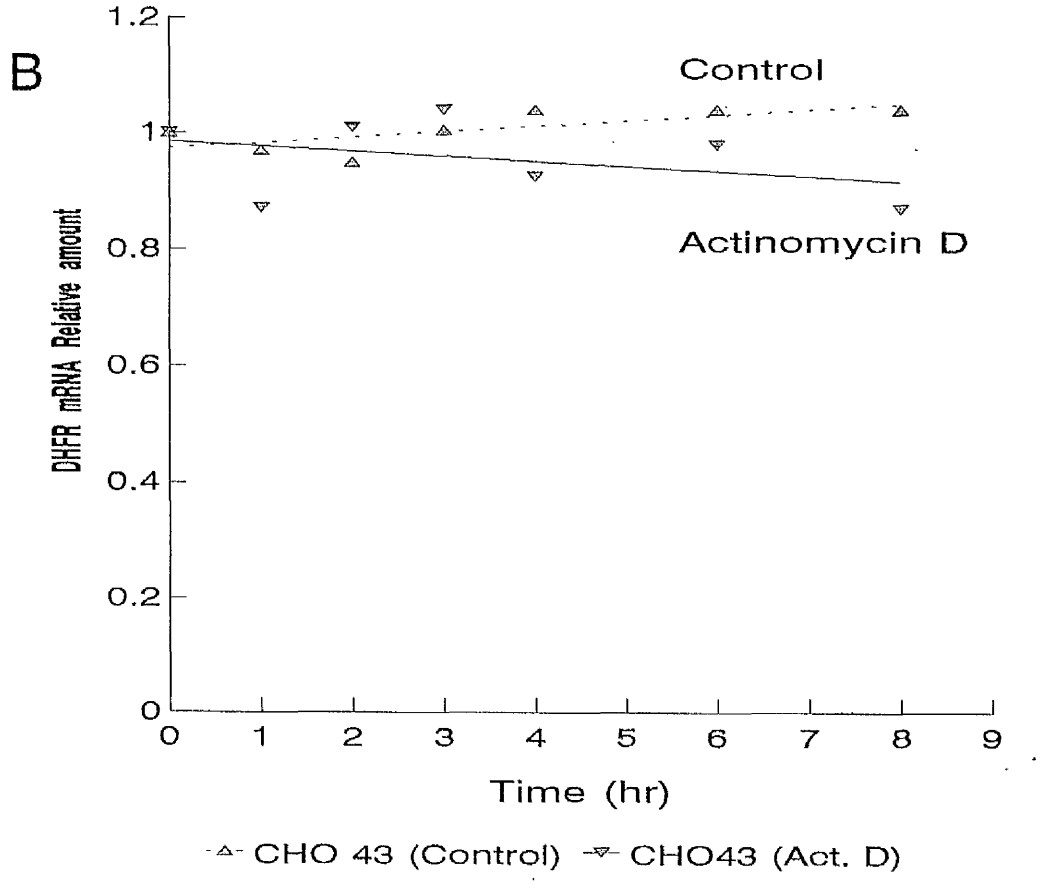

IFN-gamma mRNA Relative amount
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9
Time (hr)
T ½ > 8hr (42RNA0.98k)
T ½ > 8hr (43RNA0.9k)
T ½ = 5.3 hr
(42RNA1.2k)
42RNA0.98k
42RNA1.2k
43RNA0.9k

Figure 3.18 DHFR mRNA stability in CHO 42 and CHO 43 cells

The autoradiographs of DHFR mRNA in Fig 3.16 were scanned as described in Section 2.6.4.3, a single experiment is shown in this figure. The amount of mRNA was calculated from 15 µg of total RNA and normalised with 18S rRNA.


A
Control
Actinomycin D
DHFR mRNA Relative amount
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9
Time (hr)
CHO 42 (Control)
CHO42 (Act. D)


B
Control
Actinomycin D
DHFR mRNA Relative amount
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6 7 8 9
Time (hr)
CHO 43 (Control)
CHO43 (Act. D)

3.2.1.3 DHFR mRNA stability

Similar analyses were made for DHFR mRNA (Fig 3.16) and results are summarised in Fig 3.18. DHFR mRNA in CHO 42 and CHO 43 cells had similar stabilities with a half-life greater than 8 hr.

3.2.2 Effect of cycloheximide

To investigate the effects of translation inhibition on IFN-γ mRNA stability, CHO 42 and CHO 43 cells were treated with cycloheximide.

3.2.2.1 Effect of cycloheximide on CHO 42 and CHO 43 cells

When added to medium, at a concentration of 5 μg/ml, cycloheximide inhibited incorporation of ^{3}H-leucine into TCA-precipitable material by more than 90% (Table 3.4). After 7 hr of treatment, 70% of cells were still attached to flasks (Fig 3.19) and the morphology of the attached cells was not different from control cells (Fig 3.20).

3.2.2.2 Effects of cycloheximide on IFN-γ mRNA

After cycloheximide administration, RNA samples were collected. Northern hybridisation of IFN-γ mRNA from these experiments is presented in Fig 3.21 and standardised results are summarized in Fig 3.22. In both CHO 42 and CHO 43 cells, the level of IFN-γ mRNA was not changed by cycloheximide treatment. This is unlike the response which occurs to IFN-γ mRNA in natural IFN-γ producing cells where IFN-γ mRNA was superinduced or stabilised by addition of cycloheximide (Chan et al., 1992; Kaldy and Schmitt-Verhulst, 1991; Lebendiker et al., 1987).

Table 3.4 Effect of cycloheximide on protein synthesis in CHO 42 and CHO 43 cells

Cycloheximide concentration (µg/ml)	Synthesis rate (%)	
	CHO 42 cells	CHO 43 cells
Control	100	100
5	5.5	6.2
10	3.9	5.5
15	3.5	4.4
20	2.9	2.9

CHO cells were cultured in 24 well plates. After 24 hours incubation, cycloheximide and ^{3}H-leucine were added andincubated for 6 more hours. Then cells were harvested and lysed. Protein was precipitated by adding an equal volume of 10% trichloroacetic acid containing 10 mM leucine. Then protein precipitates were dissolved in SCN solubilizer and 2ml of scintillation cocktail was added and samples were counted by liquid scintillation counting (Section 2.7.7.1.1).

Figure 3.19 Effect of cycloheximide on CHO 42 and CHO 43 cell attachment

CHO 42 and CHO 43 cells were cultured in T_{25} flasks for two days, media were aspirated and cell sheets were washed two times with PBS. One set of cells were replenished with fresh media containing 10 µg/ml cycloheximide, and a control set of cells were replenished with fresh media containing 1 µl/ml sterile water (cycloheximide was dissolved in water to a concentration of 10 mg/ml). Cells were then incubated for up to 7 hr. At each time of sampling cells were washed with PBS, trypsinised, counted as described in Section 2.3.3. Those cells treated with cycloheximide were compared to the control set to determine the relative amount of cell attachment.

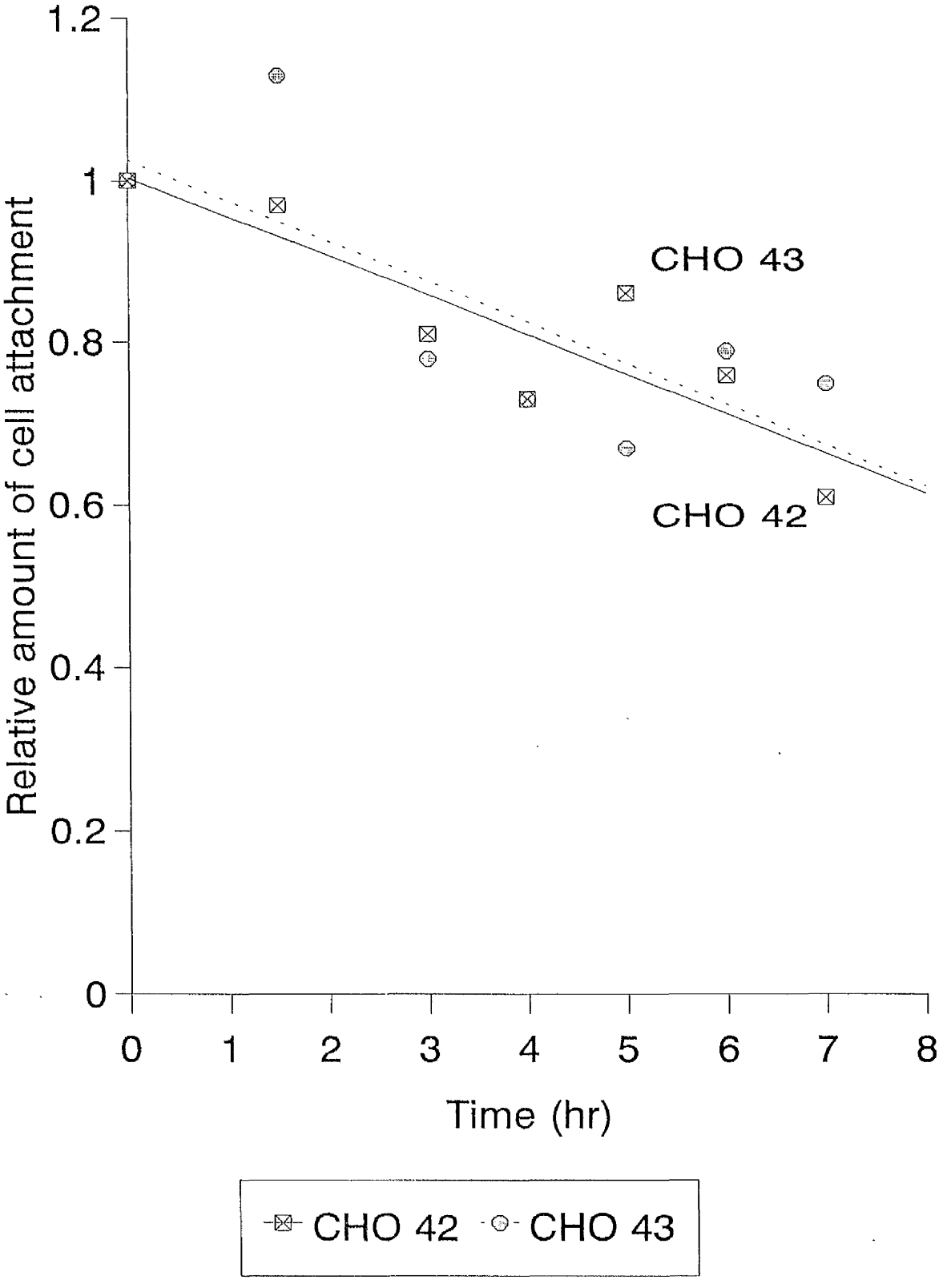
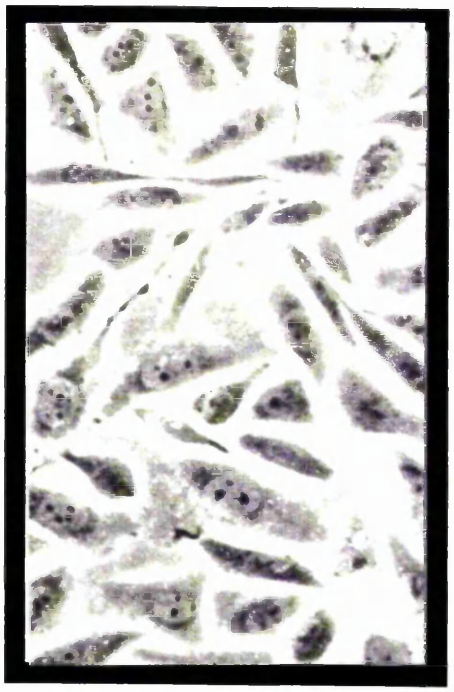
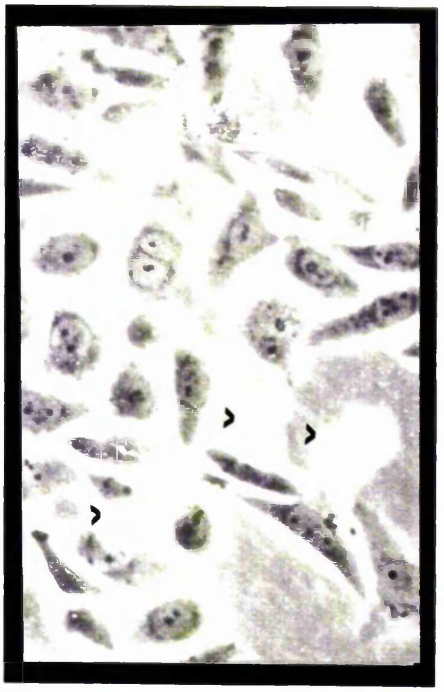

1.2
1
0.8
0.6
0.4
0.2
0
Relative amount of cell attachment
CHO 43
CHO 42
0
1
2
3
4
5
6
7
8
Time (hr)
CHO 42
CHO 43

Figure 3.20 Effect of cycloheximide on CHO 42 and CHO 43 cell morphology

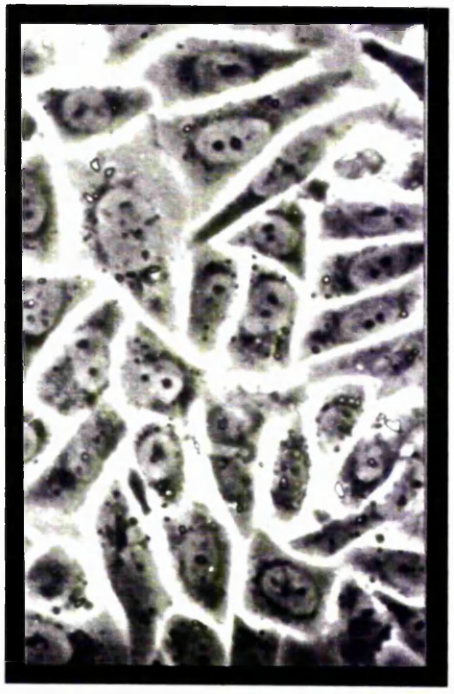
CHO 42 and CHO 43 cells were cultured in T_{25} flasks for two days, medium was aspirated and cell sheets were washed two times with PBS. One set of cells were replenished with fresh media containing 10 µg/ml cycloheximide, and the control set of cells were replenished with fresh media containing 1 µl/ml sterile water (cycloheximide was dissolved in water to a concentration of 10 mg/ml). Cells were then incubated for up to 7 hr. At the end of 7 hr incubation cells were photographed under light microscopy.



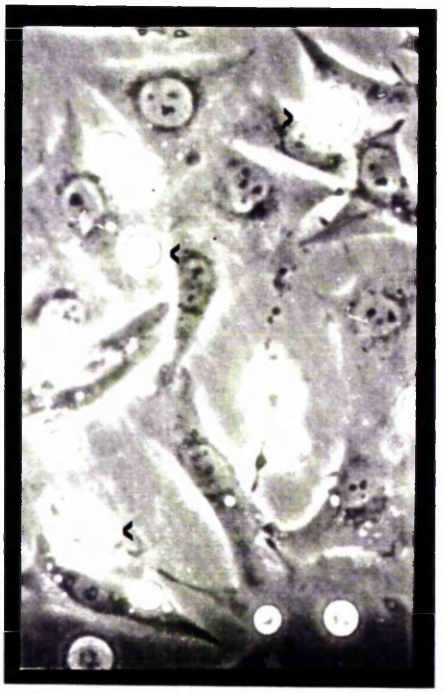
CHO 42 (control)



CHO 42 (cycloheximide)



CHO 43 (control)



CHO 43 (cycloheximide)

100 μm

Figure 3.21 Northern blotting of IFN-γ, DHFR and 18S rRNA of cycloheximide-treated cells

CHO 42 and CHO 43 cells were cultured in T_{75} flasks for two days, medium was aspirated and cell sheets were washed two times with PBS. One set of cells were replenished with fresh media containing 10 µg/ml cycloheximide, and a control set of cells were replenished with fresh media containing 1 µl/ml sterile water (cycloheximide was dissolved in water to a concentration of 10 mg/ml). Cells were then incubated for up to 8 hr.

Total RNA was extracted (Section 2.5.3.1) from both cycloheximide-treated and control cells. Fifteen µg of RNA extracts were resolved in 1.5% agarose gel (Section 2.6.1.2), Northern blotted (Section 2.6.4.5) and probed with ^{32}P-labelled cDNA (IFN-γ, DHFR and 18S rRNA) as described in Section 2.6.4. Lane A to G were from control cells and H to N were from cycloheximide-treated cells. A and H were cells from trysinization, B and I, C and J, D and K, E and L, F and M, G and N were cells incubated for 1, 2, 3, 4, 6, 8 hr, respectively.

Fig 3.21A and Fig 3.21B show autograph from CHO 42 and CHO 43, respectively.

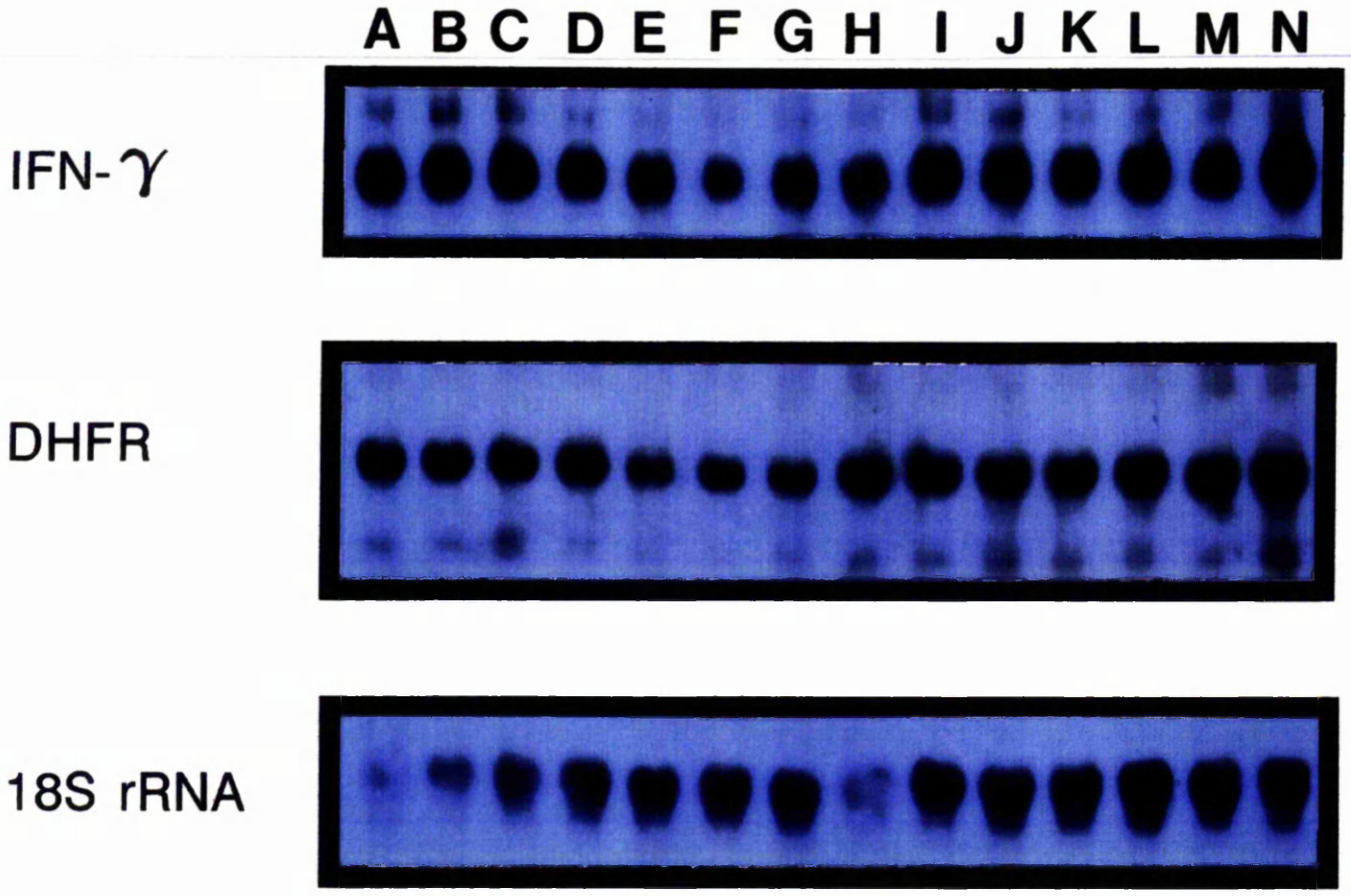


Fig 3.21 A Effect of cycloheximide on DHFR and IFN-γ mRNA (CHO 42)

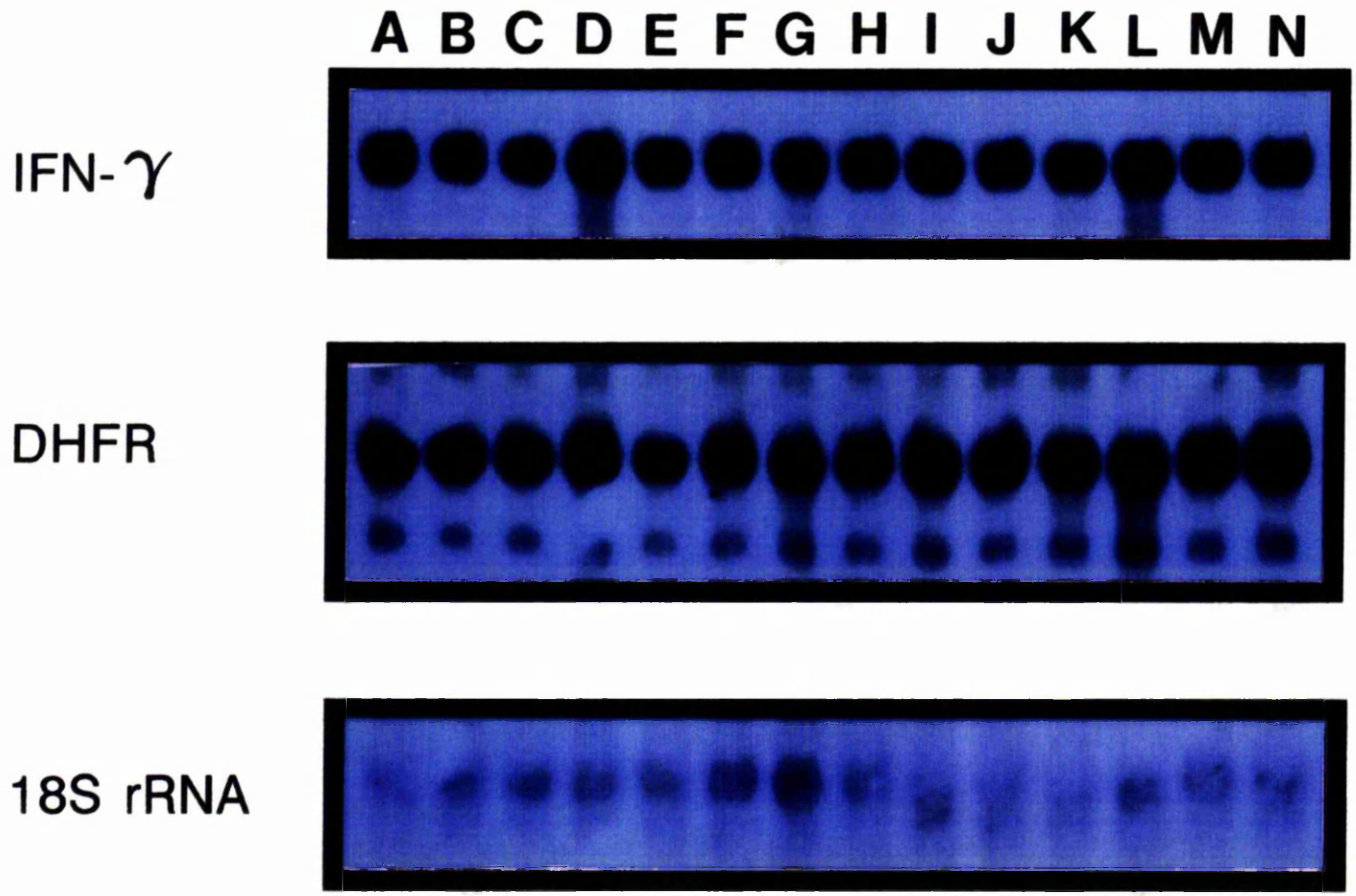
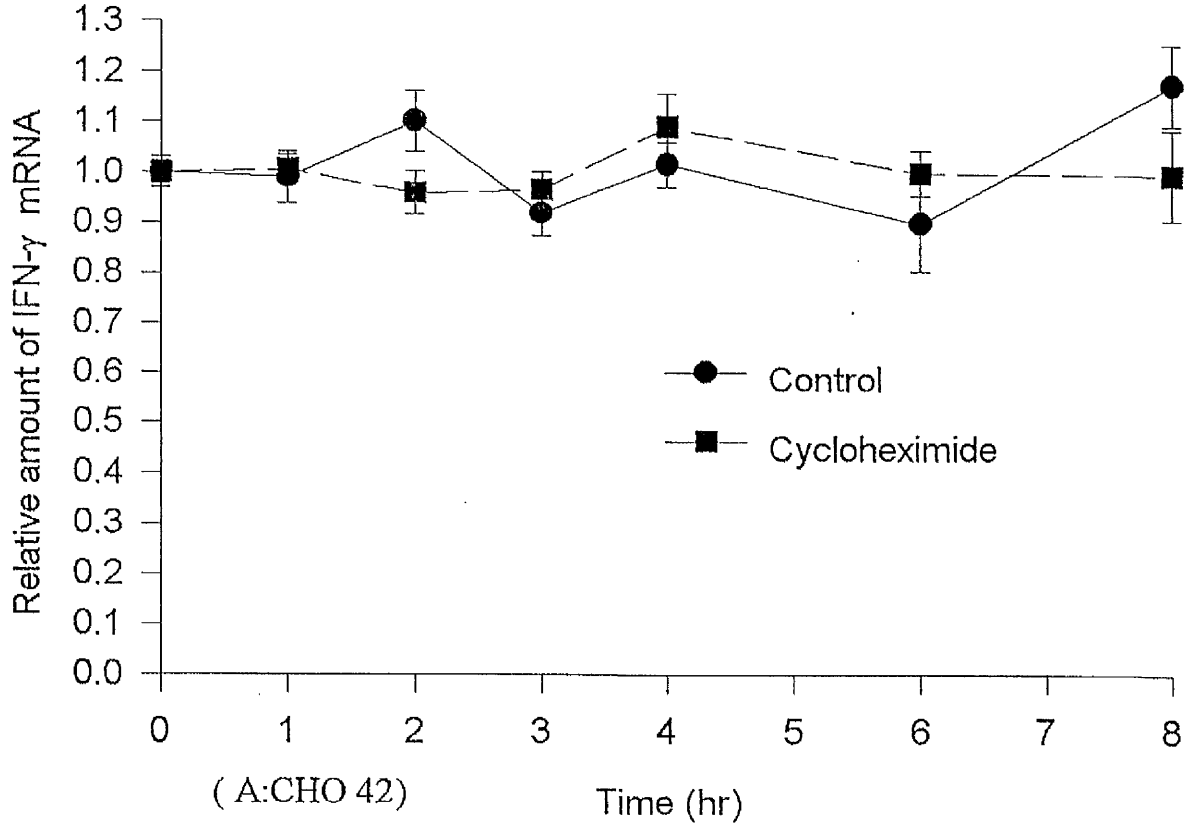


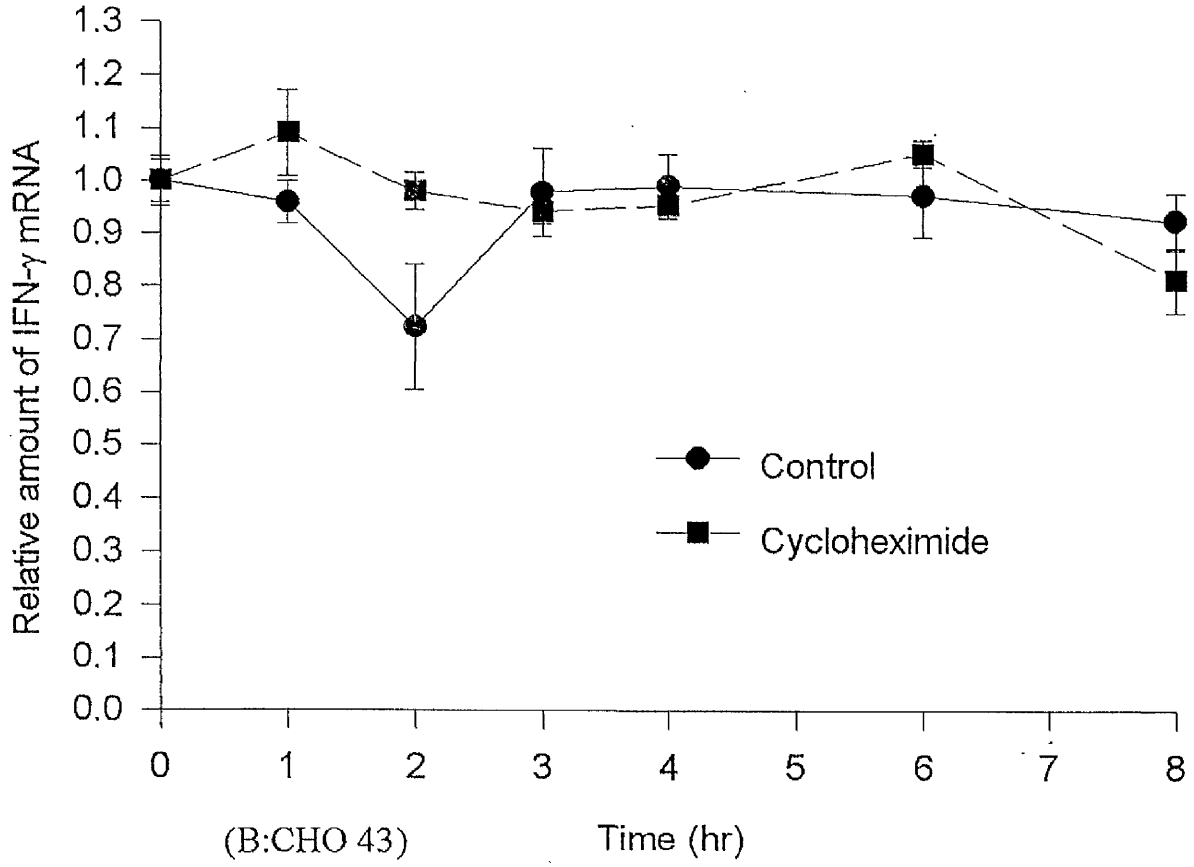
Fig 3.21B Effect of cycloheximide on DHFR and IFN-γ mRNA (CHO 43)

Figure 3.22 Effect of cycloheximide on IFN-γ mRNA in CHO 42 and CHO 43 cells

The autoradiographs of IFN-γ mRNA in Fig 3.21 were scanned as described in Section 2.6.4.3, values are means±SEM of three separate experiments. The amount of IFN-γ mRNA was calculated from 15 μg of total RNA and normalised with 18S rRNA.



