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**REGULATION OF THE HUMAN β -INTERFERON
PROMOTER**

PETER JAMES KING

A thesis submitted in partial fulfilment of the
requirements of the Open University for the
degree of Doctor of Philosophy

May 1997

Imperial Cancer Research Fund

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"The horror! The horror!"
Joseph Conrad: Heart of Darkness

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ABBREVIATIONS

Common terms

DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
mRNA	messenger RNA
ds	double stranded
ss	single stranded
utr	untranslated region
IFN	interferon
PKA	protein kinase A
PKC	protein kinase C
PKR	dsRNA dependent protein kinase
N-terminal	amino-terminal
C-terminal	carboxy-terminal
EMSA	electrophoretic mobility shift assay
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
kDa	kilodalton
bp	base pair
kb	kilobase pair
cpm	counts per minute
SV5	simian virus 5
CAT	chloramphenicol acetyl transferase

Chemicals

ATP, CTP, GTP, UTP	adenosine-, cytosine-, guanosine-, uridine triphosphate
dATP, dCTP, dGTP, dUTP	deoxyadenosine-, deoxycytosine-, deoxyguanosine-, deoxythymidine triphosphate
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid, disodium salt
TE	Tris/EDTA
TBE	Tris/borate/EDTA
TAE	Tris/acetate/EDTA
IPTG	isopropyl β -D-thiogalactopyranoside
X-GAL	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside
SDS	sodium dodecylsulphate
NP40	Nonidet-P40
Tween-20	polyoxyethylene-sorbitan monolaureate
PMSF	phenylmethylsulphonylfluoride
DTT	dithiothreitol
PBS	phosphate-buffered saline
TBS	Tris-buffered saline
DMEM	Dulbecco's modified Eagle's medium

CHX	cycloheximide
PEG	polyethyleneglycol
BSA	bovine serum albumin

One letter amino acid code

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

CHAPTER ONE - INTRODUCTION

THE INTERFERON SYSTEM

Introduction

Interferon was first identified in 1957 as a secreted factor produced by chicken egg chorioallantoic membrane fragments following infection by heat-inactivated influenza virus (Isaacs and Lindenmann 1957). When this factor was used to treat uninfected membrane fragments in culture, it was able to create a virus-resistant state and thereby interfere with subsequent influenza virus replication and in recognition of this property the factor was named interferon (IFN). Much research followed this initial discovery and it is now clear that IFN, in addition to its role in restricting viral multiplication, has profound effects on the regulation of cellular functions, such as cell growth and differentiation and the immune response (for reviews, see (Stewart 1979, Pestka et al. 1987, Samuel 1988)).

It is now known that there are two structurally and functionally distinct forms of mammalian IFN, referred to as types I and II IFN. Type I IFNs are induced by viral infection and are acid- and heat-stable, and the genes, which are intronless, are clustered on the short arm of chromosome 9 in humans at band 9p21 (Trent et al. 1982, Owerbach et al. 1981, Slate et al. 1982, Shows et al. 1982). In contrast, type II IFN is induced by mitogenic or antigenic stimulation, is neither acid- nor heat-stable, and the gene, containing three introns, maps to the long arm of human chromosome 12 at band 12q24.1 (Naylor et al. 1983, Trent, et al. 1982). The type I IFNs can be further subdivided into α - (originally known as leukocyte) and β - (originally fibroblast) IFN, the two most important members of the family, based on their sites of production. Type II IFN was originally referred to as immune IFN, because of its production by cell types involved in the immune system, particularly T lymphocytes (Kasahara et al. 1983a, b, Young and Ortaldo 1987), and is now known as γ -IFN. All forms of IFN are synthesised with secretory signal peptide leader sequences which are cleaved to form the mature proteins which are between 165 and 172 amino acids long (Sen and Lengyel 1992). With the exception of the major α -IFN species, the IFNs are often N-glycosylated (Labdon et al. 1984, Knight and Fahey 1982).

Interferon Genes and Proteins

α -Interferon

The α -IFN family can be further subdivided into at least two groups, α_1 - and

α_{II} -IFN. These are now known as α - and ω -IFN respectively. There are 13 distinct, functional, α -IFN genes in humans, and several pseudogenes (Henco et al. 1985), whereas there is only one active ω -IFN gene and 6 pseudogenes (Capon et al. 1985, Feinstein et al. 1985, Shepard et al. 1985, Hauptmann and Swetly 1985, Diaz et al. 1994). Although the α -IFNs are largely produced by leukocytes, the entire range of cell types capable of producing α -IFN *in vivo* is not known: macrophages, T and B cells and fibroblasts have all been reported as α -IFN producing cells *in vitro*. A novel member of the α -IFN family, with approximately 70% identity to ω -IFN, has been discovered in ruminant species. This is known as τ - (originally trophoblast) IFN, whose expression is restricted to the trophectoderm of the pre-implantation embryo, and it is thought to play an important role in maternal recognition of the embryo and maintenance of pregnancy (Cross and Roberts 1991) by blocking the synthesis of the oxytocin receptor and preventing regression of the corpus luteum (Spencer and Bazer 1996). All the α -IFNs are active as monomers, and the major species are non-glycosylated.

It is not clear why the α -IFN family is so large. In general, the α -IFNs are 165-166 amino acids long and across the family there is 36% identity at the amino acid level, with at least 70% identity between individual members (Henco et al. 1985). Certain differences in biological activity between different members of the family have been reported (Goren et al. 1983), for example $\alpha 2$ -IFN can stimulate natural killer cell activity whilst $\alpha 7$ -IFN cannot (Ortaldo et al. 1984), and it is therefore possible that different α -IFN subtypes could set up subtly different antiviral states specific for individual viruses or families of viruses. In support of this hypothesis, it has been reported that the IFN pool secreted by human macrophages infected with respiratory syncytial virus (RSV) was more effective at blocking the replication of RSV than was the IFN pool secreted by cells treated with influenza virus (Bell et al. 1983). The replication of influenza virus was equally affected by treatment with the IFN pools from either RSV or influenza virus treated cells. An alternative hypothesis is that the size of the α -IFN family may be required not because of a need for differences in the biological activities of the subtypes, but to allow expression of α -IFN in a wide range of tissues and at different developmental stages in response to a wide range of inducers. There has been no systematic investigation of the range of α -IFN subtypes produced in known expressing cells in response to different viral inducers, although studies have shown that several α -IFN species are produced in peripheral blood lymphocytes (Rubinstein et al. 1981) and cell lines such as Namalwa (Allen and Fantes 1980) and KG-1 (Hobbs and Pestka 1982) in response to

induction by virus. Clearly, τ -IFN is an example of tissue specific expression at a defined developmental stage.

β -Interferon

In contrast to the large α -IFN family, there is only one β -IFN gene in most mammalian species (Taniguchi et al. 1980, Derynck et al. 1980), although some species, for example the domestic cow, have multiple genes (Wilson et al. 1983). Whereas 36% of the amino acid positions are invariant across the α -IFN family, this identity drops to 20% when β -IFN is included in this analysis, and there is between 30% and 40% homology between β -IFN and individual α -IFNs (Henco, et al. 1985). It appears to be produced ubiquitously *in vivo* upon infection, and it has been shown to be the major IFN species produced by non-lymphoid cells in response to viral infection (Billiau et al. 1977, Havell et al. 1978). Biologically active β -IFN is dimeric (Pestka et al. 1983) and N-glycosylated.

γ -Interferon

As well as differing from the type I IFNs in its genomic structure and its chromosomal location, γ -IFN is also quite unrelated to the type I IFNs at the amino acid level, apart from showing limited homology to β -IFN in some regions. Even though it is not induced directly by viral infection, γ -IFN does have *bona fide* antiviral activity, inducing many of the proteins induced by type I IFNs (Revel and Chebath 1986), as well as playing a major role in the control of the immune system. γ -IFN exists as a single copy gene in humans, and appears to exert its effects as an N-glycosylated dimer or possibly a tetramer (Pestka et al. 1983). Its induction is restricted to T cells and natural killer cells.

Biological Activities of Interferons

Antiviral activity

The IFNs suppress the growth of a wide range of DNA and RNA viruses, and have been reported to affect many stages of viral infection and replication, including adsorption, uncoating, transcription and methylation of viral RNA, protein synthesis, virus assembly and release (for reviews see (Lengyel 1982, McMahon and Kerr 1983, Pestka et al. 1987, Samuel 1991, Staeheli 1990). All (animal) viruses induce type I IFN, and even though an infected cell may be killed by the virus (Takizawa et al. 1993, Hinshaw et al. 1994), the IFN released by the cell activates a protective antiviral state in neighbouring cells, thus helping to limit the extent of the

infection. This antiviral activity can be reproduced *in vitro*, and although not strictly proof of the presence of interferon, the antiviral assay is often used as a measure of IFN production. The effectiveness of IFN production in inhibiting viral infections *in vivo* has been demonstrated by injecting animals with neutralising antibodies raised against the IFNs and then challenging them with viruses. The susceptibility of mice to virally-induced disease caused by Semliki Forest virus, encephalomyocarditis virus (EMCV), herpes viruses, vesicular stomatitis virus (VSV) and various oncogenic viruses was found to be markedly enhanced when they were previously injected with anti- α/β -IFN antibodies (Gresser 1990). Subsequently, it was shown that mice carrying a targeted deletion in the gene for the type I IFN component, IFNAR1 (see below), that is required for response to IFN, show a complete lack of antiviral response to Semliki Forest virus or EMCV (Hwang et al. 1995).

Research into the mechanism by which the interferons set up an antiviral state initially determined that active RNA synthesis and protein synthesis was required (for reviews see (Pestka, et al. 1983, Lengyel 1982), and this led to the isolation of IFN-inducible proteins which were demonstrated to play a role in the inhibition of viral replication. The best characterised of these antiviral proteins are described below.

Mx proteins

An inbred laboratory mouse strain, A2G, was discovered to be completely resistant to normally lethal dose infections of influenza A virus (Lindenmann 1962). These mice were, however, fully susceptible to infection by VSV or EMCV. Analysis of this strain of mice led to the identification of the 72kDa Mx1 protein which is normally inducible by type I, but not type II, IFN and provides specific resistance to orthomyxovirus infection in mice (Haller et al. 1980, Horisberger et al. 1983). This resistance could be overcome by injection with an anti- α/β -IFN antibody (Haller et al. 1979). The mechanism of action of Mx1 is unclear, but the protein is located in the nucleus (Dreiding et al. 1985) and is thought to perhaps interfere with viral transcription (Sen and Lengyel 1992). A second IFN-inducible gene homologous to Mx1 has been found in mice, Mx2, although no Mx2 protein has been found (Staeheli and Sutcliffe 1988).

Mx genes have been found in a wide range of species from yeast to man (Rothman et al. 1990, Aebi et al. 1989, Horisberger et al. 1990). Human cells have two type I IFN-inducible genes homologous to Mx1, MxA (76kDa) and MxB (73kDa)

(Aebi et al. 1989, Horisberger et al. 1990), although these proteins are localised in the cytoplasm. When transfected into Mx-deficient 3T3 cells, MxA provides a high degree of resistance against both influenza and VSV (Pavlovic et al. 1990). Presumably, because of its different subcellular location, MxA exerts its effects via a different mechanism to Mx1, although this is not known. It has been suggested that the cytoplasmic Mx proteins may play a role in vacuolar protein sorting and somehow block the uptake and/or export of virions (Rothman et al. 1990, Obar et al. 1990).

The (2'-5')-oligoadenylate synthetase system

The (2'-5')-oligoadenylate synthetase (2,5- A_n synthetase) system is an extensively characterised enzyme pathway present in IFN-treated cells, containing three components: a synthetase that catalyses the formation of oligoadenylates containing 2'-5' phosphodiester bonds with the general structure ppp(A2'p5) $_n$ A (2,5- A_n , where n is between 2 and 15), an endoribonuclease (RNase L) activated by 2,5- A_n , and a phosphodiesterase that hydrolyses 2'-5'-linked oligonucleotides (for reviews see (Lengyel 1982, Pestka et al. 1987, Samuel 1991, Kerr et al. 1984). This system was identified following the observation that extracts from IFN-treated cells catalysed the production of a low molecular weight inhibitor of protein synthesis, 2,5- A_n (Kerr and Brown 1978, Eppstein et al. 1979). The enzyme responsible for the production of this inhibitor, 2,5- A_n synthetase, is induced by both type I and II IFN, and its activity is dependent on the presence of double stranded RNA (dsRNA), which is produced during the replicative cycle of most viruses (see below). There are four known forms of 2,5- A_n synthetase in human cells, with sizes of 40, 46, 49 and 100kDa (Benech et al. 1985a and b, Saunders et al. 1985, Wathelet et al. 1986, Chebath et al. 1987b, Marie et al. 1990). Most studies have focused on the 40kDa form of the enzyme which specifically confers resistance to infection by picornaviruses such as mengovirus and EMCV when expressed in Chinese hamster ovary (CHO), T98G or NIH3T3 cells (Chebath et al. 1987a, Rysiecki et al. 1989, Coccia et al. 1990). The spectrum of antiviral activity conferred by the other isoforms is not well understood, although it is known that the different isoforms exhibit different subcellular localisations as well as having different responses to IFN and dsRNA requirements for activation (Chebath et al. 1987b, Marie et al. 1990), suggesting that they may play different roles, not only in antiviral activity but also in the control of normal cell growth (Lengyel 1982).

The only known function of the oligoadenylates catalysed by 2,5- A_n

synthetase is to activate RNase L, a latent endoribonuclease whose activity is unaffected by IFN treatment. This RNase catalyses the cleavage of both viral and cellular single stranded RNA molecules on the 3' side of UA, UG and UU sequences to leave 3' phosphate-terminated products (Floyd-Smith et al. 1981), (Wreschner et al. 1981). Because 2,5- A_n is unstable, its degradation being catalysed by a 2'-5' phosphodiesterase which converts 2,5- A_n to ATP and AMP (Schmidt et al. 1979), the activation of RNase L is only observed in the continued synthesis of 2,5- A_n .

When cells are treated with high concentrations of IFN and low multiplicities of infection of EMCV, viral protein synthesis is specifically inhibited (Vaquero et al. 1981, Munoz and Carrasco 1981) and host protein synthesis is uninhibited. The activity of RNase L is unselective for viral RNA, but there is evidence to suggest that under these conditions, the 2,5- A_n system is activated by locally high concentrations of replicating viral dsRNA, leading to the degradation of the predominantly viral RNA in the vicinity (Nilsen and Baglioni 1979, Nilsen et al. 1980).

Protein kinase R

Protein kinase R (PKR), also previously referred to as DAI, dsI, p68 kinase, and P1, was originally identified as an activity in rabbit reticulocyte lysates which caused the inhibition of translation of exogenously added mRNAs (Farrell et al. 1977). Human PKR has subsequently been characterised as a 551 amino acid protein with a predicted molecular weight of 62kDa, although it runs as a 68kDa protein on SDS-PAGE gels (Meurs et al. 1990) (for reviews see (Williams 1995, Proud 1995, Samuel 1991, Pestka et al. 1987)). PKR is present at low levels in untreated cells, but is induced five to ten fold by type I, and to a lesser extent by type II, IFN (Laurent et al. 1985, Samuel and Knutson 1982, Samuel and Knutson 1983). It is generally activated by binding to dsRNA, although some single stranded RNAs containing secondary structures such as stem-loop regions can also activate (Henry et al. 1994)(Robertson et al. 1996). There are two RNA binding domains near the amino terminus which are both necessary for efficient RNA binding and kinase activation (see (Williams 1995, Clemens 1996) for reviews). PKR can also be activated by binding to other poly-anions such as heparin and dextran sulphate (Hovanessian and Galabru 1987), although the ability to activate RNA binding mutants of PKR with heparin suggests that this probably occurs by a different mechanism (Patel et al. 1994).

PKR binds dsRNA with very high affinity, and low concentrations of dsRNA

(100ng/ml) are maximal for activation (see (Samuel 1993) for review). Concentrations higher than this are actually inhibitory for activation, and this has suggested a mechanism for activation whereby two molecules of PKR bind to one dsRNA molecule and probably transphosphorylate each other on several serine and threonine residues (Kostura and Mathews 1989, Green and Mathews 1992) although intramolecular phosphorylation has also been suggested (Galabru et al. 1989). Once phosphorylated, PKR activity is independent of dsRNA and it is able to phosphorylate its substrates, the two known targets being eIF2 α (Clemens 1996) and I κ B (Kumar et al. 1994, Offerman et al. 1995). Phosphorylation by PKR of the protein synthesis initiation factor eIF2 α on serine residue 51 prevents the recycling of phosphorylated eIF2-GDP to eIF2-GTP by the nucleotide exchange factor eIF2B, resulting in the sequestering of eIF2B by phosphorylated eIF2 α and the inhibition of initiation of protein synthesis (De Benedetti and Baglioni 1986), (Hershey 1989, Safer 1983). PKR shows marked homology to two other kinases which also phosphorylate eIF2 α on serine 51, the mammalian heme-regulated kinase (HCR or HRI) and the *Saccharomyces cerevisiae* protein GCN2 (for reviews see (Clemens 1996, Hinnebusch 1994), not only in the kinase subdomains but also in another region between two subdomains which may be involved in substrate recognition (Chong et al. 1992). The phosphorylation of I κ B is less well understood and will be discussed below.

The antiviral nature of PKR was originally inferred from the fact that viruses have developed many and varied means of inhibiting the activation and activity of the kinase, which would result in the shut down of viral as well as host protein synthesis (see (Katze 1995, Proud 1995, McMillan and Williams 1996, Jacobs and Langland 1996), for reviews). The best example of this phenomenon is to be found in adenovirus, which synthesises large quantities of a small RNA molecule, VA_I, which due to its extensive secondary structure (Mellits and Mathews 1988) binds to PKR and inactivates it. Interestingly, VA_I is capable of activating 2,5-A_n synthetase. A mutant adenovirus, *dl* 331, which fails to synthesise VA_I, cannot inhibit PKR and is sensitive to IFN (Thimmappaya et al. 1982). A similar strategy is employed by Epstein Barr virus (EBV), which produces similarly sized RNA molecules called EBERs. These also possess secondary structure and are produced at high concentrations, with the result that they bind to PKR with a 1:1 stoichiometry, thereby blocking the dimerisation necessary for activation. EBER genes can functionally substitute for VA_I in *dl* 331 (Bhat and Thimmappaya 1983), although lack of functional EBERs in EBV mutants does not seriously affect their replication.

Other viruses prevent the activation of PKR by encoding dsRNA-binding proteins which sequester dsRNA from the kinase. Reoviruses synthesise a dsRNA binding protein, $\sigma 3$, which can also rescue adenovirus *dl* 331 replication (Huisman and Joklik 1976). Similarly, vaccinia utilises its E3L protein (Akkaraju et al. 1989), and rotavirus uses the product of its NSP3 gene. Another strategy used by vaccinia virus is to encode a protein, K3L, which can act as an eIF2 α homologue and block substrate phosphorylation and thus translational inhibition (Beattie et al. 1991). Human immunodeficiency virus-1 (HIV-1) inhibits PKR but by an as yet poorly understood mechanism, although both the above mechanisms have been suggested. All HIV-1 mRNAs contain an extensive region of secondary structure at their 5' end, known as the transactivating region or TAR. TAR is capable of both activating and inhibiting PKR, depending on its concentration (Edery et al. 1989, SenGupta and Silverman 1989). TAR is bound by a protein named Tat, which can also inhibit PKR, possibly by acting as a non-phosphorylatable substrate, in a manner analogous to K3L (McMillan et al. 1995), or perhaps, in a manner similar to $\sigma 3$, E3L and NSP3, by sequestering TAR, thus preventing PKR activation (Judware et al. 1993).

Influenza virus and poliovirus employ different strategies to inactivate PKR. Influenza virus activates a cellular 58kDa protein, P58IPK, which binds to the N-terminus of PKR and blocks its activation and substrate phosphorylation. P58IPK, a member of the tetratricopeptide family of proteins, is itself held in an inactive state in normally growing cells by direct interaction with the molecular chaperone hsp40, and influenza virus infection disrupts this to liberate the inhibitor of PKR (Melville et al. 1997). Poliovirus inactivates PKR by activating a cellular protease which acts with dsRNA to specifically degrade PKR (Black et al. 1989).

Since the cloning of the gene for PKR, experiments that have modulated the levels of expressed wild type and mutant proteins, and ablated endogenous expression, have more directly demonstrated its role in the antiviral response (Meurs et al. 1992, Yang et al. 1995).

Antiproliferative activity

As well as possessing antiviral activity, it has long been recognised that the IFNs can have effects on cell growth and differentiation. The antiproliferative activity of IFN was first noted in 1962, and was confirmed by studies first performed with purified fractions of α -IFNs (Evinger et al. 1981) and subsequently with recombinant IFNs (Rehberg et al. 1982). IFNs are sometimes referred to now as negative growth factors and have been shown to inhibit the growth of a wide

variety of normal and transformed cells (Taniguchi et al. 1995). This inhibition has been shown to affect various phases of the cell cycle, causing an extension of G1, blocking entry into S phase and lengthening both S phase and G2. The addition of anti-IFN antibodies to some cell lines can result in the stimulation of cell growth, suggesting that in some cases very low level production of IFN plays a role in the control of cell growth (Creasey et al. 1983, Moore et al. 1984).

Although certain viruses can inhibit the antiproliferative but not the antiviral activity of IFN (Aman and von Gabain 1990), it was found that all purified IFN fractions that exhibited antiviral activity also exhibited antigrowth activity (Evinger et al. 1981), suggesting that similar mechanisms may be involved in both processes, and the well characterised IFN-inducible proteins and factors involved in the induction of IFN and IFN-inducible genes have been analysed for their contribution to the regulation of cell growth. In accordance with the findings from these studies, some of these proteins have been designated as tumour suppressors and oncogenes.

PKR

When a wild-type human PKR cDNA was expressed in yeast it lead to the phosphorylation of eIF2 α and the suppression of cell growth (Chong et al. 1992, Dever et al. 1992). Conversely, when enzymatically inactive forms of human PKR were expressed in mouse NIH 3T3 cells, transformed lines resulted which were capable of forming tumours in nude mice (Koromilas et al. 1992), (Meurs et al. 1993). Interestingly, any tumours arising from the injection of cell lines expressing wild-type PKR were found to be harbouring inactive forms of PKR which had presumably arisen spontaneously. These results lead to the hypothesis that the mutant forms of PKR were acting as dominant inhibitors of normal PKR, and the classification of PKR as a tumour suppressor.

In support of this, it was shown that overexpression of the cellular inhibitor of PKR, p58, in NIH 3T3 cells causes malignant transformation (Barber et al. 1994), and that the activity of PKR was inhibited in IL-3-dependent cells upon addition of the cytokine (Ito et al. 1994). However, "knockout" mice devoid of functional PKR develop normally, showing no increase in spontaneously arising tumours, and cells derived from these animals show no increased susceptibility to transformation (Yang et al. 1995). It is thus possible that PKR is not a tumour suppressor, and the dominant inhibitor mutants of PKR are acting on other pathways.

The (2'-5')-oligoadenylate synthetase system

In addition to its antiviral effects, introduction of 2,5- A_n into cells results in the inhibition of growth rates, suggesting that it may also be involved in the anti-proliferative activity of IFNs (Hovanessian and Wood 1980). Overexpression of the 40kDa form of 2,5- A_n synthetase also reduced the growth rate of the recipient cell (Chebath et al. 1987a), (Rysiecki et al. 1989) (Coccia et al. 1990), although the other forms of the synthetase have not been analysed. To assess the contribution of the whole 2,5- A_n system, a dominant negative mutant of RNase L was overexpressed in murine SVT2 cells with the result that, as well as inhibiting the antiviral effect of IFN against EMCV, it also inhibited the antiproliferative effect of IFN on these cells (Hassel et al. 1993).

IRF-1 and IRF-2

IRF-1 and IRF-2 (Interferon Regulatory Factors-1 and -2) were originally identified as positive and negatively acting transcription factors, respectively, of the β -IFN promoter (Miyamoto et al. 1988, Harada et al. 1989) (see below). Both are IFN-inducible factors and have since been shown also to be involved in the induction of IFN-inducible genes. The transcriptional activational ability of IRF-1 on a synthetically derived reporter is antagonised by IRF-2 by virtue of its ability to bind to the same sites, and the much greater stability of IRF-2 protein results in its accumulation to much higher levels in cells than IRF-1, with the consequent repression of potential IRF-1 target genes. Following the addition of an IRF-1-inducing signal, for example IFN treatment, IRF-1 synthesis is upregulated and this can augment the induction of IFN-inducible genes (see below). As a consequence of this, IRF-2 is induced and this contributes to the post-induction repression of these genes (Harada et al 1994).

Studies on the levels of IRF-1 and IRF-2 mRNA during the cell cycle in NIH 3T3 cells have shown that whereas IRF-2 levels remain relatively constant, the levels of IRF-1 mRNA vary several fold, with the highest levels found in growth-arrested cells and the lowest following stimulation (Harada et al. 1993), and this suggested the involvement of IRF-1 in the normal control of cell growth. Furthermore, overexpression of IRF-1 can inhibit cell growth (Yamada et al. 1990, Kirchhoff et al. 1993), whereas overexpression of IRF-2 has been shown to transform NIH 3T3 cells (Harada et al. 1993). This transformation can be blocked by the co-expression of IRF-1. Similarly, embryo fibroblast cells from IRF-1 "knockout" mice

can be transformed *in vitro*. Taken together, these results indicate that IRF-2 and IRF-1 can function as an oncogene and anti-oncogene respectively.

The induction of apoptosis in cells derived from IRF-1 "knockout" mice is impaired (Tanaka et al. 1994, Tamura et al. 1995), suggesting that the antiproliferative activity of IRF-1 is mediated by its activity as a promoter of apoptosis. In support of this, IRF-1 acts as a transcriptional activator of ICE (Tamura et al. 1995), a cysteine protease that is centrally involved in the execution phase of apoptosis (see Chapter Seven for more detail). It has also been reported that the antiproliferative activity of IRF-1 is dependent upon the activity of PKR (Kirchhoff et al. 1995), which has itself been implicated in the TNF α -induced apoptosis of U937 cells (Yeung et al. 1996) see Chapter Seven).

In support of its proposed role as an anti-oncogene and inducer of apoptosis, the region of human chromosome 5 to which the IRF-1 gene maps, is frequently deleted or rearranged in leukaemia and preleukaemic myelodysplastic syndrome (Willman et al. 1993). Thus IRF-1 and IRF-2 appear to be important IFN-inducible regulators of cell growth.

p202

One of the first IFN-inducible genes cloned, 202 cDNA has been found to be a member of a gene cluster on mouse chromosome 1 with at least five other members (the gene 200 cluster). Some of these members are also IFN-inducible, and two human homologues have been found (for review see (Lengyel et al. 1995). So far, only p202 has had a function ascribed to it. In transfection assays, overexpression of p202 can inhibit cell growth, and has been shown to bind the hypophosphorylated form of the tumour suppressor pRb in IFN-treated cells (Choubey and Lengyel 1995). The consequence of this remains to be established, but it is possible that by doing so p202 can inhibit the phosphorylation of pRb, thus preventing the activation of the E2F transcription factor. In support of this, IFN treatment has been reported to impair the phosphorylation of pRb (Resnitzky et al. 1992). It has also been shown that p202 can bind E2F, possibly in a complex with pRb, although in transfection experiments p202 can inhibit the activity of one form of E2F, E2F-1, in the absence of functional pRb (Choubey and Lengyel 1995).

Immunomodulatory activity

As well as their antiviral and antiproliferative effects, the IFNs also have profound effects on the immune system, some of which may contribute to their

previously described activities. The best known example of the role of the IFNs in the immune system is their ability to upregulate major histocompatibility complex (MHC) antigens. Both type I and II IFNs induce class I murine H-2 and human HLA-A, B and C MHC antigens, as well as the associated subunit, β 2-microglobulin, in a variety of cell types. Class II MHC antigens, such as the murine I-region-associated Ia antigens and the human HLA-DR, -DP and -DQ antigens, are induced primarily by γ -IFN. This induction occurs in both immune and non-immune cells. Increased MHC expression may contribute to the antiviral and antiproliferative activities of IFNs by enhanced presentation of viral antigens and subsequent lysis by cytotoxic T cells (Pestka et al. 1987).

Some immature cell types and certain virus-infected cells, however, have low MHC expression, even following IFN stimulation, and thus present a threat because they may escape T cell surveillance even if expressing foreign antigens. However, IFN production following viral infection induces both an increase in natural killer (NK) cell number and activity (Trinchieri 1989). NK cells are involved in the defence against viral infections, the destruction of malignant cells and the regulation of haematopoiesis, and it is thought that, because NK cells are not MHC-restricted, IFN-activated NK cells may selectively eliminate cells with low MHC class I expression, including those cells that have been infected with viruses that have blocked the usual upregulation of the MHC (Bukowski and Welsh 1985).

Recently, a role for type I IFN has been postulated in promoting long term viral immunity via CD8-positive T cells. It has been shown that purified β -IFN can activate 70-80% of CD8 cells and thus potentiate the long term persistence of memory T cells by acting as a survival factor for these cells, produced during any viral infection (Tough et al. 1996).

Interferon as a therapeutic agent

The biological properties of IFNs described above have led to investigations of their use in treating viral illnesses and tumours. Although injections of IFNs, particularly α -IFN, can produce serious side effects, for example fever and fatigue and the inhibition of haematopoiesis, with the consequence that only low doses can be administered, they have proved very useful in treating certain conditions. α -IFN is used in the treatment of cancers such as hairy-cell leukaemia, Kaposi's sarcoma, non-Hodgkin's lymphoma, multiple myeloma and chronic myeloid leukaemia, whereas its antiviral activities are used to treat chronic hepatitis B and C, papillomavirus-associated genital warts and HIV infection. β -IFN is currently being

used to treat relapsing-remitting multiple sclerosis and γ -IFN has proved to be effective against chronic granulomatous disease. Recently τ -IFN has been shown to have much lower toxicity than the other forms of IFN, but it is equally effective as an antiviral agent, and the human form of τ -IFN may prove to be a much safer alternative and widen the use of IFN as a therapeutic agent (Johnson et al. 1994).

Interferon Signal Transduction

Introduction

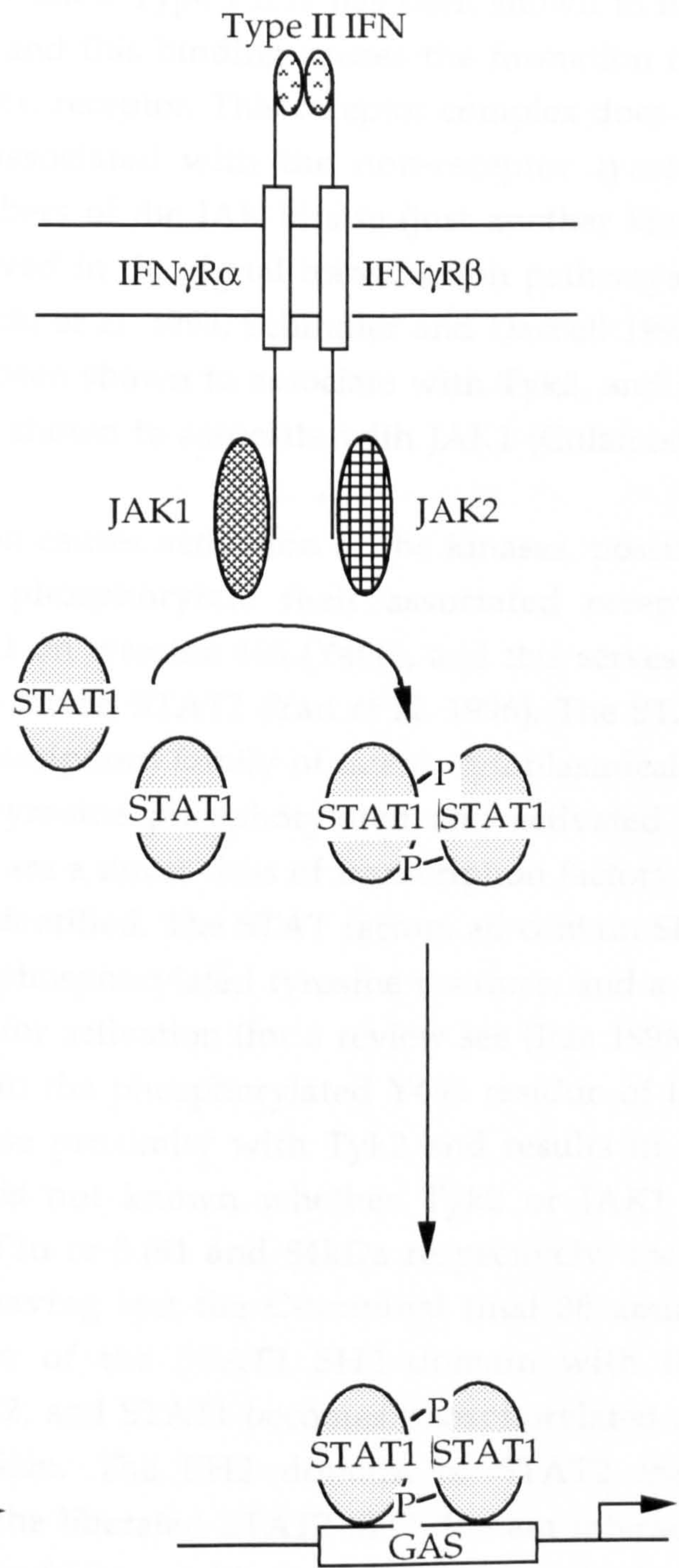
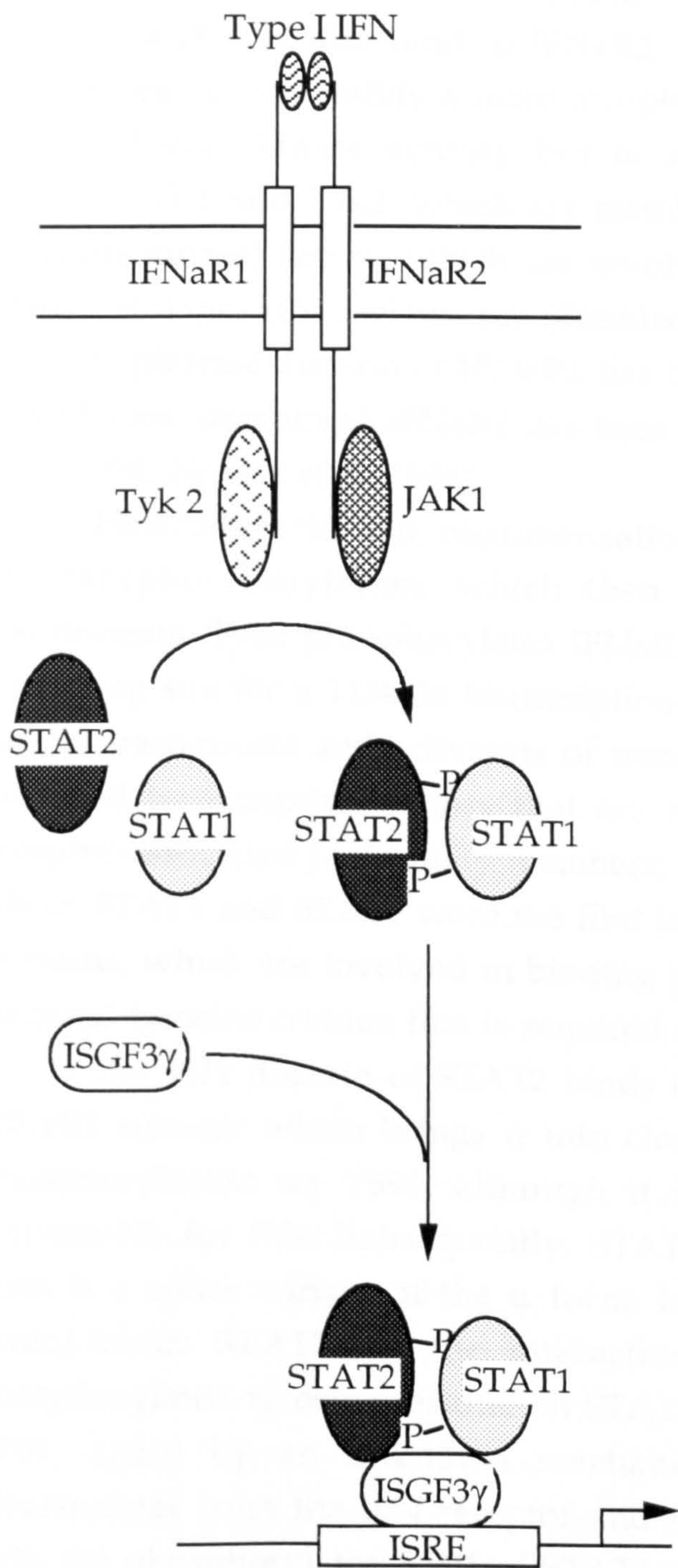
As discussed above, the biological responses to the IFNs require the activation of transcription and protein synthesis of a class of IFN-inducible proteins, some of which have already been described. There are currently fifty or more such proteins that have been identified, and studies on the transcriptional regulation of prototypical members of this family led to the identification of the promoter elements in the genes of these proteins that were responsible for type I or type II IFN induction. These two types of promoter element are named ISRE (IFN stimutable response element) and GAS (γ -IFN activated sequence), respectively, and their consensus sequences are shown below:

ISRE	G/AGGAAANNGAAACT
GAS	TTNCNNNAA

Two complementary approaches to studying the regulation of these sequences, one biochemical involving the purification of the IFN-activated binding factors, and one genetic, isolating cell lines that were unable to respond to IFN treatment, were undertaken in recent years, and by complementing the defects of the mutant cell lines from the latter study with the cDNA clones eventually isolated in the former study, a comprehensive model of the signal transduction pathways activated by each type of IFN, from IFN receptor to transcriptional initiation, has been elucidated (for reviews see (Schindler and Darnell 1995, Darnell et al. 1994, Goodbourn and King 1996)). The overall scheme is shown in Figure 1.1.

The JAK-STAT pathway

The first step in the activation of the Type I and Type II IFN signal transduction pathways is the interaction of the ligands with the extracellular domains of their receptors. Like the IFNs themselves, these receptors are encoded on different chromosomes (Epstein et al. 1982, Rashidbaigi et al. 1986).



Type I IFN

The Type I IFN receptor, which binds all the identified Type I IFN members, consists of at least two subunits, IFNaR1 (IFNAR α) and IFNaR2 (IFNAR β), with evidence for a third from cross-linking studies. Type I IFN has been shown to bind IFNaR1, and may also bind to IFNaR2, and this binding causes the formation of a heterodimeric, or possibly a more complex, receptor. This receptor complex does not have intrinsic kinase activity but is associated with the non-receptor tyrosine kinases JAK1 and Tyk2, which are members of the JAK kinase (just another kinase or Janus kinase) family, which are involved in the signal transduction pathways of many cytokines (for reviews see (Ziemiecki et al. 1994, Schindler and Darnell 1995)). The cytoplasmic domain of IFNaR1 has been shown to associate with Tyk2, and the cytoplasmic domain of IFNaR2 has been shown to associate with JAK1 (Colamonici et al. 1994, Novick et al. 1994).

Heterodimerisation/oligomerisation causes activation of the kinases, possibly by transphosphorylation, which then phosphorylate their associated receptor components. Tyk2 phosphorylates IFNaR1 on tyrosine 466 (Y466), and this serves as a docking site for a 113kDa transcription factor, STAT2 (Yan et al. 1996). The STAT (signal transducers and activators of transcription) family of latent, cytoplasmically-localised transcription factors that are tyrosine phosphorylated and activated by receptor-associated JAK family members, are a novel class of transcription factors, of which STAT1 and STAT2 were the first identified. The STAT factors all contain SH2 domains, which are involved in binding phosphorylated tyrosine residues, and a C-terminal tyrosine residue that is required for activation (for a review see (Ihle 1996)).

The SH2 domain of STAT2 binds to the phosphorylated Y466 residue of the IFNaR1 subunit which brings it into close proximity with Tyk2 and results in its phosphorylation on Y690, although it is not known whether Tyk2 or JAK1 is responsible for this. Subsequently, STAT1 α or β (91 and 84kDa respectively; the β form is a splice variant of the α form, having lost the C-terminal final 38 amino acids) binds STAT2 via the interaction of the STAT1 SH2 domain with the phosphorylated tyrosine residue on STAT2, and STAT1 becomes phosphorylated on Y701, again by an unknown mechanism. The SH2 domain of STAT2 then disassociates from the IFN receptor and the liberated STAT2 SH2 domain interacts with the phosphorylated Y701 of STAT1 to form a stable complex (Yan et al. 1996). Once released from the receptor, this heterodimer then enters the nucleus as a complex with a 48kDa DNA binding protein, ISGF3 γ , to form the mature transcriptionally active complex ISGF3, that can bind to and activate transcription

from the ISREs of type I IFN-inducible genes (Darnell et al. 1994, Goodbourn and King 1996).

Type II IFN

The type II IFN receptor also consists of at least two components, IFN γ R α and IFN γ R β , which are associated with JAK1 and JAK2, respectively (Sakatsume et al. 1995). γ -IFN binding induces heterodimerisation/oligomerisation of the receptor, which results in the phosphorylation of IFN γ R α on Y440 (Greenlund et al. 1994). JAK2 is thought to phosphorylate both itself and JAK1, which can then phosphorylate the receptor and recruit STAT1. Once bound via the interaction of its SH2 domain and the phosphorylated Y440 of IFN γ R α , STAT1 is phosphorylated on Y701, possibly by JAK2, and, by an unknown mechanism, is released and homodimerises, with the SH2 domain of each partner binding the other's phosphorylated Y701 residue (Briscoe et al. 1996). The homodimeric STAT1 translocates to the nucleus and binds to DNA directly. However, only homodimeric STAT1 α , known as GAF (γ -IFN-activated factor), not STAT1 β , can activate transcription from GAS site-containing genes (Muller et al. 1993). STAT1 α contains a residue in its C-terminal extension missing from STAT1 β , serine 727, present in a consensus site for MAP kinase phosphorylation. This residue is also phosphorylated following ligand treatment, possibly by the concomitant activation of a MAP kinase pathway, and this phosphorylation is required for maximal GAF activity (Wen et al. 1995, David et al. 1995). The C-terminus of STAT2 is thought to contain the transcriptional activation domain of ISGF3, and thus STAT1 β can participate in a productive complex following α -IFN treatment.

Cross talk between type I and type II IFN pathways

IFN-inducible genes are known to vary in their ability to respond to both type I and type II IFN. Some are responsive to each, whereas others are predominantly inducible by only one type of IFN (Revel and Chebath 1986). Analysis of the promoters of the genes responsive to both types of IFN revealed that some had both ISRE and GAS sites, whereas others had only one type of response element. The fact that type I IFN treatment results in tyrosine phosphorylated STAT1 has explained the ability of certain GAS site-containing promoters to respond to type I IFN, because as well being recruited into ISGF3, type I IFN-activated STAT1 can also homodimerise to form GAF. Conversely, γ -IFN has been shown to directly activate through an ISRE by the redirection of GAF to this sequence by complexing with

ISGF3 γ to form a transcriptionally active ISGF3-like complex (Bluyssen et al. 1995). Type II IFN can also activate indirectly through ISREs by the induction of the type II IFN-inducible ISRE-binding factor, IRF-1.

Specificity of responses via the JAK-STAT pathway

Since the discovery of the JAK-STAT pathway in IFN signal transduction, homology screens have identified other STAT family members, STATs 3, 4, 5a and b, and 6, that are activated by tyrosine phosphorylation and subsequent homo- and heterodimerisation in response to treatment by a variety of cytokines and growth factors. Although GAF can be activated by other ligands, for example EGF, PDGF and IL-6, ISGF3 has been shown to be only activated by type I IFN, presumably because STAT2 is only phosphorylated by type I IFN treatment. It is not clear whether the GAF-ISGF3 γ ISRE-activating complex can also be produced by other GAF-activating ligands.

In contrast to the situation with STAT2, the relatively small and overlapping repertoire of STAT homo- and heterodimers activated by the many other cytokines and growth factors that elicit their biological responses via JAK-STAT pathways raises the question of how specificity of gene activation is achieved. This has, to some extent, been explained by the analysis of GAS sites and their reiteration within STAT-responsive promoters. Site selection studies have revealed subtle differences between the optimal binding sites for the different STAT complexes, with the most dramatic being the discovery that the IL-4-activated STAT6 homodimer preferentially binds to a GAS site in which the two half-sites (TT and AA) are separated by six nucleotides, rather than five, as is found in a classical GAS site (Schindler et al. 1995, Seidel et al. 1995). It has also been found that the N-termini of the STAT proteins can be involved in protein/protein interaction between STAT dimers, and in such a fashion the STATs may co-operatively bind to the tandem or multiple GAS sites found in some responsive promoters, and thus achieve specific gene activation (Vinkemeier et al. 1996, Xu et al, 1996). Finally, as well as co-operative binding between STAT homo- and heterodimers, there is also evidence of co-operation between STAT dimers and other transcription factors which may be coordinately activated by ligand treatment (Schaefer et al. 1995, Perez et al. 1994).

INTERFERON GENE INDUCTION

The Nature Of Inducing Signals

The synthesis of type I IFNs is not detectable in normally growing cells, but reaches high levels after induction. *In vivo*, almost all viruses can act as inducers, whether their genome consists of DNA, single-stranded RNA, or double-stranded RNA. Furthermore, many viruses can induce IFNs *ex vivo* in isolated tissues and cell suspensions, or *in vitro* in primary fibroblast cultures and in many established fibroblastoid and lymphoblastoid cell lines. However, several viruses that are efficient IFN-inducers *in vivo*, are poor inducers, or not inducers at all, of cultured cells (Jacobs and Langland 1996).

Many years ago it was discovered that the IFN-inducing component of extracts of *Penicillium funiculosum* was dsRNA (Lampson et al. 1967), and subsequent studies have shown that synthetic dsRNA molecules, such as polyinosinic:polycytidylic acid (poly(I):poly(C)), are extremely efficient inducers of β -IFN induction *in vitro*. In contrast, DNA and single-stranded RNA are incapable of inducing β -IFN. It has been determined that to act as inducers of β -IFN, double-stranded polynucleotides need to be of a sufficiently high molecular weight, with a consequently high thermal stability and a reasonable resistance to degradation by nucleases, which may be advantageous *in vivo*. The exact sequence of the dsRNA appears to be unimportant for the inducibility of the molecule (DeClerq 1977).

Based on the finding that dsRNA is a very effective inducer of β -IFN, it has been hypothesised that the inducer present in all virus-infected cells is dsRNA, either provided by the viral genome itself (in dsRNA viruses) or produced as a replicative intermediate (in ssRNA viruses). It is believed that DNA viruses produce dsRNA as a result of overlapping transcription from both strands of the genome (for a review see (Jacobs and Langland 1996)). However, the induction pathways activated by viruses and dsRNA are clearly not identical, and at least some viruses seem to provide an inducing factor, or activate a cellular signal transduction pathway, that is different from, or additional to, those provided by dsRNA. For example, certain ssRNA viruses can induce IFN under conditions that are non-permissive for replication, and certain replication-defective mutants of reovirus do not induce IFN, even if their genome is dsRNA (Lai and Joklik 1973). It should be noted, however, that the reovirus genome remains within the inner viral capsid for the life cycle of the virus, and perhaps this prevents the exposure of the dsRNA to the cell. Furthermore, the viral induction of the α -IFN genes does not appear to be mediated solely by dsRNA, since they are inducible by NDV but not by

poly(I):poly(C) in primary human fibroblasts, whereas the β -IFN gene can be induced by both agents (Havell et al. 1978). In addition, partial induction of otherwise priming-dependent cell lines can be achieved by Sendai virus (a paramyxovirus; genome (-)ssRNA) without the need to pretreat cells with IFN (King and Goodbourn 1994) (see below and Chapter Three). A difference between Sendai virus and dsRNA has also been reported at the level of the DNA binding factors that bind to the DNA elements within the β -IFN promoter: in differentiated mouse embryonal carcinoma cells, Sendai can induce the PRD II-binding protein NF- κ B (see below), whilst dsRNA cannot (Ellis and Goodbourn 1994). The nature of the non-dsRNA component(s) provided by Sendai virus is not known, although it has been suggested that the viral protein C could function as an efficient inducer of β -IFN (Taira et al. 1987).

The signal pathway activated by dsRNA, and leading to specific gene activation, has not been well elucidated. Efforts to determine whether dsRNA acts by triggering membrane receptors or by interacting with an intracellular target, following endocytosis, have provided contradictory evidence (Pitha 1977), although the establishment of the antiviral state following microinjection of dsRNA strongly implies that dsRNA acts as an intracellular inducer (Silhol et al. 1986). The activities of the two known dsRNA-activated enzymes, PKR and 2,5- A_n synthetase have been discussed earlier, and it is interesting that β -IFN induction can be inhibited by the purine analogue 2-aminopurine (2AP) (Marcus and Sekellick 1988, Zinn et al. 1988), which, although it is a rather nonspecific kinase inhibitor, is known to inhibit PKR. Recent experiments using cell lines deficient in PKR activity have further implicated PKR in β -IFN induction (see below). It has thus been speculated that this enzyme is the intracellular receptor for dsRNA.

It has also been suggested that under some circumstances the accumulation of naturally occurring cellular dsRNAs can induce IFN production (Belhumeur et al. 1993). If this kind of endogenous induction machinery exists, it would have to be tightly regulated to prevent inappropriate IFN expression, which could inhibit cell proliferation. In light of this, it is interesting to note that several cellular inhibitors of PKR have been identified, including p58 (Barber et al. 1994) and dRF (Judware and Petryshyn 1992), which seem to inhibit by interacting directly with the kinase, and the RNA-binding proteins La (Xiao et al. 1994) and TRBP (Park et al. 1994).

Induction Cycle of β -IFN

The induction of β -IFN has been shown to occur primarily at the level of

transcriptional initiation (see Figure 3.2) (Raj and Pitha 1983, Nir et al. 1984) (for reviews see (Goodbourn 1990), (Maniatis 1988)). In uninduced cells the β -IFN mRNA is undetectable. The induction cycle begins with a lag period after the introduction of an inducer. This period does not appear to result from the delayed entry of the inducer into the cell (Hauser et al. 1982), nor does it reflect a need for the synthesis of virus- or dsRNA-induced proteins, since β -IFN mRNA is inducible in the presence of protein synthesis inhibitors (Cavalieri et al. 1977). Rather, the lag period is likely to reflect the time required to derepress the promoter to allow efficient transcription (see below). The lag period is followed by an IFN synthesis phase peaking between 3 and 12 hours after the cells have encountered the particular inducer, during which a substantial proportion of the newly synthesised mRNA is β -IFN-specific, in the order of several thousand transcripts per cell. The induction of β -IFN is transient, and at the final post-induction turn-off stage, the β -IFN mRNA level rapidly decreases back to undetectable. The lengths of different phases vary depending on the cell type and inducer. Although inductions with poly(I):poly(C) are usually effective within a few hours of addition of the inducer, different viruses can require up to 18 hours to achieve a maximal induction, possibly because viral transcription and/or replication is required for the production of dsRNA.

The dramatic changes in β -IFN expression during the induction cycle reflect the biological properties of β -IFN. The potent cytostatic effects of type I IFN make expression in uninduced cells incompatible with cellular growth (see above), and thus tight repression of the gene is required in the absence of inducer. Once this repression is overcome, rapid, high level expression of β -IFN sets up the antiviral state and helps to minimise the spread of viral infection.

The β -Interferon Gene Promoter

The transcriptional regulation of the β -IFN gene has been extensively studied as a paradigm for the regulation of highly inducible promoters, and has been greatly facilitated by the fact that the promoter constructs are appropriately regulated when transfected into cultured cell lines (Hauser et al. 1982, Ohno and Taniguchi 1982, Zinn et al. 1983). The requirement for stringent positive and negative transcriptional control of the β -IFN promoter at different stages of the induction cycle is reflected in the complexity of its organization (reviewed in (Maniatis 1988, Taniguchi 1989, Goodbourn 1990).

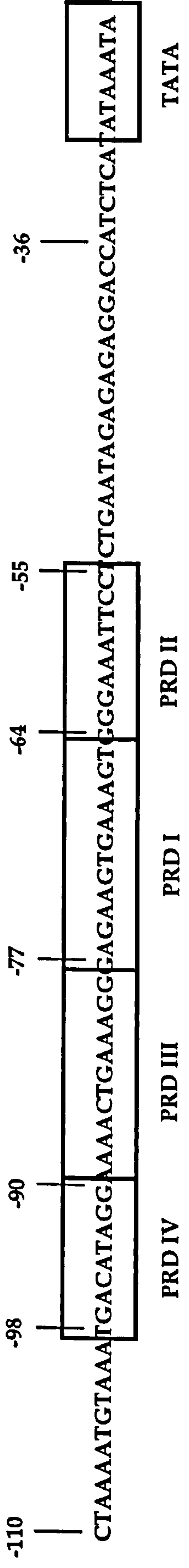
As has been described previously, transcription from the human β -IFN gene

can be transiently induced in many cell types by viral infection or treatment with synthetic double-stranded RNA (dsRNA) (Stewart 1979). The induction can occur in the presence of protein synthesis inhibitors, implying that the regulatory transcription factors involved pre-exist in uninduced cells, and their transcriptional activity or access to their respective response elements is modified upon induction. Genetic analysis utilising various deletions expressed in host cells has revealed that the approximately 200bp 5'-flanking regulatory sequence responsible for transcriptional activation consists of multiple distinct positive and negative DNA response elements, referred to as PRDs (Positive Regulatory Domains) and NRDs (Negative Regulatory Domains) (Figure 1.2, reviewed in (Goodbourn 1990)). The murine β -IFN promoter contains significant homology to the human promoter (Dirks et al. 1989).

The contribution of promoter sequences to the maximal induction of the β -IFN gene appears to differ depending on the cell line used in transfection analyses. Different cell lines may utilize different arrays of transcription factors that recognise the *cis*-acting regulatory elements of the gene. In mouse L929 cells, it was shown that the level of induction by Newcastle disease virus diminished dramatically as the 5' deletion extended from -105 to -91 (Fujita et al. 1985). Further deletion from -91 to -78 rendered the promoter uninducible. Also in HeLa cells, efficient inducibility requires sequences upstream of -91 (Du and Maniatis 1992, King and Goodbourn 1994) (see Chapter Four). On the other hand, it has been shown that the critical boundary for induction resides between -77 and -73 in mouse C127 fibroblasts transformed by episomal bovine papilloma virus vectors carrying various deletion mutants of the β -IFN gene (Zinn et al. 1983). However, while this was the requirement for substantial inducibility, the sequences upstream of -77 exhibited a modulatory effect. Specifically, the extensions of the promoter to -91 and -104 both caused further two-fold increases in inducibility. Extending the promoter even further to -210 actually decreased both the basal and induced transcription levels, suggesting that a negative regulatory element lies in these upstream sequences. A similar pattern of changes in the inducibility of the promoter variants in C127 cells was observed either in transient transfection assays, or when the deletion mutants were stably introduced into the host chromosomes, although in the latter system, the effect of the sequence between -104 and -91 was more pronounced (Goodbourn et al. 1985). It should be borne in mind that the analysis of the magnitude of transcription in these studies was performed by different groups using different

Figure 1.2 The organisation of the β -IFN promoter

The β -IFN promoter contains positive and negative regulatory domains (PRDs and NRDs) between -110 and the TATA box at -30. See text for details.



NRD II

NRD I

techniques (S1 mapping and RNase protection for the measurement of RNA levels, and luciferase quantitation for reporter assays) and the observed differences in sequence requirements might be a reflection of the sensitivity of each. The broadly similar qualitative results would argue that this was the case.

The regulatory regions of the β -IFN promoters can confer inducibility to a heterologous promoter, which has greatly facilitated the analyses of the induction events. In C127 cells the region between -77 and -36 is sufficient for this (Goodbourn et al. 1985), while in L929 cells sequences upstream of -77 are also required (Fujita et al. 1985). The reiteration of short elements from within these regions upstream of minimal promoters has also resulted in dsRNA-inducible constructs (Fujita et al. 1987, Kuhl et al. 1987, MacDonald et al. 1990). By the analysis of promoter deletion series and the effects of point mutations within the promoter on the basal and induced levels of transcription, a complex spectrum of overlapping positive and negative regulatory domains (PRDs and NRDs) has been determined

Negatively-acting promoter elements

The β -IFN promoter is tightly repressed in uninduced cells, which is necessary because of the cytotoxic properties of the protein product. The repression of the β -IFN promoter is complex and does not appear to be mediated by a single sequence element. Two negative regulatory domains (NRDs) have thus far been identified. As judged by the effect on transcription levels of progressive 5' promoter deletions, the NRD II element is located just upstream of the nucleotide position -100 (Zinn et al. 1983, King and Goodbourn 1994) (see Chapter Four). Deletion of the NRD I region located 3' to PRD II (see below) also leads to elevated basal activity (Goodbourn et al. 1986). The precise 5' end point of NRD I has been difficult to define, since the element overlaps with a positive regulatory domain, PRD II (Goodbourn et al. 1986, Nourbakhsh et al. 1993), and point mutations across this region of overlap can thus affect both basal and inducible levels of transcription. However, certain single base substitutions within PRD II cause an elevated basal expression without increasing the induced levels of expression (Goodbourn and Maniatis 1988), suggesting that the phenotype is produced by creating a weakened binding site for a pre-induction repressor protein rather than a stronger binding site for an activator protein.

Besides being a dsRNA- and virus-inducible element (see below), the PRD I element also acts as an inhibitory DNA element for enhancers, such as the viral SV40 enhancer, when positioned between them and the promoter (Kuhl et al. 1987).

This implies that the element is capable of binding active transcriptional repressor proteins, and suggests that pre-induction repressors of the β -IFN promoter can act through the PRD I element.

Positively-acting promoter elements

Upon induction of the β -IFN promoter the repression is relieved and a number of PRD sequence elements combine to stimulate expression. With the exception of PRD I and PRD III, the PRDs are not related in sequence, further suggesting that there is more than one downstream target for inducer-triggered cellular processes. The relative contribution of the various PRDs differs between cell lines, and in some cases a particular PRD is dispensable (Ellis and Goodbourn 1994). In isolation as single copies, none of the four identified PRDs can function as virus-inducible elements. However, they have been shown to be inducible, either in the context of the native β -IFN promoter (Figure 1.2), or in artificial multimeric constructs (Visvanathan and Goodbourn 1989, Fan and Maniatis 1989, Leblanc et al. 1990, Whiteside et al. 1992). In undifferentiated mouse embryonal carcinoma cells, in which the β -IFN promoter does not respond to inducers, all multimers of the individual PRD-elements remain uninducible (Ellis and Goodbourn 1994).

PRD I is a 13bp element GAGAAGTGAAAGT between -77 and -65, that is homologous to an ISRE, and indeed can act as an IFN-inducible element when multimerised (Fan and Maniatis 1989, King and Goodbourn 1994) (see Chapter Four). PRD III, located between -90 and -77, is highly homologous to PRD I at the DNA sequence level. PRD III appears necessary for efficient induction in HeLa cells (King and Goodbourn 1994) (see Chapter Four) and L cells (Fujita, Ohno et al. 1985, Fujita et al. 1987), but not in C127 cells.

The PRD II element lies between -64 and -55 of the β -IFN promoter. It seems that in most cell types PRD II activation is a key event in the induction process (Goodbourn and Maniatis 1988, Lenardo et al. 1989), Visvanathan and Goodbourn 1989) (see Chapter Six), although it has been demonstrated that β -IFN promoter constructs lacking PRD II sequences are inducible in differentiated mouse embryonal carcinoma cells (Ellis and Goodbourn 1994).

Sequences located between -104 and -87 comprise the PRD IV element, and are required for induction in L929 cells (Fujita et al. 1985), but not in C127 cells (Zinn et al. 1983). Multimers of the PRD IV element have been shown to be inducible in some situations (Du and Maniatis 1992), but not in others (Ellis and Goodbourn 1994, King and Goodbourn 1994) (see Chapter Four).

β -Interferon promoter binding proteins

Induction is thought to be brought about by a change in activity or availability of the DNA binding proteins that regulate the behaviour of the multiple elements within the β -IFN promoter. This is supported by an *in vivo* footprinting analysis, which indicated that the DNAase cleavage patterns before and after induction differ significantly (Zinn and Maniatis 1986). Several experimental approaches have been taken to identify these proteins, including electrophoretic mobility shift assay (EMSA). Complementary DNAs encoding these DNA binding activities have also been cloned by screening expression libraries. A large number of proteins that can bind to each of the four PRDs have been characterised, but in contrast, attempts to identify NRD I binding factors have not been successful. Some of the factors involved in the induction process may not be readily clonable in binding site screenings of bacterial libraries, since these factors may undergo certain post-translational modifications during the induction cycle that confer on them the ability to bind DNA. As discussed, the fact that in most cases the induction process does not require new protein synthesis clearly suggests that this may be the case. Also, the cDNAs encoding proteins that require dimerisation or oligomerisation for DNA binding would not be retrieved from such expression libraries. The factors known to bind to the β -IFN promoter will be discussed in connection with their respective DNA binding elements.

NRD I and NRD II binding factors

As mentioned earlier, the transcription of the β -IFN gene can be induced to some degree by treatment of cells with the protein synthesis inhibitor cycloheximide alone (Enoch and Maniatis 1986, Ringold et al. 1984), implying that the undetectable basal level is maintained by labile negatively regulatory factors, or by labile factors that modulate such transcriptional repressors. These repressor proteins are thought to act through specific *cis*-acting NRDs, and also through some of the PRDs. Induction is brought about by a change in the abundance and/or activities of the negatively and positively acting DNA binding proteins.

It has been shown that the ubiquitous Octamer binding protein Oct-1 can bind to NRD I (J. Eloranta, S. Goodbourn and K. Visvanathan, pers. comm.), although no convincing evidence for a negative regulatory role of Oct-1 in β -IFN expression has been shown, and two HeLa cell factors of molecular weights 95 and 100kDa have also been shown to bind to this region (Nourbakhsh, Hoffmann et al. 1993). Interestingly, a *Drosophila* protein DSP1 (Dorsal switch protein 1) can bind to NRD I

and inhibit NF- κ B-mediated activation through PRD II (see below) (Lehming et al. 1994). DSP1 was cloned in a yeast screen for Dorsal corepressor factors, and is homologous to a member of the High Mobility Group (HMG) of proteins. It should be emphasized, however, that no functional studies have indicated that DSP1 can decrease the level of transcription from the β -IFN promoter in uninduced cells, which should obviously be a necessary prerequisite for a preinduction repressor. Furthermore, NRD I can function as a negative regulatory element when isolated from the neighbouring PRD II (Nourbakhsh, Hoffmann et al. 1993), whereas the effect of DSP1 requires the native context. Also, DSP1 is a *Drosophila* protein, and no identification of functional mammalian homologues has been reported.

As is the situation with NRD I, no factors capable of negatively regulating the β -IFN promoter through the NRD II region have been reported, although Oct-1 (Du and Maniatis 1992) and two uncharacterised complexes, Un1 and Un2 (see below) have been shown to bind here (J. Eloranta, PhD. Thesis, London University, 1995). It should be noted that the 3' end of NRD II overlaps with the 5' end of the PRD IV element, thus the factors shown to bind PRD IV (see below) should also be considered candidate NRD II binding proteins.

In addition to the NRD elements, at least one of the PRD regions of the β -IFN promoter, namely PRD I, is involved in the pre-induction repression of transcription. As discussed above, besides being a virus-inducible element, PRD I can also negatively regulate the SV40 enhancer (Kuhl et al. 1987), implying that the PRD I element is capable of binding active transcriptional repressor proteins, which could also function as pre-induction repressors of the β -IFN promoter. The silencing effect is reversed upon virus-induction, implying that the constitutive PRD I-binding repressor proteins are displaced by positive regulators during the process. One such potential repressor is IRF-2 (see below). Two other putative PRD I-specific repressive DNA binding complexes, Un1 and Un2, have been identified (Whiteside et al. 1992, J. Eloranta, PhD. Thesis, London University, 1995).

PRD I and PRD III binding factors

PRD I has been shown to bind the transcription factors IRF-1 and IRF-2. The mRNAs for both of these are expressed at low levels in unstimulated cells, but are strongly inducible by viral infection or IFN-stimulation (Harada, Fujita et al. 1989). The IRF-1 cDNA was isolated from a λ gt11 library with a DNA probe containing multiple copies of a PRD I derivative (Miyamoto et al. 1988). IRF-2 was subsequently isolated by cross-hybridization with an IRF-1 probe (Harada et al. 1989), by virtue of

the fact that the amino termini of the IRF factors are homologous (62% identity). It seems likely that IRF-1 and IRF-2 loci are derived from a single ancestral gene as a result of gene duplication, and subsequent sequence divergence. The amino-terminal regions of the IRFs are responsible for DNA binding, and other members of the family, with similar DNA-binding sequence specificity, have since been identified (see (Bluyssen et al. 1996) for review). In keeping with the high degree of homology between the amino-termini of the two IRFs, the interaction of IRF-1 and IRF-2 with DNA exhibits virtually identical sequence preference (Tanaka et al. 1993). They apparently compete for the same *cis*-acting sequences, but mediate different effects.

IRF-1 is a positive regulator of β -IFN transcription, as evidenced by experiments with a human fibroblast cell line constitutively overexpressing IRF-1 mRNA in either sense or antisense orientation (Reis et al. 1992). Upon induction with dsRNA or NDV, cells harbouring the sense IRF-1 message produced more β -IFN mRNA than the control cells, whereas cells expressing the antisense IRF-1 mRNA produced neither β -IFN mRNA nor protein. High-level expression of the IRF-1 cDNA in transfected monkey COS cells results in detectable, albeit very low, induction of the endogenous β -IFN, as well as α -IFN, genes (Fujita et al. 1989), although this was not observed in GM-637 cells (Reis et al. 1992). While it is clear that IRF-1 can act as a positive regulator of the β -IFN promoter, it cannot be the primary, or essential, activator of the β -IFN gene in all cell types because it has been shown that both the intact β -IFN promoter (Pine et al. 1990) and multimers of the IRF-1 binding site, PRD I (Whiteside et al. 1992), are inducible under conditions in which IRF-1 is not detectable. Furthermore, both α - and β -IFN genes are inducible by virus in murine embryonic fibroblasts (MEFs) and embryonal stem (ES) cells, in which both IRF-1 alleles have been disrupted (Matsuyama et al. 1993), (Ruffner et al. 1993, Reis et al. 1994). Interestingly, it appears that in MEFs the β -IFN gene is uninducible by dsRNA unless the cells are primed (Matsuyama, Kimura et al. 1993).

The transcriptional activation domain of IRF-1 lies in its carboxy-terminus (Fujita et al. 1989), within which a region rich in serines and threonines can be found, although there is no evidence for a change in the levels of phosphorylation of this factor upon induction. The carboxy-terminus of IRF-2 shows only little homology (25%) with that of IRF-1; thus IRF-2 lacks the activation domain of IRF-1, although an artificial truncation of mouse IRF-2 C-terminal final 59 amino acids revealed a strong transactivation domain that is presumably silenced by the C-terminus (Yamamoto et al. 1994). Interestingly, it has been reported that IRF-2 can

activate transcription of the histone H4 gene FO108, although generally it appears to act as a transcriptional repressor, and as such negatively regulates the multimerised PRD I-like AAGTGA sequence (Harada et al. 1989), and, in embryonal carcinoma cells, inhibits the activation of α - and β -IFN promoters mediated by IRF-1 (Harada et al. 1990). Since overexpression of IRF-2 can thus block the transactivation effects of IRF-1, a model was proposed that IRF-2 could function as the pre-induction repressor of PRD I activity, and that induction would somehow bring about a change in the relative activities or abundances of IRF-1 and -2. However, a targeted disruption of IRF-2 failed to generate detectable β -IFN expression in uninduced MEFs derived from the knockout mice (Matsuyama et al. 1993).

IRF-2 is proteolytically processed during induction to leave an amino-terminal fragment missing approximately 185 amino acids, but still capable of binding DNA (Cohen and Hiscott 1992, Palombella and Maniatis 1992, Whiteside et al. 1992, Whiteside et al. 1994). It appears that the truncation product has a higher affinity for a specific DNA binding site, and is also a more potent repressor of the β -IFN promoter than the full-length precursor (Whiteside et al. 1994). Furthermore, the kinetics of production of the cleavage product lag behind that of the activation of β -IFN transcription. It has thus been proposed that the truncated IRF-2 is a postinduction repressor generated by a proteolytic event upon induction. Indeed, supporting this, the only detectable effect of IRF-2 knock out is an inefficient turn-off of expression following induction (Matsuyama et al. 1993).

Another expression library screen using a PRD I probe isolated a cDNA clone encoding a novel PRD I-binding protein, PRD I-BFI, containing five zinc fingers (Keller and Maniatis 1991). The PRD I-BFI gene is inducible by virus, and the peak in PRD I-BFI mRNA levels follows that of the β -IFN mRNA, suggesting that PRD I-BFI functions as a post-induction repressor of the β -IFN promoter. In agreement with this, overexpression of PRD I-BFI can block viral induction of either the intact β -IFN promoter or PRD I multimers.

In accordance with the fact that the sequence of PRD III is similar to PRD I, it binds an overlapping set of transcription factors. It is possible that in cells which require the PRD III element for efficient activation of the β -IFN promoter, this second binding site may allow co-operative binding of a positively-acting factor that may be present at insufficient levels to activate a promoter containing only one binding site.

As mentioned above, PRD I shows considerable homology to the ISRE:

GAGAAGTGAAAGT: PRD I

GGAAACCGAAACT: ISG15 ISRE

A/GGGAAANNGAAACT: consensus ISRE

and as a result of this PRD I can bind ISGF3 γ , the DNA binding factor of the IFN-activated transcription factor ISGF3 (see above) (Kawakami et al. 1995) Chapters 5 and 6). ISGF3 γ is a member of the IRF family of DNA-binding proteins, with a homologous DNA-binding domain but no transactivation domain (for a review see (Bluyssen et al. 1996)). Unlike the other members of the family, ISGF3 γ binds ISRE sequences more avidly than PRD I, due in part to a preference for a C at the penultimate position (Veals et al. 1993). Although PRD I cannot bind ISGF3, an extended element with the inclusion of sequences 5' to PRD I can bind this factor (S. Goodbourn, pers. comm., Yoneyama et al. 1996). It is thus possible that, even though IFN treatment cannot induce β -IFN expression, the induction achieved by dsRNA or virus may be augmented by IFN in an autocrine manner through the PRD I/PRD III region (Yoneyama et al. 1996) Chapter Three).

It has also been shown that Oct-1 can bind PRD I (S. Goodbourn, pers. comm., J. Eloranta, PhD. Thesis, London University, 1995).

PRD II binding factors

The sequence GGGAAATTCC between -64 and -55 of the β -IFN promoter represents a consensus motif for the binding of the transcription factor NF- κ B (Visvanathan and Goodbourn 1989, Fujita et al. 1989, Lenardo et al. 1989), a transcription factor originally identified as a regulator of immunoglobulin κ light chain transcription, and shown to play a central role in the regulated expression of a number of immune and inflammatory response genes (reviewed in (Baeuerle and Henkel 1994)). Point mutations that abolish the binding of a classical NF- κ B-dimer p65/p50 to PRD II result in considerable reduction in virus-induced expression (Goodbourn and Maniatis 1988, Thanos and Maniatis 1995). NF- κ B makes contacts with the major groove at the GC-rich ends of the κ B site in the β -IFN promoter, while another protein, HMG I(Y), binds to the minor groove in the central (A+T)-rich region (Thanos and Maniatis 1992).

NF- κ B is sequestered in the cytoplasm by association with an inhibitor termed I κ B, which masks the nuclear localisation sequences of the Rel subunits (reviewed in (Gilmore 1996)). NF- κ B is liberated from the inhibitor by dsRNA, but also by a variety of other inducers, such as TPA, IL-1, TNF, cAMP, bacterial lipopolysaccharides, viral transactivators, and reactive oxygen intermediates.

Nevertheless, the pathways by which dsRNA or TPA activate NF- κ B do not appear to be identical, since in mouse embryonal carcinoma cells, the former cannot induce NF- κ B binding to DNA while the latter can do so (Ellis and Goodbourn 1994).