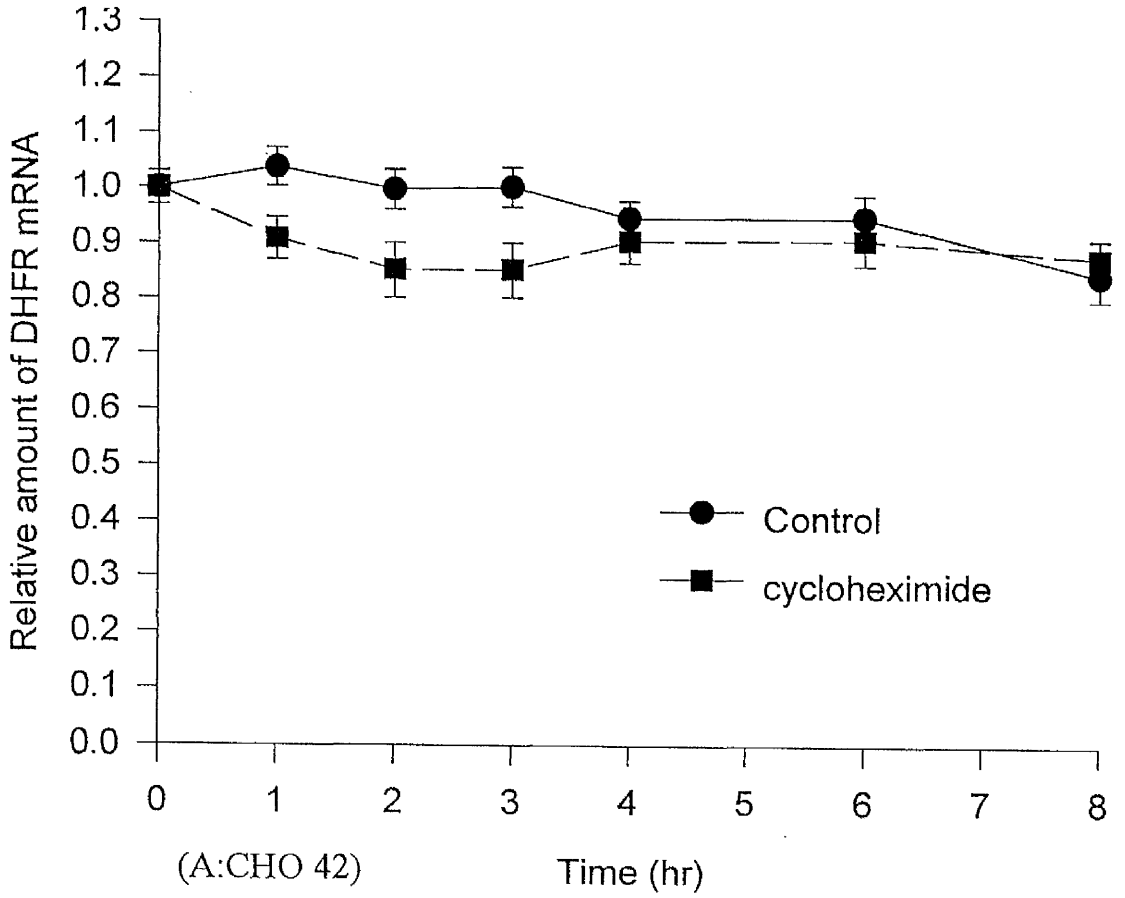
(A:CHO 42)



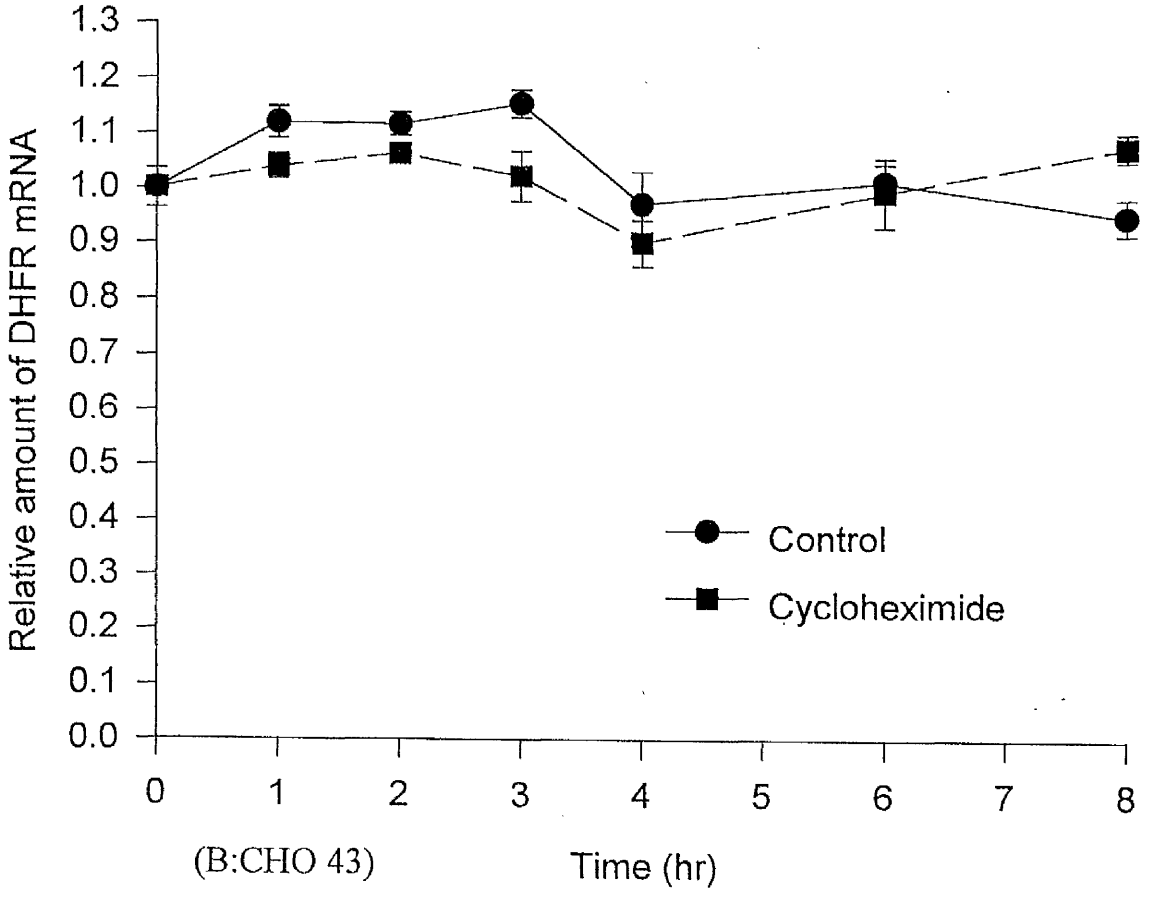
(B:CHO 43)

Figure 3.23 Effect of cycloheximide on DHFR mRNA in CHO 42 and CHO 43 cells

The autoradiographs of DHFR mRNA in Fig 3.21 were scanned as described in Section 2.6.4.3, values are means±SEM of three separate experiments. The amount of DHFR mRNA was calculated from 15 µg of total RNA and normalised with 18S rRNA.



(A:CHO 42)



(B:CHO 43)

3.2.2.3 Effects of cycloheximide on DHFR mRNA

Northern hybridisation of DHFR mRNA from cells treated with cycloheximide is shown on Fig 3.21 and standardized results are summarized in Fig 3.23. Cycloheximide had little effect on DHFR mRNA in either CHO 42 and CHO 43 cells. After 8 hr of treatment, DHFR mRNA expression was not affected by cycloheximide.

3.3 IFN-γ protein metabolism

The rate of protein translation in CHO 42 and CHO 43 cells was monitored using ^{35}S-methionine labelling and IFN-γ translation was assessed using subsequent immunoprecipitation.

3.3.1 IFN-γ translation

3.3.1.1 IFN-γ translation rates

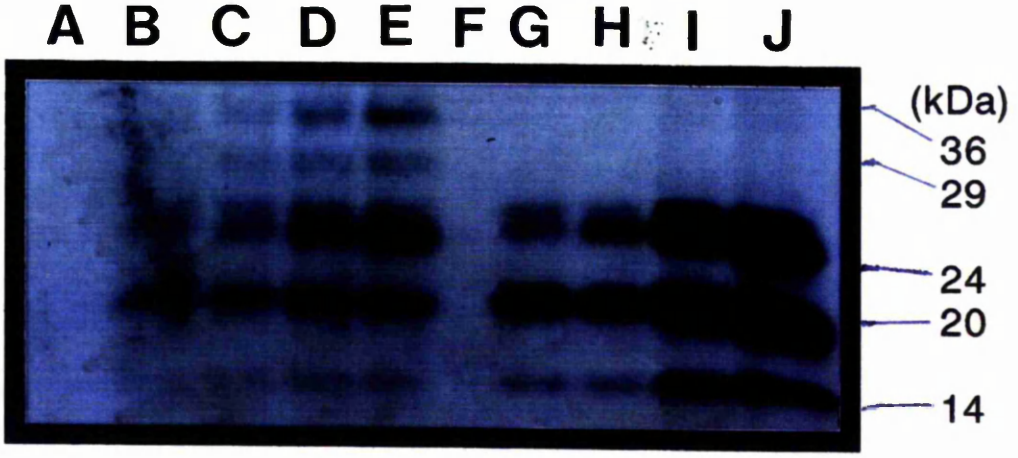
The IFN-γ translation during 15 min intervals is presented in Fig 3.24 and results are summarised in Fig 3.26A, Fig 3.27. The crude cellular extracts show that longer labelling times generated more intense protein labelling. After an equivalent pulse labelling period of 15 min (Fig 3.26A), CHO 43 cells synthesised 6-11 times more IFN-γ protein (705, 101.42 arbitrary densitometric units in two separate experiments) per cell than CHO 42 cells (125 and 8.6 arbitrary standardised densitometric units in two separate experiments). As IFN-γ protein continued to increase within each cell type at 15 min, a further 2 hr pulse labelling was

Figure 3.24 IFN-γ synthesis by CHO 42 and CHO 43 cells

CHO 42 (5 X 10^5 cells/well) and CHO 43 (2.5 X 10^5 cells/well) cells were cultured in 6-well culture dishes for two days, medium was aspirated and cell sheets were washed two times with PBS. Cells were incubated in pre-warmed labelling media minus methionine and cysteine for 15 min (Section 2.7.7.1.2), then the medium was replaced with labelling media containing 100 µCi Tran^{35}S-label for 15 min. The labelled cells were washed, lysed as described in Section 2.7.7.1.2 and cellular lysate were used in immunoprecipitation (Section 2.7.7.2).

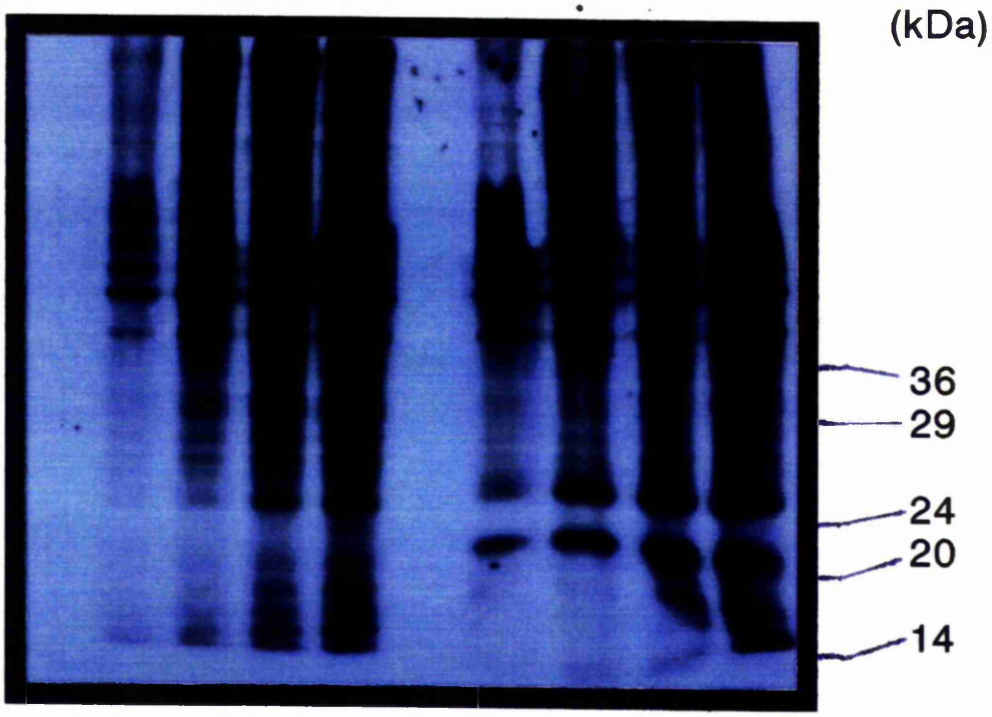
(A) The labelled IFN-γ immunoprecipitates were resolved in 12% SDS-PAGE, the gel was dried and autoradiographs were taken (Section 2.7.7.2). Lane A to E shows CHO 42 cells labelled for 0, 3, 7, 11, 15 min and F to J are CHO 43 cell samples labelled 0, 3, 7, 11, 15 min.

(B) Five µl of labelled crude cell lysates were resolved in 12% SDS-PAGE, the gel was dried and autoradiographs were taken (Section 2.7.7.2). Lanes A to J shown here correspond to the samples for IFN-γ immunoprecipitates.



CHO 42 CHO 43

A. (Anti IFN-γ immunoprecipitated)



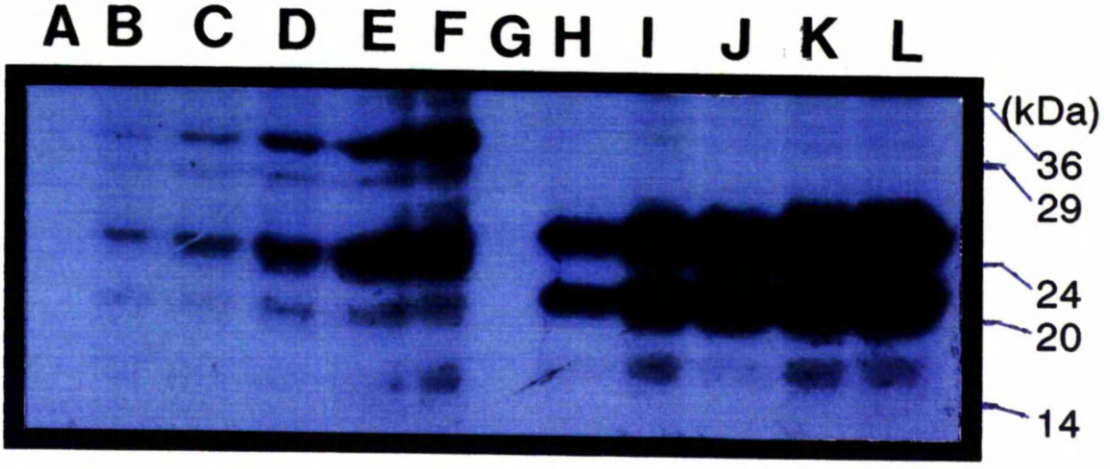
B. (Crude extracts)

Protein synthesis in 15 min of CHO 42 and CHO 43 cells

Figure 3.25 IFN-γ synthesis over a 2 hr incubation of CHO 42 and CHO 43 cells

CHO 42 (5 X 10^5 cells/well) and CHO 43 (2.5 X 10^5 cells/well) cells were cultured in 6-well culture dishes for two days, medium was aspirated and cell sheets were washed two times with PBS. Cells were incubated with pre-warmed labelling media minus methionine and cysteine for 15 min, then the media was replaced with labelling media containing 100 µCi Tran^{35}S-label for up to 2 hr. The labelled cells were washed, lysed as described in Section 2.7.7.1.2 and cellular lysate were used for immunoprecipitation (Section 2.7.7.2).

The labelled IFN-γ immunoprecipitates were resolved in 12% SDS-PAGE, the gel was dried and autoradiographs were taken (Section 2.7.7.2). Lane A to F shows CHO 42 cells labelled for 0, 0.25, 0.5, 0.75, 1 and 2 hr and G to L are CHO 43 cell samples labelled for the same times as CHO 42 cells.

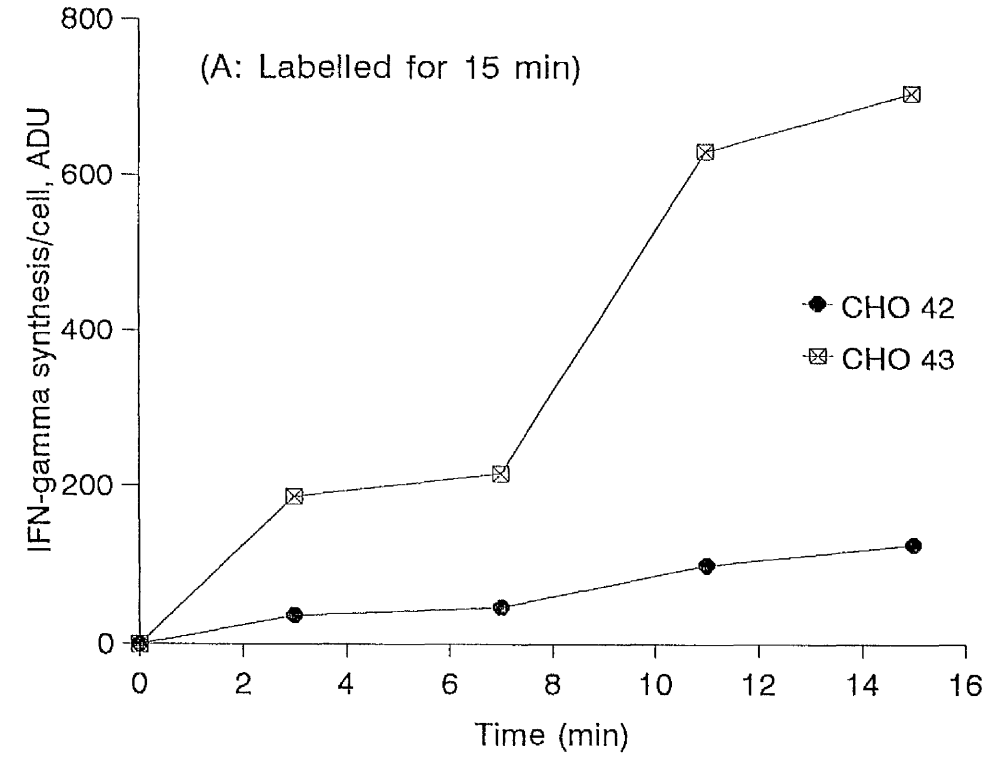


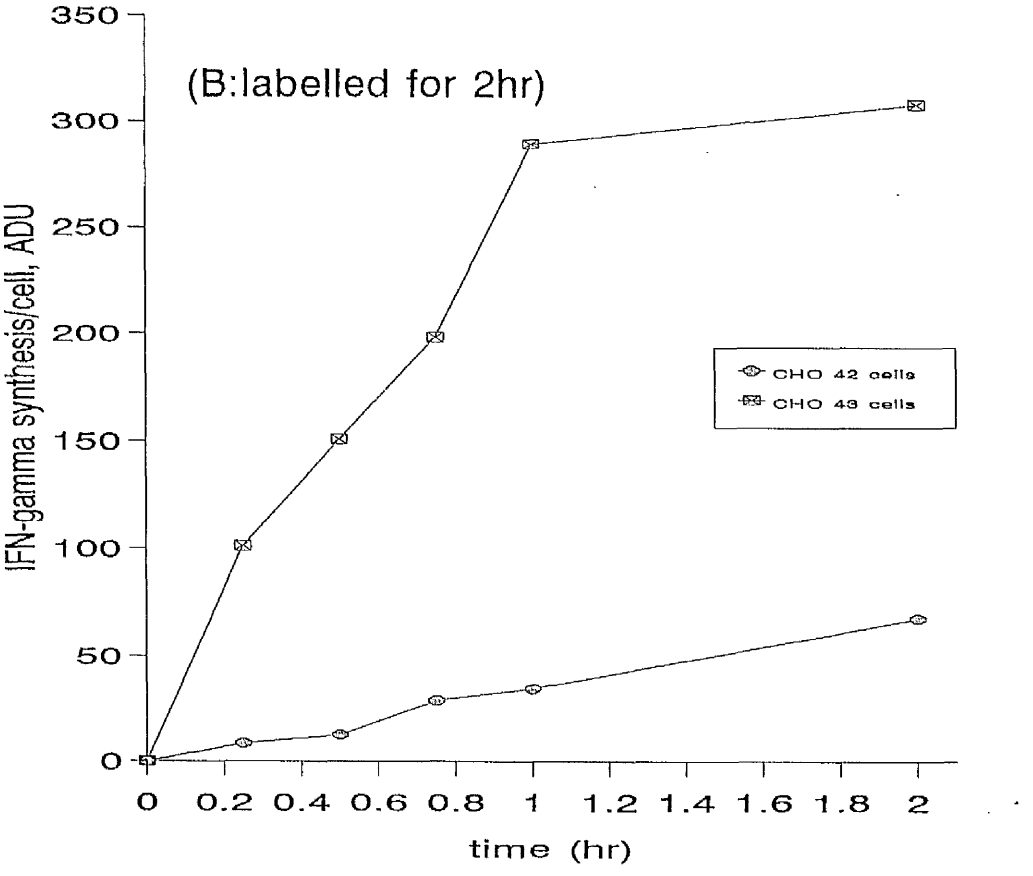
IFN-γ synthesis during 2 hr period by CHO 42 and CHO 43 cells

Figure 3.26 Total IFN-γ synthesis by CHO 42 and CHO 43 cells during 15 min and 2 hr labelling periods

(A) The autoradiograph of immunoprecipitated IFN-γ in Fig 3.24 was scanned as described in Section 2.6.4.3, integrated results from all IFN-γ isoforms were added together to generate the values shown in this figure (values are from one experiment).

(B) The autoradiograph of immunoprecipitated IFN-γ in Fig 3.25 was scanned as described in Section 2.6.4.3, integrated results from all IFN-γ isoforms were added together to generate the values shown in this figure (values are from one experiment).





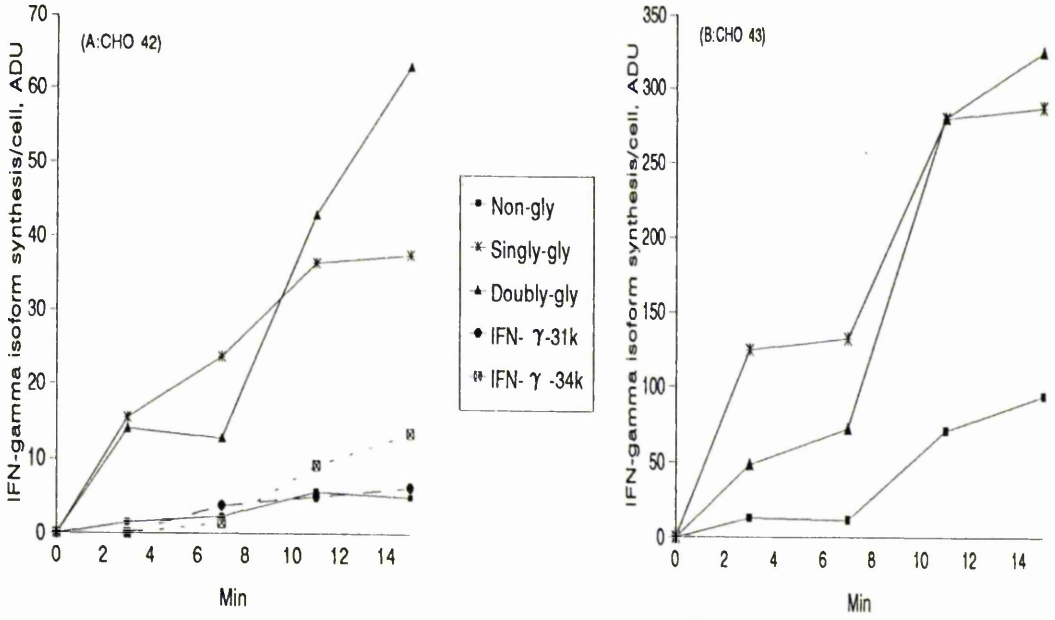
ADU: arbitrary densitometric units

Figure 3.27 IFN-γ isoforms synthesis and distribution over 15 min labelling

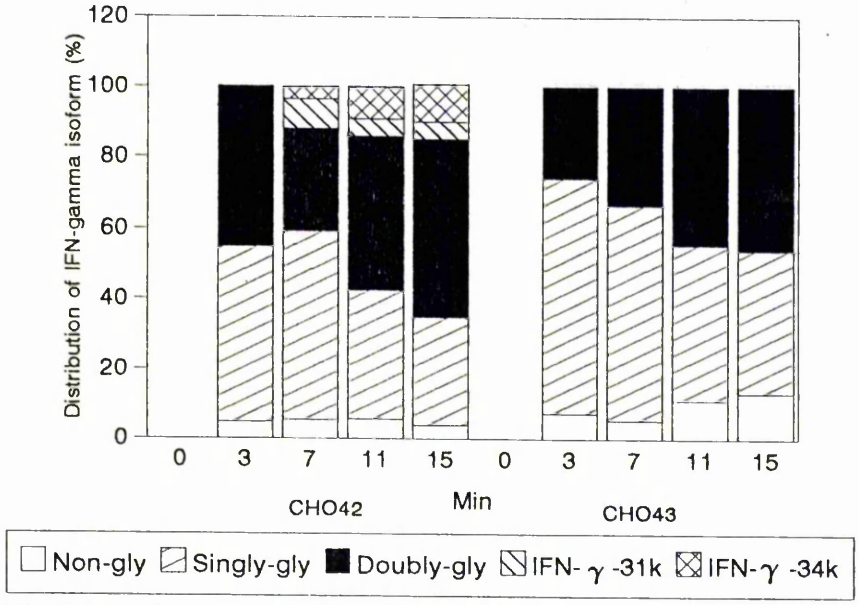
(A) Each IFN-γ isoform synthesized by CHO 42 cells in Fig 3.24 was scanned as described in Section 2.6.4.3, integrated results were plotted to generate this figure (values are from one experiment).

(B) Each IFN-γ isoform synthesized by CHO 43 cells in Fig 3.24 was scanned as described in Section 2.6.4.3, integrated results were plotted to generate this figure (values are from one experiment).

(C) The percentage of each IFN-γ isoform is expressed in relative to total IFN-γs (Fig 3.24; five in CHO 42 and three in CHO 43 cells).



ADU: arbitrary densitometric units



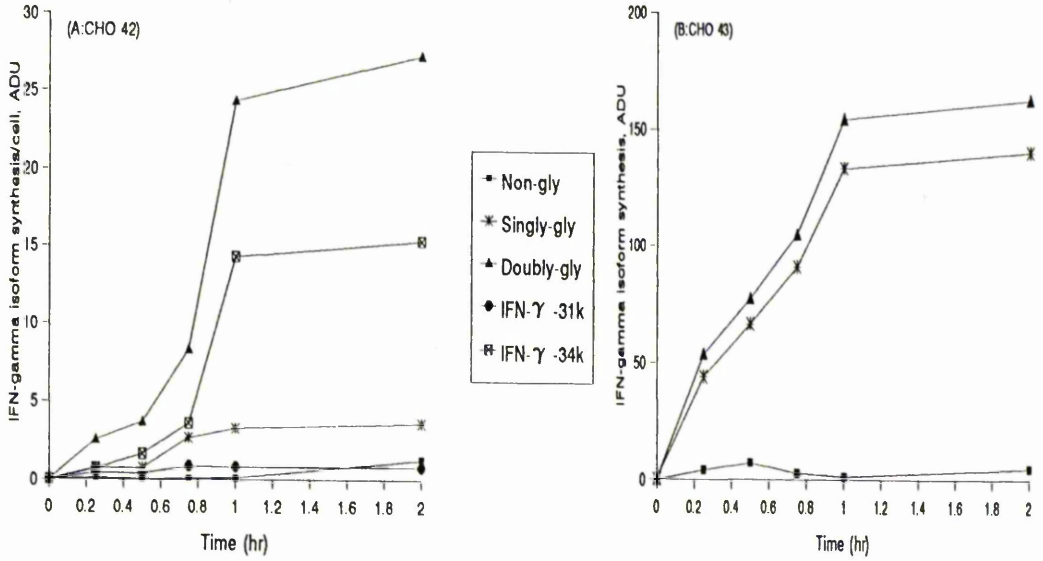
Distribution of glycosylation IFN-gamma in CHO 42 and CHO 43 cells

Figure 3.28 IFN-γ synthesis by CHO 42 and CHO 43 cells for 2hr

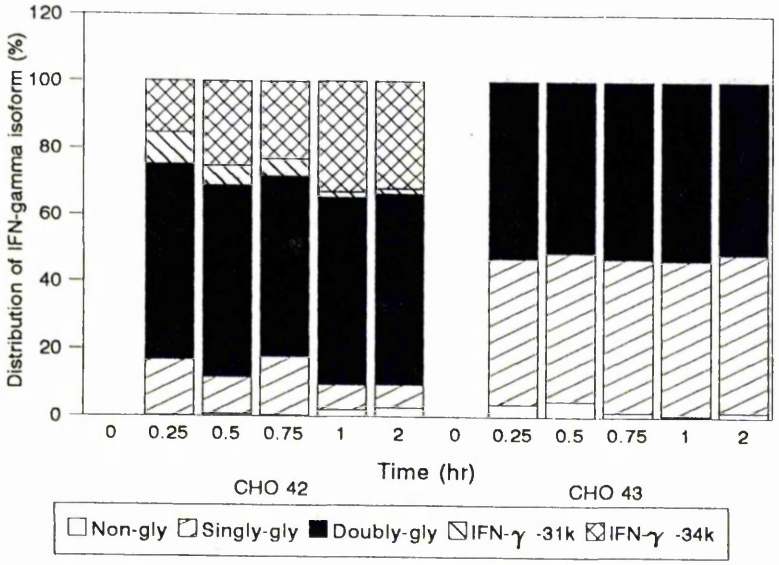
(A) Each IFN-γ isoform synthesized by CHO 42 cells in Fig 3.25 was scanned as described in Section 2.6.4.3, integrated results were plotted to generate this figure (values are from one experiment).

(B) Each IFN-γ isoform synthesized by CHO 43 cells in Fig 3.25 was scanned as described in Section 2.6.4.3, integrated results were plotted to generate this figure (values are from one experiment).

(C) The percentage of each IFN-γ isoform is expressed in relative to total IFN-γs (Fig 3.25; five in CHO 42 and three in CHO 43 cells).



ADU: arbitrary densitometric units



Distribution of glycosylation IFN-gamma in CHO 42 and CHO 43 cells

performed (Fig 3.25, Fig 3.26B). The incorporation of radioisotope into IFN-γ in CHO 43 cells reached steady-state by about 1 hour. Labelled-IFN-γ accumulated in CHO 43 cells during the first hour of labelling, but after that the level of radio-labelled IFN-γ did not change. CHO 42 cells presented a different translation pattern from CHO 43 cells. Even after labelling for 2 hr, radio-labelled IFN-γ was still increasing in CHO 42 cells. After 1 hour of labelling, CHO 43 cells produced a seven-fold greater amount (289 arbitrary densitometric units) of radio-labelled IFN-γ protein than CHO 42 cells (34.5 arbitrary densitometric units). However, after 2 hr of labelling, the difference was only 4.5-fold (308, 67.8 arbitrary densitometric units, for CHO 43 and CHO 42 respectively).

3.3.1.2 IFN-γ translation products

The relative amounts of each molecular weight "isoform" of IFN-γ are summarised in Fig 3.27A (CHO 42 cells) and Fig 3.27B (CHO 43 cells). CHO 42 and CHO 43 cells can translate IFN-γ in less than 3 min and glycosylate it soon after it has been translated (Fig 3.24A, Fig 3.27A). At this time, most of the IFN-γ is singly-glycosylated (70% CHO 43 cells and 50 % CHO 42 cells; Fig 3.27C). Then, glycosylation increases throughout a further time course and reaches a balance of singly- and doubly-glycosylated isoforms by 15 min. The relative proportion of doubly-glycosylated IFN-γ is higher in CHO 42 cells. The ratio between singly-glycosylated and doubly-glycosylated IFN-γ in CHO 42 cells was 1 : 1.7 and in

CHO 43 cells was 1 : 1.2 (Fig 3.27C).

Three forms of intracellular IFN-γ were immunologically detected in CHO 43 cells, but CHO 42 cells contained two extra forms of IFN-γ with molecular weights of 31 and 34 kDa (IFN-γ-31k, and IFN-γ-34k). These two extra forms of IFN-γ first appeared after 7 min of labelling (Fig 3.24A), later than the other three forms. The highest molecular form (IFN-γ-34k) increasingly accumulates in the cellular sample but the lower molecular form (IFN-γ-31k) is maintained at a constant amount as is the non-glycosylated form of IFN-γ (Fig 3.27A, Fig 3.28A). The IFN-γ-34k products accounted for up to 10% of total IFN-γ products of CHO 42 cells. These two high-molecular weight IFN-γ products were not found in other IFN-γ recombinant CHO cell lines (Anwar, 1994; Bulleid et al., 1990; Curling et al., 1990; Mutsaers et al., 1986; Scahill et al., 1983) and other recombinant cells (Sareneva et al., 1994).

3.3.2 IFN-γ glycosylation

3.3.2.1 ^{35}S-methionine labelling

As labelling into IFN-γ was still increasing at 15 min, further experiments were performed over a 2 hr labelling period. Results are shown in Fig 3.25 and summarized in Fig 3.26B and Fig 3.28. In both CHO 42 and CHO 43 cells, the non-glycosylated form contributed less than 10% of total IFN-γ products and the doubly-glycosylated isoform was the major product (56.4±1.7% and 52.3±3% of total IFN-γ products in CHO

42 and CHO 43 cells, respectively; values are means±SD for 4 samples). The second most abundant product in CHO 43 cells was the singly-glycosylated form of IFN-γ (45.2±1.34%; values are means±SD for 4 samples). In CHO 42 cells, at early stages of culture the second most abundant product was the singly-glycosylated form. However, after an hour of labelling the IFN-γ-34k isoform increasingly accumulated in cells and this contributed up to 30% of total labelled products (Fig 3.28C). At this stage the singly-glycosylated IFN-γ was less than 10% of total IFN-γ products.

3.3.2.2 Effect of tunicamycin

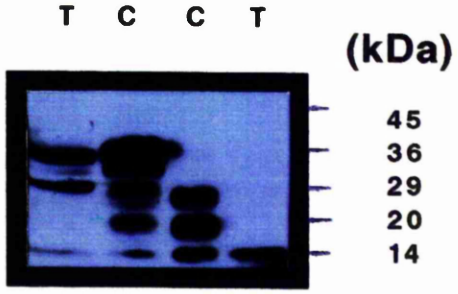
The glycosylation of IFN-γ was inhibited using tunicamycin and western blotting of IFN-γ in cellular extracts of cells treated by this regime is shown in Fig 3.29. In CHO 43 cells, there were three isoforms of IFN-γ in the cellular extracts from control conditions (17, 21, 24 kDa; Section 3.1.2.3). When cells were treated with tunicamycin, only one form (17 kDa) was found. This should be the non-glycosylated form. There were five isoforms in CHO 42 cells under control conditions (17, 21, 25, 31 and 34 kDa). However, when cells were treated with tunicamycin, the 21 and 25 kDa isoforms are missing but there were still four isoforms of IFN-γ detected (17, 26, 28 and 32.6 kDa). The three highest molecular isoforms were slightly smaller than their counterparts in untreated cells.

Figure 3.29 Western blotting of IFN-γ in tunicamycin-treated CHO 42 and CHO 43 cells

CHO 42 and CHO 43 cells were cultured in T_{75} flasks for two days, medium was aspirated and cell sheets were washed two times with PBS. One set of cells were replenished with fresh media containing 0.5 µg/ml tunicamycin, and a control set of cells were replenished with fresh media containing 2 µl/ml ethanol (tunicamycin was dissolved in ethanol to a concentration of 0.25 mg/ml), cells were then incubated for a further 6 hr.

Cells were trypsinised, washed with PBS and resuspended in PBS to give concentrations of 4 X 10^5 cells/10µl for CHO 42 and 1.5 X 10^5 cells/10µl for CHO 43 cells. Cell suspensions were treated as described in Section 2.7.5.1 and proteins were resolved in 12% SDS-PAGE (Section 2.7.5.2), Western blotted and detected by ECL (Section 2.7.6). The primary antibody used was monoclonal 20D7 (Section 2.1.1).

T: tunicamycin treated samples
C: control samples



Western blotting of IFN-γ
of tunicamycin treated CHO 42
and CHO 43 cells

3.3.3 IFN-γ secretion

3.3.3.1 IFN-γ secretion rates

Cellular protein was labelled for 1 hour, and IFN-γ secretion into media was "chased" by incubation in "cold" medium for up to 4 hour. The result of IFN-γ secretion is presented in Fig 3.30 and summarized in Fig 3.31. CHO 43 cell secretion of labelled IFN-γ reached a steady-state by 1 hour. After 1 hr, levels of radio-labelled IFN-γ in medium showed little change. CHO 42 cells secreted IFN-γ slightly more slowly. Even after 4 hr, levels of radio-labelled IFN-γ in medium was still increasing slightly. CHO 43 cells secreted much greater amounts of radio-labelled IFN-γ than CHO 42 cell. After 1 hr, CHO 43 cells secreted 13-26 times more IFN-γ than CHO 42 cells (71.1, 390.8 arbitrary densitometric units in CHO 43 and 5.47, 14.53 in CHO 42 in two separate experiments; 13, 26 times different).

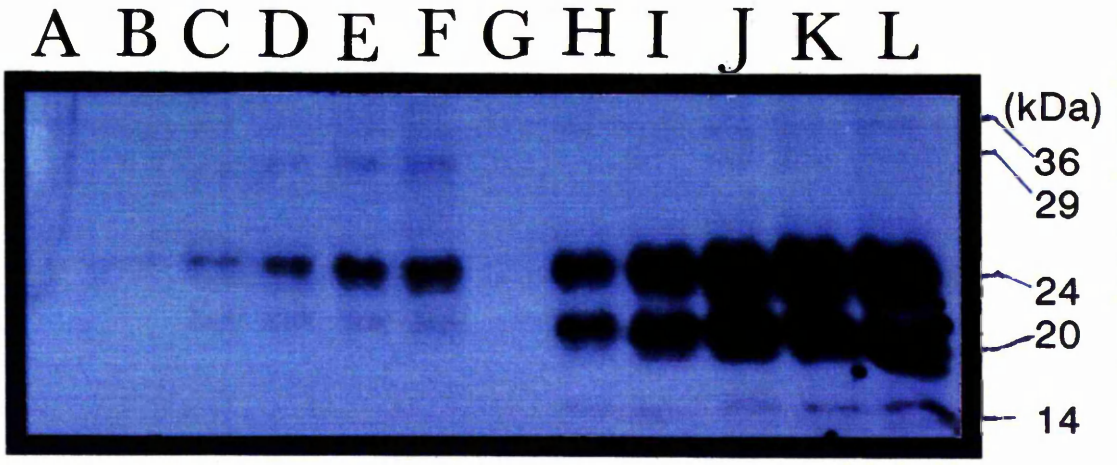
3.3.3.2 Secretion of isoforms of IFN-γ

N-glycosylation has been reported to influence the rate of glycoprotein transport through the secretory pathway (Kukuruzinska et al., 1994; Rothwell et al., 1993). As IFN-γ has two glycosylation sites (Asn 28 and Asn 97; Sareneva et al., 1994), it is important to define the relation between glycosylation and secretion. To investigate this, cells were pulse-labelled with ^{35}S-methionine for 60 min and chased with unlabelled media to maintain IFN-γ secretion to media for up to 4 hr.

Figure 3.30 IFN-γ secretion by CHO 42 and CHO 43 cells

CHO 42 (5 X 10^5 cells/well) and CHO 43 (2.5 X 10^5 cells/well) cells were cultured in 6-well culture dishes for two days, medium was aspirated and cell sheets were washed two times with PBS. Cells were incubated with warmed labelling medium (Section 2.7.7.1) minus methionine and cysteine for 15 min, then the medium was replaced with labelling media containing 100 µCi Tran^{35}S-label for 1 hr. The medium was aspirated, cell sheets were washed two times with PBS and FCS containing medium (Section 2.3.1) was added. Incubations were continued for up to 4 hr.

The labelled IFN-γ in medium was determined in immunoprecipitates resolved on 12% SDS-PAGE, gels were dried and autoradiographs were taken (Section 2.7.7.2). Lane A to F shows CHO 42 cells chased for 0, 0.25, 0.5, 1, 2 and 4hr and G to L are samples from CHO 43 cells chased for the same time intervals.

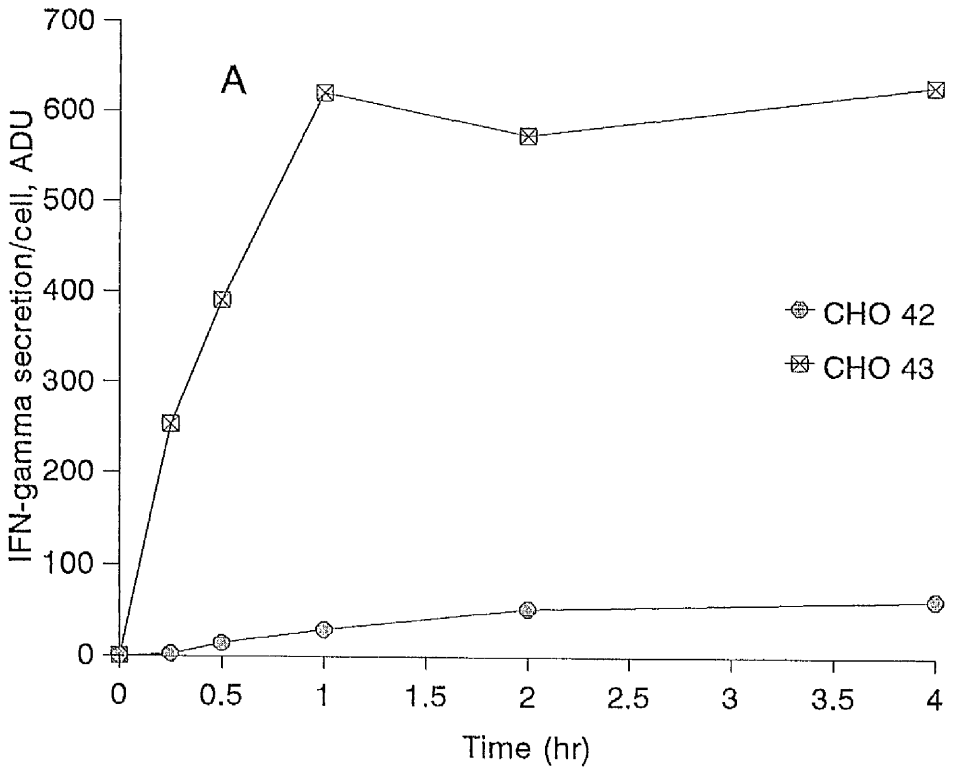


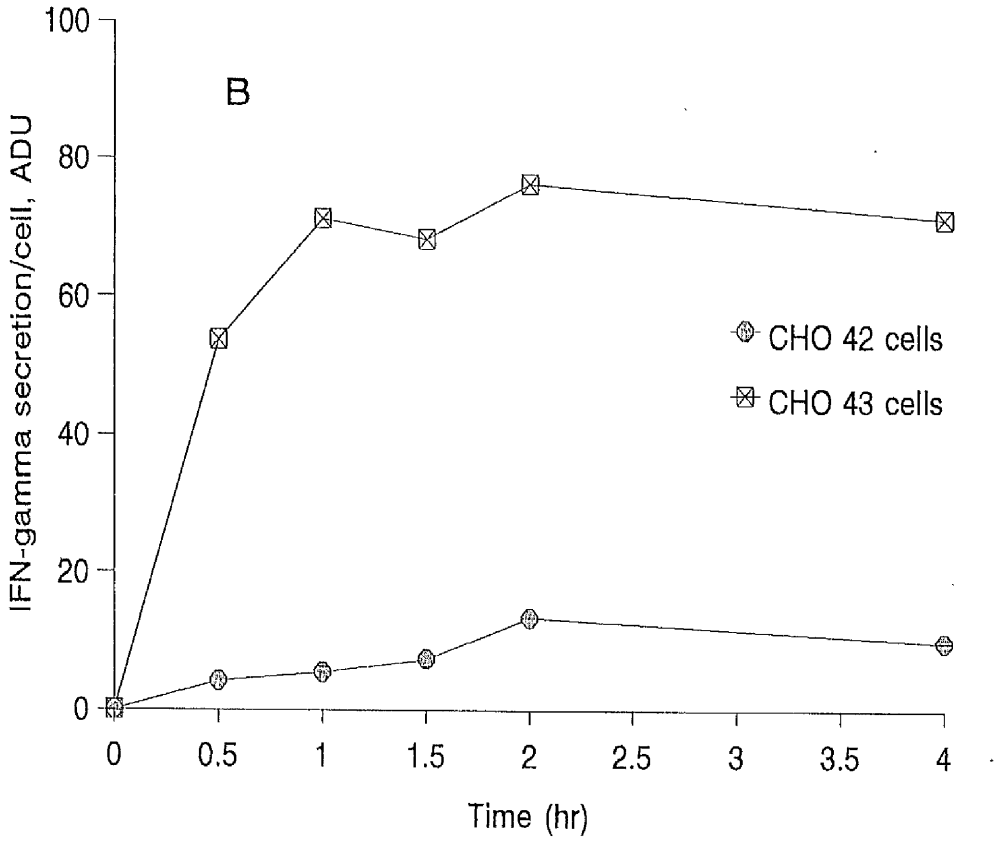
IFN-γ secretion by CHO 42 and CHO 43 cells

Figure 3.31 Total amount of IFN-γ secretion by CHO 42 and CHO 43 cells

The autoradiographs of secreted IFN-γ in Fig 3.30 were scanned as described in Section 2.6.4.3, integrated results from all bands were added together to generate values shows in this figure. Values from two separate experiments are shown in this figure.

A and B show two separate experiments



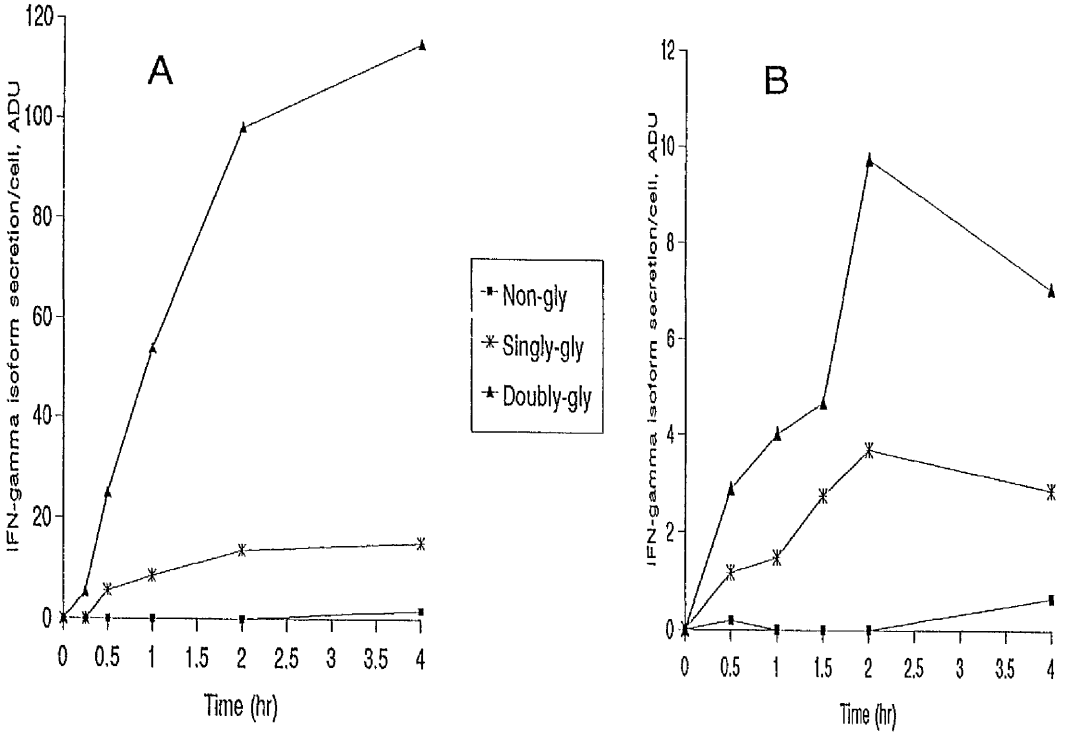


ADU: arbitrary densitometric units

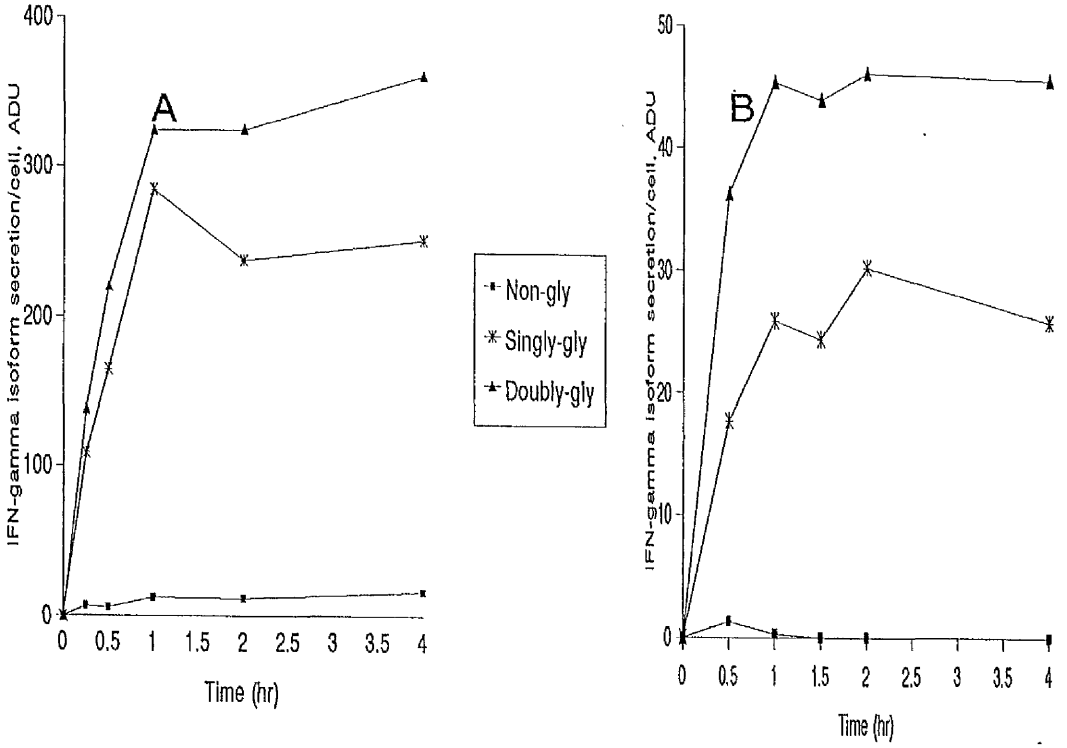
Figure 3.32 IFN-γ species and their secretion by CHO 42 and CHO 43 cells

Each of the IFN-γ isoforms secreted by CHO 42 and CHO 43 cells in Fig 3.30 was scanned as described in Section 2.6.4.3, integrated results were plotted to generate this figure.

A and B show two separate experiments



IFN-γ secretion by CHO 42 cells



IFN-γ secretion by CHO 43 cells

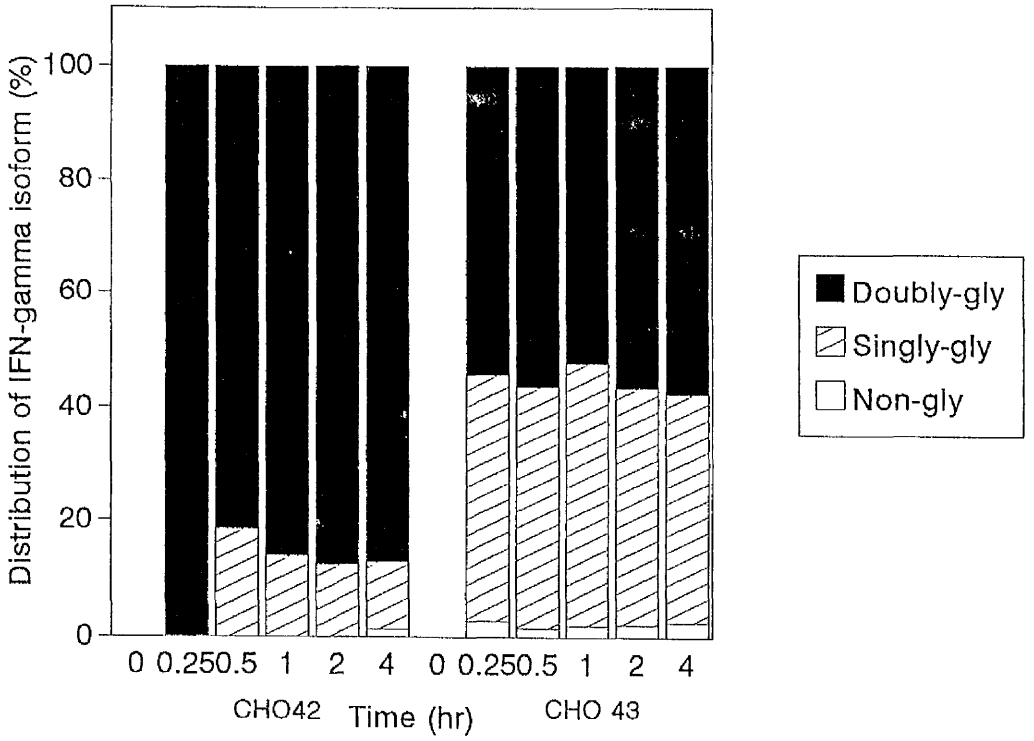
ADU: arbitrary densitometric units

Figure 3.33 Profile of each IFN-γ isoforms secreted by CHO 42 and CHO 43 cells

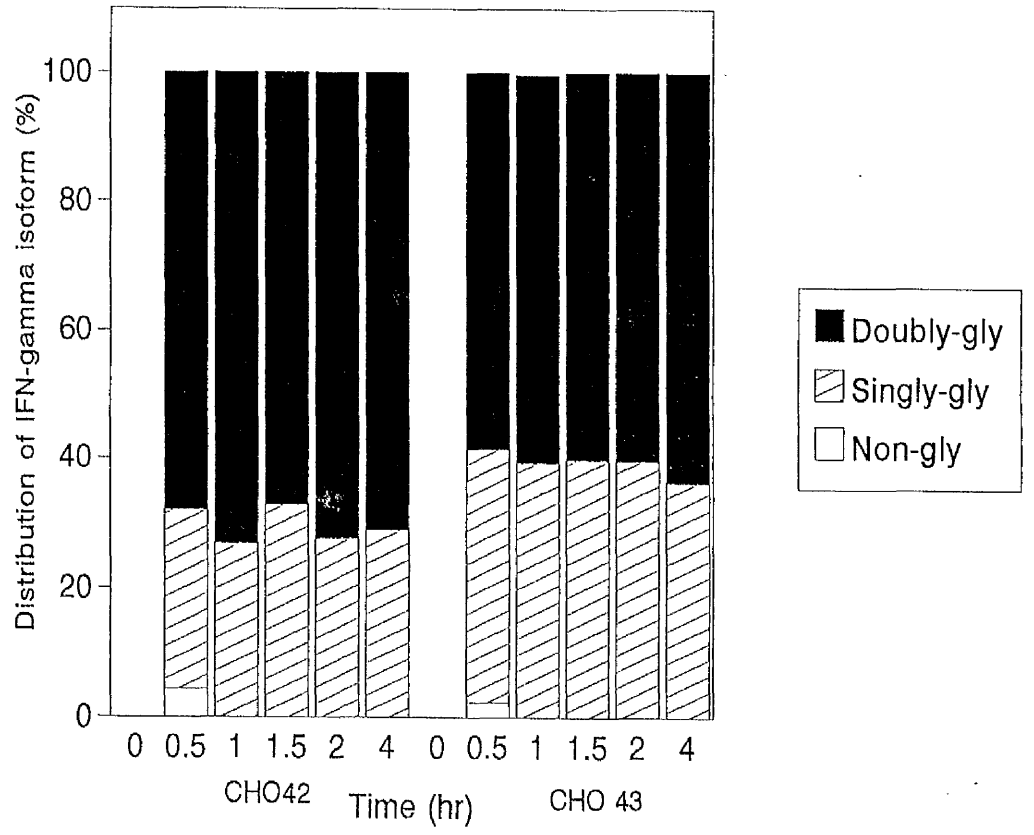
The percentage of each IFN-γ isoform is expressed in relation to total IFN-γs (Fig 3.30; three isoforms in both CHO 42 and CHO 43 cells).

A and show two separate experiments

A


Distribution of IFN-gamma isoform (%)
100
80
60
40
20
0
0 0.250.5 1 2 4 0 0.250.5 1 2 4
CHO42
Time (hr)
CHO 43
Doubly-gly
Singly-gly
Non-gly

B


Distribution of IFN-gamma isoform (%)
100
80
60
40
20
0
0 0.5 1 1.5 2 4 0 0.5 1 1.5 2 4
CHO42
Time (hr)
CHO 43
Doubly-gly
Singly-gly
Non-gly

The two high molecular (IFN-γ-31k, IFN-γ-34k; Section 3.1.2.3) IFN-γ products found within CHO 42 cells were not found in media during a 4 hr chase period and the majority of IFN-γ secreted by CHO 42 cells was the doubly-glycosylated form (Fig 3.32, Fig 3.33). The composition of secreted IFN-γ in both CHO 42 and CHO 43 cells maintained roughly the same ratio of singly- to doubly-glycosylated forms throughout the chase time. As shown for cellular samples (Section 3.3.4), only a small amount of secreted IFN-γ was non-glycosylated. IFN-γ secreted by CHO 43 cells was distributed between singly-glycosylated (42.3±2.0%, 38.6±1.6% in two experiments; values are means±SD for three samples in each experiment) and doubly-glycosylated (55.3±3.2%, 59.8±1.2%) forms. This is consistent with the results of western blotting shown in Section 3.1.2.3 and with the report of Sano et al (1988). They found that recombinant CHO cells secreted equal amounts of singly- and doubly-glycosylated IFN-γ. However, other recombinant mouse cells (C127) secreted the doubly-glycosylated form mainly (Sano et al., 1988). The situation for CHO 42 cells was different from that of CHO 43 cells. The doubly-glycosylated form of IFN-γ (68.8±13.1%, 88.4± 6% of total secreted forms in two separate experiments; values are means±SD for three samples in each experiment) was the main product, the second most abundant form was singly-glycosylated (22.2±7.7%, 13.8±3.2% in two separate experiments) and there was little of the non-glycosylated form (less than 5% of total products).

3.3.4 Cellular IFN-γ retention

As described earlier, CHO 43 cells synthesize IFN-γ at a rate 4.6 times greater than that of CHO 42 cells (Section 3.3.1) and secrete IFN-γ at a rate 13-26 times greater than that of CHO 42 cells (Section 3.3.3). To define if this reflected isoform-specific secretion, cells were pulse-labelled with ^{35}S-methionine for 1 hr and IFN-γ in cellular fractions was analyzed throughout a 4 hr pulse-chase period. IFN-γ in cellular extracts was immunoprecipitated.

3.3.4.1 IFN-γ retention in CHO 42 and CHO 43 cells

Radio-labelled IFN-γ is retained in cells during the pulse-chase (Fig 3.34) and results are summarized in Fig 3.35. CHO 43 cells showed a rapid loss of cellular IFN-γ whereas the change in CHO 42 cells was much slower. Cellular radio-labelled IFN-γ in CHO 43 cells had a half-life of about 30 min (half-lives were 20 and 34 min in two separate experiments) and most of the radio-labelled IFN-γ had been secreted within 1 hr. This was consistent with IFN-γ secretion by CHO 43 cells (Section 3.3.3). However, about half of IFN-γ translated in CHO 42 cells was retained in cells even after 4 hour of pulse chase (half-lives of retention was 4 hr and 3.4 hr in two separate experiments).

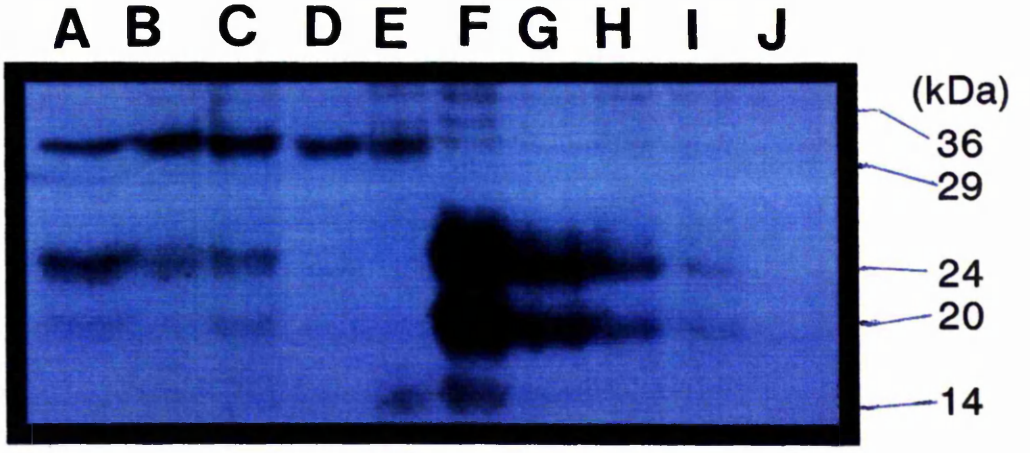
3.3.4.2 IFN-γ isoform retention

Both major IFN-γ products, singly- and doubly-glycosylated forms, disappeared rapidly from CHO 43 cells during pulse-chase with half-lives of about 30 min [20 and 34 minutes, respectively (experiment 1) and 24 and 30 min

Figure 3.34 IFN-γ retention in CHO 42 and CHO 43 cells

CHO 42 (5 X 10^5 cells/well) and CHO 43 (2.5 X 10^5 cells/well) cells were cultured in 6-well culture dishes for two days, medium was aspirated and cell sheets were washed two times with PBS. Cells were incubated with warmed labelling medium (Section 2.7.7.2) minus methionine and cysteine for 15 min, then the medium was replaced with labelling media containing 100 µCi Tran^{35}S-label for 1hr. The medium was aspirated, cell sheets were washed two times with PBS and FCS containing medium (Section 2.3.1) was added. Incubations were continued for up to 4 hr.

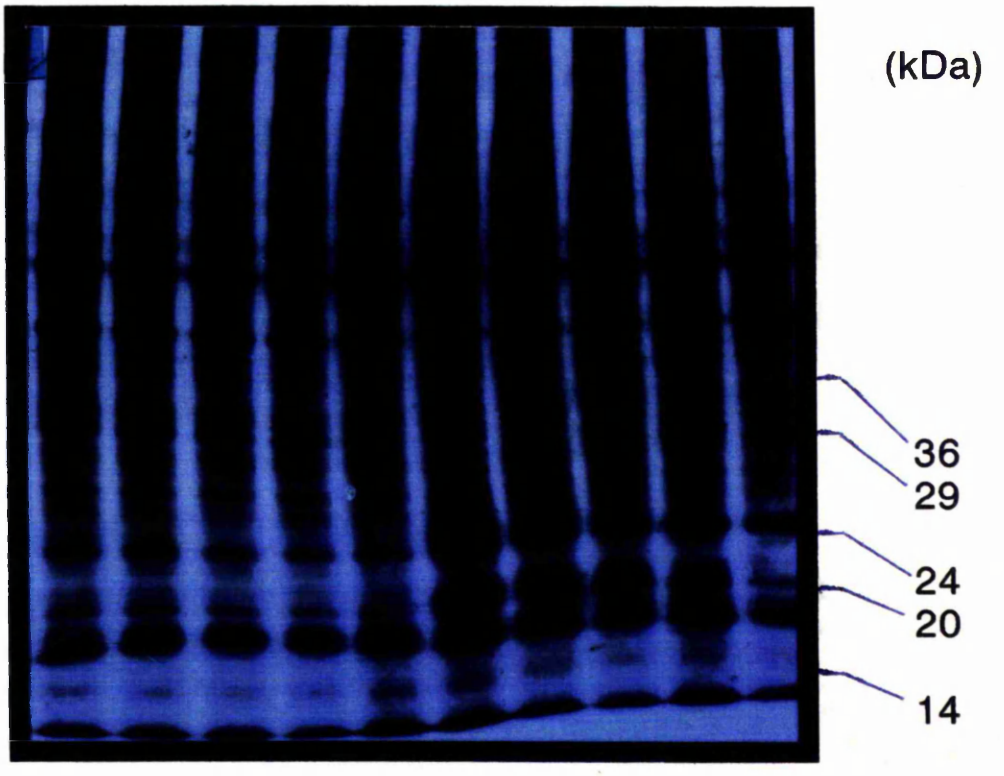
The labelled IFN-γ in cells was determined in immunoprecipitates resolved on 12% SDS-PAGE, gels were dried and autoradiographs were taken (Section 2.7.7.2). Lane A to E show CHO 42 cells chased for 0, 0.5, 1, 2 and 4hr and F to J are samples from CHO 43 cells chased for the same time periods.