The mammalian NF- κ B activity can consist of homodimers or heterodimers of the subunits belonging to the Rel family, including p50 (NF- κ B-1), p65 (RelA), c-Rel, p49, and RelB (Thanos and Maniatis 1995). All these proteins share a conserved 300 amino acid domain (Rel homology domain) required for DNA binding, dimerization, I κ B binding and nuclear localisation. Both the precise preferred DNA sequence for various dimers, and the regulatory consequences of binding particular forms of NF- κ B differ from each other. Several cDNA clones have been isolated which encode proteins with I κ B-like properties; these include MAD3 (I κ B α), and the product of the bcl-3 proto-oncogene (Gilmore 1996). Different I κ B activities have different targets for the inhibition: MAD3 prevents both the nuclear uptake and the DNA binding of p65 or c-Rel -containing NF- κ B-dimers, whereas Bcl-3 inhibits the DNA binding of the p50 homodimers. The NF- κ B subunits p50 and p49 are derived from the precursor proteins p105 and p97, respectively, through proteolytic processing. The carboxy-terminal regions of both of these precursors share a conserved putative protein-protein interaction domain, called the ankyrin repeat, with the I κ B proteins, and indeed, p105 exhibits an I κ B-like activity in that its *trans*-inhibitory carboxy-terminus can block the nuclear localisation or DNA binding of NF- κ B.

According to the current model (see (Baldwin 1996) for review) for the activation of NF- κ B, both the inhibitor MAD3 and the p50 precursor protein p105 become specifically phosphorylated upon induction with dsRNA, or the other inducers. The phosphorylation of MAD3 on serine residues 32 and 36 (Brockman et al. 1995, Brown et al. 1995, Traenckner et al. 1995) marks it for its subsequent degradation by the ubiquitin-mediated proteasome pathway (Chen et al. 1995), and releases NF- κ B, which translocates to the nucleus to associate with κ B response elements. Many kinases (e.g. PKC ζ and PKA) are capable of activating NF- κ B-I κ B complexes in cell free systems, however these are not capable of phosphorylating serines 32 and 36 of MAD3. A novel, ubiquitin- and TNF- α -activated kinase has been identified that can perform this role has been termed I κ B α kinase (Chen et al. 1996).

The demonstration that the dsRNA-activated kinase PKR is also capable of phosphorylating I κ B (although the site(s) of phosphorylation have not been determined (Lee et al. 1997), and activating NF- κ B *in vitro* (Kumar et al. 1994,

Offerman et al. 1995), has tempted further speculation that PKR is the intracellular receptor of dsRNA, and that it exerts its effects on β -IFN induction via NF- κ B activation. However, antisense studies in primed U-937 cells suggested that in the absence of PKR, β -IFN induction was still efficiently achieved by dsRNA (Der and Lau 1995), and more compellingly, both NF- κ B activation and β -IFN induction are observed in dsRNA-treated primed MEFs derived from PKR "knockout" mice (Yang et al. 1995).

PRD IV binding factors

The PRD IV element contains a binding site for the ATF/CREB family of bZip transcription factors, and it has been suggested that ATF-2 mediates the virus-inducibility through PRD IV, either as homodimers or heterodimers with c-Jun (Du and Maniatis 1992, Du et al. 1993). Accordingly, the overexpression of either ATF-2 or c-Jun antisense RNA in transfected HeLa cells decreases the inducibility of the native human β -IFN promoter. Nucleotide substitutions that interfere with *in vitro* binding of the ATF factors to PRD IV decrease the level of virus induction in mouse L929 cells, and multiple copies of PRD IV have been reported to confer inducibility by both virus and cAMP treatment on a cotransfected heterologous promoter. Whilst the entire PRD IV element appears to be required for viral induction, the flanking 5' and 3' A+T rich regions are dispensable for stimulation by cAMP. This is consistent with the suggestion that these A+T rich sequences interact with HMG I(Y) proteins, and that these protein-DNA interactions are necessary for viral induction (see below).

Contribution of HMG I(Y) proteins to induction

As previously mentioned, the HMG (I)Y protein has been reported to be required for the transcriptional activities of both NF- κ B (Thanos and Maniatis 1992, Thanos and Maniatis 1995) and ATF-2 (Du et al. 1993) in the context of the β -IFN promoter. HMG I(Y) is a basic, low-molecular weight protein that binds to double-stranded DNA with a limited sequence specificity for A tracts (reviewed in Bustin et al 1990). The mechanisms by which HMG I(Y) may act on PRD II and IV appear rather similar. HMG I(Y) makes minor groove contacts with both the central (A+T)-rich region of PRD II and the two (A+T)-rich regions flanking PRD IV (Thanos and Maniatis 1992) (Du, Thanos et al. 1993). It appears that the degree of HMG I(Y) binding to any of these sites correlates with the extent of the virus induction of the β -IFN gene, and that the overexpression of antisense HMG I(Y) RNA can block the

virus induction.

The mechanism of action of the HMG I(Y) protein appears to involve the bending of DNA upon its binding, which then leads to the enhancement of binding of both NF- κ B and ATF-2 to their respective binding sites, as well as to the physical interaction between the DNA-bound NF- κ B and ATF-2 proteins (Falvo et al, 1995, Thanos and Maniatis 1995). Furthermore, even in the absence of DNA, HMG I(Y) can interact directly with both NF- κ B and ATF-2, and as it has been shown that NF- κ B and members of the bZip family, including ATF-2, can interact in solution (Nolan 1994), this could extend the opportunity for co-operative binding still further. Also, in a cell-free assay in the presence of high concentrations of specific proteins, the association between HMG I(Y) and a particular splicing isoform of ATF-2 in solution triggers an equilibrium shift towards dimerization of this ATF-2 variant (Du and Maniatis 1994), which could contribute to the observed stimulation of the ATF-2 binding activity.

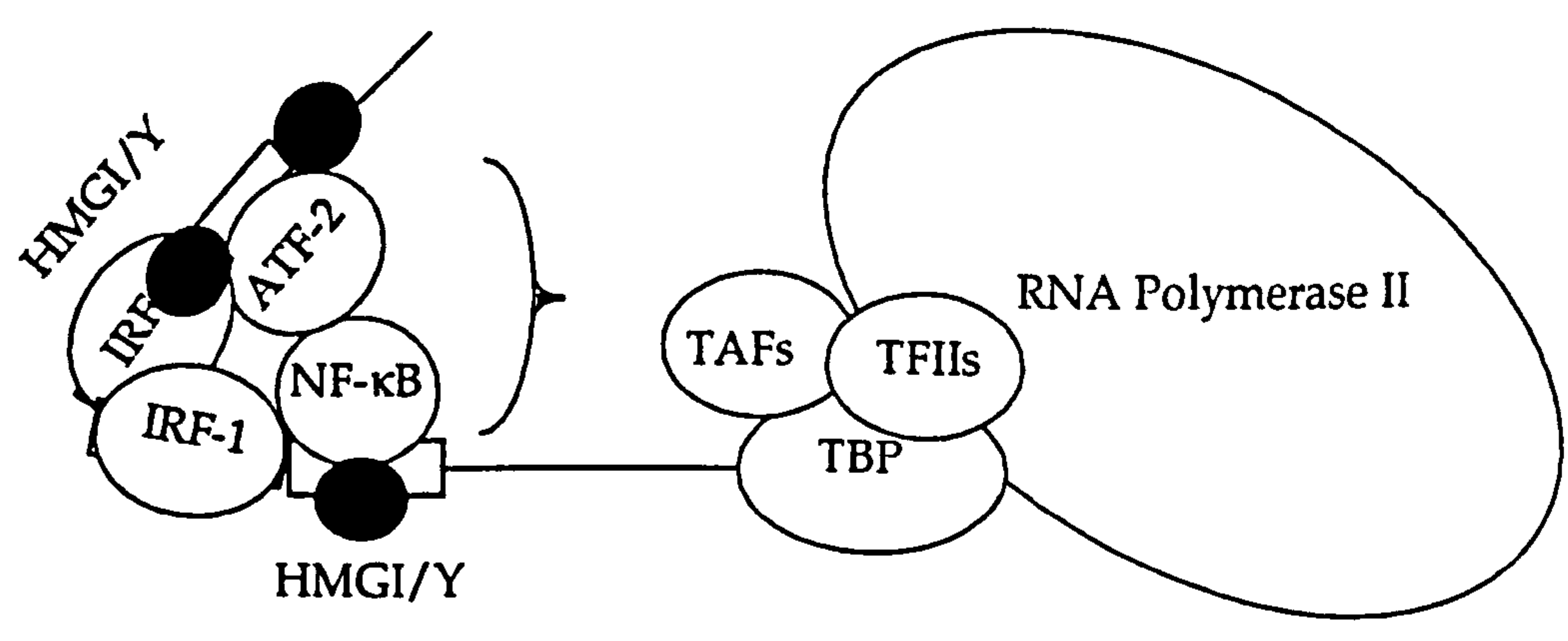
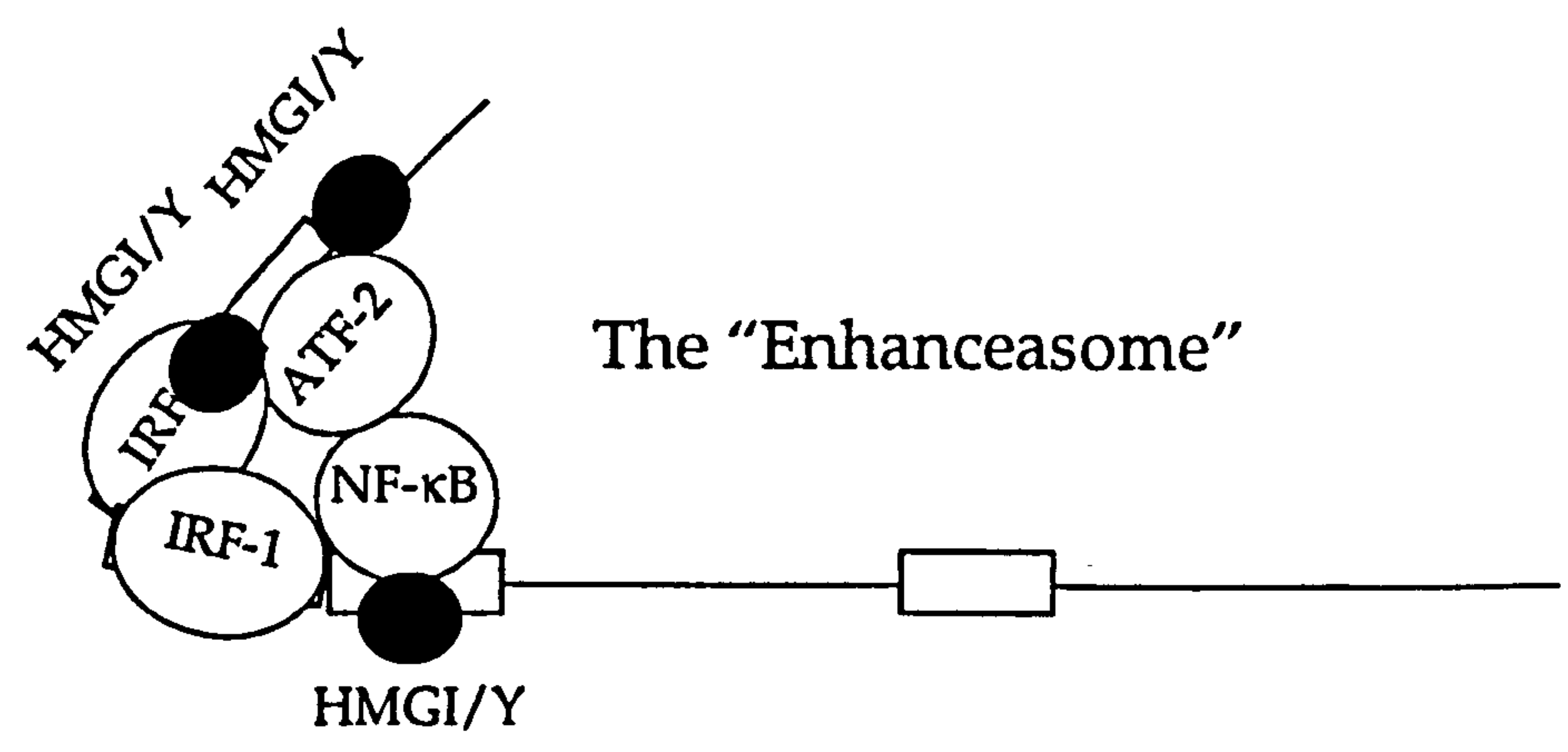
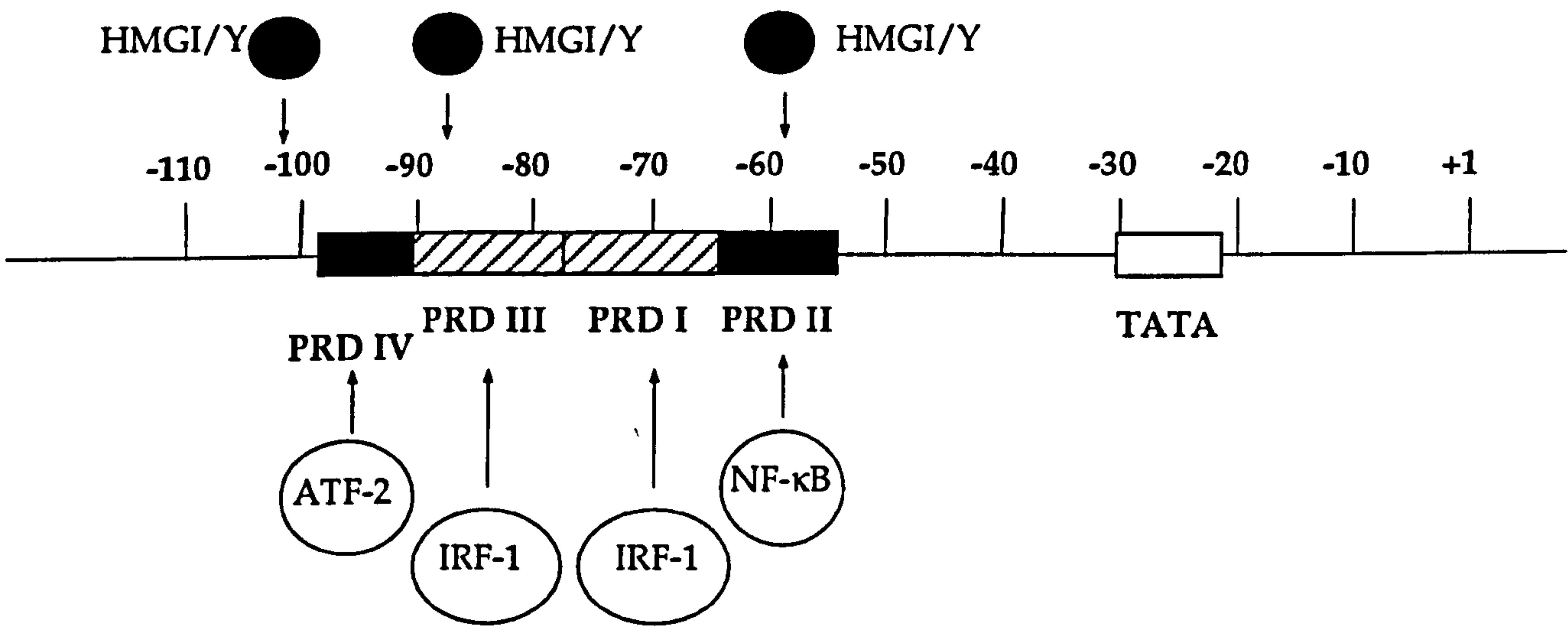
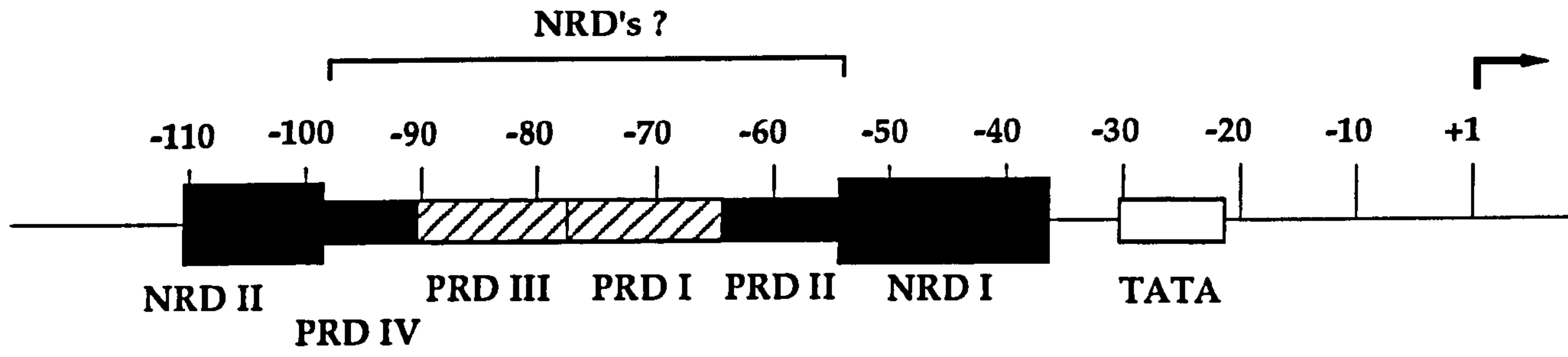
In conclusion, the function of HMG I(Y) may be to contribute to the assembly of an inducible multiprotein complex on the β -IFN promoter, referred to as an enhanceosome (Thanos and Maniatis 1995) (see Figure 1.3), by facilitating both protein-DNA and protein-protein interactions. This may serve to allow productive activation by NF- κ B and ATF-2 through relatively weak binding sites, which individually would be inefficiently activated by their respective inducers, for example TNF and cAMP. Interestingly, a similar role for HMG I(Y) has been demonstrated in the regulation of the E-selectin promoter by TNF α . This promoter contains multiple NF- κ B sites and a binding site for ATF-2, and the binding of these factors is enhanced in vitro by HMG I(Y), which binds specifically to the A-T-rich regions of the NF- κ B sites and 3' of the ATF-2 site (Whitley et al. 1994).

Post-induction repression

The post-induction repression of the β -IFN promoter also appears to occur at the level of transcription, and requires yet another distinct set of transcription factors. In mouse C127 cells, the postinduction shut-off is caused, in part, by changes in the rate of transcription initiation (Whittemore and Maniatis 1990a, b). In transfection analyses, the virus-inducible IRE (Interferon gene Regulatory Element) region from the β -IFN promoter, that contains the elements PRD I, PRD II, and NRD I, can be appropriately turned off when fused to a heterologous gene, thus this region must contain the regulatory elements involved in post-induction repression. NRD I is not a likely candidate for the post-induction repressor binding site, since

Figure 1.3 The enhanceosome

Induction of the β -IFN promoter by dsRNA has been proposed to occur through the assembly of a multi-protein complex, mediated by HMG I(Y), which can lead to bending of the promoter and the further accessibility of PRDs I and III for IRF-1 binding. The resultant functional transcriptional unit is called the enhanceosome. See text for details.



the kinetics of the induction cycle are not affected by a deletion that removes most of the NRD I, and therefore the factors involved in pre- and post-induction repression appear to be distinct. Interestingly, virus-inducible multimers of either PRD I or PRD II exhibit the characteristic post-induction repression, indicating that both elements can serve as binding sites for regulatory proteins capable of mediating the effect. Two PRD I-binding factors, IRF-2 and PRD I-BFI, have been proposed to mediate post-induction shutoff (see earlier).

Although the β -IFN gene is turned off at the transcriptional level, rapid degradation of the transcript also contributes to the post-induction decrease in β -IFN mRNA (Whittemore and Maniatis 1990a). Analysis of fusion gene constructs in stably transfected mouse C127 cells indicates that there are two distinct destabilisers in the β -IFN transcript. One destabiliser is located in the 3' untranslated region and is similar to the AU-rich motif found in many other highly inducible mRNAs with short half-lives, such as the c-myc, c-fos, and GM-CSF mRNAs. The other destabilizer within the β -IFN mRNA is located 5' to the translation stop codon and does not resemble AU-rich destabilisers. Rapid decay of β -IFN transcripts occurs constantly, and is not regulated during the induction cycle.

The post-induction shut-off in expression can be delayed in the presence of cycloheximide, so that the rate of transcription remains high for up to 24 hours after induction (Whittemore and Maniatis 1990b), suggesting the existence of a mechanism dependent, in part, on protein synthesis.

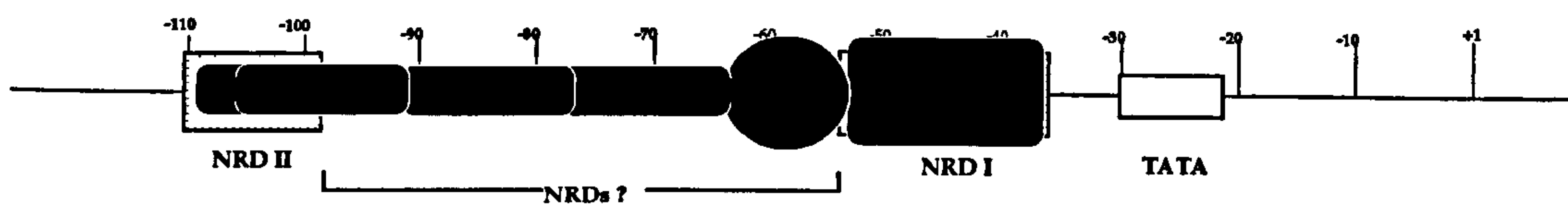
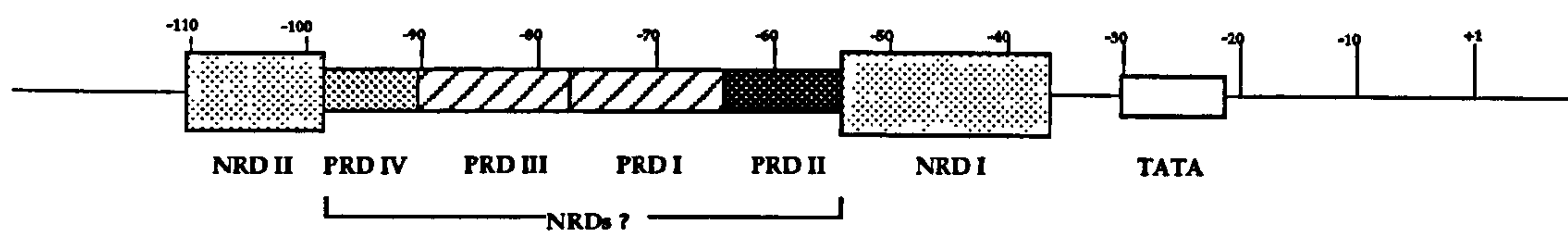
The complexity of the β -interferon promoter

As outlined above, the correct transcriptional regulation of the β -IFN promoter requires the co-ordinated function of regulatory transcription factors acting through multiple response elements. The changes in the DNA binding activities at all the stages of the induction process are summarised in Figure 1.4. The ultimate aim in understanding the transcriptional regulation of the β -IFN promoter is to determine the roles of individual transcription factors during the different stages of the induction cycle. It seems likely that the promoter is organised into distinct, stage-specific, complex, nucleoprotein structures, but despite years of investigation by several groups, it still seems a formidable challenge to elucidate all the protein-DNA and protein-protein interactions mediating these changes in promoter architecture.

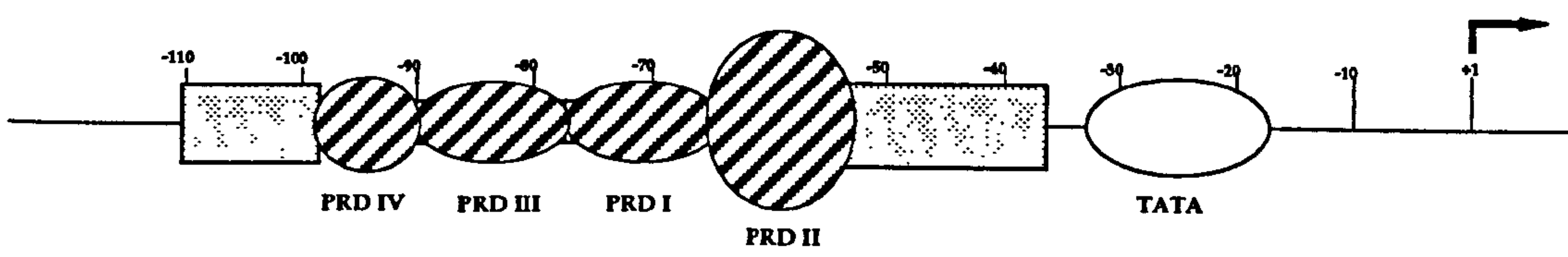
An induction response that requires multiple events is likely to serve to prevent fortuitous transcriptional activation of a gene encoding such a potent

Figure 1.4 The induction cycle of the β -IFN promoter

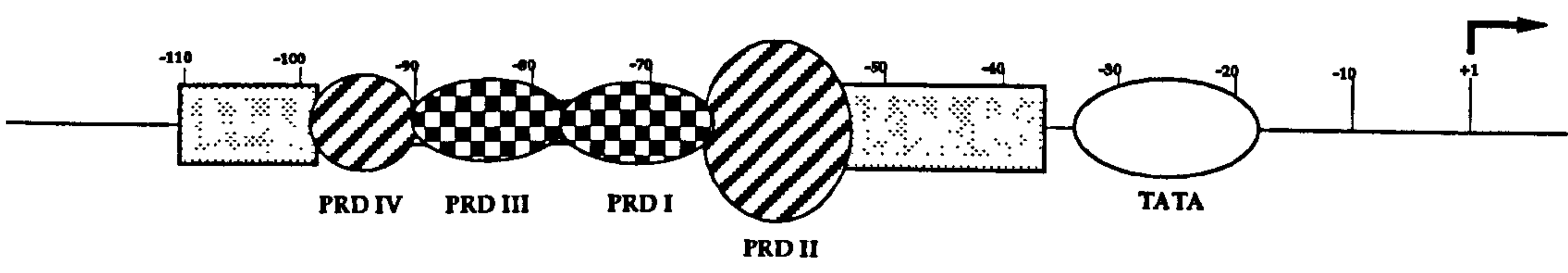
DNA binding proteins with different properties bind to the promoter at defined stages of induction. See text for details.



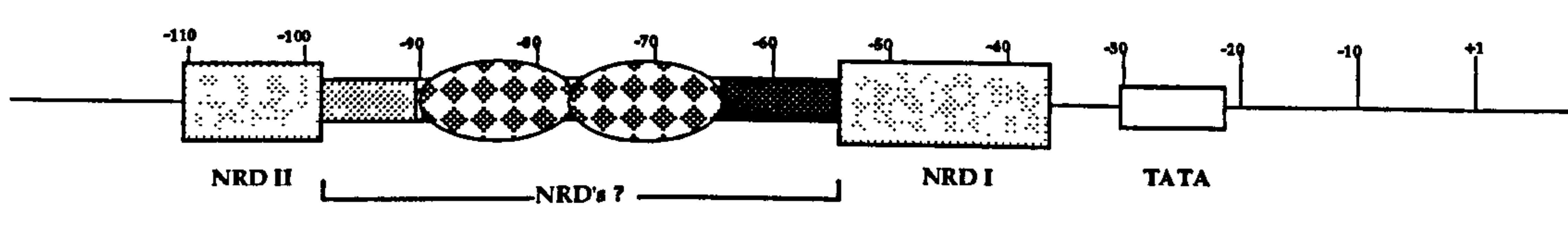
Uninduced



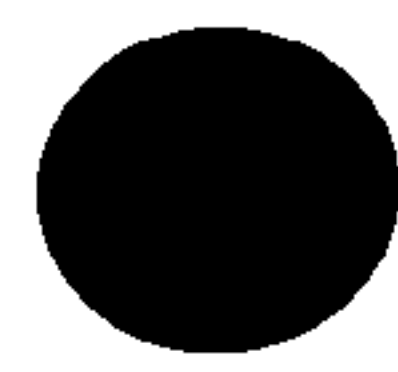

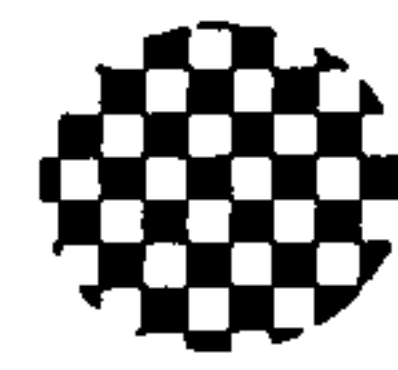
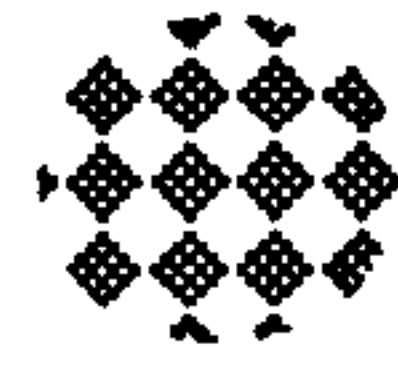
Early induction



Late induction



Post Induction

-  Pre-induction repressors
-  Primary activators
-  Secondary activators
-  Post-induction repressors

cytostatic protein. This becomes apparent when one considers the possibility that one or more of the regulatory elements may be affected by other signal transduction pathways. Indeed, in addition to dsRNA, NF- κ B can be activated by a number of cellular signals (see earlier), none of which appears to induce the β -IFN gene (Lenardo et al. 1989, Lacoste et al. 1990, Watanabe et al. 1991). Also, as discussed above, PRD I is related to an ISRE, which is sufficient to mediate induction of a heterologous gene by IFNs, and both the reiterated AAGTGA sequence, a PRD I-like element, and multimerised PRD I, have the same property. However, it is important to bear in mind that the intact β -IFN promoter cannot be induced by IFNs. This does not exclude the possibility that the induction can be enhanced by IFNs after the primary signal, for example dsRNA, has initiated the necessary events for induction. It is possible that the β -IFN produced from the gene itself further contributes to the induction in a positively autoregulatory manner (Yoneyama et al. 1996) (see Chapter Six). Nevertheless, the production of IFN cannot be obligatory, since protein synthesis is not required for induction (see above). Furthermore, it has been demonstrated that a transfected β -IFN gene is inducible when introduced into a cell line that lacks its own type I genes (Mosca and Pitha 1986).

Although many of the promoter-binding factors have been identified, it is still not clear how virus infection or dsRNA treatment bring about the described changes in the activity of these factors. The activation of NF- κ B binding is the best understood event in the induction of the β -IFN gene, but it is clear that this can be brought about in the absence of PKR, the only obvious candidate for a transducer of the signal provided by dsRNA, as can the induction of the β -IFN gene itself. The modulation of the activities of the PRD I binding factors IRF-1 and -2 remains unexplained and the roles of the factors HMG I(Y) and ATF-2 are controversial, and no progress has been made in understanding the molecular basis of the pre-induction repression. Clearly, these are important avenues of research.

Priming

As mentioned above, induction can occur in most cell types without the requirement for *de novo* protein synthesis, indicating that all the factors necessary for induction pre-exist in these cells in some form (Cavalieri, Havell et al. 1977), and that the entire induction cycle can take place with merely the addition of a dsRNA trigger. In fact, simultaneous treatment of dsRNA-induced cells with inhibitors of protein synthesis, such as cycloheximide, causes an enhancement in the degree of β -IFN induction, a phenomenon referred to as superinduction (Havell and Vilcek

1972).

However, in many cultured cell lines the extent of β -IFN induction can be enhanced by pre-treating the cells with type I IFN before induction, a phenomenon known as priming (Stewart et al. 1971). Some cell lines are readily inducible by dsRNA and respond only slightly to priming, whereas other cell lines are largely uninducible in the absence of priming, whereupon they can be induced very efficiently (Enoch and Maniatis 1986). It has been shown that γ -IFN, as well as type I IFN, can prime cells, although not as efficiently (Toth et al. 1985), and that α -IFN genes and some other cytokine genes are also more readily induced by dsRNA following priming (Rosztoczy et al. 1992, Rosztoczy and Pitha 1993). The required length of exposure to IFN for priming has been reported to vary between 2 and 24 hours, and one study has reported that the primed state is very long lived, lasting up to eighteen days following withdrawal of the IFN, and surviving up to three cell divisions (Chen 1985). Although IFN pre-treatment can enhance the induction of β -IFN following virus or dsRNA treatment, it should be stressed that this IFN treatment itself cannot induce β -IFN expression.

Early studies indicated that MM virus induction of β -IFN expression in mouse L cells could only take place if the cells were primed, and it was observed that the magnitude of the effect was dose-dependent, and that at high doses IFN could accelerate the kinetics of induction. This has also been suggested by others (Abreu et al. 1979, Content et al. 1980, Fujita and Kohno 1981). By run-on transcription assays, priming has been shown to operate at the level of transcription (Nir et al. 1985). An early attempt to localise the priming effect to a specific sequence element within the β -IFN promoter indicated that the region containing PRD III and PRD I is two-fold responsive to priming, although other regions were not investigated (Dron et al. 1990), but a more in depth analysis has shown that the priming requirement cannot be localised to a particular promoter element, and is correlative with the inducibility of a particular element (King and Goodbourn 1994) (see Chapter Four). This also appears to be the case for Sendai virus-induction of the murine α 4-IFN promoter (Rosztoczy and Pitha 1993).

The exact nature of the priming phenomenon is unclear. Studies to investigate the protein synthesis requirement of priming have generated conflicting data (Stewart et al. 1971, Fujita and Kohno 1981, Enoch and Maniatis 1986) and may be obscured by the capability of protein synthesis inhibitors such as cycloheximide not only to superinduce the β -IFN gene, but also to cause a slight induction of the gene in the absence of dsRNA or virus (Enoch and Maniatis 1986, Ringold et al.

1984). However, priming generally takes a long time to establish, and this observation, together with the finding that a priming-dependent cell can be induced in the absence of IFN pre-treatment by fusing it with a priming-independent heterologous cell (Enoch and Maniatis 1986), suggests that priming provides an IFN-inducible factor(s) required for dsRNA induction that is constitutively present in priming-independent cells. It is formally possible that the requirement for priming is a consequence of the inability of unprimed cells to take up the inducer, although the presumably different methods of entry into the cell of dsRNA and viruses, and the unimpaired nature of some dsRNA-inducible events (Dron et al. 1990), argue against this. As mentioned earlier, in some cases virus infection is capable of inducing the β -IFN gene in unprimed cells whereas dsRNA cannot (Matsuyama et al. 1993, King and Goodbourn 1994) (see Chapter Three), suggesting that the two inducers are not equivalent.

As well as being poorly understood at the molecular level, the physiological basis for priming is also unclear. Viral infection of tissue results in the production of IFN from infected cells which is secreted and can prime uninfected neighbouring cells (Figure 1.5). It is likely that if the IFN produced in the first wave of infection, from the readily inducible cells, is insufficient to set up the antiviral state in the infected cells and halt the infection at this stage, subsequent infection by newly synthesised virus particles or a new wave of infection would elicit a greater IFN induction in the now primed, readily inducible cells, and a secondary wave of IFN induction from the previously poorly inducible cells. In this way it may be possible to rapidly limit the extent of viral infection with the minimum amount of IFN induction, thereby protecting the tissue from unnecessary damage caused by the cytostatic and immunomodulatory activities of IFN.

Heterogeneous expression

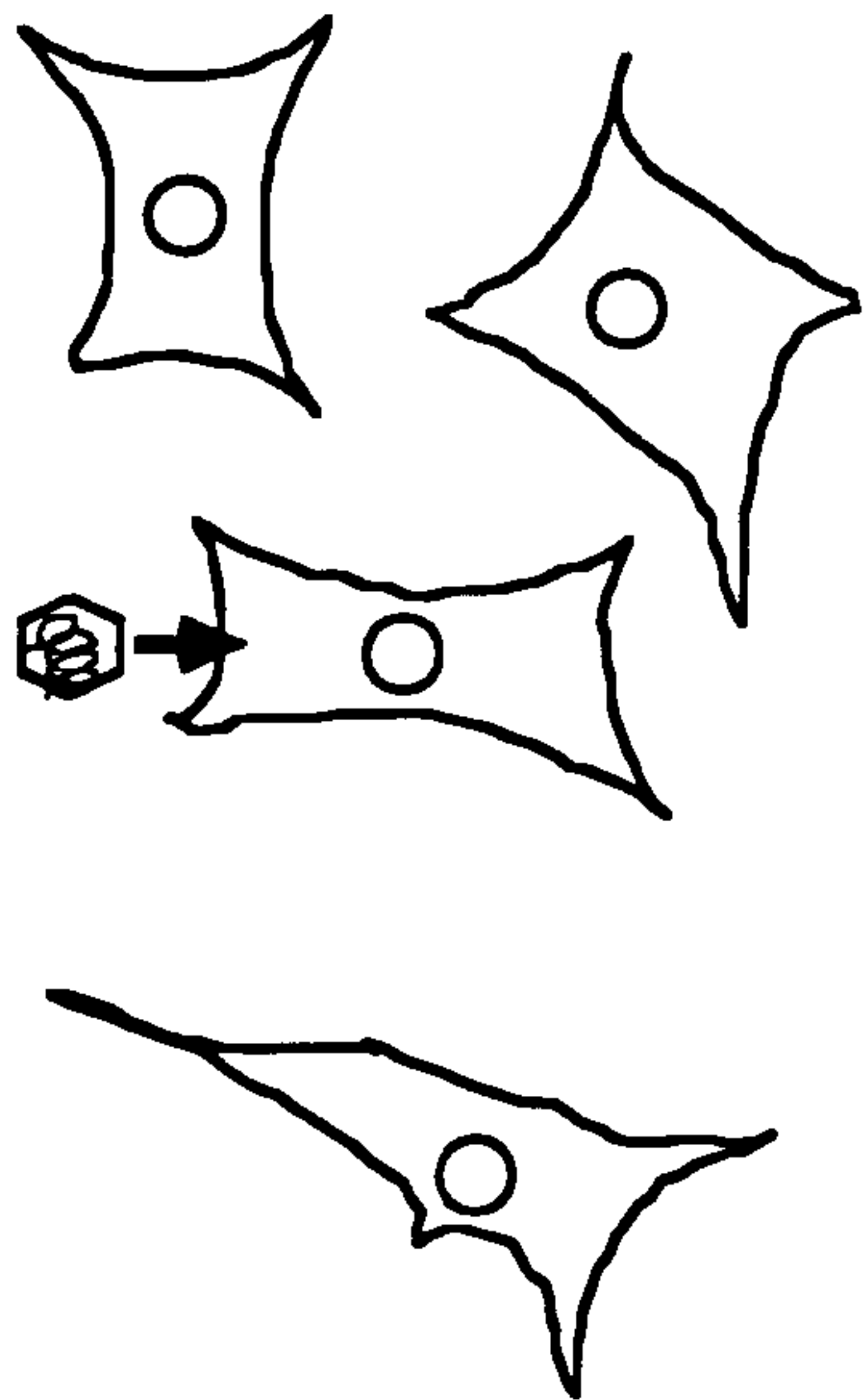
It should be noted that cells respond to the β -IFN inducers in a heterogeneous manner, so that only approximately 10% of cells are stimulated to produce β -IFN mRNA (Enoch and Maniatis 1986). If this percentage is factored into the calculation of the number of β -IFN transcripts induced following the treatment of a plate of cells with dsRNA, it would appear that up to half the newly synthesised message in an induced cell is β -IFN-specific. The molecular basis for this heterogeneity in cellular response is not known, but it does not appear to reflect the ability of cells to respond to an inducer at only certain stages of the cell cycle (S. Goodbourn and T. Enoch, pers. comm.).

Figure 1.5 The role of priming

See text for details.

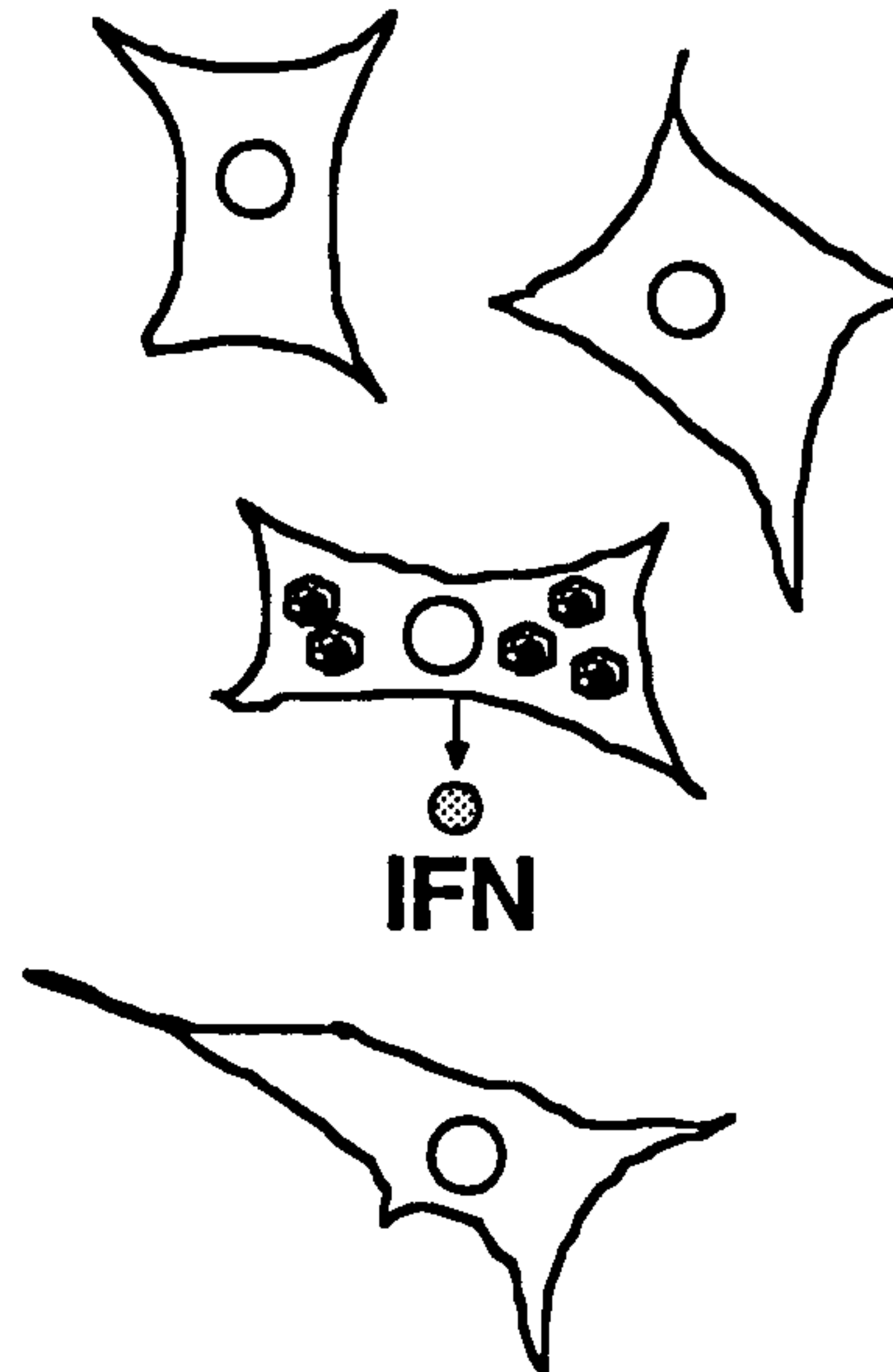
1

All unprotected - virus susceptible



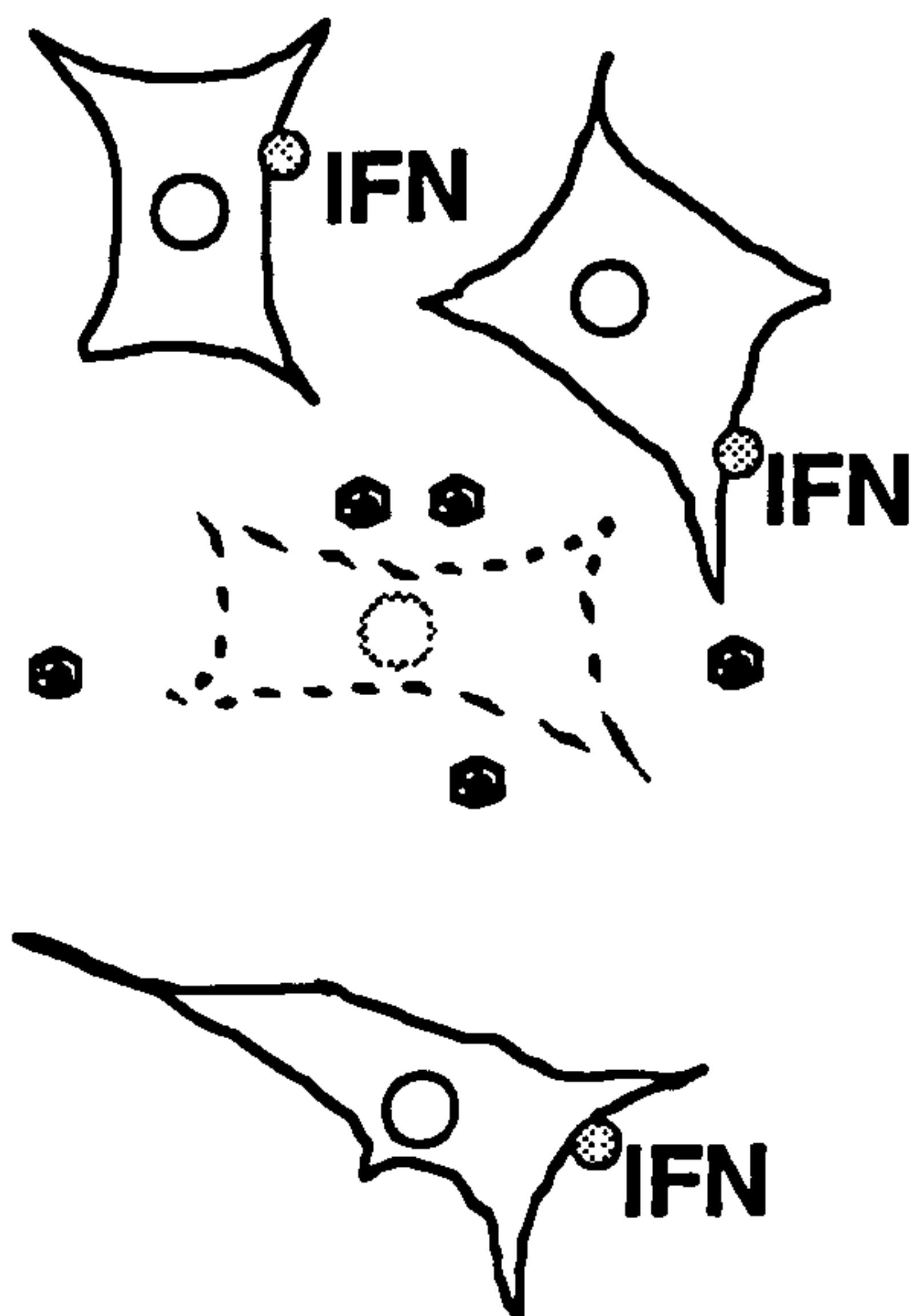
2

Viral replication and IFN induction



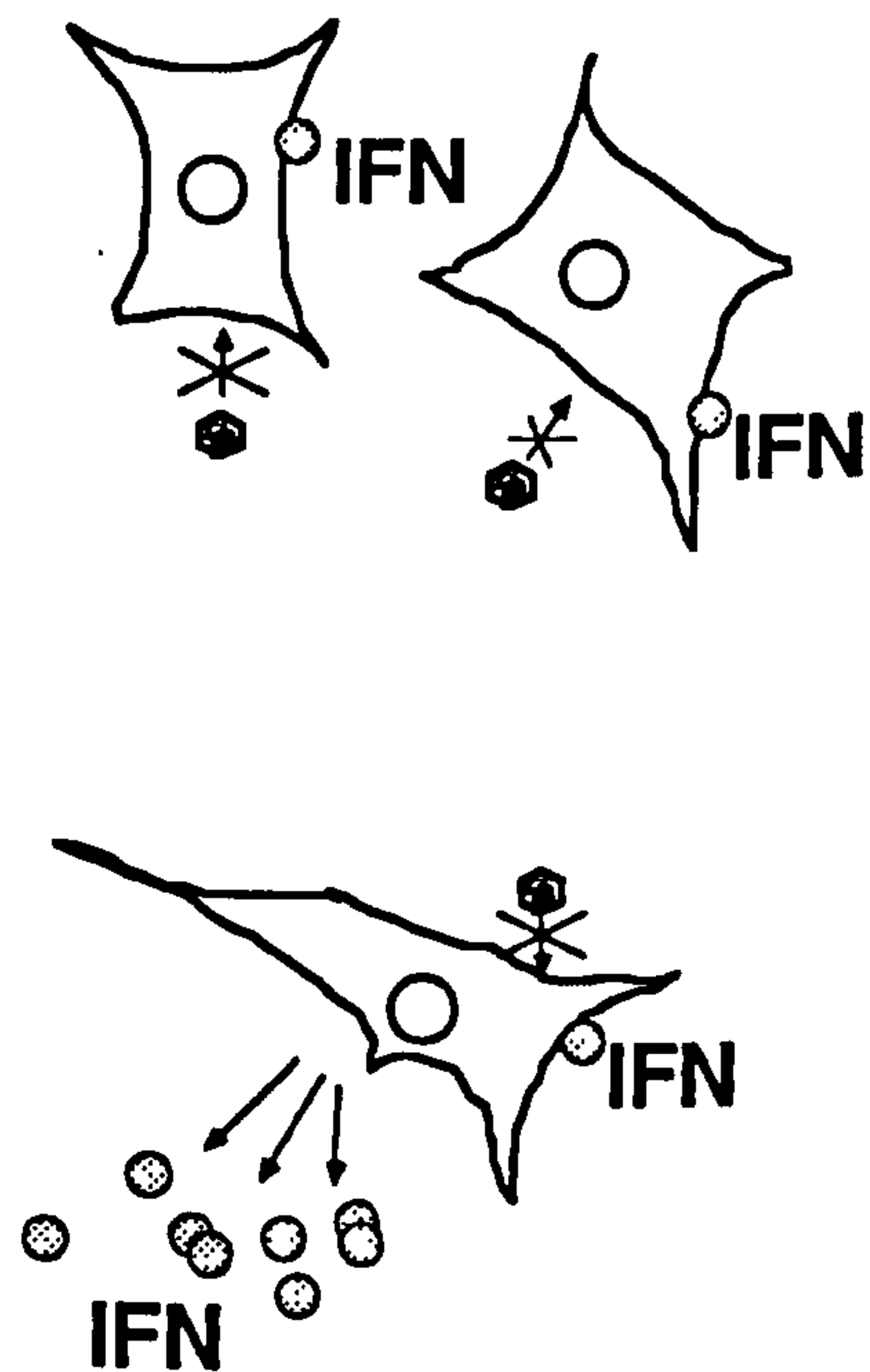
3

Virus release/ IFN action on uninfected cells



4

Interferon elicits anti-viral response - Cells resist infection, and are primed



Transcriptional Regulation of α -IFN Genes

The transcriptional regulation of the α -IFN genes has not been as well characterised as the regulation of the β -IFN gene. Even though α -IFN promoters share some homology with the β -IFN promoter, they lack NF- κ B binding sites, and their more tissue-restricted expression also suggests that they are regulated differently (MacDonald et al. 1990). Virus-inducible elements have been identified in the human α 1-IFN (Ryals et al. 1985) and murine α 4- (Raj et al. 1989) and α 11-IFN genes (Genin et al. 1995). These have IRF binding sites and potential ATF/CREB sites, as well as sites for factors not found binding to the β -IFN promoter, including AF-1 (Raj et al. 1991) and TG protein (MacDonald et al. 1990, Naf et al. 1991), Genin et al. 1995). As with β -IFN induction, the virus-inducibility of the α -IFN genes was not affected by the disruption of the IRF-1 gene in knockout cells (Matsuyama et al. 1993), (Ruffner et al. 1993).

Induction of Other Genes by dsRNA

Type I IFN-inducible genes

Several type I IFN-inducible genes are also directly inducible by dsRNA (Tiwari et al. 1987, Wathelet et al. 1986, Tiwari et al. 1988). In the case of the 561 gene, this induction has been shown to occur through the ISRE, because mutations in this sequence that abolish α -IFN induction also abolish dsRNA induction (Bandyopadhyay et al. 1995), and it has been shown that antisense IRF-1 blocks the induction, and that functional STAT1 α , but not β , can restore inducibility in STAT1-negative cells (Bandyopadhyay et al. 1995), but that other ISGF3 components do not seem to be involved. The activation by dsRNA of potentially novel proteins binding the ISRE of ISG15 has been reported, but these have not been characterised further (Daly and Reich 1995).

Analysis of the induction of the 561 gene in HeLaM cells has indicated that ongoing protein synthesis is required for α -IFN-induction, which has been postulated to provide a factor missing in normally growing HeLaM cells that is required for induction. Once this first signal, referred to as signal 1, has been provided, a second signal, signal 2, which is sufficient for induction of type I IFN-inducible genes in most cell types, brings about the induction of the gene. Interestingly, this is reminiscent of the priming-dependence of the β -IFN gene in some cells, including HeLa (Enoch and Maniatis 1986) (see Chapter Three), and in support of this, signal 1 can be provided by α -IFN and signal 2 by dsRNA (Tiwari et al. 1987). These observations suggest that the induction of genes by type I IFN and

the induction of the β -IFN gene by dsRNA may well involve the activation of common pathways and factors.

VCAM-1

It has been reported that the cellular adhesion molecules VCAM-1, ICAM-1 and E-selectin are induced by dsRNA in endothelial cells (Marui et al. 1993, Yang et al. 1994). The promoters of these genes all contain consensus NF- κ B binding sites, and it has been shown that a 63bp region of the human VCAM-1 promoter, containing the tandem NF- κ B sites, is responsible for dsRNA-induction.

It has also been shown that c-fos, c-myc, JE and IRF-1 are directly activated by dsRNA (Haines et al. 1991), and that c-fos and c-myc activation can be blocked by 2AP, suggesting a role in the induction for PKR.

CHAPTER TWO - MATERIALS AND METHODS

Bacterial Strains and Culture

E. coli SCS-1 (F^- , *endA1*, *gyrA96*, *thi-1*, *hsdR17* [r_k^- , m_k^+], *supE44*, *recA1*, *relA1*) was used routinely for DNA sub-cloning manipulations. Manipulations involving bacteriophage M13-based vectors were performed using *E. coli* JM101 (*supE*, *thi-1*, $\Delta(lac-proAB)$, [F' *traD36*, *proAB*, *lacI^qZ* Δ M15]) to enable a blue/white test to be employed that facilitated identification of insert-bearing recombinants. BL21(DE3) [F^- , *ompT* *hsdS_B*(r_B^- , m_B^-) *gal dcm* (DE3)] were used for expression of proteins in bacteria.

Bacteria were plated out on L-Broth/1.5% agar plates (Sambrook et al. 1989). Ampicillin was added to 100 μ g/ml when antibiotic selection was required. For manipulations requiring a blue/white test, plates were supplemented with 0.0017% X-GAL (Sigma) and 0.002% IPTG (Sigma). Large-scale bacterial cultures were grown in Brain Heart Infusion (Difco) at 37°C with good aeration, cultures containing 100 μ g/ml ampicillin where applicable. Brain Heart Infusion supports bacterial growth to a higher density than L-Broth.

DNA Preparation

Preparation of plasmid DNA

Large-scale preparations of plasmid DNA were prepared by the alkaline lysis method followed by centrifugation on CsCl gradients (Sambrook et al. 1989).

Preparation of miniprep DNA

Colonies were picked and used to inoculate 3ml of L Broth containing 100 μ g/ml ampicillin. Cultures were incubated overnight at 37°C with vigorous shaking. Half the culture volume was transferred to 1.5ml microfuge tubes and the bacteria harvested by centrifugation in a benchtop microfuge for 15 seconds. Plasmid DNA was prepared by alkaline lysis as described (Sambrook et al. 1989).

Rapid microtitre plate plasmid minipreps

Many colonies were rapidly screened for the presence and size of a short insert (150bp or less) by a microtitre plate procedure. Single colonies were picked into 1ml "Micronic" 45 x 8.8 mm tubes (Flow) containing 500 μ l LB plus 100 μ g/ml ampicillin.

These tubes were placed in a "Micronic" systems tube holder with spacing equivalent to that of a standard 96 well microtitre plate, and shaken overnight at 37°C. The following day, 250µl of each culture were transferred to a 96 well microtitre dish with a Titertek Digital Multichannel pipette, which was used for all subsequent manipulations. The cells were pelleted at 1500g and the supernatants were discarded by shaking off the liquid. Bacterial pellets were resuspended in 60µl of a 1:2 (v/v) pre-mix of solutions I + II (alkaline lysis mini-prep procedure (Sambrook et al. 1989)), then 30µl of solution III were added and mixed. Debris was pelleted at 1500g and the supernatants were transferred to a new microtitre plate. Eighty microlitres of isopropanol were then added and mixed to precipitate nucleic acids.

Following centrifugation, the pellets were washed by gently flooding the dishes with 70% ethanol and, after air drying, subsequently dissolved in 20µl of a solution containing the appropriate restriction enzyme buffer, 0.5 units of each required enzyme, 80µg/ml RNase A, 0.1µCi $\alpha^{32}\text{P}$ -dNTP (with 0.1mM each of the other three cold nucleotides) and 0.1 units of Klenow. After 1 hour at 37°C, 20µl of formamide dyes (90% deionised formamide in 1xTBE (Sambrook, Fritsch et al. 1989) with 0.1% w/v bromophenol blue and xylene cyanol dyes) were added and the plate was heated in an 85°C oven for 15 minutes to denature the DNA.

The deletions were subsequently analysed on a 6% denaturing polyacrylamide gel. In addition to labelling the ends produced by digestion, the prolonged incubation of impure miniprep DNA with Klenow and $\alpha^{32}\text{P}$ -dNTP leads to the production of a "ladder" of labelled fragments which differ in size by a single nucleotide. These are probably caused by nicking events and are useful in allowing the size differences between clones analysed in adjacent gel lanes to be accurately assessed.

Preparation of genomic DNA

Cells ($2-5 \times 10^6$) were harvested from a 9cm dish into a microfuge tube, washed with PBS and resuspended in 400µl of 0.5M EDTA. Proteinase K was added to 250ng/ml and SDS to 0.5%, and the solution was incubated at 50°C for 1hr. The lysate was extracted with phenol/chloroform and then phenol, and the nucleic acid in the sample was ethanol precipitated. The resultant pellet was resuspended in TE with the addition of RNase A to 80µg/ml and incubated at 37°C for 15 minutes. Following phenol/chloroform and then phenol extraction as before, the genomic DNA was ethanol precipitated and resuspended in 1ml of TE (10mM Tris, pH 8.0,

1mM EDTA).

For preparation of DNA from apoptotic cells, following washing and harvesting the cells were resuspended in a cytoplasmic lysis buffer (50mM Tris pH 7.9, 10mM EDTA, 1% Triton X-100). The lysates were incubated on ice for 10 minutes and then the insoluble fraction was pelleted by centrifugation in a microfuge for 5 minutes. Proteinase K and SDS were added to the supernatant as above and incubated at 37°C for 15 minutes. Following this treatment the DNA preparation proceeded as above.

The concentration of DNA in the samples from each method was determined by measurement of its absorbance at 260nm using the equation:

$$1 A_{260} \text{ unit} = 50 \mu\text{g/ml DNA.}$$

DNA Manipulations

Restriction enzyme digestion

For general manipulations and the construction of plasmids, 5µg of plasmid DNA were digested with the appropriate enzymes according to the manufacturers' specifications, typically in a 10µl reaction volume. Reactions were routinely analysed by agarose gel electrophoresis (Sambrook et al. 1989) and DNA fragments purified by extraction into low melting temperature agarose (Sea Kem) (see agarose gel electrophoresis). For cloning steps involving partial digestion, the optimal conditions for digestion were determined in each case by restricting 10µg of DNA with 5 units of enzyme and analysing the extent of digestion over a range of 1 to 15 minutes.

Phosphatase treatment of DNA

To remove 5' phosphates from DNA molecules, 1 unit of Calf Intestinal Alkaline Phosphatase (Boehringer) was added to the restriction enzyme reaction and incubation continued for a further 30 minutes at 37°C before fragment purification.

Treatment of DNA with Mung Bean Nuclease

To digest 5' or 3' overhangs with Mung Bean Nuclease (MBN) following restriction digestion, the DNA was phenol extracted, ethanol precipitated and washed extensively with 70% ethanol to rid the pellet of any remaining salt. The DNA was then digested with 50 units of MBN for 5 minutes at 16°C in 50µl 30mM sodium acetate (pH 4.6), 50mM NaCl, 1mM ZnCl₂ before reactions were stopped

with the addition of Tris pH 9.0 to 10mM, LiCl to 0.5M and SDS to 1%. The resulting mixture was then phenol extracted and ethanol precipitated.

Filling recessed 3' ends of double-stranded DNA with Klenow enzyme

Double-stranded DNA molecules with protruding 5' ends were made blunt-ended by incubation with 5 units of Klenow fragment of DNA polymerase I (Boehringer) in the presence of 200 μ M dNTPs for 5 minutes at room temperature in a volume of 10 μ l. Reactions were stopped by phenol extraction and the products precipitated.

Exonuclease digestion of large regions of DNA

Digestion of large stretches of DNA were performed using Exo III (Stratagene), Bal 31 nuclease or T4 DNA polymerase (both New England Biolabs) as described (Sambrook et al. 1989), or as outlined in Chapter Four.

Ligation of DNA

Ligation reactions contained up to 200ng of DNA in a 20 μ l reaction with a molar ratio of vector:insert of 1:3. Ligations were performed using FPLC-pure T4 DNA ligase (Pharmacia) at 16 $^{\circ}$ C overnight using the manufacturer's buffer conditions.

Fragments isolated into LMT agarose were heated to 65 $^{\circ}$ C to melt the agarose before setting up the ligation reaction. Buffer and enzyme were added last to the reaction once it had cooled down to 37 $^{\circ}$ C, and the final agarose concentration was not allowed to exceed 0.5%.

Phenol extraction of DNA

Reactions requiring phenol extraction were made up to a minimum of 100 μ l with TE and an equal volume of Tris-buffered phenol was added. The mixture was vortexed vigorously for at least 30 seconds before centrifugation. Typically 90% of the aqueous layer was taken for subsequent precipitation. If phenol/chloroform was used, an equal volume of a 1:1 premix of Tris-buffered phenol and chloroform was added to the reaction mixture and extraction was performed as above.

Precipitation of DNA

To precipitate DNA from solution, sodium acetate (pH 5.3) was added to 0.3M followed by 2.5 volumes of absolute ethanol. Volumes containing less than 10 μ g of

DNA were supplemented with 10µg of yeast tRNA or glycogen (both Gibco/BRL) before addition of ethanol. To precipitate synthetic oligonucleotides or short restriction fragments (less than 50 base pairs), MgCl₂ was added to a final concentration of 10mM before ethanol addition. Reactions were left on dry ice for 15 minutes before centrifugation in a microfuge for 5 minutes. The resulting pellets were routinely washed with 100ml 70% ethanol to rid the DNA of excess salt contamination. To remove unincorporated nucleotides from labelling reactions, 2M ammonium acetate was used instead of 0.3M sodium acetate.

Agarose gel electrophoresis

Restriction digestions, plasmid preparations and PCR products were routinely analysed on 1%-2% agarose (Sea Kem) gels electrophoresed at 10 V/cm in 1xTBE or TAE as described (Sambrook et al. 1989). Samples (typically 10µl) were loaded following the addition of 5µl of loading buffer (40% sucrose in TE with 0.1% w/v Orange G dye). If the DNA was to be visualised by UV irradiation, 0.5µg/ml ethidium bromide was incorporated in the gel.

For extraction of DNA from gels for use in ligations or further manipulations, a trough was cut from the agarose just below the desired band with a razor blade and filled with 1.4% low melting temperature (LMT) agarose (Sea Kem). The band was then run into the LMT plug and excised in the smallest possible volume of agarose.

Transformation of DNA into *E. coli*

E. coli that were competent for transformation (typical efficiencies ranging between 5×10^6 and 5×10^7 colonies per µg of supercoiled plasmid DNA) were prepared as a frozen stock (Hanahan 1985), and DNA was transformed according to this protocol. Ligations containing any LMT agarose were heated to 68°C for 10 minutes before transformation. Transformation mixtures were spread onto LB/1.5% Agar plates containing ampicillin.

Electroporation

If greater transformation efficiencies than could be achieved by the previous method were required, the products of ligation were transformed into *E. coli* by electroporation. *E. coli* SCS-1 was streaked out onto an LB agar plate and incubated overnight at 37°C. Three fresh colonies were picked and inoculated into 100ml of LB (in a 500ml flask), and the culture was aerated vigorously at 37°C until an OD₅₉₅ of

0.4 was reached. The cultures were rapidly chilled in an ice-salt water bath, and the cells were pelleted by centrifugation in a preparative centrifuge at 1500g. The cell pellets were resuspended in 100ml of ice-cold 1mM Hepes, pH7.5, and then recovered by centrifugation as before. This procedure was repeated, resuspending successively in 50ml of ice-cold 1mM Hepes, pH7.5, 1ml of ice-cold H₂O then finally 200µl of H₂O.

Forty microlitres of electrocompetent cells were used per transformation in a Bio-Rad Gene Pulser and Pulse Controller system. Great care was taken to keep the cells ice-cold at all times by pre-chilling the cuvettes and electroporation chamber and minimising the handling time. Electroporation at 2.5kV and 25µF with a pulse controller setting of 200Ω typically gave a time constant of between 4.5 and 5msecs. Following electroporation, 1ml of SOC (Sambrook et al. 1989) was added as rapidly as possible to the cuvette and the cells were allowed to recover for 1 hour at 37°C, with gentle aeration, before plating. Under these conditions, a transformation efficiency of between 2×10^{10} and 10^{11} colonies per µg of pBR322 was obtained.

Cloning into bacteriophage M13-based vectors

To prepare cells competent for transformation by M13-based vectors, 1ml of a fresh overnight culture of *E. coli* JM101 cells was used to inoculate a 100ml preparative culture. Cells were grown at 37°C with vigorous shaking until they reached OD₅₉₅ 0.3, whereupon they were harvested by centrifugation in a preparative centrifuge at 1000g for 5 minutes. The resulting cell pellet was resuspended in 50ml of 50mM CaCl₂ and incubated for 15 minutes on ice. The cells were then harvested as before and resuspended in 5ml of 50mM CaCl₂.

Three hundred microlitres of competent JM101 cells were mixed with a maximum 1/10th volume of DNA and incubated on ice for 45 minutes. The reaction mixture was heat-shocked for 2 minutes at 42°C and then added to 3ml molten top agar (LB/0.8% agar) supplemented with 0.0017% X-GAL (Sigma) and 0.002% IPTG (Sigma). The mixture was poured onto a LB/1.5% agar plate, left to set and incubated at 37°C overnight.

Insert-bearing (white) plaques were picked into 3ml of a 1/100 dilution of a fresh overnight culture of *E. coli* JM101 and incubated at 37°C with vigorous aeration for 5 hours. One and a half millilitres of each culture was transferred to a microfuge tube and cells were pelleted by centrifugation for 5 minutes in a microfuge. The supernatant was removed to a fresh tube and 0.2ml of PEG/NaCl (20% PEG 6000, 2.5M NaCl) added. The mixture was vortexed and left to precipitate

at room temperature for 10 minutes. After centrifugation the supernatant was discarded, with care being taken to remove all traces of PEG/NaCl. The pellet was resuspended in TE, phenol/chloroform and phenol extracted and ethanol precipitated. After centrifugation the single-stranded DNA pellet was resuspended in 50µl TE, ready for further analysis.

Enzymatic sequencing of DNA

Single-stranded DNA prepared from bacteriophage M13 DNA was sequenced with a Sequenase Kit 2.0 (USB) according to the manufacturer's instructions.

For sequencing double-stranded DNA, 3µg of purified plasmid DNA, or half of a plasmid miniprep, was ethanol precipitated, washed, resuspended in 20µl TE and treated with 200mM NaOH and 200µM EDTA for 5 minutes at room temperature to collapse the supercoils. The solution was neutralised by the addition of ammonium acetate (pH 4.6) to 275mM and the DNA was reprecipitated. The template was then annealed to 5ng of the relevant primer and sequenced as for single-stranded DNA.

Amplification of DNA by the Polymerase Chain Reaction

DNA was amplified as described (Kawasaki and Wang 1989). Approximately 50ng of DNA were amplified in a 50µl reaction with 25pmoles of upstream and downstream primers (approximately 100ng of a 17mer). For each reaction the T_m of the oligonucleotides and template was calculated, and a hybridisation temperature of $T_m-10^{\circ}\text{C}$ was used. Thirty cycles were performed in a Techne PHC-3 thermal cycler.

Polyacrylamide gel electrophoresis

Electrophoresis was carried out using vertical gel-running apparatus as described (Sambrook et al. 1989). Gels were routinely run in 1xTBE buffer (Sambrook et al. 1989) unless otherwise stated. For reactions that required electrophoresis under denaturing conditions, 8M urea was added to gel mixtures.

End-labelling ladder DNA from apoptotic cells

To visualise the cytoplasmic fragments of DNA found in apoptotic cells, 1µg of nucleic acid purified from cytoplasmic extracts was end labelled with $\alpha^{32}\text{P}$ -dATP using the terminal transferase activity of Taq polymerase (Eldadah et al. 1996).

Oligonucleotide synthesis

Oligonucleotides were synthesised by Iain Goldsmith (ICRF, Clare Hall Laboratories) and Dr. Vincent Ang (St. George's Hospital Medical School).

Annealing oligonucleotides

Oligonucleotides were annealed to target sequences by allowing the reaction to cool slowly from 65°C to room temperature in 40mM Tris pH 7.5, 20mM MgCl₂ and 50mM NaCl in a typical reaction volume of 10µl.

Cell Culture Conditions

Cell lines and culture conditions

HeLaE (ATCC CCL 2) (a gift from E. Laufer, ICRF), MG63 (ATCC CRL 1427), HT1080 (ATCC CCL 121) and its sub-types (gifts from I. Kerr and G. Stark, ICRF), C127 (ATCC CRL 1616) and L929 cells (ATCC CCL 1) were routinely cultured on 90mm plastic tissue culture dishes (Nunc) in 10ml Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL) supplemented with 10% Foetal Bovine Serum (DMEM/FBS) (Gibco/BRL). Cells were frequently checked for mycoplasma contamination. For experiments using hormone binding domain fusion proteins, cells were grown in phenol red-free DMEM (Gibco/BRL) and serum free from oestrogen (a gift from D. Hancock, ICRF).

Induction of β -interferon

Human cells were grown to 80% confluence and induced with poly(I).poly(C) (Pharmacia) at a concentration of 100µg/ml in 3ml DMEM. Cells were washed twice with serum free medium before the addition of the induction medium. When primed, unless stated otherwise, cells were treated for at least 16 hours with 500U/ml Wellferon (Wellcome, Lot 72, a mixture of natural human α interferons) in 10ml DMEM/10% FBS. Mouse cells (C127 and L929) were primed with recombinant human α A/D-IFN and induced with a dsRNA/DEAE-dextran precipitate prepared by slowly adding poly(I):poly(C) to a 0.6mg/ml DEAE-dextran (Pharmacia) solution, in DMEM, to a final concentration of 25µg/ml. The resultant fine precipitate was incubated at 37°C for 5 minutes and then 3ml of this mix were added to each washed 9cm plate of cells. Viral inductions were carried out with 7500 haemagglutination units of Sendai Virus (Wellcome) at a 1:10 dilution in 3ml DMEM.

For all inductions, the induction mix was left on the cells for 2 hours and

replaced with DMEM/10% FBS for the remainder of the experiment. Where applicable, cycloheximide (CHX) was added to the induction medium at a concentration of 50µg/ml.

Transiently transfected cells were treated with dsRNA, as described above, for a total of five hours unless otherwise stated.

Transfection of DNA into tissue culture cells

DNA was transfected into HeLa, C127 and HT1080-derived cells by co-precipitation with calcium phosphate as described (Sambrook et al. 1989). The precipitate was left on the cells overnight and then removed by aspiration to be replaced by DMEM/10% FCS.

For Lipofectamine transfection of HeLaE cells, the manufacturer's recommendations were followed (Gibco/BRL). Briefly, a 2µg final amount of DNA per transfection was diluted into 100µl of DMEM and this was supplemented by 100µl of an 8% solution of lipofectamine in DMEM. Following a 45 minute incubation at 37°C, the transfection mixes were made up to 1ml with serum free medium and then added to washed 80% confluent cells grown in 6 well dishes (Nunc). After a 5-6 hour incubation at 37°C, the transfection mix was removed by aspiration and replaced with DMEM/10% FCS.

In both protocols cells were left to grow for up to 48 hours after the beginning of transfection before harvesting for the assay of RNA or protein levels.

CD2-selection of transiently transfected cells

To select for transiently transfected cells, three 9cm dishes of HeLaE cells per sample were each transfected with 5µg pKSCD2 and 10µg test plasmid using calcium phosphate co-precipitation. Cells were primed and induced as appropriate. Plates were then washed twice with PBS, and cells harvested by scraping with a rubber policeman in a final volume of 5ml of ice-cold PBS + 2% FBS per sample. The cell suspension was transferred to a 15ml snap-cap tube, and 4 x 10⁷ magnetic beads conjugated to a monoclonal antibody specific for human CD2 (Dyna) in a 100µl volume were added with vigorous mixing. The mixture was incubated on a rotating wheel at 4°C for 15 minutes. The CD2⁺ and CD2⁻ cell populations were then separated using multiple rounds of magnetisation. For the first magnetisation, a Dynal MPC-1 magnetic particle concentrator was used for the 15ml snap-caps. The CD2⁺ population was then resuspended in 1ml ice-cold PBS + 2% FCS and transferred to a 1.5ml microfuge tube, and subsequent magnetisations were

performed using a Dynal MPC-E magnetic particle concentrator. This reduction in volume led to smaller cell losses. Rounds of magnetisation were continued until the supernatant was clear of cells for the CD2⁺ population, and until no beads could be detected in the CD2⁻ population. Cells in the CD2⁺ population were then pelleted and RNA or nuclear extracts were prepared as described below. Under these conditions magnetic beads can be observed binding to all of the cells in the CD2⁺ population, with about 97% of cells having in excess of 20 beads per cell. Using enzyme markers, it appears that the frequency of co-transfection is about 94%, and that between 2% and 40% of the HeLaE cells are in the CD2⁺ population.

Analysis of Gene Expression

Isolation of cellular RNA

RNA was routinely prepared by cytoplasmic lysis. Cells were washed twice with cold PBS, harvested into 1ml PBS and collected by a 15 second centrifugation in a microfuge. The supernatant was removed and the cell pellet was resuspended in 375 μ l of cytoplasmic RNA lysis buffer (50mM Tris pH8.0, 100mM NaCl, 5mM MgCl₂, 0.5% Nonidet P40). After a five minute incubation on ice, nuclei and membrane fractions were pelleted by a two minute spin in a microfuge. The supernatant, representing the cytoplasmic fraction, was supplemented with SDS to 0.5% and then incubated with 250ng/ml of proteinase K for 15 minutes at 37^oC. Following this treatment the cytoplasmic prep was extracted with phenol/chloroform and then phenol, and the RNA in the sample was precipitated by addition of 2.5 volumes of ethanol. After centrifugation the RNA pellet was washed with 70% ethanol and resuspended in 100 μ l of TE supplemented with 0.1% SDS (TES). The concentration of RNA in the sample was determined by measurement of its absorbance at 260nm using the equation:

$$1 A_{260} \text{ unit} = 40 \mu\text{g/ml RNA.}$$

RNAse protection analysis of cellular RNA

Steady state levels of RNA expressed from endogenous or transfected genes were quantitated using the RNAase protection assay (Zinn et al. 1983). Linearised templates (all from SP6-based vectors) were *in vitro* transcribed using SP6 RNA polymerase in the presence of 0.5mM ATP, CTP and UTP and 50 μ Ci α^{32} P GTP (Amersham, 800Ci/mmol) to create probes. The γ -actin probe was synthesised in the presence of 0.1mM unlabelled GTP to lower its specific activity. The probes for human β -IFN, mouse β -IFN and γ -actin are as described (Enoch and Maniatis 1986);

(Goodbourn et al. 1985). The human IRF-1 probe has also been described (S. Whiteside, PhD thesis, London university, 1992). The protected products were analysed on 6% denaturing polyacrylamide gels.

Quantitation of protein expressed from transfected genes

Luciferase

Extracts for the quantitation of luciferase were prepared by lysing cells in 300µl Buffer A (25mM Tris Phosphate, pH 7.8, 8mM MgCl₂, 1mM DTT, 1mM EDTA and 1% Triton X-100). Cell lysates were carefully transferred into a microfuge tube and an equal volume of Buffer B (Buffer A supplemented with 30% Glycerol, 2% BSA and 0.8µM rATP) was added. Any cell debris transferred from the plates was pelleted by centrifugation in a microfuge for 1 minute. 250µl of the supernatant was placed into a cuvette containing 100µl of a 1:1 pre-mix of Buffer A and Buffer B. D-luciferin (Sigma) was injected to a concentration of 150µM and luciferase levels were assayed using a LKB 1251 luminometer or a Berthold LB 9501 luminometer.

Chloramphenicol Acetyl Transferase (CAT)

Extracts for the quantitation of CAT as the reporter were prepared and assayed as described (Sleigh 1986) and the CAT activity in the samples was assessed by liquid scintillation following the ethyl acetate-extraction of (³H) acetylated chloramphenicol. In experiments in which luciferase was the reporter and CAT the transfection control, extracts were prepared as described for luciferase assays and used as normal for CAT quantitation.

Gel Retardation Analysis

Preparation of crude nuclear extracts

Crude nuclear extracts were prepared by a variation of a published method (Schreiber et al. 1989). Typically 10⁶ cells were washed twice with cold PBS, and then scraped off the plate into 1ml cold PBS with a rubber policeman and harvested by centrifugation in a benchtop microfuge for 15 seconds. Following aspiration of the PBS, cells were resuspended in 400µl cold Buffer A (Dignam et al. 1983) to which protease inhibitors had been added (Visvanathan and Goodbourn 1989). Cells were left to swell for 5 minutes before the addition of Nonidet P40 to 0.1% and the mixture was immediately vortexed for 10 seconds. Nuclei were isolated by centrifugation in a microfuge at 4°C for 2 minutes and extracted at 4°C, with continual agitation, for 1hr into 50µl of Buffer C (Dignam et al. 1983) supplemented with protease inhibitors as before. This mixture was then centrifuged for 10 minutes

at 4°C in a microfuge, and the resulting supernatant, containing a crude nuclear extract, was collected and stored at -70°C.

The protein concentrations of the extracts were determined using Bradford reagent (Bio-Rad).

Labelling of oligonucleotide probes

Double-stranded oligonucleotides to be used as probes for gel retardation assays were end-labelled using the Klenow fragment of DNA polymerase I (Boehringer) in the presence of a 2-fold molar excess of $\alpha^{32}\text{P}$ -dATP (Amersham, >3000Ci/mmol) and unlabelled dCTP, dGTP and dTTP at concentrations of 200 μM . Labelled oligonucleotides were purified by electrophoresis on a 15% native polyacrylamide gel, and eluted from the acrylamide by incubation of the gel slice in 500 μl of 0.5M ammonium acetate, 1mM EDTA and 0.1% SDS at 37°C overnight.

The sequence of probes used for EMSA analysis in this thesis are shown below:

PRD I top strand:	5' GATCGAGAAGTGAAAGT 3'
PRD I bottom strand:	5' GATCACTTTCACCTTCTC 3'
PRD II top strand:	5' GATCGGGAAATTCC 3'
PRD II bottom strand:	5' GATCGGAATTTCCC 3'

Electrophoretic mobility shift assay

To analyse cellular activities capable of binding to DNA, crude cellular extracts were analysed by the gel retardation assay, or electrophoretic mobility shift assay (EMSA). Briefly, 10 μg of extract was incubated for 5 minutes at room temperature in 20 μl binding buffer (20mM Tris pH 8.0, 2mM MgCl_2 , 60mM KCl and 12% v/v glycerol) in the presence of carrier DNA (poly(dI).poly(dC) or poly(dI.dC).poly(dI.dC), (Pharmacia) depending on the probe used). To prevent non-specific binding to PRD I or related probes by an unknown component of these crude extracts, it was necessary to pre-heat the poly(dI).poly(dC) at 98°C for 10 minutes, followed by quenching on ice, or to omit Mg^{2+} from all buffers. After the 5 minute incubation, 2×10^4 c.p.m. of end-labelled probe were added to the reaction and the reaction mixture was incubated at 30°C for a further 15 minutes. Reaction products were loaded onto pre-electrophoresed 5% native polyacrylamide gels and electrophoresed at 14V/cm in 0.5 x TBE buffer (Sambrook et al. 1989). If blocking or supershifting antibodies were included in the experiment, 2 μl of specific or preimmune antisera were added to the reaction mixture and incubated on ice for at

least 1 hour prior to addition of the probe .

Analysis of Proteins

Production of antibodies

The anti-IRF-1 and anti-ISGF3 γ antibodies were raised against the respective full-length, C-terminally histidine tagged, human proteins expressed in *E.coli* from pET-21 (Novagen). Competent BL21DE3 (Novagen) were transformed with the His-tagged constructs and a 4ml overnight culture of a single colony was added to 400ml of LB/AMP. This culture was grown at 37°C with shaking until it reached an OD₅₉₅ of 0.6. Expression of the fusion protein was then induced by the addition of 1mM IPTG and the cells were grown for a further 3 hours. After harvesting by centrifugation, the bacteria were lysed and the His-tagged proteins were purified by batch affinity chromatography using a nickel-agarose resin (Qiagen) following the Novagen protocol for purification under denaturing conditions (including 6M urea in all buffers). Following elution, positive fractions were assessed by SDS-PAGE and Coomassie staining and these were pooled and dialysed against saline (0.9% NaCl) with successively decreasing concentrations of urea. If the protein reprecipitated at low concentrations of urea, it was resolubilised by the addition of SDS to a maximum of 0.1%.

Rabbits were immunised with seven injections of alum precipitates of 50 μ g aliquots of each protein over a period of three months. To make the alum precipitates the protein aliquot was diluted to 50 μ g/ml in saline and 300 μ l of 10% potassium aluminium sulphate (alum) were added. The pH of the solution was adjusted to pH 8.0 by the addition of 1M NaOH (about 200 μ l) and the precipitate was allowed to form at room temperature for 20 minutes. The precipitate was then pelleted with a two minute spin in a microfuge, washed with 1ml saline and then resuspended in a maximum volume of 0.5ml. The rabbits were sacrificed 10 days after the final injection.

Caprylic acid purification of antibodies

To partially purify the IgG component of the rabbit bleeds, 5ml of serum were adjusted to pH 5.0 by the addition of 0.5ml 1M sodium acetate, pH 5.0 in a glass beaker. 250 μ l of caprylic (octanoic) acid were added slowly with constant stirring and the solution was stirred for a further 30 minutes in a fume hood to allow precipitation of the non-IgG component of the serum. Following a 30 minute

centrifugation at 10,000rpm in a preparative centrifuge the IgG fraction was precipitated from the supernatant by the dropwise addition of one volume of saturated $(\text{NH}_4)_2\text{SO}_4$ with constant stirring. The precipitate was pelleted by centrifugation at 10,000rpm for 10 minutes and was washed with 45% saturated $(\text{NH}_4)_2\text{SO}_4$ solution. The pellet was drained thoroughly, allowed to air dry and then resuspended in 3ml H_2O . After overnight dialysis against PBS, the IgG preparation was stored in aliquots at -70°C .

Preparation of whole cell protein extracts

Whole cell extracts for use in immunoprecipitations and Western Blotting experiments were prepared by washing the cells twice with cold PBS. They were then scraped off the plate into 1ml cold PBS with a rubber policeman and harvested by centrifugation in a benchtop microfuge for 15 seconds. Following aspiration of the PBS, cells for Western blotting were resuspended in SDS sample buffer (10% SDS, 250mM Tris pH6.8, 10% β -mercaptoethanol, 0.1% bromophenol blue) at a concentration of 10^6 cells/100 μl , and those for immunoprecipitation were resuspended in RIPA buffer (150mM NaCl, 50mM Tris pH8.0, 1% NP40, 0.5% deoxycholate, 0.1% SDS) at 10^6 cells/100 μl . The DNA in the samples was sheared by passing the sample six times through a 25g needle and the samples were stored at -70°C until analysed.

SDS-polyacrylamide gel electrophoresis

Protein extracts were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970)) using a BioRad Mini Protean II apparatus. The acrylamide content of the stacking gel was 5% and the content of the resolving gel ranged from 7-12%.

Gels were stained with Coomassie blue to visualise protein by soaking in staining solution (20% methanol, 10% acetic acid, 0.1% Coomassie blue) for up to 1 hour and then in several changes of destaining solution (20% methanol, 10% acetic acid) for up to 2 hours.

Western blotting

Protein extracts were resolved by SDS-PAGE and then blotted onto 0.45 μ PVDF membrane (Pierce) using a BioRad Mini Protean II Transfer Cell according to the manufacturer's instructions using transfer buffer (25mM Tris base, 192mM glycine, 20% methanol). For Western blots with rabbit polyclonal antibodies, the

filters were blocked either overnight at 4°C in 50mM Tris pH7.5, 50mM NaCl, 1mM EDTA and 1mM DTT supplemented with 5% Marvel or for 1 hour at room temperature in PBS, 5% Marvel, 0.2% Tween-20. After washing three times for 10 minutes each in PBS plus 0.2% Tween-20, filters were probed with the primary antibody in PBS, 5% Marvel, 0.2% Tween-20 for 1 hour at room temperature. Filters were washed as before and probed with a horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham) in PBS, 5% Marvel, 0.2% Tween-20 for 45 minutes at room temperature. Following washing, filters were developed using enhanced chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

If a mouse monoclonal antibody was used as the primary antibody, the secondary antibody was a sheep anti-mouse Ig (Amersham). In the case of the monoclonal anti-phosphotyrosine antibody PY20, filters were blocked overnight at 4°C or for 1 hour at room temperature in TBS, 5%BSA (immunoglobulin free, Sigma), 0.1% Tween-20. Both primary and secondary antibody incubations were performed for 1 hour in TBS, 1% BSA, 0.1% Tween-20 and all washes used TBS, 0.1% Tween-20. The secondary antibody used was a horseradish peroxidase-conjugated sheep anti-mouse IgG F(ab')₂ fragment (a gift from D. Guschin, ICRF, London).

Following exposure to X-ray film, Western filters were kept damp by storage at 4°C wrapped in Saran wrap, to allow subsequent reprobing. If this was required, the filters were first stripped in 50mM Tris pH6.8, 100mM β-mercaptoethanol and 1% SDS at 50°C for 30 minutes. Following extensive washing in the appropriate buffer, the filters were then blocked and probed as above.

Immunoprecipitation

RIPA extracts were pre-cleared by incubation with 5µl pre-immune serum for 30 minutes at 4°C. 30µl of 50% v/v Protein A sepharose (Pharmacia) was then added and the mixture incubated for a further 30 minutes at 4°C. Non-specific immune complexes that adsorbed to the protein A sepharose were pelleted with the beads by centrifugation in a microfuge for 10 minutes. Supernatants were removed to fresh tubes and incubated with the appropriate amount of specific antiserum for 60 minutes at 4°C. Immune complexes were collected by centrifugation for 1 minute and the beads were washed three times in 1ml of RIPA buffer. Supernatants were removed using a syringe and 23g needle as described (Harlow and Lane 1988). The Protein A sepharose beads were resuspended in 20µl SDS loading buffer, heated at 85°C for 10 minutes and analysed by SDS-PAGE.

Protease and phosphatase inhibitors

The following protease and phosphatase inhibitors were included in Buffers A and C for the preparation of nuclear extracts and in RIPA buffer for immunoprecipitations:

Protease inhibitors: PMSF, 0.5mM, Aprotinin, 5µg/ml, Leupeptin, 30µg/ml, Pepstatin, 5µg/ml.

Phosphatase inhibitors: 1mM sodium orthovanadate, 5mM NaF.

CHAPTER THREE - IDENTIFICATION AND CHARACTERISATION OF A PRIMING-DEPENDENT SYSTEM FOR β -IFN INDUCTION

Introduction

A key strategy in determining the molecular mechanisms of the regulation of the β -IFN gene is the identification and characterisation of cell lines that are defective in this process. Extensive studies have shown that β -IFN is inducible in virtually all cell types (see Chapter One), but two classes of cells have been found to be poorly inducible. Firstly, β -IFN is not inducible in the early embryo, or in undifferentiated cell lines derived from embryonal carcinomas (Ellis and Goodbourn 1994). Secondly, several cell lines have been shown to be poorly inducible unless they are pre-treated with IFN, a phenomenon known as priming (see Chapter One). In the latter case, inducibility could be restored by fusion with highly inducible cells, demonstrating that IFN treatment leads to the provision of an essential factor normally present in priming-independent cells (Enoch and Maniatis 1986). Priming-dependent cells can thus be considered as a system in which to study the conditional inducibility of the β -IFN gene, and the identification of the essential factor(s) provided by IFN treatment would be a considerable advance in the understanding of the regulation of this gene. At the outset of this work, none of the experimental systems for studying β -IFN induction showed complete dependence upon priming, and this could have lead to difficulties in the interpretation of experiments. This Chapter describes the establishment of a fully priming-dependent system and characterises the process in further detail.

HelaE cells are absolutely dependent upon priming for induction by dsRNA

To initiate studies on the role that priming plays in the induction of the β -IFN gene, the response of a series of cell lines to pre-treatment with type I IFN was investigated. Without IFN pre-treatment, the β -IFN genes of a human osteosarcoma cell line, MG63, and a mouse fibroblast cell line, C127, were found to be inducible by dsRNA, as judged by RNase protection (Fig 3.1). Following priming, both cell lines were approximately two to five fold more inducible. This was also found to be the case for two other mouse lines, L929 (data not shown) and retinoic acid-differentiated F9 cells (Ellis and Goodbourn 1994). In contrast, a HeLa subline, HeLaE, was found to be uninducible by dsRNA unless the cells were pre-treated overnight with type I IFN.

Figure 3.1 MG63 and C127 cells are more inducible following priming

MG63 and C127 cells were grown to near confluence and induced with dsRNA for 4 hours in the presence or absence of priming. The cellular RNA was mapped by RNase protection using β -IFN (5' IF) (A and B) and γ -actin (A) probes.

(A). MG63 cells were treated as follows:

Lane 1: unprimed, uninduced

Lane 2: unprimed, induced

Lane 3: primed, uninduced

Lane 4: primed, induced

(B). C127 cells were treated as follows:

Lane 1: unprimed, uninduced

Lane 2: unprimed, induced

Lane 3: primed (with α A/D-IFN), induced

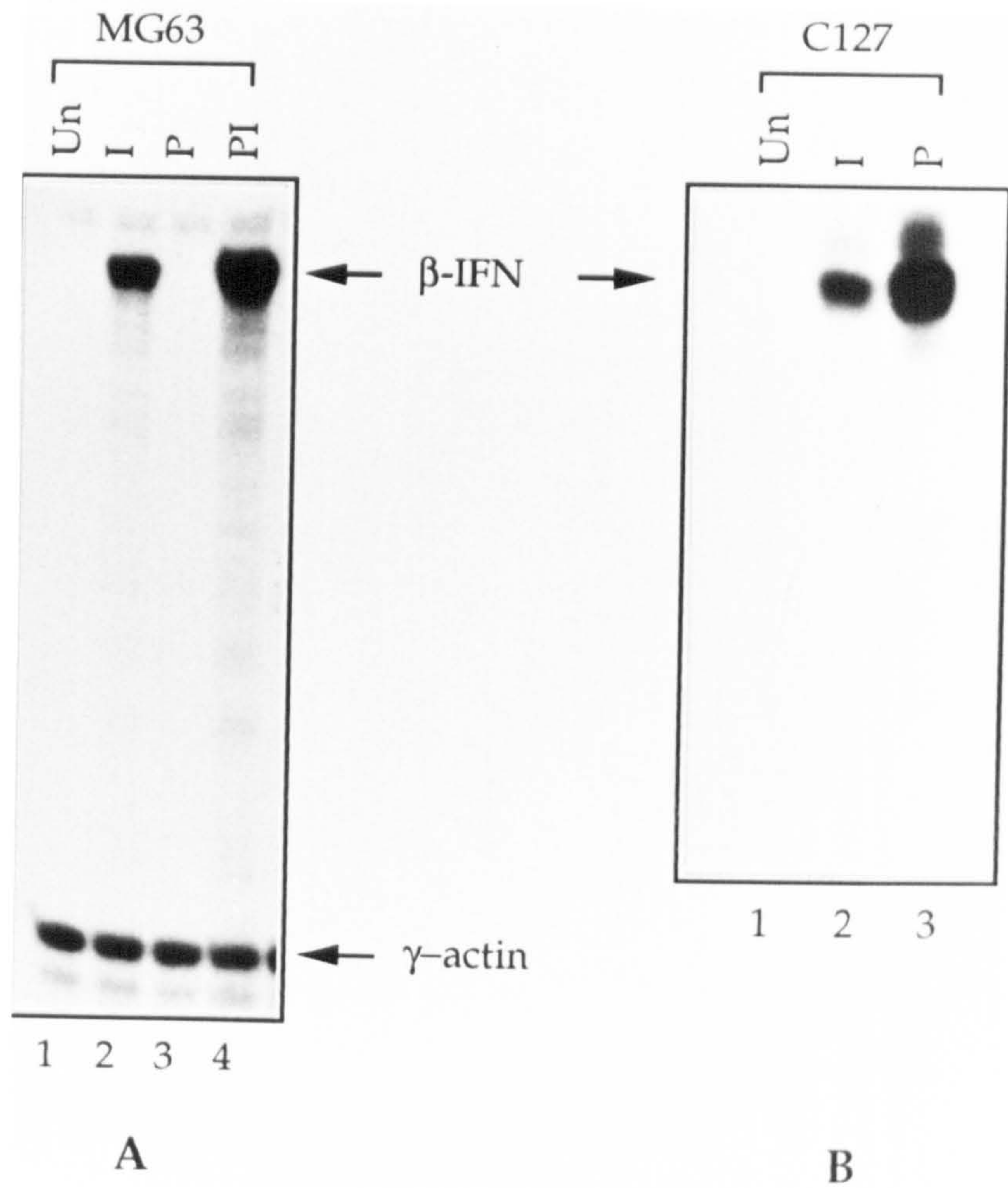


Figure 3.2 The β -IFN gene is absolutely dependent on priming for induction by dsRNA in HeLaE cells

HeLaE cells were induced by dsRNA, in either the primed (18 hours of Wellferon treatment) or the unprimed state, for the length of time indicated above the autoradiograph. RNA was prepared and analysed by RNase protection, using β -IFN (5' IF) and γ -actin probes. The autoradiograph was overexposed to demonstrate the absence of β -IFN-specific protected fragments in the absence of priming.

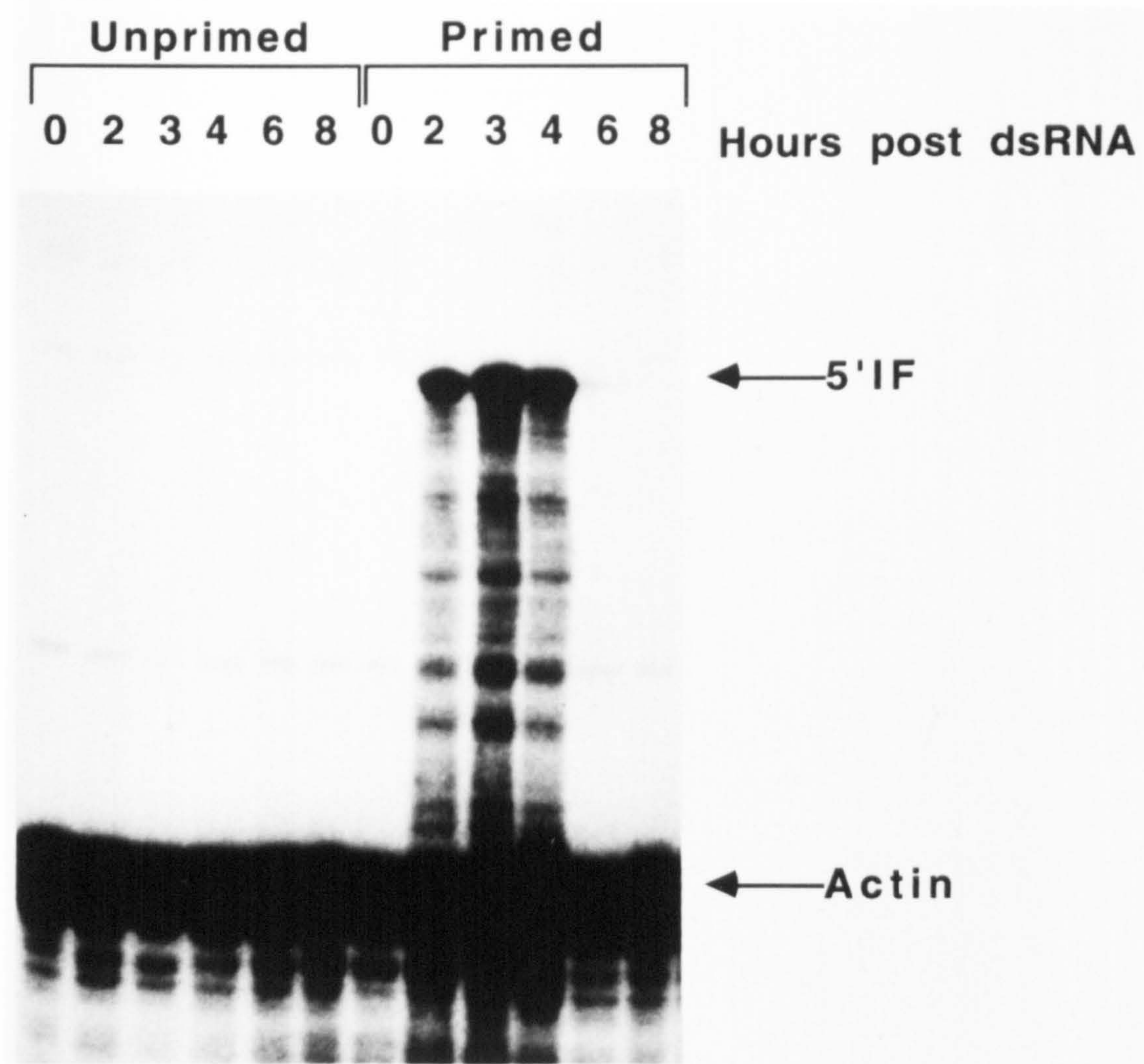


Figure 3.2 shows an RNase protection assay of RNA prepared from a time course of dsRNA induction of unprimed and primed HeLaE cells. No β -IFN-specific RNA can be seen in the unprimed, induced samples in this heavily overexposed autoradiogram, whereas it is easily detectable in the primed and induced samples. As well as demonstrating the priming-dependence of this cell line, Figure 3.2 also illustrates the typical profile of β -IFN induction by dsRNA: there is a lag period of approximately 2 hours before the production of detectable β -IFN RNA, mRNA levels peak at around 3 hours in this cell line and then decay again to become undetectable after approximately 8 hours. It is important to stress that priming itself cannot induce the β -IFN gene, as evidenced by the absence of β -IFN-specific RNA in the primed, uninduced sample in this experiment.

This HeLa subline was obviously of great interest because it appeared to be absolutely dependent upon priming for induction of the β -IFN gene by dsRNA, and further studies were undertaken to investigate the nature of the priming response in these cells.

The kinetics of the establishment of the primed state

In order to characterise the priming effect in HeLaE cells in more detail, first of all the optimal length of time of the pre-incubation of HeLaE cells with type I IFN was determined. RNA was isolated from a time course of dsRNA induction of HeLaE cells, following exposure to Wellferon for varying lengths of time, and mapped for β -IFN and γ -actin specific messages. The results, as analysed by densitometry, are shown in Figure 3.3. This experiment shows that the onset of the priming response is very slow, with effects not being visible until 4 hours after treatment with 500 units per ml of Wellferon, but continuing to increase in magnitude at all times tested. It was also noted in similar experiments that HeLaE cells could be primed with lower concentrations of type I IFN, a process requiring longer periods of time, and could be primed with type II IFN, using 500 units γ -IFN/ml of medium for 18 hours (data not shown). In all subsequent experiments, cells were routinely treated for approximately 18 to 20 hours with 500 units per ml of Wellferon.

Treating HeLaE cells with dsRNA in the presence of cycloheximide leads to priming-independent induction

The relatively slow time course of the development of the response to priming suggests that IFN may be acting to induce the synthesis of new proteins

which are required for the induction of β -IFN mRNA, consistent with the observations of others (Fujita and Kohno 1981, Enoch and Maniatis 1986). Attempts to test this directly by priming HeLaE cells in the presence of cycloheximide were unsuccessful due to the pronounced cytotoxicity of this inhibitor over a prolonged incubation. However, inclusion of cycloheximide in the induction mixture lead to the production of detectable β -IFN-specific mRNA in the absence of priming, although priming did significantly enhance these levels (Figure 3.4A). Cycloheximide alone had no detectable effect in primed or unprimed cells under the conditions used here (data not shown). This experiment demonstrates that cycloheximide can provide a function that cannot be provided by dsRNA alone in priming-dependent cells.

Sendai virus treatment of HeLaE cells can induce β -IFN without the requirement for priming

In contrast to the effect of dsRNA alone, β -IFN mRNA can be detected in unprimed HeLaE cells when Sendai virus is used as the inducer, although approximately 5-fold more RNA is produced if the cells are primed (Figure 3.4B). This result was unexpected, as it has been generally assumed that the inducing signal provided by the transcription and replication of the negative-stranded paramyxovirus genome, in common with other viruses, is dsRNA (see Chapter One).

It is also interesting to note that the β -IFN induction profiles observed in HeLaE cells in Figures 3.4 and 3.5 are very similar in both the unprimed and the primed states (i.e the peaks of induction are at 4 hours in Fig 3.4, and 2 hours in Fig 3.5), indicating that priming does not alter the kinetics of induction in these cells.

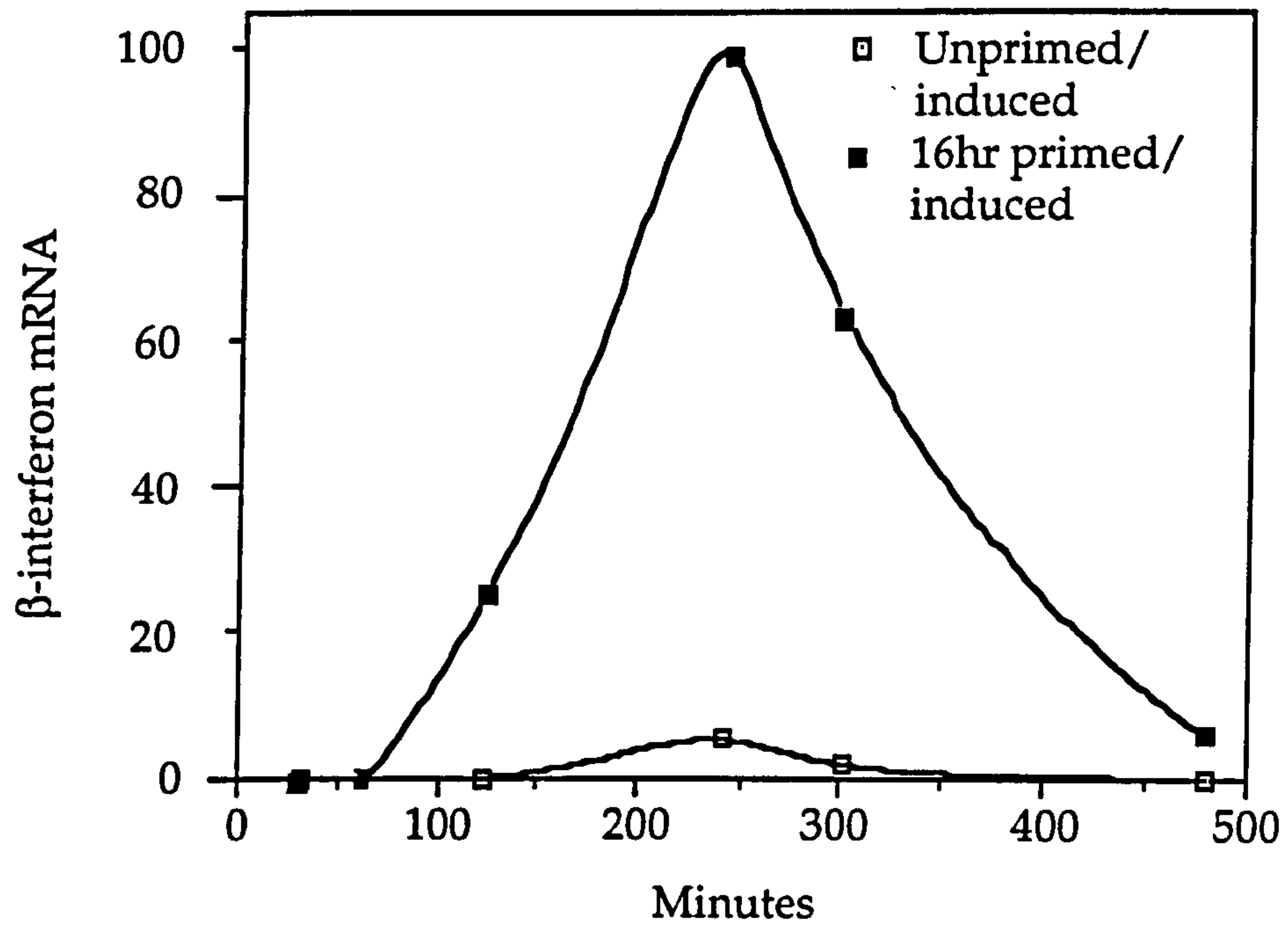
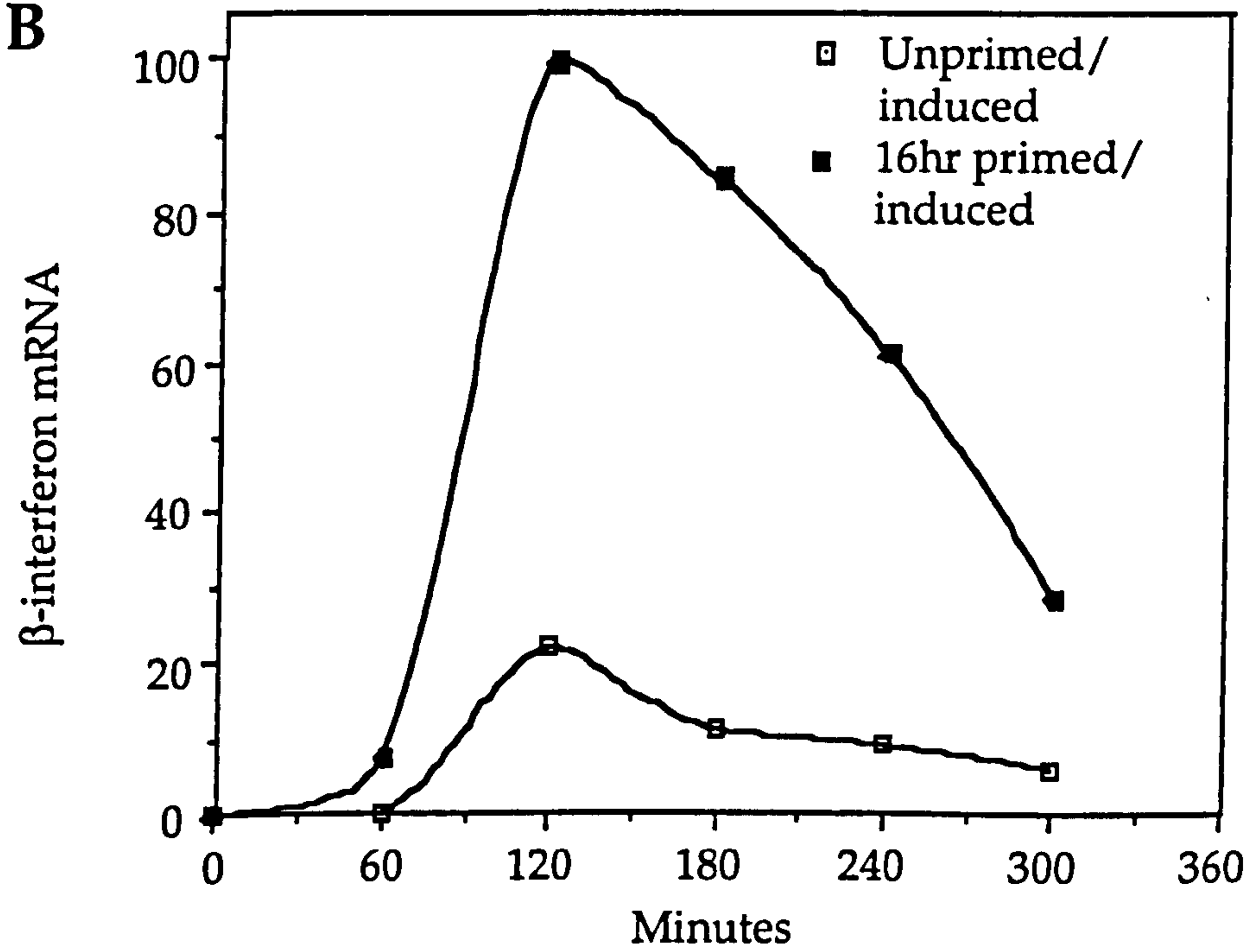
The primed state persists after IFN withdrawal

In the experiments described so far, cells have been exposed to interferon for up to twenty hours and then induced at the end of this incubation. In an effort to determine whether the effects of priming can be observed following the removal of Wellferon and continued growth of HeLaE cells in culture, that is to see if priming has a "memory", HeLaE cells were primed for eighteen hours and the Wellferon-containing growth medium was then replaced with normal medium. Cells were allowed to grow for up to two days further, and then induced with dsRNA (Figure 3.5). Although the level of induction observed is not as great as that obtained immediately following an overnight incubation with Wellferon, the clearly

Figure 3.4 Priming-independent induction in HeLaE cells with the inclusion of cycloheximide or the use of Sendai virus as inducer

(A). HeLaE cells were treated with dsRNA and cycloheximide (50 μ g/ml) over the time course shown, in the unprimed and primed (16 hours Wellferon treatment) states. The RNA from each time point sample was analysed by RNase protection and the autoradiograph was analysed by densitometry, with the β -IFN signal being corrected to the corresponding actin signal. The relative β -IFN expression was then plotted.

(B). HeLaE cells were treated with Sendai virus (7500 haemagglutination units/ml induction medium) over the time course shown, in the unprimed and primed (16 hours Wellferon treatment) states. The β -IFN expression was analysed and depicted as described in A.

A**B**

detectable β -IFN RNA in lanes 7 and 10 indicate that although the primed state is slow to establish, once primed, HeLaE cells can respond to dsRNA treatment some days later. It was further shown that the conditioned medium from primed HeLaE cells allowed to grow in culture following the removal of Wellferon cannot prime untreated HeLaE cells, indicating that the primed state is not being maintained by a secreted factor (data not shown). Figure 3.5 also indicates that HeLaE cells can be primed by the addition of Wellferon for a short period of time, in this case one hour, if then left in culture for a further 17 hours, although the magnitude of the subsequent induction is not as great as that seen with a continuous exposure to IFN (compare lanes 3 and 4).

Discussion

The experiments described in this chapter have established that whereas some cell lines, such as MG63, are inducible by dsRNA in the absence of priming, a HeLa subline, HeLaE, is completely uninducible by dsRNA unless primed. The length of incubation with IFN required to achieve a fully primed state, up to 20 hours, strongly suggests that this process requires protein synthesis, although this has not been shown directly due to the cytotoxicity of cycloheximide in this cell line. The primed state lasts for at least 48 hours following an overnight incubation with IFN, and it has been reported that it is even transmissible to daughter cells in human foreskin fibroblast cells (Chen 1985). Conditioned medium from primed HeLaE cells failed to prime other HeLaE cells, indicating that a secreted factor is not involved in maintaining the primed state, and this would suggest that any intracellular factor(s) induced during the priming process that make HeLaE cells permissive for induction by dsRNA would be very stable.

In contrast to the absolute dependence upon priming for induction by dsRNA in HeLaE cells, some priming-independent β -IFN expression is seen when either cycloheximide is included in the induction mix with dsRNA or Sendai virus is used as the inducer. The effects of protein synthesis inhibition upon induction ("superinduction") have been well documented (reviewed in (Goodbourn 1990)), and at least part of this effect operates at the level of transcription (Ringold et al. 1984). One explanation for the effect of cycloheximide is that it allows derepression of the β -IFN promoter by blocking the synthesis of labile repressor molecules. It is possible that dsRNA alone might be inefficient at accomplishing this in the absence of a priming-dependent enzyme.

Figure 3.5 Primed HeLaE cells can respond to dsRNA treatment up to two days later.

HeLaE cells were treated with Wellferon for 18 hours, after which the IFN was removed, the cells washed extensively with DMEM and then replaced with DMEM/10% FCS. At this point the cells were either treated with dsRNA or left in culture for a further 24 or 48 hours before induction. The autoradiograph of the RNase protection is shown.

On day 1 (i.e. immediately following IFN withdrawal) the cells were treated as follows:

Lane 1: uninduced

Lane 2: induced

Lane 3: induced following 1 hour Wellferon treatment and then 17 hours recovery in DMEM/10% FCS

Lane 4: primed and induced

On day 2 (i.e. 24 hours following IFN withdrawal) the cells were treated as follows:

Lane 5: uninduced

Lane 6: induced

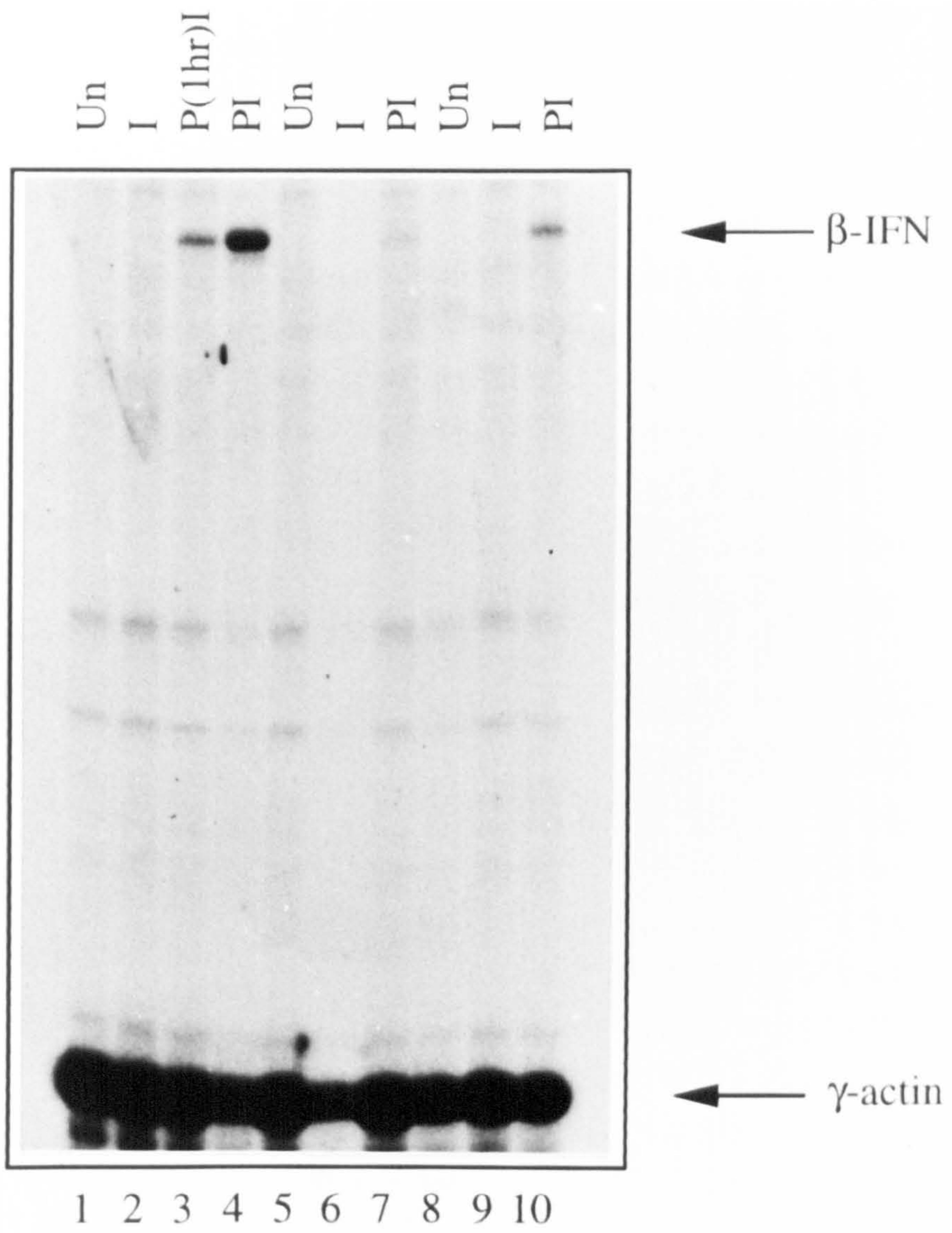
Lane 7: primed and induced

On day 3 (i.e. 48 hours following IFN withdrawal) the cells were treated:

Lane 8: uninduced

Lane 9: induced

Lane 10: primed and induced



The ability of Sendai virus to induce β -IFN expression without a requirement for priming is surprising, although the magnitude of induction can be enhanced by IFN treatment. This fundamental difference in the requirements for induction indicates that Sendai virus can provide an alternative or additional signal to dsRNA. Since some degree of induction is seen in the absence of priming when dsRNA is used together with cycloheximide, it is possible that priming-independent induction by Sendai virus simply reflects a combination of the ability of the virus to block cellular protein synthesis and to provide dsRNA. However, this is unlikely, since Sendai virus is a more efficient inducer in unprimed cells than the mixture of dsRNA and cycloheximide, which can block protein synthesis under these conditions by more than 99% (Whiteside et al. 1992). It is therefore more likely that Sendai virus can provide a second signal for induction. Because cycloheximide has the effect of rendering cells at least partially independent of the need for priming, it has proved difficult to investigate whether priming-independent induction by Sendai virus requires protein synthesis. However, it should be noted that the permissiveness for dsRNA induction takes a long time to establish following IFN treatment, whereas certain batches of Sendai virus, including the one used in Figure 3.5, rapidly induce β -IFN without the need for priming, suggesting that no new protein synthesis is required for induction.

Two types of mechanism may be envisaged for a direct involvement of Sendai virus. Firstly, the Sendai virion may provide a function that can substitute for the effect of priming. Secondly, the virus might trigger a signal transduction pathway that can substitute for priming. Since Sendai virus binds to target cells through a relatively non-specific interaction with sialic acid groups on a variety of surface proteins (Kingsbury 1991), it is unlikely that the putative activation event involves a membrane-linked signal. Similarly, the intracellular processing of absorbed Sendai virus offers no clear mechanism for the activation of cellular signalling events. If the priming independent induction by Sendai virus does depend upon protein synthesis, it is possible that a virally-encoded protein can substitute for a cellular protein that is normally provided by IFN treatment, and indeed the C protein of Sendai has been described as being an inducer of β -IFN (Taira et al. 1987). Alternatively, viral infection may induce the synthesis of cellular factors that can substitute for priming, or may induce the same factor(s) that are normally induced by priming. In support of the latter hypothesis, it has been observed that adenoviral particles can activate the synthesis of genes that are normally induced by IFN treatment (Reich et al. 1988).

These lines of investigation have not been pursued further due to the variability between different batches of Sendai virus. The reason for this variability is not well understood but it was observed that the effects on the kinetics of induction and on the level of priming-independent induction observed in HeLaE cells could be profound, in extreme cases with the peak of induction of the β -IFN gene varying from two hours to up to eight hours or longer (data not shown). It is possible that the nature of the batch of virus reflects the ratio of live virus to defective interfering particles within it, but this was not an easy parameter either to verify or to control, and the absence of such information made comparisons between experiments using different batches of virus very difficult.

On the other hand, the ability of different batches of dsRNA to induce the β -IFN gene was found to be extremely reproducible (data not shown), and this characteristic of dsRNA, together with its total inability to induce the β -IFN gene in unprimed HeLaE cells led us to concentrate on this combination of cell line and inducer to study the role of priming in the induction of β -IFN in the experiments presented in this thesis.

CHAPTER FOUR - THE β -IFN PROMOTER RESPONDS TO PRIMING THROUGH MULTIPLE INDEPENDENT REGULATORY ELEMENTS

Introduction

The experiments described in Chapter Three show that in HeLaE cells the inducibility of the endogenous β -IFN gene by dsRNA is completely dependent on priming. As it has previously been suggested that the priming effect operates at the level of transcription (see Chapter One), it is thus reasonable to consider the priming effect a means by which to study the transcriptional regulation of the β -IFN gene in a conditionally inducible manner, and therefore hopefully gain an insight into the mechanisms by which this regulation is controlled. In this Chapter the transcriptional nature of priming was investigated, and the contribution of different regions of the promoter to the priming response was assessed.

Priming dependence is a property of the β -IFN promoter

In an initial experiment to verify whether the priming-dependence was a property of the β -IFN promoter, a fragment of the β -IFN promoter spanning sequences -116 to -12 (relative to the cap site at +1) was fused to a β -globin transcription unit (Figure 4.1A). This construct was linked to a G418 resistance gene, and transfected into HeLaE cells. Several hundred G418-resistant colonies were selected and pooled, and the pools were analysed, using RNase protection, for their ability to respond to induction. Figure 4.2A shows that expression of the β -globin mRNA is significantly enhanced by, although not completely dependent upon, priming for inducibility by dsRNA, and thus, the -116 to -12 fragment behaves in a similar manner to the endogenous β -IFN gene, although the priming dependence is not absolute. The effect of priming was further localised, since induction was still enhanced in response to Wellferon pre-treatment when the -116 to -36 region was fused upstream of a heterologous TATA box from the HSV thymidine kinase promoter, which is itself unaffected by these treatments (Figure 4.2B, plasmid depicted in Figure 4.1B). The inclusion of cycloheximide in the induction mix in these experiments leads to superinduction of both the endogenous β -IFN gene and the transgene.

Figure 4.1 Plasmids used in Figures 4.2 and 4.3

(A). ptk Δ [-105] β -globin.neo contains HSV tk promoter sequences from the Bam HI site (-105) to the MluI site (-15) of pBLCAT2 (Luckow and Schutz 1987) fused upstream of a β -globin gene. This promoter/transcription unit is inserted into the EcoR I site of pKO.neo (Sambrook et al. 1989).

(B). pIF[-116/-12] β -globin.neo was created by replacing the tk promoter sequences in A with β -IFN sequences from -116 to the Nco I site at -12.

(C). pIF[-116/-36] β -globin.neo was created by fusing β -IFN sequences from -116 to -36 upstream of the tk promoter in ptk Δ [-39] β -globin.neo, which is equivalent to the plasmid in A except that the promoter only extends as far as -39.

(D). pIF[-116/+72]lucifer contains β -IFN sequences from -116 to 72 fused to position -17 of a firefly luciferase gene (DeWet et al. 1987). Upstream of the β -IFN sequences is a dimeric SV40 terminator element that helps to block read-through transcription.

(E). ptk Δ [-105]lucifer has a -105 to -15 tk promoter fragment fused to -17 of the luciferase gene, again with the SV40 terminator elements upstream.

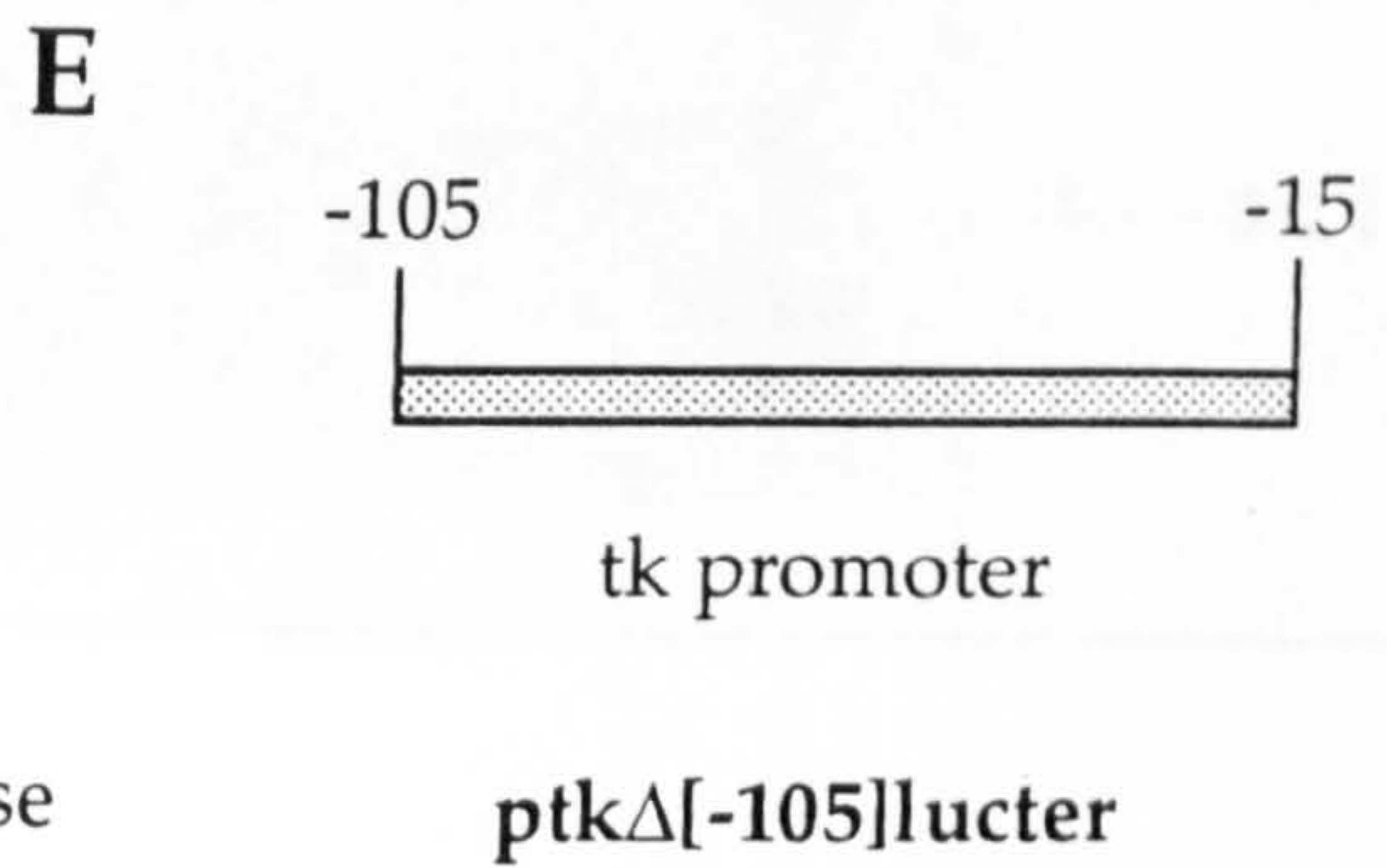
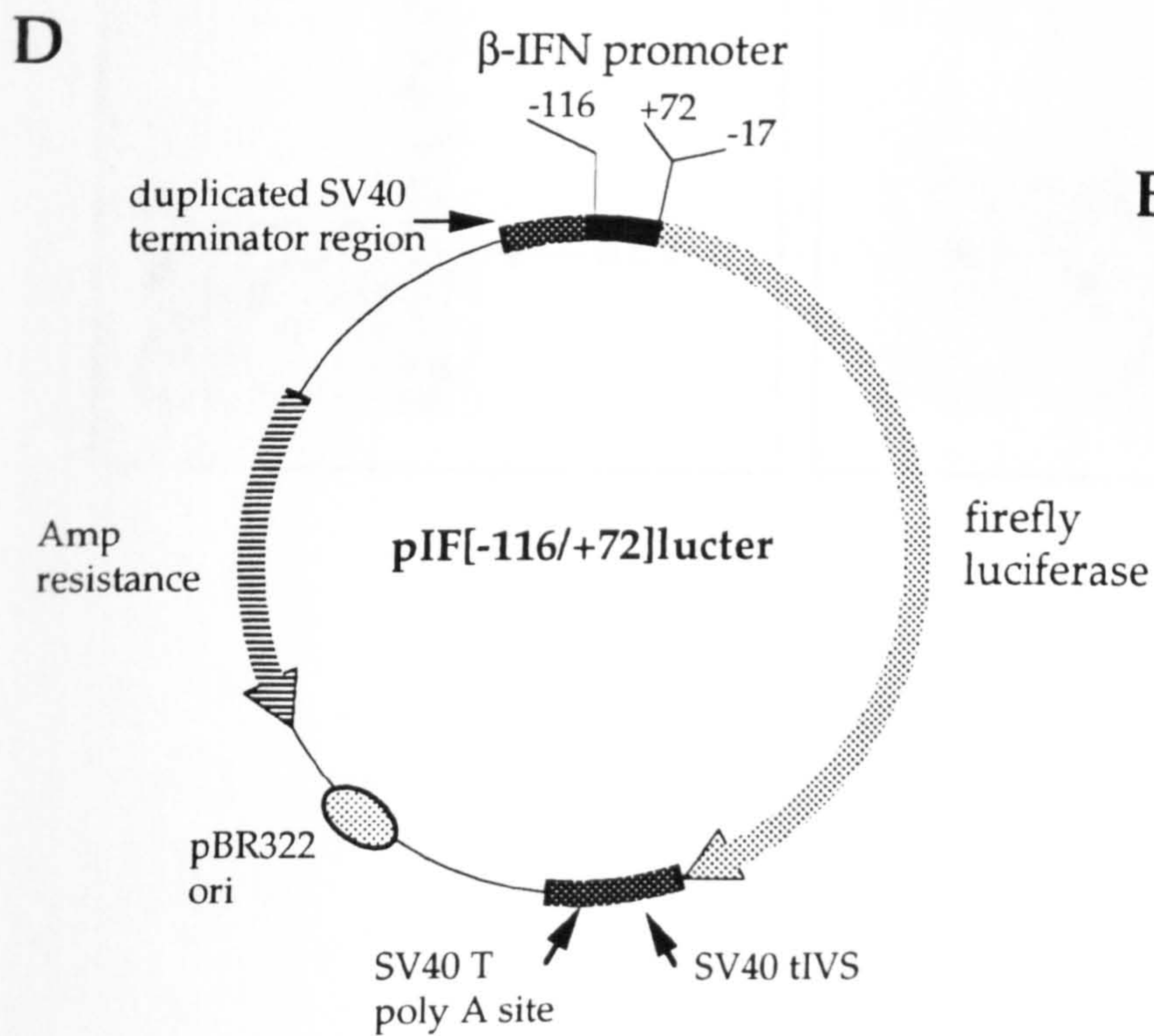
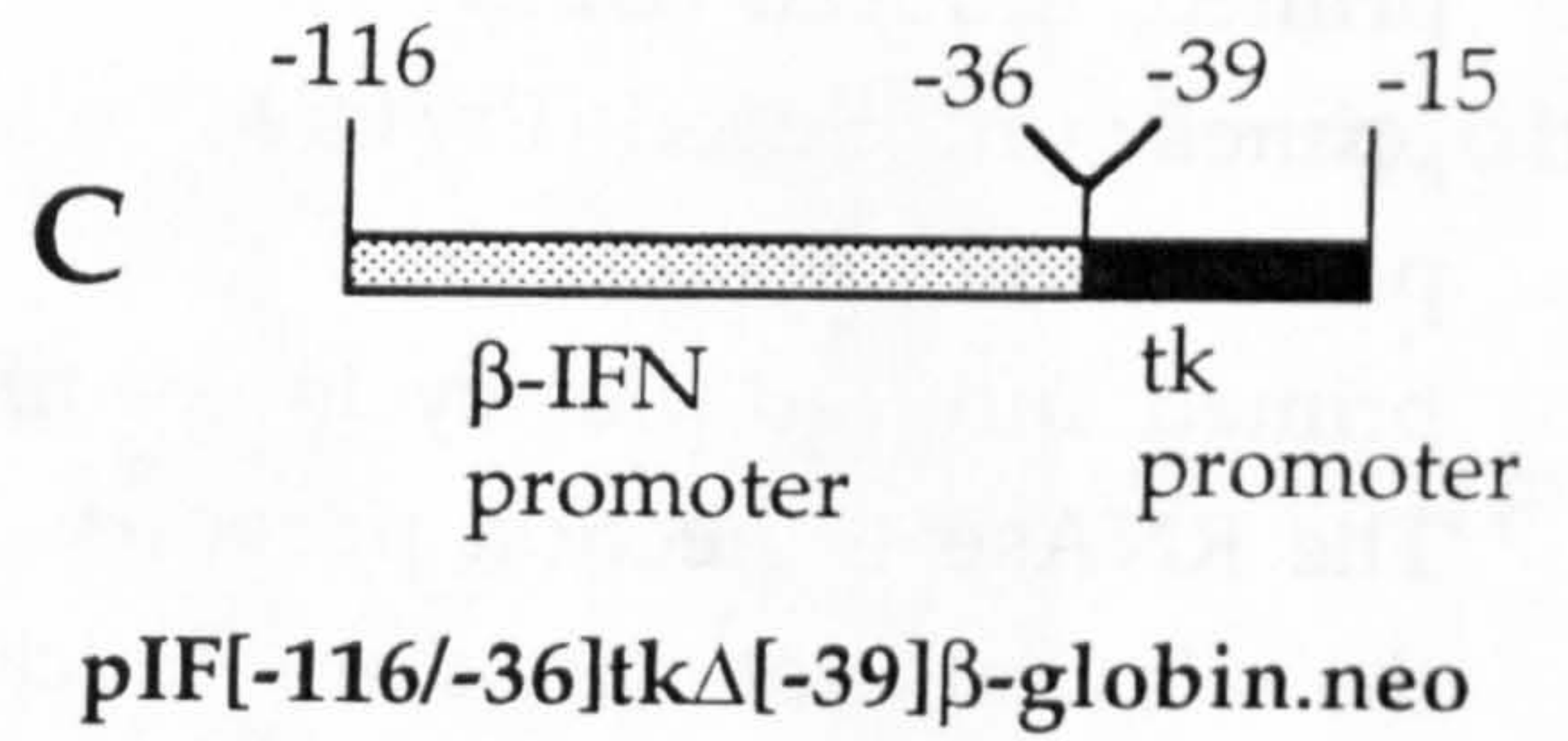
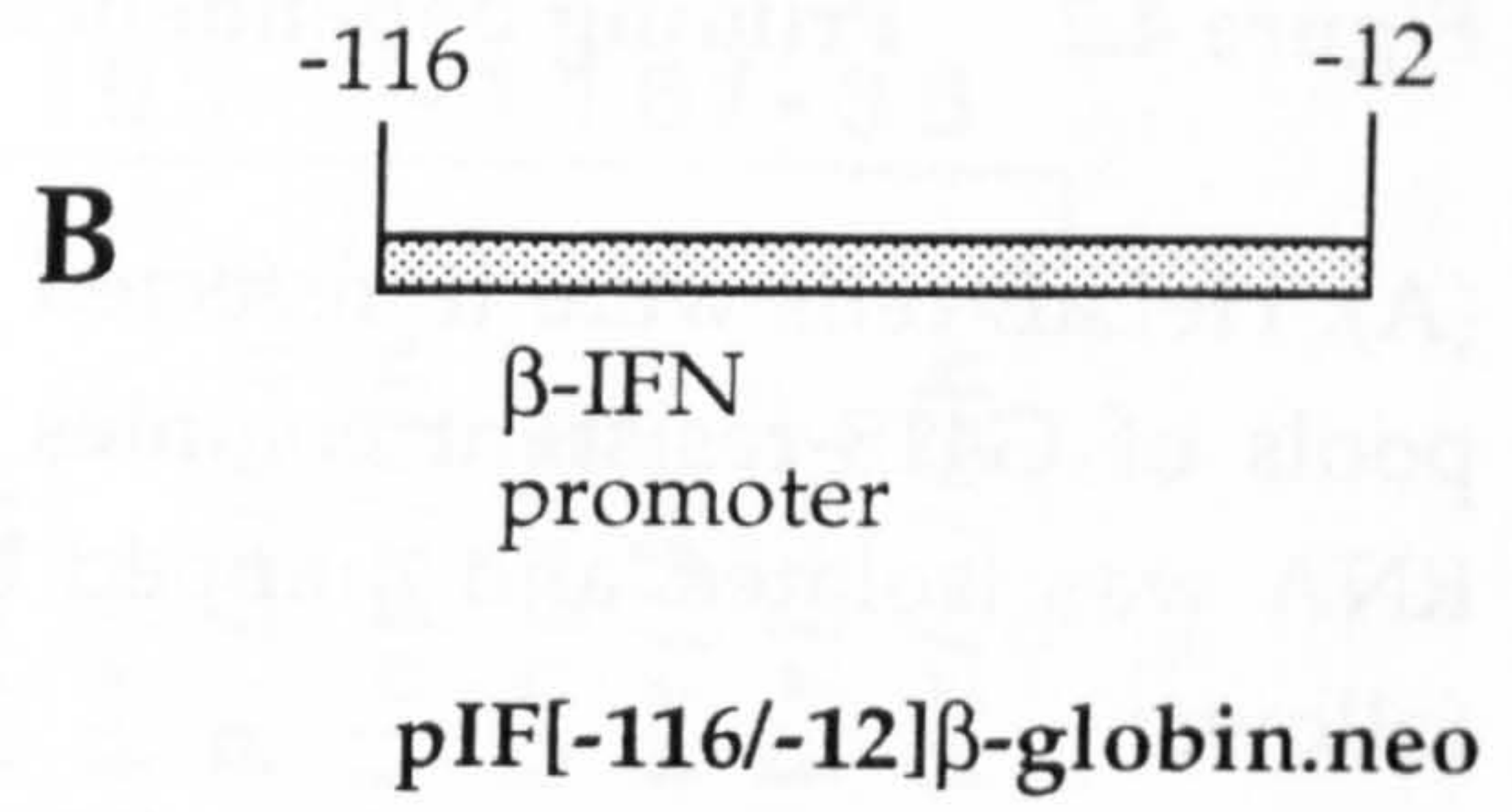
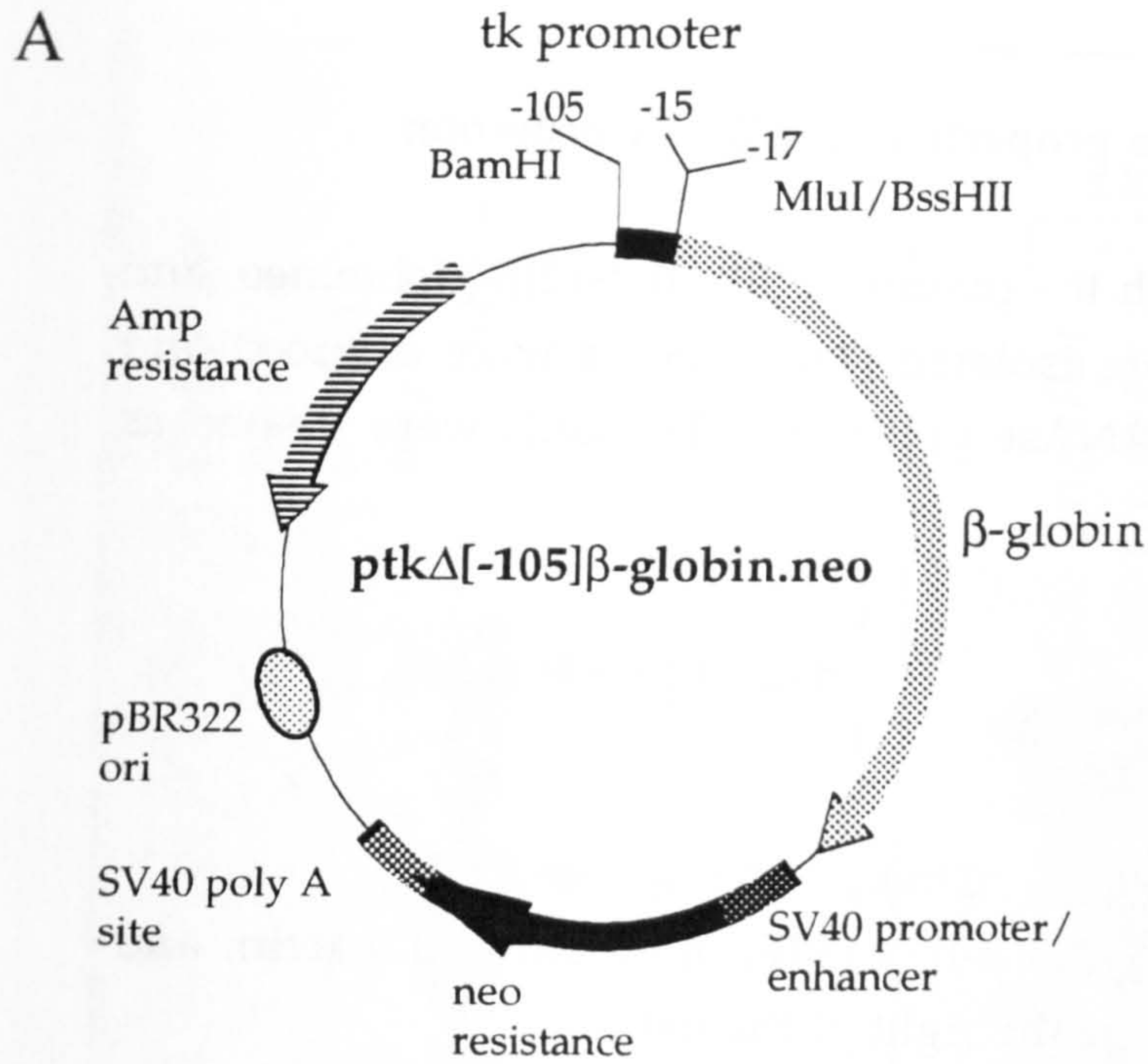


Figure 4.2 Priming dependence is a property of the β -IFN promoter

(A). HeLaE cells were transfected with the plasmid pIF[-116/-12] β -globin.neo, and pools of G418-resistant colonies were isolated. These pools were induced and RNA was isolated and mapped by RNase protection. The cells were treated as follows:

unprimed, uninduced (Unpr/Un),

primed, induced (Unpr/+),

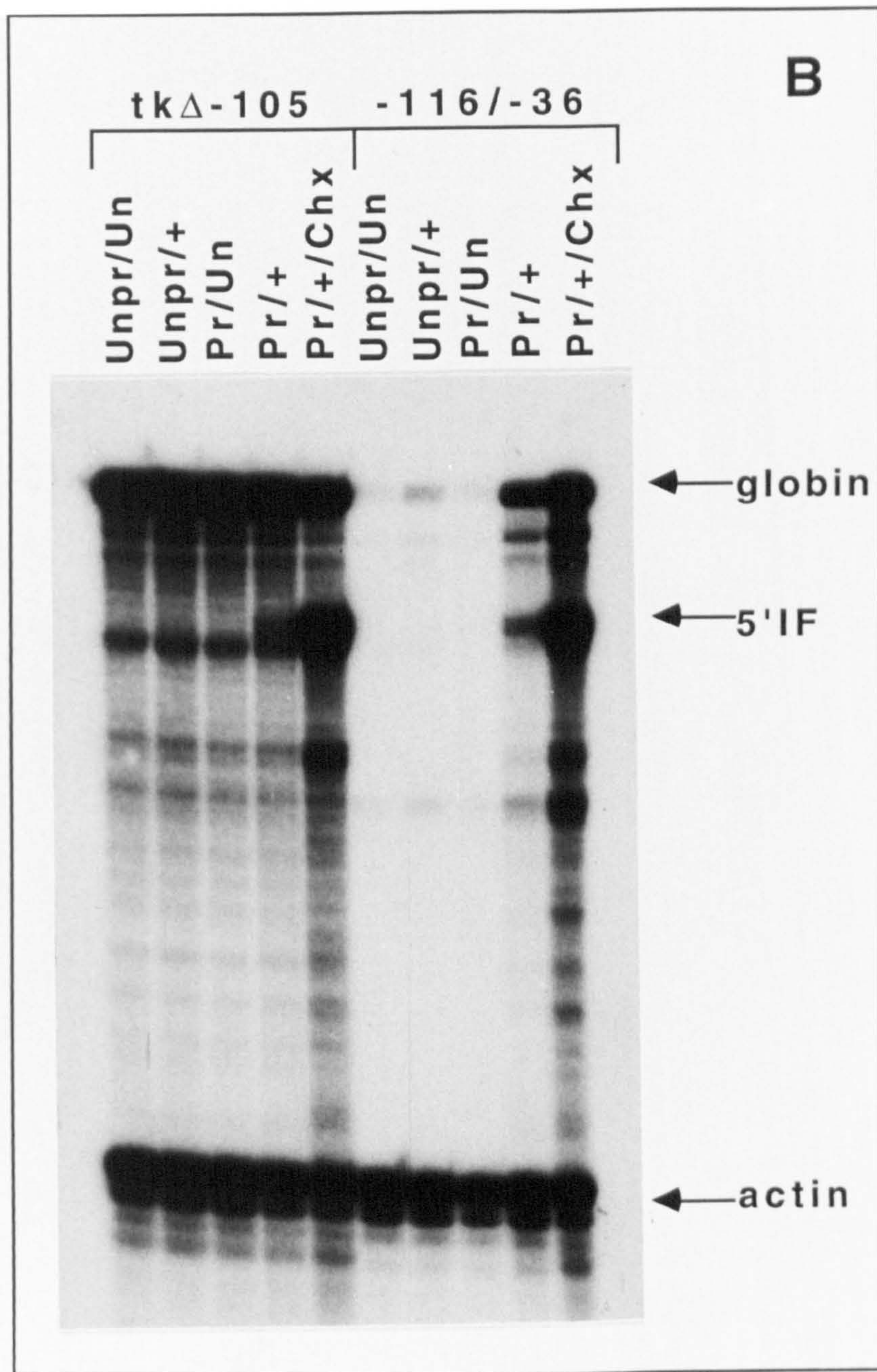
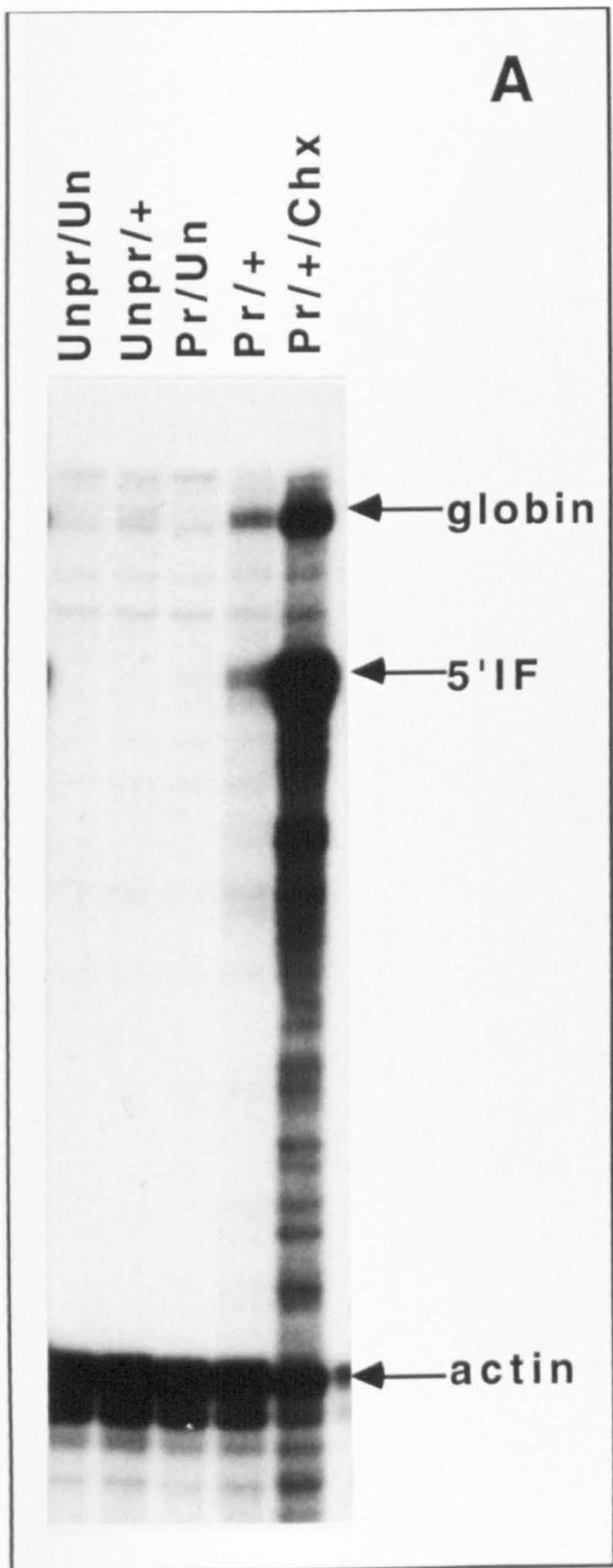
primed, uninduced (Pr/Un),

primed, induced (Pr/+),

primed, induced plus cycloheximide (Pr/+/Chx).