CHO 42 CHO 43

A: IFN-γ retention in CHO 42 and CHO 43 cells

(Anti IFN-γ immunoprecipitated)



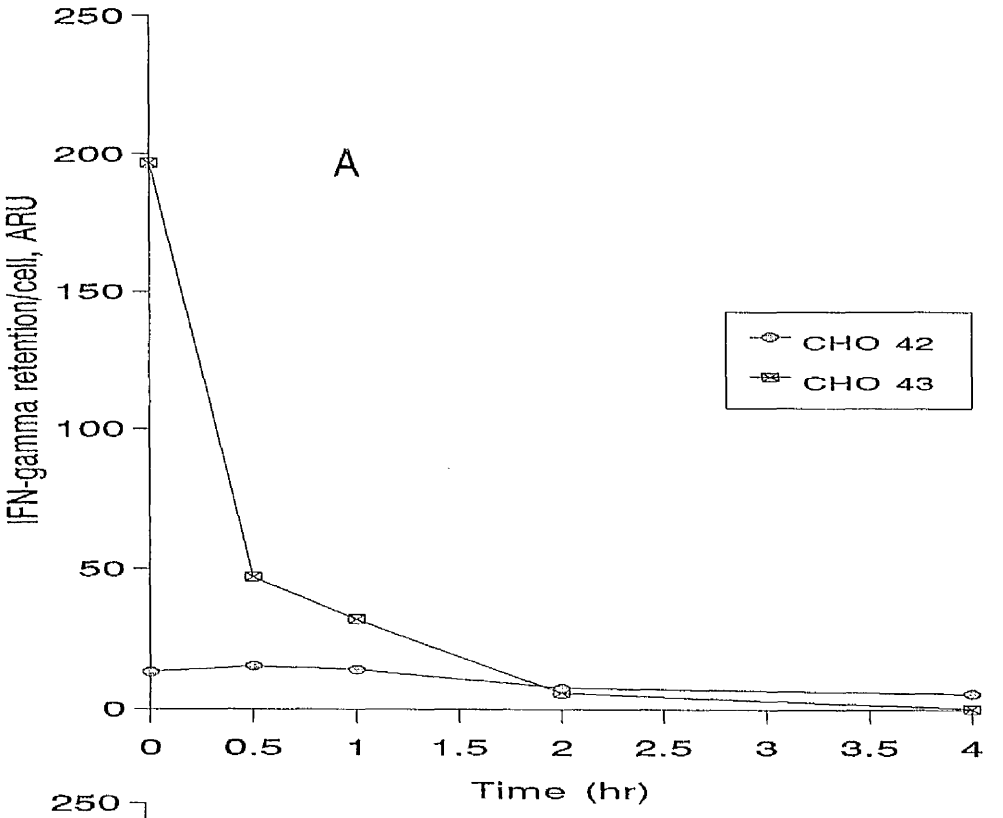
(Crude extracts)

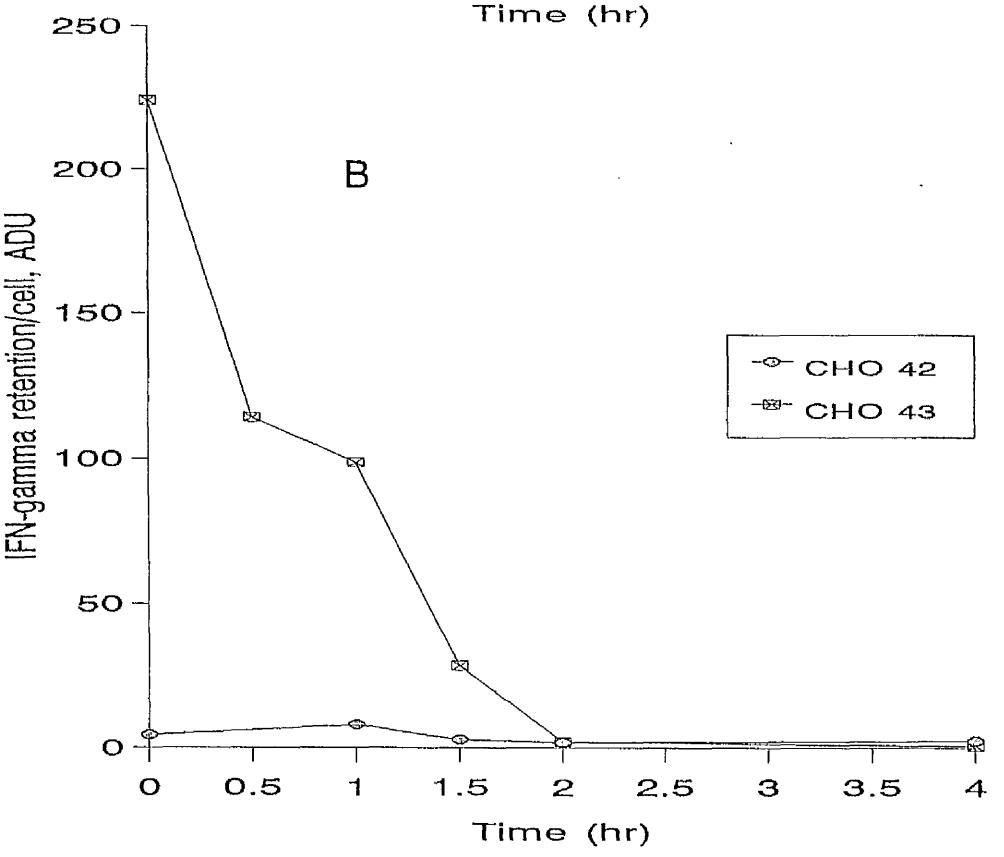
Protein metabolism in CHO 42 and CHO 43 cells

Figure 3.35 Total amount of IFN-γ retention in CHO 42 and CHO 43 cells

The autoradiographs of secreted IFN-γ in Fig 3.34 were scanned as described in Section 2.6.4.3, integrated results from all bands were added together to generate values shown in this figure. Values from two separate experiments are shown in this figure.

A and B show two separate experiments



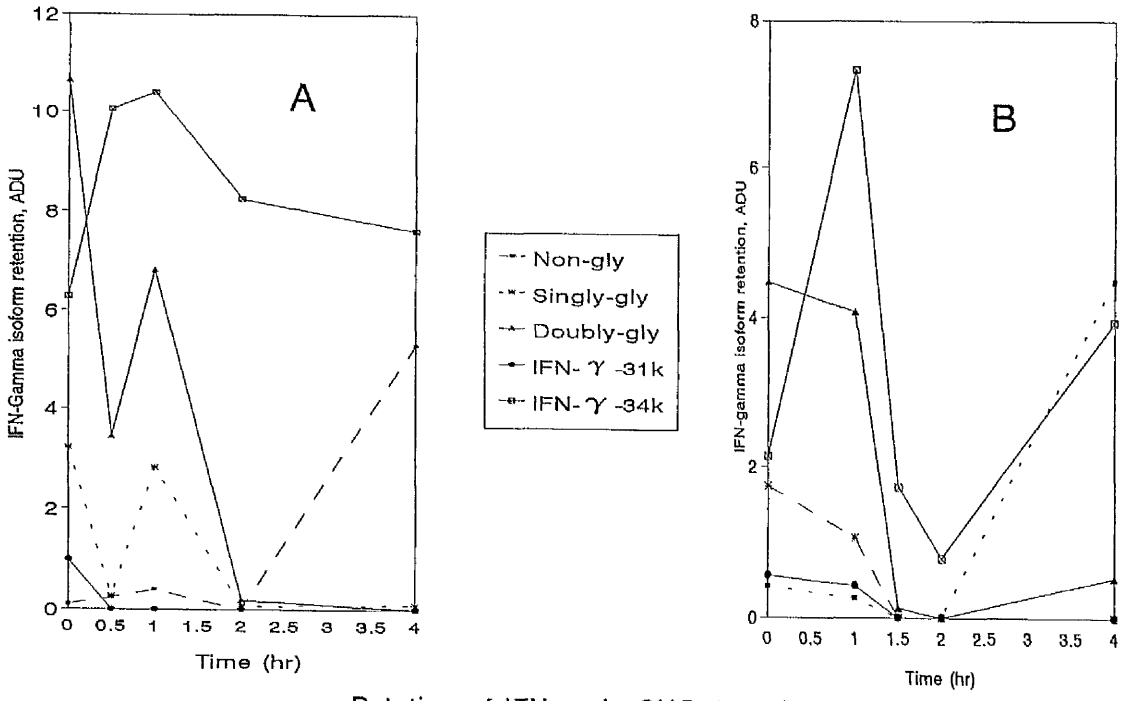


ADU: arbitrary densitometric units

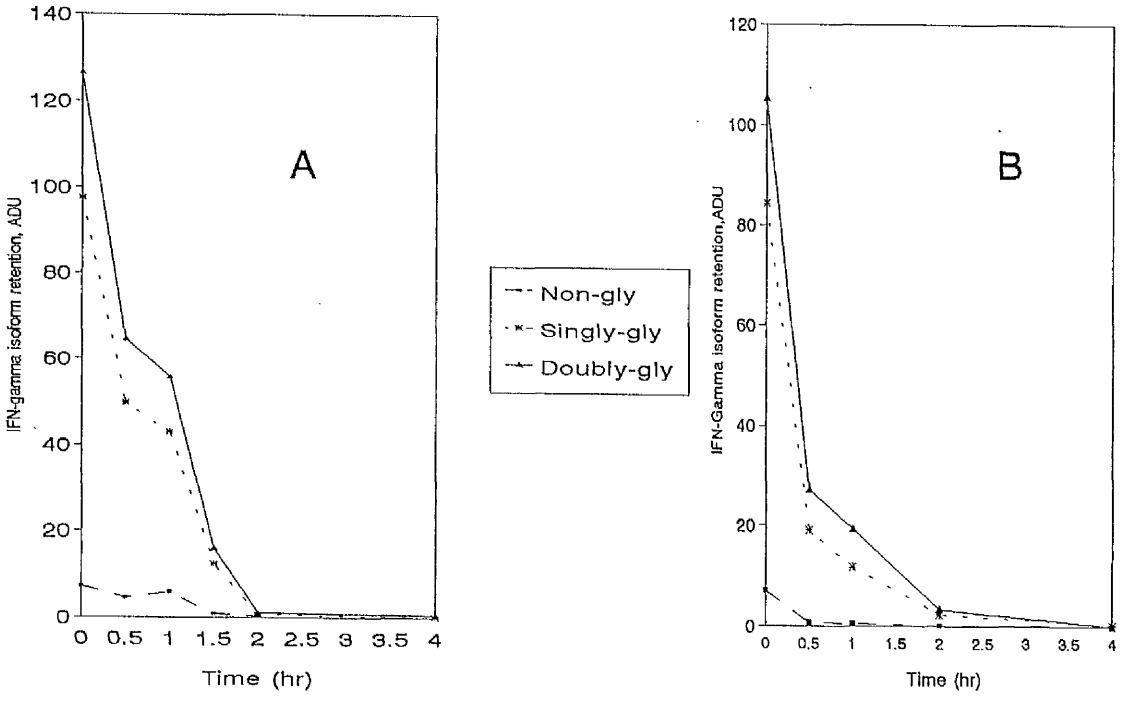
Figure 3.36 IFN-γ species and their retention by CHO 42 and CHO 43 cells

Each IFN-γ isoform secreted by CHO 42 and CHO 43 cells in Fig 3.34 was scanned as described in Section 2.6.4.3, integrated results were plotted to generate this figure.

A and B show two separate experiments



Retetion of IFN-γ in CHO 42 cells



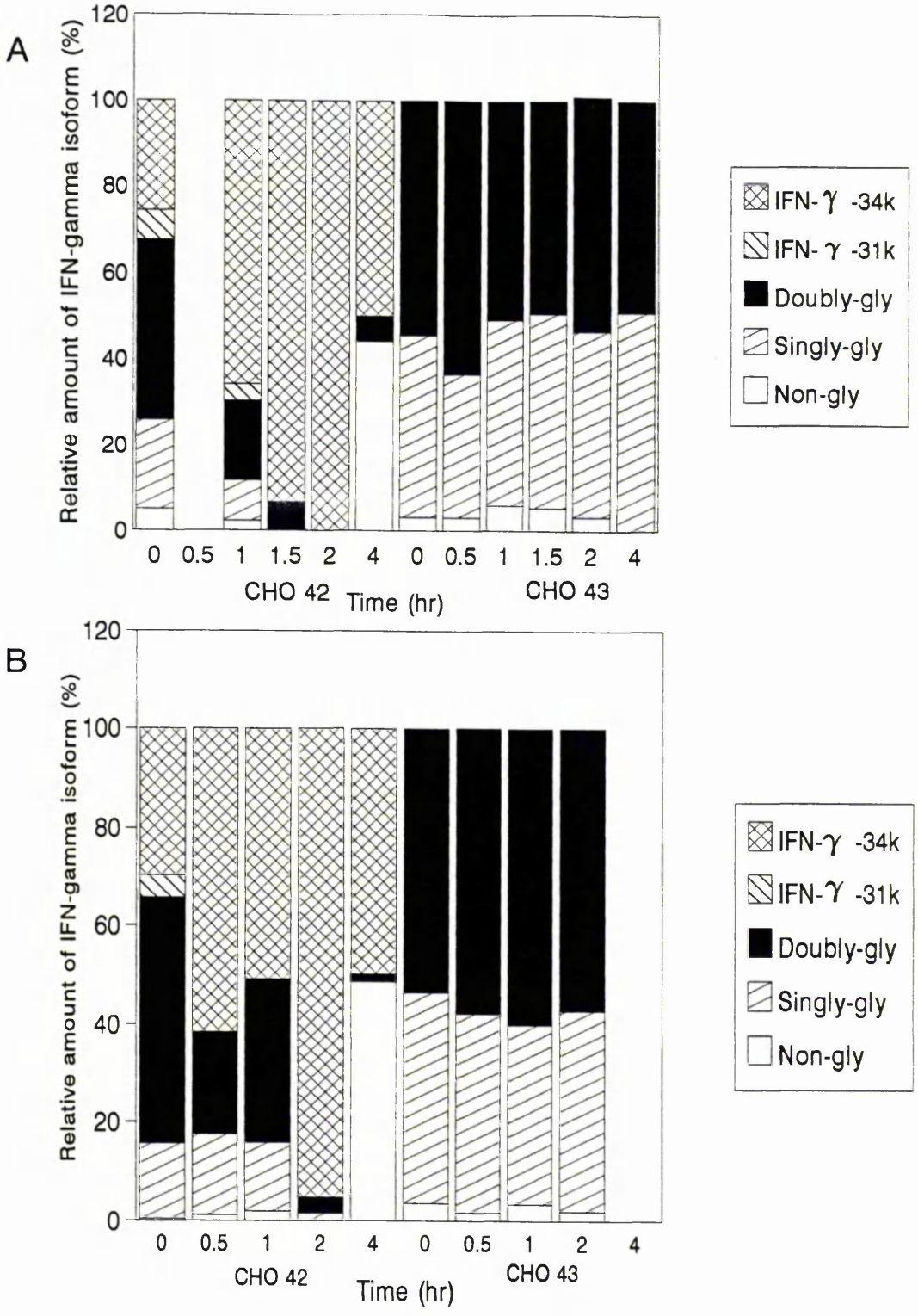
Retetion of IFN-γ in CHO 43 cells

ADU: arbitrary densitometric units

Figure 3.37 Composition of IFN-γ isoform-specific retention in CHO 42 and CHO 43 cells

The percentage of each IFN-γ isoform was expressed relative to total IFN-γs (Fig 3.34; five isoforms in CHO 42 cells and three isoforms in CHO 43 cells).

A and B show two separate experiments



IFN-γ retention in CHO 42 and CHO 43 cells

respectively (experiment 2); Fig 3.36]. However, in CHO 42 cells the distribution of each form of IFN-γ had changed dramatically during the time period of the "chase" (Fig 3.36). The proportion of non-glycosylated and IFN-γ-34k forms increased during the pulse-chase whereas the other three forms of IFN-γ were rapidly lost from the cells. The IFN-γ-34k isoform increased in abundance during the first hour of pulse-chase (by 1.5 to 3.3 times). The non-glycosylated form was maintained at a low level for the first 2 hr of the chase period but increased in concentration slightly at later stages of the chase. The singly- and doubly-glycosylated forms declined through the chase with a half-life of 53 min (38, 65 min and 45, 60 min for singly- and doubly-glycosylated IFN-γ isoforms, respectively in two separate experiments). The half-lives are greater than those obtained for the same species in CHO 43 cells.

The ratio of doubly- to singly-glycosylated IFN-γ isoforms was constant (Fig 3.37; 1.26, 1.43; in two separate experiments) throughout the 4 hour chase period for CHO 43 cells. The ratio of doubly- to singly- glycosylated IFN-γ in CHO 42 cells was also constant with a higher ratio of doubly-glycosylated IFN-γ (3.09, 2.5; in two separate experiments) throughout the 4 hr chase. However, the two higher molecular isoforms and the non-glycosylated isoform changed throughout the chase period in CHO 42 cells. When pulse labelling stopped the doubly-glycosylated form of IFN-γ was the major product (41-50% of the total IFN-γ products) then it declined to less than 10% of total amount. The IFN-γ-34k was the second most abundant form (25-30% of total) at

the beginning of the chase and it replaced the doubly-glycosylated form as the major product in CHO 42 cells (more than 90% of total) after 2 hr of chase. However, after a 4 hr chase the non-glycosylated form increased dramatically and it was equivalent to IFN-γ-34k.

3.4 IFN-γ mRNA processing

Two facts from the work presented in previous sections (Section 3.1.2.1, 3.2.1.2) present novel features for regulatory phenomena in recombinant cells. Firstly, the means by which heterogeneity has been generated in CHO 43 and CHO 42 cells in terms of product (protein, RNA) has implication for general recombinant product fidelity. Secondly, the unexpected stability of IFN-γ mRNA in CHO 42 cells, associated with size differences from expected mRNA species requires characterisation. These aspects are examined in detail below.

Protein factors have been described which bind to AU-rich sequences in the mRNA encoding lymphokines and cytokines (Hamilton et al., 1993; Katz et al., 1994). The binding of these factors to AU-rich sequences may affect mRNA stability and transport between nucleus and cytoplasm and may result in a differential IFN-γ mRNA pattern in nucleus and cytoplasm. This may be of relevance to CHO 43 and CHO 42 cells.

In this section, I will concentrate on characterisation of IFN-γ RNA species in nucleus and cytoplasm, on the polyadenylation of IFN-γ transcripts and on IFN-γ mRNA sequence determination.

3.4.1 Cytosolic and nuclear RNA

Roop et al (1978) used a Northern blotting method to compare ovalbumin mRNA in cytosol and nucleus and were able to observe a range of hybridising species including, not only the primary transcript and the fully spliced RNA prior to transport to cytoplasm, but also a series of intermediate-sized RNAs from which some of the intervening sequences had still to be removed.

I prepared RNA from cytoplasm and nucleus as well as total cellular RNA and probed all with IFN-γ and DHFR cDNA to examine RNA processing and transport during batch culture in relation to possible differences between CHO 42 and CHO 43 cells.

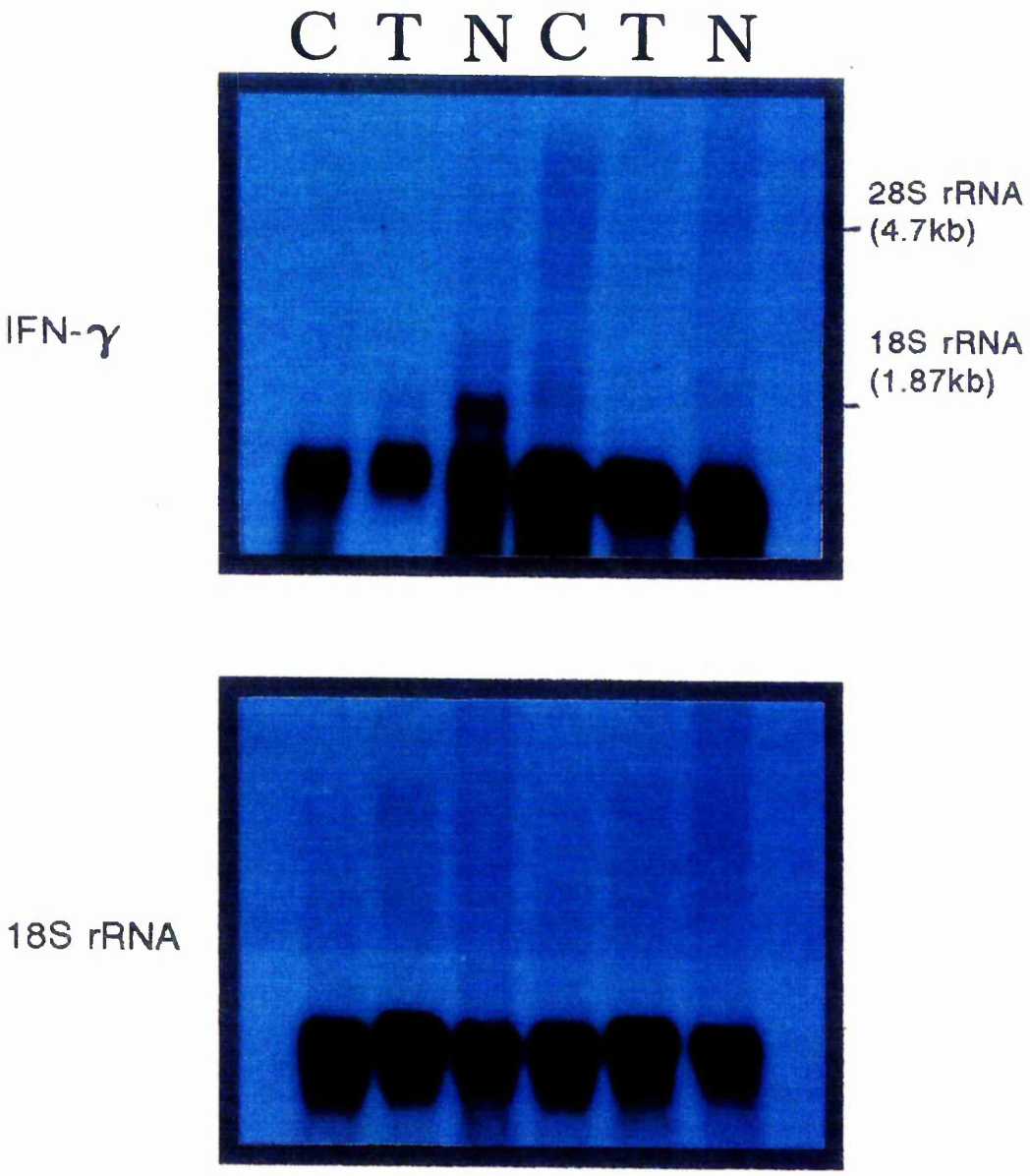
3.4.1.1 IFN-γ RNA species

RNA samples were taken from cells in the mid-log phase of growth and these were resolved by 1.5% formaldehyde denaturation agarose gel. Northern hybridization for IFN-γ species is shown in Fig 3.38 and the results are summarized in Fig 3.39 and 3.40. There were three major IFN-γ hybridisation bands in nuclear extracts from CHO 42 cells and two bands from CHO 43 cells. There was also a smear of hybridisation signal below the major bands in cytoplasmic and nuclear extracts. In CHO 43 cells, IFN-γ mRNA 0.9 kb is the major species in cytoplasmic extracts, nuclear and total RNA

Figure 3.38 Northern hybridisation of cytoplasmic, total, and nuclear IFN-γ mRNA, and 18S rRNA of CHO 42 and CHO 43 cells at mid-log phase

CHO 42 and CHO 43 cells were cultured for 2 days (mid-log phase). Cytoplasmic, total and nuclear RNA were extracted as described in Section 2.5.3. Extracted RNA was resolved in 1.5% agarose RNA gels (Section 2.6.1.2), Northern blotted and probed with ^{32}P-labelled IFN-γ, 18S rRNA cDNA.

C,T,N are RNA from cytoplasm, total cell and nuclei, respectively. The first three lanes shows RNA from CHO 42 cells, the next three lanes shows RNA from CHO 43 cells.

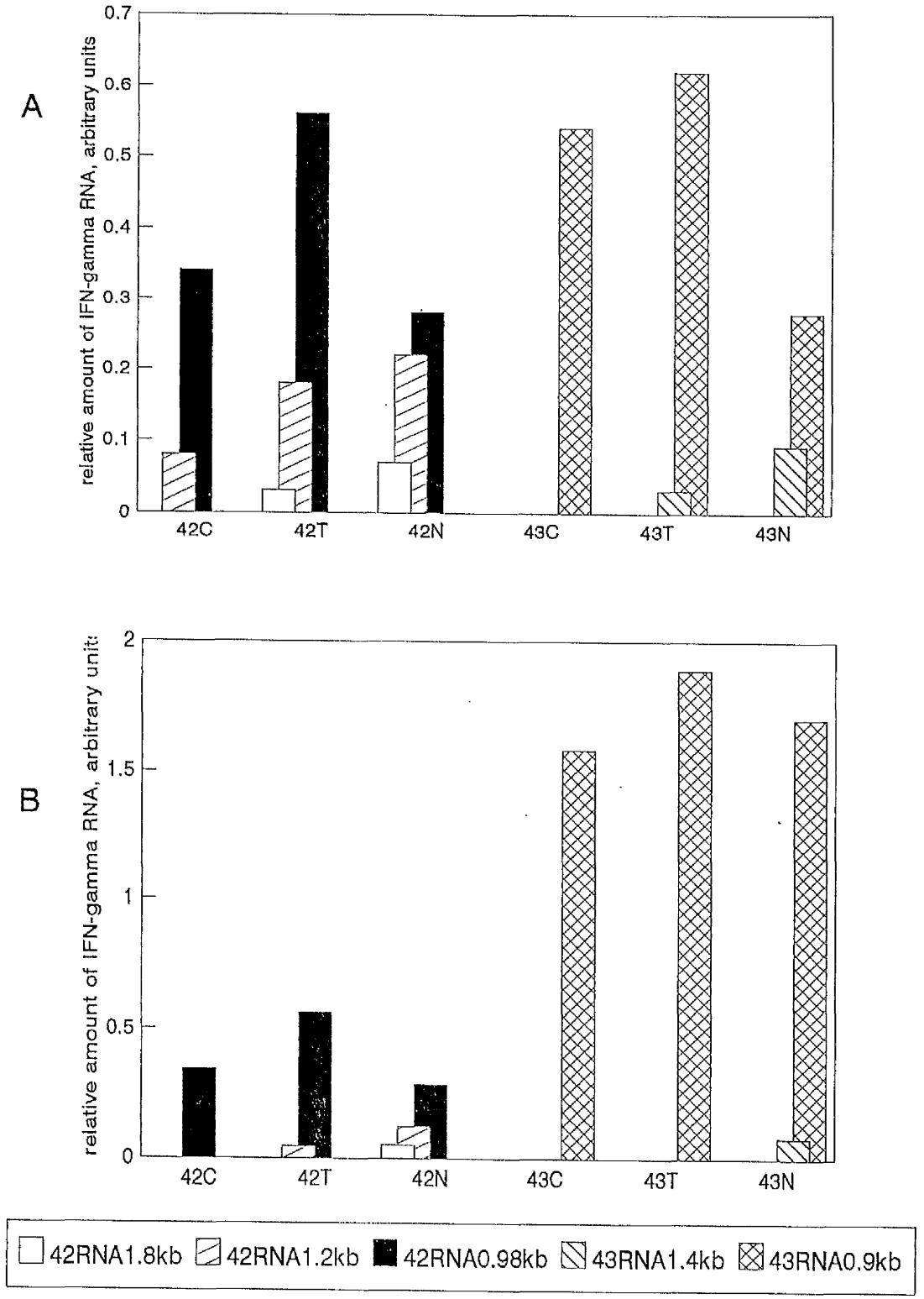


Cytoplasmic, total and nucleus RNA of CHO 42 and CHO 43 cells

Figure 3.39 Distribution of IFN-γ RNA in cytoplasm and nuclei in CHO 42 and CHO 43 cells

The autoradiographs of IFN-γ RNAs in Fig 3.38 were scanned as described in Section 2.6.4.3, and two separate experiments are shown in this figure. The amount of IFN-γ RNAs was calculated from 15 μg of RNA and normalised with 18S rRNA. Legends show the mRNA of CHO cells and its size (eg.: 42 0.98kb means IFN-γ mRNA of CHO 42 and its size is 0.98 kb).

Data shown are from two separate experiments

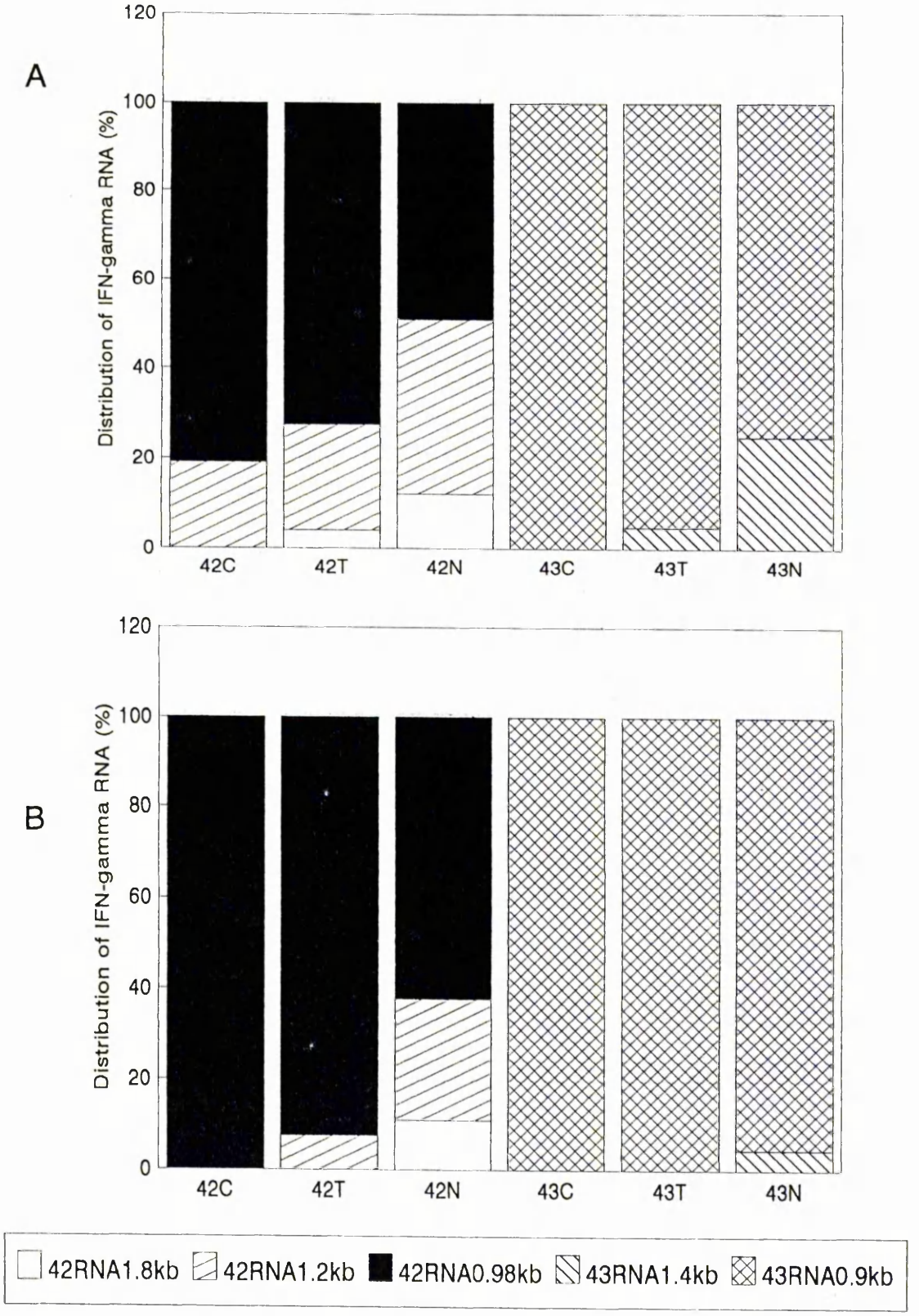


C: cytoplasmic RNA
N: nuclear RNA
T: total RNA

Figure 3.40 Composition of IFN-γ RNA in cytoplasm and nuclei in CHO 42 and CHO 43 cells

The percentage of each IFN-γ RNA isoform is expressed in relation to total IFN-γ RNAs (Fig 3.38; three isoforms in CHO 42 and two in CHO 43 cells).