The RNase protection probes used were β -IFN (5'IF), β -globin and γ -actin, and the relevant protections are indicated at the right of the gel.

(B). HeLaE cells were transfected with either ptk Δ [-105] β -globin.neo (tk Δ -105) or pIF[-116/-36]tk Δ [-39] β -globin.neo (-116/-36) as indicated, and pools of G418-resistant colonies were isolated and analysed as described in (\).



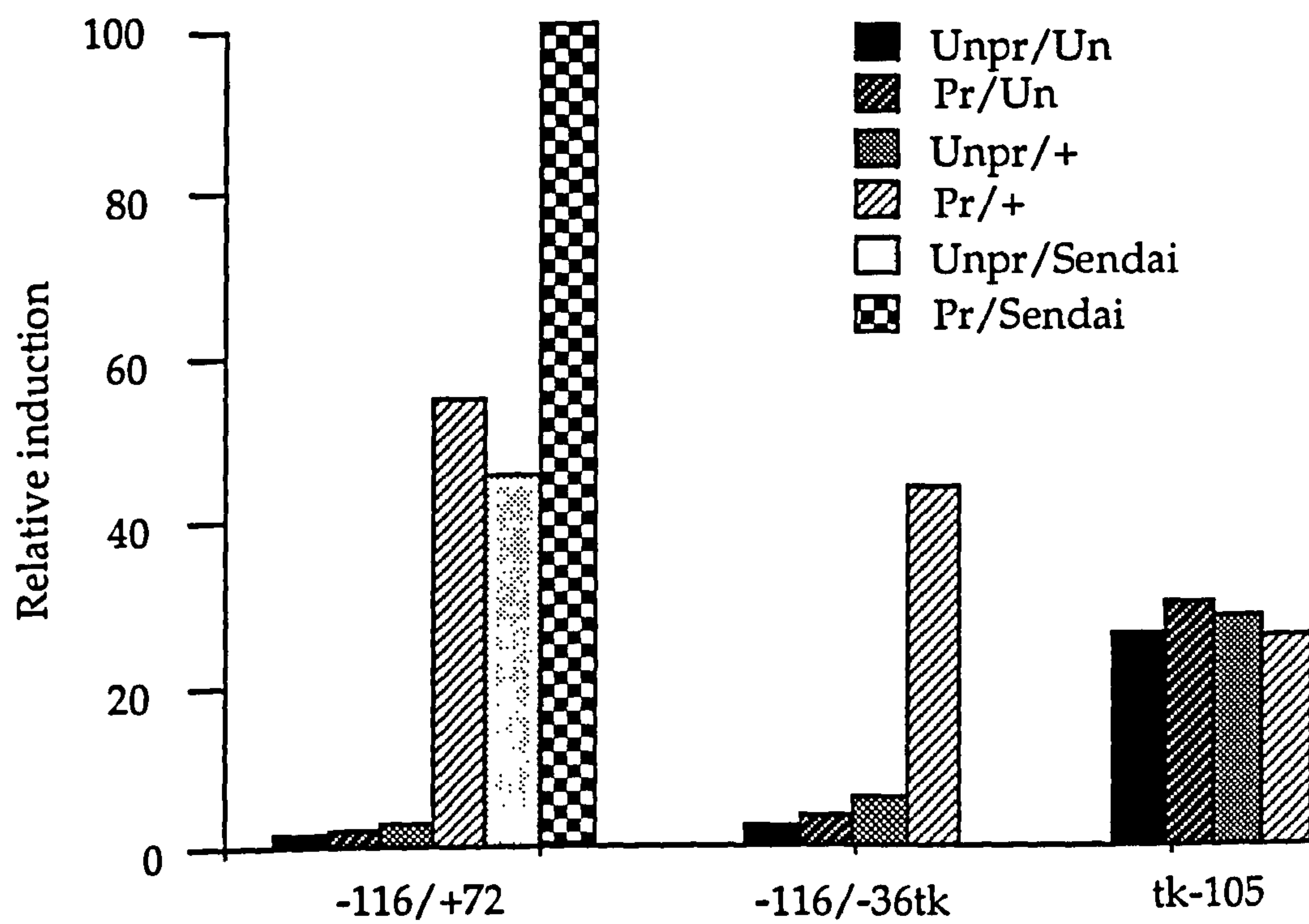
Transiently transfected promoter constructs are priming-dependent

The stable transfection experiments presented above demonstrate that a fragment of the β -IFN promoter as small as 80 base pairs still requires priming for maximal induction in HeLaE cells and indicated that the analysis of promoter deletion mutants was a potentially successful approach for the identification of an element within the promoter responsible for the requirement for priming. Previous studies of the β -IFN promoter had utilised cell lines to map the mRNA transcribed from the stably introduced promoter constructs (Zinn et al. 1983, Goodbourn et al. 1985). In order to simplify the analysis of a large number of deletion mutants and avoid the necessity for the creation of stable lines, which is both time consuming and expensive, a transient transfection system was established using firefly luciferase as a reporter gene. Several reporter genes were tested, including chloramphenicol acetyl transferase (CAT), β -galactosidase (β -GAL) and ornithine transcarbamylase (OTC), but luciferase was chosen for several reasons, chiefly because it was the most sensitive but also because there is essentially no background activity in mammalian cells, unlike CAT and β -GAL, and the protein is relatively unstable and so does not accumulate over the time course of a transient transfection experiment, which can be a problem with both CAT and, to a lesser extent, β -GAL. The stability of the CAT protein, however, made it an attractive choice for a transfection control, because its levels were less affected by changes in translation presumably caused by the modulation of PKR levels and activity during the priming and induction steps in these experiments (see Chapter One), and thus gave a more reliable indication of the efficiency of transfection in a particular experiment.

The stimulatory effects of priming on the inducibility of β -IFN constructs were therefore investigated in transient transfection assays. In a pilot experiment, the -116 to +72 region of the β -IFN promoter was fused to a reporter luciferase gene and transfected, using the calcium phosphate co-precipitation technique, into HeLaE cells, along with a second reporter gene (pBLCAT2) as a transfection control. The construction of this vector (pIF[-116/+72]lucifer) is shown in Figure 4.1C. Figure 4.3 shows that luciferase activity from the β -IFN promoter, normalised to the CAT activity present in each extract, is indeed priming-dependent in response to dsRNA, whereas it is largely independent of priming when Sendai virus is used as the inducer. This result is in line with the observation in Chapter Three that priming-independent induction of β -IFN could be observed when Sendai virus was used as the inducer. As was also seen for the stable transfection experiment (Figure 4.2), the β -IFN sequences upstream from the TATA box (-116 to -36) are sufficient to confer

Figure 4.3 Transiently transfected β -IFN promoter constructs are priming dependent in HeLaE cells

HeLaE cells were transiently transfected, using calcium phosphate, with the following mixtures of plasmids; 15 μ g of pIF[-116/+72]lucifer and 5 μ g pBLCAT2 (-116/+72); 15 μ g of pIF[-116/-36]tk Δ [-39]lucifer and 5 μ g pBLCAT2 (-116/-36tk); 15 μ g of ptk Δ [-105]lucifer and 5 μ g pBLCAT2. The cells were treated as follows: unprimed, uninduced (Unpr/Un), primed, uninduced (Pr/Un), unprimed, induced (Unpr/+), primed, induced (Pr/+), unprimed, Sendai virus induced (Unpr/Sendai), primed, Sendai virus induced (Pr/Sendai), and cellular lysates were analysed for luciferase and CAT activities. The expression is calculated by correcting the luciferase value to the CAT value to normalise for variations in transfection efficiency, and are plotted on a scale of 0 - 100% relative to that achieved by Sendai virus induction of a -116 β -IFN promoter in primed cells.



priming dependence on a heterologous promoter in a transient assay, whilst a heterologous promoter containing no β -IFN sequences (the HSV thymidine kinase -105 promoter, tk Δ [-105]lucifer) does not respond to priming or induction by dsRNA (Figure 4.3).

As observed in Figure 4.3, a transiently transfected [-116/+72] promoter fragment retains priming dependence, and deletions downstream of -73 are uninducible in HeLaE cells (data not shown). It was therefore decided to create a comprehensive deletion series with endpoints that mapped between -116 and -73. Previous attempts to create such a comprehensive deletion series using standard exonuclease digestion procedures had been unsuccessful in generating mutations in certain areas of the promoter. For example, nucleotides between -90 and -79 had been difficult to target using Bal 31 (S. Goodbourn, personal communication). One possible explanation for this is that exonucleases possess strong sequence specificity. In this regard it had previously been noted that Bal 31 digests A-T rich regions of DNA at a much faster rate than G-C rich regions (data not shown) and the -90/-79 region is highly A-T rich (see Figure 4.4A). For this reason it was necessary to develop a method for exonuclease digestion with reduced sequence specificity.

Nuclease digestion patterns of the β -IFN promoter are sequence-specific

In a preliminary experiment, the exonuclease digestion patterns of Bal 31, T4 DNA polymerase and Exonuclease III (Exo III) were compared on the human β -IFN promoter (Figure 4.4B). Over the region shown (-116 to -62), each enzyme produces a non-uniform pattern of digestion products, indicating that the enzymes do indeed display sequence specificity. Bal 31 primarily generates molecules that have 5' ends which map to G residues, while T4 DNA polymerase and Exo III both give a digestion pattern with products highly enriched for molecules with 5' ends which map to A residues. Since the latter enzymes are 3' exonucleases, these data suggest that they are inefficient at removing T residues.

Development of a new method for generating deletion series

Although a range of deletion mutants might have been obtained by combining the Bal 31 and T4 DNA polymerase or Exo III digestion products, it should be noted that many positions are heavily over-represented in the patterns

Figure 4.4. Exonuclease digestion patterns of the β -interferon promoter.

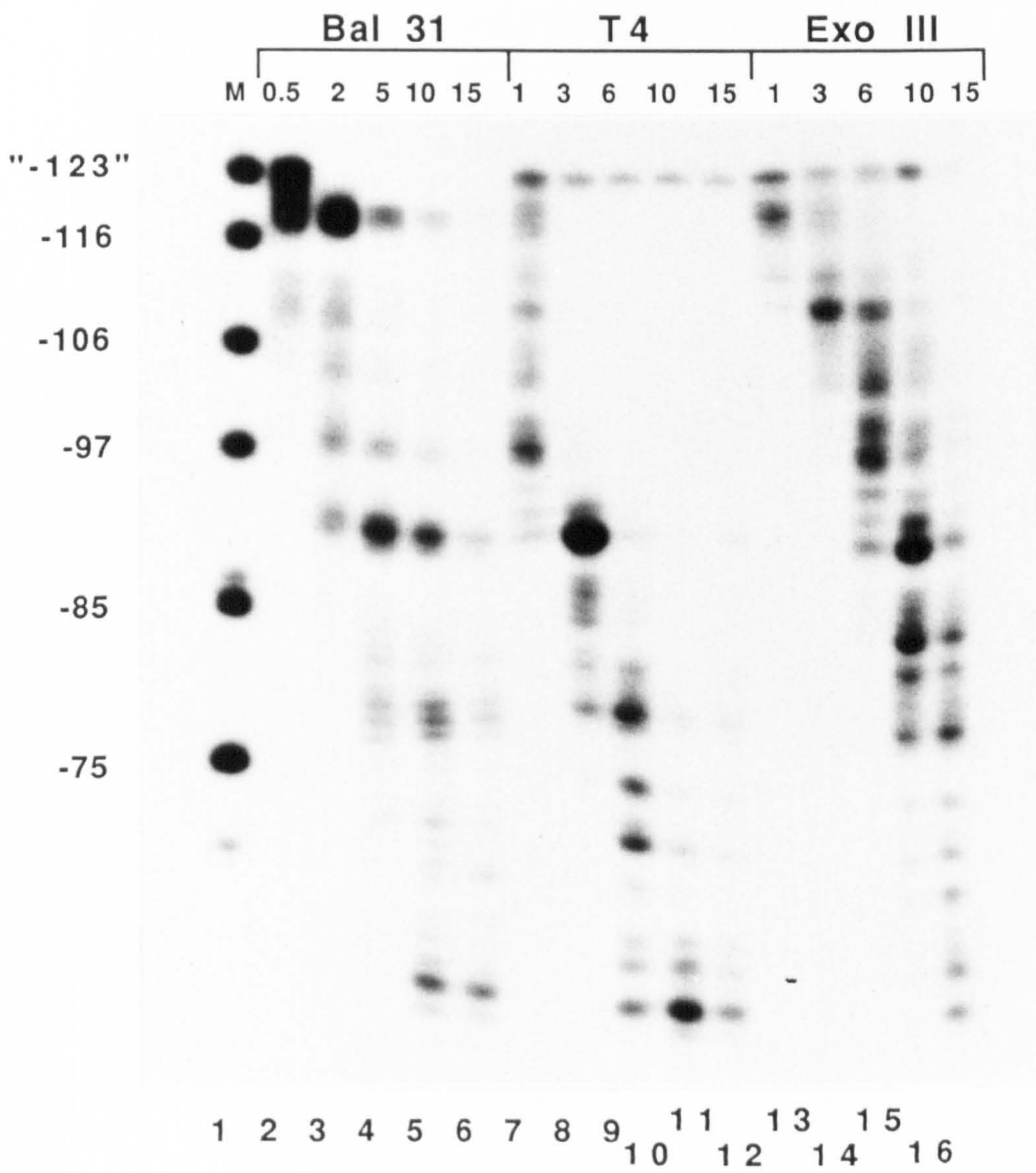
(A). Sequence of the -116/-62 region of the human β -interferon promoter. The GATCCCG sequence between "-123" and -116 is from a BamH I linker introduced at -116. The co-ordinates beneath the sequence correspond to the effective endpoints of clones used as markers in (B).

(B). Bal 31 and T4 DNA polymerase digestions were performed at room temperature on 10 μ g aliquots of pIF[-116/+72]lucifer linearised with BamH I (cutting at -116), using 0.5 units of Bal 31 and 2.5 units of T4 DNA polymerase, respectively, in 200 μ l reaction volumes. For Exo III, a 10 μ g aliquot of BamH I-cut pIF(-116/+72)lucifer was digested with 2.5 units of enzyme in a 125 μ l reaction volume at 37°C. At this temperature Exo III is non-processive (Thomas and Olivera 1978) and the rate of digestion can be controlled by reducing the amount of enzyme used. To assess the extent of nuclease digestion, reaction aliquots from specific timepoints, indicated in minutes above each lane, were restricted with Nco I (cutting at -12) and radioactively labelled with α^{32} P-dCTP and Klenow. Since Exo III and T4 DNA polymerase are 3'-5' exonucleases, it should be noted that it is the non-digested strand which is radio-labelled. In order to reduce the labelled strand to the size of the nuclease digestion products, the Exo III and T4 DNA polymerase reactions were treated with MBN prior to Nco I cleavage. The reaction products were fractionated on a 6% denaturing polyacrylamide gel alongside size markers (M, lane 1). BamH I-cut pIF[-116/+72]lucifer was used to create the largest size marker, "-123" (see above). Other size markers were generated by BamH I digestion of additional deletion clones (pIF[-x/+72]lucifer), and are also indicated on the gel by their effective endpoints.

A

GATCCCGAAACTACTAAAATGTAAATGACATAGGAAAAGTAAAGGGAGAAGTGAAAGTGGG
"-123" -116 -106 -97 -85 -75

B



shown in Figure 4.4B, with the result that the generation of a clone with a specific deletion endpoint could still be laborious. To facilitate the generation of a saturating range of deletion mutants over a given region of the β -IFN promoter, a procedure was developed which generated a much more even distribution of digestion products and which does not show the sequence-specificity associated with standard exonuclease procedures. The principle of this procedure is outlined in Figure 4.5.

Since the technique is based upon a primer extension reaction, it was necessary to prepare the target template in a single-stranded form. This was achieved by cloning the fragment of interest into an M13 vector, but comparable results were also obtained using collapsed supercoiled DNA, prepared under the conditions described for standard DNA sequencing procedure (data not shown). The restriction fragment (delineated by sites A and B) was chosen so that site A (Nco I in these studies) is adjacent to, and site B (Bam HI in these studies) is distal to, the primer binding site. In this representation it was site B that served as the initiation point for deletion reactions.

The first step in the procedure is to anneal a specific primer to the single-stranded template (Figure 4.5, step 1). One advantage of using M13 vectors is that the "universal primer" can be used for any insert. To radiolabel the reaction products for subsequent analysis, an initial labelling reaction is performed with $\alpha^{32}\text{P}$ -dATP and a limiting concentration of the other nucleotides, such that the products do not extend into the region intended for deletion (Figure 4.5, step 2). The labelled products were then extended using DNA polymerase and a dNTP mix containing a single specific α -thio-dNTP (A, G, C or T) which, like in a standard dideoxy sequencing reaction, is incorporated at random (Figure 4.5, step 3). Unlike the dideoxy reaction, the incorporation of the α -thio-dNTP does not terminate the extension reaction (Gupta et al. 1984). The primer extension product is then cleaved at site B (Figure 4.5, step 4) and the resulting product digested with Exo III (Figure 4.5, step 5). This enzyme progressively removes DNA from the 3' end of double stranded DNA, but is blocked by the thioester linkage formed at the position of the α -thio-dNTP incorporation (Putney et al. 1981) giving rise to a base-specific series of products. Indeed this approach has been proposed as a method for sequencing DNA (Labeit et al. 1986).

To convert the reaction products into a form suitable for cloning, the 5' overhanging top strand is very carefully digested with mung bean nuclease (MBN) to produce blunt-ended molecules (Figure 4.5, step 6). In addition, since

Figure 4.5. Deletion mutagenesis scheme

Step 1: The primer, shown as an arrow to indicate the direction of synthesis, is hybridised to a single-stranded template and extended through the region of interest in the presence of a limiting concentration of nucleotides, including one which is radio-labelled. This radiolabelled sequence is shown as a broken line.

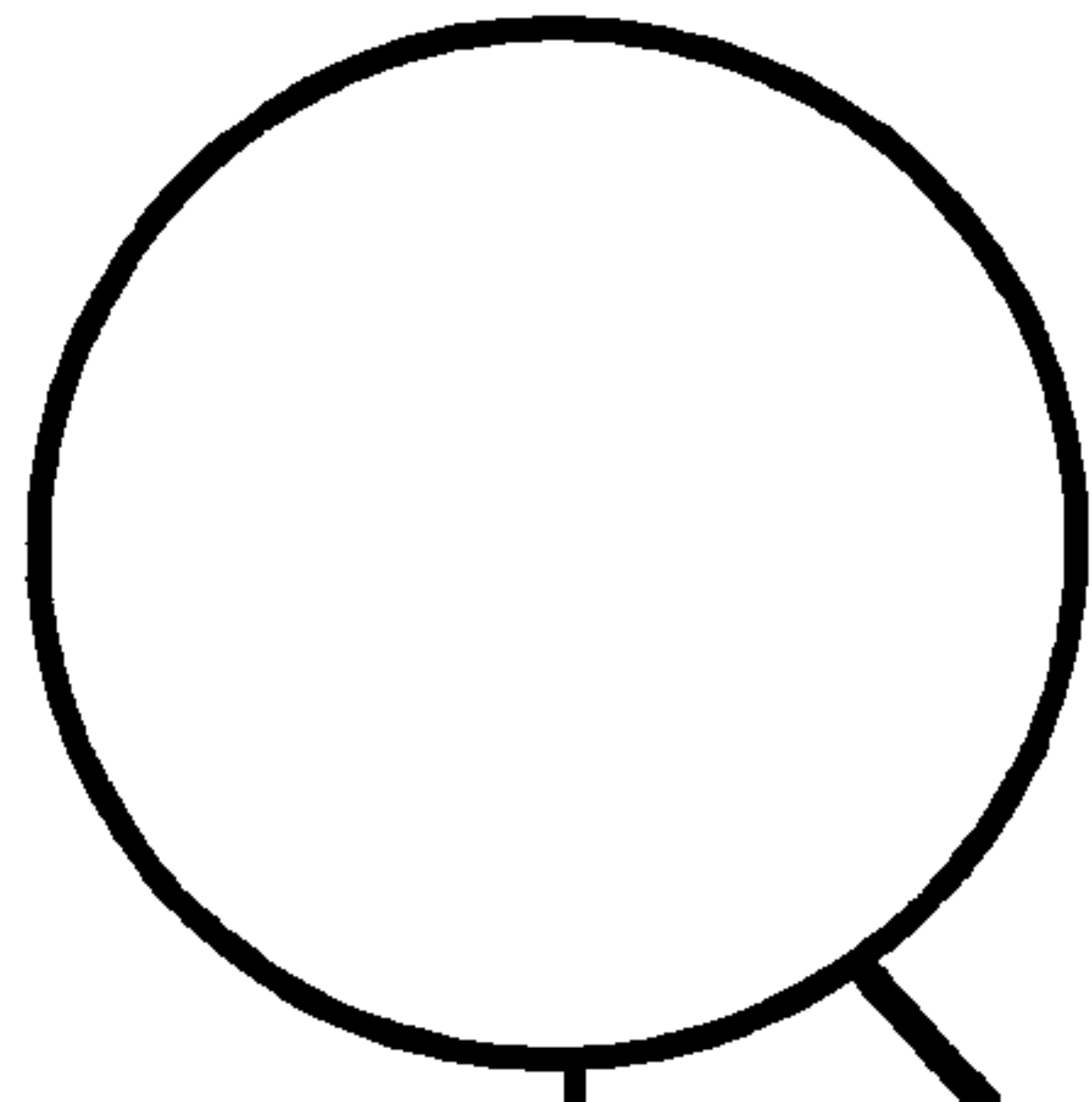
Step 2: The partially extended products are fully extended in the presence of an excess of nucleotide, including a base-specific α -thio-dNTP, indicated as an enclosed S.

Step 3: The four base-specific reactions are cleaved with enzyme B.

Step 4: The cleaved templates are digested with Exo III which is blocked by the α -thio-deoxynucleotides.

Step 5: The reaction products are digested with MBN to generate templates which are now blunt-ended.

Step 6: Reaction products are digested with enzyme A to generate molecules with one blunt (a linker can be ligated to this end if desired) and one unique end which can be subcloned into the vector of choice.



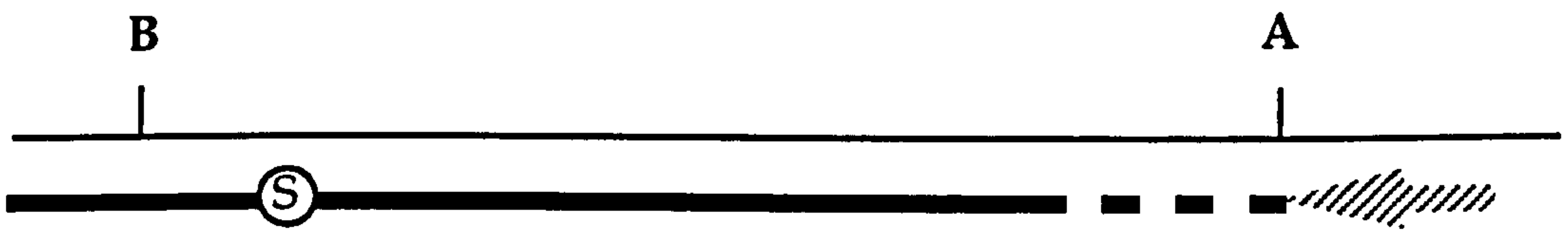
Clone restriction fragment A/B into M13 or other single-stranded vector

1



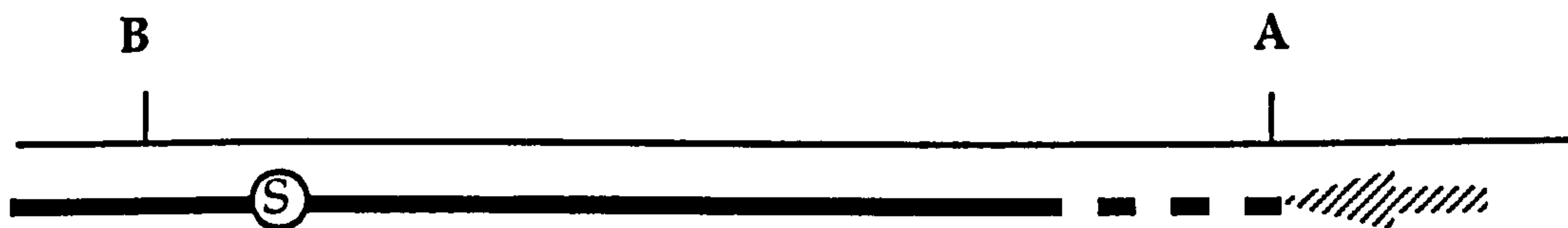
Hybridise template to universal or appropriate primer and extend with labelled nucleotide mix

2



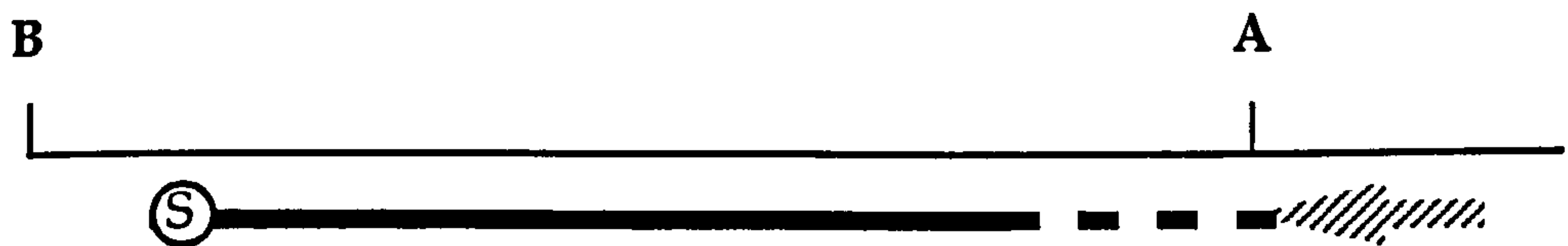
Extend with excess nucleotide in the presence of (alpha-thio) dNTPs(S)

3



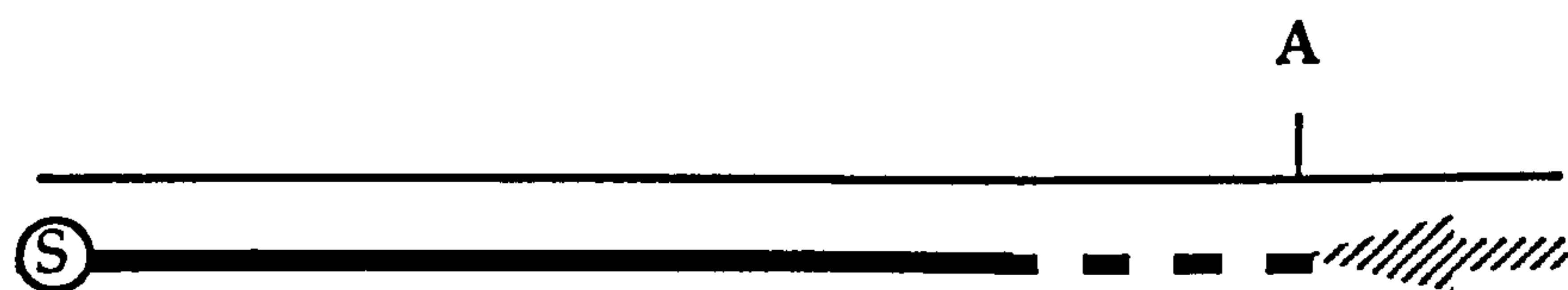
Restrict product with enzyme B to generate startpoint for 5' deletions

4



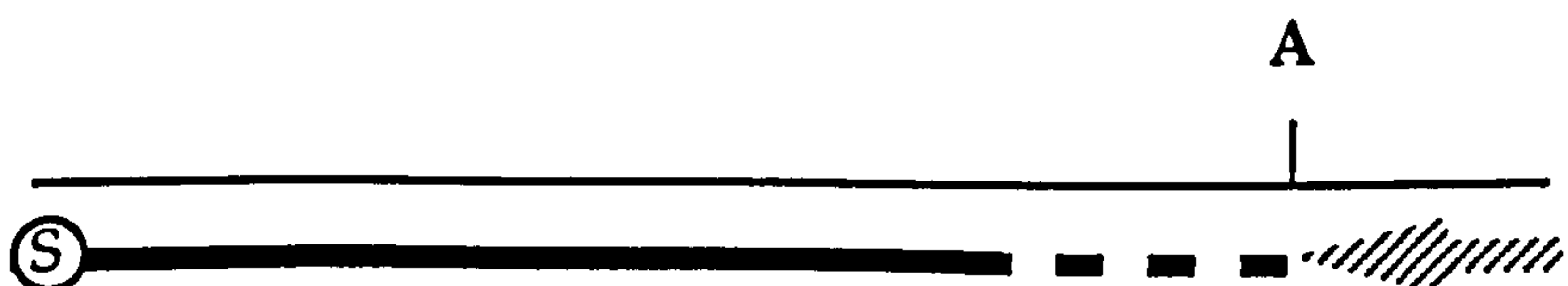
Remove 3' overhang with exonuclease III - enzyme is blocked by thio-ester linkage

5



Remove 5' overhang with mung bean nuclease - deletions with defined end points are generated

6



Product can be cloned by digesting with A (a linker can be added to the blunt end if desired)

the primer ends of the molecules have a substantial 3' overhang on the non-labelled strand, which cannot serve as templates for Exo III, the 5' end of the labelled strand is protected from digestion. The templates are then digested at site A and cloned, following the addition of a linker to the blunt end if desired (Figure 4.5, step 7).

Figure 4.6 shows an experiment performed under conditions described in Materials and Methods. Under these conditions a sequencing ladder is obtained from -116 to around -20. Preliminary analysis of clones obtained from the processed material (lanes 14-17) indicated that the deletion endpoints corresponded to the base-specific α -thio-dNTP used, and were distributed randomly throughout the region encompassed by the sequencing ladder (data not shown).

Since the generation of clones in the region -116 to -73 was of primary interest, the possibility of varying the target area for digestion products was investigated. Although it is possible to modify the concentration of nucleotides in the labelling reaction so as to alter the position at which the initial α -thio-dNTP gets incorporated, more reproducible results were obtained by altering the ratio of α -thio-dNTP to dNTP in a specific mix. Figure 4.7 shows the results of an experiment in which the dNTP to α -thio-dNTP ratio was systematically varied. The optimal ratio to target the -116/-77 region of the human β -interferon promoter is 1:1 for A, G and C (*i.e.* 40 μ M dNTP, 40 μ M α -thio-dNTP, lanes 9-11) and 3:1 for T (*i.e.* 60 μ M dTTP, 20 μ M α -thio-dTTP, lane 16). The requirement for a lower concentration of α -thio-dTTP presumably reflects the over-abundance of A residues on the top strand of this region of the β -interferon promoter. It should be noted that the α -thio-dNTP to dNTP ratios required to target a 40 base-pair region are higher than would be expected if both types of nucleotide are incorporated into the template with equal efficiency. However, a limited degree of Exo III digestion was observed when all the nucleotides incorporated in the primer extension reaction were thio-substituted (lanes 1-4), suggesting that at least part of the requirement for high α -thio-dNTP concentrations was due to the ability of Exo III to digest thioester linkages under these conditions.

To investigate the fidelity of the manipulations described above, the products of an experiment designed to target digestion products to the -116 to -77 region were cloned into the vector pIF8, using Bgl II linkers, and transformed into *E. coli* using an electroporation procedure. Since each deletion was generated in relatively small quantities in the above procedure, it was found that this subcloning was the most difficult step in the protocol. Three steps in the procedure were particularly

Figure 4.6. Generation of base-specific termination products.

An example of an experiment following the protocol depicted in Figure 4.5 is shown.

M13IF[-116/+72] was constructed by inserting the BamH I/EcoR I [-116/+72] fragment from pIF[-116/+72]lucifer into BamH I/EcoR I cut M13mp19. One microgram of ssDNA from this clone was used in the experiment, annealed to 5ng of primer complementary to the [-10/+7] region of the insert.

Lane 1: Primer extension reaction in the presence of limiting nucleotide concentrations to label templates. (This is Step 2 from Figure 4.5)

Lanes 2-5: Extension reactions with base-specific α -thio-dNTP mixes. Since the products are extended well beyond the BamH I site at -116 they migrate very slowly and have been removed from the autoradiograph. (Step 3 from Figure 4.5)

Lanes 6-9: Extended products were digested with BamH I (lanes 6-9). (Step 4 from Figure 4.5)

Lanes 10-13: Reaction products were digested with Exo III. (Step 5 from Figure 4.5)

Lanes 14-17: Reaction products were digested with MBN. (Step 6 from Figure 4.5)

Experimental conditions are as described in the text.

1 2 3 4 5
A G C T | A G C T | A G C T | A G C T

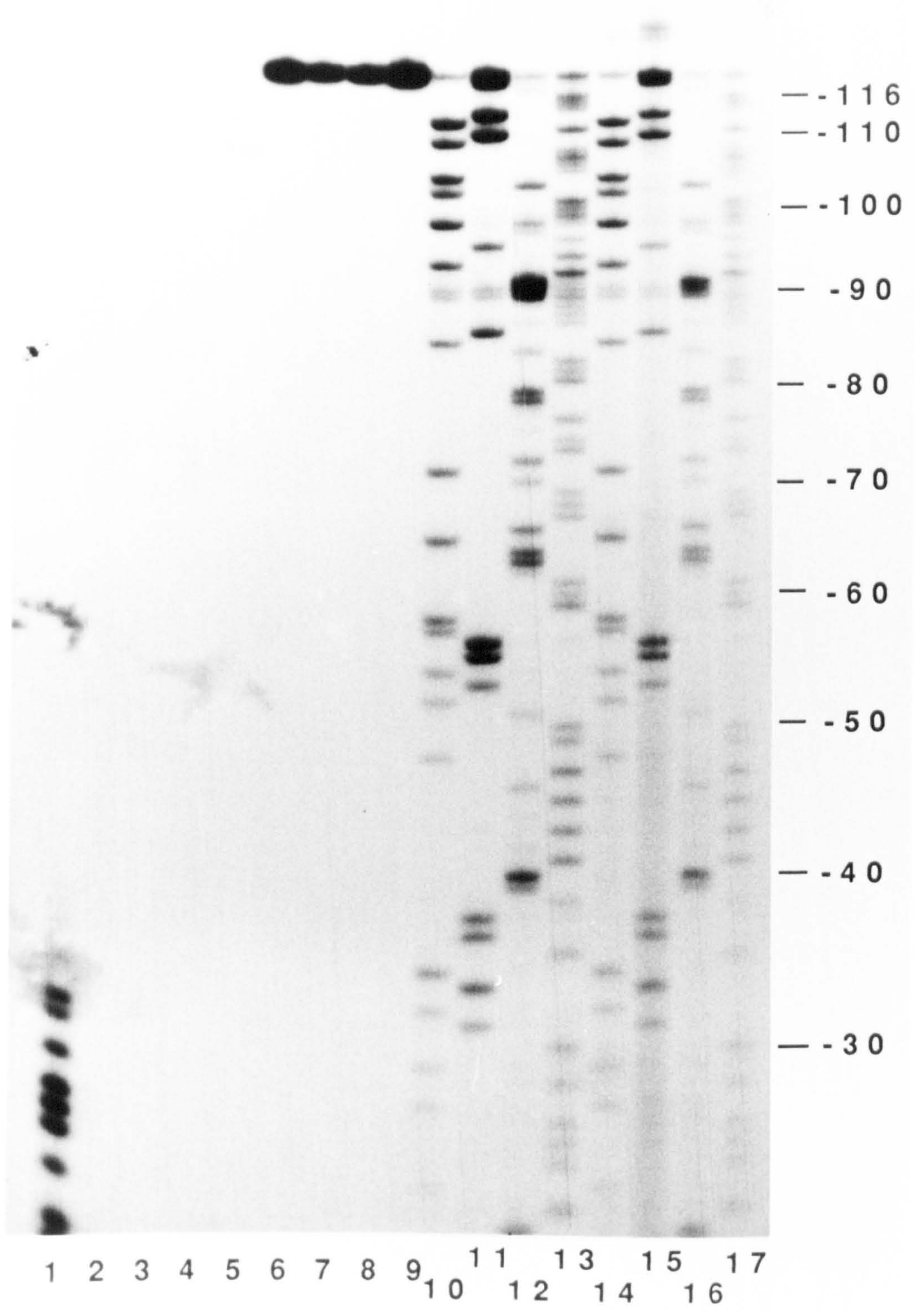


Figure 4.7 Effect of varying α -thio-dNTP concentration on the extent of Exo III digestion.

Exo III reactions were performed on labelled and BamH I-cut template, prepared as described in Figure 4.6, previously extended in the presence of:

Lanes 1-4: 80 μ M α -thio-dNTP/0 μ M dNTP

Lanes 5-8: 60 μ M α -thio-dNTP/20 μ M dNTP

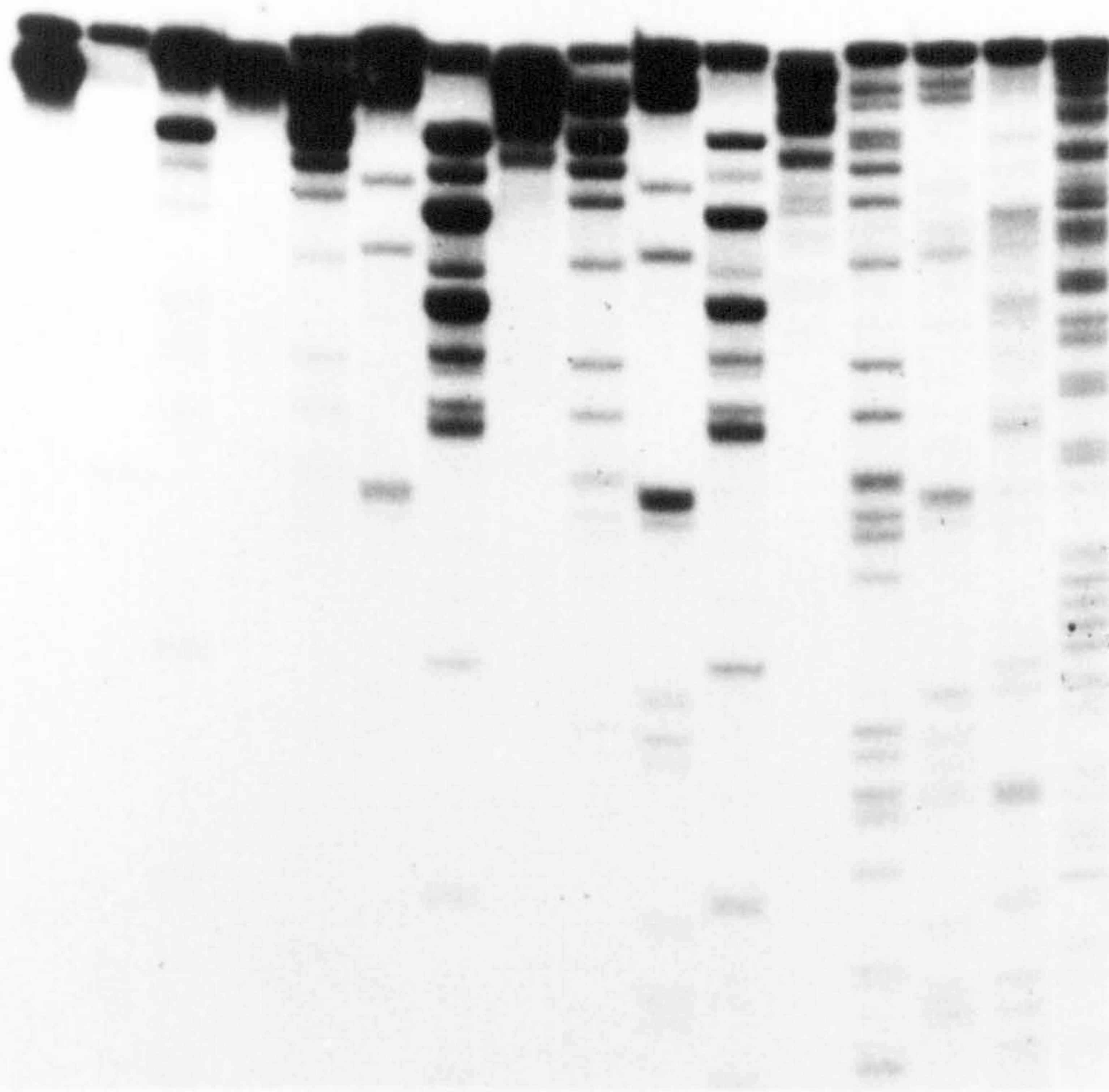
Lanes 9-12: 40 μ M α -thio-dNTP/40 μ M dNTP

Lanes 13-16: 20 μ M α -thio-dNTP/60 μ M dNTP

for the indicated nucleotide. Non-terminating nucleotides were present at 80 μ M.

1 2 3 4

A G C T A G C T A G C T A G C T



— -116
— -110
— -100
— -90
— -80
— -70
— -60
— -50
— -40
— -30

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

important in generating a maximal number of recombinant subclones. Firstly, careful generation of vector backbone by double restriction enzyme digestion and sucrose density purification was necessary to reduce the background of parental pIF8. Secondly, optimisation of backbone to insert ratios ensured the maximum degree of ligated product (data not shown), and thirdly, electroporation of these products into bacteria resulted in a very large yield of recombinant clones. Also, the rapid screening of large numbers of bacterial colonies (up to one hundred colonies a day) was facilitated by developing a micro-titre dish mini-prep procedure (see Chapter Two).

Clones with differing endpoints were grown up and the exact position of their deletions was determined by sequencing. The results are shown in Figure 4.8. Deletion products with endpoints at all but 11 positions between -116 and -77 were isolated in one experiment (Figure 4.8A), demonstrating the efficiency of the technique. Of the 74 plasmids sequenced only 8 have a 5' endpoint inconsistent with the specific base used in the reaction mix (Figure 4.8B), indicating a base-specific fidelity of approximately 90%. Six of these mutants appear to result from the removal of one additional nucleotide by MBN. The deletions are distributed throughout the region, although there are a few 'hot spots' (for example, -107 A and -79 G). In addition, some regions appear to be hard to target (for example, the sequence AACT starting at -87).

All of the 11 clones missing from the "shotgun" collection have endpoints which correspond to digestion products which are present in the sequencing ladder produced by Exo III digestion of the thio-substituted template (see Figure 4.6, lanes 10-13 and Figure 4.7, lanes 13-16). It is not clear why these mutants were not isolated, although it should be noted that there is a degree of variability of the intensity of bands in the sequencing ladder. For example, only the most 5' G in a run of 2 or 3 (positions -91 and -79) was isolated, and this is reflected in the C Exo III track, where the bands in these positions are more intense than the following ones in the run (Figure 4.6, lane 12).

In contrast to the C track, the T track showed little variability in intensity between positions, although the "hot-spot" at -107A could be explained by the increased intensity of the corresponding fragment in the T reaction (Figure 4.6, lane 13). Despite this lack of variability, A-specific deletions at -105, -87 and -86 were not isolated. In each case these would be derived from T residues at or near the end of a run of four consecutive Ts.

Figure 4.8 Distribution and analysis of deletion products.

The deletions produced in an experiment similar to that shown in Figure 4.6 were ligated to Bgl II linkers, following MBN treatment, and then excised from the backbone by digestion with Bgl II and Nco I. The deletion fragments were ligated to Bgl II- and Nco I-cut pIF8 (pIF8 contains a human β -IFN gene fragment [-77/+1302] flanked by a Bgl II linker (-77) and an Xho I linker (+1302) cloned into the vector pGC2). Following ligation the products were transformed into *E. coli* by electroporation.

(A). Seventy four plasmids containing deletion products were isolated from this experiment and the end points were determined by sequencing. The frequency of isolation of a particular 5' deletion is shown.

(B). The fidelity of the procedure is shown by comparing the number of clones obtained with a 5' end point of a particular nucleotide with the number of clones in which that end point was the expected nucleotide from the particular base-specific reaction.

The A and G reactions (Figure 4.6, lanes 10 and 11) also show no variable intensity. There is no explanation for the missing T and C endpoints, although very few G reaction products were screened. It is probable that many of the missing endpoints would have been isolated by screening more recombinant products.

The deletion procedure described here allows the generation of base specific deletions over a pre-determined length of DNA, in this case over a 40 to 50bp region of the β -interferon promoter. Analysis of the products of a single reaction demonstrated that most end-points are represented. However, certain products did not seem to be represented in the cloned material, and in addition, there may be circumstances in which an entire series of deletions is not required. To further simplify the generation of specific deletions the possibility of gel-purifying individual sequence-specific products and cloning these directly was investigated. Following MBN treatment and digestion with Nco I, the products were fractionated on a native gel. It has been well documented (reviewed in (Travers and Klug 1987)) that DNA can show anomalous migration on native gels which is a reflection of structural heterogeneity (e.g. A-T-rich regions can "bend" and show retarded mobility). Many of these structures are removed by heating, which led to the investigation of the behaviour of reaction products on a heated non-denaturing gel.

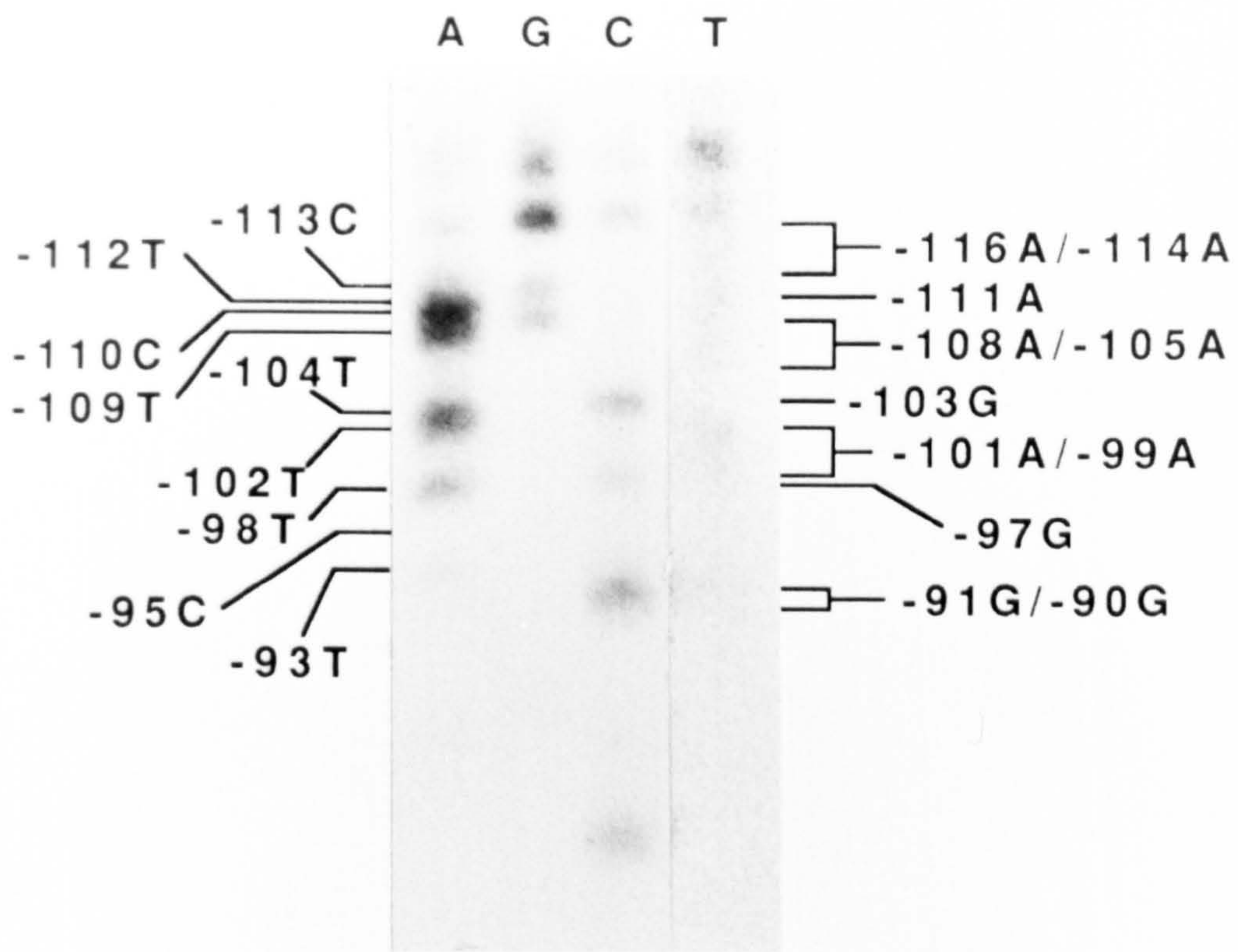
Figure 4.9 shows an experiment in which reaction products were fractionated on a 10% native polyacrylamide gel at 55°C. Under these conditions fragments had a mobility commensurate with their length. Individual fragments were isolated from these gels and were used to generate specific deletion clones. The poor resolution observed below 55°C (data not shown) is probably due to the effects of secondary structure, while above the optimal temperature the loss in resolution may reflect a local denaturation phenomenon. It is expected that the optimal temperature for resolution will be strongly sequence dependent.

Analysis of the deletion series in HeLaE cells

The 5' deletion series of the β -interferon promoter was transferred from the pIF8 vector into the pIF[-x/+72]lucifer test background. Each of these constructs was transfected into HeLaE cells as a calcium phosphate precipitate, along with a second reporter gene (pBLCAT2 or pSV₂CAT) as a transfection control. Initially, the entire set of deletion plasmids was compared in a single experiment with each deletion assayed for its basal and induced levels of expression in the primed and unprimed states. Subsequently, constructs were tested in groups of five to eight plasmids with each set of transfections including a -210 β -IFN promoter standard, pIF[-

Figure 4.9 Resolution of MBN-treated reaction products on native polyacrylamide gels.

The reactions from an experiment performed as in Figure 4.6 were taken through to the MBN stage, cut with Nco I and fractionated on a 10% non-denaturing polyacrylamide gel performed on a water jacket-heated electrophoresis apparatus operated at 55°C. The Figure shows the sequence of the β -interferon promoter between -116 and -90 at the sides of the autoradiograph.



210/+72]lucifer, which allowed a correction to be made for variations in the efficiency of induction and priming.

Each construct was tested from two to five times in separate transfections, and Figure 4.10A shows a typical set of results of these analyses. Several points emerge from these experiments. Firstly, treatment with IFN alone has little effect on the basal level of expression (Figure 4.10B). However, this basal level is not constant. Most notable is an increase in basal level when nucleotides between -103 and -101 are removed, suggesting that -103 marks the 5'-most boundary of a novel negative regulatory element. Secondly, in primed cells inducibility declines in an incremental manner (Figure 4.10C), suggesting that there are several discrete sequence elements in the upstream part of the promoter. The most 5' of these maps to -114, with a second boundary at -108. Neither of these boundaries map to a previously determined element. Downstream of -108, the incremental decrease in inducibility occurs with the successive mutation of PRD IV (5' boundary at -98), PRD III (-90) and PRD I (although there is no -77 deletion in this series of experiments the drop in induction between -83 and -73 is consistent with the inactivation of PRD I). Thirdly, the dependence upon priming is not constant across the whole promoter. To quantitate this, the ratio of expression in primed cells versus unprimed cells is presented as a "priming index", such that the greater the effects of priming upon the inducibility of a promoter construct, the greater the priming index (Figure 4.10D). There is a strong link between the priming index and the degree of induction for any given deletion product, and this correlation suggests that there may not be one specific element that responds to priming.

Multimerised PRD I and PRD II elements are priming-dependent

Although promoters that have inactivated PRD IV (deletions 3' to -98) are still at least five-fold responsive to priming, they are only weakly inducible in HeLaE cells (Figure 4.10A), and this is probably a reflection of the importance of PRD IV in this line. It is still possible however that the regulatory elements downstream of PRD IV are responsive to priming but that the inducibility is too weak to observe a priming effect. To investigate this further, synthetic promoters were constructed containing multimers of either PRD I, PRD II or PRD IV and examined in transient transfection assays. Figure 4.11 shows that the activity of PRD I is markedly affected by priming. However, IFN treatment alone activated PRD I-containing promoters. This observation is consistent with the known IFN-inducible properties of PRD I multimers (Fan and Maniatis 1989, S. Goodbourn, pers. comm.). Figure 4.11 shows

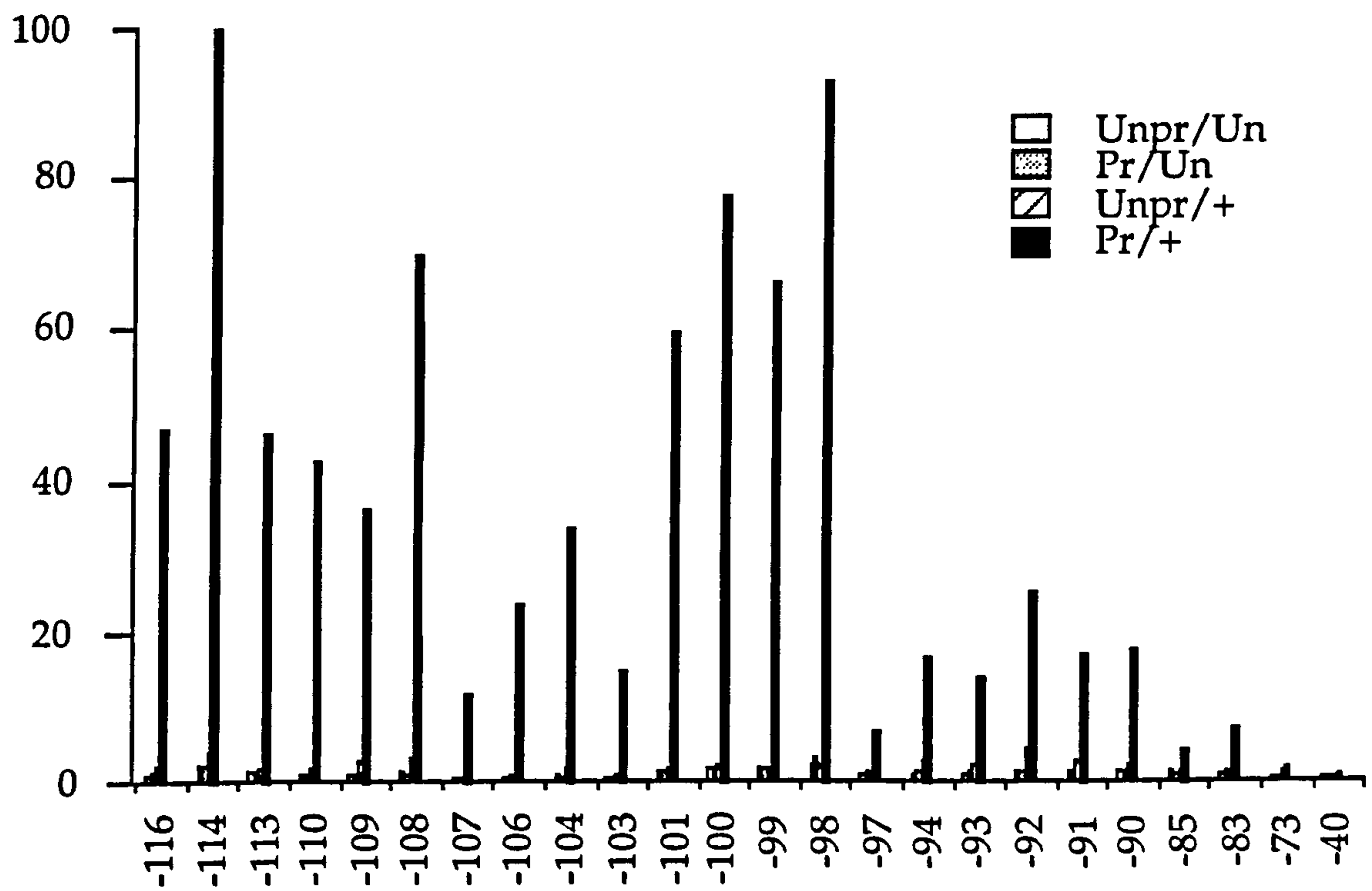
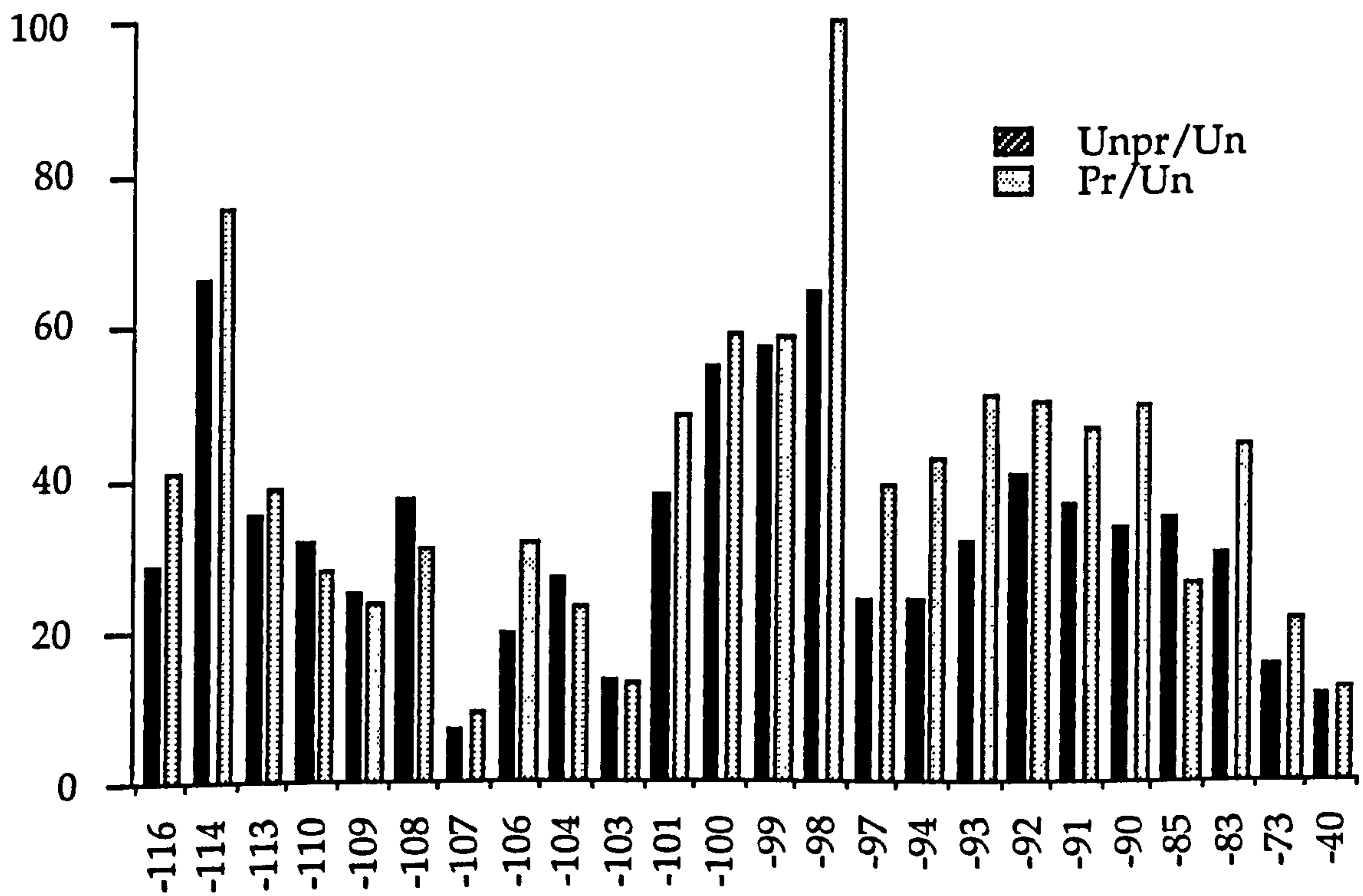
Figure 4.10 Analysis of a deletion series of the β -IFN promoter

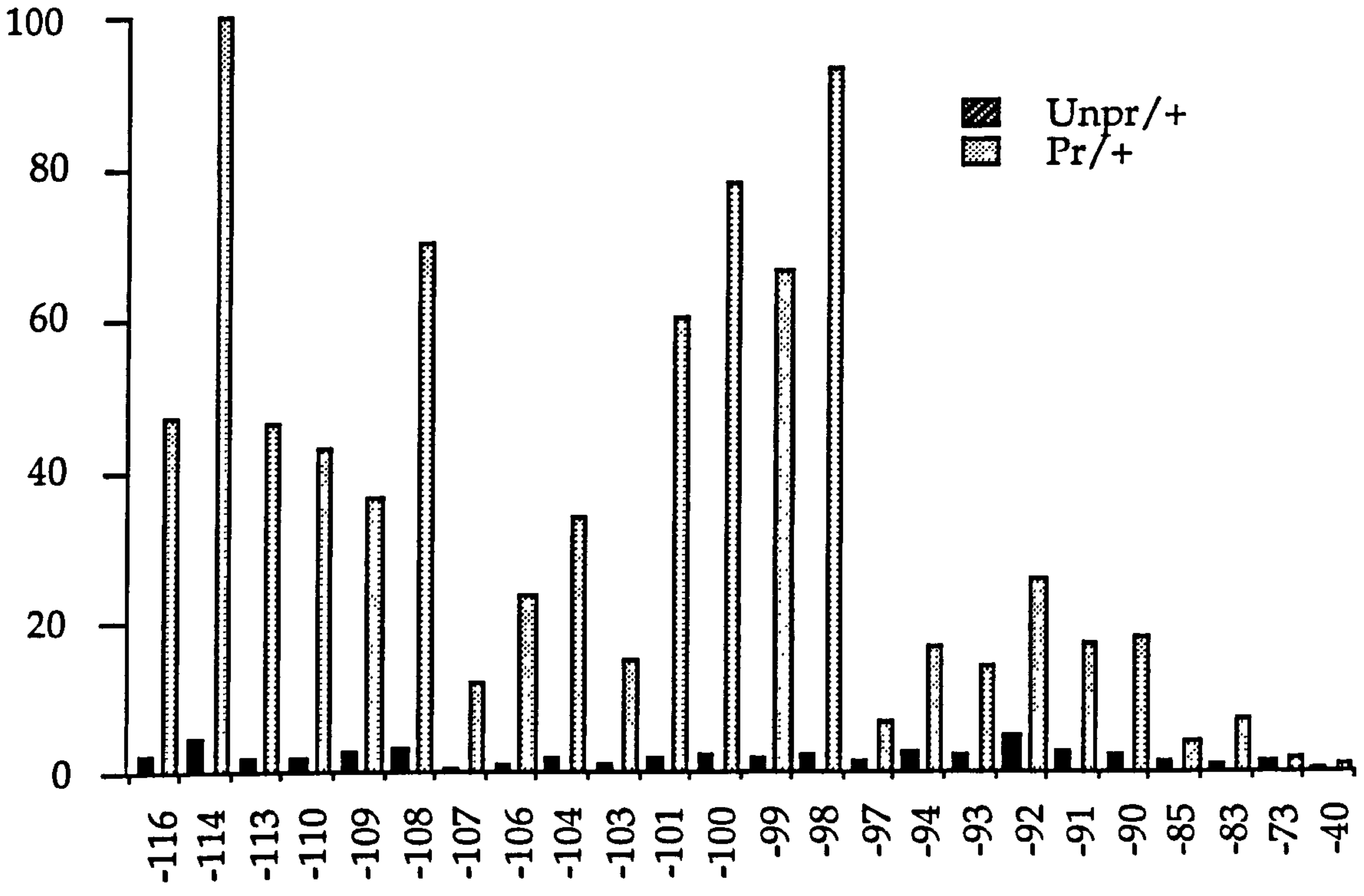
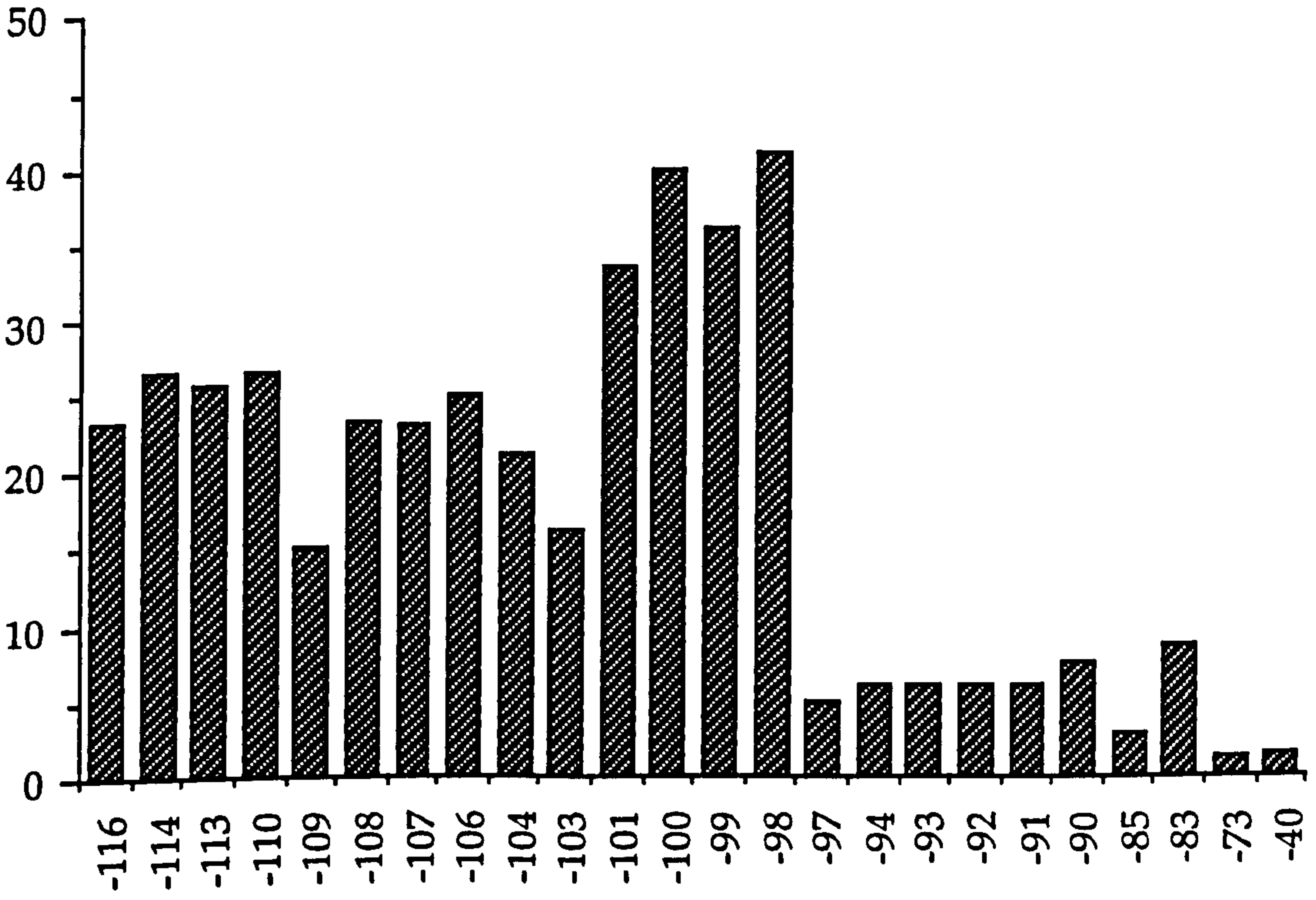
The 5' deletions created and displayed in Figure 4.8 A were isolated from the pIF8 vector as [-x/-12] fragments by digestion with Bgl II and Nco I and gel purification on 2% agarose gels. The pIF[-x/+72]lucifer series was then created by ligating these fragments into a backbone generated by digesting pIF[-116/+72]lucifer with BamH I and Nco I. This deletion series was transfected into HeLaE cells by calcium phosphate precipitation and the transfections were treated as follows:

unprimed, uninduced,
primed, uninduced,
unprimed, induced,
primed, induced.

The luciferase values were normalised to the respective CAT values in each case, and the results are displayed as follows:

- (A). The entire data set is shown
- (B). The basal level activities are shown (Un and P values)
- (C). The induced levels are shown (I and PI values)
- (D). The ratio of the primed, induced values to uninduced values for each deletion is shown to give a "priming index".

A**B**

C**D**

that the activity of PRD II also responds dramatically to priming. It has previously been reported that PRD II is inducible by dsRNA without the need for priming in MG63 cells and in mouse C127 and L929 fibroblasts (Visvanathan and Goodbourn 1989). In contrast to PRD I, PRD II does not function as an IFN-inducible element. Surprisingly, a synthetic promoter containing PRD IV is only weakly active in HeLaE cells, and does not respond to induction, even if cells are primed.

Discussion

The purpose of the experiments described in this Chapter was to investigate the promoter sequences responsible for the need to prime HeLaE cells in order to render them permissive for β -IFN induction. Because priming serves to allow transcriptional initiation in response to the inducer, it was possible that the inability of unprimed cells to respond could be due to the failure to activate transcription factors. Since β -IFN induction depends upon at least four positive regulatory elements (Figure 1.2), the requirement for priming might be further localised to a need to activate a single defined positive factor. Alternatively, unprimed cells may lack the means to derepress a specific negative regulatory domain.

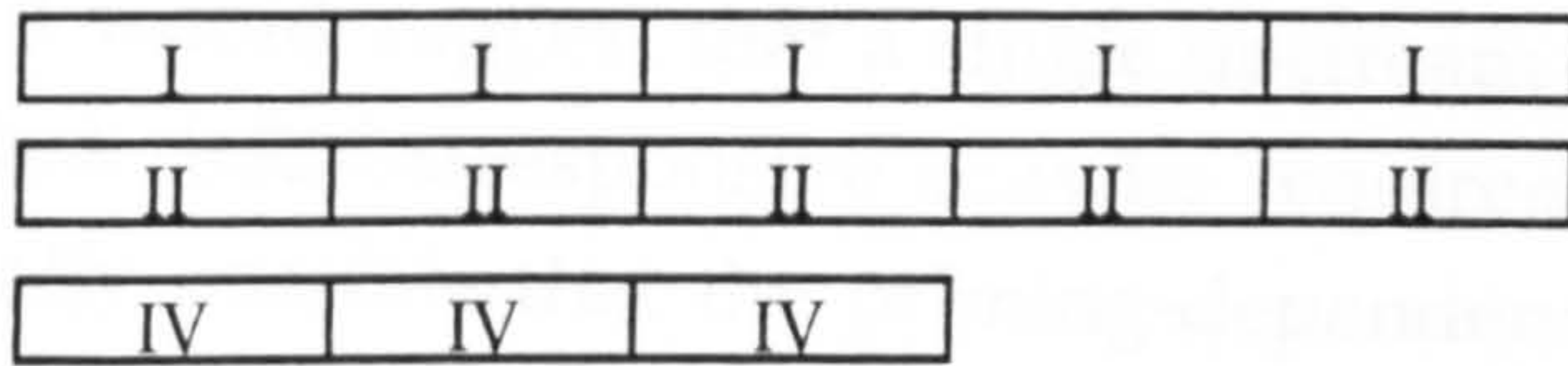
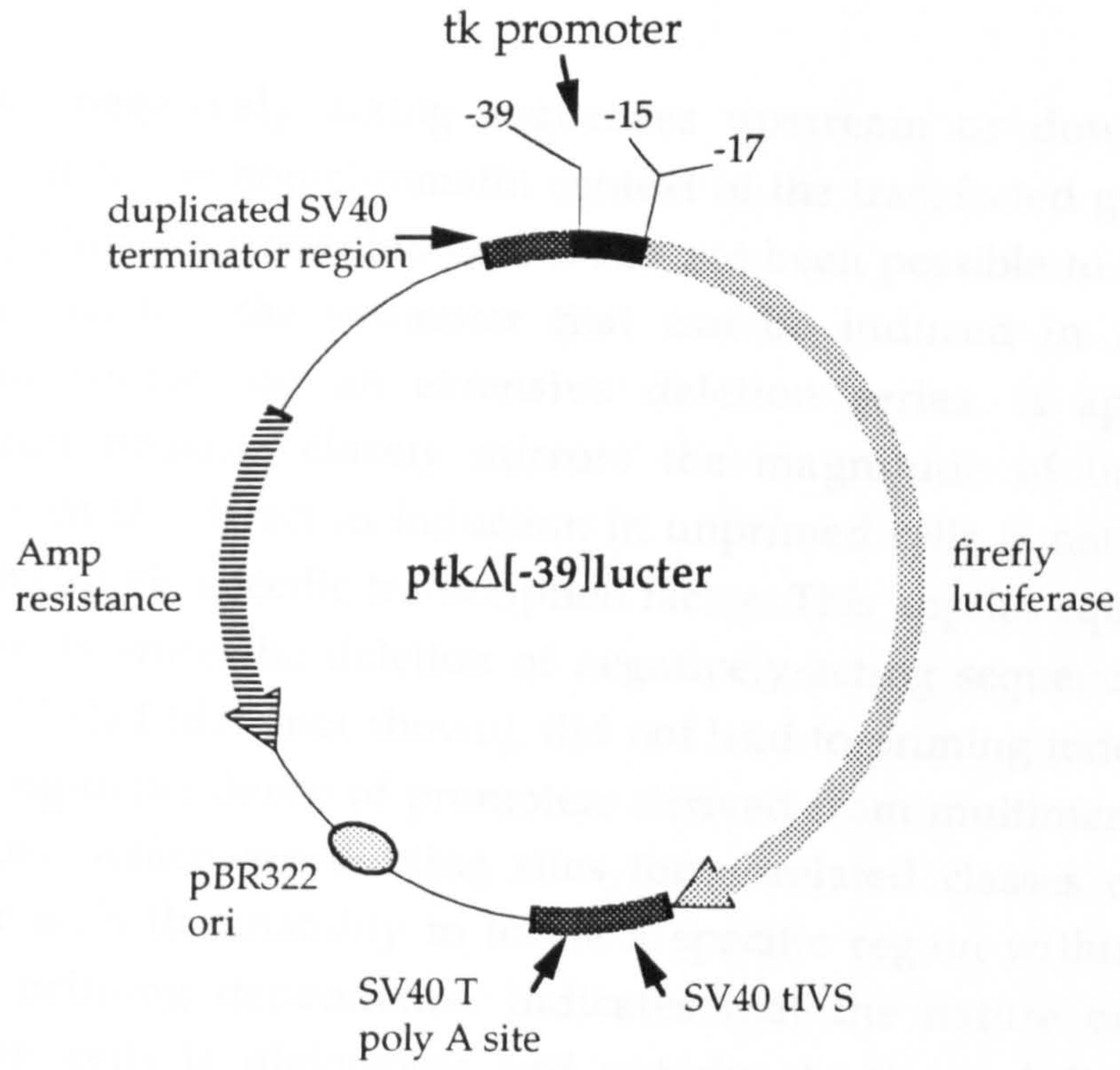
Stable transfection experiments presented in Figures 4.2A and 4.2B show that a fragment of the β -IFN promoter as small as 80 base pairs still requires priming for maximal induction in HeLaE cells and therefore demonstrated that the analysis of promoter deletion mutants was a potentially successful approach for the identification of an element within the promoter responsible for the requirement for priming.

The β -IFN promoter/luciferase reporter system gave very reproducible results and hugely expedited the analysis of the deletion series created in the experiments presented in this Chapter. The inducibility of 5' deletion mutants that had previously been analysed in stable transfections in C127 cells were qualitatively similar between the two systems, although interestingly, in the transient transfection experiments, basal level activity is readily detectable from the 5' β -IFN promoter constructs (Figure 4.10B). This is in contrast to the RNase protection results shown in Chapter Three performed on the endogenous β -IFN genes in these cells, where absolutely no β -IFN expression is observed in the absence of priming and induction. The reason for this difference could be due to the highly sensitive nature of the luciferase assay used in the transient transfection system, although it is possible to see some basal expression from the stably expressed constructs in Figure 4.2 by RNase protection, or possibly because of the relief of repression due to the

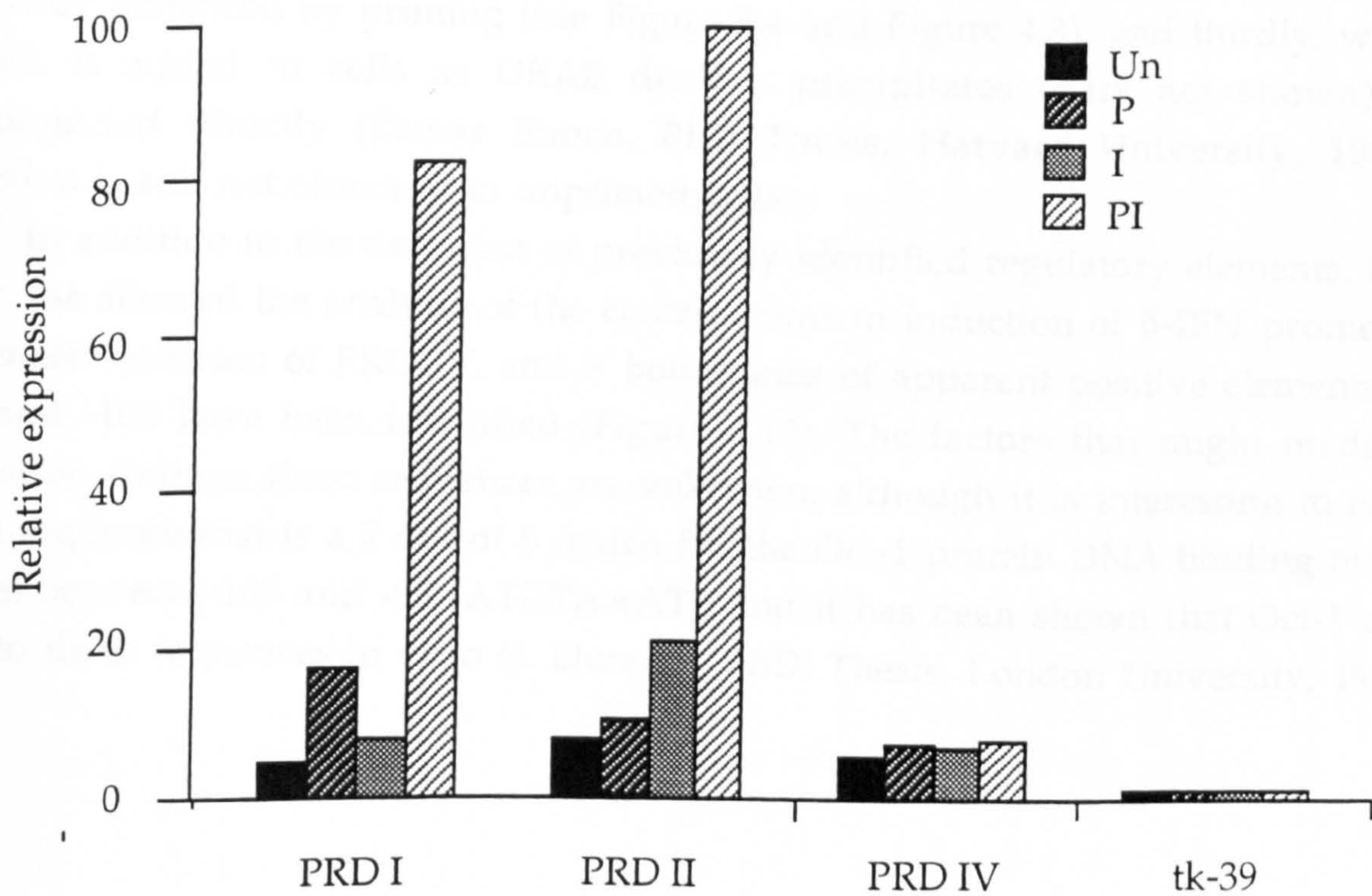
Figure 4.11 Priming dependence is a property of both PRD I and PRD II

(A). The PRD I-, PRD II- and PRD IV-driven promoters were created by inserting multimers of phosphorylated double stranded oligonucleotides, synthesised with BamH I-compatible 5' overhanging ends, into the BamH I site at position -39 of ptk Δ [-39]lucifer. As shown in the Figure, clones containing 5 copies of PRD I, 5 copies of PRD II and 3 copies of PRD IV were obtained to give p[PRD I]₅tk Δ [-39]lucifer, p[PRD II]₅tk Δ [-39]lucifer and p[PRD IV]₃tk Δ [-39]lucifer respectively.

(B). HeLaE cells were transiently transfected with 15 μ g of ptk Δ [-39]lucifer or the β -IFN promoter derivatives, PRD I, PRD II and PRD IV, along with 5 μ g of pBLCAT2. The constructs were tested under four conditions as indicated, and the cellular lysates were tested for luciferase and CAT activity. The relative expression of each plasmid was calculated by normalising the luciferase value to the respective CAT value, and these values were further corrected to any variation observed from the minimal tk promoter construct, ptk Δ [-39]lucifer.

A

p[PRD I]5tkΔ[-39]lucifer
p[PRD II]5tkΔ[-39]lucifer
p[PRD IV]3tkΔ[-39]lucifer

B

loss of putative negatively acting sequences upstream or downstream of the constructs used, or to the non-chromatin context of the transfected genes.

In the experiments presented here it has not been possible to identify a single specific element within the promoter that can be induced in unprimed cells. Furthermore, in studies on an extensive deletion series, it appears that the dependence upon priming closely mirrors the magnitude of induction. These results indicate that the defect in induction in unprimed cells is not due to the lack of function of any single specific transcription factor. This applies equally to negative regulatory elements since the deletion of negatively-acting sequences identified in this study, or of NRD I (data not shown), did not lead to priming independence.

The priming-dependence of promoters derived from multimerised PRD I and PRD II sequences, which are binding sites for unrelated classes of transcription factors, together with the inability to locate a specific region within the promoter responsible for priming dependence, indicates that the nature of the defect in unprimed HeLaE cells is pleiotropic and not due to the inability to activate a particular positively acting factor or derepress a particular negatively acting factor. Rather it would suggest that a single upstream factor is absent in unprimed cells, for example a dsRNA-responsive enzyme required to transduce the induction signal. It is formally possible that the priming-dependence of HeLaE cells is a consequence of the permeability of the cells to dsRNA, although this is unlikely for several reasons. Firstly, dsRNA is capable of mediating some dsRNA-dependent events in unprimed HeLaE cells (for example, the activation of apoptosis-see Chapter Seven). Secondly, although unprimed HeLaE cells are, to an extent, inducible by Sendai virus infection (which uses a different mechanism of entry into cells than dsRNA), induction can be greatly enhanced by priming (see Figure 3.4 and Figure 4.3), and thirdly, when dsRNA is added to cells as DEAE dextran precipitates (data not shown) or microinjected directly (Tamar Enoch, PhD Thesis, Harvard University, 1986), induction is still not observed in unprimed cells.

In addition to the detection of previously identified regulatory elements, this study has allowed the analysis of the contributions to induction of β -IFN promoter sequences upstream of PRD IV, and 5' boundaries of apparent positive elements at -114 and -108 have been identified (Figure 4.10). The factors that might mediate expression through these sequences are unknown, although it is interesting to note that a sequence that is a 7 out of 8 match for the Oct-1 protein DNA binding motif resides between -105 and -98 (ATGTAAAT), and it has been shown that Oct-1 can bind to these sequences in vitro (J. Eloranta, PhD. Thesis, London University, 1995,

K. Visvanathan and S. Goodbourn, pers. comm., Du and Maniatis 1992). After the -108 boundary is crossed, the basal and induced levels of expression remain relatively low until the sequences between -103 and -101 are deleted, suggesting that the 5' boundary to a negative element resides in this region. The next boundary that is observed resides downstream of -98. This probably corresponds to the requirement for ATF-2 binding to the PRD IV element (Du and Maniatis 1992), although it is interesting to note that the upstream HMG I/Y binding site proposed to be important for induction (Du et al. 1993) is not required, at least when downstream sequence elements are present. When the behaviour of PRD IV oligonucleotides was examined in the absence of other β -IFN promoter sequences, it was found to be uninducible (Figure 4.11). These results are consistent with previous observations (Du and Maniatis 1992) that showed that in HeLa cells, unlike in L929 cells, PRD IV is a constitutively active element that is unresponsive to induction. In addition to the changes observed at -114, -108, and -103 a number of smaller changes are also seen (for example between -93 and -92). Again these have not been analysed further, but given the already complex nature of the β -IFN promoter it would not be surprising if these also indicate regulatory effects.

The deletion series analysed in this Chapter was created using a novel method for the generation of a comprehensive range of deletions in a region of DNA of interest. Generally, deletion mutants are generated either by digestion from a fixed point, using nuclease-based enzymatic procedures, or by using sequence-specific primers. The former has the advantage of being technically easy and inexpensive and can be used to generate a number of mutations across a large region of DNA. However, the enzymes commonly used exhibit some sequence specificity (e.g. Bal 31 preferentially degrades A-T-rich regions), making it almost impossible to target some regions of the template and therefore making it difficult to generate defined substitutions. By contrast, the use of specific primers does allow accurately targeted mutagenesis, but the cost of synthesising such primers renders the generation of a large set of deletions impractical. The technique developed here allows the construction of complete deletion series differing by single base increments. The procedure is rapid and inexpensive, and the accuracy and saturation of this procedure should significantly aid mutational analysis and facilitate the generation of defined substitution mutants for the many applications where it is necessary to use deletions with precisely defined breakpoints, for example, in the production of so-called "linker-scanning" mutations.

CHAPTER FIVE -THE NATURE OF THE PRIMING FACTOR - A NOVEL ROLE FOR IRF-1

Introduction

The data presented in Chapter Four indicate that the priming requirement of HeLaE cells cannot be localised to a single region of the β -IFN promoter, and that unrelated inducible elements within the promoter display priming-dependence when analysed in the context of heterologous promoters. Because these elements are known to respond to induction by dsRNA by different mechanisms, involving different classes of trans-acting factors (see Chapter One), this suggests that the requirement for priming in these cells is not simply the absence of a single factor acting downstream at the level of the promoter. On the contrary, the pleiotropic nature of the defect in unprimed HeLaE cells may suggest that a single, crucial, upstream factor is missing, for example a dsRNA-responsive enzyme. This chapter describes attempts to identify an upstream priming-induced factor.

Protein kinase R (PKR) is not the priming factor

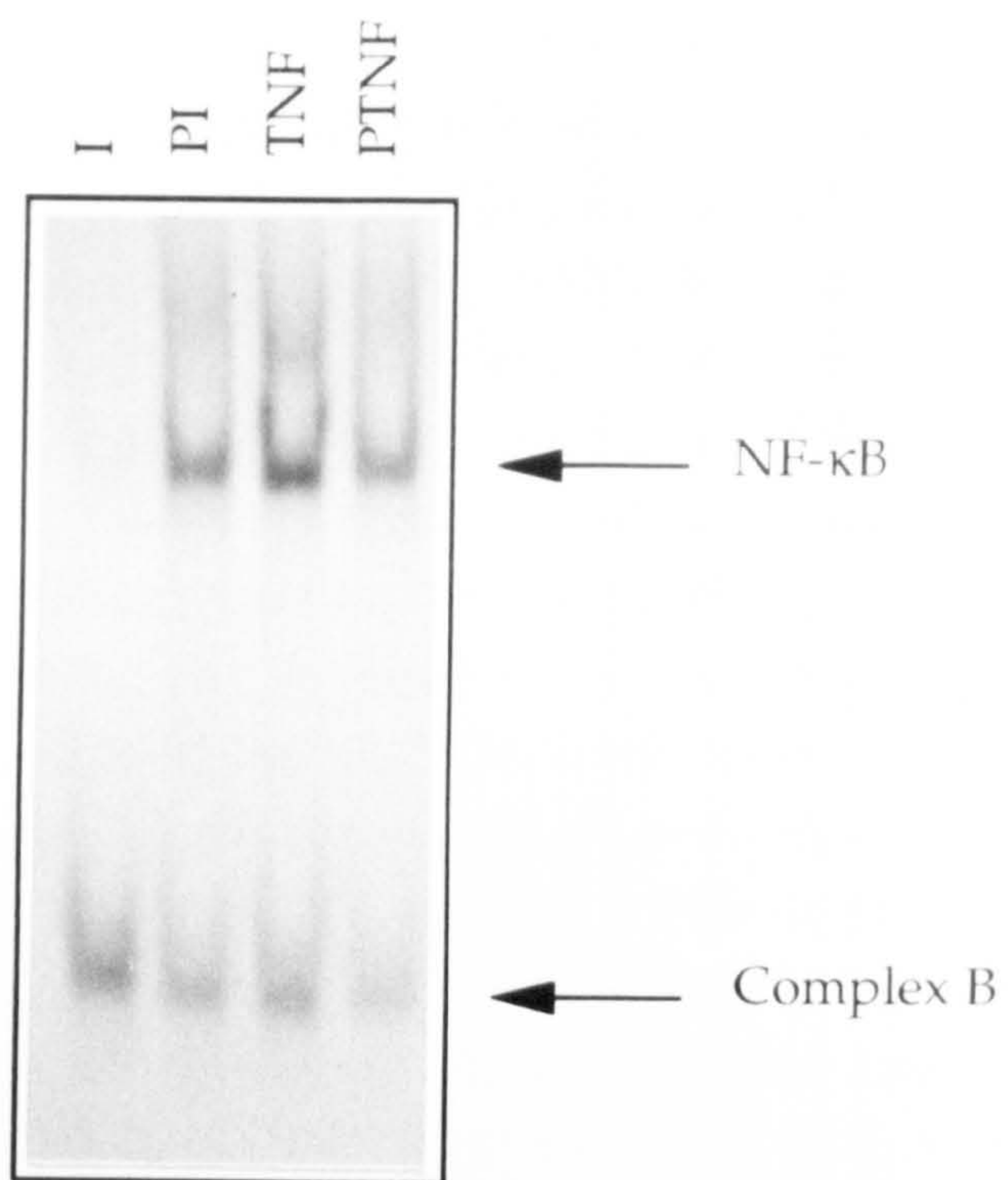
Protein kinase R (PKR) plays a well-defined role in the anti-viral response, by mediating translational shut-down in response to viral dsRNA. However, it has been specifically proposed that PKR also plays a role in the induction of the β -IFN gene (see Chapter One). Most notably, PKR has been shown to phosphorylate I κ B (Kumar et al. 1994, Offerman et al. 1995), and that such a phosphorylation may play a role in NF- κ B activation. Mouse EF cells that are deficient in PKR are unable to activate NF- κ B in response to dsRNA (Yang et al. 1995), while the introduction into cells of a peptide synthesised against the phosphorylation site of one of the characterised substrates of PKR, eIF2 α , blocks the activation of NF- κ B binding to PRD II by dsRNA (S. Goodbourn, pers. comm.). It was demonstrated in Chapter Four that induction of transcription through PRD II is dependent upon priming, and this is reflected by the specific failure of dsRNA to induce NF- κ B in unprimed cells, as judged by EMSA analysis using a PRD II probe (Figure 5.1A). The ability of TNF α treatment to activate NF- κ B binding is unimpaired in unprimed HeLaE cells, indicating that the cells are not deficient in NF- κ B, and since the levels of PKR are enhanced by IFN treatment (see Chapter One), it was possible that the need for priming reflects an insufficiency of PKR.

Figure 5.1

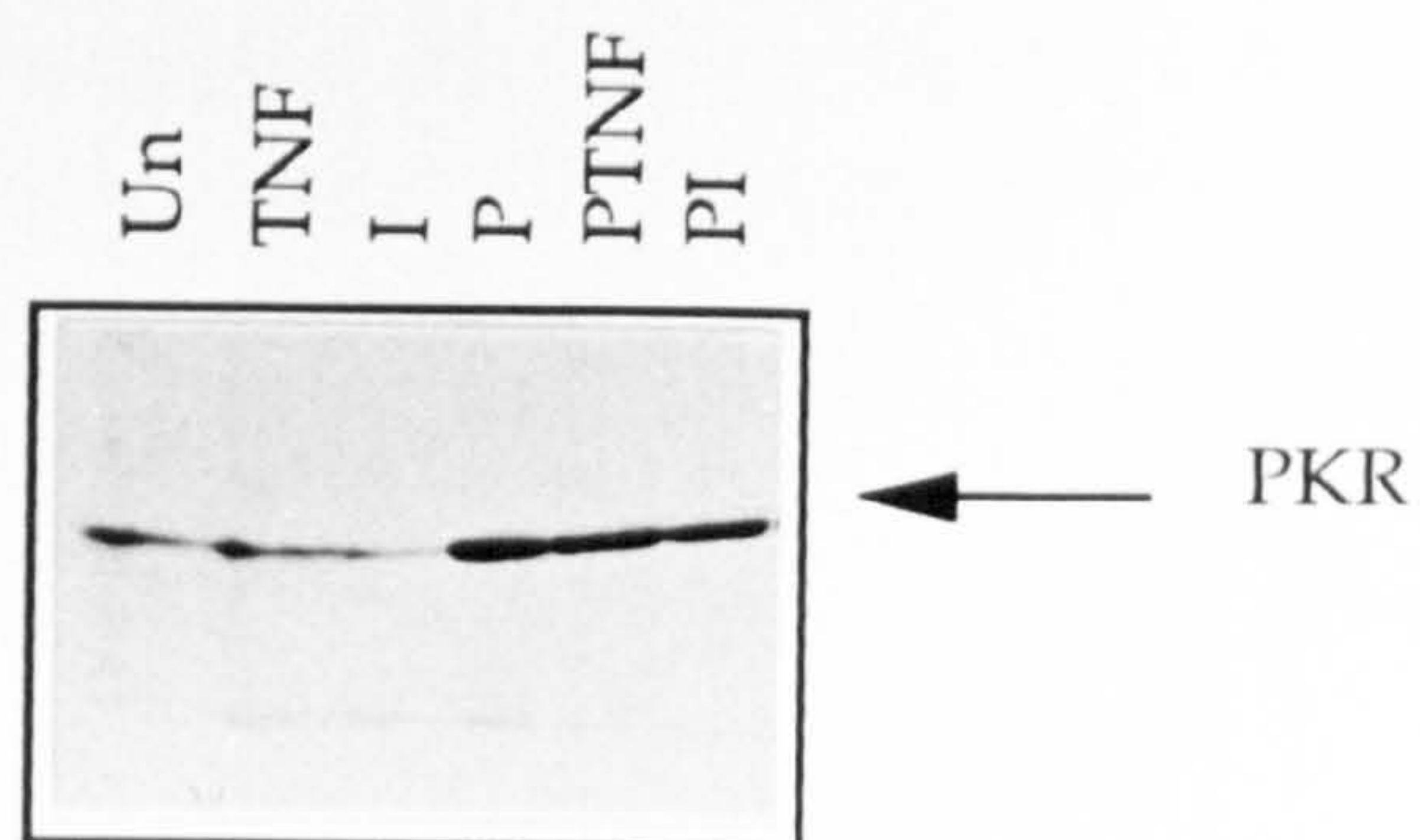
(A). HeLaE cells were treated with dsRNA (90 minutes) (lanes 1 and 3) and TNF α (10ng/ml, 30 minutes) (lanes 2 and 4) in the unprimed (lanes 1 and 2) and primed (lanes 3 and 4) states. Nuclear extracts were made from the cells and these were analysed by EMSA using a radiolabelled PRD II probe. The positions of NF- κ B and complex B are indicated to the right of the autoradiograph.

(B). HeLaE cells were untreated (lanes 1 and 4), treated with TNF α (10ng/ml, 30 minutes) (lanes 2 and 5) or dsRNA (90 minutes) (lanes 3 and 6) in the unprimed (lanes 1 to 3) and primed (lanes 4 to 6) states. The extracts were analysed by Western blotting, using an anti-PKR monoclonal antibody (Laurent et al. 1985).

A



B



The levels of PKR in unprimed and primed HeLaE cells were determined by a Western analysis of extracts (Figure 5.1B; courtesy of J. Brown). Although the amount of PKR in HeLaE cells is increased by priming, it is clearly present in unprimed cells. It is, however, possible that this level is insufficient for induction by dsRNA. In order to address this possibility, expression plasmids for PKR and two dominant-negative mutant forms, M2 and M6, which have mutations in the first dsRNA binding domain and catalytic domain, respectively (Meurs et al. 1990, Feng et al. 1992), were constructed (Figure 5.2) and transfected into HeLaE cells.

Figure 5.3A shows that increasing the levels of wild-type PKR significantly upregulates the activity of the cotransfected [-108/+72] β -IFN promoter construct, but this is not only seen in primed and induced cells, but also in uninduced and unprimed induced cells. Importantly, raised PKR levels were completely unable to rescue induction in unprimed cells. In the course of performing experiments with PKR, it was noted that the apparent transfection efficiencies were very much reduced, and since it has been previously reported that inhibiting PKR dramatically stimulates translation (see Chapter One), it is probable that the lowered transfection efficiencies are a result of translational inhibition by raised PKR levels. To counter this, the data shown in Figure 5.3A are corrected by comparing the effect of PKR on luciferase levels from the β -IFN promoter with that obtained from a non dsRNA-responsive promoter (ptk Δ [-39]lucifer). In contrast to the effect of wild type PKR, the overexpression of the M6 dominant interfering form of PKR raised overall translation, but caused a marked inhibition of the activity of the β -IFN promoter. Figure 5.3B shows the results of a similar transient transfection experiment using the NF- κ B reporter construct, p(PRDI₅)tk Δ [-39]lucifer, together with plasmids directing the expression of wild type PKR and both the dominant interfering forms, M2 and M6. Wild-type PKR has little noticeable effect on the activity of the PRD II-driven promoter, but once again M6, and also M2, inhibit the observed induction. Taken together, the results shown in Figures 5.1 and 5.3 indicate that although PKR activity is important for the induction of β -IFN by dsRNA, overexpression of PKR in HeLaE cells is not sufficient to prime them.

Figure 5.2 Expression plasmids for PKR

Expression plasmids for PKR were created by inserting HindIII/SalI cDNA fragments of wild-type human PKR (Meurs et al. 1990), mutant M2, missing amino acids 58-69 from the dsRNA binding domain 1 (Feng et al. 1992), and mutant M6, which has a K296/R amino acid substitution in the catalytic domain (Meurs et al. 1990), into the polylinker of pCO2 to create pCO β PKR, pCO β PKRM2 or pCO β PKRM6, respectively.

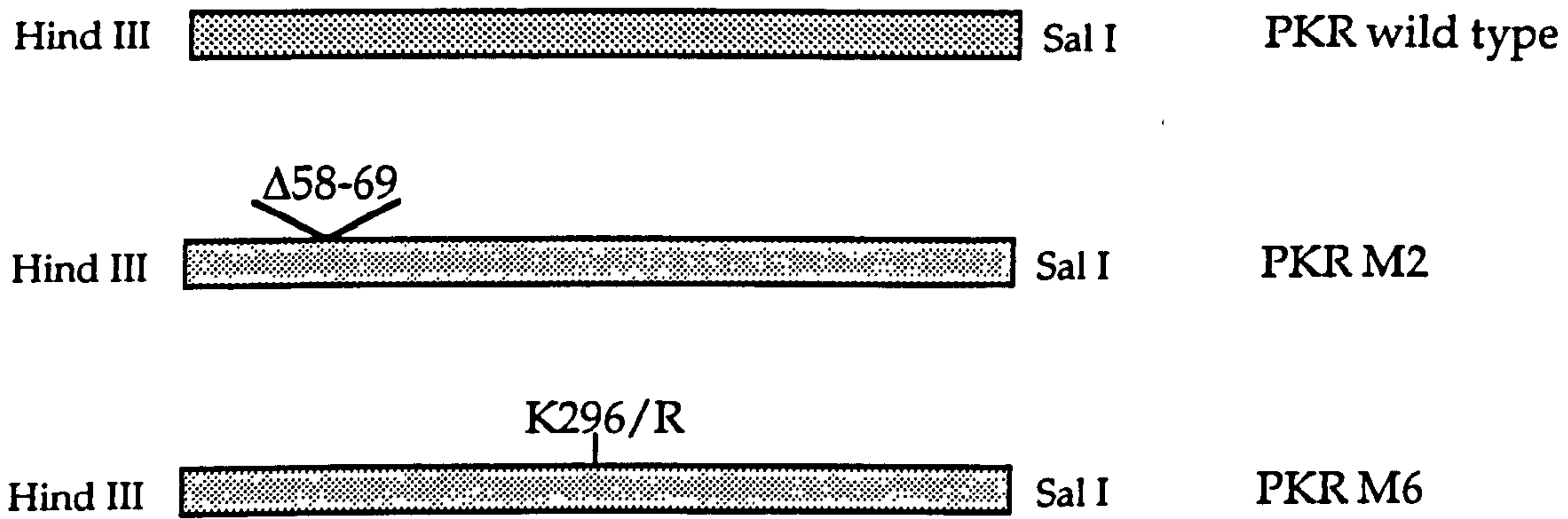
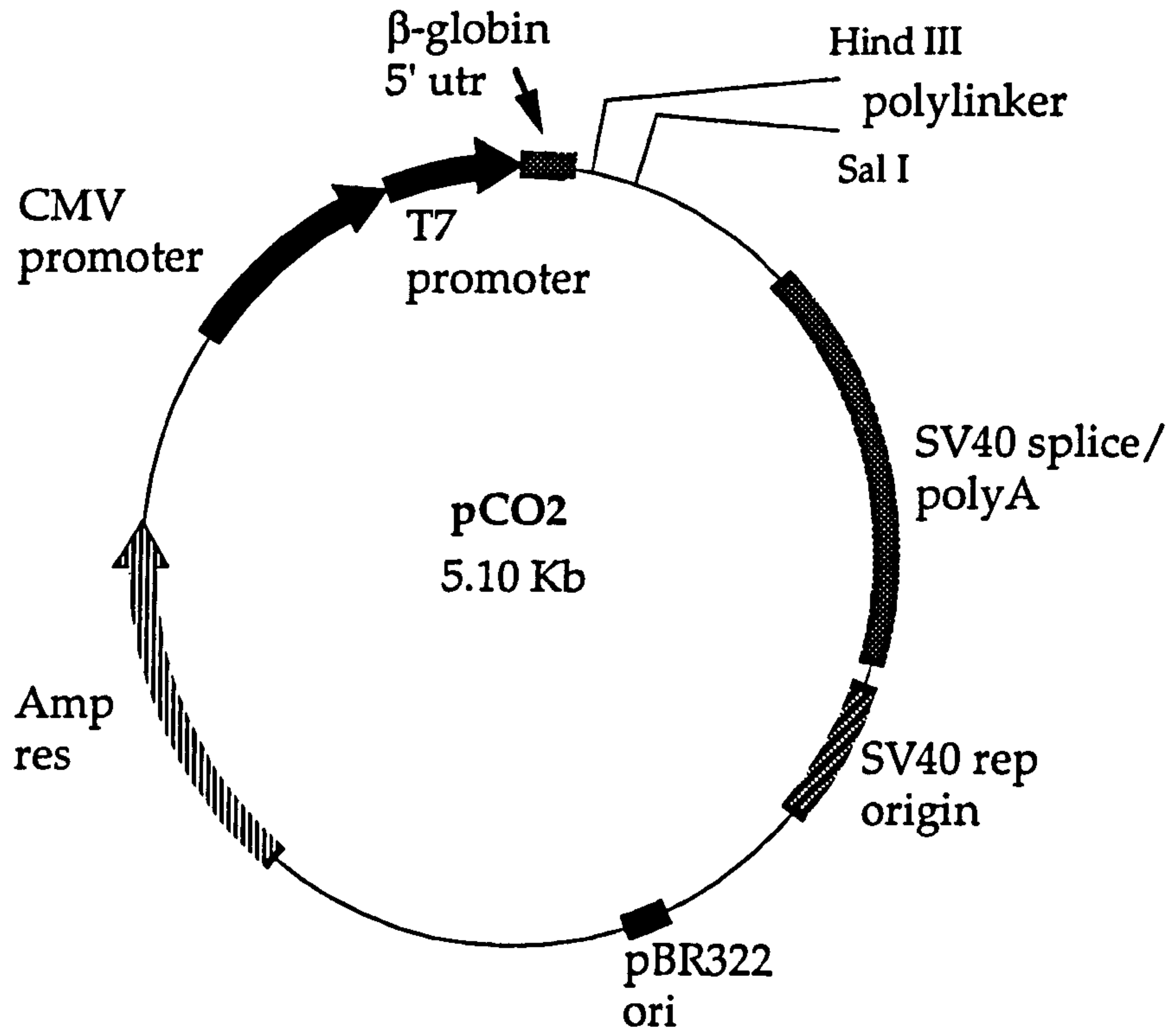


Figure 5.3 Overexpression of PKR does not prime HeLaE cells

(A). HeLaE cells were transiently transfected, using calcium phosphate co-precipitation, with 10 μ g of pIF[-108/+72]lucifer and 10 μ g of pCO₂, pCO β PKR or pCO β PKRM6, along with 2 μ g of pSV₂CAT (Gorman et al. 1982), and the cells were treated as follows:

unprimed, uninduced (Un)

unprimed, induced (I)

primed, induced (PI)

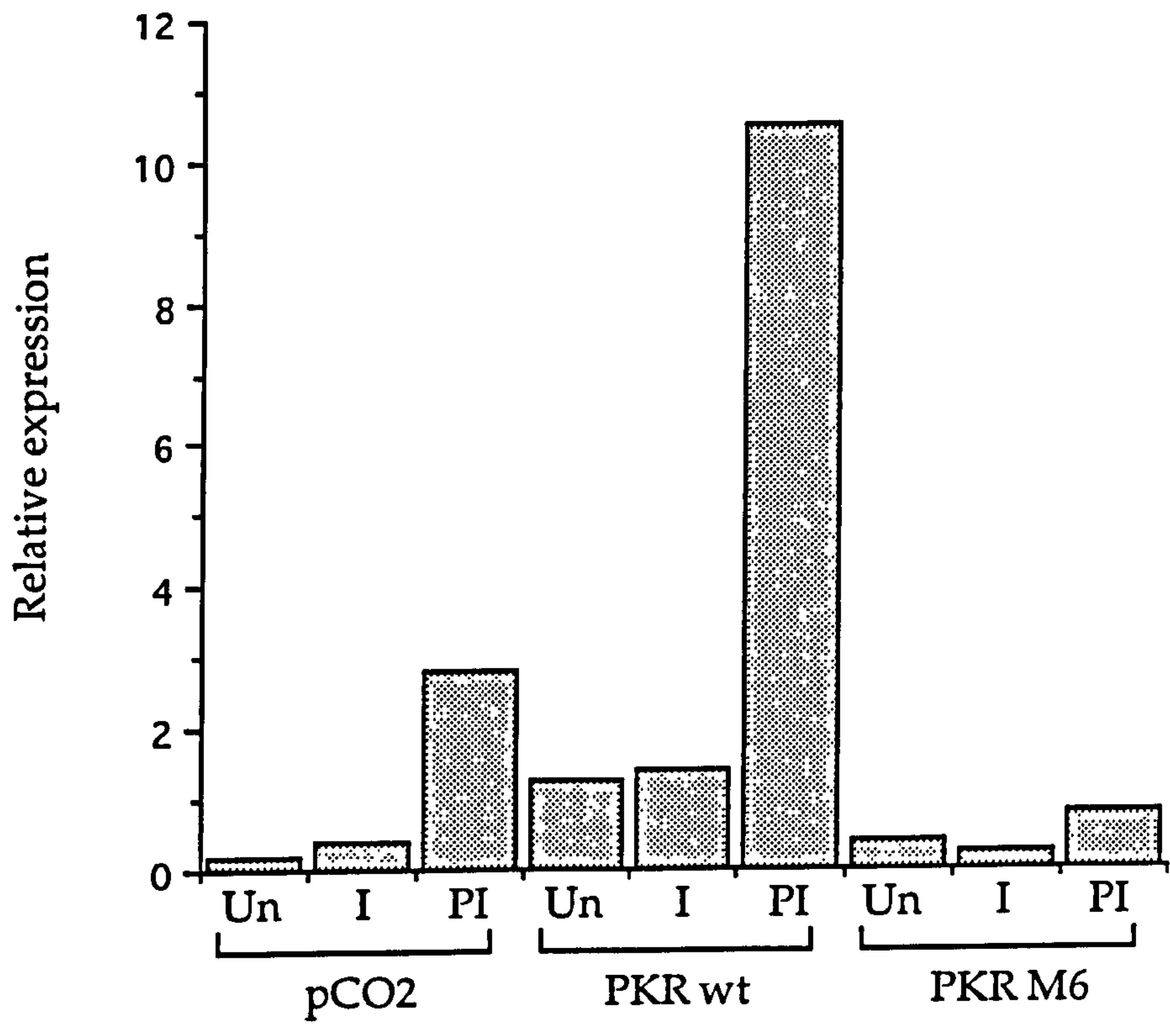
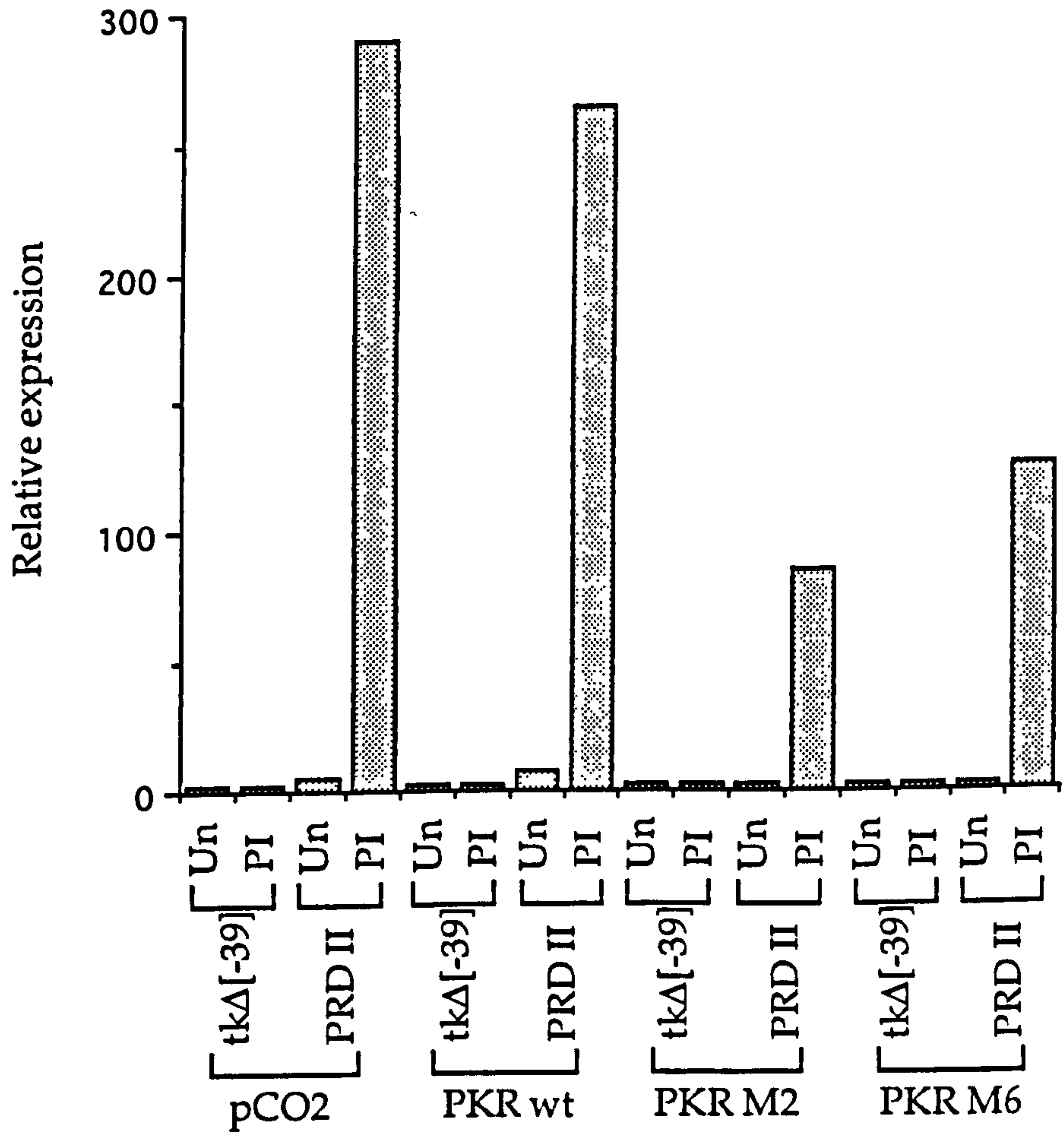
The relative expression levels were calculated by correcting to CAT and to the effect of the PKR construct on the activity of tk Δ [-39]lucifer in separate transfections (data not shown).

(B). HeLaE cells were transiently transfected, using calcium phosphate co-precipitation, with 10 μ g of p[PRD II]5tk Δ [-39]lucifer or ptk Δ [-39]lucifer along with 10 μ g of pCO₂, pCO β PKR, pCO β PKRM2 or pCO β PKRM6, and 2 μ g of pSV₂CAT, and the cells were treated as follows:

unprimed, uninduced (Un)

primed, induced (PI)

The relative expression levels were calculated by correcting to CAT and to the effect of the PKR construct on the activity of tk Δ [-39]lucifer.

A**B**

Other candidates for priming factor(s)

In addition to PKR, there is a second IFN-inducible enzyme that is dependent on dsRNA for activation, namely (2'-5')-oligoadenylate synthetase (2,5- A_n synthetase). This enzyme generates (2'-5')-oligoadenylates (2,5- A_n) that bind to and activate ribonuclease L (RNase L) (see Chapter One). The role of 2,5- A_n in β -IFN induction was investigated by overexpressing a dominant negative mutant of RNase L that could sequester 2,5- A_n without RNase activation. In NIH3T3 cells, this mutant blocked the antiviral activity of type I IFN against EMCV (Hassel et al. 1993), but failed to block β -IFN induction in response to dsRNA (S. Goodbourn, pers. comm.), suggesting that the 2,5- A_n system is not involved in β -IFN induction, and therefore unlikely to be involved in the priming response.

Since IFN is able to upregulate the levels of at least 50 other proteins, there are many candidates for a priming factor. A number of IFN-inducible cDNAs have been obtained and expressed in HeLaE cells with the aim of rendering them priming-independent. The IFN-inducible factors HLA-A, HLA-C, metallothionein, 1-8, ISG15 (all data not shown), ISGF3 γ and STAT1 (see Chapter Six) were not able to prime HeLaE cells. Another IFN-inducible protein, IRF-1, was originally identified as a DNA binding protein that specifically recognises a multimer of the sequence AAGTGA and transactivates expression from promoters containing this motif (Taniguchi et al. 1995). Since AAGTGA multimers are related to PRD I, it was hypothesised that IRF-1 could play a direct role in β -IFN induction. Consistent with this, drastic overexpression of IRF-1 in COS cells led to weak induction of the endogenous β -IFN gene (Fujita et al. 1989), while treatment of cells with antisense IRF-1 (Reis et al. 1992) or disruption of the IRF-1 gene by "knockout" (Matsuyama et al. 1993) impaired the magnitude of β -IFN induction by dsRNA. Significantly, for the purposes of this thesis, HeLaE cells do not contain detectable IRF-1 even after they have been primed (Whiteside et al. 1992). It was therefore possible that HeLaE cells are unable to induce IRF-1, resulting in their priming-dependence, and this was tested directly.

Priming causes a transient induction of IRF-1 protein synthesis in HeLaE cells

In a preliminary experiment to analyse the levels of IRF-1 protein present in HeLaE cells during the development of the primed state, nuclear extracts were prepared from HeLaE cells which had been treated with Wellferon for up to 24 hours, and the amount of IRF-1 present in these extracts was assessed by its

Figure 5.4 IRF-1 is induced in HeLaE cells during priming

HeLaE cells were treated with Wellferon and nuclear extracts were prepared at the times following IFN addition indicated in the Figure. The extracts were analysed by EMSA, using a radiolabelled PRD I probe. Each sample from the time course was analysed by the preincubation of the extract with 2 μ l of:

rabbit pre-immune serum (lanes 1, 4, 7, 10, 13, 16)

rabbit polyclonal anti-IRF-2 antiserum (lanes 2, 5, 8, 11, 14, 17)

rabbit polyclonal anti-IRF-1 and anti-IRF-2 antisera (lanes 3, 6, 9, 12, 15, 18)

The positions of identified complexes are indicated to the right of the autoradiograph (n. s. = non specific complex), and they are discussed in the text.

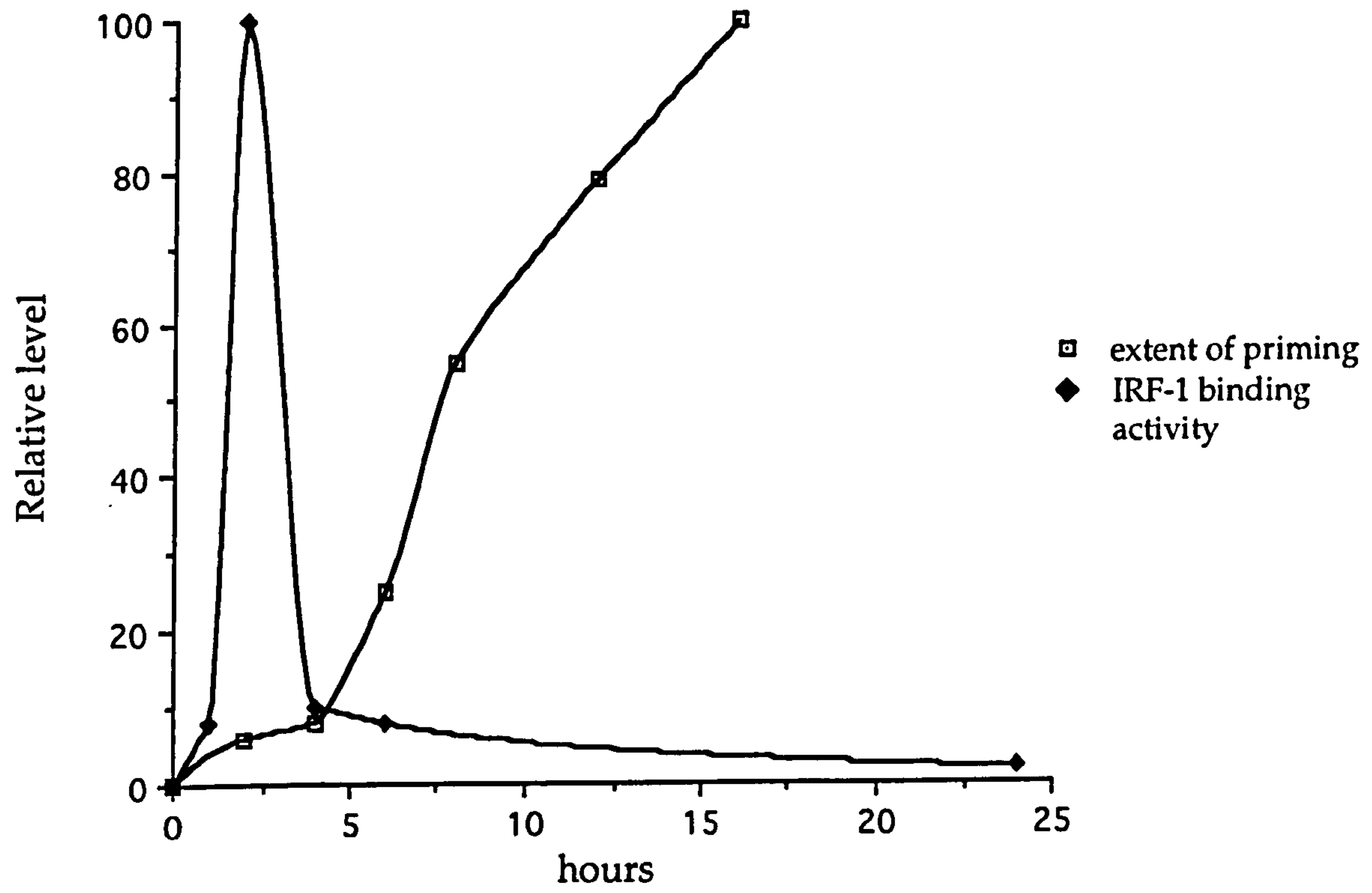
ability to bind to a radiolabelled PRD I probe in an EMSA experiment. Figure 5.4 shows the results of this analysis. Because complexes containing the abundant IRF-1-related protein, IRF-2, have a similar mobility to IRF-1-bound PRD I, the extracts were run in groups of three, treated with either pre-immune serum, anti-IRF-2 polyclonal antibody (Whiteside et al. 1992), or both anti-IRF-2 and anti-IRF-1 (produced as described in Chapter Two) antibodies. The anti-IRF-1 and -2 antibodies block the binding of their respective target proteins to the PRD I probe. Treating the extracts with the anti-IRF-2 antibody reveals the presence of a residual binding activity whose identity as IRF-1 is confirmed by the inclusion of the anti-IRF-1 antibody.

Figure 5.4 confirms that IRF-1 is not present in unprimed HeLaE cells but demonstrates that it is rapidly induced by Wellferon treatment, its levels peaking at around 4-6 hours after IFN addition before declining to very low levels between 6 and 24 hours, by which time the cells are fully primed (see Chapter Three). By plotting the profile of induction of IRF-1 by Wellferon against the kinetics of establishment of the primed state in HeLaE cells, it can clearly be observed that the brief spike of IRF-1 induction precedes the establishment of priming by several hours (Figure 5.5). Interestingly, a complex that runs with a faster mobility than IRF-1, which is not affected by either anti-IRF-1 or anti-IRF-2 antibodies, is induced with kinetics that mirror those of the establishment of the primed state. This complex was shown to contain the 48kDa binding component of the type I IFN-activated ISGF3 transcription factor complex ISGF3 γ (see Chapters One and Six).

The above result indicates that one consequence of priming HeLaE cells is a transient induction of IRF-1 protein synthesis. Interestingly, IRF-1 has been shown to be able to bind to and transactivate other IFN-inducible genes through their ISRE sequences (see Chapter One), and it is possible that it is through this property that the transiently raised IRF-1 levels lead to priming. Alternatively, the raised IRF-1 levels might be merely coincidental to the synthesis of other IFN-inducible factors. If the role of IRF-1 in priming is to upregulate other factors required for induction, then two predictions follow from this hypothesis. Firstly, the overexpression of IRF-1 in priming-dependent cells should abrogate the need for priming, and secondly, that IRF-1 may be constitutively present in priming-independent cells, and if this is the case the disruption of IRF-1 synthesis in these cells should make them priming-dependent.

Figure 5.5 IRF-1 induction precedes the development of the primed state

The data from Figures 5.4 and 3.3 were plotted to show the relative kinetics of IRF-1 induction and the development of the primed state. The amount of IRF-1 binding in Figure 5.4 was quantified by densitometry and presented as a percentage of the maximum level (in the 2 hour sample), and the development of priming was shown by plotting the maximum observed inducibility of the β -IFN gene, following varying lengths of Wellferon treatment, as a percentage of the maximum observed level (seen with 16 hours of treatment).



Transfection of HeLaE cells with IRF-1 expression plasmids renders them priming-independent

To test whether overexpression of IRF-1 could render the endogenous β -IFN gene inducible in a priming-independent manner in HeLaE cells, a transient transfection system was used because it had not proved possible to create stable lines expressing IRF-1, probably because of the antiproliferative activity of this factor (data not shown, and see Chapter One). However, to observe the effect of IRF-1 on the small number of transfected cells above the background of untransfected cells in a transient transfection, the cells were co-transfected with a CMV promoter-driven IRF-1 expression vector (Figure 5.6) and a plasmid capable of expressing the T-cell-specific CD2 cell surface marker, and the transfected cells were selected by their ability to bind to immobilised anti-CD2 antibodies. After induction, the transfected cells were selected on anti-CD2 antibody-coated magnetic beads and the RNA extracted from these cells was mapped for β -IFN-specific mRNA. Cells transfected with a control vector, pCO2, remain priming-dependent (lanes 1-8), whereas those transfected with the IRF-1 expressing plasmid can be induced in the absence of priming (lanes 9 - 16). Interestingly, the presence of IRF-1 at the time of induction by dsRNA also leads to an acceleration of the induction of β -IFN expression, with specific mRNA being detectable as early as one hour after induction (lanes 10 and 14).

Since the overexpression of IRF-1 was capable of priming cells, it was interesting to ask whether IRF-1-expressing HeLaE cells demonstrated other properties seen in primed cells. Specifically, since it was shown in Chapter Four that induction through PRD II was priming-dependent, the ability of IRF-1-expressing cells to activate PRD II function was tested in the absence of priming. Figure 5.7A demonstrates that a reporter gene containing PRD II is indeed responsive to dsRNA in a priming-independent fashion when IRF-1 is overexpressed, as is a [-108/+72] β -IFN promoter construct. Furthermore, when IRF-1-expressing cells are isolated using CD2 selection they are able to induce NF- κ B in response to dsRNA in a priming-independent manner (Figure 5.7B, lanes 1-4). Since the CD2-ve cells remain priming-dependent in this experiment (lanes 5-8), secreted factors cannot be responsible for the priming effect of IRF-1. These results demonstrate that IRF-1 can act indirectly to prime cells since it is incapable of binding to the PRD II element (data not shown).

Figure 5.6 Transient transfection of IRF-1 renders the endogenous β -IFN gene in HeLaE cells priming-independent

(A). pCO β IRF-1 was constructed by inserting a cDNA for human IRF-1 into the polylinker of pCO2 (Whiteside et al. 1994). The presence of the β -globin 5' untranslated region (5' utr) upstream of the cDNA allows efficient translation of the transcribed message driven from the cytomegalovirus (CMV) promoter.

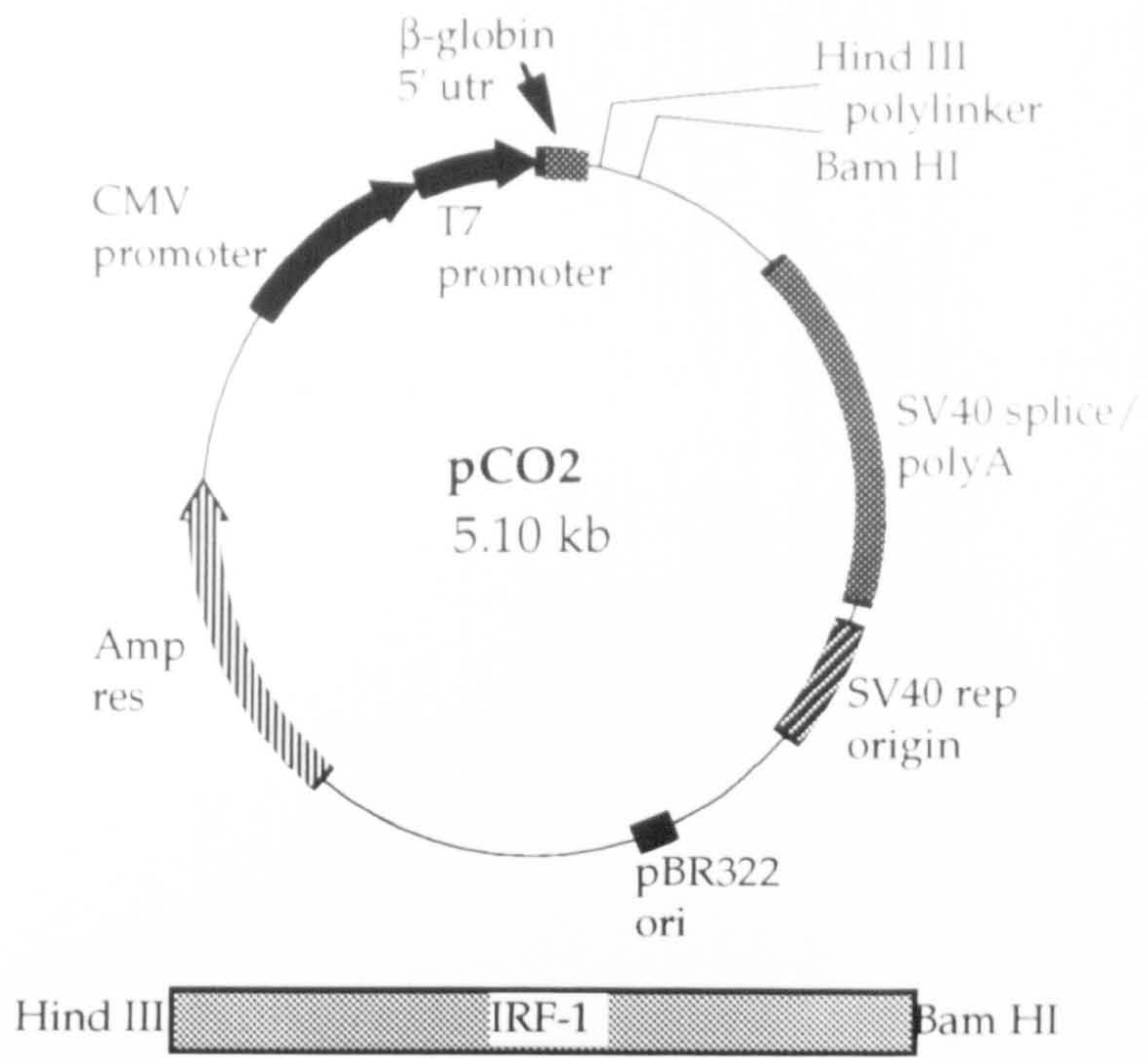
(B). HeLaE cells were co-transfected, by calcium phosphate co-precipitation, with 5 μ g of pKSCD2 (Whiteside et al. 1994), a CMV promoter-driven expression plasmid for the T-cell-specific surface marker, CD2, and 10 μ g of either pCO2 or pCO β IRF-1. In each case, the cells were treated thus:

induced with dsRNA over a 4 hour time course (lanes 1-4 and 9-12)

primed and induced over a 4 hour time course (lanes 5-8 and 13-16)

and following selection for CD2-positive cells (see Chapter Two), RNA was isolated and mapped for β -IFN- and γ -actin-specific messages, which are indicated to the left of the autoradiograph. The length of exposure to dsRNA, in hours, is indicated above each lane.

A



B

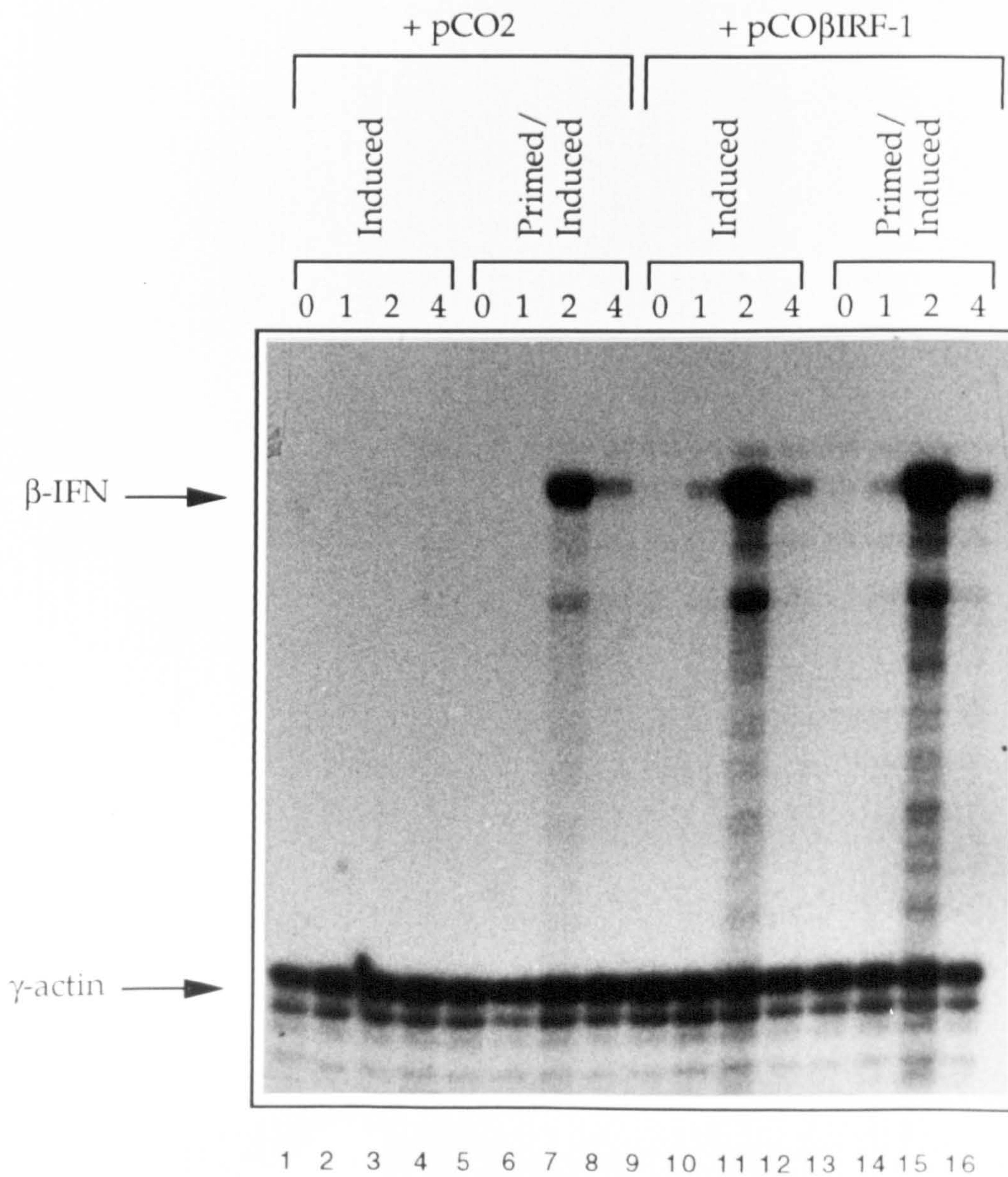


Figure 5.7 A transiently transfected [-108 /+72] β -IFN promoter fragment and a PRD II-driven promoter are rendered priming-independent by IRF-1

(A). HeLaE cells were co-transfected, by calcium phosphate co-precipitation, with 10 μ g of pIF[-108/+72]lucifer or p[PRD II]_{5tk} Δ [-39]lucifer, 10 μ g of pCO₂ (- IRF-1) or pCO β IRF-1 (+ IRF-1), and 2 μ g of pSV₂CAT. The transfected cells were treated as indicated (Un = unprimed, uninduced, P = primed, uninduced, I = unprimed, induced and PI = primed, induced), and the luciferase activity from each condition was normalised to the respective CAT activity.

(B). HeLaE cells were co-transfected, by calcium phosphate co-precipitation, with 5 μ g of pKSCD2 and 10 μ g of pCO₂ or pCO β IRF-1 and treated as follows:

unprimed, uninduced (lanes 1 and 5)

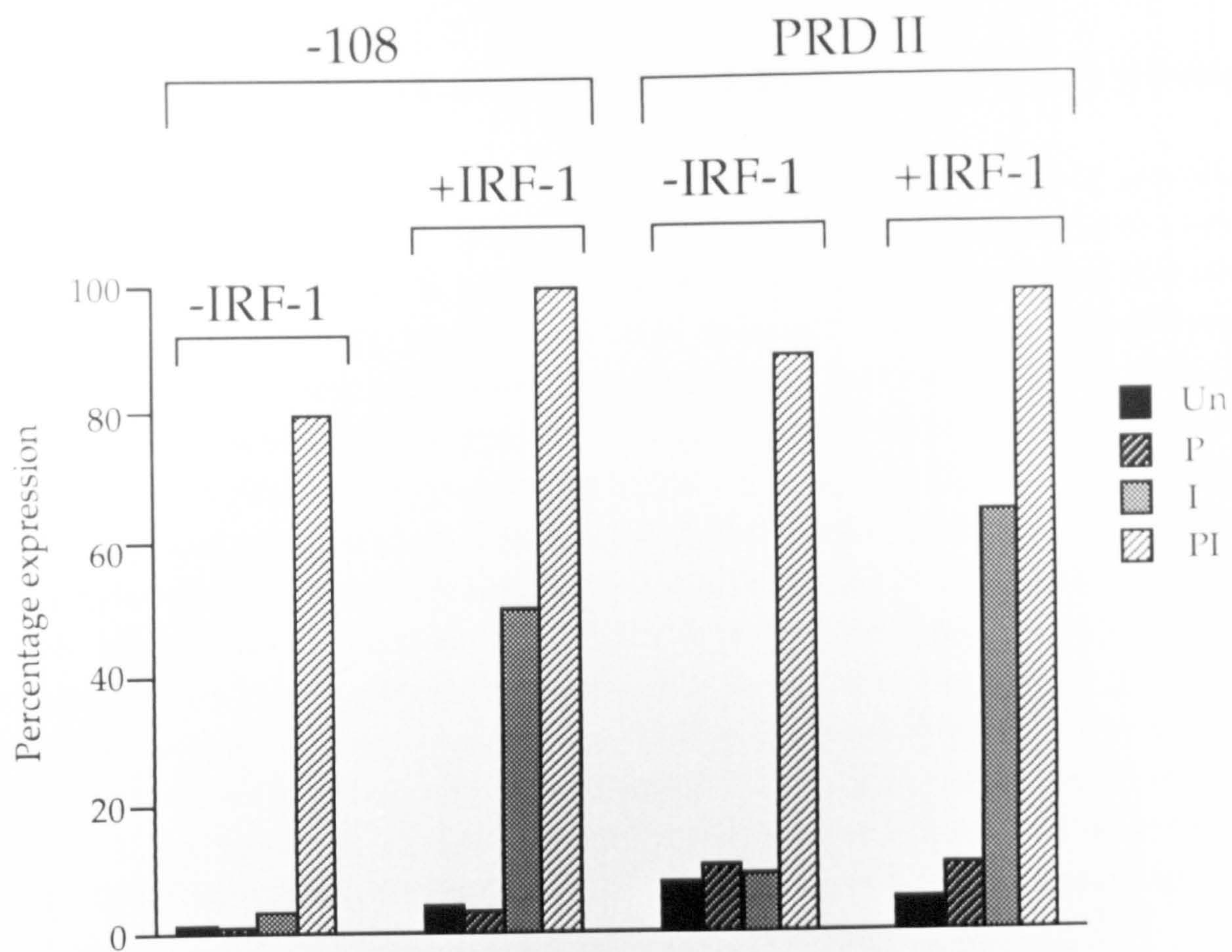
unprimed, induced (lanes 2 and 6)

primed, uninduced (lanes 3 and 7)

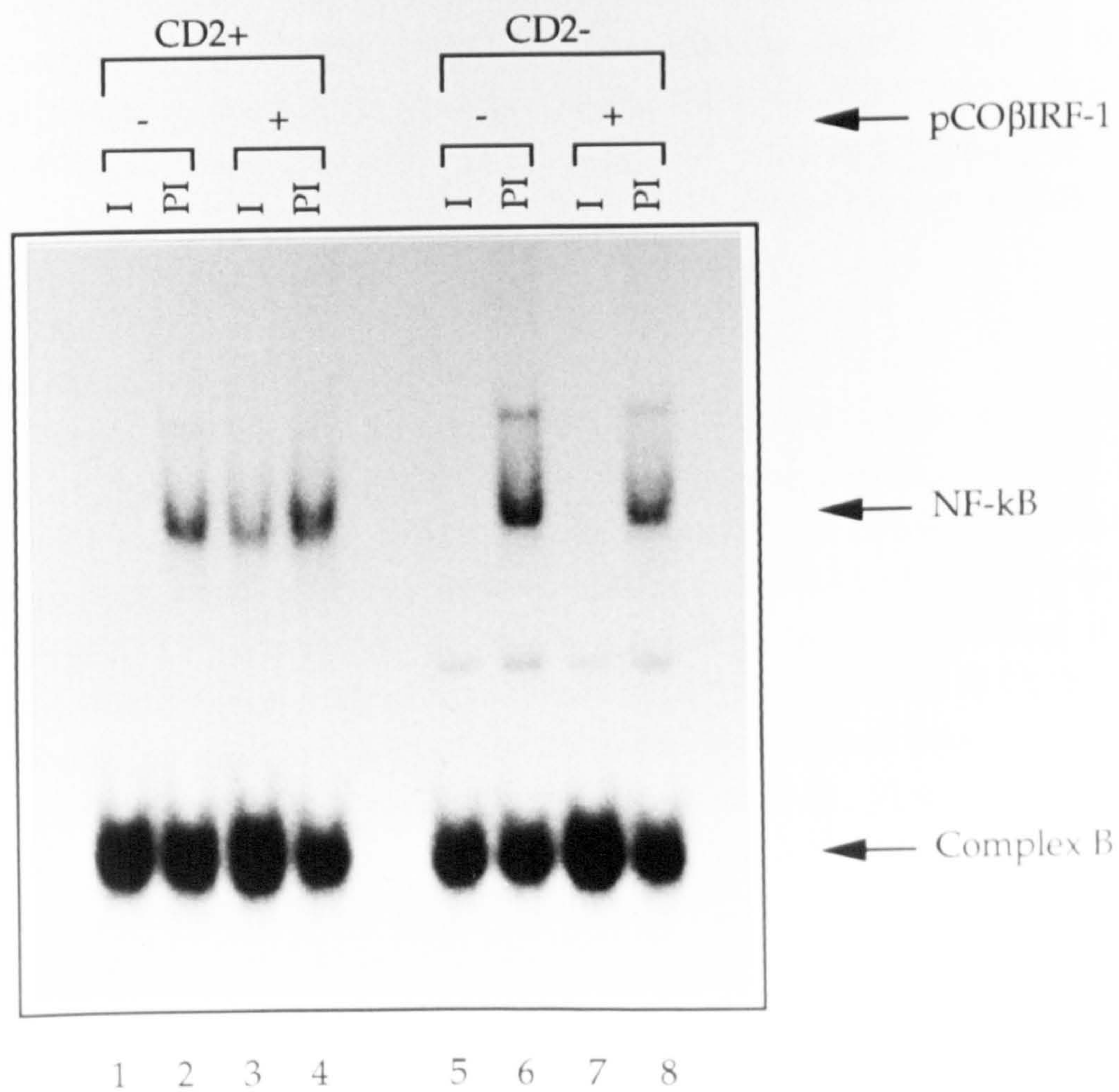
primed, induced (lanes 4 and 8)

Following treatment the cells were separated into CD2-positive (lanes 1-4) and negative (lanes 5-8) fractions and nuclear extracts were made from both pools. The nuclear extracts were subjected to EMSA analysis on a radiolabelled PRD II probe to assess the extent of NF- κ B activation. The position of NF- κ B and complex B (Visvanathan and Goodbourn 1989) are indicated.