Data shown are from two separate experiments



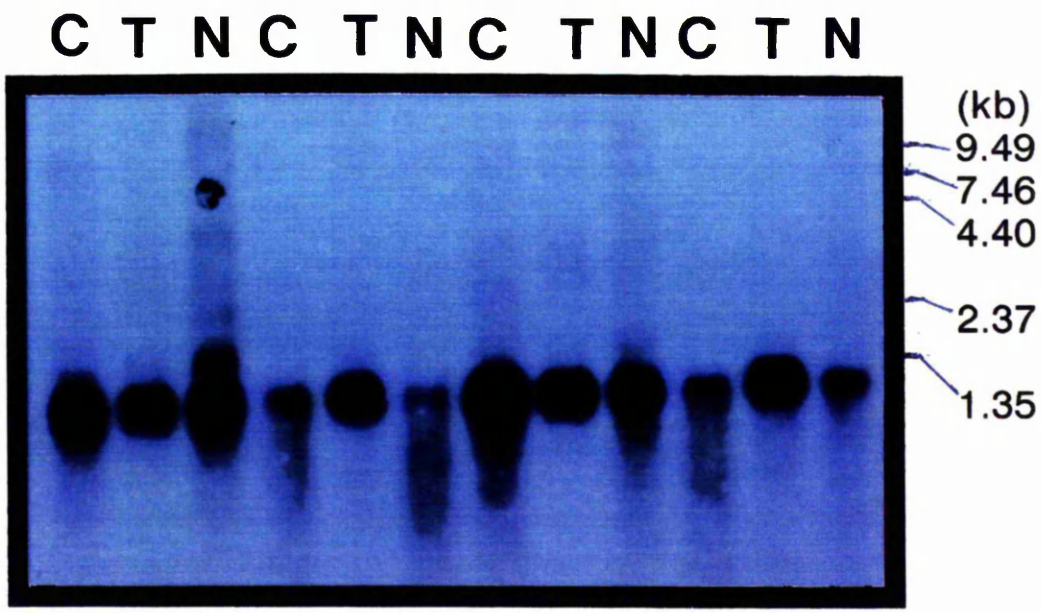
C: cytoplasmic RNA N: nuclear RNA T: total RNA

Figure 3.41 Northern hybridisation of cytoplasmic, total, and nuclear IFN-γ mRNA, and 18S rRNA of CHO 42 and CHO 43 cells at mid- and late-log phase

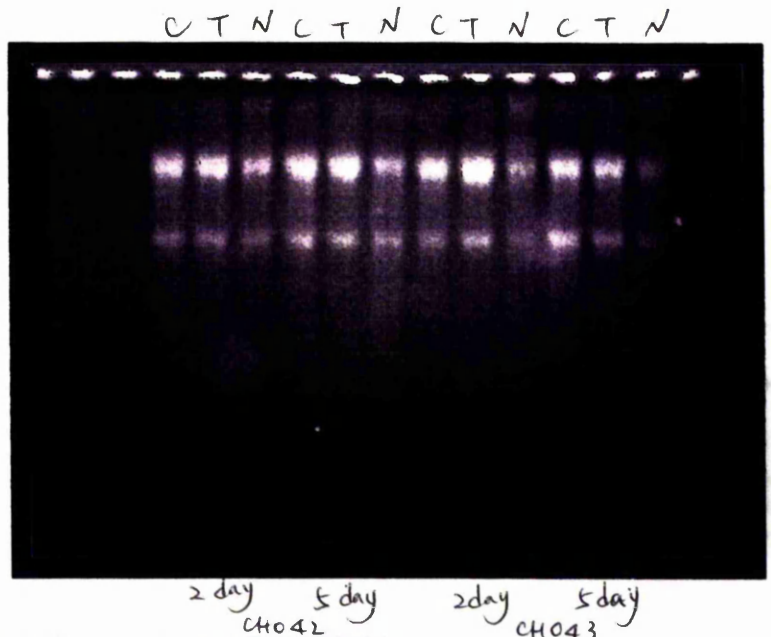
(A) CHO 42 and CHO 43 cells were cultured for 2 days (mid-log phase) and 5 days (late-log phase), cytoplasmic, total and nuclear RNA were extracted as described in Section 2.5.3. Extracted RNA was resolved in 1.5% agarose RNA gels (Section 2.6.1.2), Northern blotted, and probed with ^{32}P-labelled IFN-γ.

C,T,N are RNA from cytoplasm, total cell and nuclei, respectively. The first three lanes show RNA from 2 and next three lanes show 5 days of CHO 42 cell culture, the third and fourth set of three lanes show RNA from 2 and 5 days of CHO 43 cell culture.

(B) RNA gel was stained with ethidium bromide and equivalence of 18S and 28S rRNA loading is shown.



A:Northern hybridisation of IFN-γ



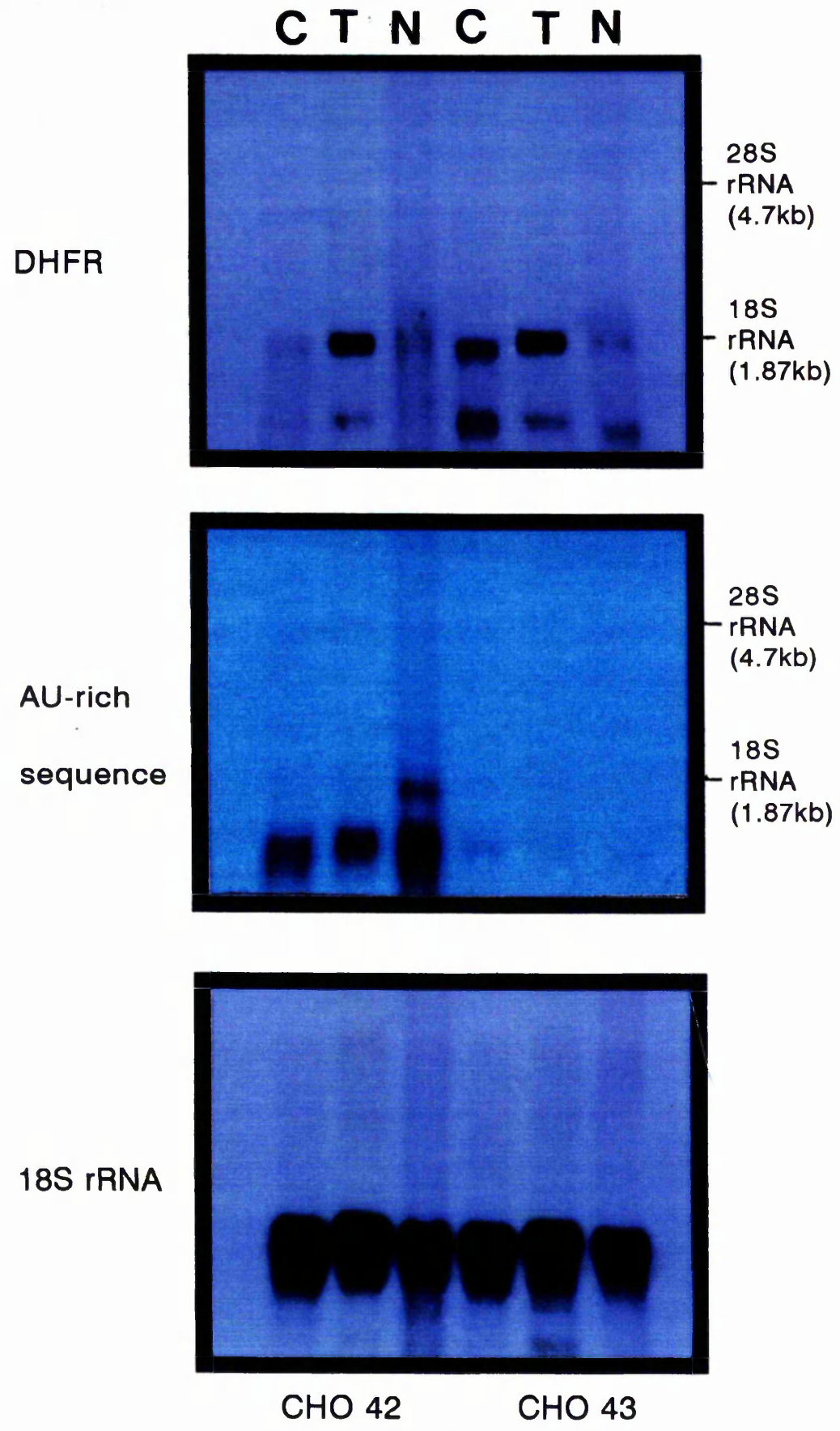
B:18S and 28S rRNA

Cytoplasmic and nucleus IFN- γ RNA

Figure 3.42 Northern hybridisation of cytoplasmic, total, and nuclear AU-rich containing IFN-γ, DHFR RNA and 18S rRNA of CHO 42 and CHO 43 cells

CHO 42 and CHO 43 cells were cultured for 2 days (mid-log phase). Cytoplasmic, total and nuclear RNA were extracted as described in Section 2.5.3. Extracted RNA was resolved in 1.5% agarose RNA gel (Section 2.6.1.2), Northern blotted, and probed with AU-rich sequence of IFN-γ (Section 2.6.2.3) and 18S rRNA cDNA.

C,T,N are RNA from cytoplasm, total cell and nuclei, respectively. The first three lanes show RNA from CHO 42 cells, the next three lanes show RNA from CHO 43 cells.


C T N C T N
DHFR
28S rRNA (4.7kb)
18S rRNA (1.87kb)
AU-rich sequence
28S rRNA (4.7kb)
18S rRNA (1.87kb)
18S rRNA
CHO 42
CHO 43

samples. There was a second species (about 4-25% of total IFN-γ RNA) of 1.49 kb in nuclear samples. This corresponds to the size of the expected initial transcript of the IFN-γ sequences in p1043.

The major IFN-γ RNA product in CHO 42 cell is one of 0.98 kb which accounts for more than 70% of total IFN-γ mRNAs in cytoplasm and total RNA samples. The 1.22 kb band (the expected size of IFN-γ mRNA product from p1042; Section 2.2.1) was present but accounted for only about 39% of all hybridising species in nuclear extracts (43-78% of the amount of the 0.98 kb mRNA) but it made up no more than 15% of all species in total RNA and less than 5% of the cytoplasmic species. The 1.78 kb IFN-γ transcript accounts for about 10% of IFN-γ RNA in nuclear samples.

During earlier analysis of IFN-γ mRNA (Section 3.1.4.1), only one IFN-γ mRNA (0.98 kb) was found at stationary phase. The distribution of IFN-γ RNAs in cytoplasm, nucleus and total RNA were also investigated in cells at stationary phase (Fig 3.41A). The amount of the 1.22 kb mRNA in nuclear samples at different stages of growth varied. The 1.22 kb IFN-γ mRNA species in CHO 42 cells was observed in nuclear samples of mid-log phase as seen in Fig 3.38. However, this IFN-γ mRNA was not found in nuclear extracts obtained from cells in stationary phase (Fig 3.41A). The 18S and 28S rRNA staining with ethidium bromide (Fig 3.41B) showed that this difference was not the result of unequal loading.

3.4.1.2 DHFR RNA species

IFN-γ expression in CHO 42 and CHO 43 cells is coamplified with a recombinant DHFR gene (Section 2.1.1.1). It is important to measure DHFR RNA in nucleus and cytoplasm in comparison to IFN-γ RNA. Northern hybridisation of DHFR mRNA is shown in Fig 3.42. CHO 42 and CHO 43 cells have the same pattern of DHFR RNA in cytoplasm, total and nuclear RNA, respectively. In the nuclear RNA, both CHO 42 and CHO 43 have faint DHFR cDNA hybridisation bands at 1.40 kb and 1.35 kb. These are likely to be the hybridisation to the recombinant DHFR primary transcript (1.40 kb species) and the product obtained after splicing the 50 bp intron (1.35 kb; from SV40 small t antigen; Nable et al., 1986). It was difficult to integrate their amount separately. These faint bands were about 10% in amount of the total DHFR mRNA and 25% of cytoplasmic RNA. Comparing this pattern with the pattern of IFN-γ RNAs in CHO 42 cells, the accumulation of a large amount of one RNA product (eg. 1.22 kb IFN-γ species; Section 3.4.1.1) in nucleus does not happen for DHFR pre-mRNA.

3.4.1.3 AU-rich IFN-γ RNA species

To investigate the integrity of the IFN-γ AU-rich sequence in CHO 42 mRNA, the AU-rich sequence was isolated from plasmid p1042 and used as a hybridisation probe (Section 2.6.4).

The AU-rich sequence of the recombinant IFN-γ gene in p1043 was removed, thus IFN-γ mRNA from CHO 43 cells should

not hybridise with this sequence and this was confirmed with blotting (Fig 3.42). For CHO 42 cells two bands (corresponding to 1.22 kb and 0.98 kb) were found in nuclear extracts hybridised to the "AU-probe", but only one band (0.98 kb) was found in either total and cytoplasmic samples in CHO 42 cells. The relative strength of hybridisation to the AU-rich sequence for the 1.22 kb and 0.98 kb nuclear IFN-γ RNA species is 3 to 10. This is consistent to the results of hybridisation of these species to probes containing the IFN-γ coding sequence (Section 3.4.1.1).

3.4.2 Poly(A)$^+$ containing IFN-γ RNA

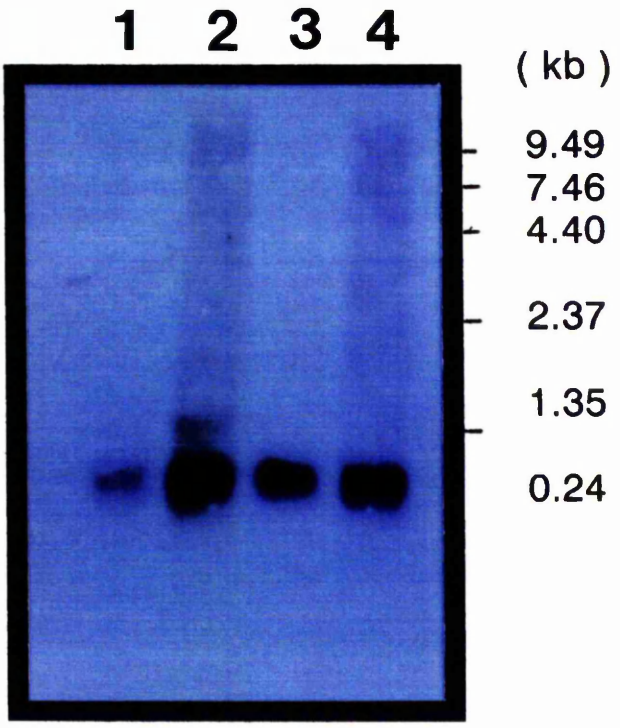
IFN-γ productivity of CHO 42 cells was lower than CHO 43 cells (Section 3.1.2.2) and there were two forms of IFN-γ mRNA in CHO 42 cells (1.22 kb and 0.98 kb; Section 3.1.2.1). It was important to define if either or both of these two IFN-γ mRNA species in CHO 42 cells were polyadenylated. Poly(A)$^+$-containing RNA in extracts of CHO 42 and CHO 43 cells were concentrated using oligo-d(T) cellulose column chromatography (Sambrook et al., 1989; Section 2.5.3). The photograph of 18S and 28S rRNA (Fig 3.43B) and Northern hybridisation reveals that IFN-γ mRNA was enriched in poly(A)$^+$ RNA samples (Fig 3.43A). This enrichment was about 20-fold (on a total 2 μg basis). All three forms of IFN-γ RNA (as seen in nuclear RNA extracts; 1.78 kb, 1.22 kb and 0.98 kb) in CHO 42 cells were enriched and thus they all contained poly(A)$^+$ tracts. Only a single form of IFN-γ RNA was found for both total RNA and enriched poly(A)$^+$ RNA of CHO 43 cells and this was of identical size (0.9 kb).

Figure 3.43 Northern hybridisation of IFN-γ poly(A)$^+$ RNA

(A) CHO 42 and CHO 43 cells were cultured for 2 days (mid-log phase). Total RNA was extracted as described in Section 2.5.3. Extracted RNA was purified through an oligo(dT) cellulose column as described in Section 2.5.4. The poly(A)$^+$ RNA were resolved in 1.5% agarose RNA gel (Section 2.6.1.2), Northern blotted and probed with IFN-γ cDNA (Section 2.6.4.1).

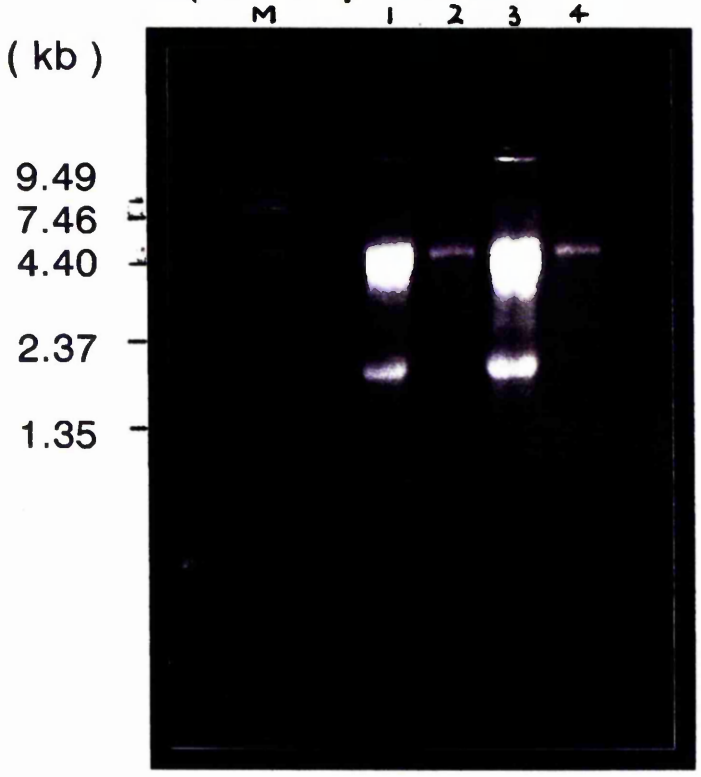
(B) The RNA gel was stained with ethidium bromide and the presence of bands corresponding to 18S and 28S rRNA was confirmed.

Lanes 1 and 2 are RNA samples from CHO 42 cells and 3 and 4 are from CHO 43 cells (lanes 1 and 3 are total and lanes 2 and 4 are poly(A)$^+$ RNA).



CHO 42 CHO 43

(A: IFN-γ poly(A)$^+$RNA)



(B: 18S and 28S rRNA)

3.4.3 IFN-γ mRNA and genomic DNA sequence

3.4.3.1 Synthesis of first strand cDNA

Poly(A)$^+$-enriched RNA purified from CHO 42 and CHO 43 cells, as described above (Section 3.4.2), was used as a template to generate first strand cDNA and the reaction was monitored by ^{32}P-dATP labelling (Section 2.6.3.2.1). Reverse transcription of poly(A)$^+$ RNA was successful with an incorporation efficiency of 10% (Table 3.5).

The first strand cDNA generated in the above reactions and genomic DNA of CHO 42 and CHO 43 cells, digested with Hind III, were used as templates for PCR amplification of IFN-γ sequences.

Table 3.5 Efficiency of reverse transcription

Counts					
CHO 42			CHO 43		
Filter	Washed	%	Filter	Washed	%
1177± 234	135±23	11.47	1413±169	161±38	11.43

Poly(A)$^+$-containing RNA was purified as described in Section 2.5.4. For 15µl reaction solution of CHO 42 cells, 2µg of poly(A)$^+$ RNA were used and for CHO 43 cells 1µg of poly(A)$^+$ RNA were used. After mixing the first strand cDNA synthesis buffer (Section 2.6.3.2.1), 1µl of reaction solution was transferred to a vial containing 1µl of ^{32}P-labelled dCTP and the reaction proceeded. The acid-precipitated fraction was counted using a liquid scintillation counter.

3.4.3.2 PCR product

Oligomers ACB6 and ACB8 have been successfully used to amplify part of IFN-γ fragment from CHO 43 cell genomic DNA (producing a fragment of 830 bp) and also from total RNA from CHO 43 cells (producing a fragment of 255 bp; Anwar, 1994).

Figure 3.44 PCR products generated using IFN-γ cDNA and genomic DNA isolated from CHO 42 and CHO 43 cells as templates

(A) Poly(A)$^+$ RNAs (cytoplasmic and total RNA; 0.11 µg of CHO 42, 0.028 µg of CHO 43 cells) were used to make first strand cDNA (Section 2.6.3.2.1). The synthesised first strand cDNA and Hind III digested genomic DNA (2.5 µg of CHO 42 and 1 µg of CHO 43 cells) and plasmid (1.2 ng of p1042 and 1 ng of p1043) were PCR amplified as described in Section 2.6.3. Lane 1 and 5 (shown as C in brackets) are PCR products of cytoplasmic poly(A)$^+$ mRNA (CHO 42 and CHO 43, respectively). Lane 2 and 6 (shown as T in brackets) are total RNA RT-PCD products. Lane 3 and 7 are PCR products generated from plasmids as template (p1042 and p1043, respectively). Lane 4 and 8 are PCR products of genomic DNA.

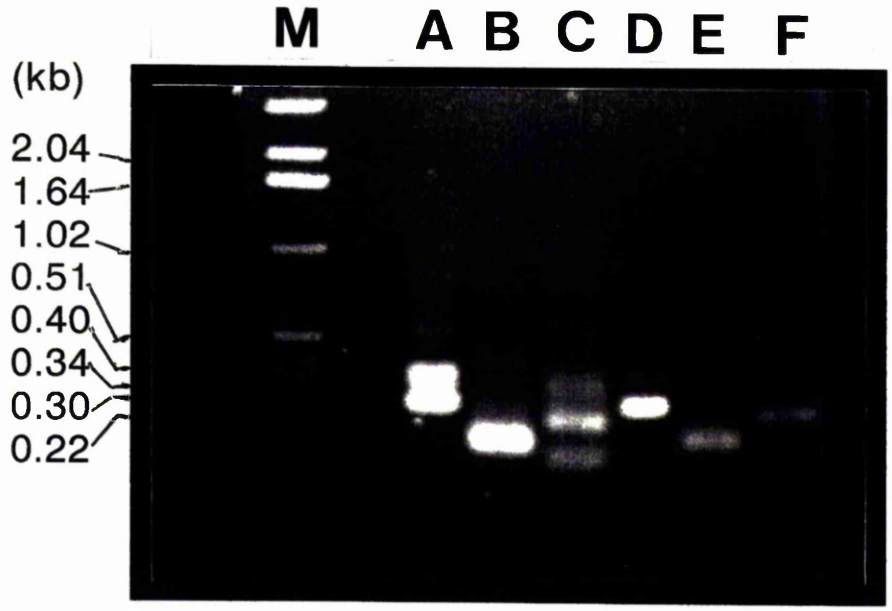
Lane	Source	Expected fragments (kb)	Amplified fragments (kb)
M	1kb ladder		
CHO 42			
1	RT-PCR(C)	0.52	0.29, 0.31
2	RT-PCR(T)	0.52	0.29, 0.31
3	p1042	1.14	1.14
4	DNA	1.14	1.14
CHO 43			
5	RT-PCR(C)	0.25	0.25
6	RT-PCR(T)	0.25	0.25
7	p1043	0.83	0.83
8	DNA	0.83	0.83

(B) The PCR products were digested with EcoR I or BamH I as described in Section 2.6.2. Lane A and D are RT-PCR products from total RNA (CHO 42 and CHO 43, respectively), and their digested products (lane B and E were digested with BamH I; lane C and E were digested with EcoR I).

Lane	PCR product source	Fragments (bp)
M	1kb ladder	
CHO 42		
A	RT-PCR(T)	**296, 308**
B	BamH I	**139, 147, 169 (152)**
C	EcoR I	**72, 72, 214, 236**
CHO 43		
D	RT-PCR(T)	**255**
E	BamH I	**116, 139 (130)**
F	EcoR I	**72, 183**

Bands shown on photograph (Fig 3.44B) are bold and BamH I digested sample showed one band in CHO 42 (B) and CHO 43 (E) are in brackets (average size of bands; 3 in CHO 42 cells and 2 in CHO 43 cells).

A:RT-PCR products of IFN- γ in CHO 42 and CHO 43 cells

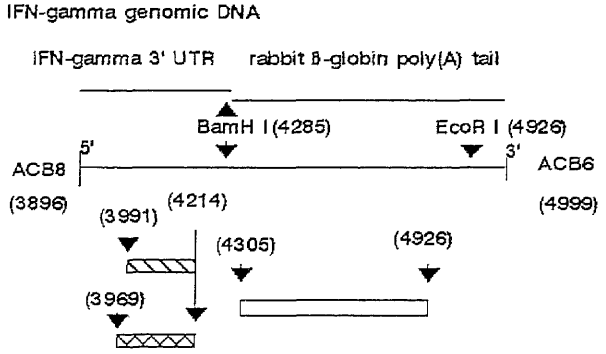


B:EcoR I and BamH I digestion

Figure 3.45 IFN-γ mRNA processing of CHO 42 and CHO 43 cells and the restriction sites of RT-PCR products

A: RNA42U and RNA42L processing of CHO 42 cells
The difference of bases composition between the genomic DNA and IFN-γ mRNA sequencing data (Fig 3.46) was used to compare the sequences of plasmid p1042 (Appendix II) to generate this figure.

B: RNA43 processing of CHO 43 cells
The difference of bases composition between the genomic DNA and IFN-γ mRNA sequencing data (Fig 3.47) was used to compare the sequences of plasmid p1043 (Appendix III) to generate this figure.



IFN-gamma mRNA of CHO 42 cells

RNA42U: and spliced

RNA42L: and spliced

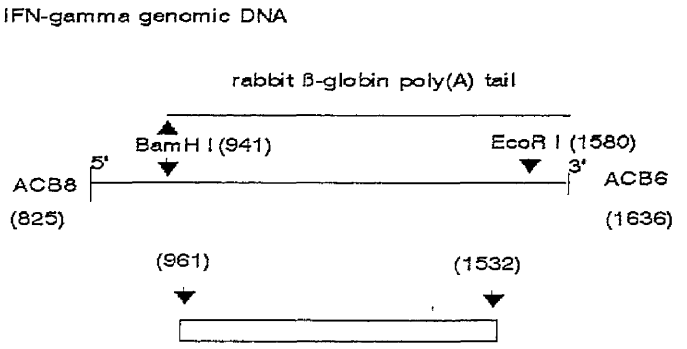
Cryptic intron 1 in IFN-gamma 3'UTR

Cryptic intron 2 in IFN-gamma 3'UTR

Intron from rabbit ß-globin poly(A) tail

Position of restriction site and splicing site are shown in brackets

A: RNA42U and RNA42L processing of CHO 42 cells



IFN-gamma mRNA of CHO 43 cells

spliced

Intron from rabbit ß-globin poly(A) tail

Position of restriction site and splicing site are shown in brackets

B: RNA43 processing of CHO 43 cells

These two oligomers were used to PCR amplify regions of IFN-γ from genomic DNA and cDNA generated from CHO 42 and CHO 43 cells. The PCR products were resolved in 1% agarose gels and the gels were stained with ethidium bromide (Fig 3.44A). Only one band was amplified from each of the genomic and cDNA samples from CHO 43 cells. However, for CHO 42 two bands were obtained from cDNA and one band was obtained from genomic DNA. The fragments amplified from genomic DNA were the same sizes as the respective fragments amplified from plasmid p1043 (830 bp) or p1042 (1.12 kb). The single band (255 bp) from CHO 43 cDNA is consistent with the previous report of Anwar (1994). The two bands amplified from CHO 42 cDNA were about 200 bp shorter than expected from plasmid p1042 and had molecular sizes of 308 bp and 286 bp (RNA42U and RNA42L, respectively).

The RT-PCR products were digested with restriction enzymes BamH I and EcoR I (Fig 3.44B), and all bands from CHO 42 and CHO 43 cDNA had both BamH I and EcoR I digestion sites as predicted from the structure of the recombinant IFN-γ gene in plasmids p1042 and p1043. When the RT-PCR products from CHO 42 cells were digested with BamH I restriction enzyme, both RNA42U and RNA42L generated a 239 bp (a single high density band was found) fragment. However, when digested with EcoR I restriction enzyme, RNA42U gave a 226 bp fragment and RNA42L gave a 204 bp fragment (a size differential identical to their precursors). All the BamH I and EcoR I digested fragments of both RNA42U and RNA42L are smaller than the expected sizes from plasmid p1042 digestion (404 bp and 474

bp, BamH I and EcoR I digestion, respectively).

As the BamH I site in ACB8 and ACB6 is located in the ligation junction of IFN-γ cDNA and rabbit β-globin poly(A) tail (as shown in Fig 2.1), the intactness of the BamH I site revealed that the missing bases are either in the IFN-γ cDNA 3'UTR area or in rabbit β-globin poly(A) tail. The EcoR I site in the ACB8 and ACB6 area is located in rabbit β-globin poly(A) sequence, the localisation of an EcoR I site suggests that the 200 "missing" base pairs (as described in earlier this Section) are not a result of splice removal of the rabbit β-globin poly(A) tail sequence. The BamH I and EcoR I digestion patterns revealed that the difference in sequences between these two frements was located in the IFN-γ AU-rich sequences area rather than in the poly(A) tail. The source of the two IFN-γ mRNA species are shown in Fig 3.45A and the positions of restriction sites, 5' and 3' splicing sites are given based on sequencing data of genomic DNA and RT-PCR products described in Section 3.4.3.3.

3.4.3.3 Genomic DNA and mRNA sequence

3.4.3.3.1 CHO 42 cells

Sequences were determined for two IFN-γ mRNA species, RNA42U and RNA42L, and for IFN-γ genomic DNA (Fig 3.46). Comparing the sequences of genomic DNA and both RNA42U and RNA42L, the two mRNAs were products of differential intron splicing. One of the introns spliced in these two IFN-γ mRNAs is the natural intron originating from the rabbit

Figure 3.46 IFN-γ genomic DNA and IFN-γ mRNA sequences of CHO 42 cells

The IFN-γ PCR and RT-PCR products of CHO 42 cells (Fig 3.44) were cloned into T-vector as described in Section 2.6.3.3 and the sequences of IFN-γ mRNA and genomic DNA were determined by sequencing as described in Section 2.6.4.4.

A: sequences of IFN-γ genomic DNA (1.14 kb)
B: sequences of RNA42U (308 bp IFN-γ mRNA species)
C: sequences of RNA42L (286 bp IFN-γ mRNA species)

A: IFN-γ DNA sequence shown here are
5'-GAGTCAGATGCTGTTTCGAGGTCGAAGAGCAT CCCAGTAATGGTTGTCCTACAATATTTGA-3'
* L — * U

B:Sequence of IFN-γ mRNA (RNA42U)
5'-TCCCAGTAATGGCAAGGCTA...CCCATGCGG GATCCTGAGAACTTCAGGCTCCTGGG-3'
@U

C:Sequence of IFN-γ mRNA (RNA42L)
5'-TGTTTCGAGGCAAGGCTATGTGATTAC...CC TGAAGAACTTCAGGCTCCTGGGCAACG-3'
@L

—— :Stop codon

* L :missing sequence in RNA42L

* U :missing sequences in RNA42U

@L :base in RNA42L differ from genomic DNA

@U :base in RNA42U differ from genomic DNA

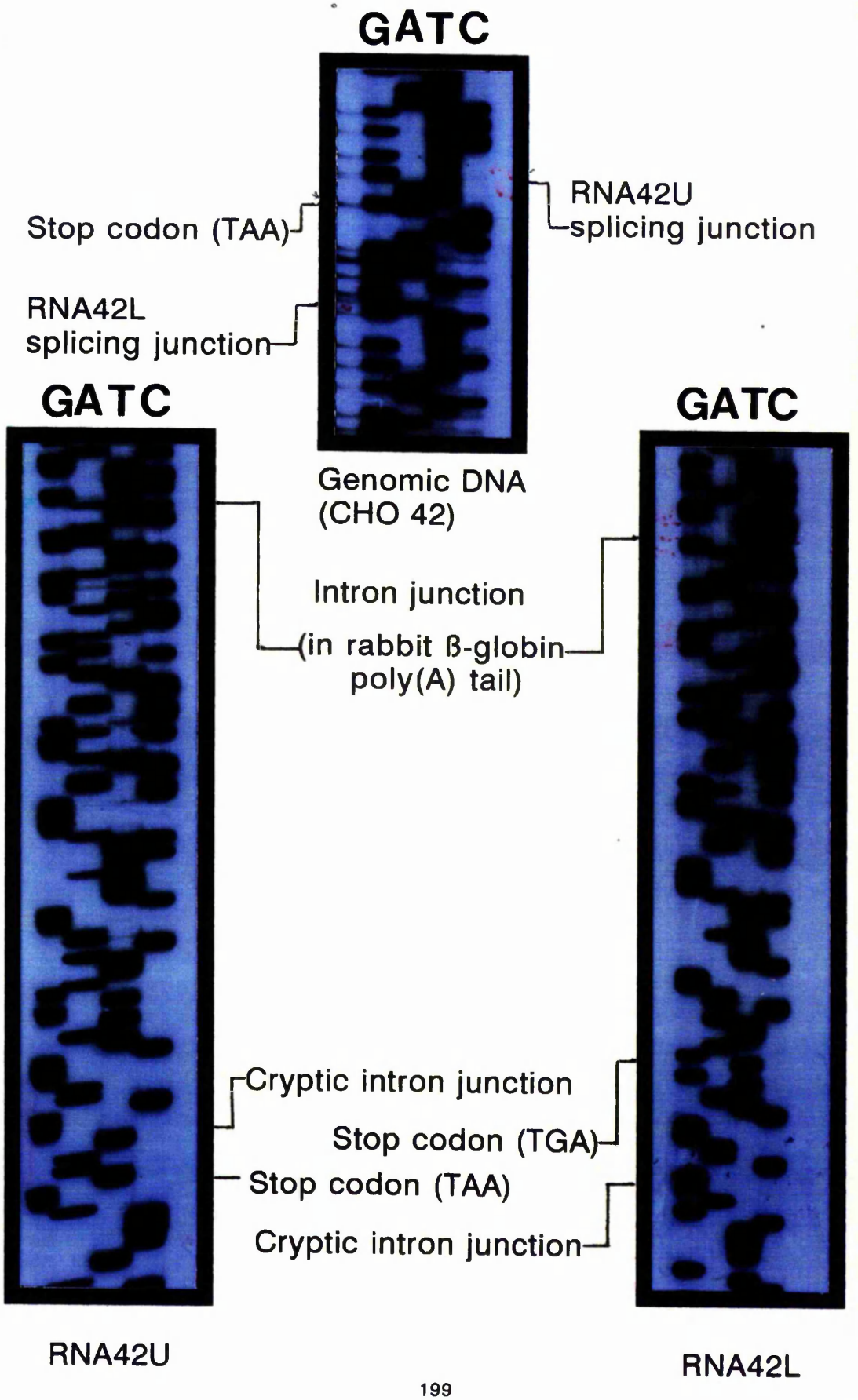

GATC
Stop codon (TAA)
RNA42L
splicing junction
RNA42U
splicing junction
Genomic DNA
(CHO 42)
GATC
GATC
Intron junction
(in rabbit ß-globin
poly(A) tail)
Cryptic intron junction
Stop codon (TGA)
Stop codon (TAA)
Cryptic intron junction
RNA42U
RNA42L

Figure 3.47 IFN-γ genomic DNA and IFN-γ mRNA sequences of CHO 43 cells

The IFN-γ PCR and RT-PCR products of CHO 43 cells (Fig 3.44) were cloned into T-vector as described in Section 2.6.3.3 and the sequences of IFN-γ mRNA and genomic DNA were determined by sequencing as described in Section 2.6.4.4.