A



B



Treating MG63 cells with antisense IRF-1 oligonucleotides renders them priming-dependent

In order to test whether the loss of IRF-1 function renders normally priming-independent cells dependent upon priming, antisense oligonucleotides were introduced into MG63 cells. To achieve this, a complementary phosphorothioate-substituted oligonucleotide was chosen that spanned the initiator methionine codon in the IRF-1 mRNA, and preliminary experiments indicated that loss of IRF-1 function could be obtained by simple addition of this oligonucleotide to the growth medium at 10 μ M. The cells were exposed to the oligonucleotide for 2, 3 or 4 days and then induced with dsRNA. The results of the RNase protection analysis of RNA samples prepared from these cells are shown in Figure 5.8. The presence of the antisense oligonucleotide renders the MG63 cells largely dependent upon priming after two days, and totally dependent after three. In all cases, the level of induction achieved following priming is unaffected. This was also found to be the case in priming-independent 2fTGH cells (see Chapter Six). These results indicate that when IRF-1 expression is impaired, β -IFN induction can be restored to cells by priming. Taken together with the results of IRF-1 overexpression, it would appear that priming and IRF-1 expression are functionally redundant.

The results presented so far in this Chapter suggest that although the presence of IRF-1 protein in cells at the time of addition of dsRNA is not necessary for the induction of β -IFN, a prior exposure to this protein is sufficient to render the cells permissive for induction by dsRNA. It would appear unlikely that IRF-1 needs to act directly on the β -IFN promoter to achieve this result because it can prime the induction of a PRD II-driven promoter which does not contain a binding site for IRF-1. It is therefore likely that the role of IRF-1 is to effect the upregulation of another gene(s) required for priming.

β -IFN promoter requirements for IRF-1 effects

Despite the fact that IRF-1 can act indirectly, it is possible that it can play a more direct role, since the β -IFN promoter contains strong binding sites for this factor (PRD I and PRD III). Two separate approaches have been made in an attempt to address the question of the direct or indirect nature of IRF-1 in its role in priming HeLaE cells. Firstly, the inducibility of β -IFN promoter mutants lacking IRF-1 binding sites was tested in the presence and absence of overexpressed IRF-1. To this end, linker-scanning mutants of pIF[-116/+72]lucifer were constructed using the deletion mutagenesis procedure described in Chapter Four. Since preliminary

Figure 5.8 Antisense IRF-1 oligonucleotides render MG63 cells priming-dependent

MG63 cells were grown under normal conditions (lanes 1 and 2) or in the presence of 10 μ M phosphorothioate-linked antisense IRF-1 oligonucleotides for 2 (lanes 3 and 4), 3 (lanes 5 and 6) or 4 (lanes 7 and 8) days. The cells were treated as follows:

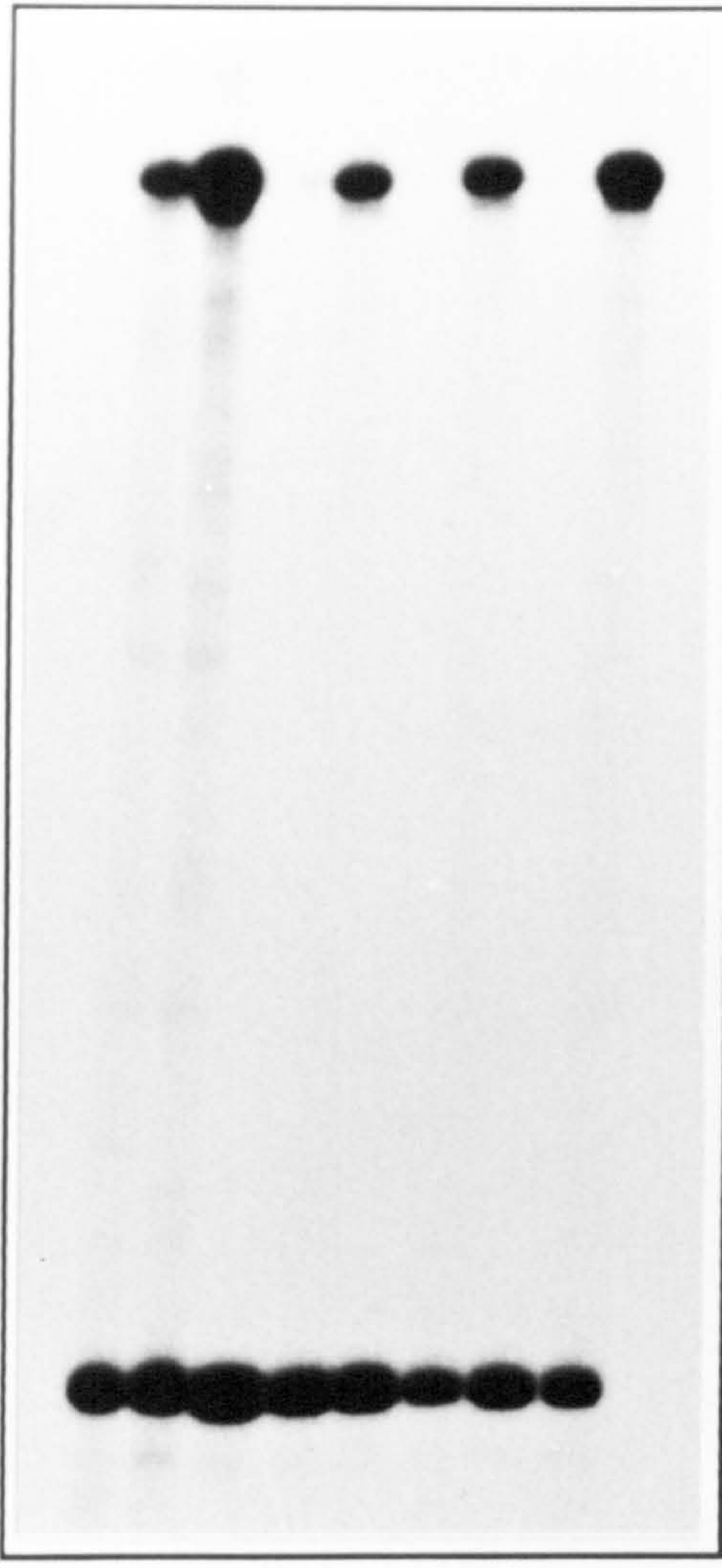
induced with dsRNA for 4hrs (lanes 1, 3, 5 and 7)

primed and induced (lanes 2, 4, 6 and 8)

and the RNA isolated from each sample was mapped with probes specific for β -IFN and γ -actin. The position of the protected bands is indicated to the side of the autoradiograph.

0 2 3 4
┌──┐ ┌──┐ ┌──┐ ┌──┐
I PI I PI I PI I PI

← Days antisense
IRF-1 treatment



← β -IFN

← γ -actin

1 2 3 4 5 6 7 8

experiments had determined that removing β -IFN promoter sequences seriously impairs the activity of the promoter (data not shown), a highly efficient liposome-based method of transfection was used in this experiment, rather than the calcium phosphate co-precipitation method used in Chapter Four and Figure 5.7A.

Figure 5.9 shows the results of this experiment. Strikingly, as well as leading to priming-independent induction of the wild type [-116/+72] promoter, the inclusion of pCO β IRF-1 in the transfection caused an approximately 7-fold enhancement of luciferase activity in the uninduced and primed/induced cases, an effect that is more marked than that previously seen in Figure 5.7A. This elevation of promoter activity seen with IRF-1 may reflect a direct contribution to β -IFN gene expression.

When PRD II was substituted with a linker sequence (LS[-64/-55]) the β -IFN promoter is completely uninducible, either after priming, or in the presence of overexpressed IRF-1. This result demonstrates that PRD II is essential for induction by dsRNA. Strikingly, this mutant is also transactivated approximately 7-fold by the inclusion of the IRF-1-expressing plasmid. Since this elevation is not a function of PRD II, IRF-1 must be contributing in some other manner to gene expression, perhaps by direct binding to the PRD I and PRD III elements.

When PRD I was substituted with a linker sequence (LS[-74/-65]), β -IFN induction is impaired but remains inducible in a priming-dependent manner. IRF-1 overexpression enhances β -IFN promoter activity, and confers priming-independence upon the promoter. Interestingly, β -IFN promoter activity is not stimulated to the same degree as either the intact promoter or the PRD II knockout, and such a result is consistent with the loss of one binding site for IRF-1. The priming-dependence of this promoter may reflect the requirement for priming for PRD II activation. Surprisingly, the PRD III knockout construct, LS[-90/-82], is uninducible in the absence of co-expressed IRF-1, although the inclusion of IRF-1 does lead to a slight restoration of inducibility, which is priming-independent. Once again there is a general enhancement of luciferase activity upon IRF-1 cotransfection which is comparable to that observed with the PRD I knockout construct.

A mutant that removes the function of both PRD I and PRD III (LS[-82/-73]) is completely uninducible even when cotransfected with pCO β IRF-1. These results demonstrate that induction of the β -IFN promoter absolutely requires

Figure 5.9 Analysis of the priming-dependence of β -IFN promoter constructs lacking PRD I, PRD II and PRD III

(A). The linker scan constructs pIF[-116/+72]LS[-64/-55], [-74/-65], [-90/-82] and [-82/-73] target the regions PRD II, PRDI, PRD III and both PRD I and PRD III respectively (see the top depiction of pIF[-116/+72]lucifer). They were created by combining deletion mutants from a 5' deletion series of pIF[-116/+72]lucifer (-55, -65, -82 and -73 endpoints) with those from a 3' deletion series, generated from the Nco I site at -12 (-64, -74, -90 and -82 endpoints), via a Bgl II linker, which gives the sequence CAGATCTG. These deletion series were generated as described in Chapter Three.

(B). The wild type and linker scan mutant forms of pIF[-116/+72]lucifer (0.9 μ g) were transiently transfected into HeLaE cells, using Lipofectamine, with 0.9 μ g of either pCO2 or pCO β IRF-1, and 0.2 μ g pSV2CAT. The transfected cells were treated as follows:

unprimed, uninduced (Un)

unprimed, induced (I)

primed, induced (PI)

The luciferase activities were normalised to CAT and the relative expression levels are shown.

PRD II, and at least one of the PRD I or PRD III elements. Since the dsRNA-independent and priming-independent enhancement of expression seen with IRF-1 overexpression is impaired when either PRD I or PRD III is mutated, and entirely lost when both elements are damaged, these results strongly suggest that IRF-1 acts directly through these sequences.

The extent to which overexpression of IRF-1 can prime HeLaE cells is dose-dependent

Further evidence to support a direct role for IRF-1 in β -IFN induction was provided by experiments which tested the effect of concentration of co-transfected pCO β IRF-1 on the establishment of priming-independence. pIF[-116/+72]lucifer was cotransfected with a range of pCO β IRF-1 concentrations, and the luciferase activities were determined in the uninduced, induced and primed and induced cases. Figure 5.10 shows the results of this titration. At a 1:1 ratio of pIF[-116/+72]lucifer to pCO β IRF-1 (0.9 μ g of each plasmid in the transfection mix) there is a large increase in the uninduced and primed/induced levels, compared to pIF[-116/+72]lucifer alone (Figure 5.10A). There is also a noticeable degree of priming-independent induction (approximately 50% of the primed and induced level). Surprisingly, this priming-dependent induction is almost completely lost as the ratio of pIF[-116/+72]lucifer to pCO β IRF-1 is increased to 5:1 (0.18 μ g of pCO β IRF-1), with only a slight decrease in the elevated uninduced and primed/induced levels. These levels decline steadily as the ratio is increased to 50:1, with a further increase to 100:1 having little effect, leaving a residual elevation of luciferase levels and a priming-dependent promoter.

This result suggested that to maximise the priming effect of the overexpressed IRF-1, the amount of IRF-1 expression plasmid in the transfection mix needed to be increased. Figure 5.10B shows the results obtained when a constant amount of pIF[-116/+72]lucifer (0.1 μ g) was included in the transfection mix with an increasing amount of pCO β IRF-1 over a range of ratios of 1:1 to 18:1 of pCO β IRF-1 to pIF[-116/+72]lucifer. At a ratio of 1:1 (0.1 μ g of each plasmid), an equivalent elevation of the luciferase levels to that in the 1:1 case in A was observed, although the priming effect observed at this ratio in Figure 5.10A was not seen, suggesting that this is a consequence of the overall levels of IRF-1 in the cell rather than the ratio of IRF-1 expressing plasmid to β -IFN promoter plasmid. As the ratio of IRF-1 expressing plasmid to IFN promoter plasmid increases, the uninduced and primed/induced levels reach a maximum at 5:1, with a slight degree of priming-independent induction appearing. At higher ratios no further increase in the uninduced and

Figure 5.10 The degree of priming-independent induction achieved by IRF-1 overexpression is dose-dependent

(A). HeLaE cells were transiently transfected, using Lipofectamine, with 0.9 μ g of pIF[-116/+72]lucifer and varying amounts of pCO β IRF-1, between 0.009 μ g and 0.9 μ g (100:1 to 1:1 ratios), as shown, and 0.2 μ g of pSV2CAT. The amount of DNA in the transfection mix was made up to 2 μ g with pCO2, as appropriate.

(B). A similar experiment to that shown in A was performed, but using a constant 0.1 μ g of pIF[-116/+72]lucifer, and varying amounts of pCO β IRF-1, between 0.1 μ g and 1.8 μ g (1:1 to 1:18 ratios), as shown.

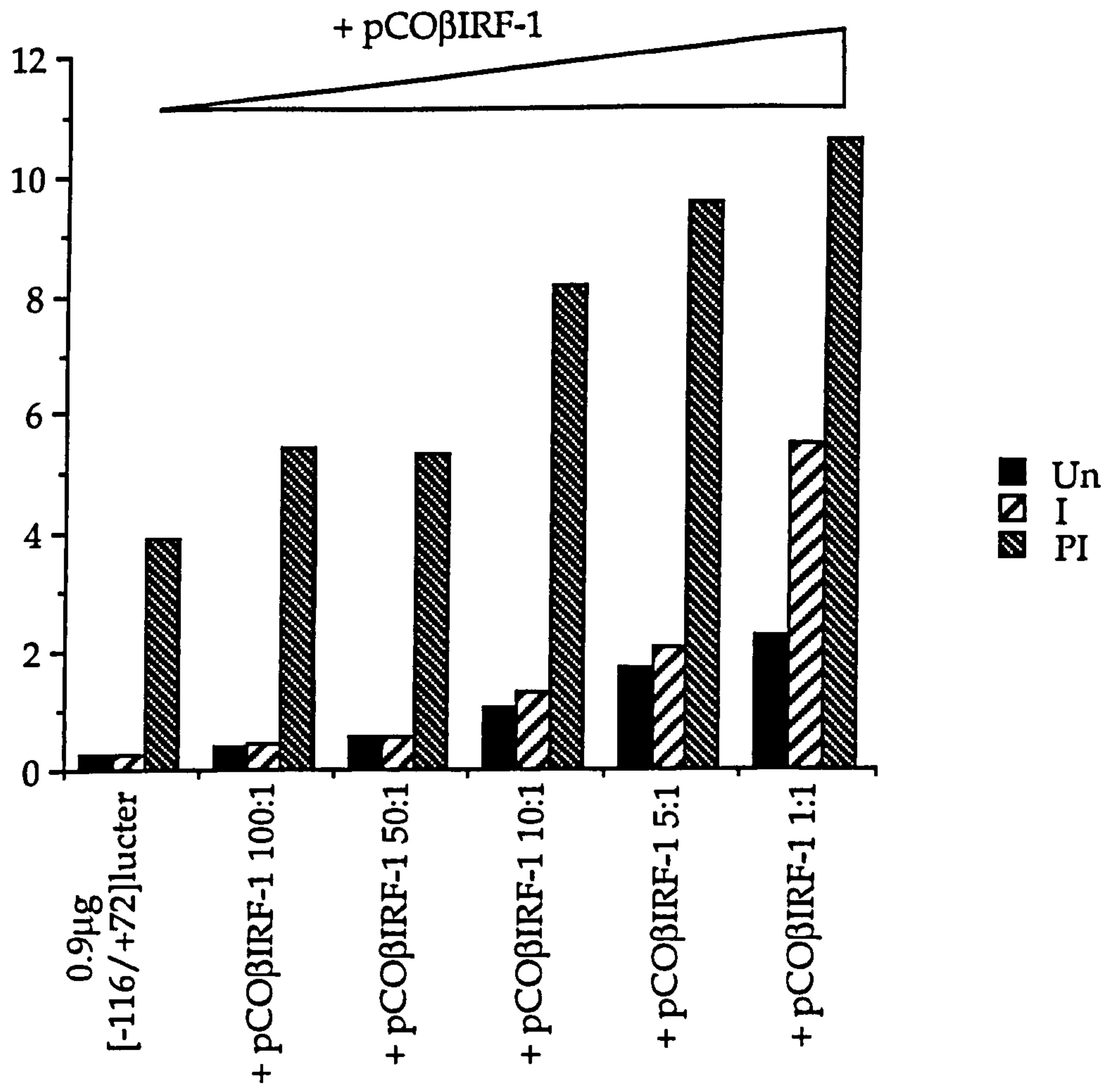
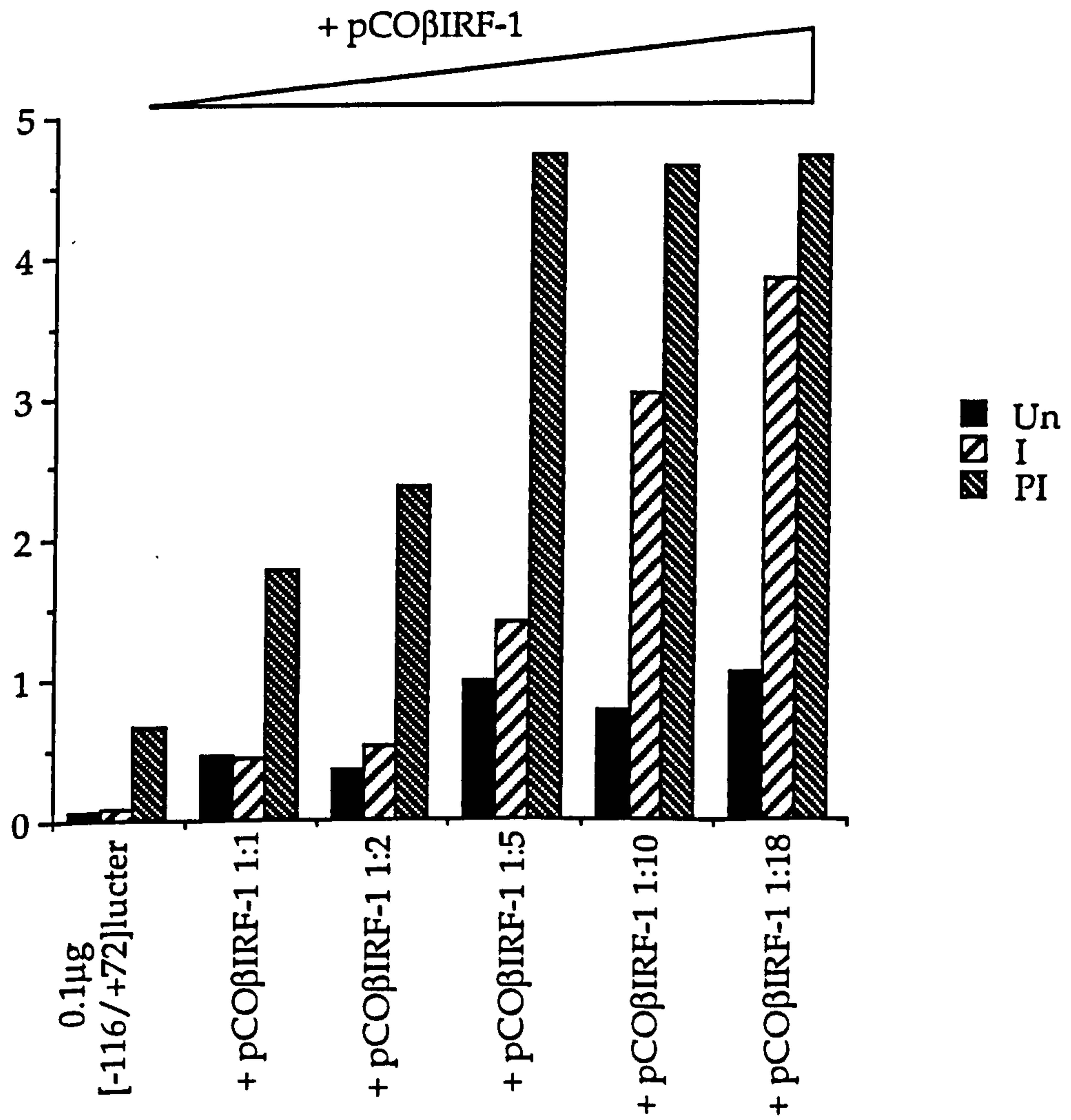
In each figure the transfected cells were treated thus:

unprimed, uninduced (Un)

unprimed, induced (I)

primed, induced (PI)

The luciferase activities were normalised to CAT and the relative expression levels are shown.

A**B**

primed/induced levels was observed, suggesting that this effect has saturated, whereas the degree of priming-independence continues to increase until at 18:1 it is approximately 85% of the primed and induced level.

The above results demonstrate that there are two effects of IRF-1 on β -IFN promoter function that show different concentration dependence. The effect on expression in the absence of induction or priming shows the ability to be saturated, in contrast to the priming effect which continues to be enhanced at all concentrations tested. This suggests that the two effects are distinguished by the affinity of IRF-1 for different promoters. Since the higher affinity effect requires IRF-1 binding sites in the β -IFN promoter, it is tempting to speculate that this effect is direct.

HeLaE cells expressing an IRF-1/HBD fusion protein can be primed by the addition of oestrogen

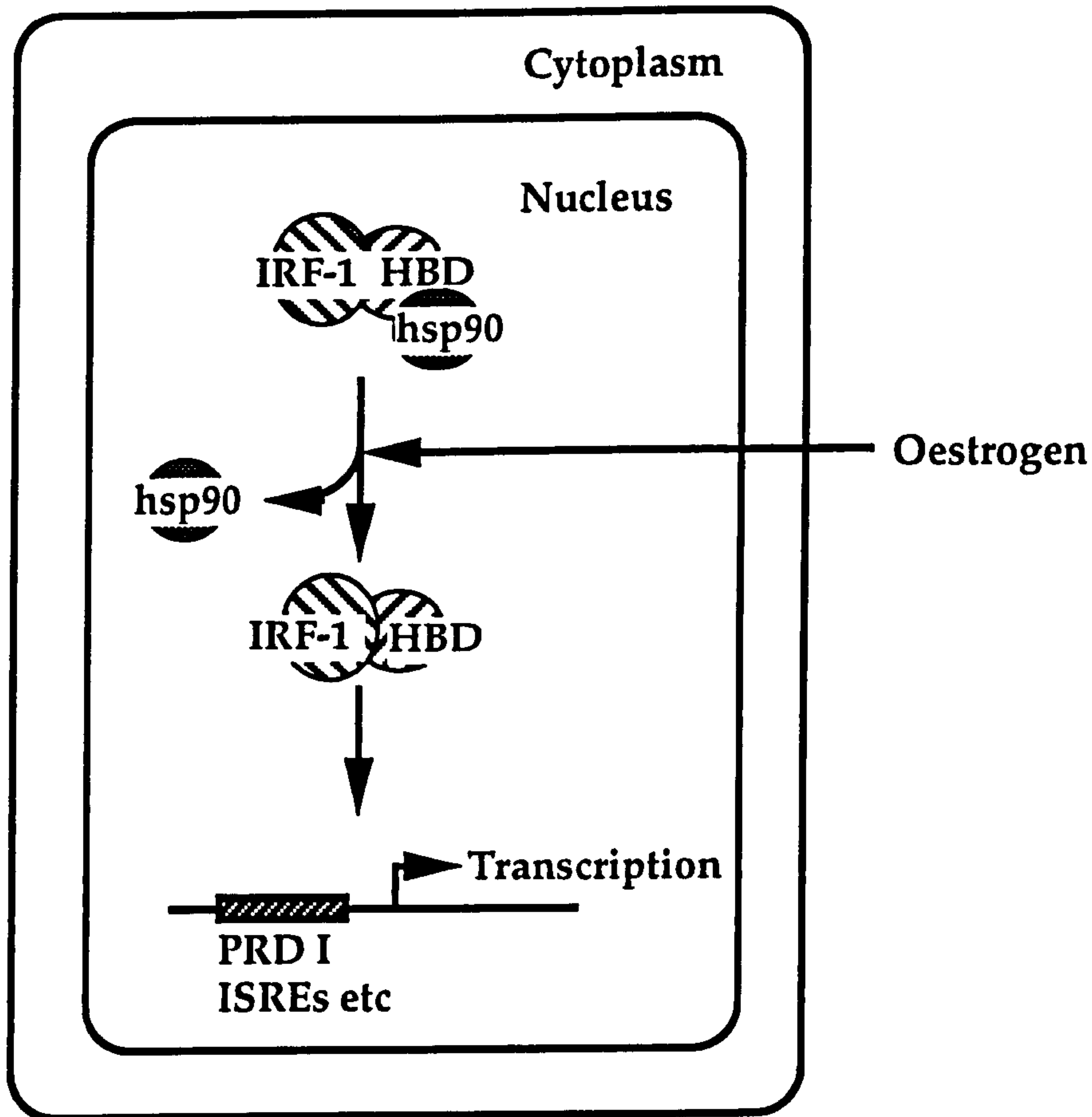
In a second approach to investigate the mode of action of IRF-1 in priming HeLaE cells, a system was established whereby IRF-1 protein could be produced in cells in a transcriptionally inert form, which could be converted to an active form without the requirement for protein synthesis. To achieve this, constructs were made which express IRF-1 as a fusion protein with a hormone binding domain (HBD) from the human oestrogen receptor (hER) fused to the C-terminus. The presence of the HBD causes the fusion protein to be sequestered in the nucleus in a non-DNA bound state due to the association between the HBD and the heat shock factor hsp90. This strategy has been used to generate conditional forms of several transcription factors (Littlewood et al. 1995) (see Figure 5.11). The fusion protein remains bound to hsp90 until the cells are treated with oestrogens such as 17β -oestradiol or tamoxifen. These agents cause rapid disassociation of the complex allowing the fusion proteins access to the DNA. If IRF-1 is acting in a direct manner, then liberation of the IRF-1/HBD fusion protein from hsp90 following oestrogen treatment should lead to priming-independent induction of the β -IFN gene by dsRNA even in the presence of protein synthesis inhibitors. Conversely, if IRF-1 acts in an indirect manner by inducing other factors, then treatment of cells harbouring the IRF-1/HBD fusion with oestrogen should render them priming-independent only if protein synthesis is allowed to proceed.

Figure 5.11 Creating an IRF-1/HBD fusion protein.

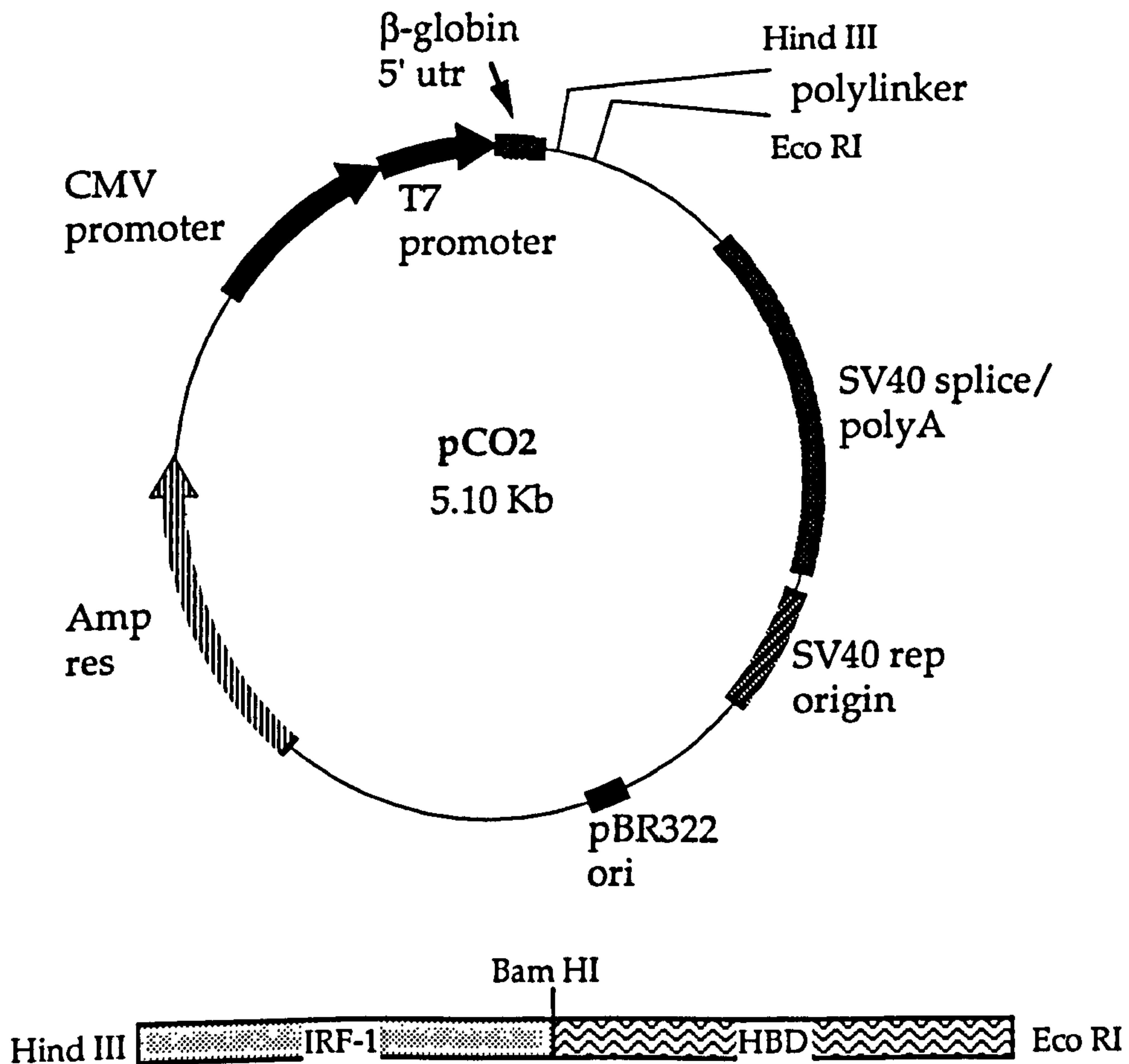
(A). The experimental scheme for the regulation of an oestrogen-responsive IRF-1 fusion protein is shown. The fusion protein is sequestered in the nucleus away from its binding sites by the interaction of the C-terminally fused hormone binding domain, from the human oestrogen receptor, and the hsp90 factor. Upon treatment of the cells with oestrogen, the fusion protein is rapidly liberated from hsp90 and can then bind its target sites to activate transcription.

(B). pCO β IRF-1hER was constructed by fusing the hormone binding domain of the human oestrogen receptor (amino acids 282-595), isolated from pMU7.mycER (a kind gift from T. Littlewood, ICRF) as a BamH I/EcoR I fragment in frame to the C-terminal BamH I site in the human IRF-1 cDNA in pCO β IRF-1.

A



B



In an initial experiment to test whether the IRF-1/HBD fusion protein could act as an oestrogen-inducible transcription factor, pCO β IRF-1hER was cotransfected into HeLaE cells along with an IRF-1-responsive promoter-driven luciferase construct, p(AAGTGA)₄tk Δ [-39]lucifer (Figure 5.12A). In the absence of 17 β -oestradiol the IRF-1/HBD fusion product causes a slight transactivation of the reporter construct, but following addition of the inducer, which has no effect in the absence of pCO β IRF-1hER, the reporter construct is activated approximately 8-fold. These levels are about 20% of the levels that are achievable when the pCO β IRF-1 wild-type IRF-1-expressing plasmid is included in the experiment.

Having established the oestrogen-dependence of the transactivational ability of the IRF-1/HBD fusion protein, it was thus possible in effect to prime HeLaE cells cotransfected with the pCO β IRF-1hER expression plasmid by the addition of oestrogen. Figure 5.12B shows the results from an experiment in which HeLaE cells were cotransfected with pCO β IRF-1hER and pIF[-108/+72]lucifer. Following an overnight incubation of the cells with 17 β -oestradiol the β -IFN promoter shows constitutive stimulation in a manner that is reminiscent of the stimulation seen with the overexpression of IRF-1 (Figures 5.9 and 5.10). However, under these conditions, induction of the β -IFN promoter by dsRNA can now be seen in the absence of priming. This effect requires longer than 2 hours to develop indicating that the priming-substitution effect of IRF-1 overexpression is not direct, because the quantitative release of proteins from hsp90 in response to 17 β -oestradiol takes only 5 minutes (M. Parker, ICRF, pers. comm.). Interestingly, if cells are primed, there is a marked decrease in expression in the presence of prolonged exposure to 17 β -oestradiol. The molecular basis of this effect is not understood, but the combination of priming and 17 β -oestradiol treatment has been observed to inhibit the β -IFN promoter in the absence of cotransfected pCO β IRF-1hER (data not shown).

Discussion

This Chapter has set out to investigate the roles of some of the better characterised IFN-inducible proteins in the establishment of the primed state. The two dsRNA-activatable IFN-inducible proteins were initially considered. The dsRNA-activated 2,5-A_n synthetase has a variety of forms of different sizes and subcellular localisations (see Chapter One), and any differences in activities between these proteins have yet to be determined, allowing the formal possibility

Figure 5.12 The IRF-1/HBD fusion protein can render HeLaE cells priming-independent in an oestrogen-dependent manner

(A). HeLaE cells were transiently transfected, using calcium phosphate co-precipitation, with 10 μ g of p[AAGTGA₄]_{5tk} Δ [-39]lucifer, a PRD I-like reporter plasmid (Whiteside et al. 1994), and 10 μ g of either pCO₂, pCO β IRF-1, or pCO β IRF-1hER, along with 2 μ g of pSV₂CAT. The transfections were either untreated or treated for 18 hours with 2 μ M 17 β -oestradiol, and the luciferase activities were normalised to CAT.

(B). HeLaE cells were transiently transfected, using calcium phosphate co-precipitation, with 10 μ g of pIF[-108/+72]lucifer, 10 μ g of pCO β IRF-1hER and 2 μ g of pSV₂CAT. The transfected cells were treated as indicated:

Un = unprimed, uninduced

I = unprimed, induced with dsRNA

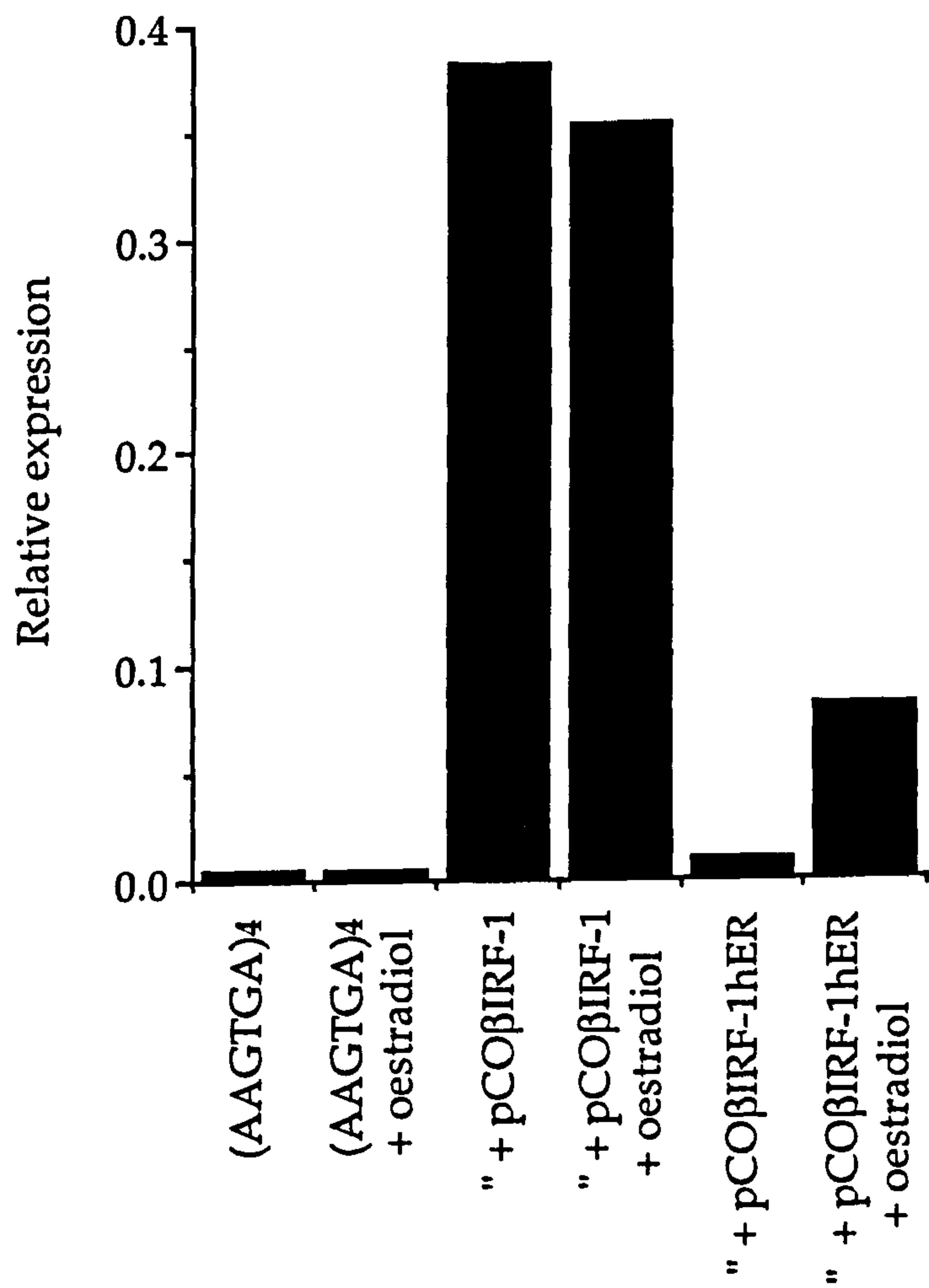
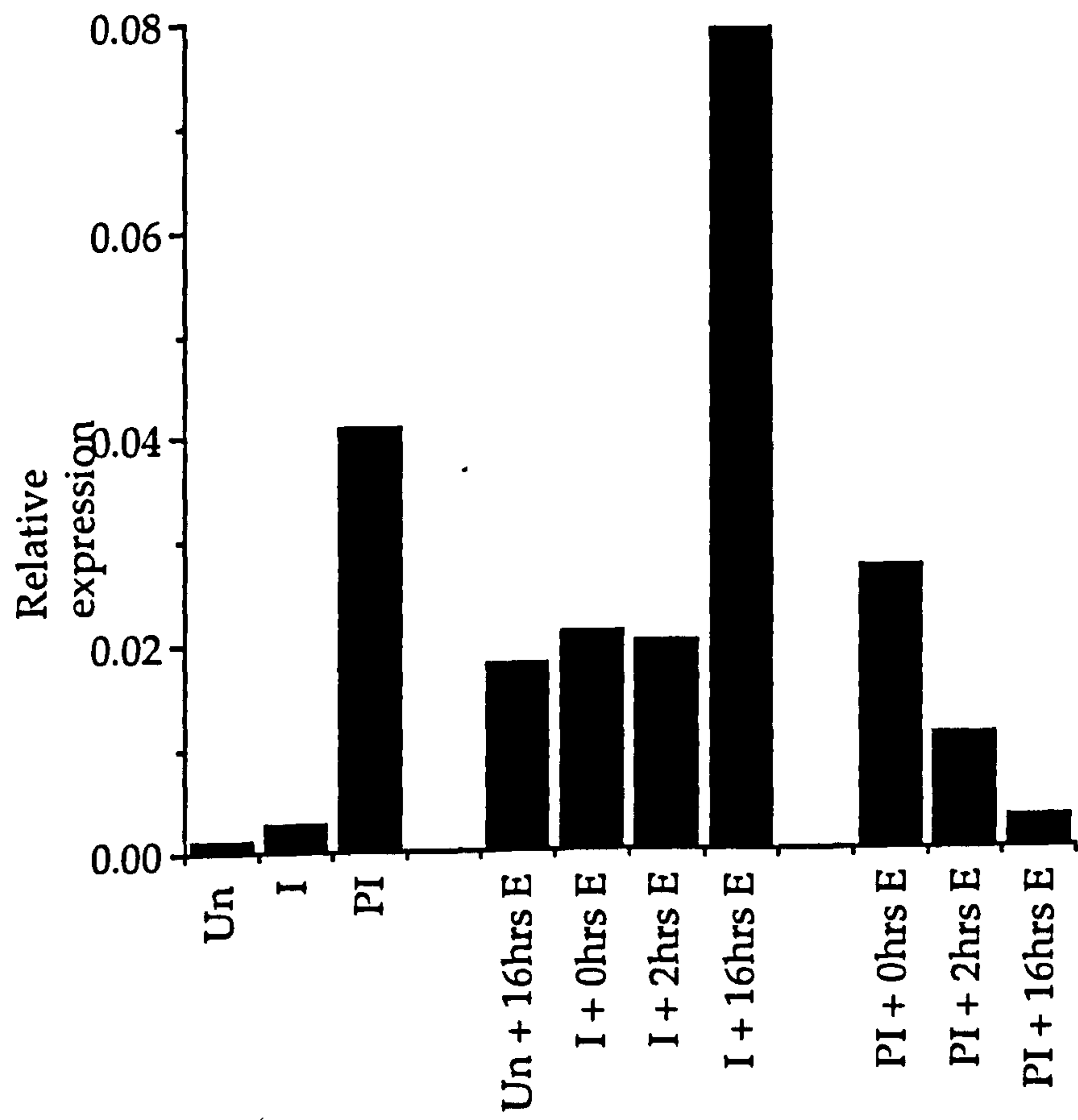
PI = primed, induced

Un + 16hrs E = unprimed, uninduced with 16 hours treatment with 2 μ M 17 β -oestradiol

I + xhrs E = unprimed, induced following 2 and 16 hours pre-treatment with 17 β -oestradiol, or with the addition of 17 β -oestradiol at the time of induction (I + 0hrs E)

PI + xhrs E = primed, induced following 2 or 16 hours pre-treatment with 17 β -oestradiol, or with the addition of 17 β -oestradiol at the time of induction (PI + 0hrs E).

The luciferase activities were normalised to CAT.

A**B**

that one or more of these forms may play an as yet unidentified role in β -IFN induction. In the absence of cDNAs for these forms of the synthetase no direct assessment of the involvement of these proteins could be made. However, the fact that HeLa cells are known to have high basal levels of synthetase (Baglioni et al. 1979), together with the inability of a dominant-negative inhibitor of RNaseL, which is activated as a consequence of the only known catalytic activity of the synthetase, namely the production of 2,5- A_n , to block induction in NIH3T3 cells appears to rule out a role for this enzyme.

On the other hand, it is widely believed that the IFN-inducible, dsRNA-activated protein kinase, PKR, does play a role in β -IFN induction (see Chapter One), and the results presented here confirm that PKR is involved in the activation of NF- κ B by dsRNA, and demonstrate that dominant interfering mutants of PKR can impair β -IFN induction in primed HeLaE cells. However, even though there is clearly less PKR in unprimed HeLaE cells, it is present. Furthermore, it has been previously reported that in GM7267 cells and other human fibroblasts, which have very low levels of PKR even after IFN-treatment, dsRNA-mediated induction events are unimpaired (Holmes and Gupta 1982). Importantly, overexpression of PKR cannot render HeLaE cells priming-independent. During the course of these studies, PKR "knockout" mice (mice devoid of functional PKR) were bred and embryo fibroblasts from these animals were analysed to determine the requirement for PKR in both NF- κ B activation and β -IFN induction in response to dsRNA treatment. Interestingly, both responses were absent in these cells unless they were primed, after which they were almost fully restored (Yang et al. 1995). These results have been interpreted to indicate the existence of an IFN-inducible alternative pathway for dsRNA induction which does not require PKR. The results presented in this Chapter demonstrate that PKR is necessary but not sufficient for β -IFN induction.

In contrast to the inability of overexpressed PKR to relieve the priming-dependence of HeLaE cells, overexpression of another IFN-inducible protein, the transcription factor IRF-1, leads to priming-independent induction of the endogenous β -IFN gene in transiently transfected HeLaE cells (Figures 5.6 and 5.7). This result, together with that shown in Figure 5.8, which shows that priming-independent MG63 cells can be rendered priming-dependent by their growth in the presence of antisense IRF-1 oligonucleotides, provides compelling evidence that it is the absence of IRF-1 protein in normally growing HeLaE cells that is responsible for their priming-dependence.

The role of IRF-1 in the induction of β -IFN by dsRNA has been controversial. It was originally proposed to be the primary activator of β -IFN induction on the basis that it could bind to the promoter and activate promoter constructs, and also activate the endogenous gene in COS cells (albeit to a very small degree) and in EC cells (although not in GM-637 cells). Furthermore, when antisense IRF-1 constructs were stably introduced into priming-independent GM-637 cells (Reis et al. 1992), the β -IFN gene in these cells was no longer inducible by dsRNA and only to a small extent by NDV. However, contrary evidence showed that β -IFN induction could be achieved both in the absence of detectable IRF-1 and ongoing protein synthesis (Pine et al. 1990, Whiteside et al. 1992), indicating that IRF-1 was not necessary for induction, at least in these cell lines.

The data presented in this Chapter on the role of IRF-1 in the priming response of HeLaE cells resolve the controversy over the importance of IRF-1 for dsRNA induction of β -IFN. It appears that even though IRF-1 is produced during priming, it is produced only transiently and has declined to low or undetectable levels by the time the HeLaE cells would normally be induced (see Figures 5.4 and 5.5). It is important to remember that primed HeLaE cells are inducible in the presence of cycloheximide (Whiteside et al. 1992), and that HeLaE cells remain inducible at least 2 days after the removal of IFN from the cells (Figure 3.6), presumably long after the spike of IRF-1 synthesis has decayed. It would therefore appear that although the presence of IRF-1 protein is not required at the time of addition of dsRNA for induction of the β -IFN gene, a prior exposure to the protein is sufficient to render the cells competent for induction. Conversely, the ablation of IRF-1 from MG63 cells renders them uninducible by dsRNA, although importantly this can be reversed by priming. This result is analogous to the observation that GM-637 cells harbouring antisense IRF-1 constructs are uninducible by dsRNA (Reis et al. 1992). Although these workers did not attempt to prime these cells, one would hypothesise that priming would restore the inducibility of the β -IFN gene in these lines.

During the course of these experiments, evidence in support of the theory proposed here, that the priming requirement of cells for β -IFN induction by dsRNA is a consequence of the absence of IRF-1, was obtained from the study of embryonal stem (ES) cells in which both IRF-1 alleles had been disrupted, and from cells derived from mice generated by injection of these cells into blastocysts (IRF-1 "knockout" mice). In the absence of both IRF-1 alleles, mouse embryo fibroblasts (MEFs) were largely uninducible by dsRNA, although this inducibility could be

restored by priming the cells (Matsuyama et al. 1993). Taken together, the results obtained from the IRF-1 overexpression experiments in this Chapter and from the MEFs indicate that IRF-1 and IFN treatment can activate alternative pathways leading to the permissiveness of cells for β -IFN induction by dsRNA. Thus IRF-1 expression and priming are functionally redundant. Interestingly, disruption of both IRF-1 alleles did not seriously impair the induction of β -IFN by NDV (Ruffner et al. 1993, Matsuyama et al. 1993). As was seen in the experiments shown in Chapters Three and Four, Sendai virus is also capable of substantial β -IFN induction in the absence of priming, and thus at least two types of paramyxovirus are capable of inducing β -IFN through a pathway that appears neither to depend upon priming or IRF-1.

The observation that IRF-1 is not present in primed HeLaE cells strongly suggests that the mode of action of IRF-1 in priming cells is indirect and presumably a consequence of its ability to transactivate other genes. IRF-1 has been shown to transactivate type I IFN-inducible genes (Taniguchi et al. 1995) and presumably one or more of these IFN- and IRF-1-inducible genes is responsible for establishing the primed state. It is important to stress that a trivial explanation for the ability of IRF-1 to prime cells can be ruled out. Even though it has been documented that IRF-1 can induce type I IFN genes, the mechanism by which IRF-1 is priming the HeLaE cells does not involve a secreted factor, because the CD2-ve cells in Figure 5.7 are not primed in this experiment. The involvement of other IFN- and IRF-1-inducible genes will be investigated in Chapter Six.

The experiments presented in this Chapter also suggest that IRF-1 can play a direct role in the induction of β -IFN, since it can accelerate the kinetics of induction (Figure 5.6) and stimulate the extent of overall expression in a manner that depends upon the presence of IRF-1 binding sites in the β -IFN promoter (Figures 5.9 and 5.10). Furthermore, the degree of transcriptional activation observed from the β -IFN promoter in the absence of induction can be saturated, in contrast to the effect on priming. Since IRF-1 does seem to be able to act in a direct manner, it is important to stress that it has not been possible to formally rule out an essential role for trace amounts of IRF-1 in the induction of HeLa cells, although the results discussed above for IRF-1 knockout cells would argue against this. Antisense IRF-1 oligonucleotides appear to be very efficient at converting MG63 cells into priming-dependent cells, presumably by the ablation of IRF-1 protein synthesis due to the destabilising of the IRF-1 mRNA. However, it is possible that the block to IRF-1 protein synthesis effected by the oligonucleotides can be overcome, however briefly,

by the transient activation of the IRF-1 gene during the course of priming, and this could be directly responsible for the restoration of inducibility. This possibility will be investigated further in Chapter Six. The experiments presented in Figures 5.6 and 5.7A, in which IRF-1 is overexpressed in a constitutively active form, show the results of induction of the β -IFN promoter in the presence of IRF-1, and so a direct involvement of IRF-1 in primary transcriptional activation is obviously possible in these cases. However, in Figure 5.7, even though IRF-1 is present at the time of induction, the PRD II-driven promoter is not capable of binding IRF-1, and thus the role of IRF-1 in establishing priming-independent induction from this promoter must in this case be indirect.

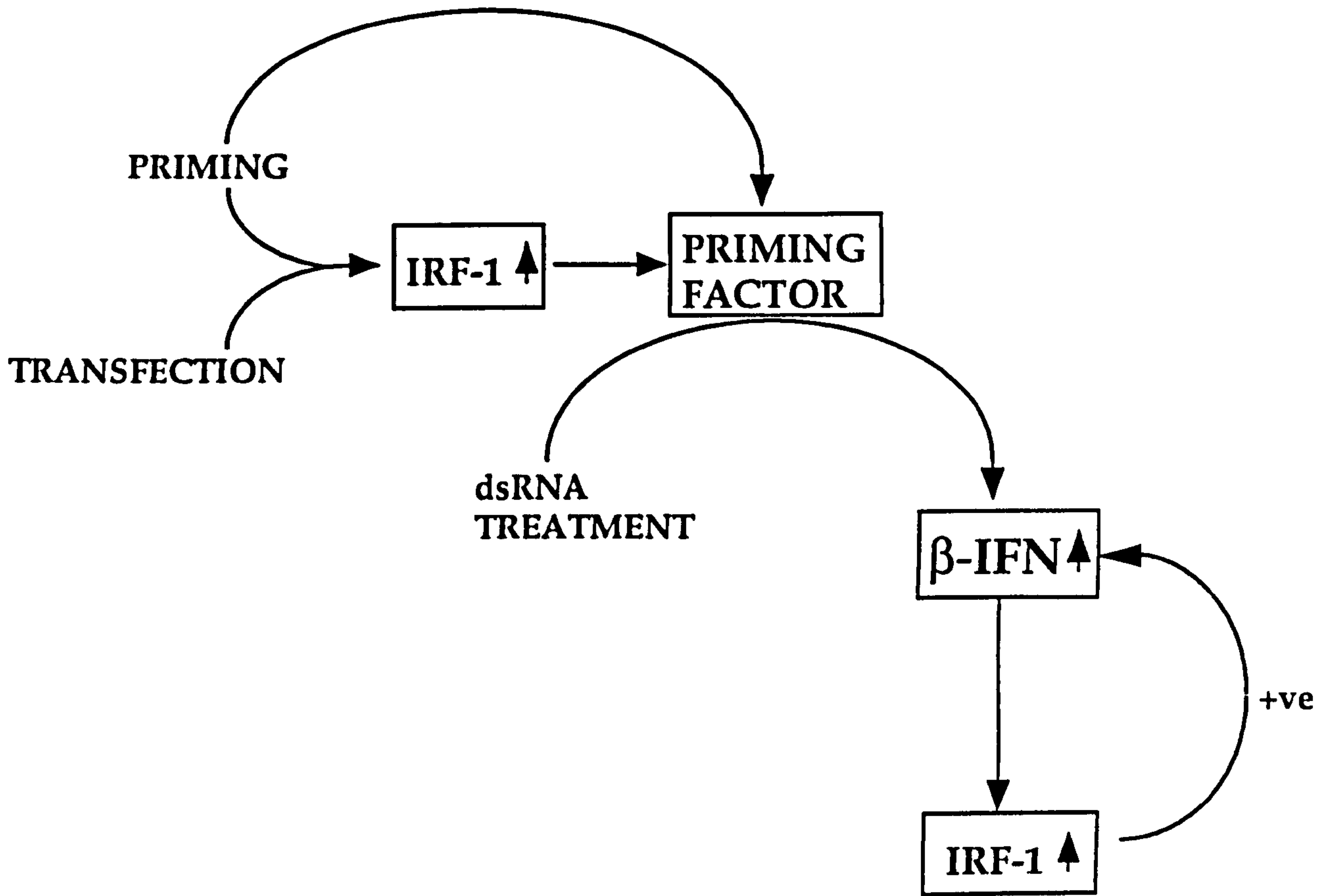
One final set of data suggesting that the priming effects of IRF-1 are indirect is provided by studies on the behaviour of an IRF-1/HBD fusion protein (Figure 5.12). This construct can clearly transactivate the β -IFN promoter, but in two quite distinct ways. Firstly, there is an increase in expression that is dependent on treatment with 17β -oestradiol, which releases the transactivator from hsp90. In the absence of this treatment, dsRNA is unable to stimulate gene expression. However, when 17β -oestradiol and dsRNA are used as co-inducers, the enhancement of stimulation due to dsRNA is not seen unless a significant amount of time is allowed to elapse after 17β -oestradiol addition. It is most likely that this lag is due to the requirement for the translation of transcripts that have been induced by IRF-1. Because the results from the transient transfection system are quantitated by the assay of accumulated reporter enzyme levels which necessarily require ongoing protein synthesis, it has not been possible to examine this hypothesis directly. To test this further, stable lines were created containing the IRF-1/HBD fusion protein, so that the priming effect could be assessed by analysing the transcriptional activation of the endogenous β -IFN gene by RNase mapping, both in the presence and absence of cycloheximide. Unfortunately, none of the stable lines created expressed the IRF-1 fusion protein (data not shown). Previous attempts to create stable lines overexpressing wild-type IRF-1 have also failed (data not shown), probably because of the antiproliferative activity of IRF-1 (see Chapter One). It is possible that the leakiness of the HBD system observed in Figure 5.12A allows sufficient unsequestered IRF-1 access to the chromatin to have a deleterious effect on cell growth. A CD2-selection experiment with the transiently transfected IRF-1/HBD fusion protein-expressing plasmid, similar to that performed in Figure 5.6, may prove to be the best way to answer this question.

A model for the priming response in HeLaE cells is presented in schematic

form in Figure 5.13. HeLaE cells lack IRF-1, and as shown above, this can be provided either by priming or by transfection of IRF-1 expression plasmids. Because Wellferon-primed HeLaE cells contain very little detectable IRF-1, it is likely that the role of IRF-1 is to induce other factors, here depicted as a single putative Priming Factor. The action of priming in IRF-1-knockout cells must work through a similar mechanism. This Priming Factor then supports dsRNA-induction of the β -IFN gene. However, if IRF-1 is present at the time of induction, for example in MG63 cells or in overexpression experiments as shown here, it could also play a direct role in the induction of β -IFN, as indicated by data in this Chapter.

Figure 5.13 A model for the role of IRF-1 in priming

The β -IFN gene in HeLaE cells is uninducible by dsRNA in the absence of priming. One effect of priming is to transiently induce the synthesis of IRF-1, which is undetectable in normally growing cells, and the introduction of IRF-1 into these cells by transfection can also render them priming-independent. Evidence provided in this Chapter suggests that IRF-1 does not need to act directly on the β -IFN promoter, but probably primes cells by virtue of its ability to function as a transcriptional activator of IFN-stimulated genes, shown here as a single putative Priming Factor. As priming can be achieved in IRF-1-negative cells, created by antisense IRF-1 oligonucleotide treatment of MG63 cells or gene knockout experiments, this factor can also be induced directly by IFN treatment. If the induction is performed under conditions permissive for protein synthesis, the induction of β -IFN could be augmented by newly synthesised IRF-1, which is present at the time of induction in overexpression experiments. For more details see text.



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CHAPTER SIX - THE ROLE OF ISGF3 COMPONENTS IN β -INTERFERON INDUCTION

Introduction

Experiments outlined in Chapter Five indicate that IRF-1 can have both direct and indirect effects on β -IFN induction. However, in primed HeLaE cells that are fully competent for induction in response to dsRNA there is no detectable IRF-1, and yet a linker-scan mutagenesis study (Figure 5.9) shows that the binding sites for IRF-1 are essential for efficient induction. Furthermore, in Chapter Four it was demonstrated that induction through the PRD I element is priming-dependent. Taken together, these results indicate that priming is capable of providing a transcription factor that can bind to these elements that is distinct from IRF-1. The experiments described in this Chapter attempt to identify the nature of this factor.

ISGF3 γ can bind to PRD I in a priming-dependent manner

Previous experiments in this laboratory had shown that priming induces the synthesis of a PRD I-binding factor (Pr1) that is distinct from IRF-1 or IRF-2 (Whiteside et al. 1992). Although the nature of this factor was not determined, the similarity between PRD I and an ISRE led to the hypothesis that Pr1 might contain well-characterised components of transcription factors that are induced by type I IFN.

It has been shown that the transcriptional regulation of type I IFN and type I IFN-inducible genes show several similarities, for instance some type I IFN-inducible genes are also dsRNA-inducible in a protein synthesis-independent manner (see Chapter One), suggesting that the signal transduction pathways involved in induction by both type I IFN and dsRNA may utilise common components. Transcriptional induction in response to type I IFN involves the activation of a heterotrimeric complex called ISGF3, the DNA binding component of which, ISGF3 γ , is a member of the IRF family of DNA binding proteins (see Chapter One). Significantly, whereas treatment of cells with type I IFN normally leads to a very transient transcriptional activation of genes through the activation of ISGF3, ISGF3 γ has been reported to be inducible by IFN treatment and shown to persist for several hours after treatment (Pine et al, 1994), and thus might be expected to be present in primed cells.

To test the hypothesis that Pr1 contains ISGF3 γ , an EMSA experiment was performed on nuclear extracts from HeLaE cells using a polyclonal anti-ISGF3 γ

Figure 6.1 Primed HeLaE cells contain ISGF3 γ and STAT1

(A). HeLaE cells were treated as follows:

unprimed, uninduced (Un, lanes 1 and 5),

unprimed, induced with dsRNA, 3 hours (I, lanes 2 and 6),

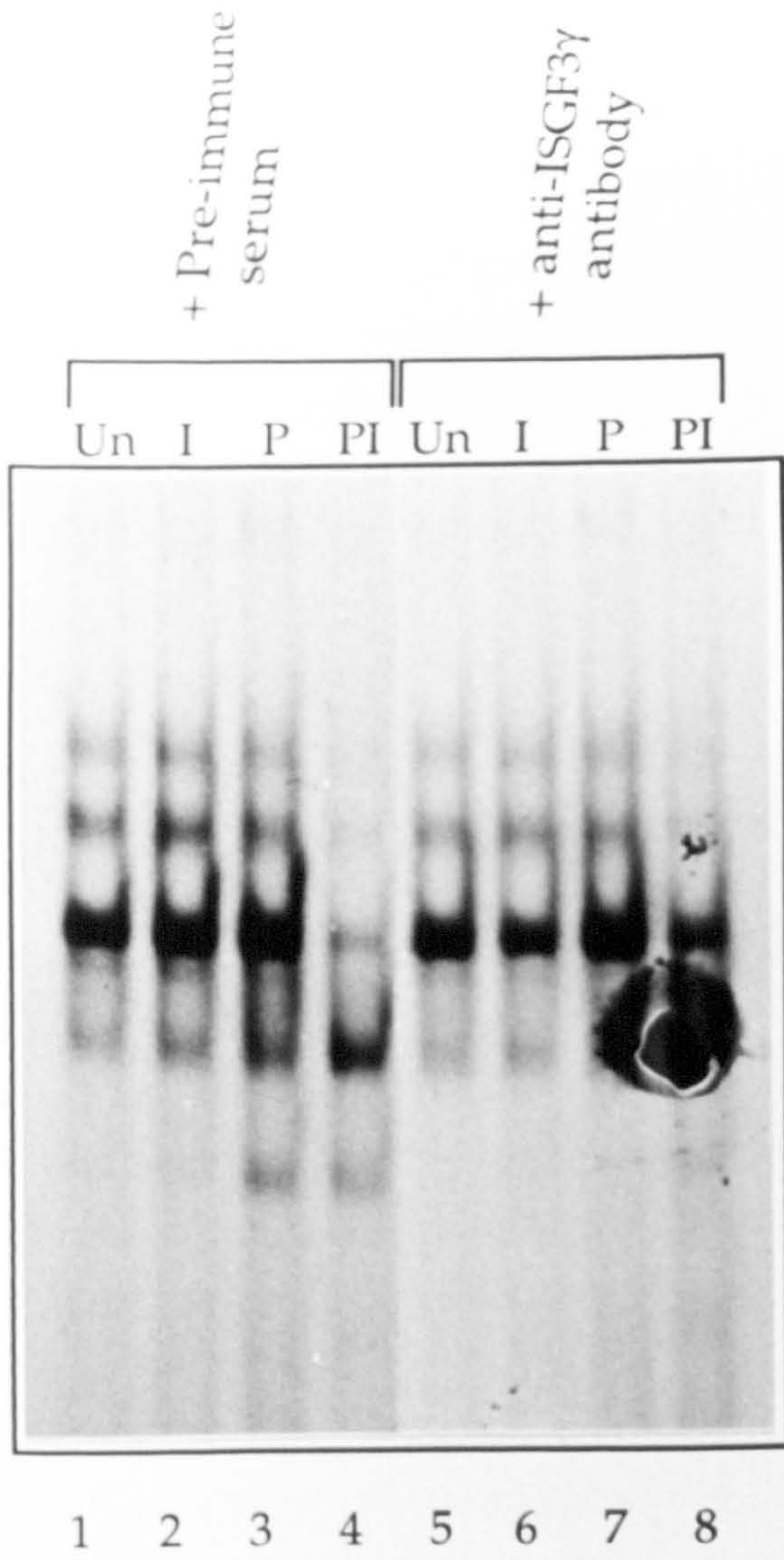
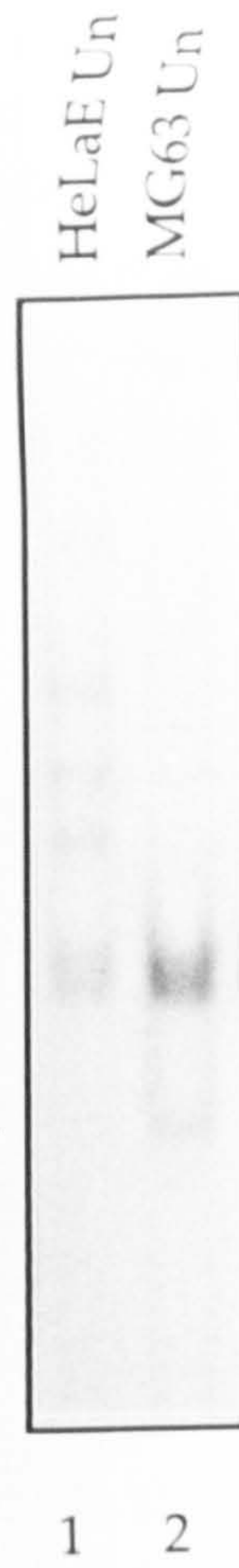
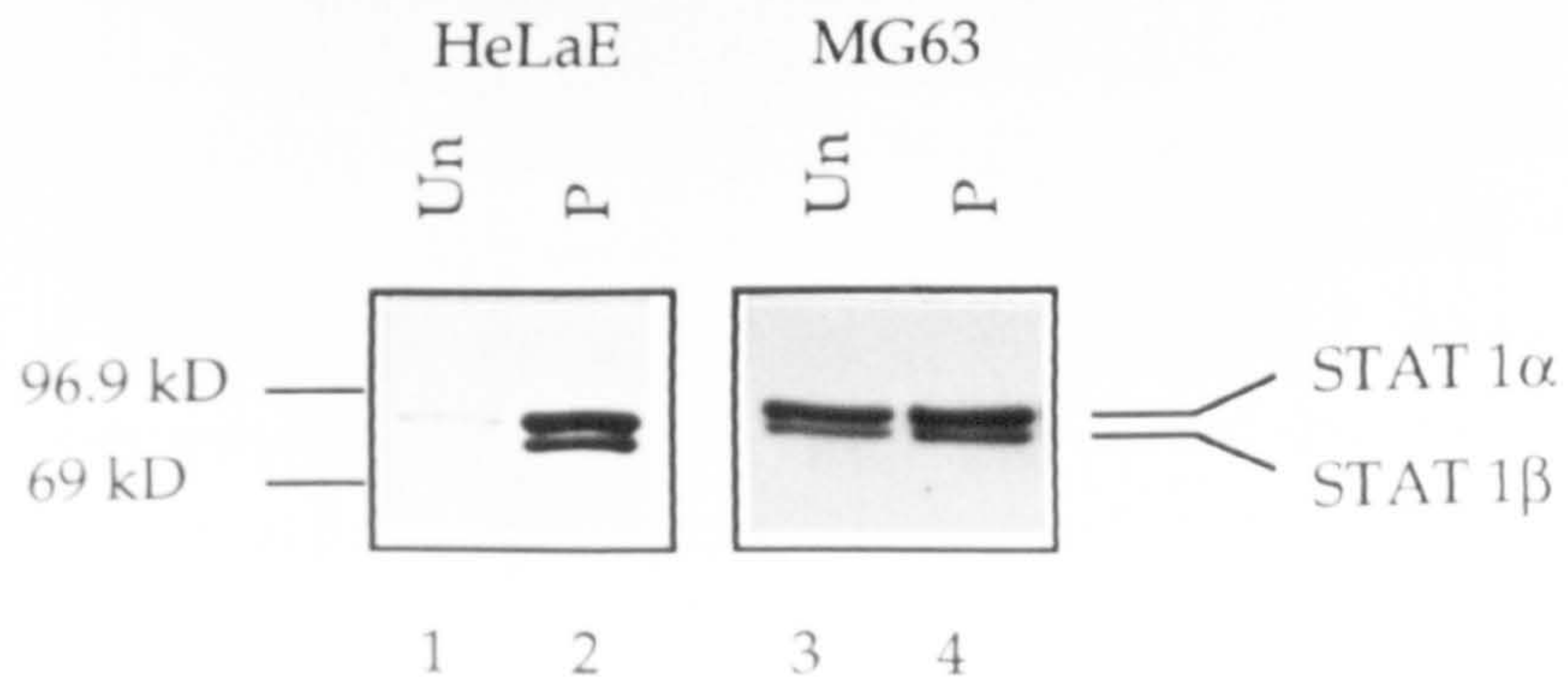
primed, uninduced (P, lanes 3 and 7),

primed, induced with dsRNA, 3 hours (PI, lanes 4 and 8).

The cells were harvested and nuclear extracts were prepared which were analysed by EMSA, using a radiolabelled PRD I probe. Before incubation with the probe, the extracts were incubated either with 2 μ l of rabbit pre-immune serum (lanes 1-4), or 2 μ l of anti-IRF-1 polyclonal antibody.

(B). Nuclear extracts were prepared from unprimed, uninduced HeLaE (lane 1) and MG63 (lane 2) cells, and these were analysed by EMSA on a PRD I probe as in A. The position of ISGF3 γ , IRF-1 and -2, Un 1 and Un 2 and the non-specific complex (n.s.) in A and B are indicated by arrows.

(C). (J. Brown) HeLaE (lanes 1 and 2) and MG63 (lanes 3 and 4) cells were harvested in the unprimed state (lanes 1 and 3, Un) or following priming (lanes 2 and 4, P), and whole cell protein extracts were prepared for Western blotting. The samples were electrophoresed on a 7.5% SDS-PAGE gel and the blot was probed with a 1:1000 dilution of polyclonal anti-STAT1 antibody raised against the N-terminal 194 amino acids of human STAT1 (Transduction Laboratories). The positions of STAT1 α and β and the size markers are shown.

A**B****C**

antibody (see Chapter Two). Figure 6.1A shows that a PRD I-binding complex absent in normally growing HeLaE cells is induced by priming and is present during induction. Pre-incubation of the nuclear extracts with a polyclonal anti-ISGF3 γ antibody abolishes this complex, demonstrating that it contains ISGF3 γ (compare lanes 3 and 4 with lanes 7 and 8). This complex is in fact the same as that induced with kinetics mirroring those of the establishment of priming in Figure 5.4. The same complex is present constitutively in priming-independent MG63 cells (Figure 6.1B). Interestingly, priming also led to increased levels of the ISGF3 component STAT1 (Figure 6.1C). The Western blot shows that primed HeLaE and MG63 cells contain equivalent levels of STAT1 α and β , but unprimed HeLaE cells contain much less STAT1 α and almost undetectable levels of STAT1 β . The significance of this will be discussed below.