A: sequences of IFN-γ genomic DNA (0.83 kb)
B: sequences of RNA43 (255 bp IFN-γ mRNA species)

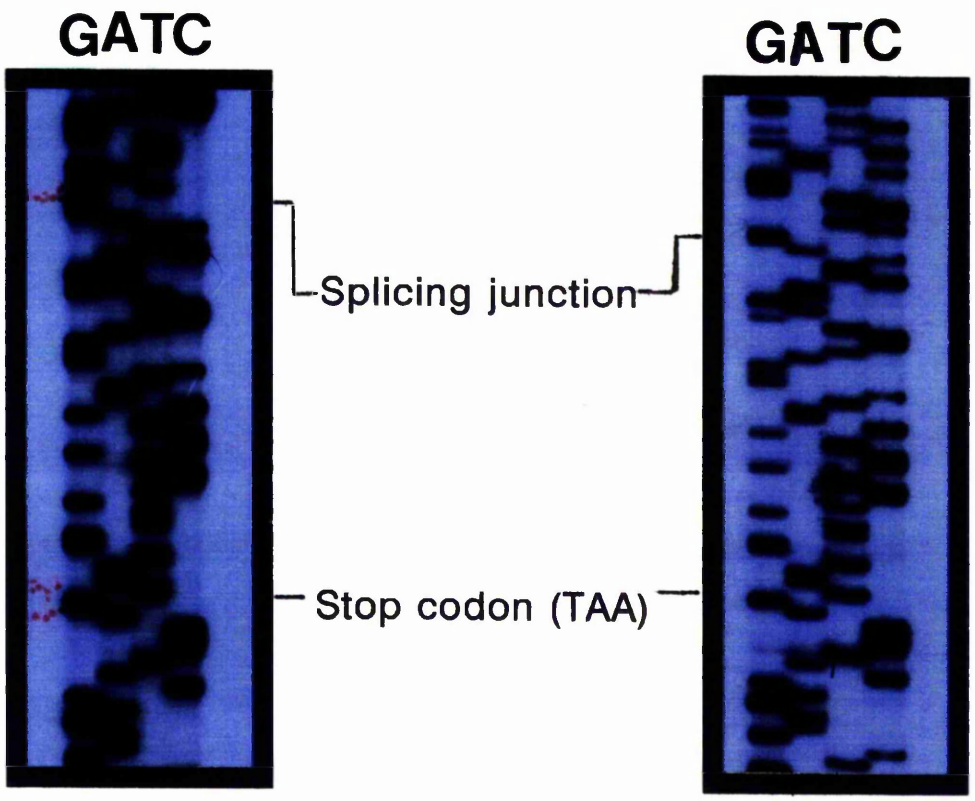
A: IFN-γ DNA sequence shown here are
5'-AGCATCCCAGTAATGGTTGTCCTGCCTGCAATCGGGATCCTGAGAAACTTCAGGGTGAG-3'
*

B:Sequence of IFN-γ mRNA (RNA43)
5'-AGCATCCCAGTAATGGTTGTCCTGCCTGCAATCGGGATCCTGAGAAACTTCAGGCTCCT-3'
@

—:Stop codon

* :base missing in RNA43

@ :base differ from genomic DNA


GATC
GATC
Splicing junction
Stop codon (TAA)
Genomic DNA
(CHO 43)
RNA43

poly(A) tail (bases 4305-4999 in plasmid p1042; van Ooyen et al., 1979). RNA42U and RNA42L differ from each other due to a further alternative splicing which occured at a cryptic intron which is located in the 3'UTR of IFN-γ pre-mRNA, containing the AU-rich sequence, and this site is not used by cells which produce IFN-γ naturally (Section, 2.1.1; Gray and Goeddel, 1982). RNA42U and RNA42L share the same 3' acceptor splicing site (at base 4214 in plasmid p1042) but use different 5' donor splicing site. The RNA42U species resulted from use of a 5' splicing site at base 3991, just 2 bases after the translation stop codon, TAA (bases 3987-3989). The RNA42L species resulted from use of another 5' splicing site at base 3969, 17 bases 5' of the translation stop codon. The whole sequences of IFN-γ mRNAs, RNA42U and RNA42L, of CHO 42 cells and their translation IFN-γ polypeptide sequences (IFN-γ and ab-IFN-γ) are also shown in Appendix II .

3.4.3.3.2 CHO 43 cells

The sequences of both IFN-γ genomic DNA and mRNA of CHO 43 cells are shown in Fig 3.47. Comparing the sequences of genomic DNA and IFN-γ cDNA, the 255 base pair fragment from RT-PCR of CHO 43 cell mRNA (Section 3.4.3.2; Fig 3.45B) results from splicing of an intron from the rabbit β-globin poly(A) tail (base 959 to base 1532 of plasmid p1043; Appendix III). The whole sequences of IFN-γ mRNAs, RNA43, of CHO 43 cells and its translational IFN-γ polypeptide sequences (IFN-γ) are also shown in Appendix III.

3.4.3.4 IFN-γ mRNA secondary structure prediction

Messenger RNA secondary structure has been reported to influence protein translation and mRNA stability (Konings et al., 1987). It is important to understand the secondary structure of each form of IFN-γ mRNA in CHO 42 and CHO 43 cells.

Using the computer program, FOLDRNA (based on lowest free energy structure; Zuker and Stiegler, 1981), the predicted secondary structures of IFN-γ mRNAs defined in CHO 42 and CHO 43 cells have been drawn (Fig 3.48). IFN-γ mRNA from CHO 43 cells (RNA43) has a predicted free energy of -210.5 kcal (-0.215 kcal/base), and contains 11 loops (75.4 base/loop). IFN-γ mRNAs of CHO 42 cells (RNA42U and RNA42L) both have similar prediction conformations around the initiation codon (starting at 67 base) to that determined for RNA43. RNA42U has a predicted free energy of -221.5 kcal (-0.215 kcal/base) and contains 11 loops (93.8 base/loop) and RNA42L has a predicted free energy of -220.1 kcal (-0.218 kcal/base) and contains 12 loops (84.2 base/loop). However, using the IFN-γ mRNA structure predicted to arise from direct transcription of p1042 (AU-rich containing mRNA) generates a predicted molecule with a different secondary structure around the initiation codon. This species also has more loops (15 loops, 79.1 base/loop) and a lower free energy (-242.4 kcal, -0.204 kcal/base).

3.5 IFN-γ isomer sequence and structure prediction

In an earlier section (3.4.3), I characterised the existence of an alternatively spliced IFN-γ mRNA species

Figure 3.48 IFN-γ mRNA secondary structure and folding energy prediction

IFN-γ mRNA sequences, containing 150 poly(A) tail, from the CHO 42 and CHO 43 cells were used as input for a computer program, FOLDRNA (based on minium energy; Zuker and Stiegler, 1981), to predict their RNA secondary structure.

A: IFN-γ mRNA of CHO 43 cells (RNA43)
B: IFN-γ mRNA of CHO 42 cells (RNA42U)
C: IFN-γ mRNA of CHO 42 cells (RNA42L)
D: IFN-γ mRNA of CHO 42 cells with the AU-rich sequence intact (RNA42)

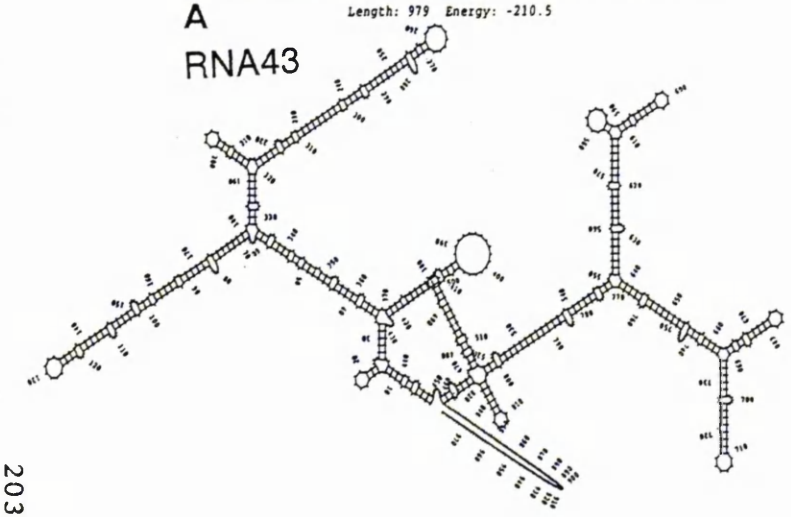
RNA species	RNA43	RNA42	RNA42U	RNA42L
Free energy (kcal)	-210.5	-242.4	-221.5	-220.1
Free energy per base	-0.215	-0.204	-0.215	-0.218
Loops	11	15	11	12

RNA43: 0.829 kb (ARE deleted)
RNA42: 1.187 kb (contining ARE)
RNA42U: 1.132 kb (ARE spliced)
RNA42L: 1.010 kb (ARE spliced)

SQUIGGLES of: rna43.connect October 12, 1994 11:29
FOLDRNA of: rna43 Check: 8761 from: 1 to: 979 October 12, 1994 11:22
Length: 979 Energy: -210.5

A

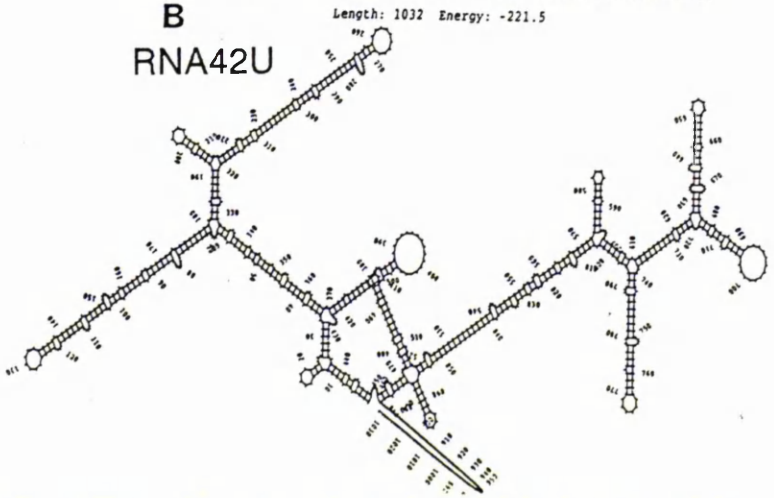
RNA43



SQUIGGLES of: rna42u.connect October 12, 1994 11:12
FOLDRNA of: rna42u Check: 5914 from: 1 to: 1032 October 12, 1994 11:04
Length: 1032 Energy: -221.5

B

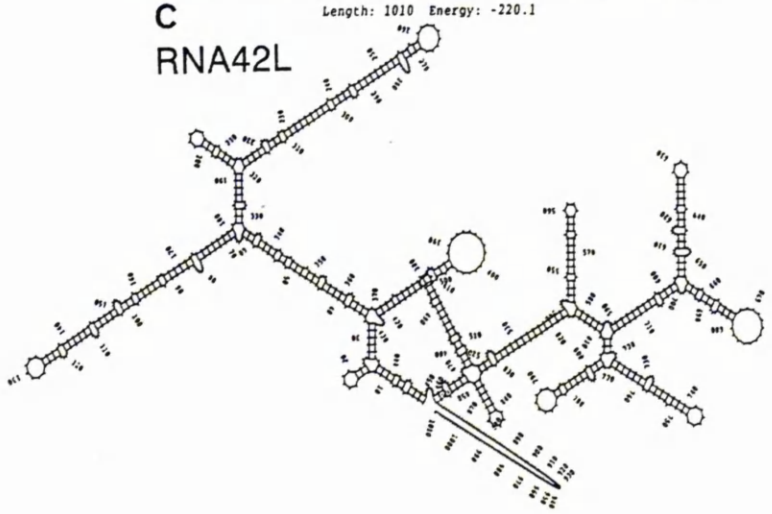
RNA42U



SQUIGGLES of: rna421.connect October 12, 1994 11:21
FOLDRNA of: rna421 Check: 3953 from: 1 to: 1010 October 12, 1994 11:12
Length: 1010 Energy: -220.1

C

RNA42L



SQUIGGLES of: rna42us.connect June 30, 1995 09:56
FOLDRNA of: rna42us Check: 7671 from: 1 to: 1187 June 29, 1995 16:31
Length: 1187 Energy: -242.4

D

RNA42
(containing ARE)

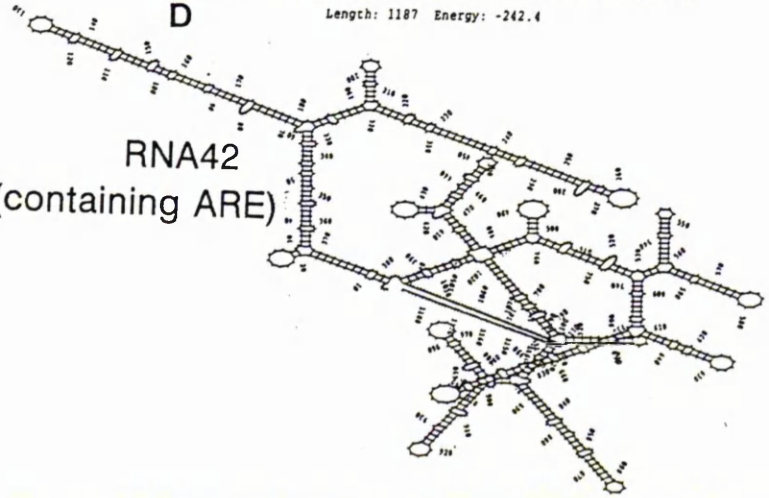


Figure 3.49 IFN-γ mRNA processing and translation of IFN-γ mRNA products from CHO 42 and CHO 43 cells

Sequences data from Fig 3.46 and Fig 3.47 were used to generated this figure to show IFN-γ RNA processing by CHO 42 and CHO 43 cells. IFN-γ mRNA of CHO 42 (RNA42U and RNA42L) and CHO 43 cells (RNA43) were used as input for a computer program, SEQAID, to define the polypeptide sequence.

A: processing of RNA42U

Two introns are splicing in this IFN-γ mRNA, one intron (base 4305-4879 in plasmid p1042; Appendix II) is located in rabbit β-globin poly(A) tail and the other one in IFN-γ 3' UTR (base 3992-4214; two base 3' to stop codon, UAA base 3987-3989). This mRNA translated a natural form IFN-γ (IFN-γ) and has six amino acid (amino acid 160-166) differ from translation product (ab-IFN-γ) of RNA42L.

B: processing of RNA42L

Two introns are splicing in this IFN-γ mRNA, one intron (base 4305-4879 in plasmid p1042; Appendix II) is located in rabbit β-globin poly(A) tail and the other one in IFN-γ 3' UTR (base 3970-4214; including stop codon, UAA base 3987-3989). When translation, the translation macinary readthrough to a new stop codon, UGA (base 4225-4227) and produced an abnormal form of IFN-γ (ab-IFN-γ) which has four amino acid (amino acid 160-164) differ from natural IFN-γ.

C: processing of RNA43

One intron is splicing in this IFN-γ mRNA (base 961-1532 in plasmid p1043; Appendix III) the intron is located in rabbit β-globin poly(A) tail. This mRNA translated a natural form IFN-γ (IFN-γ) and has six amino acid (amino acid 160-166) differ from translation product (ab-IFN-γ) of RNA42L.


A: processing of RNA42U (CHO 42 cell)
genomic IFN-γ DNA
AT-rich sequence
Intron
5'- TTTCGAGGT CGAAGAGCATCCCAGTAATG GT TG--- AG GCAAGGCTATGTGA---GG GT ---AG CTCCT--- 3'
Intron splicing
RNA42U
5'- UUUCGAGGUCGAGCAUCCCAGUAAUGGCAAGGCUAUGUGA---GGCUCCU- 3'
IFN-γ
N'- PheArgGlyArgArgAlaSerGln>>> (Natural IFN-γ protein)
B: processing of RNA42L (CHO 42)
genomic IFN-γ DNA
AT-rich sequence
Intron
5'- TTTCGAGGT CGAAGAGCATCCCAGTAATGGT TG--- AG GCAAGGCTATGTGA ---GG GT ---AG CTCCT--- 3'
Intron splicing
RNA42U
5'- UUUCGAGGCAAGGCUAUGUGA---GGCUCCU -3'
ab-IFN-γ
N'-PheArgGlyLysAlaMet >>> (Abnormal IFN-γ protein)
C: Processing of RNA43 (CHO 43)
genomic IFN-γ DNA
Intron
5'- TTTCGAGGT CGAAGAGCATCCCAGTAATGGTTGTCCTGCCTAC... GGT ---AG CTCCT--- 3'45
Intron splicing
RNA43
5'- UUUCGAGGUCGAGCAUCCCAGUAAUGGUUGUCCUGCCUAC---GGCUCCU -3'
IFN-γ
N'-PheArgGlyArgArgAlaSerGln>>> (Natural IFN-γ protein)

Figure 3.50 IFN-γ polypeptide secondary structure prediction and hydrophobicity

IFN-γ proteins (natural and abnormal) were used as input for a computer program, PLOTSTRUCTURE (based on Chou-Fasman protein structure prediction; Chou and Fasman, 1978) to predict their secondary structure and hydrophobicity (based on Hopp and Wvoels, 1981).

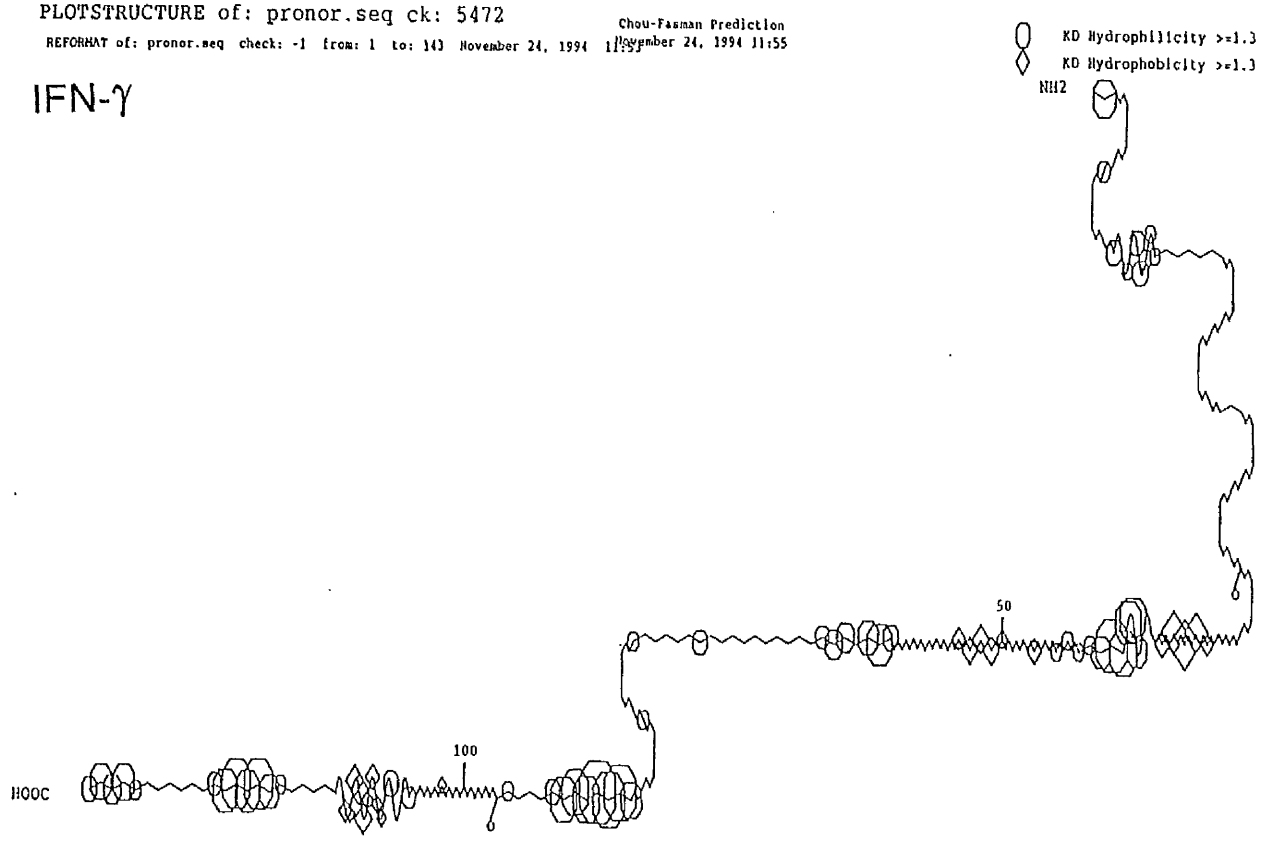
A: IFN-γ (Natural IFN-γ; products of RNA42U and RNA43)
B: ab-IFN-γ(Abnormal IFN-γ; products of RNA42L)

PLOTSTRUCTURE of: pronor.seq ck: 5472

REFORMAT of: pronor.seq check: -1 from: 1 to: 143 November 24, 1994 11:55

Chou-Fasman Prediction
November 24, 1994 11:55

IFN-γ



PLOTSTRUCTURE of: prounn.seq ck: 506

REFORMAT of: prounn.seq check: -1 from: 1 to: 141 November 24, 1994 11:56

Chou-Fasman Prediction
November 24, 1994 11:56

ab-IFN-γ

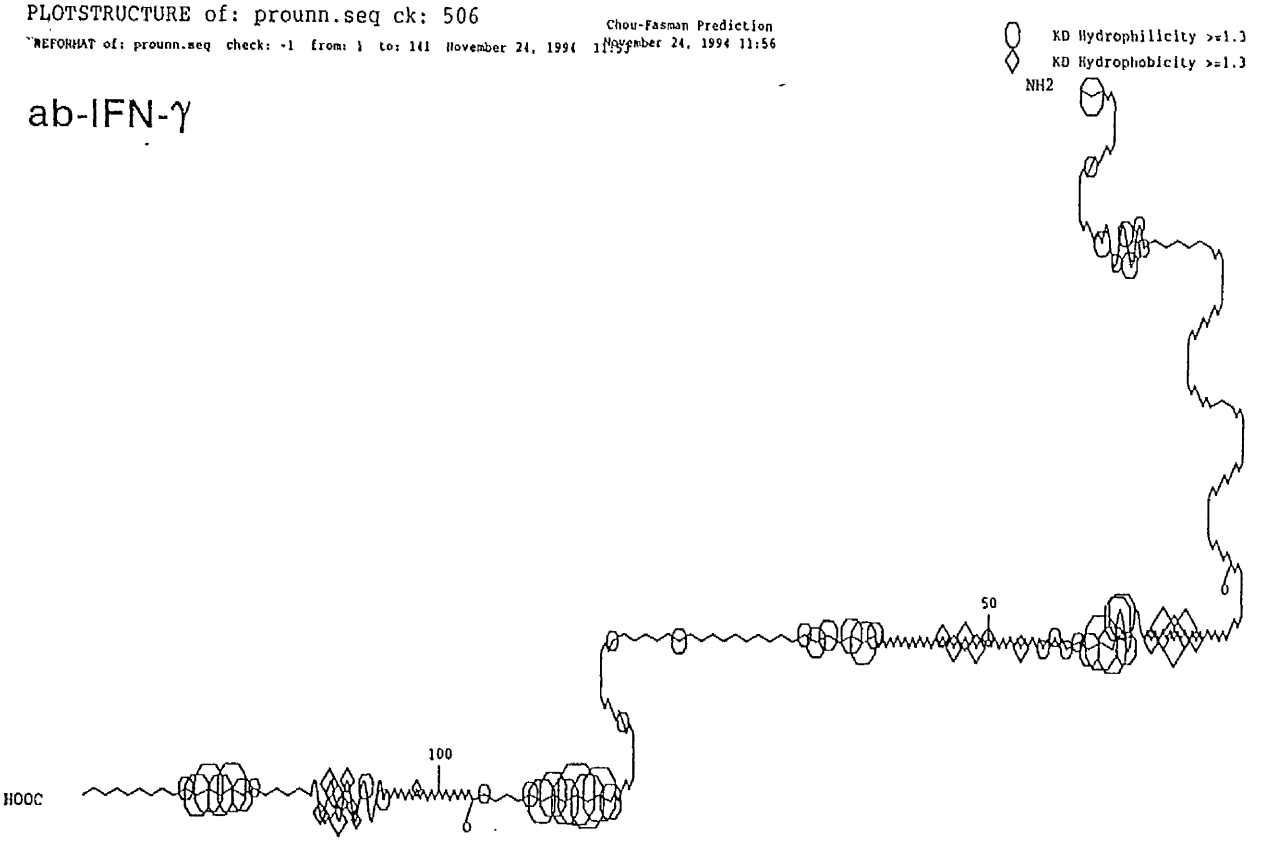
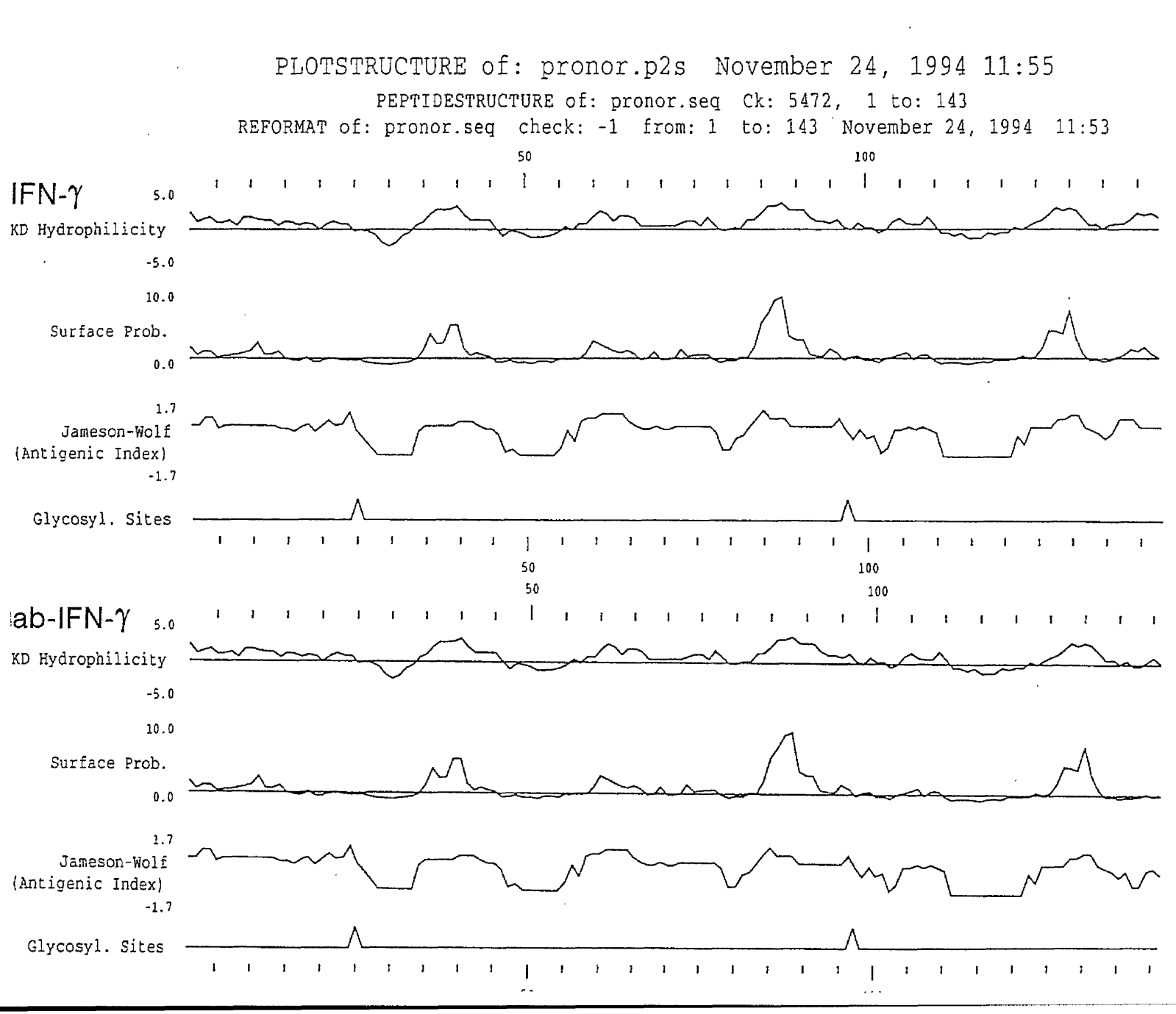


Figure 3.51 IFN-γ surface probility and antigenic index prediction

IFN-γ proteins (natural and abnormal) were used as input for a computer program, Plotstructure to predict their surface probility (based on Emini et al., 1985) and antigen index (based on Jameson and Wolf, 1981).

A: IFN-γ (Natural IFN-γ; products of RNA42U and RNA43)
B: ab-IFN-γ (Abnormal IFN-γ; products of RNA42L)


PLOTSTRUCTURE of: pronor.p2s November 24, 1994 11:55
PEPTIDESTRUCTURE of: pronor.seq Ck: 5472, 1 to: 143
REFORMAT of: pronor.seq check: -1 from: 1 to: 143 November 24, 1994 11:53
IFN-γ
KD Hydrophilicity
5.0
-5.0
Surface Prob.
10.0
0.0
Jameson-Wolf
(Antigenic Index)
1.7
-1.7
Glycosyl. Sites
50
100
ab-IFN-γ
KD Hydrophilicity
5.0
-5.0
Surface Prob.
10.0
0.0
Jameson-Wolf
(Antigenic Index)
1.7
-1.7
Glycosyl. Sites
50
100

(RNA42L) in which the natural stop codon was spliced (Section 3.4.3.3.1) and which may be generated a new stop codon for translation. When the RNA42L mRNA is translated (Fig 3.50), the translation machinery could readthrough the natural stop codon position until next stop codon, UGA, appeared and this is located 13 bases downstream of the splicing junction. The protein (ab-IFN-γ) translated from this RNA42L mRNA would lose the last 6 amino acids (N'-GlyArgArgAla-SerGln) of natural IFN-γ (translated from both RNA42U and RNA 43) to be substituted with 4 new amino acids (N'-GlyLysAlaMet; Fig 3.49). However, these two forms of IFN-γ have only five amino acids different and exhibit an overall 97% homology.

Using this new protein sequence, I have compared ab-IFN-γ to natural IFN-γ in terms of secondary structure, hydophilicity (Fig 3.50), surface probility and antigenic index (Fig 3.51). The two forms of IFN-γ have very similar predicted secondary structure. They also have very similar surface probility and antigenic index throughout the whole sequence. However, the two IFN-γ species have different hydrophilicity at the C terminus. IFN-γ has a strong hydophilic tail but the Ab-IFN-γ tail shows neither hydophilic nor hydrophobic characteristics.

CHAPTER FOUR : DISCUSSION

Plasmids p1042 and p1043 share common DHFR and IFN-γ cDNA expression cassettes but differ in the IFN-γ 3'UTR. Plasmid p1042 contains an AU-rich sequence at the 3'UTR of the IFN-γ cDNA but this sequence was deleted from p1043. Transfection of these two plasmids into CHO Duk cells allowed the establishment of two cell lines, CHO 42 cells (generated from plasmid p1042) produced only 4% of the amount of IFN-γ/cell of CHO 43 cells (generated from plasmid p1043; Section 3.1.2.2). Comparisons of the two cell lines are shown in Fig 4.1 and Table 4.1. In this thesis, I have shown that, compared to CHO 43 cells, CHO 42 cells have:

(1) less IFN-γ mRNA (22.2%)

(2) lower IFN-γ translation (22.2%)

(3) less DHFR mRNA (21.7%)

(4) lower IFN-γ gene copy number per cell (20%)

These differences cannot explain the 20-fold lower rate of IFN-γ production by CHO 42 cells. There are a number of other significant findings from my studies:

(1) there are two IFN-γ mRNA species in CHO 42 cells but only one in CHO 43 cell (Section 3.1.2.1)

(2) the stability of IFN-γ mRNA of CHO 42 cells is comparable to that of CHO 43 cells (Table 4.1)

(3) there are five IFN-γ protein isoforms in CHO 42 cells but only three in CHO 43 cells (Section 3.1.2.3)

(4) IFN-γ protein secretion is lower in CHO 42 cells than CHO 43 cells (5.5% of CHO 43 cells; Section 3.3.3.1)

(5) most of the IFN-γ secreted by CHO 42 cells is the doubly-glycosylated form whilst nearly equal amount of singly- and doubly-glycosylated forms are secreted by CHO 43 cells (Section 3.3.3.2)

Table 4.1 Comparison of CHO 42 and CHO 43 cells

	CHO 42	CHO 43
Cell volume (X 10^{-2} µl)[1]	1.12 (0.33)	3.40 (1)
IFN-γgene copy number[2]	250 (0.17)	1500 (1)
IFN-γ mRNA		
AU-rich sequence[3]	Yes	No
Amount[4]	0.24 (0.21)	1.14 (1)
Species[5]	2	1
size (kb)	0.98, 1.22	0.9
Stability[6]	>8 (0.98 kb)	>8 (0.9 kb)
half-life (hr)	5.2 (1.22 kb)	
IFN-γ protein		
Production[7]	8.2 (0.04)	202 (1)
Cellular[8]	1.89 (0.59)	3.21 (1)
Species[9]	5	3
size (kDa)	17, 21, 25, 31,34	17, 21, 25
Translation[10]	67.8 (0.22)	308 (1)
Ratio (D- to S-gly)[11]		
Cellular	1.7	1.2
Secreted	4.4	1.4
Secretion rates[12]	20 (0.098)	203 (1)
DHFR mRNA amount[13]	0.29 (0.26)	1.15 (1)

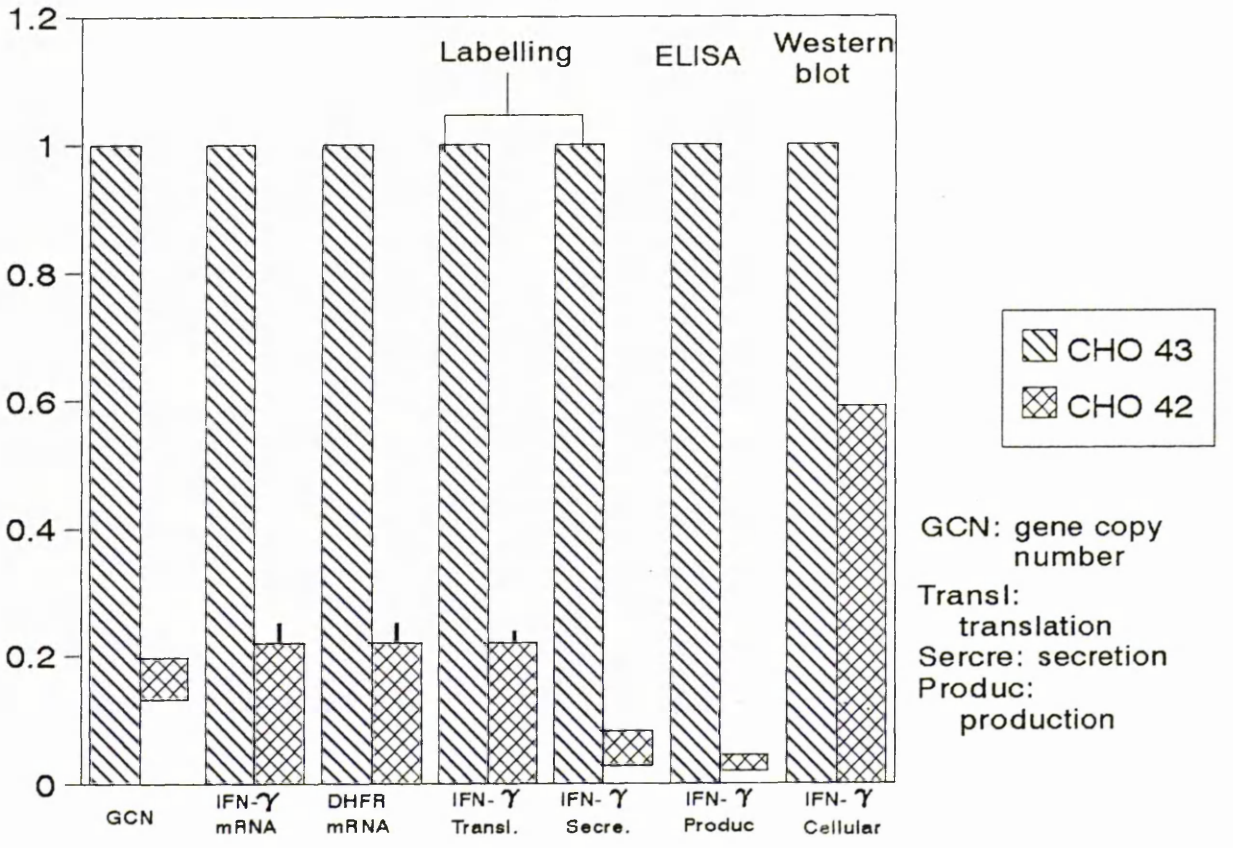
Ratio shown in brackets are based on ratio of CHO 42 to CHO 43 cells (as 1) except data in IFN-γ mRNA stability which show the mRNA size.

1. Data from Section 3.1.1.1; 2. Courtesy of Alison Bate; 3. Described in Fig 2.1; 4. Data show are arbitrary densitometric unit/cell. Data from Section 3.1.2.1; 5. Described in Section 3.1.2.1; 6. Described in Section 3.2.1.2; 7. Data based on (X 10^{-5} units/cell/hr); Data from Section 3.1.2.2; 8. Data shown are Western arbitrary units; Data from Section 3.1.2.3; 9. Described in Figs 3.6, 3.24, 3.25.; 10. Based on arbitrary densitometric unit/cell (labelled for 2 hr); Described in Section 3.3.1.1; 11 D- and S-gly represent doubly- and singly-glycosylated isoforms. Described in Sections 3.3.2.1 and 3.3.3.1; 12. Data based on arbitrary densitometric units/cell (1 hr); Described in Section 3.3.3.1; 13. Data shown are arbitrary densitometric units/cell; Described in 3.1.4.2.

Figure 4.1 Comparison of gene expression of IFN-γ and DHFR of CHO 42 and CHO 43 cells

The IFN-γ and DHFR gene expression data of CHO 42 cells in Chapter three are used to compare with data of CHO 43 cells (1) to generate this figure. IFN-γ gene copy number, secretion and production are shown as high and low from two separate experiments. IFN-γ in cellular sample is from single experiment and others are from more than three experiments (standard error bars are shown).

A. IFN-γ Gene copy number is courtesy of Alison Bate.
B. IFN-γ mRNA data from Section 3.1.2.1.
C. DHFR mRNA data shown is described in 3.1.4.2.
D. IFN-γ translation is described in Section 3.3.1.1.
E. IFN-γ secretion rate is described in Section 3.3.3.1.
F. IFN-γ production in culture medium is described in Section 3.1.2.2.
G. IFN-γ in CHO cells are described in Section 3.1.2.3.


1.2
1
0.8
0.6
0.4
0.2
0
Labelling
ELISA
Western blot
GCN
IFN-γ mRNA
DHFR mRNA
IFN-γ Transl.
IFN-γ Secre.
IFN-γ Produc
IFN-γ Cellular
CHO 43
CHO 42
GCN: gene copy number
Transl: translation
Sercre: secretion
Produc: production

In the sections which follow, I will concentrate on discussing IFN-γ mRNA species and stability, IFN-γ pre-mRNA splicing, IFN-γ protein isoforms and IFN-γ secretion.

4.1 IFN-γ mRNA species and stability

Many cytokine and lymphokine mRNAs are rapidly degraded in their natural producing cells and have half-lives of less than 30 min (Stoecklin et al., 1994; Henics et al., 1994; Section 1.2.2.2.2.1). Reiterated ARE found within the 3' UTR of these labile mRNAs are responsible for mRNA lability. However, Henics et al. (1994) reported that the stability of IL-2 mRNA (a cytokine) was enhanced in type C retrovirus-containing cells. The IL-2 mRNA was stabilised because the ARE was spliced. This splicing results from the IL-2 pre-mRNA structure being changed by proviral insertion with an internal deletion within the ARE of 3'UTR. CHO cell lines contain such a type C retrovirus (Liptrot and Gull, 1992; Lie et al., 1994) and this raises the possibility that the IFN-γ mRNA of CHO 42 cells might be stabilised through a similar mechanism. To address this issue, the IFN-γ gene in both plasmid DNA and genomic DNA from CHO 42 and CHO 43 cells was PCR-amplified with primers ACB9 and ACB10. The IFN-γ-specific sequence PCR-amplified products showed that the IFN-γ genes in CHO 42 and CHO 43 cells had identical sizes to those from p1042 and p1043 (Fig 3.7). In addition, Southern hybridisation of genomic DNA with IFN-γ cDNA showed that the recombinant IFN-γ genes of CHO 42 and CHO 43 cells have

similar restriction digestion patterns and restriction fragment sizes to those obtained from plasmids p1042 and p1043 (Section 3.1.2.4). The PCR and Southern hybridisation results showed no evidence for this insertion or of the deletion events that might have occurred in CHO 42 cells to modify IFN-γ mRNA structure and hence half-life. Thus enhanced stability of IFN-γ mRNA must be caused by mechanisms other than proviral insertion or gross gene rearrangement.

Change of IL-2 gene structure, by viral insertion and deletion, caused a cryptic splicing site within 3'UTR of IL-2 pre-mRNA to be utilised and as a consequence all the AREs were deleted from the IL-2 mRNA (Henics et al., 1994). The 3' terminal and poly(A) signals of the recombinant IFN-γ gene in plasmids 1042 and p1043 did not originate from the IFN-γ gene but were derived from sequences from the rabbit β-globin gene. This change to the 3'UTR of the IFN-γ gene could change the secondary structure of IFN-γ pre-mRNA and consequently a cryptic intron in the ARE may be recognised and spliced, thus resulting in a stabilised IFN-γ mRNA.

A further possibility exists that specific bases in the IFN-γ gene may have mutated and created splice sites at the ARE which result in ARE splicing. To investigate these issues, the 3' end of the IFN-γ coding region and 3'UTR of both the 0.98 kb IFN-γ mRNA and IFN-γ gene were sequenced (Section 3.4.3.3). The RT-PCR results and sequence data of IFN-γ mRNA and IFN-γ gene showed that the 0.98 kb mRNA species was a mixture of two IFN-γ mRNAs in which the ARE was subject to usage of alternative 5' splice sites. These

results suggest the source of 0.9 kb mRNA species and suggest that the enhanced IFN-γ mRNA stability results from removal of 240 bp region containing the ARE. My sequencing of recombinant IFN-γ in genomic DNA showed that mutation did not generate new splice sites in the IFN-γ gene in CHO 42 cells (Section 3.4.3.3.2). This suggested that splicing may result from events such as the structure of recombinant IFN-γ pre-mRNA structure exposing cryptic splicing sites to permit the binding of splicing factors. The possible causes of alternative splicing will be discussed later (Section 4.2).

4.2 Mechanism of ARE alternative splicing

There are several questions raised from my observation of splicing variants for IFN-γ RNA transcripts. Firstly, what makes splicing occur at the cryptic sites which were not used by naturally producing cells? Is this splicing cell-specific or a result of changes to IFN-γ gene structure? Secondly, what determines the balance in the alternative splicing?

The formation of a spliceosome depends on the binding of snRNPs to the consensus sequence of a mRNA 5' splicing site, branch point, pyrimidine tract and 3' splicing site (Green, 1991). For eukaryotic cells the sequence of a branch point, which is 18-38 nucleotides and contains the sequence UNCURAC (A is the branch point), and the presence of a pyrimidine tract near the 3' splice site are the most important factors for splice site selection (Sharp, 1994). Analysis of the

IFN-γ sequence of plasmid p1042 around the 3' cryptic splicing site, shows high homology to the consensus splicing sequence (shown in Fig4.2). In the recombinant IFN-γ gene of CHO 42 cells, there are 3 possible branch consensus sequences at 9, 28, 36 bp (with 71, 86 and 86% homology, respectively) upstream from the 3' cryptic splicing site. The sequence next to the 3' cryptic site, would require a pyrimidine consensus tract for splicing. A polypyrimidine tract containing 14 pyrimidines out of 18 bases is immediately 5' to the cryptic site. The 3' cryptic splicing site is 100% homologous to a consensus 3' splicing sequence. However, the two cryptic 5' sites have only 44 and 56% homologies to 5' consensus splicing sequence.

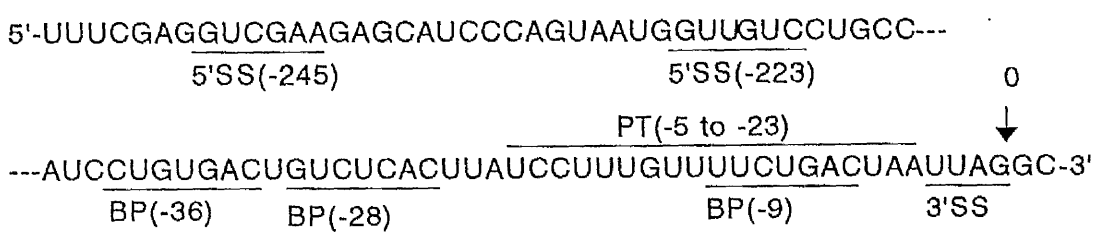
An SV40 promoter may also contribute in part towards the splicing of IFN-γ transcripts. The strong transcriptional activity of SV40 promoters have been reported to result in localisation of splicing factors and hnRNPs around transcripts derived from this promoter (Jimenez-Garcia and Spector, 1993). The high concentration of splicing factors and hnRNPs around IFN-γ transcripts would increase the possibility that a cryptic site would be exposed to splicing factors and therefore may be spliced.

Alternative splicing requires more than one splicing site and branch point (Noble et al., 1988) and splicing is mediated by specific trans-acting factors (Mayeda and Krainer, 1992; McKeown, 1992). I discussed earlier in this Section how there are three possible branch points in the IFN-γ pre-mRNA. However, other factors are required in the

selection of alternative splicing site. The AU-rich binding protein may play a part in alternative splicing. Hamilton et al. (1993) found that two, hnRNP A1 and C, of the five known AU-rich binding factors are also splicing factors which bind to the polypyrimidine stretch (Mayeda and Krainer, 1992). hnRNP A1 can modulate AU-rich mRNA degradation (Hamilton et al., 1993) and select for 5' splicing sites which are quite distal to 3' splicing site (Fu et al., 1992) and hnRNP C is needed for 3' splicing-site definition (Swanson and Dreyfus, 1988). Another splicing factor, SF2, can also modulate 5' splicing site selection being more selective for a site which is proximal to the 3' splicing site (Horowitz and Krainer, 1994). The 5' splicing site competition is dependent on the concentrations of hnRNP A1 and SF2 (Mayeda and Krainer, 1992). The concentration of SF2 around the IFN-γ transcripts may have been enriched due to the SV40 promoter activity as described earlier (Jimenez-Garcia and Spector, 1993).

The expression of hnRNPs is cell-dependent (McKeown, 1992) and the expression of hnRNP A1 and C in CHO cells has not been described and is beyond the aims of this thesis to consider in detail. However, a similar pattern of splicing of recombinant IFN-γ has also been observed in another recombinant CHO cells (CHO 320 cell line; Anwar, 1994). The CHO 320 cells have the same SV40 promoter and IFN-γ cDNA sequence but differ from CHO 42 cells in that the 3' terminal and poly(A) signals were obtained from the SV40 early gene (Anwar, 1994). It is interesting that CHO 320 cells also used one of the same 5' splicing sites as CHO 42 cells (the -223 up

Primary transcript sequences of IFN-γ in CHO 42 cell



Elements	Consensus[A] seqence	Sequence in[B] IFN-γ gene	Homology (%)	Site[C]
5′ Splicing site	C/AAGGURAGU	GAGGUCGAA	44	-245
		AUGGUUGUC	44	-223
Branch point	YNCUGAC	CUGUGAC	71	-36
		GUCUCAC	86	-28
		UUCUGAC	86	-9
Pyrimidine tract		UCCUUUGUUU-[D] UCUGACUAA	74	-5
3′ Splicing site	NYAG	UUAG	100	-1

A: Bases underlined are splicing junction and branch point
N: any base, R: purine, Y: pyrimidine.
B: The sites of underline base are shown in "site" column
C: The splicing junction of 3′ site as site 0 (AG\GC; G is at position -1)
D: Bases underline are pyrimidine

5'SS :5' Splicing site

BP :Branch point

PT :Pyrimidine tract

3'SS :3' Splicing site

Fig 4.2 Elements of splicing site selection

the 3' receptor site; Fig 4.2) but used sequences of SV40 as a 3' receptor site. Thus it would be of great interest to examine the expression of protein factors involved in the regulation of splicing of recombinant mRNA species in CHO cell lines.

4.3 IFN-γ isoforms and IFN-γ secretion

4.3.1 Sources of IFN-γ isoforms

CHO 42 cells express two additional IFN-γ isoforms of IFN-γ (31 and 34 kDa) when compared to CHO 43 cells and those forms are not seen for natural IFN-γ. This raises the question of the source of these two isoforms. These two isoforms could arise from trivial reasons such as the false binding of the 20D7 antibody in Western blotting, or due to other reasons such as the expression of an endogenous IFN-γ gene, read-through of the stop codon when IFN-γ mRNA is translated, IFN-γ binding to other cellular factors, such as ubiquitin or chaperones or due to oligomerisation to produce a structure which is more compact and could resist dissociation in SDS. Firstly, the 31 and 34 kDa forms of IFN-γ from CHO 42 cells were recognised by anti-IFN-γ antibody from two different sources in the Western blotting (Fig 3.6). This approach confirms that these two protein are related to the IFN-γ superfamily. Secondly, because mouse IFN-γ has a smaller molecular size (136 amino residue, 14.4 kDa; Kiener and Spitalny, 1987; Nagata et al., 1987) than huIFN-γ, this suggests that these two IFN-γ proteins do not originate from

endogenous CHO IFN-γ. Thirdly, these two proteins could be generated by translation through mechanisms involving RNA editing or frame-shift which causes read-through of the stop codon. However, RNA editing is sequence- and organelle-specific (Hodges and Scott, 1993) and frame-shift requires special mRNA sequences (de Smith et al., 1994). There are no such sequences around the stop codon of IFN-γ mRNA suggesting this may not be the source of these two proteins. Given idealised conditions cells should be able to synthesize a 34 kDa protein in 3 min (synthesis rate was estimated to be 2.8 amino acids/s and a 34 kDa peptide would need about 2 min for synthesis; Sareneva et al., 1994; Braakman et al, 1991), however the 31 and 34 kDa IFN-γ products seen in the ^{35}S-methionine labelling experiments (Fig 3.24) first appeared at 7 min but not at 3 min whereas the 17, 20, 25 kDa isoforms appeared by 3 min. This result suggests that the 31 and 34 kDa IFN-γ species might not be primary translation products. If these proteins are not primary translation products, they could be IFN-γ isoform(s) oligomers, or arise from further processes such as aggregation with or without linkage to other cellular factors.

Analysis of the IFN-γ mRNA species showed that one form has 11 bases spliced before the translation stop codon and as a consequence IFN-γ would be translated through the original stop codon sequence until the translation machinery read a new stop codon (Fig 3.49; Section 3.6). A protein translated from this mRNA would have four amino acids at its C-terminal which differed from those of the natural IFN-γ (N-

PheArgGlyLysAlaMet, N-PheArgGlyArgArgAla-SerGln, ab-IFN-γ form and natural form, respectively, underlining shows the differences; Fig 3.49) and this would result a slightly hydrophobic C-terminal (Fig 3.50). A change to the C-terminal region of IFN-γ may result in changes to the protein conformation and changes to interactions within the secretory vesicles. As a consequence it is possible that cells might recognise such an isoform as an incorrectly folded protein. The changes could, in turn, increase the possibility that it could undergo stringent binding with chaperones in the secretory pathway (McMillan et al., 1994) or to ubiquitin in the cytosol (Hershko and Ciechanover, 1992) or even form aggregates within cells (Bergeron et al., 1994). As chaperone proteins are larger than 34 kDa (the size of the largest IFN-γ isoform in CHO 42 cells; McMillan et al., 1994), the presence of a 34 kDa IFN-γ species is unlikely to be due to chaperone binding. Ubiquitin, a 76 amino acid protein, covalently ligates to the N-terminal of proteins and channels protein into degradation pathways at rates dependent on the nature of the amino acid residue exposed at their N-terminal position (Hershko and Ciechanover, 1994). Proteins with Val, Met, Gly, Ala, Ser, Thr and Cys residues at their N-terminal regions are extremely stable with half-lives of more than 20 hr. The two possible N-terminal amino acid residues of IFN-γ are Met (before signal peptide lysis) or Cys (after signal peptide lysis). If ubiquitin were bind to either of these two amino acids of IFN-γ, the new conjugated form would be stable with a half-life of more than 20 hr. The molecular weight

would be 27 kDa or 25 kDa, with and without the signal peptide on the protein, values which are near to the molecular weight of one of the unknown IFN-γ species. The half-lives of IFN-γ-31k and IFN-γ-34k retained in CHO 42 cells are greater than 4 hr (Section 3.3.4) and this raises the possibility that the source of those protein species could be from the binding of ubiqutin or related molecules.

In addition to the possibility of ubiquitin ligation, there are other possible sources of the large IFN-γ isoforms. As IFN-γ exists as dimers in cells as a result of non-covalently-linked subunits (Ealick et al., 1991; Farrar and Schreiber, 1993), these two higher molecular weight proteins may be dimers with a different binding force resulting from alterations to the C-terminal amino acids. Seven minutes is sufficient time for IFN-γ dimerisation (Sareneva et al., 1994), a time when they first appeared during ^{35}S-methionine radio-labelling (Fig 3.24). Higher glycosylation can cause the glycoproteins to be more compact (Imperiali and Rickert, 1995) and glycosylation of IFN-γ was suggested to increase dimerisation (Sareneva et al., 1994). The higher ratio doubly- to singly-glycosylated forms of IFN-γ in CHO 42 cells together with the changes to the protein C-terminus could contribute to the formation of a more rigid dimer conformation and make it more difficult for SDS to cause dissociation.

A dramatic increase in the amount of the 17 kDa protein found after four hours of retention (Section 3.3.4.2) could be due to the partial degradation or deglycosylation of

intermediate products of higher molecular forms of IFN-γ. Whereas this 17 kDa species increased in CHO 42 cells, such an event was not observed in CHO 43 cells. However, I could not identify the source of the high molecular isoform and further experiments would be required.

4.3.2 IFN-γ protein secretion

Although the rate of IFN-γ translation measured on the basis of radioactive amino acid incorporation in CHO 42 cells is 4.5 times less than that of CHO 43 cells (Section 3.3.2.1), the rate of appearance of IFN-γ in medium is about 18-fold (13- and 22-fold in two separate experiments) lower in CHO 42 cells then in CHO 43 cells. It would appear that CHO 42 cells may be unable to secrete IFN-γ efficiently and have a low secretion rate for IFN-γ or perhaps that IFN-γ protein is rapidly degraded after synthesis through processing aberrations in the CHO 42 cell line. To address these issues, IFN-γ secretion and retention were monitored in both CHO 42 and CHO 43 cells. The results show that secretion of IFN-γ in CHO 43 cells was 22 times greater than that of CHO 42 cells (Section 3.3.3.1) and that a high molecular weight protein (34 kDa) reactive to IFN-γ antibody was retained in cells. These results suggest that poor secretion rather than accelerated degradation is responsible for the low production of IFN-γ by CHO 42 cells. This raises another question: why is secretion poor in CHO 42 cells and how and where is IFN-γ protein retained?

Analysis of IFN-γ mRNA sequences indicates that CHO 42

cells contain a transcript which encodes an IFN-γ isoform with a more hydrophobic C-terminal than the natural form of IFN-γ (Section 3.5). This isoform may fold incorrectly and thus be recognized by the cell as aberrant. Therefore inappropriate folding may affect the IFN-γ secretion as was described for cholesteryl ester transfer protein (CETP; Quinet et al., 1993). These authors reported that an exon-9 deleted protein was poorly secreted and inhibited the secretion of normal, full length CETP by forming an heteromeric complex between full-length and exon-9 deleted polypeptides. IFN-γ is secreted as a dimer (Walter et al., 1995) and this increases the possibility that a mechanism similar to that for CEPT may be responsible for the impaired secretion of IFN-γ by CHO 42 cells.

Impaired secretion could occur through recognition events in the endoplasmic reticulum (ER) or/and the Golgi apparatus. Fries et al. (1984) compared four secretory proteins and concluded that the movement from ER to Golgi complex is the limiting step for overall protein transportation. However, Yeo et al. (1985) compared the secretion rate for three kinds of secreted glycoprotein and found that each glycoprotein had a different retention time in each compartment of the intracellular transport pathway. Some were retained in the ER, some were retained in the cis to medial Golgi, others were retained in the medial to trans Golgi. The retention in each intracellular compartment also affects the pattern or extent of glycosylation (Lodish et al., 1995). For example, the initial addition of carbohydrate occurs in the ER lumen

whereas the modification and elongation of carbohydrate chains occur in the Golgi. Retention of a multi-glycosylation site protein in the ER could increase the number of sites of carbohydrate chain attachment to the protein. Retention of a glycoprotein in the Golgi complex could result in a more extensive carbohydrate chain. IFN-γ has two potential glycosylation sites, Asn-25 and Asn-97 (Sareneva et al.,1995). Asn-25 is glycosylated co-translationally whereas Asn-97, which is probably less exposed than Asn-25, is less efficiently glycosylated and may be glycosylated post-translationally (Sareneva et al., 1995). IFN-γ secreted (Section 3.3.3.2) and pulse-labelling (Section 3.3.1.2) by CHO 42 cells is mostly detected as the doubly-glycosylated form whereas in CHO 43 cells and in other recombinant IFN-γ producing CHO cells (Sano et al.,1988) secretion of singly- and doubly-glycosylated forms occurs in equivalent amounts. The patterns of glycosylation of IFN-γ from CHO 42 and CHO 43 cells suggest that it takes a greater time to transport IFN-γ from ER to Golgi in CHO 42 cells than in CHO 43 cells.

4.4 Conclusion and future work

4.4.1 Conclusion

From my data, I have shown that recombinant IFN-γ mRNA is subjected to alternative splicing of a cryptic intron covering the ARE (Fig 3.49). One of the spliced mRNA products would encode a protein with a hydrophobic C-terminal region (Fig 3.50). I have suggested that these results in retention of IFN-γ in the cellular secretory compartments and that this

affects the secretion of all IFN-γ. However, similar problems were not encountered when the 3' UTR of IFN-γ cDNA was deleted and the subsequent recombinant CHO cell line generated (CHO 43) showed a 20 times greater production of IFN-γ than CHO 42 cells. This suggests that gene structure and sequence of cDNA may affect recombinant protein production at the level of RNA processing, protein editing and secretion.

4.4.2 Future work

In this thesis, I report how different sequences (and thus different structures) of 3' UTR of recombinant IFN-γ mRNA in CHO 42 and CHO 43 cells can result in a 20-fold different rate recombinant IFN-γ production. The difference in recombinant IFN-γ production may be due to a cryptic intron splicing which translates a non-secreted IFN-γ protein and which affects the whole IFN-γ protein secretion process.

As many protein factors bind to the specific sequences of a pre-mRNA to direct splicing site selection (Sections 1.2.2.2.1 and 4.2), protein factors which bind to the recombinant pre-mRNA need to be characterized. It would be of interesting to know the pattern and characteritics of protein factors which bind to the specific sequences of recombinant IFN-γ pre-mRNA of CHO 42 cells so that a model could be developed to examine the relationship between protein factor binding and recombinant pre-mRNA processing. This model could be used as a tool to investigate the importance of specific sequences in a recombinant cassette or could be used to select suitable producing cell lines from initial screens.

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Appendix I

Sources of materials

1 Bacteria and Cells

1.1 *E. Coli*

A. DH5α was purchased from Gibco BRL, Paisley, UK.

B. XBlue 101 was purchased from Promega

1.2 Mammalian cell lines

A. DUK X and CHO 43 cell lines were obtained from Dr. M. Page, Wellcome Research Labs, Beckenham, Kent.

B. CHO 42 was from A. Bate. University of Manchester.

2 Plasmids, Oligonucleotides

2.1 Plasmids

A. pHIFN-γ was a gift from Dr. A. Morris, University of Warwick, Coventry.

B. p1042 and p1043 were obtained from Dr. M. Page, Wellcome Research Labs, Beckenham, Kent.

C. pSV2-dhfr purchased from Gibco, Paisley, UK.

D. p100-D9 (Containing the mouse 18S rRNA cDNA as a 220bp Pst1 insert) was a gift from Dr. D.R. Edwards, University of Calgary, Canada.

E. pBluescript was purchased from Stratagene, Cambridge, UK.

F. pGemT was purchased from Promega, Southampton, UK.

2.2 Oligonucleotides

A. Oligo ACB6 was synthesised by Dr. J. Rosamond, University of Manchester.

B. Oligo ACB8 was synthesised by Oswell DNA Service, Edinburgh.

C. Oligo ACB9 and ACB10 were synthesised by Dr. M. Humphrey, University of Manchester.

D. KS primer and SK primer were purchased from Stratagene, Cambridge, UK.

E. SP6 and T7 promoter sequence were purchased from Promega, Southampton, UK.

3 Antibodies

A. The monoclonal 20B8, 20D7 and R1PA (10 mg/ml) antibody binds all forms (include proteolytically cleave and complete) of natural, CHO and *E. coli* derived human IFN-γ. All three antibodies were raised in mouse and were obtained from Celltech, Slough, UK.

B. The polyclonal S1DM antibody was a gift of Dr. Dave Milson, University of Manchester.

C. The polyclonal antimouse conjugated with peroxidase was purchased from Sigma Chemical Company, Dorset, UK.

4 Chemicals

Inorganic chemicals and organic solvents were purchased from BDH Chemicals Limited. Other chemicals and kits were supplied as follows.

A. Amersham International plc, Buckinghamshire, UK.
   Hybond-N (nylon membrane).
B. BDH Chemicals Limited, Speke, Liverpool, UK.
   Acrykamide/bis-acrymide 30% solution.
C. Difco Labs, Detroit, Michigan, USA.
   Bacto-agar
   Bacto-tryptone
   Bacto-yeast extract
D. Gibco BRL, Paisley, Renfrewshire, UK.
   1Kb ladder, Fragments are 12 repeats of 1018bp plus the following bp:1636, 517, 506, 396, 344, 298, 220, 201, 154, 134 and 75.
   Agarose
   DMEM metabolite medium (10X)
   DMEM solution (10X)
   Foetal Calf Serum.
   Glutamine solution (200mM)
   Glucose
   PBS (minus calcium , magnesium and bicarbonate)
   Sodium bicarbonate solution (7.5%)
   Sodium pyruvate solution (100 mM)
   Trypsin-EDTA solution (1X)
E. National Diagnostics, Manville, New Jersey, USA.
   Eco-Scint-A
F. Pharmacia Limited, Hounslow, Middlesex, UK.
   dNTP set: 100 mM solution of each dATP, dCTP, dGTP and dTTP.
   $pd(N)_6$ random hexamers (50 A_{260} units)
G. Schleicher & Schuell, Dassel, Germany
   DEAE membrane
   Nitrocellulose membrane
H. Sigma Chemical Company, Poole, Dorset, UK.