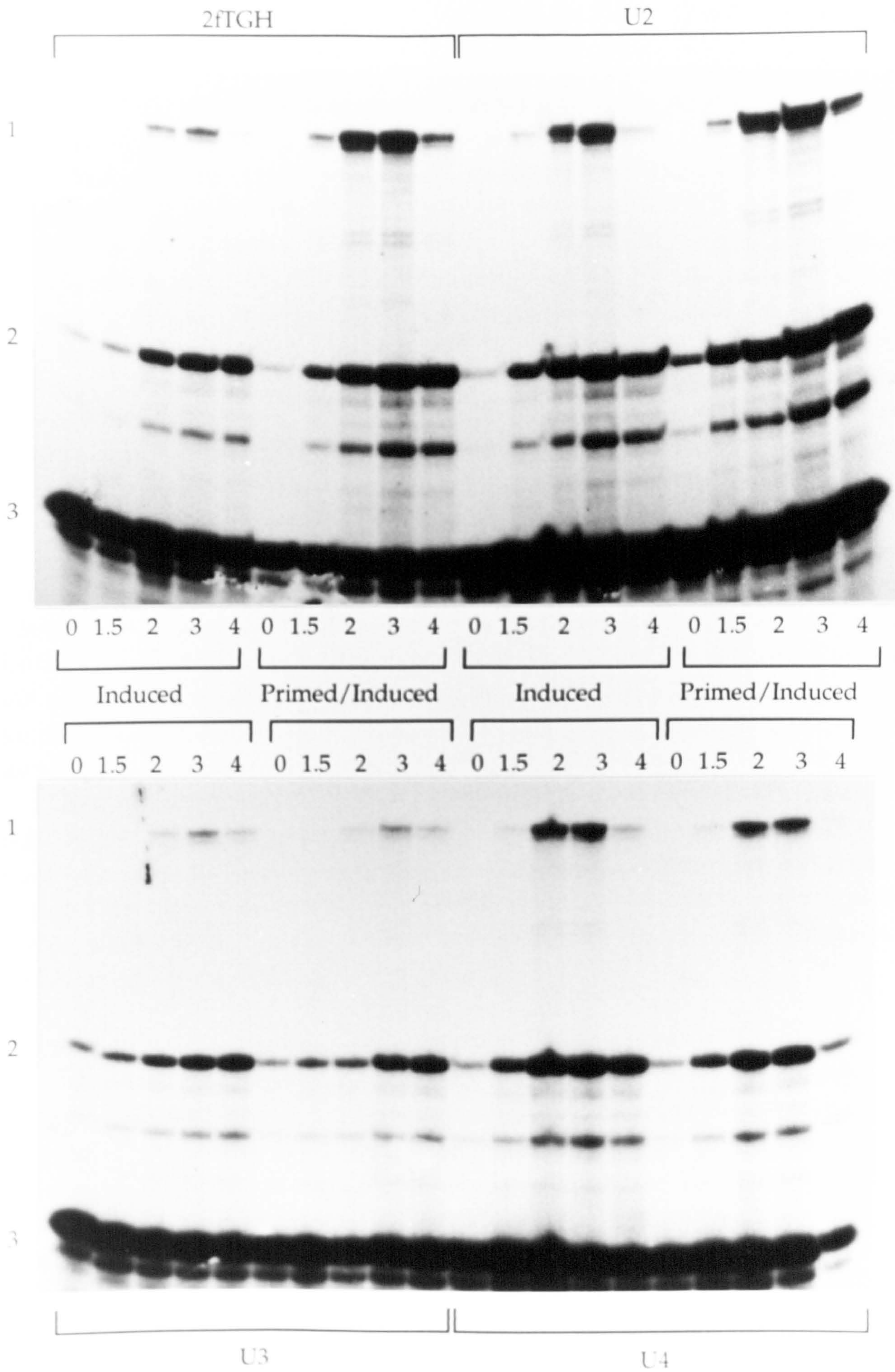
The roles of ISGF3 γ , STAT 1 and JAK 1 in β -IFN induction

The availability of cell lines that are defective in ISGF3 γ as well as other components of the IFN response pathway allowed the importance of these factors to be tested directly. The inducibility of 2fTGH cells, and U2, U3 and U4 cells derived from this cell line (which are defective in ISGF3 γ , STAT1, and JAK 1, respectively - see (Darnell, Kerr et al. 1994), and Chapter One), were initially analysed by treating them with dsRNA in both the primed and unprimed states. RNA was prepared and the levels of β -IFN, IRF-1 and γ -actin mRNA in each sample were analysed by RNase mapping.

As can be seen from Figure 6.2, upper panel, the β -IFN gene in 2fTGH cells is inducible with kinetics similar to those observed in HeLaE cells, and although it is inducible in the absence of priming, the degree of induction can be enhanced by pre-treatment with type I IFN. U2 cells, which lack functional ISGF3 γ , can also be induced by dsRNA in a priming-independent manner, indicating that in this cell line at least, ISGF3 γ does not play an essential role in induction. Their induction is also slightly enhanced by priming, which was unexpected given the crucial role of ISGF3 γ in the signal transduction pathway of type I IFN. U3 cells are impaired in their induction by dsRNA in this experiment (Figure 6.2, lower panel), perhaps suggesting that STAT1 is involved at some stage in the induction of β -IFN. U4 cells, on the other hand, do not appear to be impaired in their induction by dsRNA, and so if STAT1 is involved in the induction of β -IFN, JAK 1 would not seem to be necessary for this process. As expected, given the central role of activated STAT1 in the signal transduction pathway of type I IFN, both U3 and U4 cells are unaffected by

Figure 6.2 Kinetics of induction of cells defective in IFN signalling

2fTGH cells and cell lines derived from this parent, U2, U3 and U4, defective in ISGF3 γ , STAT1 and JAK 1, respectively, were uninduced (0hrs) or induced with dsRNA for the length of time shown (1.5, 2, 3 and 4hrs) in either the unprimed or primed states. RNA was prepared from the harvested cells and analysed by RNase mapping, using probes for β -IFN (1), IRF-1 (2) and γ -actin (3).



priming.

2fTGH and the mutant cell lines contain constitutive levels of IRF-1 which is also inducible by dsRNA

The observation that U2 cells respond to induction by dsRNA at first sight might argue against a role for ISGF3 γ in the induction of β -IFN. However, the inclusion of a probe for the protection of IRF-1 in Figure 6.2 shows that all four lines contain readily detectable IRF-1 RNA in their unprimed, uninduced states, which, as seen in Chapter Five, is not the case in HeLaE cells. Furthermore, Western blotting experiments demonstrated that each of the four cell lines contain equivalent amounts of IRF-1 protein prior to induction (data not shown). The presence of IRF-1 in these cells should render them priming-independent, and this result is observed. Furthermore, the fact that IRF-1 is present would mean that a primary transcription factor is available for PRD I function. It has previously been reported that the IRF-1 is a dsRNA-inducible gene (Miyamoto et al. 1988), and that this induction is direct (Pine et al. 1990). In each of the four cell lines tested, IRF-1 mRNA is induced by dsRNA treatment (Figure 6.2). Since the mutant cell lines are unable to respond to any secreted type I IFN, this result suggests that the induction by dsRNA in these cells is indeed direct. The inducibility of the IRF-1 gene in each cell type mirrors the corresponding induction of β -IFN, with the peak of induction for IRF-1 in most of the lines being around 3 hours, although over this time course the induction is sustained and does not exhibit the shut off observed with β -IFN.

It is important to stress that U3 cells appear to contain equivalent amounts of IRF-1 to the other cell lines, and that IRF-1 appears to be inducible by dsRNA in these cells, albeit to a lesser extent. One trivial explanation for the poor inducibility of β -IFN by dsRNA in U3 cells is that the deficiency of STAT1 could lead to decreased IRF-1 levels. The fact that this does not appear to be the case would suggest a more direct role for STAT1 in β -IFN induction.

2fTGH and U2 cells are priming-dependent in the absence of IRF-1

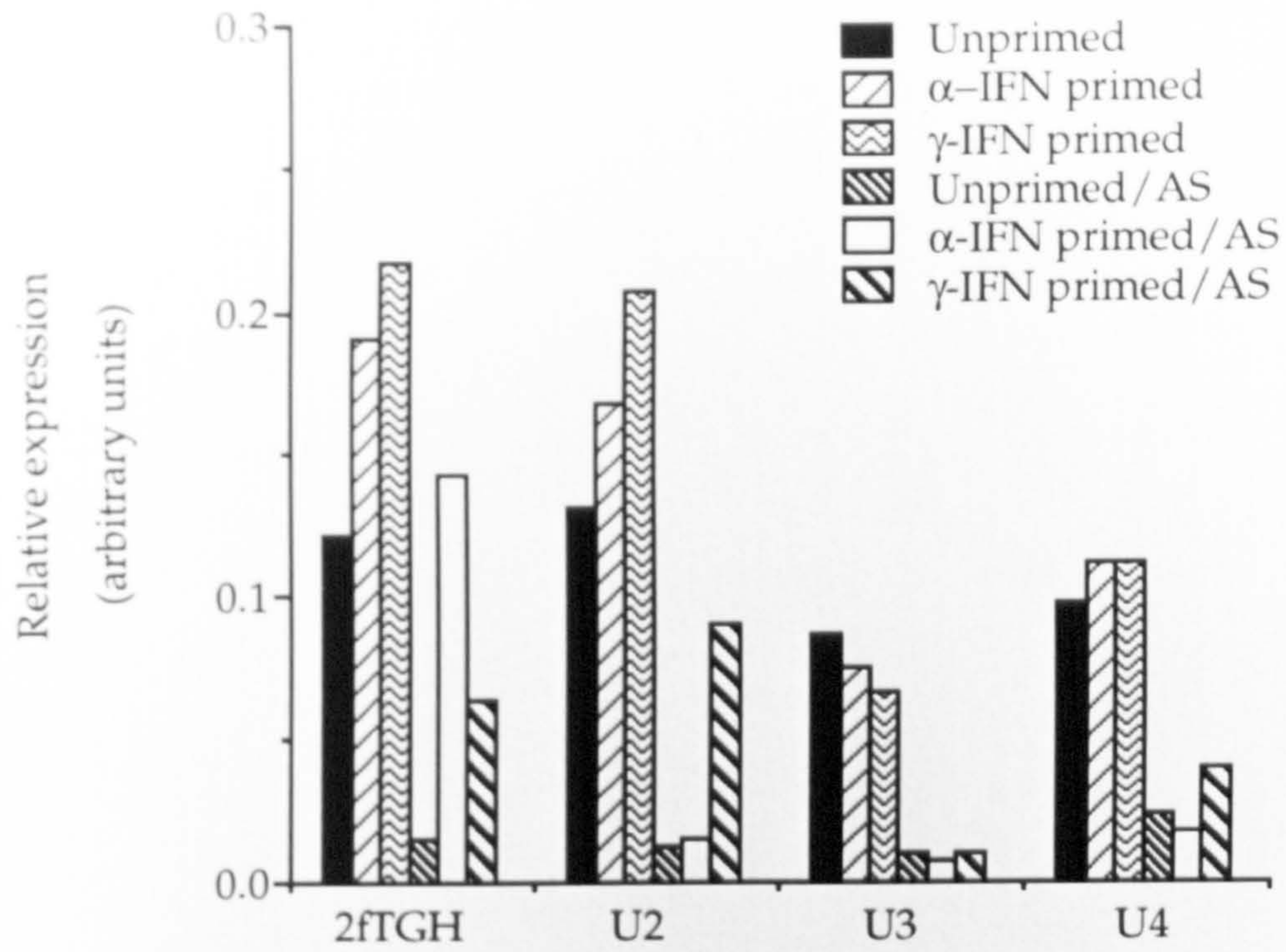
Since U2 cells contain IRF-1, it was not possible to determine the role of ISGF3 γ in the induction of β -IFN in these cells. In an attempt to circumvent the problem of constitutive IRF-1 expression, the four cell lines were grown in the presence of 10 μ M phosphorothioate-linked antisense IRF-1 oligonucleotides for 48 hours and then treated with dsRNA. The RNase protection gel was analysed on a phosphorimager and the results are depicted in Figure 6.3A. As previously shown

Figure 6.3 Induction of the mutant cells in the presence of antisense IRF-1 oligonucleotides.

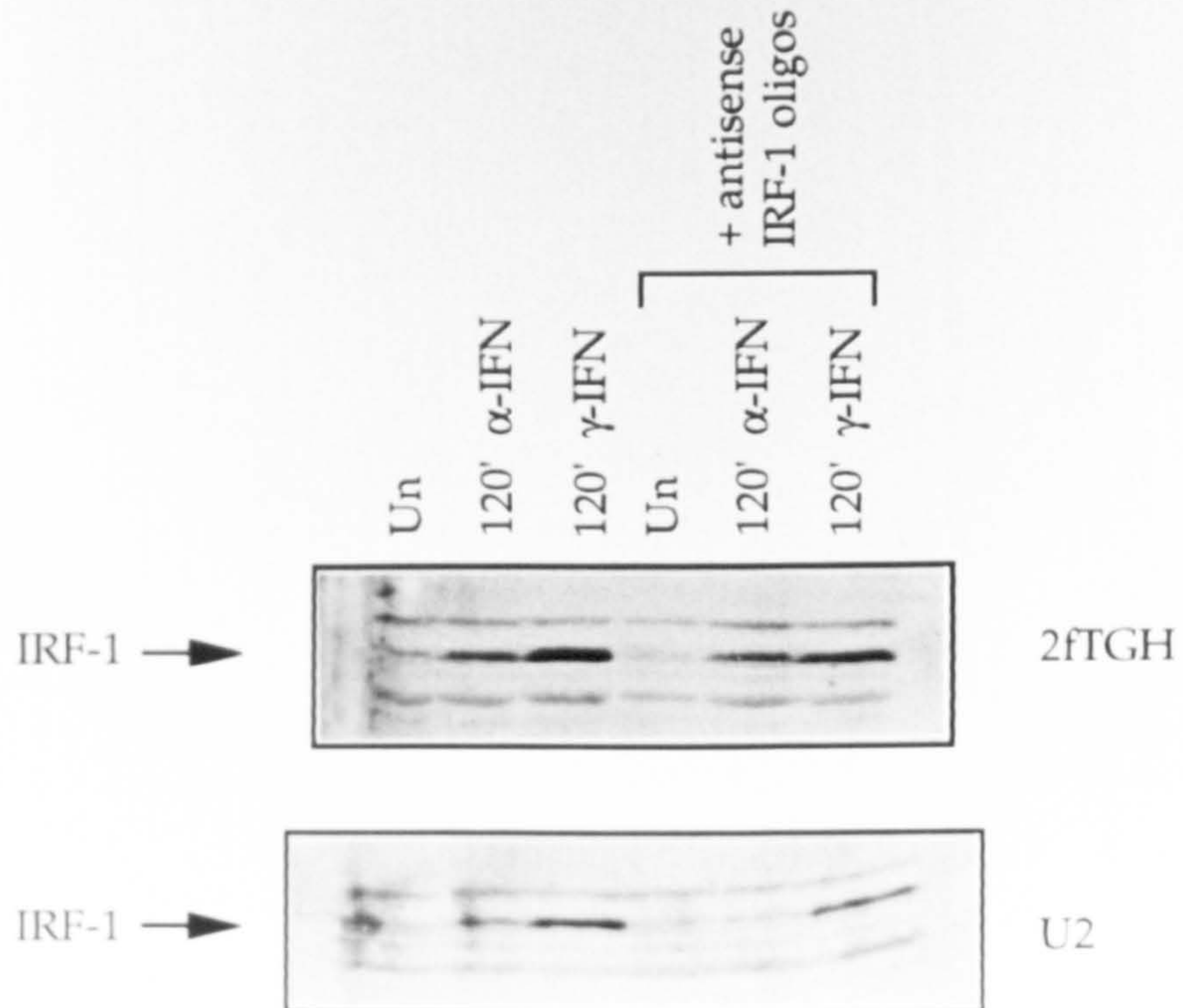
(A). 2fTGH, U2, U3 and U4 cells were grown normally or in the presence of 10 μ M phosphorothioate-linked antisense IRF-1 oligonucleotides (AS) for 48 hours. The cells were induced with dsRNA for 3 hours, either in the absence of priming or following priming with either α - or γ -IFN. The RNA from these cells was analysed by RNase mapping, using probes for β -IFN and γ -actin. Following electrophoresis, the RNase protection gel was analysed on a Molecular Dynamics phosphorimager. The β -IFN signal was corrected to the γ -actin signal for each sample and the results were plotted.

(B). 2fTGH and U2 cells were grown normally or in the presence of 10 μ M phosphorothioate-linked antisense IRF-1 oligonucleotides for 48 hours. The cells were treated with α - or γ -IFN for 2 hours as indicated, harvested and whole cell protein extracts were prepared for Western blotting. The samples were electrophoresed on a 10% SDS-PAGE gel and the blot was probed with a 1:1000 dilution of polyclonal anti-IRF-1 antibody. The position of the IRF-1 protein is indicated for both the 2fTGH cells (upper panel) and U2 cells (lower panel).

A



B



(Figure 6.2) all four cell lines are inducible by dsRNA in the absence of antisense oligonucleotides. The induction in both 2fTGH and U2 cells is enhanced by priming with both α - and γ -IFN, with γ -IFN being more effective in each case, and as expected U3 and U4 cells are not responsive to priming by either type of IFN. When grown in the presence of the phosphorothioate-substituted antisense IRF-1 oligonucleotides, all four cell lines are rendered uninducible, a result previously seen with MG63 cells (Figure 5.8), again demonstrating the importance of IRF-1 in the induction process. Analysis of the effects of the antisense oligonucleotides shows that the IRF-1 protein levels are reduced to about 30% of the level in untreated cells (Figure 6.3B), while RNase protection analysis shows that the IRF-1 mRNA levels are reduced to about 10% (data not shown).

This result on its own cannot address a role for ISGF3 γ , since 2fTGH cells and their derivatives do not constitutively express this factor (data not shown). In the case of MG63 cells, the antisense block to inducibility had been rescued by priming, and so this possibility was investigated for 2fTGH and the mutant lines (Figure 6.3A). The block to induction imposed by the antisense oligonucleotides in 2fTGH cells could be almost fully overcome by priming with type I IFN, and partially abrogated with type II IFN. In contrast, type I IFN was unable to rescue U2 cells, whereas type II IFN effected a partial rescue. Neither type I nor type II IFN had any effect on U3 or U4 cells.

An initial consideration of the above data might suggest that they are merely reflecting the expected properties of these lines. In 2fTGH cells, which possess fully functional type I- and type II-responsive pathways, either class of IFN can lead to rescue of induction. The involvement of STAT1 and JAK1 in both type I- and type II-responsive pathways would explain the inability of either cytokine to rescue U3 or U4 cells, while in U2 cells, the restriction of ISGF3 γ to the type I pathway would mean that only type II IFN could rescue induction. However, the rescue of β -IFN induction by type II IFN in antisense oligonucleotide-treated U2 cells would contradict the proposal that ISGF3 γ is an essential factor for induction of the β -IFN promoter in the absence of IRF-1, because both ISGF3 γ and IRF-1 are missing in these cells. It is therefore possible that during priming with γ -IFN, some IRF-1 protein synthesis can occur as a consequence of the high level transcriptional induction being able to overcome the antisense block. To test this, a Western analysis of IRF-1 was performed in the 2fTGH and U2 cells treated for 2 hours with type I and II IFN. Figure 6.3B shows that even in the presence of the antisense oligonucleotides an induction of IRF-1 protein levels is observed with γ -IFN

treatment in these cell lines. In contrast, the IRF-1 protein levels are not upregulated by γ -IFN treatment in U3 and U4 cells as expected (data not shown). This result suggests that the rescue of induction in U2 cells by γ -IFN is a result of residual IRF-1 synthesis which appears to be able to occur to the same extent as in 2fTGH cells.

In the light of this result it is instructive to consider the ability of type I IFN to rescue induction of IRF-1 antisense-treated cells. In fact, like γ -IFN, α -IFN can induce IRF-1 in an ISGF3 γ -independent manner through the induction of GAF (transcriptionally active homodimeric STAT1 α , see Chapter 1), and therefore it should prime U2 cells if overcoming the IRF-1 block is sufficient to rescue cells from the antisense effect. This is clearly not the case, although α -IFN is slightly less effective at inducing IRF-1 than γ -IFN (see the relative inductions in 2fTGH, Figure 6.3B) (Pine et al. 1994). By contrast, not only can α -IFN rescue 2fTGH cells from the IRF-1-antisense-imposed block to induction, but it can do so more effectively than γ -IFN. These results clearly demonstrate an absolute requirement for ISGF3 γ for β -IFN induction in the absence of sufficient IRF-1, although they do not prove that this is a requirement for direct binding to the β -IFN promoter.

The role of ISGF3 γ in β -IFN induction in HeLaE cells

In the cell lines described above, the presence of constitutive IRF-1 meant that it was not possible to test directly the role of ISGF3 γ in β -IFN induction. Because HeLaE cells do not contain significant levels of IRF-1, either before or after priming, the effect of overexpression of ISGF3 γ was tested in these cells. A cDNA for ISGF3 γ was obtained by PCR, cloned under the control of the powerful EF1 α promoter to create pEFISGF3 γ (Figure 6.4A) and tested for function by the ability to complement the type I IFN response of U2 cells (data not shown). Unprimed HeLaE cells do not contain ISGF3 γ (Figure 6.1) so the ability of this factor to confer priming-independence was examined. Figure 6.4B shows that ISGF3 γ cannot function in this way when cotransfected with pIF[-108/+72]lucifer. However, it should be stressed that unlike IRF-1, ISGF3 γ cannot by itself stimulate transcription, and so would be unable to provide the indirect components that are required for priming and are provided by IRF-1 expression, such as the provision of a signal transduction pathway for the activation of NF- κ B by dsRNA.

In contrast to the failure to prime cells, ISGF3 γ overexpression does lead to an increase in β -IFN expression in a dose-dependent manner (Figure 6.4B) suggesting that it can play a positive role in expression, but by itself is insufficient to allow induction. These experiments do not, however, address whether ISGF3 γ is essential

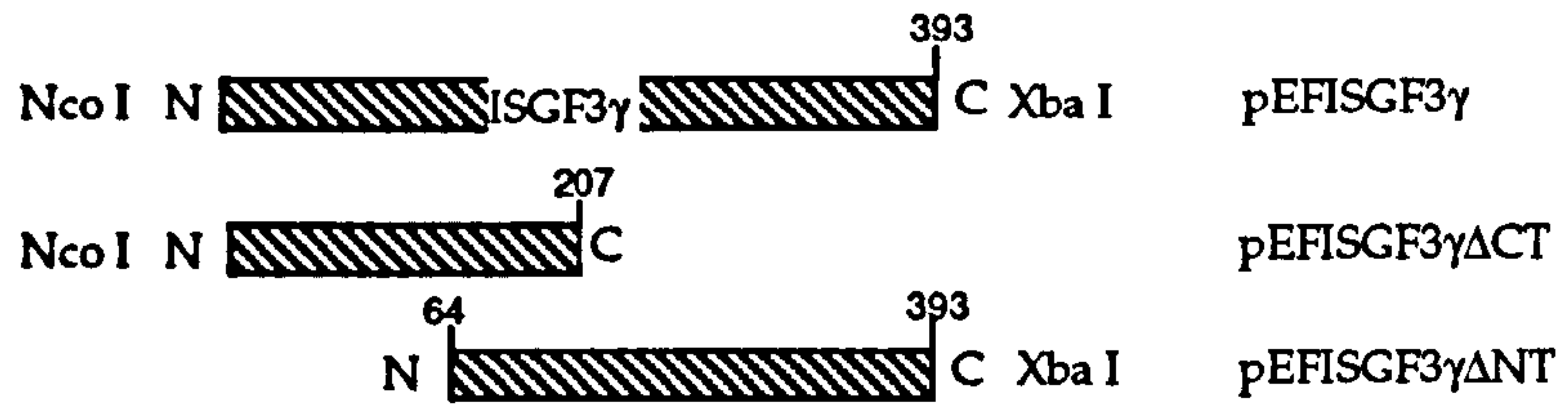
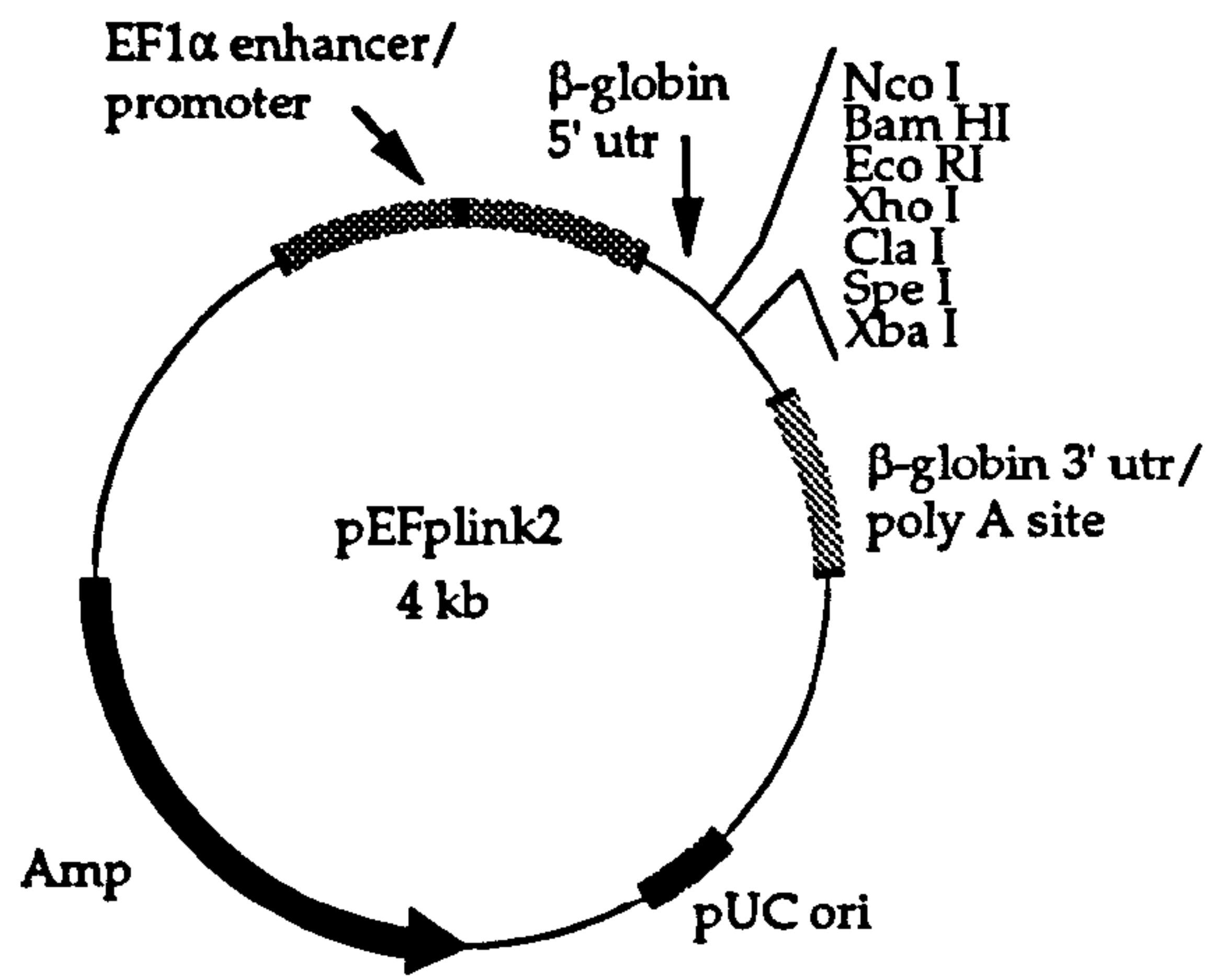
Figure 6.4 ISGF3 γ can stimulate induction in HeLaE cells

(A). A cDNA for human ISGF3 γ was obtained by PCR from reverse transcribed MG63 RNA and verified by partial sequencing and the ability to complement U2 cells. The cDNA was inserted into the polylinker of pEFplink2 (a kind gift from R. Treisman, ICRF), a plasmid containing the extremely powerful human EF-1 α polypeptide elongation chain factor promoter and enhancer region, to construct pEFISGF3 γ . pEFISGF3 γ Δ CT and pEFISGF3 γ Δ NT, which lack the C-terminal 186 amino acids and N-terminal 64 amino acids, respectively, were constructed from this plasmid by standard sub-cloning procedures.

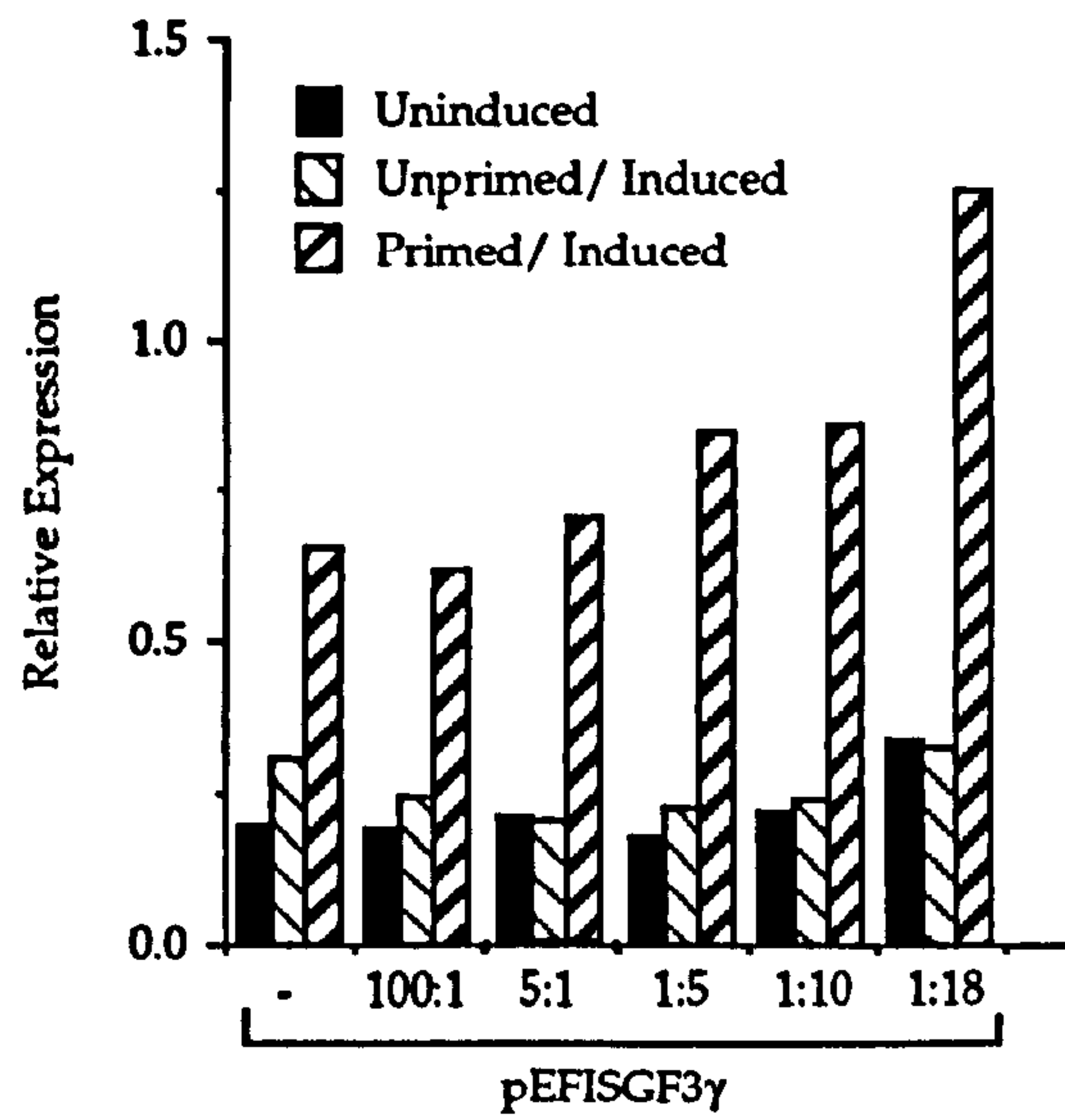
(B). HeLaE cells were transiently transfected, using Lipofectamine, with 0.1 μ g pIF[-108/+72]lucifer and pEFISGF3 γ , over a range of ratios from 100:1 (0.001 μ g) to 1:18 (1.8 μ g) along with 0.1 μ g pSV₂CAT, and pEFplink2 to make up the total amount of DNA in the transfection mix to 2 μ g as appropriate. The cells were treated with dsRNA as indicated, and the luciferase values were normalised to the CAT activities for each sample.

(C). HeLaE cells were transiently transfected, using Lipofectamine, with 0.1 μ g pIF[-108/+72]lucifer and 1.8 μ g pEFISGF3 γ , pEFISGF3 γ Δ CT or pEFISGF3 γ Δ NT, along with 0.1 μ g pSV₂CAT. The cells were treated with dsRNA as indicated, and the luciferase values were normalised to the CAT activities for each sample.

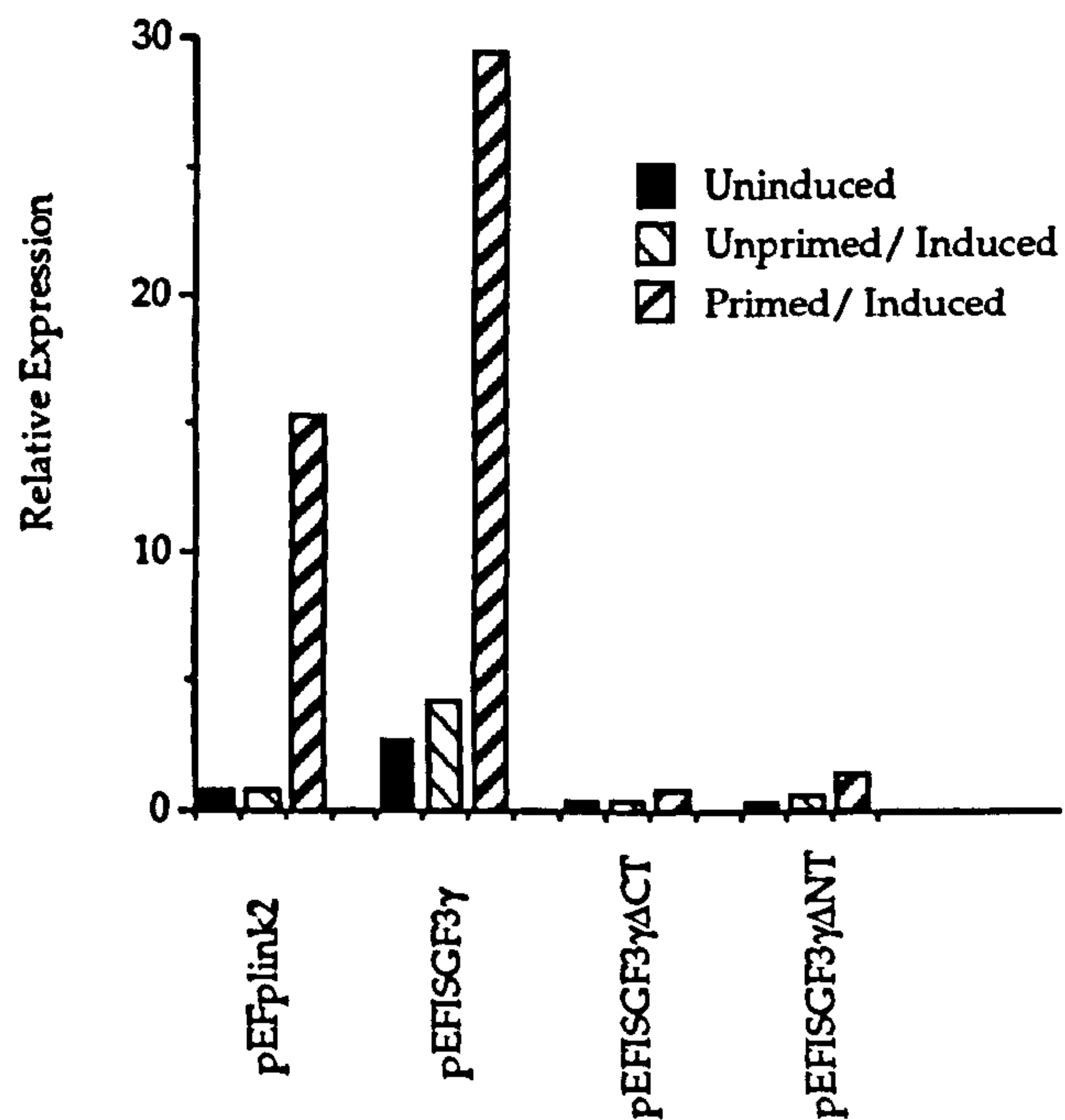
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B



C



for β -IFN induction in primed HeLaE cells. To test this directly, variants of ISGF3 γ were constructed that might be expected to function as interfering mutants, and their effects on β -IFN expression were assessed by overexpression in HeLaE cells with pIF[-108/+72]lucifer (Figure 6.4C). An ISGF3 γ variant lacking the C-terminus (which is the region that interacts with the other ISGF3 components, (Veals et al. 1993) is able to completely block the induction of β -IFN expression in primed HeLaE cells, suggesting that ISGF3 γ is essential for β -IFN induction. However, this mutant protein would still be able to bind to DNA, and thus may function as a competitive inhibitor for binding to PRD I, and exclude another positive activator, although in the absence of IRF-1 in these cells it is not clear what this might be. Interestingly, an ISGF3 γ variant lacking the N-terminus, which disrupts the DNA binding domain, is also able to completely block the induction of β -IFN expression in primed HeLaE cells. This mutant is unable to bind to DNA, and so cannot be interfering with β -IFN induction by competing for PRD I binding. The variant factor, however, still contains the motifs for interacting with STAT proteins, and is presumably interfering with β -IFN induction by sequestering such factors. The blockage is not simply due to preventing priming in these cells, since the cells were primed with type II IFN (which works in an ISGF3 γ -independent manner) in this experiment, and the overexpression of the variant is unable to block type II IFN responses through a GAS reporter (data not shown). Thus it appears that ISGF3 γ plays an essential role in β -IFN induction in cells that lack IRF-1, and it is most likely that this is a direct consequence of binding to PRD I.

The role of STAT1 in β -IFN induction

The results described above suggest that ISGF3 γ plays an essential role in β -IFN induction, but that it is insufficient to overcome the requirement for priming and would appear to recruit essential co-factors through its C-terminus. This result is consistent with the role of ISGF3 γ in ISGF3, since it is unable to stimulate transcription unless it is associated with STAT1 and STAT2. It is thus tempting to speculate that the role for STAT1 in β -IFN induction implied by the impaired induction in U3 cells (Figure 6.2) is similar to its role in IFN-inducible transcription, namely recruitment to the promoter through association with ISGF3 γ .

To test a role for STAT1 in more detail, cDNAs for both the α - and β -forms were obtained by PCR, cloned under the control of the EF1 α promoter and tested for function by the ability to complement IFN responses in U3 cells. As expected, STAT1 α was able to complement both type I and type II responses, whilst STAT1 β

Figure 6.5 Overexpression of STAT1 can rescue dsRNA induction in U3 cells

(A). 2fTGH and U3 cells were transiently transfected, using calcium phosphate, with 10µg of pIF[-108/+72]lucifer and 10µg of pEFplink2 or 10µg of pEFSTAT1α, along with 2µg pSV₂CAT. The cells were treated as follows:

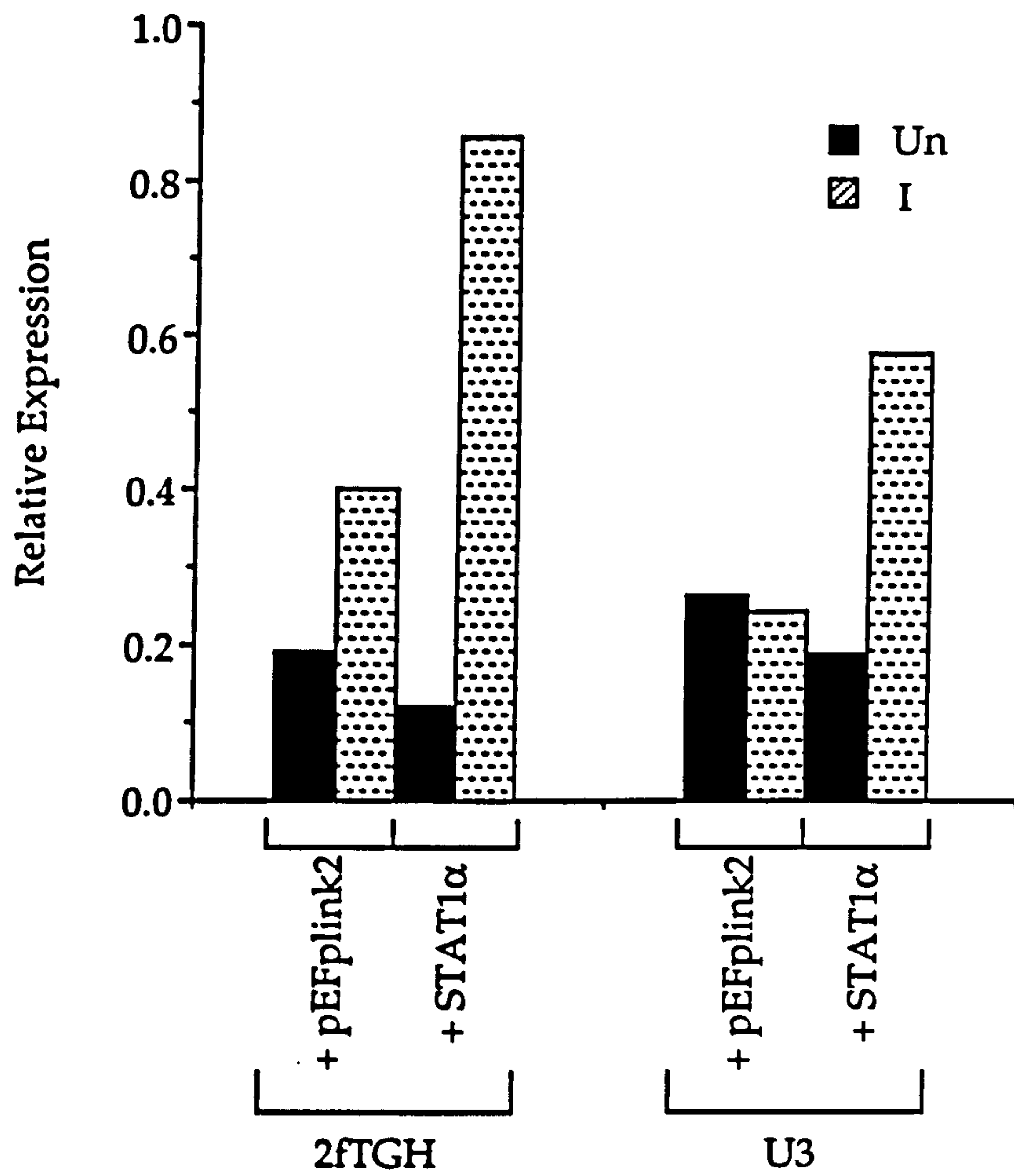
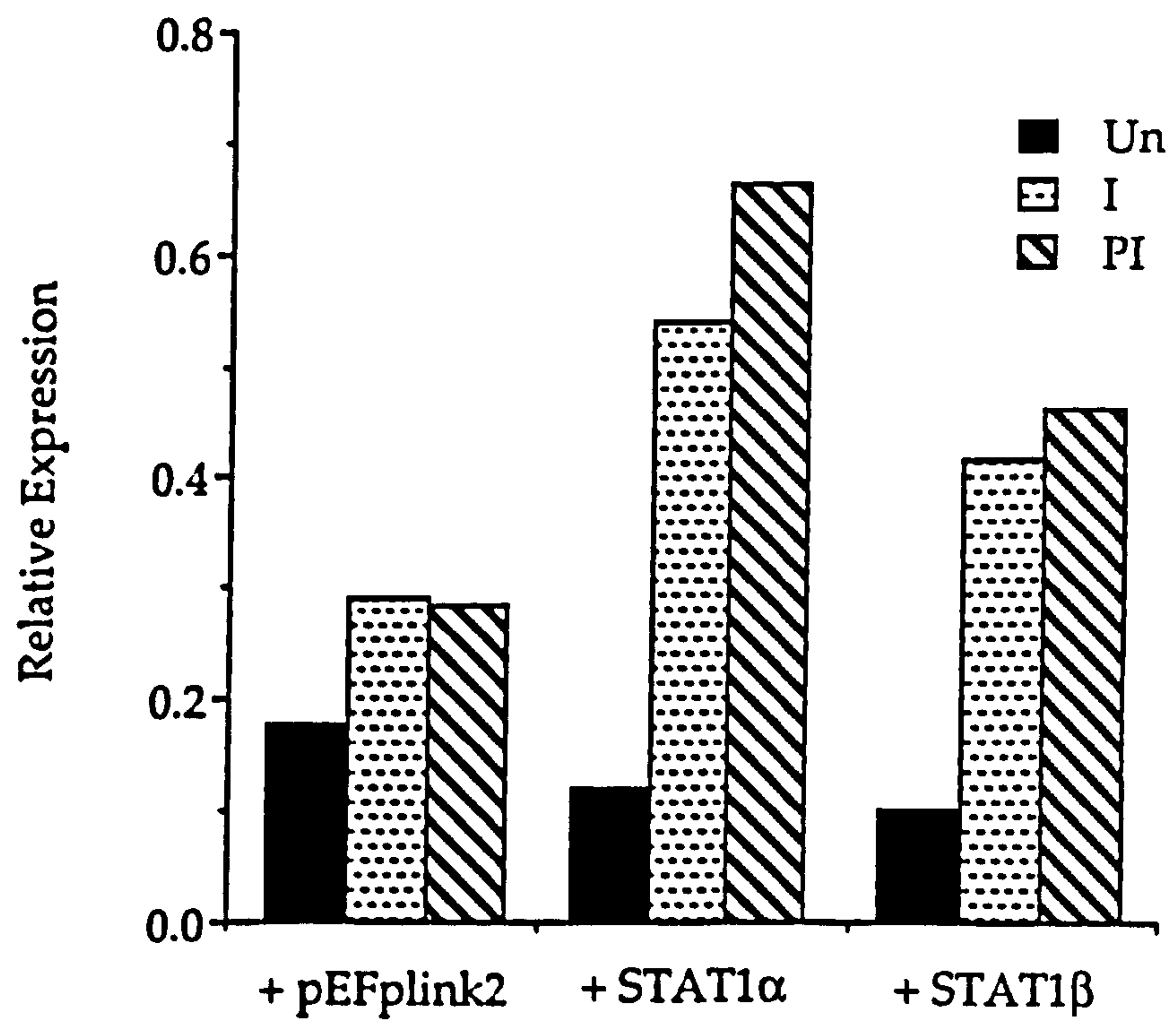
- + pEFplink2, unprimed, uninduced (un),
- + pEFplink2, unprimed, induced with dsRNA (I),
- + pEFSTAT1α, unprimed, uninduced (+ STAT1 un)
- + pEFSTAT1α, unprimed, induced with dsRNA (+ STAT1 I).

The luciferase values were normalised to the CAT values.

(B). U3 cells were transiently transfected, using calcium phosphate, with 10µg of pIF[-108/+72]lucifer and 10µg of pEFplink2 (+ pEFplink2), 10µg of pEFSTAT1α (+ STAT1α) or 10µg of pEFSTAT1β (+STAT1β), along with 2µg pSV₂CAT. The cells were treated as follows:

- unprimed, uninduced (Un),
- unprimed, induced with dsRNA (I),
- primed, uninduced (P),
- primed, induced with dsRNA (PI).

The luciferase values were normalised to the CAT values. For plasmid details, see Figure 6.6

A**B**

could complement only a type I IFN response (data not shown). The cDNAs were then overexpressed in U3 cells and their effects on dsRNA inducibility of β -IFN were examined. Figure 6.5A shows that in contrast to the result seen in uncomplemented cells, the overexpression of STAT1 α caused the β -IFN promoter to respond to induction by dsRNA at a level similar to that seen in 2fTGH cells. Figure 6.5B demonstrated that STAT1 β could also complement the defect in dsRNA response to some extent. This result suggests that STAT1 is unlikely to be acting directly on the β -IFN promoter, since STAT1 β is not thought to be able to activate transcription on its own (Muller et al. 1993).

The effects of STAT1 α overexpression on the induction of pIF[-108/+72]lucifer were also examined in HeLaE cells. As seen above for ISGF3 γ , STAT1 α is unable to confer priming-independence on these cells (Figure 6.6B). In contrast to ISGF3 γ , STAT1 α overexpression did not lead to enhanced expression in primed cells, and in fact had a marginally inhibitory effect. When the consequences of overexpressing ISGF3 γ and STAT1 α together were investigated in this experiment, STAT1 α also lowered the enhancement effect seen with ISGF3 γ alone. These results were surprising, but it should be pointed out that priming itself leads to a significant enhancement of STAT1 levels (see Figure 6.1C), and it is thus possible that when even higher levels are achieved in the transfection experiment there may be some inhibition of the signal initiated in response to dsRNA, perhaps due to the formation of functionally inactive homodimers.

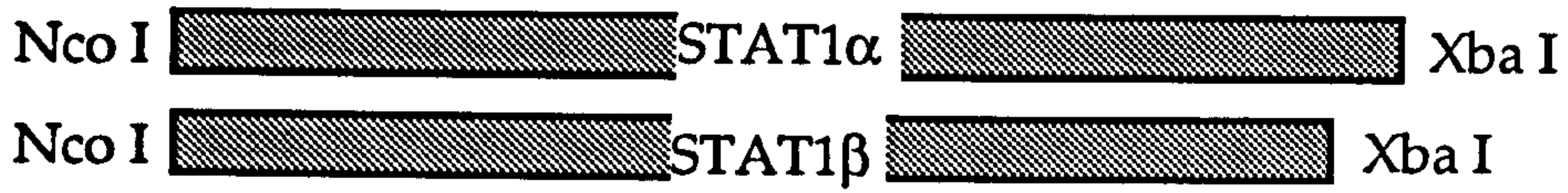
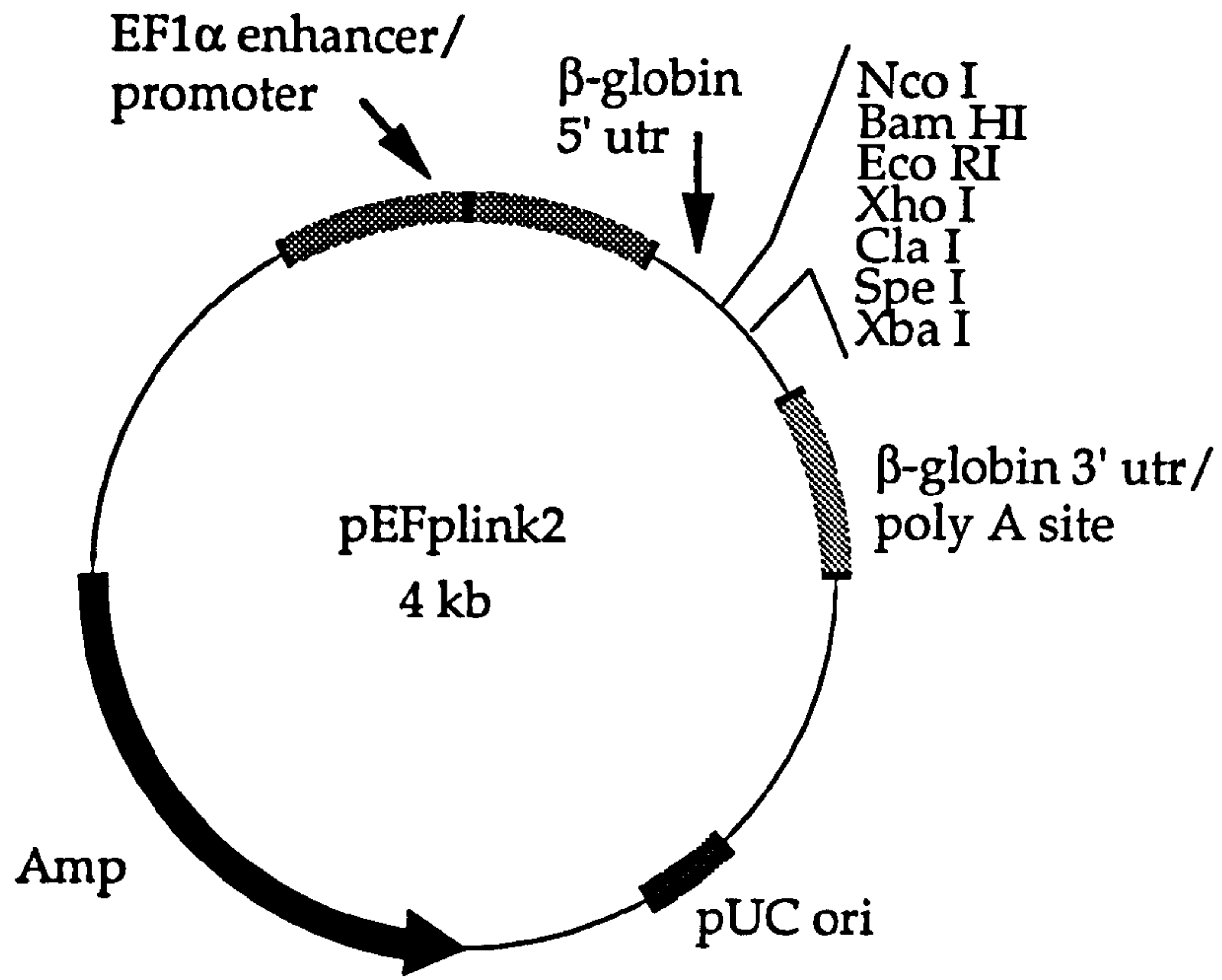
The failure of both ISGF3 γ and STAT1 α to rescue HeLaE cells from the need for priming may reflect the lack of a STAT1 activating signal. In order to test this, the inducibility of the β -IFN promoter was also analysed with the inclusion of 500 units/ml of human γ -IFN, a potent activator of STAT1, in the induction mix. The inclusion of γ -IFN causes a general activation of the promoter construct, both before and after induction, but again does not prime cells (Figure 6.6). The probable explanation of this result is that priming is required to provide indirect components, a process that requires prolonged protein synthesis, and the inclusion of γ -IFN for only 5 hours in the induction mix is an insufficient length of time for the priming effect to occur. Strikingly, when expression is examined in primed cells, γ -IFN causes a significant enhancement of β -IFN induction in response to dsRNA, this effect being most marked when both ISGF3 γ and STAT1 α cDNAs are overexpressed (Figure 6.6). This effect is fully dependent on dsRNA treatment, which presumably functions to activate NF- κ B. Furthermore, whatever the basis of the weak inhibition of dsRNA inducibility seen when STAT1 α is overexpressed,

Figure 6.6 Overexpression of ISGF3 γ and STAT1 in HeLaE cells.

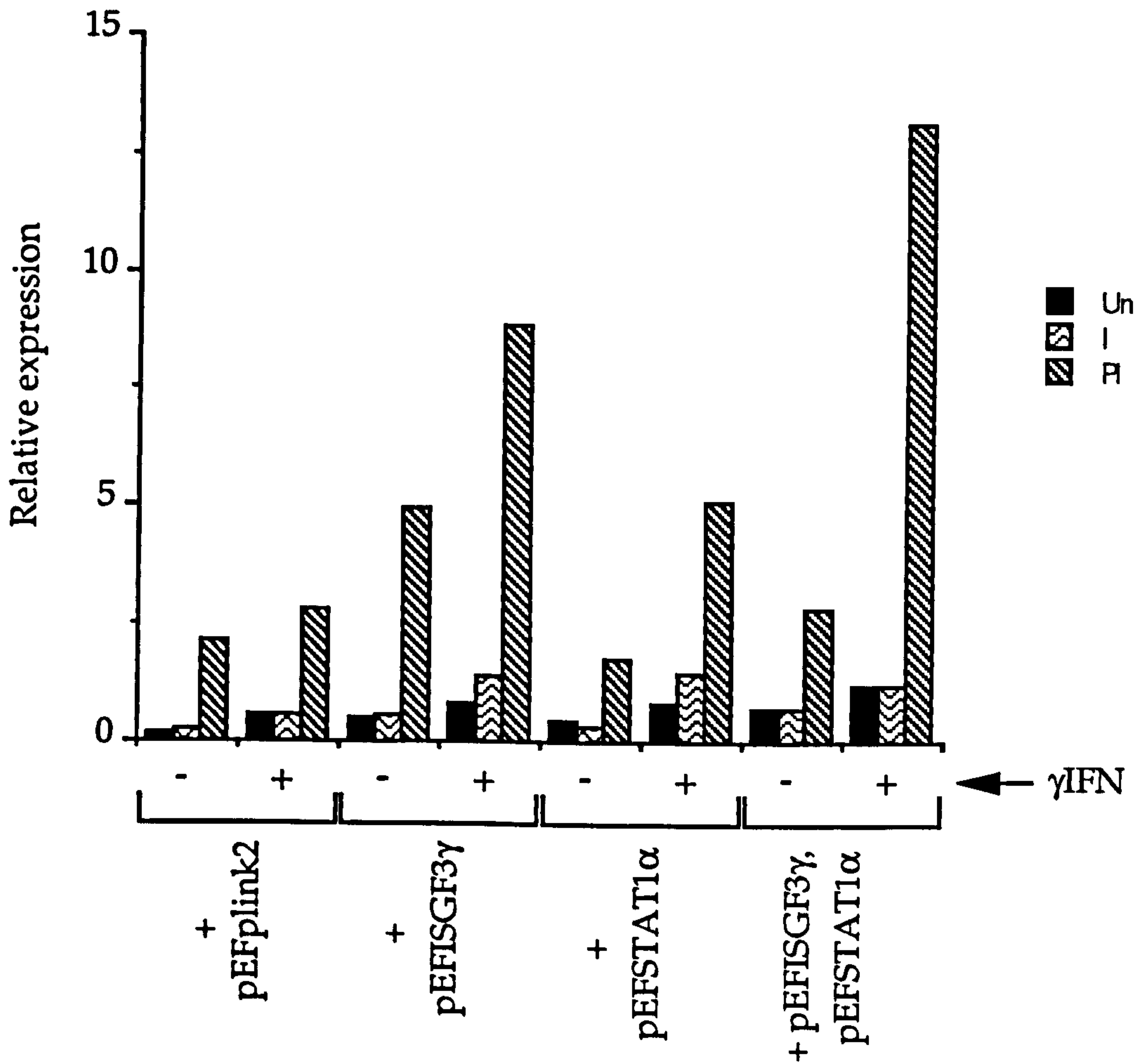
(A). cDNAs for human STAT1 α and STAT1 β were obtained by PCR from reverse transcribed MG63 RNA and verified by partial sequencing. The cDNAs were inserted into the polylinker of pEFplink2 (a kind gift from R. Treisman, ICRF), a plasmid containing the extremely powerful human EF-1 α polypeptide elongation chain factor promoter and enhancer region, to construct pEFSTAT1 α and pEFSTAT1 β .

(B). HeLaE cells were transiently transfected, using Lipofectamine, with 0.2 μ g of pIF[-108/+72]lucifer and 0.8 μ g of pEFISGF3 γ , 0.8 μ g of pEFSTAT1 α or 0.8 μ g of both, along with 0.2 μ g of pSV₂CAT. The amount of DNA in the transfection mix was made up to 2 μ g with pEFplink2 as necessary. The cells were treated as follows:
unprimed, uninduced (Un),
unprimed, induced (I),
primed, induced (PI).
The treatments were performed in the absence (-) or presence (+) of γ -IFN as indicated.

A



B



this is not seen if a strong STAT1 activator is included in the induction medium.

Whereas a role for ISGF3 γ could be inferred from the behaviour of interfering mutants, a similar experiment is unlikely to be useful for assessing the role of STAT1 α , since the results discussed so far demonstrate that the priming effect is dependent on the presence of functional STAT1 α .

IFN production uses a positive autoregulatory loop

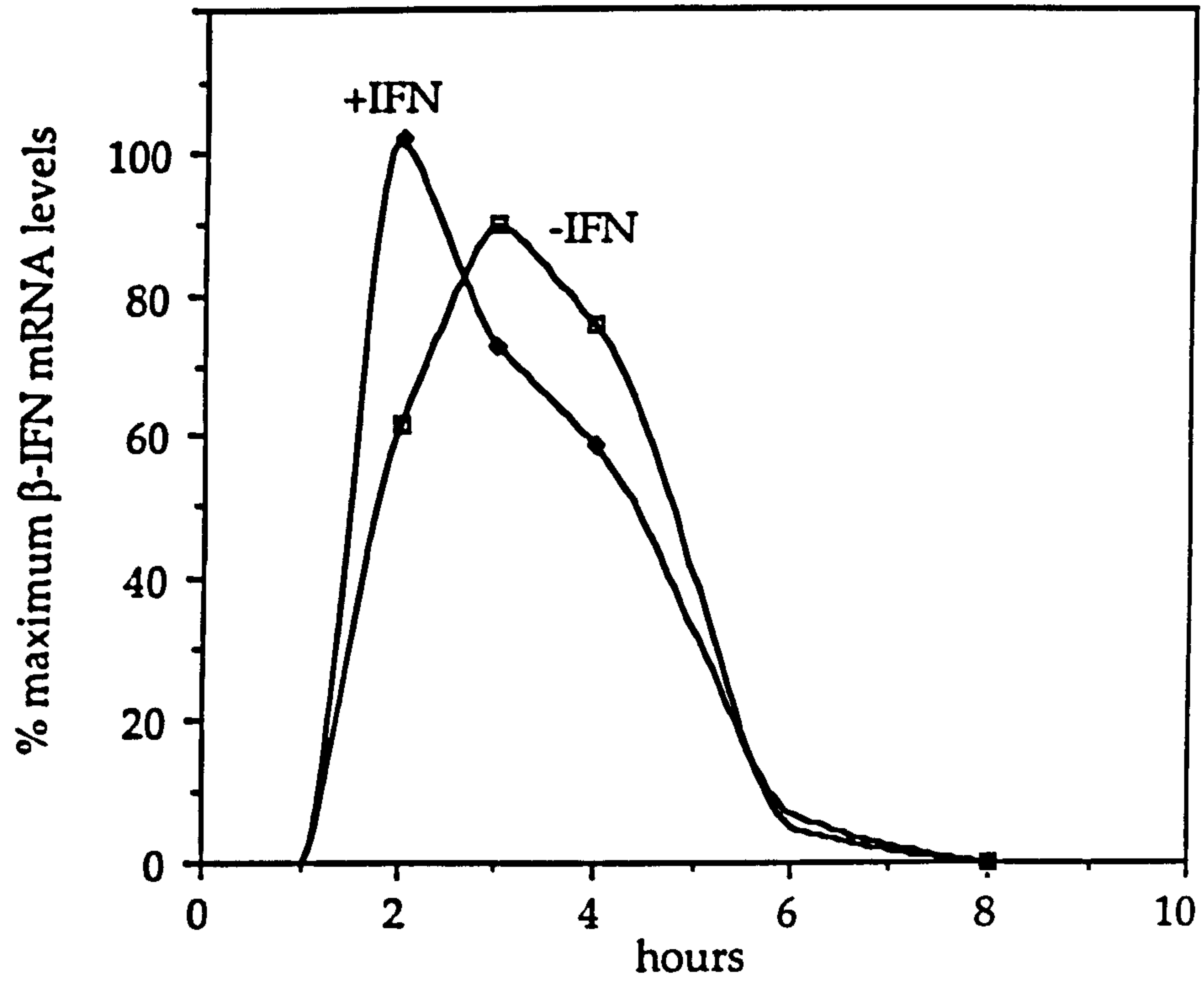
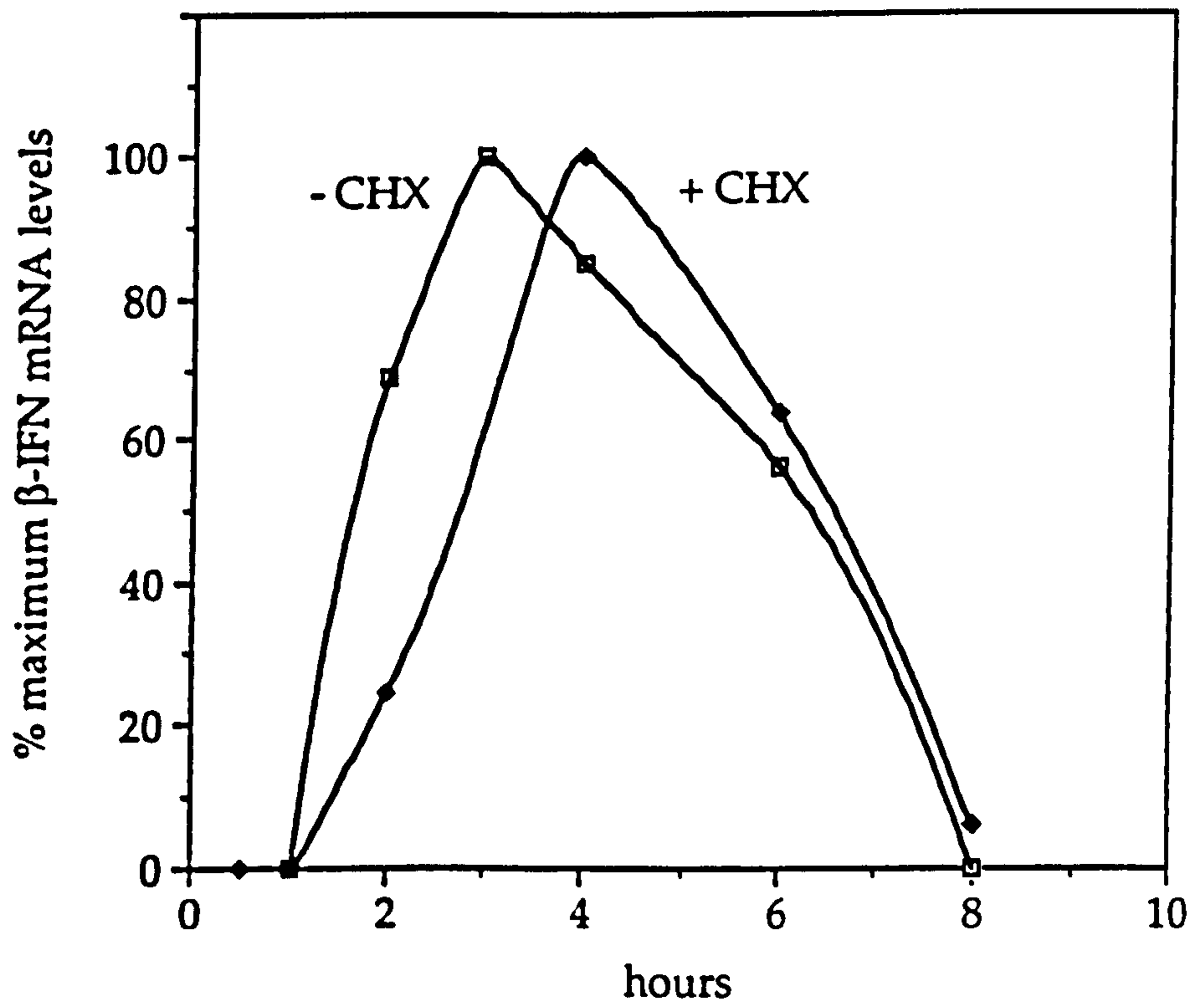
The results discussed above strongly suggest that although they are not capable of priming cells, ISGF3 γ and STAT1 play a positive role in the induction of β -IFN expression. The nature of the signal that activates STAT1, however, is unclear. Experiments in HeLaE cells demonstrate that STAT1 overexpression does not lead to enhancement of β -IFN induction, but for reasons outlined above, this may reflect the fact that primed cells already contain sufficient STAT1 to mediate a maximal response to dsRNA. A surprising result from Figure 6.6 was that β -IFN induction could be stimulated by type II IFN. One prediction from this observation is that the β -IFN gene might be capable of being superinduced in the presence of IFN. It is likely that extracellular IFN would be present during viral infections *in vivo*, secreted by previously infected cells, and a stimulatory role for IFN would lead to an amplification of IFN production. It may even be possible that low levels of type I IFN production could stimulate β -IFN expression in an autocrine manner.

In order to investigate this further, primed HeLaE cells were induced by dsRNA with and without the inclusion of γ -IFN or α -IFN in the induction mix. The results of an RNase protection experiment on the effects of α -IFN, as analysed by densitometry, are shown in Figure 6.7A. This figure shows that, as observed previously, the peak of induction of β -IFN by dsRNA in HeLaE cells is seen at around 3 hours. However, when induced in the presence of α -IFN, the kinetics of induction are accelerated, with the peak of induction brought forward to 2 hours, although the overall extent of induction remains unchanged. Similar results were obtained using γ -IFN, and have also been seen in MG63 cells (data not shown).

Whilst these results show that extracellular IFN can accelerate the induction of β -IFN, they do not address whether IFN can positively regulate its own synthesis in an autocrine manner. If this is so, blockage of protein synthesis should delay the onset of induction. When cycloheximide is added to the medium during induction in a similar experiment, this is indeed the case (Figure 6.7B), although it should be pointed out that the presence of cycloheximide leads to eventual superinduction of expression.

Figure 6.7 The effect of IFN and CHX on the kinetics of induction

HeLaE cells were induced with dsRNA, with or without the inclusion of Wellferon (A) or cycloheximide (CHX) (B) in the induction mix, and cells were harvested over the time course shown. The RNA from each sample was mapped for β -IFN- and γ -actin-specific transcripts, and the autoradiographs were analysed by densitometry, with the β -IFN signals being corrected for variations in the γ -actin signals. The results were plotted as a percentage of the maximum signal recorded for each condition.

A**B**

Can dsRNA directly activate STAT1?

Although the results presented in the previous sections demonstrate that STAT1 can play a role in the induction of β -IFN, they suggest that this may be a result of targeting signals from extracellular IFN. However, the kinetics and magnitude of β -IFN induction are such that it is difficult to imagine that this is a pre-requisite event for activation by dsRNA. Since PRD I is an essential target for β -IFN induction, and can clearly be activated by dsRNA in a protein synthesis-independent manner in primed HeLaE cells (Whiteside et al. 1992), it is likely that ISGF3 γ is a recipient of a signal that is directly activated by dsRNA. It is therefore possible that STAT1, or an alternative STAT factor, is activated in such a manner. The following experiments were designed to test this possibility.

Transcription through a STAT1-binding site is dsRNA responsive

During the course of experiments on the induction of other genes by dsRNA, it was noted that several required priming for induction in HeLaE cells. This is clearly demonstrated by the IRF-1 gene (Figure 6.8), although in contrast to the β -IFN gene there is a detectable IRF-1 signal in uninduced cells which is slightly inducible by dsRNA in the absence of priming. The observation that both β -IFN and IRF-1 require priming for induction by dsRNA suggested that they may share common promoter elements. The two promoters are displayed in schematic form in Figure 6.9. Both contain an NF- κ B site which is likely to be a target for a dsRNA response (see Chapters One, Four and Five), but single binding sites for transcription factors are usually insufficient for induction, and therefore full induction probably requires co-operation with at least one other dsRNA-inducible element. One possible site for the second dsRNA-responsive element in the IRF-1 promoter is the consensus CRE (cyclic-AMP response element (Du and Maniatis 1992), although the equivalent site in the β -IFN promoter (PRD IV) does not appear to be dsRNA-responsive in HeLaE cells (Chapter Four and J. Eloranta, PhD. thesis, London University, 1995). Interestingly, the IRF-1 promoter also contains a GAS site (Figure 6.9-see Chapter One), and given the apparent involvement of STAT1 in β -IFN induction, this was a potential target for a second dsRNA-responsive element. As mentioned previously, IRF-1 is inducible in response to dsRNA in U2, U3 and U4 cells, indicating that this response cannot be acting in response to IFN production during induction, and the limited inducibility of the IRF-1 gene in U3 cells compared to the other cell lines suggests that STAT1 is involved in the induction by

Figure 6.8 IRF-1 induction by dsRNA in HeLaE cells is priming-dependent

HeLaE cells were untreated (lanes 1 and 7) or treated with dsRNA in the unprimed (lanes 1-6) or primed (lanes 7-12) states, for the length of time indicated, in hours, above the autoradiograph. RNA from these cells was analysed by RNase mapping, using probes for β -IFN, IRF-1 and γ -actin. The position of the protected fragments is indicated to the right of the autoradiograph.