Ampicillin
Bovine serum Albumin (fraction V)
Bromophenol Blue
Caesium chloride
Diethyl pyrocarbonate
Dithiothreitol
DMSO
DNA (salmon sperm)
Ethidium Bromide
Formaldehyde
Guanidine thiocyanate
2-Mercaptoethanol
Methotrexate (MTX)
MOPS [3-(N-Morpholino) propanesulfonic acid]
Oligo(dT)-cellulose
Protease inhibitors: Leupeptin, Pepstatin, PMSF and TLCK.
Protein A sepharose CL-4B
Sarkosyl sulphate
SDS
Sephadex G-50 (fine)
TEMED
Tetracycline

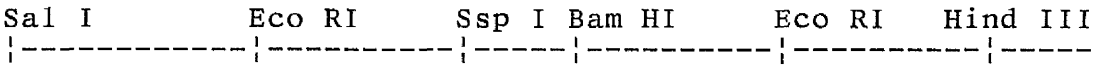
Tris-base
Trypan blue
Xylene cyanole

5.Enzymes and Commercial kits
A.Amersham, Buckinghamshire, UK.
ECL Western Blotting analysis system
B.Boehringer-Mannheim, (London) Lewes, Sussex, UK
Murine Leukaemia Reverse transcriptase
Nick translation kit
Restriction enzyme BamH I, Bgl II, EcoR I, EcoR V, Hind III, Msp I, Pst I, Sau 3A, Ssp I.
T4 DNA Ligase
Tag DNA polymerase
C.Pharmacia Limited, Hounslow, Middlesex, UK.
First-strand cDNA synthesis kitD.Promega Limited, Southampton, UK.
pGEM-T vector system kit

Appendix II Nucleic Acid sequences of Plasmid p1042, IFN-γ mRNAs (RNA42U and RNA42L) and IFN-γ polypeptide (IFN-γ and ab-IFN-γ, translated from RNA42U and RNA42L, respectively)

A: sequences of plasmid p1042

The restriction map of p1042 (Fig 2.1) shown in linear form is:



The sequences presented is of the whole plasmid and begins at the first Sal I site. The restriction sites and oligomers in the sequences are underlined. The letter N represents unknown sequence.

All nucleotide sequence data for plasmid p1042 was collected from Gray and Goeddel (IFN-γ cDNA, 1982), van Ooyen et al. (rabbit β-globin tail; 1979), Chang et al. (DHFR cDNA; 1978), Everett et al. (SV40 early gene promoter; 1983), Nable et al. (SV40 poly(A) tail; 1986) and Anwar (rest of sequences; 1994).

Sequences of IFN-γ gene (3089-5209) are bolded.

Position 4980 to 4999 using primer ACB6

Position 3896 to 3915 using primer ACB8

Position 3307 to 3321 using primer ACB9

Position 5119 to 5133 using primer ACB10

```
          V         V         V         V         V
GTCGACCGATGCCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGG   50
Sal I

GCGCGGGGCATGACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCAT  100
```

GCAACTCGTAGGACAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGG 150

ACCGCTTTCGCTGGAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTC 200

GGAATCTTGCACGCCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAA 250

ACGTTTCGGCGAGAAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGC 300

TGGGCTACGTCTTGCTGGCGTTCGCGACGCGAGGCTGGATGGCCTTCCCC 350

ATTATGATTCTTCTCGCTTCCGGCGGCATCGGGATGCCCGCGTTGCAGGC 400

CATGCTGTCCAGGCAGGTAGATGACGACCATCAGGGACAGCTTCAAGGAT 450

CGCTCGCGGCTCTTACCAGCCTAACTTCGATCACTGGACCGCTGATCGTC 500

ACGGCGATTTATGCCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGAT 550

TGTAGGCGCCGCCCTATACCTTGTCTGCCTCCCCGCGTTGCGTCGCGGTG 600

CATGGAGCCGGGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTA 650

ACGGATTCACCACTCCAAGAATTGGAGCCAATCAATTCTTGCGGAGAACT 700

GTGAATGCGCAAACCAACCCTTGGCAGAACATATCCATCGCGTCCGCCAT 750

CTCCAGCAGCCGCACGCGGCGCATCTCGGGCAGCGTTGGGTCCTGGCCAC 800

GGGTGCGCATGATCGTGCTCCTGTCGTTGAGGACCCGGCTAGGCTGGCGG 850

GGTTGCCTTACTGGTTAGCAGAATGAATCACCGATACGCGAGCGAACGTG 900

AAGCGACTGCTGCTGCAAAACGTCTGCGACCTGAGCAACAACATGAATGG 950

TCTTCGGTTTCCGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCAGCGCCC 1000

TGCACCATTATGTTCCGGATCTGCATCGCAGGATGCTGCTGGCTACCCTG 1050

```
          V          V          V          V          V
TGGAACACCTACATCTGTATTAACGAAGCGCTCTTCCGCTTCCTCGCTCA 1100

CTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC 1150

TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAA 1200

GAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCG 1250

CGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAA 1300

AATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGTAA 1350

CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCC 1400

TGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCG 1450

CTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCG 1500

CTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCG 1550

CCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTA 1600

TCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGT 1650

AGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTA 1700

GAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGA 1750

AAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGG 1800

TGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTC 1850

AAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAA 1900

AACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCAC 1950

CTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATAT 2000
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          V          V          V          V          V
ATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCT 2050

ATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGT 2100

CGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTG 2150

CAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATA 2200

AACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATC 2250

CGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTT 2300

CGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTGCAGGCATCGTG 2350

GTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACG 2400

ATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCT 2450

CCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCA 2500

CTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGT 2550

AAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAAT 2600

AGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAAT 2650

ACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTC 2700

TTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGA 2750

TGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACC 2800

AGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGG 2850

AATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAAT 2900

ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTT 2950
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```
          V          V          V          V          V
GAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCG 3000

AAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCT 3050

ATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCCTGTGG 3100
                                        EcoR I

AATGTGTGTCAGTTAGGGTGTGGAAATGCCCCAGGCTCCCAGCAGGCAGA 3150

AGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGCTCCCCAGCAG 3200

GCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCG 3250

CCCCTAACTCCGACCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTC 3300

TCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAAGGCCGAGGCCGC 3350
    ACB9

CTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCC 3400

TAGGCTTTTGCAAAAAGCTTCCGGACCTGATCAGCTTGATACAAGAACTA 3450

CTGATTTCAACTTCTTTGGCTTAATTCTCTCGGAAACGATGAAATATACA 3500

AGTTATATCTTGGCTTTTCAGCTCTGCATCGTTTTGGGTTCTCTTGGCTG 3550

TTACTGCCAGGACCCATATGTACAAGAAGCAGAAAACCTTAAGAAATATT 3600

TTAATGCAGGTCATTCAGATGTAGCGGATAATGGAACTCTTTTCTTAGGC 3650

ATTTTGAAGAATTGGAAAGAGGAGAGTGACAGAAAAATAATGCAGAGCCA 3700

AATTGTCTCCTTTTACTTCAAACTTTTTAAAAACTTTAAAGATGACCAGA 3750

GCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATGAATGTCAAGTTT 3800

TTCAATAGCAACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAATTA 3850
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```
          V          V          V          V          V
TTCGGTAACTGACTTGAATGTCCAACGCAAAGCAATACATGAACTCATCC 3900
                                                 ACB8

AAGTGATGGCTGAACTGTCGCCAGCAGCTAAAACAGGGAAGCGAAAAAGG 3950

AGTCAGATGCTGTTTCGAGGTCGAAGAGCATCCCAGTAATGGTTGTCCTG 4000
                    RNA42L                  RNA42U
                            Cryptic intron 5' donor site
CCTACAATCAATATTTGAATTTTAAATCTAAATCTATTTATTAATATTTA 4050
                                            Ssp I

ACATTATTTATATGGGGAATATATTTTTAGACTCATCAATCAAATAAGTA 4100

TTTATAATAGCAACTTTTGTGTAATGAAAATGAATATCTATTAATATATG 4150

TATTATTTATAATTCCTATATCCTGTGACTGTCTCACTTATCCTTTGTTT 4200
Cryptic intron 3' receptor site
      <-----¦
TCTGACTAATTAGGCAAGGCTATGTGATTACAAGGCTTTATCTCAGGGGC 4250

CAACTAGGCAGCCAACCTAAGCAAGATCCCATGCGGGATCCTGAGAACTT 4300
                                        BamH I

CAGGGTGAGTTTGGGGACCCTTGATTGTTCTTTCTTTTTCGCTATTGTAA 4350
    ¦----> Intron from rabbit 6-globin poly(A) tail

AATTCATGTTATATGGAGGGGGCAAAGTTTTCAGGGTGTTGTTTAGAATG 4400

GGAAGATGTCCCTTGTATCACCATGGACCCTCATGATAATTTTGTTTCTT 4450

TCACTTTCTACTCTGTTGACAACCATTGTCTCCTCTTATTTTCTTTTCAT 4500

TTTCTGTAACCTTTTTCGTTAAACTTTAGCTTGCATTTGTAACGAATTTT 4550

TAAATTCACTTTTGTTTATTTGTCAGATTGTAAGTACTTTCTCTAATCAC 4600

TTTTTTTTCAAGGCAATCAGGGTATATTATATTGTACTTCAGCACAGTTT 4650

TAGAGAACAATTGTTATAATTAAATGATAAGGTAGAATATTTCTGCATAT 4700

AAATTCTGGCTGGCGTGGAAATATTCTTATTGGTAGAAACAACTACATCC 4750

TGGTCATCATCCTGCCTTTCTCTTTATGGTTACAATGATATACACTGTTT 4800
```

```
          V          V          V          V          V
GAGATGAGGATAAAATACTCTGAGTCCAAACCGGGCCCCTCTGCTAACCA 4850
Intron from rabbit ß-globin poly(A) tail
                          <------'
TGTTCATGCCTTCTTCTTTTTCCTACAGCTCCTGGGCAACGTGCTGGTTA 4900

TTGTGCTCTCTCATCATTTTGGCAAAGAATTCACTCCTCAGGTGCAGGCT 4950
                           EcoR I

GCCTATCAGAAGGTGGTGGCTGGTGTGGCCAATGCCCTGGCTCACAAATA 5000
                           ACB6

CCACTGAGATCTTTTTCCCTCTGCCAAAAATTATGGGGACATCATGAAGC 5050

CCCTTGAGCATCTGACTTCTGGCTAATAAAGGAAATTTATTTTCATTGCA 5100
                           Poly(A) signal

ATAGTGTGTTGGAATTTTTTGTGTCTCTCACTCGGAAGGACATATGGGAG 5150
                          ACB10

GGCAAATCATTTAAAACATCAGAATGAGTATTTGGTTTAGAGTTTGGCAA 5200

CATATGCCNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 5250

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 5300

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 5350

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 5400

NNNNNNNNNNNNNNNNNNNNNNNNNNNNAACTTGTTTATTGCAGCTTATA 5450

ATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTT 5500

TTTTCACTGCCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTT 5550

ATCATGTCTGGATGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACC 5600

GGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGG 5650

GGAAGATCGGGCTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGG 5700

GTATGGTGGCAGGCCCGTGGCCGGGGGACTGTTGGGCGCCATCTCCTTGC 5750
```

```
          V          V          V          V          V
ATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTACTG 5800

GGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCC 5850

CTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGA 5900

CTATCGTCGCCGCACTTATGACTGTCTTCTTTATCATGCAACTCGTAGGA 5950

CAGGTGCCGGCAGCGCTCTGGGTCATTTTCGGCGAGGACCGCTTTCGCTG 6000

GAGCGCGACGATGATCGGCCTGTCGCTTGCGGTATTCGGAATCTTGCACG 6050

CCCTCGCTCAAGCCTTCGTCACTGGTCCCGCCACCAAACGTTTCGGCGAG 6100

AAGCAGGCCATTATCGCCGGCATGGCGGCCGACGCGCTGGGCTACGTCTT 6150

GCTGGCGTTCGGATCCAGACATGATAAGATACATTGATGAGTTTGGACAA 6200
          BamH I

ACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGA 6250

TGCTATTGCTTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACA 6300

ACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAGGTGTGGAGG 6350

TTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATAGC 6400

TTCCAGGGACACTTGTTTAGTTTCCTCTGCTTCTTCTGGATTAAAATCAT 6450

GCTCCTTTAACCCACCTGGCAAACTTTCCTCAATAACAGAAAATGGATCT 6500

CTAGTCAAGGCACTATACATCAAATATTCCTTATTAACCCCTTTACAAAT 6550

TAAAAAGCTAAAGGTACACAATTTTTGAGCATAGTTATTAATAGCAGACA 6600

CTCTATGCCTGTGTGGAGTAAGAAAAAACAGTATGTTATGATTATAACTG 6650

TTATGCCTACTTATAAAGGTTACAGAATATTTTTCCATAATTTTCTTGTA 6700
```

```
          V          V          V          V          V
TAGCAGTGCAGCTTTTTCCTTTGTGGTGTAAATAGCAAAGCAAGCAAGAG 6750

TTCTATTACTAAACACAGCATGACTCAAAAAACTTAGCAATTCTGAAGGA 6800

AAGTCCTTGGGGTCTTCTACCTTTCTCTTCTTTTTTGGAGGAGTAGAATG 6850

TTGAGAGTCAGCAGTAGCCTCATCATCACTAGATGGCATTTCTTCTGAGC 6900

AAAACAGGTTTTCCTCATTAAAGGCATTCCACCACTGCTCCCATTCATCA 6950

GTTCCATAGGTTGGAATCTAAAATACACAAACAATTAGAATCAGTAGTTT 7000

AACACATTATACACTTAAAAATTTTATATTTACCTTATAGCTTTAAATCT 7050

CTGTAGGTAGTTTGTCCAATTATGTCACACCACAGAAGTAAGGTTCCTTC 7100

ACAAAAGATCAAGTCCAAACCACATTCTAAAGCAATTAAAGCCAGCAAAA 7150

GTCCCATGGTCTTATAAAAATGCATAGCTTTAGGAGGGGAGCAGAGAACT 7200

TGAAAGCATCTTCCTGTTAGTCTTTCTTCTCGTAGACTTCAAACTTATAC 7250

TTGATGCCTTTTTCCTCCTGGACCTCAGAGAGGACGCCTGGGTATTCTGG 7300

GAGAAGTTTATATTTCCCCAAATTCATGGCTTCCTGGTAAACAGAACTGC 7350

CTCCGACTATCCAAACCATGTCTACTTTACTTGCCAATTCCGGTTGTTCA 7400

ATAAGTCTTAAGGCATCATCCAAACTTTTGGCAAGAAAATGAGCTCCTCG 7450

TGGTGGTTCTTTGAGTTCTCTACTGAGAACTATATTAATTCTGTCCTTTA 7500

AAGGTCGATTCTTCTCAGGAATGGAGAACCAGGTTTTCCTACCCATAATC 7550

ACCAGGGGTAGGTCTCCGTTCTTGCCAATCCCCATATTTTGGGACACGGC 7600

GACGATGCAGTTCAATGGTCGAACCATGATGGCAGCGGGGATAAAATCCT 7650
```

```
          V          V          V          V          V
ACCAGCCTTCACGCTAGGATTGCCGGCAAGTTTGGCGCGAAATCGCAGCC 7700

CTGAGCTGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 7750

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 7800

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 7850

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 7900

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 7950

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 8000

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 8050

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNAAGCTTTTT 8100
                                                Hind III

GCAAAAGCCTAGGCCTCCAAAAAAGCCTCCTCACTACTTCTGGAATAGCT 8150

CAGAGGCCGAGGCGGCCTCGGCCTGCATAAATAAAAAAAATTAGTCAGCC 8200

ATGGGGCGGAGAATGGGCGGAACTGGGCGGAGTTAGGGGCGGGATGGGCG 8250

GAGTTAGGGGCGGGACTATGGTTGCTGACTAATTGAGAGT
```

B: IFN-γ mRNA sequence

(1) RNA42U sequences

Translation start from position 67 and stop at position 565 and this coding region is bold .

Sequences of oligomer ACB8 (473-487) and ACB6 (762-781) and restriction sites of BamH I (641) and EcoR I (709) are underlined.

The sequences (548-567) differ from RNA42L are italicized and

sequences differ from RNA43 (570-641) are also shown.

```
          V          V          V          V          V
GGACCUGAUCAGCUUGAUACAAGAACUACUGAUUUCAACUUCUUUGGCUU    50

AAUUCUCUCGGAAACGAUGAAAUAUACAAGUUAUAUCUUGGCUUUUCAGC   100
                Translation start

UCUGCAUCGUUUUGGGUUCUCUUGGCUGUUACUGCCAGGACCCAUAUGUA   150

CAAGAAGCAGAAAACCUUAAGAAAUAUUUUAAUGCAGGUCAUUCAGAUGU   200

AGCGGAUAAUGGAACUCUUUUCUUAGGCAUUUUGAAGAAUUGGAAAGAGG   250

AGAGUGACAGAAAAAUAAUGCAGAGCCAAAUUGUCUCCUUUUACUUCAAA   300

CUUUUUAAAAACUUUAAAGAUGACCAGAGCAUCCAAAAGAGUGUGGAGAC   350

CAUCAAGGAAGACAUGAAUGUCAAGUUUUUCAAUAGCAACAAAAAGAAAC   400

GAGAUGACUUCGAAAAGCUGACUAAUUAUUCGGUAACUGACUUGAAUGUC   450

CAACGCAAAGCAAUACAUGAACUCAUCCAAGUGAUGGCUGAACUGUCGCC   500
                        ACB8

AGCAGCUAAAACAGGGAAGCGAAAAAGGAGUCAGAUGCUGUUUCGAGGUC   550

GAAGAGCAUCCCAGUAAUGGCAAGGCUAUGUGAUUACAAGGCUUUAUCUC   600
  Stop codon (UAA) ¦---> differ from RNA43

AGGGGCCAACUAGGCAGCCAACCUAAGCAAGAUCCCAUGCGGGAUCCUGA   650
                    differ from RNA43 <---¦ BamH I

GAACUUCAGGCUCCUGGGCAACGUGCUGGUUAUUGUGCUCUCUCAUCAUU   700

UUGGCAAAGAAUUCACUCCUCAGGUGCAGGCUGCCUAUCAGAAGGUGGUG   750
          EcoR I

GCUGGUGUGGCCAAUGCCCUGGCUCACAAAUACCACUGAGAUCUUUUUCC   800
            ACB6

CUCUGCCAAAAAUUAUGGGGACAUCAUGAAGCCCCUUGAGCAUCUGACUU   850

CUGGCUAAUAAAGGAAAUUUAUUUUCAUUGCAAAAAAAAAAAAAAAAAAA   900
```

```
          V          V          V          V          V
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  950

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1000

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

(2) RNA42L sequence

Translation start from position 67 and stop at Position 559 and this coding sequences are in bold .

Sequences of oligomer ACB8 (473-487) and ACB6 (740-759) and restriction sites of BamH I (620) and EcoR I (686) are underlined.

```
          V          V          V          V          V
GGACCUGAUCAGCUUGAUACAAGAACUACUGAUUUCAACUUCUUUGGCUU   50

AAUUCUCUCGGAAACGAUGAAAUAUACAAGUUAUAUCUUGGCUUUUCAGC  100
                Translation start

UCUGCAUCGUUUUGGGUUCUCUUGGCUGUUACUGCCAGGACCCAUAUGUA  150

CAAGAAGCAGAAAACCUUAAGAAAUAUUUUAAUGCAGGUCAUUCAGAUGU  200

AGCGGAUAAUGGAACUCUUUUCUUAGGCAUUUUGAAGAAUUGGAAAGAGG  250

AGAGUGACAGAAAAAUAAUGCAGAGCCAAAUUGUCUCCUUUUACUUCAAA  300

CUUUUUAAAAACUUUAAAGAUGACCAGAGCAUCCAAAAGAGUGUGGAGAC  350

CAUCAAGGAAGACAUGAAUGUCAAGUUUUUCAAUAGCAACAAAAAGAAAC  400

GAGAUGACUUCGAAAAGCUGACUAAUUAUUCGGUAACUGACUUGAAUGUC  450

CAACGCAAAGCAAUACAUGAACUCAUCCAAGUGAUGGCUGAACUGUCGCC  500
                        ACB8
```

V V V V V

AGCAGCUAAAACAGGGAAGCGAAAAAGGAGUCAGAUGCUGUUUCGAGGCA 550

AGGCUAUGUGAUUACAAGGCUUUAUCUCAGGGGCCAACUAGGCAGCCAAC 600
Stop codon

CUAAGCAAGAUCCCAUGCGGGAUCCUGAGAACUUCAGGCUCCUGGGCAAC 650
BamH I

GUGCUGGUUAUUGUGCUCUCUCAUCAUUUUGGCAAAGAAUUCACUCCUCA 700
EcoR I

GGUGCAGGCUGCCUAUCAGAAGGUGGUGGCUGGUGUGGCCAAUGCCCUGG 750
ACB10

CUCACAAAUACCACUGAGAUCUUUUUCCCUCUGCCAAAAAUUAUGGGGAC 800

AUCAUGAAGCCCCUUGAGCAUCUGACUUCUGGCUAAUAAAGGAAAUUUAU 850

UUUCAUUGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 900

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 950

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1000

AAAAAAAAAA

C: IFN-γ protein sequences

(1) IFN-γ (translation from RNA42U)

Residues indicated by 3-letter abreviations

Signal peptide sequences (1-23) are underlined and the sequences (161-166) differ from ab-IFN-γ (translation from RNA42L) are bold .

V V V V
MetLysTyrThrSerTyrIleLeuAlaPheGlnLeuCysIleValLeuGlySerLeuGly
20 Signal peptide (1-23)

CysTyrCysGlnAspProTyrValGlnGluAlaGluAsnLeuLysLysTyrPheAsnAla
40

GlyHisSerAspValAlaAspAsnGlyThrLeuPheLeuGlyIleLeuLysAsnTrpLys
60

V V V V
GluGluSerAspArgLysIleMetGlnSerGlnIleValSerPheTyrPheLysLeuPhe
80

LysAsnPheLysAspAspGlnSerIleGlnLysSerValGluThrIleLysGluAspMet
100

AsnValLysPhePheAsnSerAsnLysLysLysArgAspAspPheGluLysLeuThrAsn
120

TyrSerValThrAspLeuAsnValGlnArgLysAlaIleHisGluLeuIleGlnValMet
140

AlaGluLeuSerProAlaAlaLysThrGlyLysArgLysArgSerGlnMetLeuPheArg
160

GlyArgArgAlaSerGln (Sequences differ from ab-IFN-γ)

(2) ab-IFN-γ (translation from RNA42L)

Residues indicated by 3-letter abreviations

Signal peptide sequences (1-23) are underlined and the sequences (161-164) differ from IFN-γ (translation from RNA42U and RNA43) are bold .

V V V V
MetLysTyrThrSerTyrIleLeuAlaPheGlnLeuCysIleValLeuGlySerLeuGly
20 **Signal Peptide (1-23)**

CysTyrCysGlnAspProTyrValGlnGluAlaGluAsnLeuLysLysTyrPheAsnAla
40

GlyHisSerAspValAlaAspAsnGlyThrLeuPheLeuGlyIleLeuLysAsnTrpLys
60

GluGluSerAspArgLysIleMetGlnSerGlnIleValSerPheTyrPheLysLeuPhe
80

LysAsnPheLysAspAspGlnSerIleGlnLysSerValGluThrIleLysGluAspMet
100

AsnValLysPhePheAsnSerAsnLysLysLysArgAspAspPheGluLysLeuThrAsn
120

TyrSerValThrAspLeuAsnValGlnArgLysAlaIleHisGluLeuIleGlnValMet
140

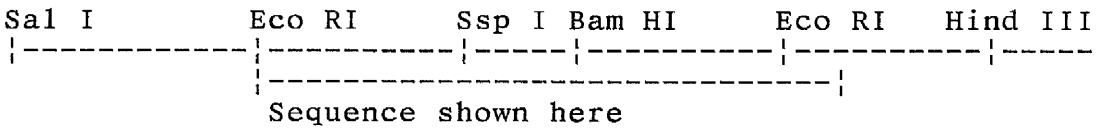
AlaGluLeuSerProAlaAlaLysThrGlyLysArgLysArgSerGlnMetLeuPheArg
160

GlyLysAlaMet (Sequences differ from IFN-γ)

Appendix III Nucleic Acid sequences of Plasmid p1043, IFN-γ mRNAs (RNA43) and IFN-γ polypetide (IFN-γ)

A: IFN-γ sequences of plasmid p1043

The restriction map of p1043 (Fig 2.1) shown in linear form is:



The sequences presented is part of plasmid and begins at the first EcoR I site. The restriction sites shown are underlined in the sequences and oligomer are shown in bold.

All nucleotide sequence data for plasmid p1043 was collected from Gray and Goeddel (IFN-γ cDNA, 1982), van Ooyen et al. (rabbit β-globin tail; 1979), Everett et al. (SV40 early gene promoter; 1983).

Position 1634 to 1653 using primer ACB6

Position 825 to 844 using primer ACB8

Position 236 to 250 using primer ACB9

Position 1773 to 1787 using primer ACB10

```
          V          V          V          V          V
GAATTCCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAATGCCCCAGGCTC    50
EcoR I

CCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCA   100

GGTGTGGAAAGTCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGC   150

ATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGACCATCCCG   200

CCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAAT   250
                                    ACB9
```

```
          V          V          V          V          V
TTTTTTTATTTATGCAAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCA  300

GAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTTC  350

CGGACCTGATCAGCTTGATACAAGAACTACTGATTTCAACTTCTTTGGCT  400

TAATTCTCTCGGAAACGATGAAATATACAAGTTATATCTTGGCTTTTCAG  450

CTCTGCATCGTTTTGGGTTCTCTTGGCTGTTACTGCCAGGACCCATATGT  500

ACAAGAAGCAGAAAACCTTAAGAAATATTTTAATGCAGGTCATTCAGATG  550

TAGCGGATAATGGAACTCTTTTCTTAGGCATTTTGAAGAATTGGAAAGAG  600

GAGAGTGACAGAAAAATAATGCAGAGCCAAATTGTCTCCTTTTACTTCAA  650

ACTTTTTAAAAACTTTAAAGATGACCAGAGCATCCAAAAGAGTGTGGAGA  700

CCATCAAGGAAGACATGAATGTCAAGTTTTTCAATAGCAACAAAAAGAAA  750

CGAGATGACTTCGAAAAGCTGACTAATTATTCGGTAACTGACTTGAATGT  800

CCAACGCAAAGCAATACATGAACTCATCCAAGTGATGGCTGAACTGTCGC  850
                         ACB8

CAGCAGCTAAAACAGGGAAGCGAAAAAGGAGTCAGATGCTGTTTCGAGGT  900

CGAAGAGCATCCCAGTAATGGTTGTCCTGCCTACAATCGGGATCCTGAGA  950
                                          BamH I

ACTTCAGGGTGAGTTTGGGGACCCTTGATTGTTCTTTCTTTTTCGCTATT 1000
        ¦----> Intron from rabbit 6-globin
               poly(A) tail
GTAAAATTCATGTTATATGGAGGGGGCAAAGTTTTCAGGGTGTTGTTTAG 1050

AATGGGAAGATGTCCCTTGTATCACCATGGACCCTCATGATAATTTTGTT 1100

TCTTTCACTTTCTACTCTGTTGACAACCATTGTCTCCTCTTATTTTCTTT 1150

TCATTTTCTGTAACCTTTTTCGTTAAACTTTAGCTTGCATTTGTAACGAA 1200
```

```
          V          V          V          V          V
TTTTTAAATTCACTTTTGTTTATTTGTCAGATTGTAAGTACTTTCTCTAA 1250

TCACTTTTTTTTCAAGGCAATCAGGGTATATTATATTGTACTTCAGCACA 1300

GTTTTAGAGAACAATTGTTATAATTAAATGATAAGGTAGAATATTTCTGC 1350

ATATAAATTCTGGCTGGCGTGGAAATATTCTTATTGGTAGAAACAACTAC 1400

ATCCTGGTCATCATCCTGCCTTTCTCTTTATGGTTACAATGATATACACT 1450

GTTTGAGATGAGGATAAAATACTCTGAGTCCAAACCGGGCCCCTCTGCTA 1500
Intron from rabbit ß-globin poly(A) tail
                                <-----¦
ACCATGTTCATGCCTTCTTCTTTTTCCTACAGCTCCTGGGCAACGTGCTG 1550

GTTATTGTGCTCTCTCATCATTTTGGCAAAGAATTCACTCCTCAGGTGCA 1600
                                     EcoR I

GGCTGCCTATCAGAAGGTGGTGGCTGGTGTGGCCAATGCCCTGGCTCACA 1650
                                    ACB6

AATACCACTGAGATCTTTTTCCCTCTGCCAAAAATTATGGGGACATCATG 1700

AAGCCCCTTGAGCATCTGACTTCTGGCTAATAAAGGAAATTTATTTTCAT 1750

TGCAATAGTGTGTTGGAATTTTTTGTGTCTCTCACTCGGAAGGACATATG 1800
                           ACB10

GGAGGGCAAATCATTTAAAACATCAGAATGAGTATTTGGTTTAGAGTTTG 1850

GCAACATATGCC
```

B: IFN-γ mRNA sequence

Translation start from position 67 and stop at position 565 and this coding region is bold .

Sequences of oligomer ACB8 (473-487) and ACB6 (762-781) and restriction sites of BamH I (641) and EcoR I (709) are underlined.

The sequences (548-567) differ from RNA42U are italicized and

sequences differ from RNA42L (548-588) are also shown.

```
          V          V          V          V          V
GGACCUGAUCAGCUUGAUACAAGAACUACUGAUUUCAACUUCUUUGGCUU    50

AAUUCUCUCGGAAACGAUGAAAUAUACAAGUUAUAUCUUGGCUUUUCAGC   100
                Translation start

UCUGCAUCGUUUUGGGUUCUCUUGGCUGUUACUGCCAGGACCCAUAUGUA   150

CAAGAAGCAGAAAACCUUAAGAAAUAUUUUAAUGCAGGUCAUUCAGAUGU   200

AGCGGAUAAUGGAACUCUUUUCUUAGGCAUUUUGAAGAAUUGGAAAGAGG   250

AGAGUGACAGAAAAAUAAUGCAGAGCCAAAUUGUCUCCUUUUACUUCAAA   300

CUUUUUAAAAACUUUAAAGAUGACCAGAGCAUCCAAAAGAGUGUGGAGAC   350

CAUCAAGGAAGACAUGAAUGUCAAGUUUUUCAAUAGCAACAAAAAGAAAC   400

GAGAUGACUUCGAAAAGCUGACUAAUUAUUCGGUAACUGACUUGAAUGUC   450

CAACGCAAAGCAAUACAUGAACUCAUCCAAGUGAUGGCUGAACUGUCGCC   500
                       ACB8

AGCAGCUAAAACAGGGAAGCGAAAAAGGAGUCAGAUGCUGUUUCGAGGUC   550
                                differ from RNA42L ¦->

GAAGAGCAUCCCAGUAAUG GUUGUCCUGCCUGCAAUCGGGAUCCUGAGAA   600
Stop codon (UAA) differ from RNA42L<-¦BamH I

CUUCAGGCUCCUGGGCAACGUGCUGGUUAUUGUGCUCUCUCAUCAUUUUG   650

GCAAAGAAUUCACUCCUCAGGUGCAGGCUGCCUAUCAGAAGGUGGUGGCU   700
      EcoR I

GGUGUGGCCAAUGCCCUGGCUCACAAAUACCACUGAGAUCUUUUUCCCUC   750
         ACB6

UGCCAAAAAUUAUGGGGACAUCAUGAAGCCCCUUGAGCAUCUGACUUCUG   800

GCUAAUAAAGGAAAUUUAUUUUCAUUGCAAAAAAAAAAAAAAAAAAAAAA   850

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA   900
```

```
          V          V          V          V          V
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  950

AAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

C: IFN-γ protein sequences (IFN-γ)

Protein Sequence coded in RNA43 is same as the sequences of RNA42U.