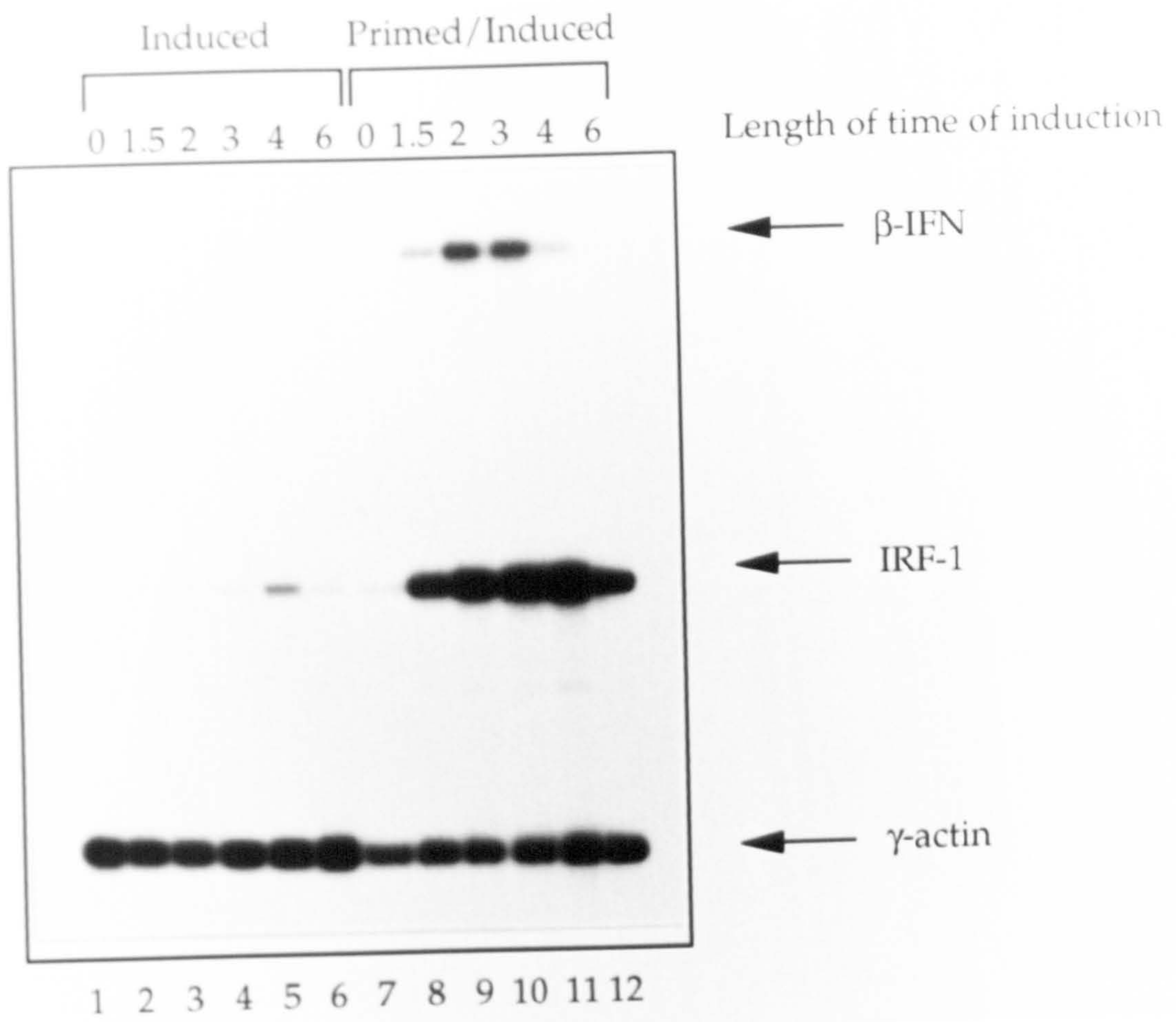


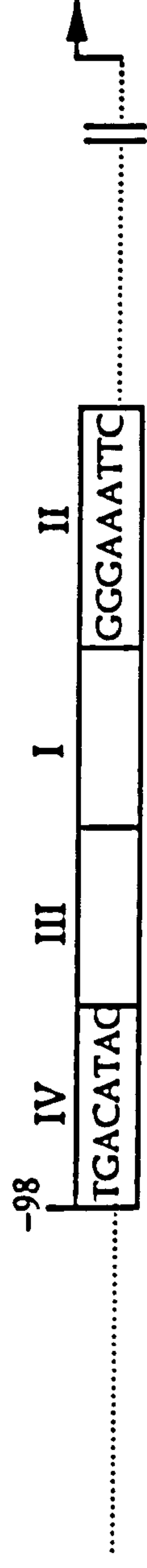
Figure 6.9 Comparison of the β -IFN and IRF-1 promoters

The promoters for β -IFN and IRF-1 are shown in schematic form.

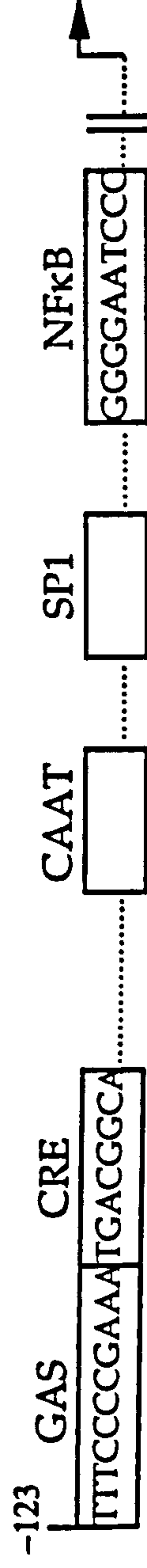
The PRD regions of the β -IFN promoter are depicted, and the sequences of PRD IV, and PRD II are shown.

Potential binding sites for GAF, ATF/CREB, SP1, NF- κ B and CAAT box binding factors in the human IRF-1 promoter are depicted (Sims et al. 1993) and the sequences of the GAS, CRE and NF- κ B sites are shown.

β -IFN promoter



IRF-1 promoter



dsRNA.

To analyse the dsRNA-responsive nature of the IRF-1 promoter, a fragment spanning co-ordinates -138/+12 was obtained by PCR from genomic DNA, and fused upstream of a luciferase gene in the construct pIRF-1[-138/+12] Δ 5'lucifer (Figure 6.10A). Unlike the endogenous IRF-1 gene (Figure 6.8) this construct shows a high degree of constitutive activity in HeLaE cells, presumably because the endogenous gene is under negative control by sequences outside the region included in the construct. dsRNA treatment causes a slight induction in unprimed cells, although surprisingly this induction, rather than being enhanced, appears to be blocked in primed cells (Figure 6.10B). This effect is also seen with γ -IFN induction, and the basis for this result is not immediately clear. In order to assess directly the contribution of the GAS site to the observed activation of the IRF-1 promoter by dsRNA, complementary oligonucleotides were synthesised to create the IRF-1 GAS site and these were multimerised by ligation upstream of the HSV tk TATA box in ptk Δ [-39]lucifer. A clone containing a dimer of the GAS site was analysed in transfection experiments. The constitutive level of activity observed from pIRF-1[GAS]₂tk Δ [-39]lucifer is much lower than from the IRF-1 promoter fragment, and the inducibility of this construct by both dsRNA and Sendai virus can be observed quite clearly (Figure 6.11). Interestingly, genestein, an inhibitor of tyrosine kinases, which has been shown to be able to block type I and II IFN signalling and β -IFN induction, blocks the induction, as does a PKA inhibitor, H89. However, as seen for the longer promoter fragment, induction by dsRNA remained independent of the need for priming.

dsRNA activation of a STAT1-binding site does not depend upon STAT1

The dsRNA-inducibility of pIRF-1[GAS]₂tk Δ [-39]lucifer seen in unprimed HeLaE cells is unlikely to be caused by a type I IFN-mediated event because no β -IFN induction can be detected under these conditions, and α -IFN is poorly inducible by dsRNA in non-lymphoid cells (see Chapter One). This result suggests that the effect of dsRNA may well be direct. Since the induction of this construct is inhibited by genestein, it is tempting to speculate that, in common with many cytokines and some growth factors, dsRNA treatment may result in the phosphorylation of STAT1 on tyrosine 701, leading to activated transcription of dsRNA-inducible genes.

To determine whether dsRNA treatment of HeLaE cells could lead to the activation of STAT1, EMSA experiments were performed with radiolabelled IRF-1 GAS site probes and dsRNA-induced nuclear extracts from both unprimed and

Figure 6.10 The IRF-1 promoter is dsRNA-inducible

(A). pIRF-1[-138/+7] Δ 5'lucifer was created from a fragment of the human IRF-1 promoter, isolated by PCR from MG63 genomic DNA using a 5' primer incorporating the -138/-115 with a Bgl II site immediately 5' to -138, and a 3' primer +18/-6 which incorporates the natural Sac I site. This was initially subcloned into BamH I/Sac I digested SP64, and then from there into BamH I/Nhe I digested ptk Δ [-105] Δ 5'lucifer as an Xho II/EcoR I fragment.

(B). HeLaE cells were transiently transfected, using calcium phosphate co-precipitation, with 10 μ g of pIRF-1[-138/+7] Δ 5'lucifer and 2 μ g of pSV₂CAT. The cells were untreated or treated with γ -IFN or dsRNA, for the length of time indicated, in the unprimed or primed states. The luciferase activities were normalised to the corresponding CAT activities.

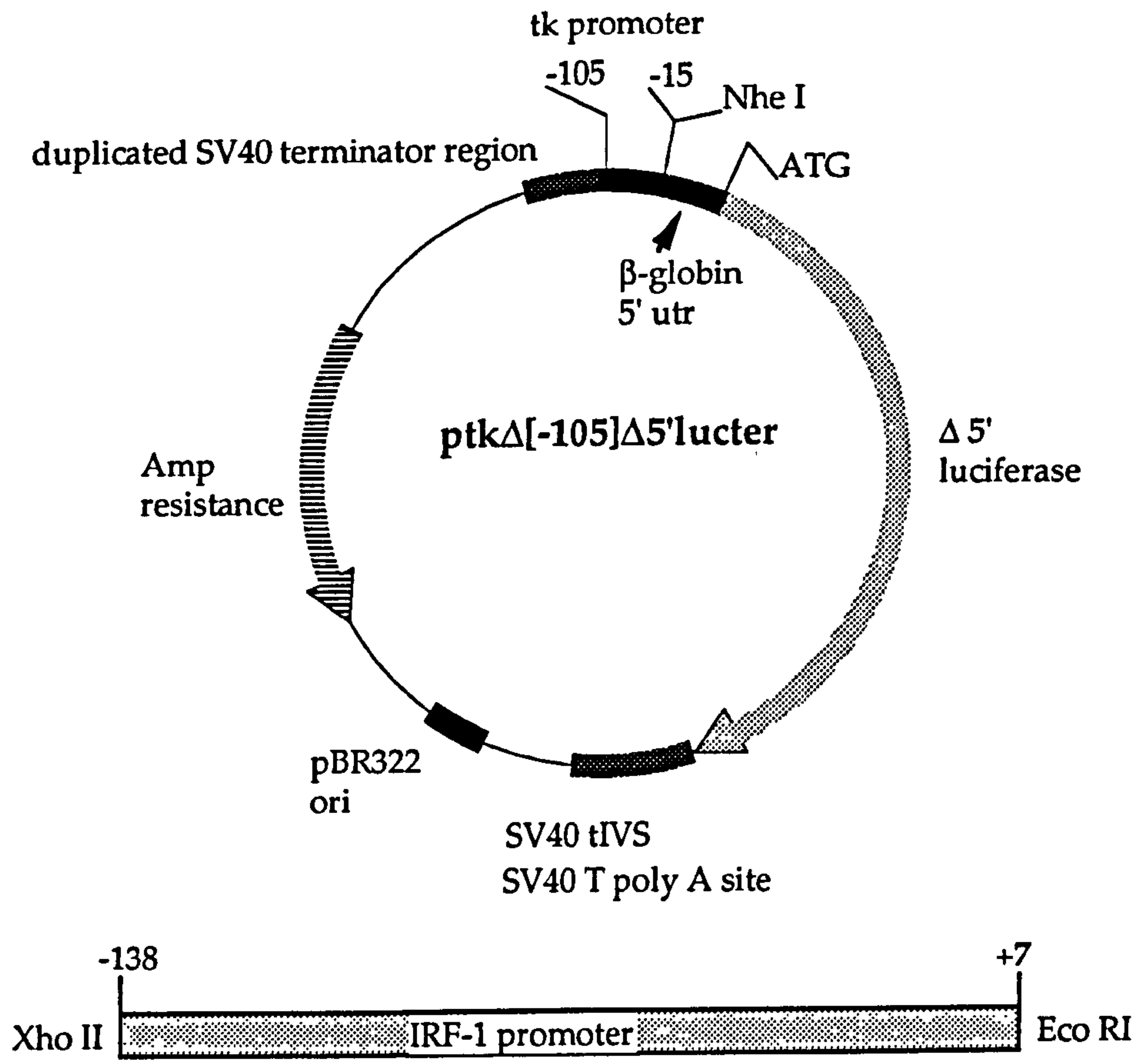
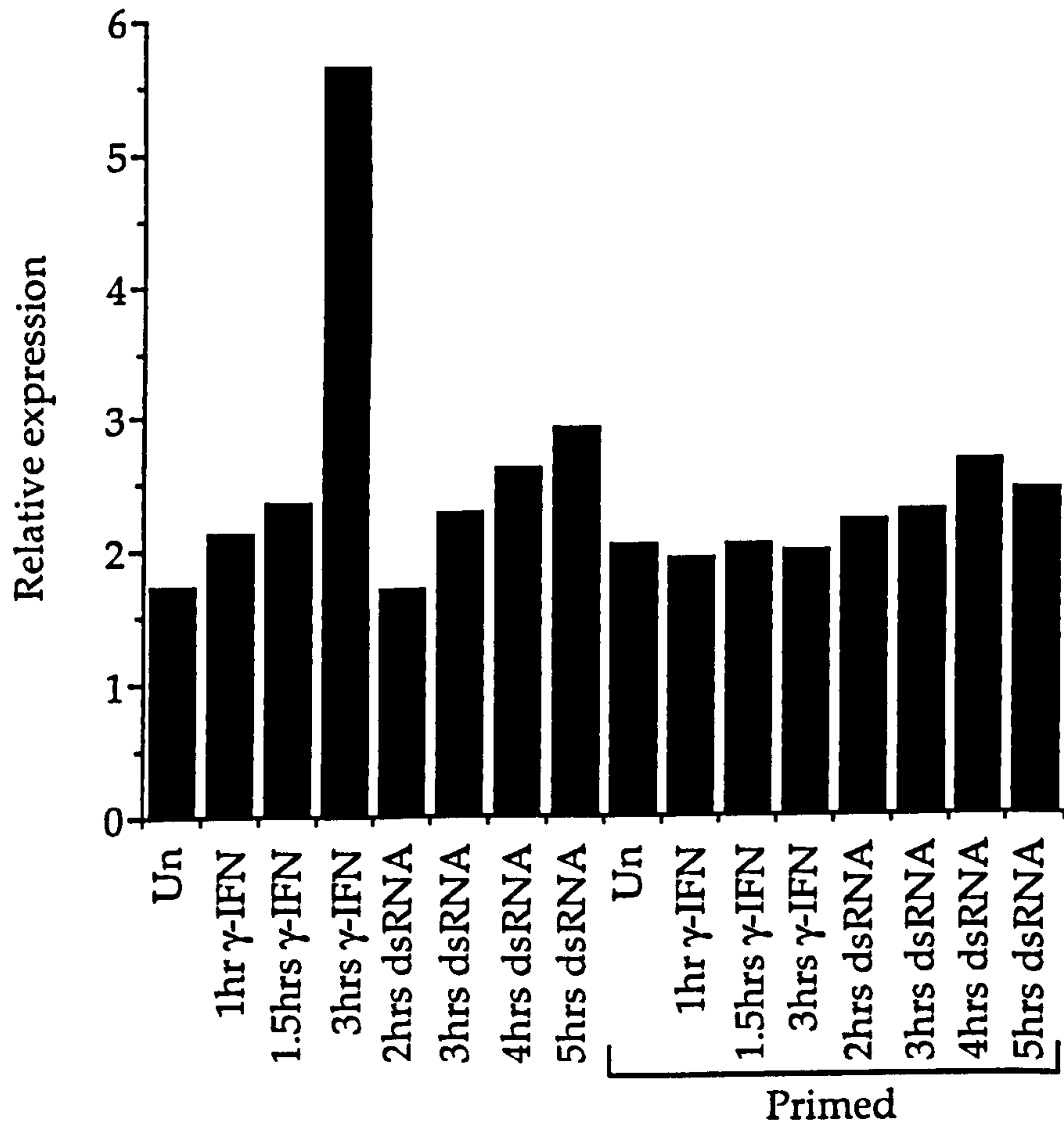
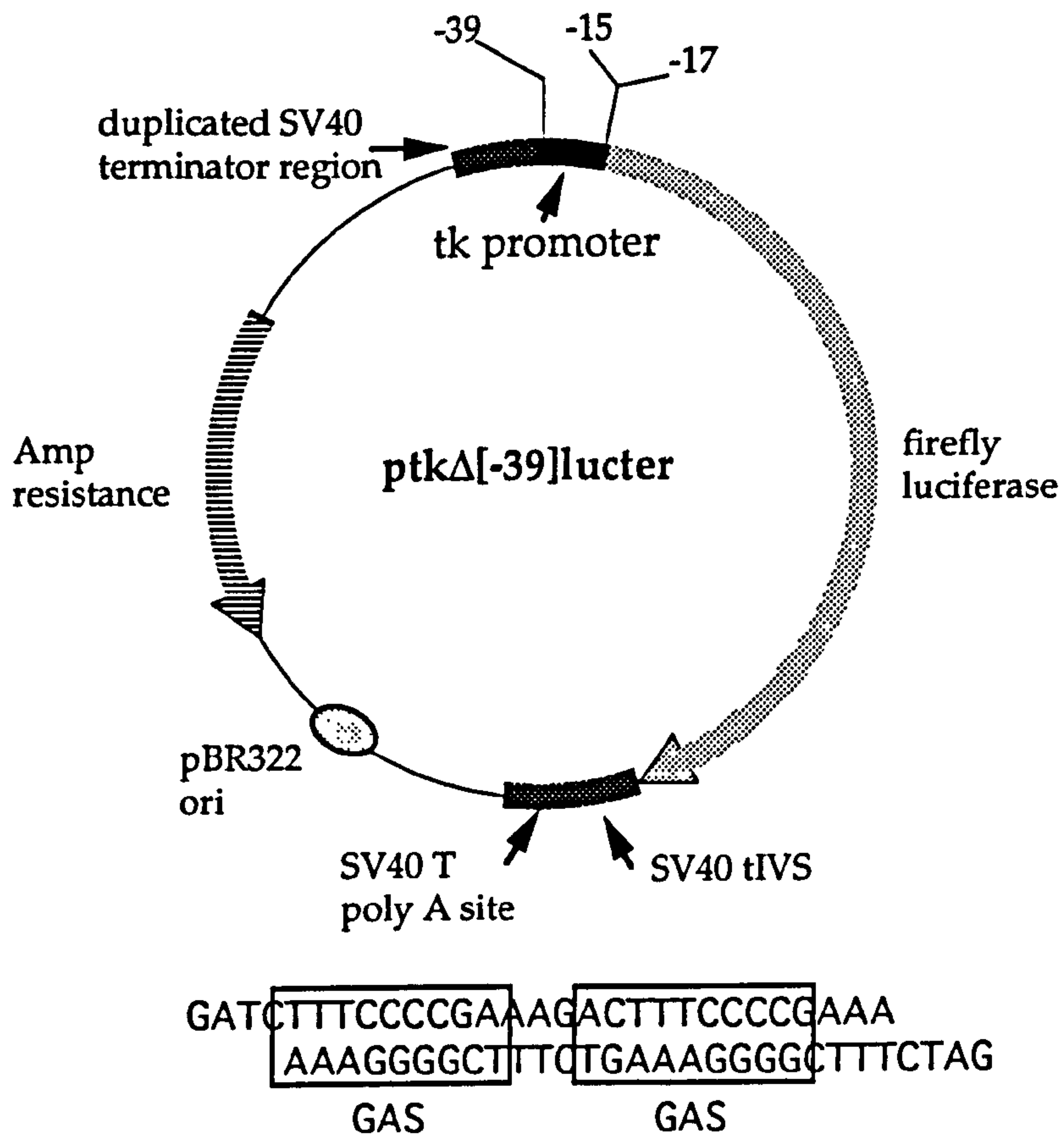
A**B**

Figure 6.11 A dimerised IRF-1 GAS site is dsRNA-inducible

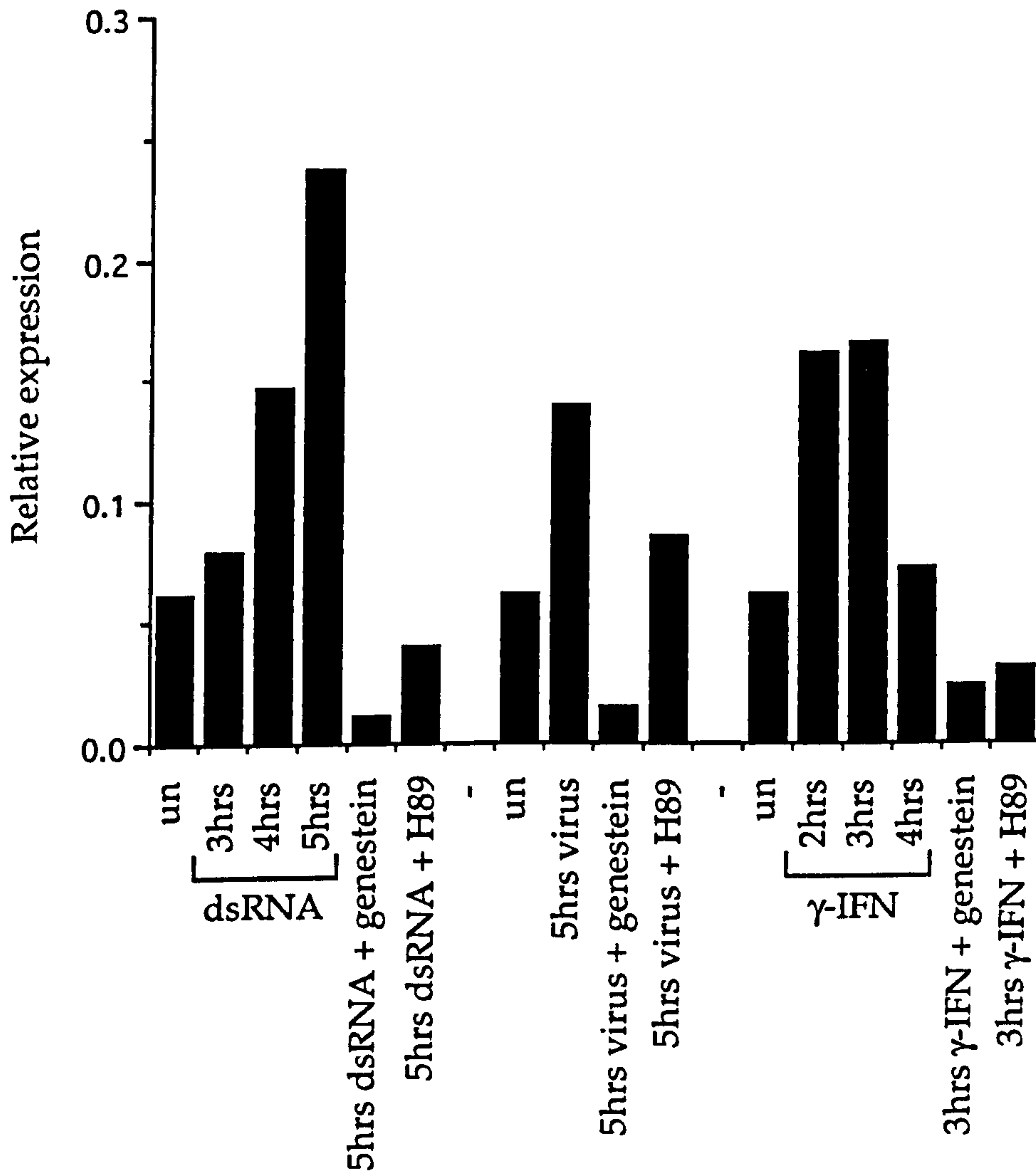
(A). pIRF-1[GAS]₂tkΔ[-39]lucifer was constructed by ligating phosphorylated IRF-1 GAS oligonucleotides (TTTCCCCGAAA), via their GATC 5' overhangs, into the BamHI site of ptkΔ[-39]lucifer. Positive clones were sequenced to determine the number and orientation of inserts. The GAS site dimer used had lost a nucleotide from the central GATC fusion as shown.

(B). HeLaE cells were transiently transfected, using calcium phosphate co-precipitation, with 10μg of pIRF-1[GAS]₂tkΔ[-39]lucifer and 2μg of pSV₂CAT. The cells were untreated or treated with dsRNA, Sendai virus or γ-IFN for the length of time indicated, in the unprimed state. The kinase inhibitors genestein and H89 (both 10μg/ml) were included with each inducer, as indicated. The luciferase activities were normalised to the corresponding CAT activities.

A



B



primed HeLaE cells. No dsRNA-activated GAS binding factors could be observed in any extract (data not shown). In a more direct test of the ability of dsRNA to lead to phosphorylation of STAT1, STAT1 was immunoprecipitated from dsRNA-treated MG63 cells (chosen because of their high constitutive level of STAT1, Figure 6.1C), and the immunoprecipitates were probed on a Western blot with an anti-phosphotyrosine monoclonal antibody. This analysis of the STAT1 immunoprecipitation shows that although tyrosine-phosphorylated STAT1 α and β can be detected following treatment of MG63 cells with γ -IFN, no such signal can be detected following dsRNA treatment (Figure 6.12).

The results so far presented on the dsRNA-inducibility of the IRF-1 GAS site would suggest that STAT1 is not involved in this induction, and in an attempt to confirm this, the experiment was repeated in 2fTGH and U3 cells. Although the extent of dsRNA induction of pIRF-1[GAS]₂tk Δ [-39]lucifer observed in Figure 6.13 is very low in 2fTGH cells, the inducibility is much greater in the STAT1-negative U3 cells, suggesting that not only is STAT1 unnecessary for the induction of this construct, its presence may even be inhibitory. As expected, γ -IFN-mediated induction of the GAS construct is not observed in U3 cells. The induction of pIRF-1[GAS]₂tk Δ [-39]lucifer by dsRNA in U3 cells indicates that the activation of the GAS site is not a consequence of IFN production in these cells.

Discussion

The experiments presented in this Chapter were performed to assess the roles of ISGF3 γ and STAT1 in the priming and induction of β -IFN. The loss of ISGF3 γ and JAK1 from U2 and U4 cells, respectively, does not seem to impair their dsRNA-inducibility, although the loss of STAT1 appears to render U3 cells less inducible, nor does the absence of any of these factors lead to priming-dependent induction. However, all the cell lines contain IRF-1 mRNA and, as was seen with MG63 cells in Chapter Five, blocking the synthesis of IRF-1 protein by incubation with antisense oligonucleotides renders them uninducible.

The analysis of the rescue of the inducibility of these antisense oligonucleotide-treated cells by priming with both type I and II IFN yielded some very interesting results (Figure 6.3). 2fTGH cells are fully rescued from IRF-1 antisense inhibition by priming with α -IFN, whereas none of the other lines are. Treatment of cells with α -IFN leads to the activation of ISGF3, but can also mediate a GAF response, and it is via this latter mechanism that it induces IRF-1 (Pine et al. 1994). However, α -IFN is less efficient at inducing IRF-1 than γ -IFN, as can be seen in

Figure 6.12 STAT1 is not tyrosine-phosphorylated by dsRNA

MG63 cells were untreated (lane 2) or treated with γ -IFN (lanes 3 and 4) or dsRNA (lanes 5-7) for the lengths of time indicated, in minutes. Cells were harvested and whole cell protein extracts were prepared for immunoprecipitation with a polyclonal anti-STAT1 antibody (Transduction Laboratories, 4 μ g/9cm plate extract). The immunoprecipitated material was fractionated on a 7.5% SDS/PAGE gel and the blot was probed with a 1:1000 dilution of an anti-phosphotyrosine antibody, PY20 (Transduction Laboratories). The positions of STAT1 α and β , the anti-STAT1 antibody and the size markers (lane 1) are indicated to the sides of the autoradiograph.

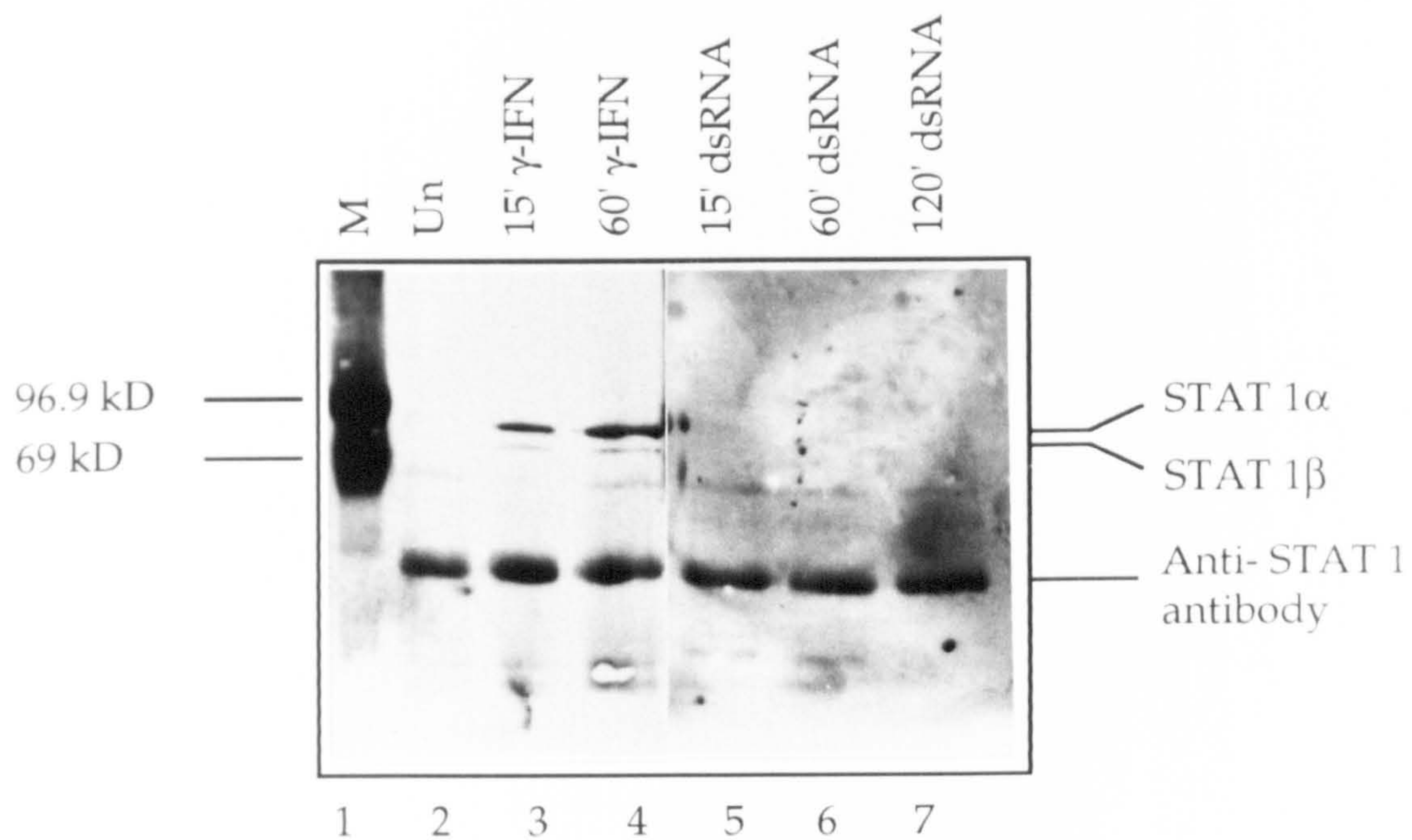
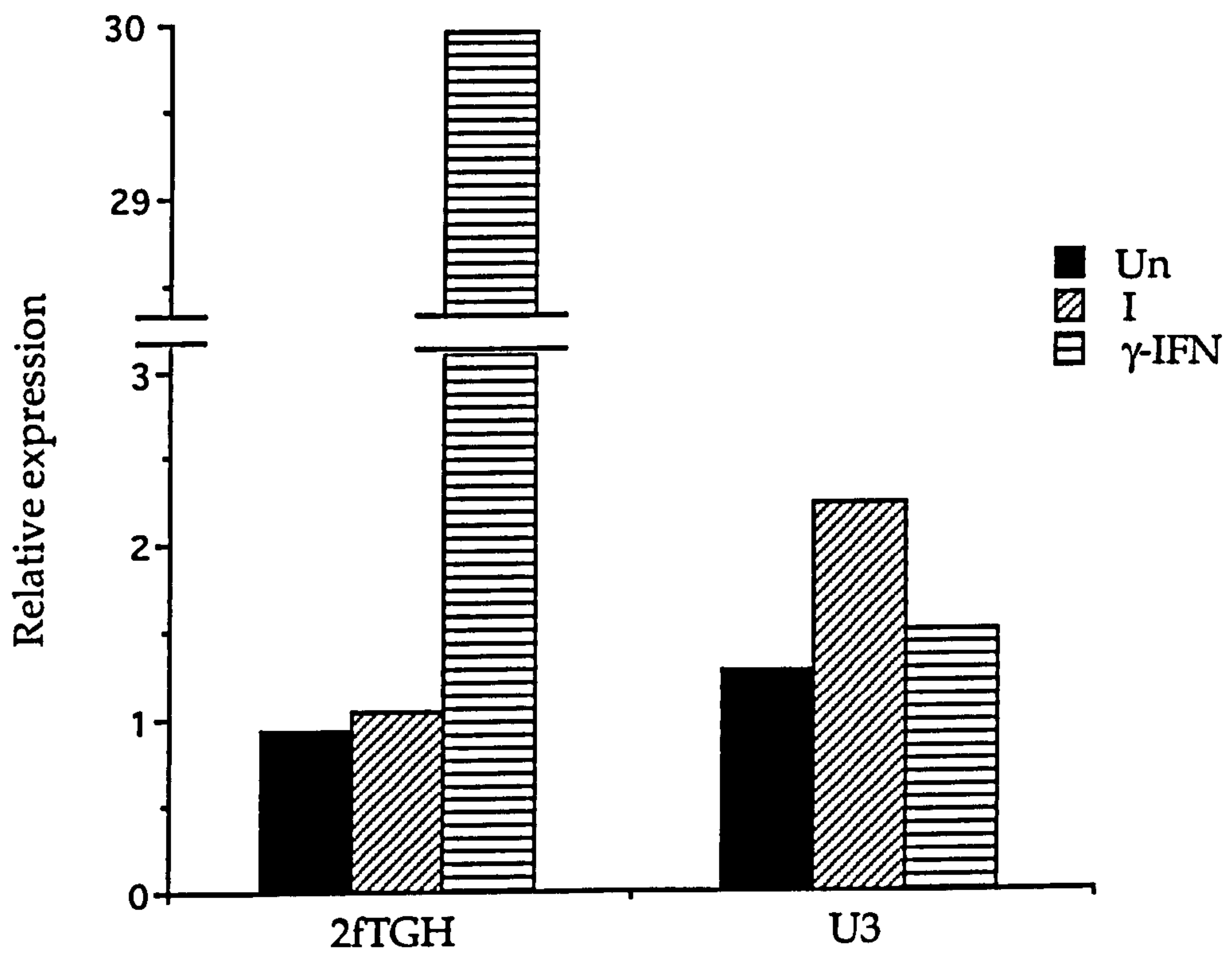


Figure 6.13 The IRF-1 GAS site is dsRNA-inducible in U3 cells

2fTGH and U3 cells were transiently transfected, using calcium phosphate, with 10 μ g pIRF-1[GAS]₂tk Δ [-39]lucifer and 10 μ g pEFplink2 or pEFSTAT1 α , along with 2 μ g pSV₂CAT. The cells were treated with dsRNA (I) and γ -IFN (3 hours) as indicated.



2fTGH cells in Figure 6.3B. The fact that priming with α -IFN is better at rescuing β -IFN induction in 2fTGH cells than priming with γ -IFN suggests that this rescue is working through an ISGF3-mediated, rather than through a GAF mediated, response, and is not simply a consequence of overcoming the block to IRF-1 protein synthesis. This is supported by the fact that dsRNA induction in oligonucleotide-treated U2 cells cannot be rescued by priming with α -IFN, even though a level of IRF-1 protein comparable to that present in normally growing, priming-independent U2 cells, is produced by the type I IFN incubation. U3 and U4 cells are unresponsive to priming by either type of IFN because of the central role of activated STAT1 in both processes and similarly are not rescued from uninducibility by IFN pre-treatment.

It should be noted that the Western analysis in Figure 6.2B provides only a "snapshot" of the IRF-1 protein levels during priming, namely after two hours of IFN treatment, when IRF-1 induction would normally be maximal. It is possible that in the presence of the antisense oligonucleotides the production of IRF-1 protein is very short lived. IRF-1 is known to have a short half life (approximately 30 minutes), and it may be that if the induction of IRF-1 protein synthesis is indeed transitory, the length of exposure of the cells to IRF-1 protein may not be sufficient for the rescue of dsRNA-inducibility of β -IFN. This may be analogous to the result shown in Figure 3.5 which shows that a one hour exposure to Wellferon, even though this should be capable of inducing IRF-1 (see Figure 5.3), is not as efficient at priming HeLaE cells as is exposure to IFN for the full course of the priming period.

Even though it does appear that the block to IRF-1 protein synthesis is overcome during IFN treatment of the cells in this experiment (and also, presumably, in the similar experiment with MG63 cells), nevertheless the striking difference between the ability of α -IFN to rescue induction in oligonucleotide-treated 2fTGH cells compared with its inability to do so in U2 cells suggests that ISGF3 γ is required for this process. Although these results do not prove that ISGF3 or an ISGF3-like complex is directly involved in β -IFN induction, they do indicate that in the absence of maximal IRF-1 protein levels, one or more genes that are inducible by α -, but not γ -IFN, are needed to be able to respond to dsRNA in these cells.

When ISGF3 γ was overexpressed in HeLaE cells, it was unable to convert them to priming-independence, in contrast to the result seen for IRF-1. However, IRF-1 possesses two activities important for β -IFN induction, the ability to transcriptionally activate a subset of IFN-stimulated genes, including that of the

priming factor(s), and the ability to contribute directly to β -IFN induction through the PRD I and PRD III regions, if present at the time of induction (see Chapter Five). The failure of ISGF3 γ to prime cells may be a consequence of an inability to provide the first function, since unlike IRF-1, ISGF3 γ requires co-factors to stimulate transcription. The fact that addition of γ -IFN to cells during the dsRNA treatment stimulated induction, and that induction was enhanced when ISGF3 γ was overexpressed, suggests that ISGF3 γ is a target for signals that can be transmitted to the β -IFN promoter. Further evidence for the involvement of ISGF3 γ was provided by the observations that variant forms of this factor are capable of completely inhibiting β -IFN induction by dsRNA.

Data presented in this Chapter also provide evidence for an essential role for STAT1 in the induction of β -IFN expression. U3 cells that lack STAT1 are poorly inducible by dsRNA, a phenotype that can be corrected by the overexpression of this factor. The fact that STAT1 overexpression allows superinduction of β -IFN in the presence of dsRNA indicates that it will transduce signals to the β -IFN promoter. Furthermore, it has been shown that β -IFN can be regulated by a positive autoregulatory loop, which by definition would involve STAT1 (Figure 6.7) (Yoneyama et al. 1996). However, it is not clear whether STAT1 is a target for direct activation in response to dsRNA. A STAT1 binding site (GAS) is indeed activated in response to dsRNA treatment of cells, and this event depends upon tyrosine phosphorylation, as would be expected from a role for STAT1. Strikingly, however, U3 cells mediate a better GAS reporter response to dsRNA than 2fTGH cells, indicating that STAT1 is not essential for this effect.

The interpretation of the STAT1 results may be complicated by the fact that primed cells dramatically induce STAT1 levels, including that of STAT1 β , which may function as a negative regulator of STAT1 function (Ihle 1996). The consequence of further overexpressing STAT1 as a result of transfection appears to be inhibitory to β -IFN induction. This does not mean that STAT1 is not involved in a primary transcriptional response. It is notable that not only can the addition of type I or type II IFN accelerate the kinetics of induction, but also that STAT1 overexpression enhances induction when IFN is added. The most likely explanation of this result is that STAT1 is recruited to the β -IFN promoter in an activated form by ISGF3 γ , and this laboratory has recently identified a β -IFN promoter-binding complex that contains ISGF3 γ and STAT1 (S. Goodbourn, pers. comm.). Furthermore, although ISGF3 is formed very rapidly and transiently in cells as a result of treatment of cells with type I IFN, studies in HeLaE cells have revealed that

there is a second phase of production of an ISGF3-like complex, (S. Goodbourn, pers. comm.). This second phase complex appears slowly with kinetics that match those of the development of the priming response.

It is likely that the formation of this complex requires protein synthesis, reflecting a need for raised levels of ISGF3 γ . Since priming can also be achieved by γ -IFN, this complex must be able to be formed without the contribution of STAT2. Although this has not been directly tested, it is interesting to note that a form of ISGF3 composed of ISGF3 γ and homodimeric STAT1, referred to as STAT1-ISGF3 γ , can activate transcription through an ISRE (Bluyssen et al. 1995), and it has been noted that the binding affinity of STAT1-ISGF3 γ to the ISRE is weaker than that of ISGF3, and only observed under conditions where both ISGF3 γ and STAT1 levels are relatively abundant (Bluyssen et al. 1996). Such requirements would exactly match the situation seen in primed cells. It is thus possible that persistent levels of such ISGF3-like complexes, present in primed cells, would act as primary activators of the β -IFN promoter. The effect of such complexes on transcription would still require dsRNA to be present, in order to activate other regions of the promoter, such as PRD II. Furthermore, since it has been shown that induction of PRD I still absolutely requires dsRNA (Whiteside et al. 1992), it is possible that de-repression of this element must occur in order for this ISGF3-like complex to function. In this context it is interesting to note that two complexes Un1 and Un2 have been reported to have the properties of transcriptional repressors and to lose their affinity for PRD I in a dsRNA- and priming-dependent manner.

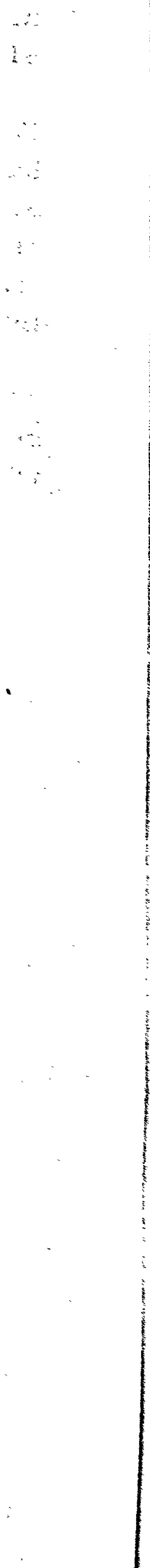
If the above model is correct, it imposes no requirement for direct activation of STAT1 by dsRNA. Nevertheless, it is clear that some GAS binding factor other than STAT1 is activated by dsRNA, and this factor might still participate in β -IFN induction. This factor is unlikely to be STAT2, since this has not been shown to be able to bind to GAS sites, and STAT4 and STAT5 are not present in HeLaE cells (data not shown). STAT6 is very abundant in HeLaE cells, but is unable to bind to the GAS element used in these studies (data not shown). STAT3 is also present in HeLaE cells (see Chapter Seven), and this factor shows some overlap in its spectrum of activation with STAT1 (for example it can be activated by γ -IFN). STAT3 is a candidate for activation, not least because it shows much enhanced affinities for target sites when serine phosphorylated (Wen et al. 1995), and the results obtained with the PKA inhibitor shown in Figure 6.10 show that dsRNA activation of the GAS site is affected by such phosphorylation. A role for STAT3 in dsRNA-mediated transcriptional activation remains to be investigated. The ability of STAT3 to

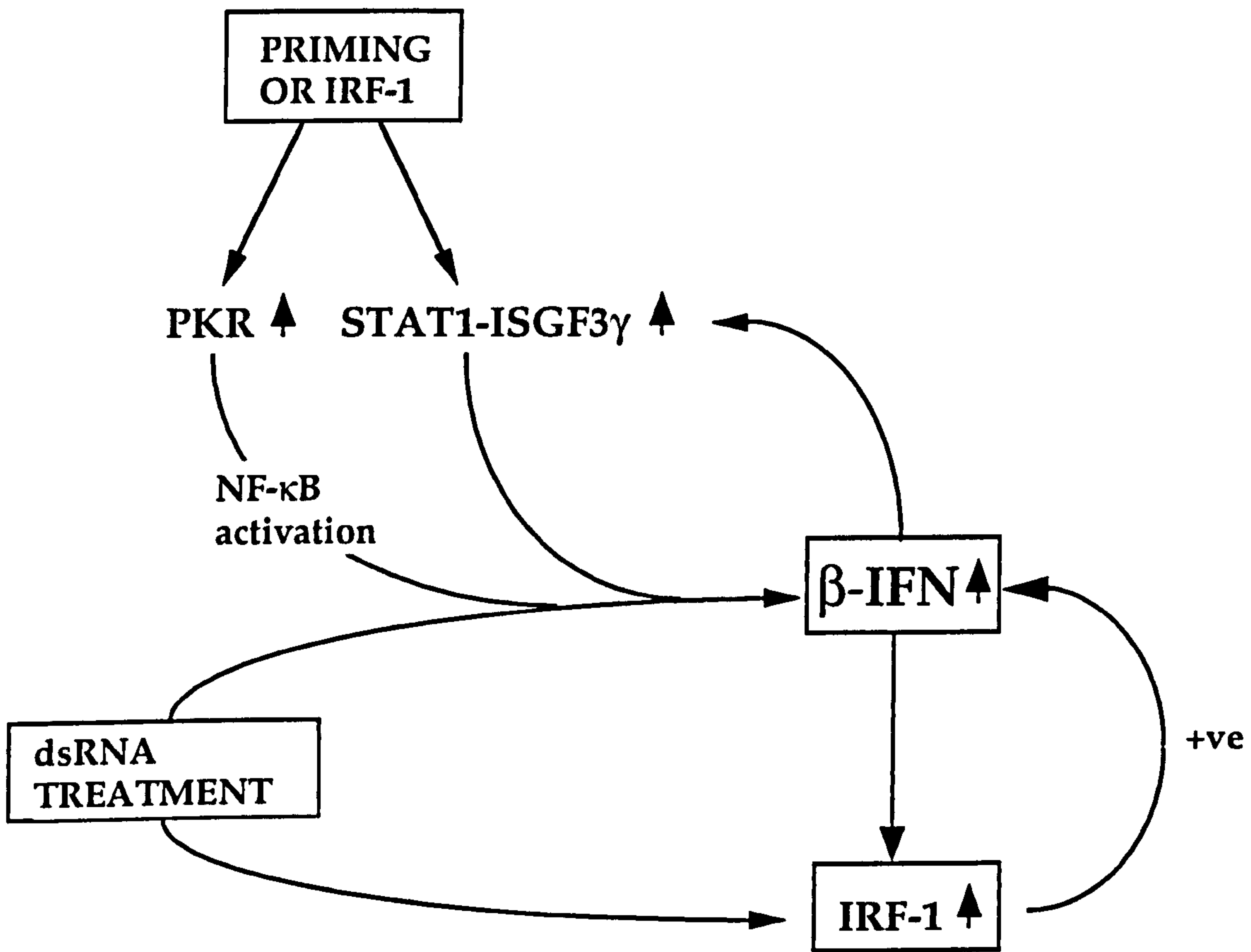
heterodimerise with STAT1 could explain the inhibitory effects of overexpressed STAT1 on dsRNA activation if the heterodimer downregulates this response.

A model for β -IFN activation in primed HeLaE cells by dsRNA is proposed in Figure 6.14. Upon dsRNA treatment, PKR is activated and phosphorylates I κ B, leading to the liberation of NF- κ B, which is then able to bind to PRD II. ISGF3 γ and STAT1 can act in concert to activate transcription through the PRDI/PRD III region of the promoter, following de-repression of the promoter. Together these complexes can lead to efficient induction of the β -IFN gene. Activation of STAT1-ISGF3 γ by IFN, either synthesised during induction or added as above, can accelerate the induction through PRDI/PRD III, and this acceleration can also be achieved in situations where IRF-1 is present at the time of induction. IRF-1 is able to prime cells because it can upregulate the levels of PKR and the STAT1-ISGF3 γ components, which are both essential for efficient induction in the absence of IRF-1.

Figure 6.14 A model for β -IFN induction in HeLaE cells

See text for further details.





CHAPTER SEVEN - THE ROLE OF ICE-LIKE PROTEASES IN β -IFN INDUCTION

Introduction

In Chapter Six, a novel activator of β -IFN induction was identified, involving ISGF3 γ and STAT1, and the direct activation of STAT1 by dsRNA was investigated. Although no evidence for tyrosine phosphorylation of STAT1 was obtained in response to induction, Western analysis of extracts from a dsRNA-induction time course of HeLaE cells using an anti-STAT1 antibody revealed the production of a protein that ran slightly faster than STAT1 β . The experiments presented in this final Chapter were performed in an attempt to determine the nature of this novel protein, and these shed light on the mechanism of production of a previously identified breakdown product of IRF-2 during induction.

A faster migrating STAT1-related complex is formed during dsRNA induction of primed HeLaE cells

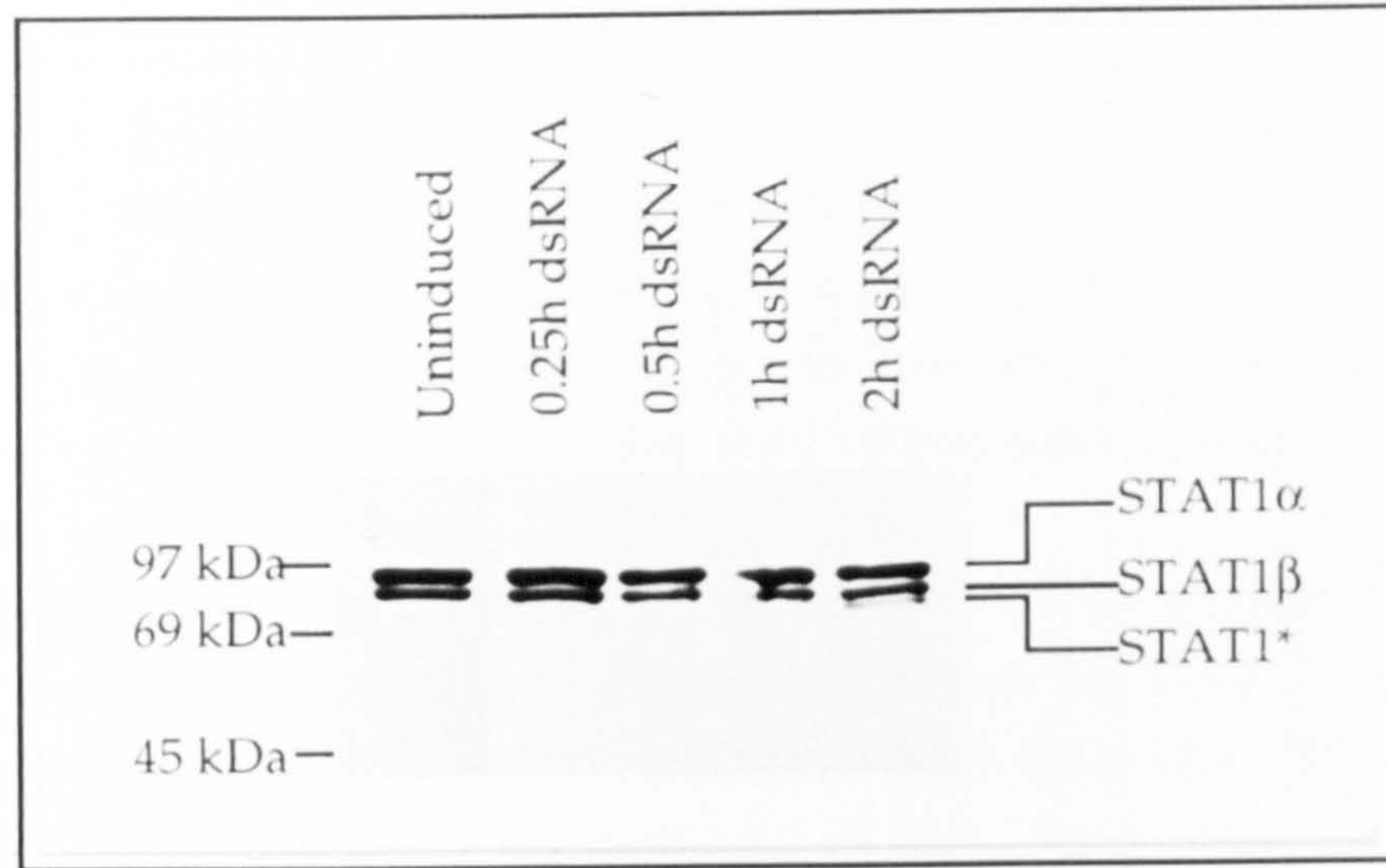
Primed HeLaE cells were treated with dsRNA for up to 2 hours and extracts from these cells were fractionated on an SDS-PAGE gel. By probing the Western blot of this gel with a polyclonal antibody raised against the N-terminal peptide of human STAT1 (Transduction Laboratories), it can be observed that after exposure of the cells to dsRNA for 2 hours a novel antibody-reactive protein is produced, migrating with slightly faster mobility than the 84kDa form, STAT1 β (Figure 7.1A). This was assumed to be a novel form of STAT1 and was named STAT1*. To determine whether the production of STAT1* required *de novo* protein synthesis, the induction of HeLaE cells with dsRNA was repeated in the presence of cycloheximide. The inclusion of the protein synthesis inhibitor in the induction medium leads to the enhanced production of STAT1* after 2 hours (Figure 7.1B) and is accompanied by the loss of both STAT1 α and β , which is almost complete by 3 hours. These results suggest that STAT1* is derived from STAT1 α and β by post-translational modification. The Western blot in Figure 7.1B was stripped and reprobed with monoclonal antibodies specific for the highly related STAT family members, STAT2 and STAT3 (Figure 7.1B, lower panels) and STAT6 (data not shown), without seeing equivalent events.

Figure 7.1 A post-translationally modified form of STAT1 (STAT1*) is generated in response to apoptosis

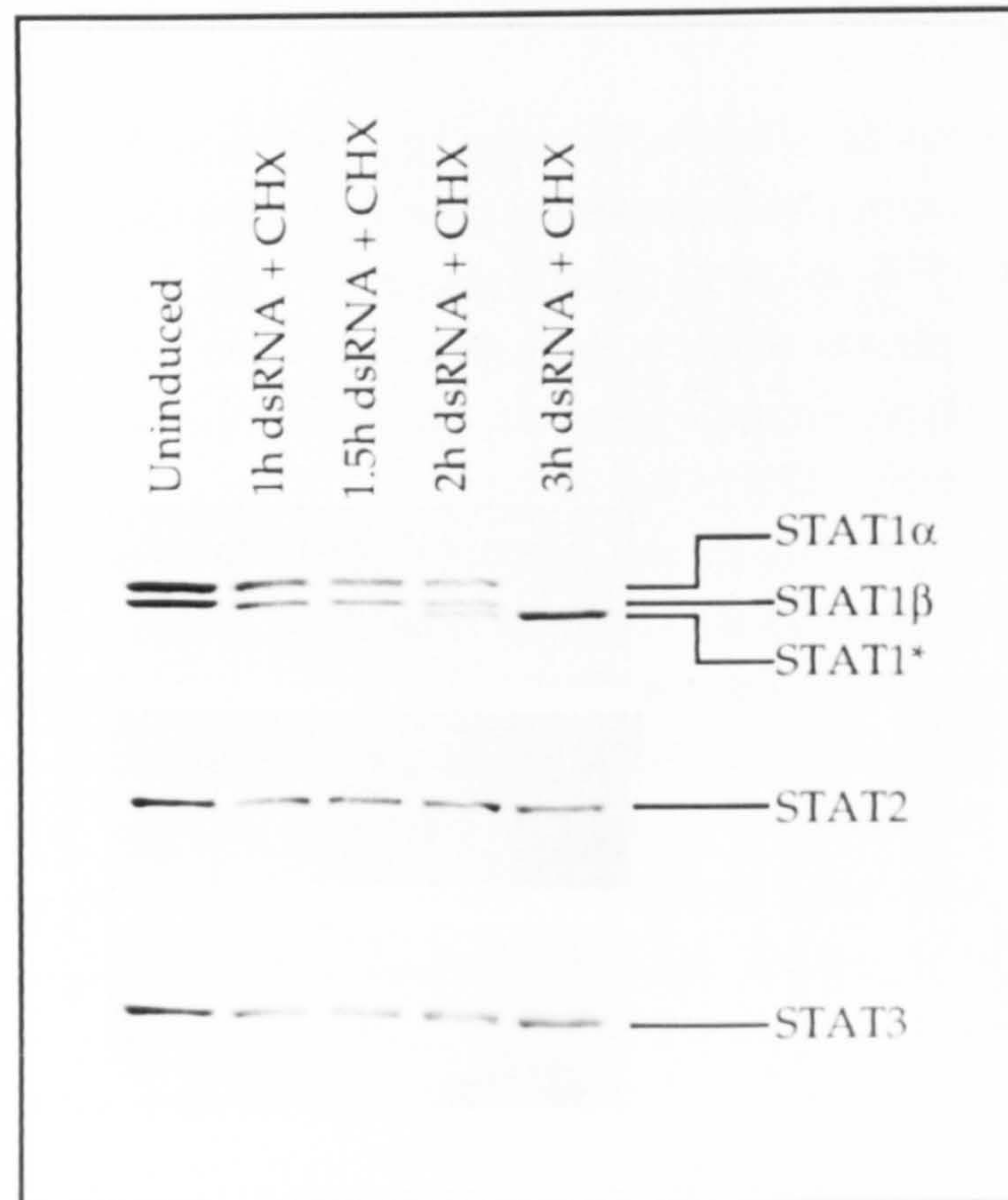
(A). Primed HeLaE cells were treated with dsRNA for the lengths of time indicated and whole cell extracts were analysed for STAT1 by Western blotting using a polyclonal antibody raised against the N-terminal 194 amino acids of human STAT1 (Transduction Laboratories). In addition to STAT1 α and STAT1 β , a novel form of STAT1 (STAT1*) is detected in dsRNA-treated cells, and the position of these factors is indicated to the right of the panel. The position of standard size markers is indicated to the left of the panel.

(B). Primed HeLaE cells were treated with dsRNA and cycloheximide (CHX) for the length of time indicated. Extracts from these cells were analysed as above for STAT1 (top panel), and the blot was stripped and reprobbed for STAT2 (middle panel) and then STAT3 (lower panel) using the polyclonal antibody for STAT1 described above and monoclonal antibodies for STATs 2 and 3 (raised against the N-terminal 178 and 175 amino acids, respectively, Transduction Laboratories).

A



B



Treatment of HeLaE cells with dsRNA causes apoptosis, and this is enhanced by cycloheximide

It had been observed throughout the course of the work presented in this Thesis that treatment of HeLaE cells with dsRNA and cycloheximide causes a significant amount of cell death within two hours, as judged by the rounding up and detachment of the cells from the tissue culture dish (data not shown), and it was possible that this may be the result of activating apoptosis. Apoptosis is the process of programmed cell death, and is a common occurrence in embryogenesis and in the destruction of infected, damaged or senescent cells (reviewed in (Fraser and Evan 1996, Takahashi and Earnshaw 1996)).

The biochemical processes involved in apoptosis can be divided into two discrete phases. A variable period of time elapses following exposure to apoptosis-inducing signals before cells become committed to undergo apoptosis. This phase is followed by the "execution" phase (more correctly a suicide phase, since apoptosis is cell autonomous) which, unlike the commitment phase, is irreversible. The execution phase is the best understood, and is characterised by changes in the cytoplasmic membrane, breakdown of the nuclear envelope, condensation of chromatin structure and eventual destruction of the chromatin, which is indicated by the appearance of a characteristic nucleosomal ladder of genomic DNA which leaks into the cytoplasm.

Execution is associated with the activation of a family of cysteine-proteases, including CPP32, which are related to the enzyme required to process interleukin-1 β (IL-1 β) from its precursor form (IL-1 β Converting Enzyme, or ICE). It is not clear how this protease cascade is activated, although studies on the Fas and TNF receptors indicate that upon activation the receptors recruit adaptor molecules through cytoplasmic "death domains" (Itoh and Nagata 1993, Tartaglia et al. 1993, Boldin et al. 1995, Chinnaiyan et al. 1995) which in turn recruit additional molecules containing ICE-like protease motifs (Boldin et al. 1996, Muzio et al. 1996, Duan and Dixit 1997). Once activated these molecules activate the downstream ICE-like protease cascade. It is not clear whether any single ICE-like protease (or Caspase) can cause cell death, since the ICE-like proteases appear to be arranged in a complex network. Furthermore, to date, although several substrates have been identified for ICE-like proteases, no single substrate cleavage has been shown to be essential for apoptosis, and it has been postulated that cell death occurs as a consequence of accumulated protein damage (Martin and Green 1995).

In addition to activation through occupation of surface receptors such as Fas

or TNF, apoptosis can also be induced by intracellular signals such as DNA damage, inhibition of protein synthesis and viral infection. Apoptosis of virally infected cells is thought to be important since it allows macrophages to destroy a cell before the completion of the viral life cycle and the release of infectious particles (Savill et al. 1993). The significance of this is reflected by the fact that many viruses encode specific inhibitors of ICE-like proteases (Ray et al. 1992, Clem and Miller 1994, Tewari and Dixit 1995, Bump et al. 1995). One of the best viral inducers of apoptosis is influenza (Hinshaw et al. 1994, Mori et al. 1995, Takizawa et al. 1993), and it has been proposed that the dsRNA generated during viral replication is central to this process (Takizawa et al. 1995, Takizawa et al. 1996).

Because of the apparent role of dsRNA in induction of apoptosis by influenza virus, it was an intriguing possibility that dsRNA treatment of HeLaE cells was activating apoptosis, and to investigate whether the presumed cell death observed with the inclusion of cycloheximide in the induction medium was indeed a consequence of this, DNA was prepared from the cytoplasm of induced cells. Figure 7.2A shows that a nucleosomal ladder characteristic of cells undergoing apoptosis is produced in response to dsRNA, and the degree of laddering is enhanced by the addition of cycloheximide. Inclusion of the ICE-like protease inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD) (Fearnhead et al. 1995) in the induction mix prevented DNA laddering and completely blocked the observed alteration of cell morphology (data not shown).

The production of STAT1* can be blocked by ZVAD

The production of STAT1* occurs with similar kinetics to the activation of apoptosis, and thus it was possible that STAT1* generation was associated with the activation of ICE-like proteases. Figure 7.2B shows that STAT1* generation can be completely blocked by treating cells with ZVAD, whereas the serine protease inhibitor, TPCK, is unable to affect cleavage. By contrast, it has been previously demonstrated that TPCK completely inhibited the dsRNA-dependent activation of NF- κ B under the same conditions (Mellits et al. 1993). To test whether STAT1* could be generated by other inducers of apoptosis, HeLaE cells were treated for 18 hours with the DNA topoisomerase II inhibitor, etoposide (Walker et al. 1991). This treatment also results in the production of STAT1*, although not as efficiently as dsRNA and cycloheximide (Figure 7.2B), and it also seems to lead preferentially to the loss of STAT1 β . Taken together, these results indicate that the dsRNA/cycloheximide induction mix triggers apoptosis in HeLaE cells and that

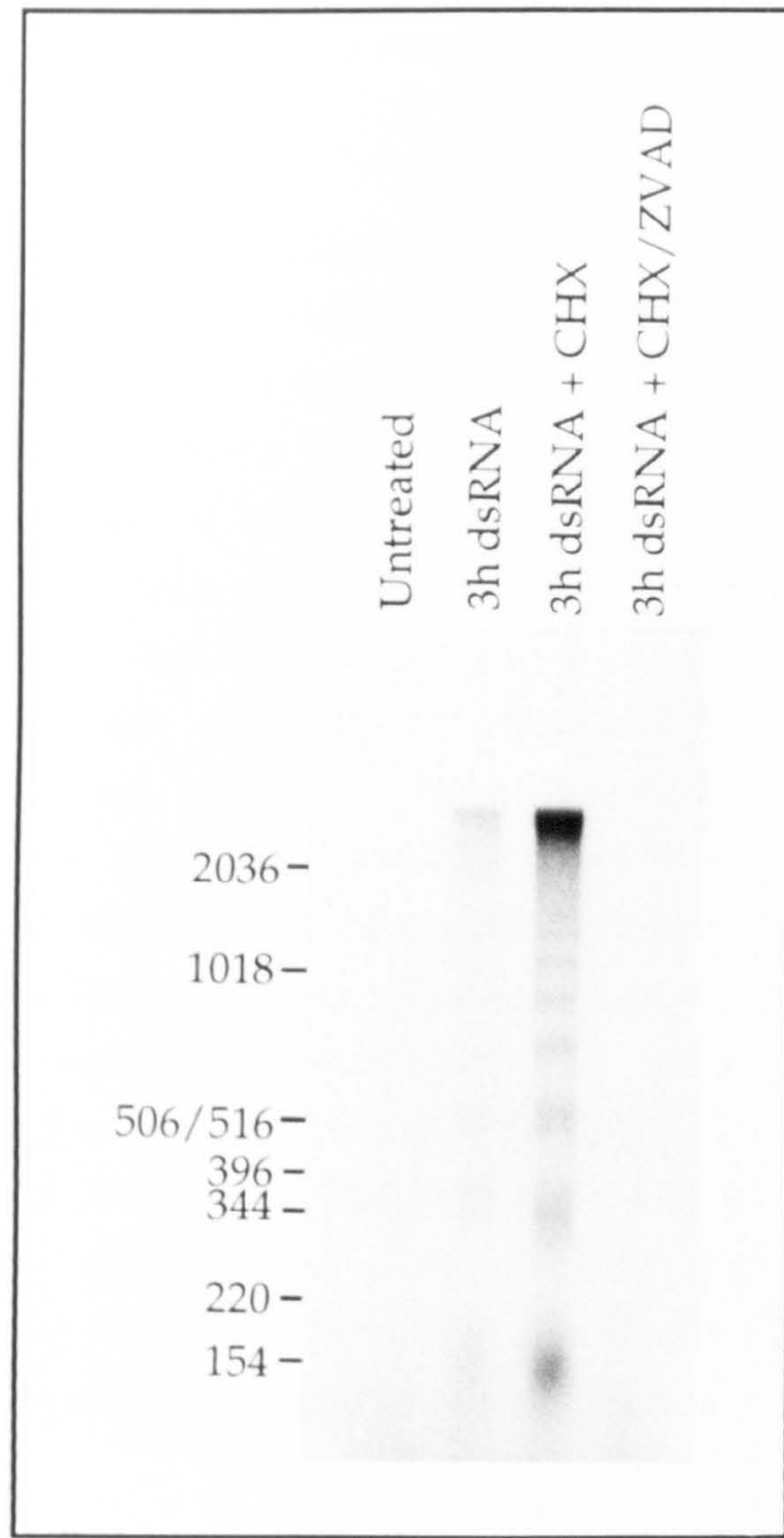
Figure 7.2 Double-stranded RNA can induce apoptosis in HeLaE cells.

(A). Cytoplasmic DNA extracts were prepared from primed HeLaE cells in the normal state (Untreated) or following dsRNA treatment for 3 hours, with the inclusion of cycloheximide and ZVAD in the induction mix as indicated. One microgram of each nucleic acid sample was assayed for DNA laddering. The migration of molecular weight markers is indicated to the left of the panel and the sizes are given in base-pairs.

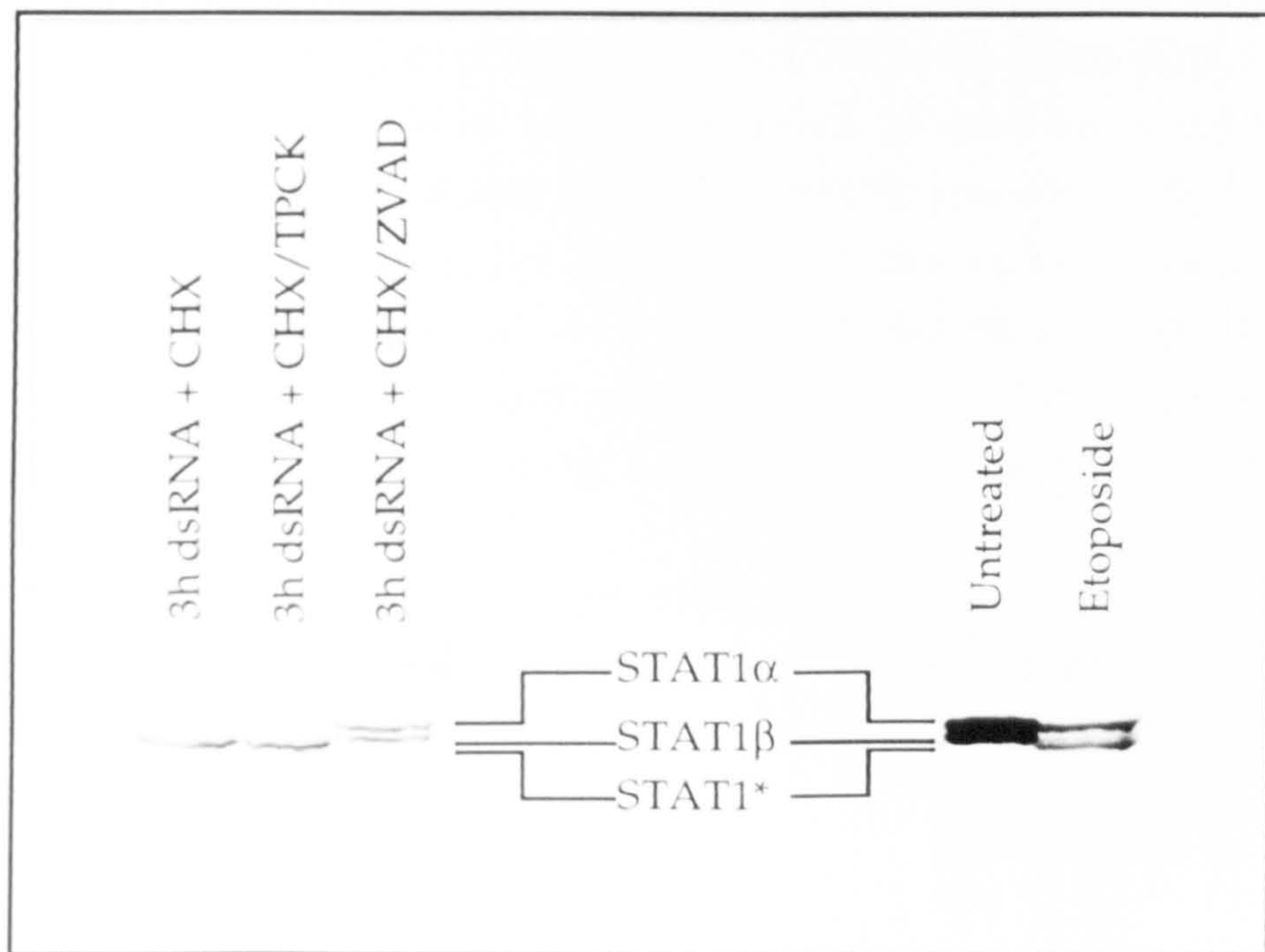
(B). Primed HeLaE cells were treated with dsRNA and CHX for 3 hours, and with the inclusion of 100 μ M ZVAD or 100 μ M TPCK in the induction mix as indicated above the left panel, and whole cell extracts were prepared and analysed by Western blotting, using the polyclonal antibody for STAT1.

In the right hand panel extracts from primed HeLaE cells and those treated with 50 μ M etoposide for 18 hours (added to the growth medium at the same time as Wellferon) were analysed by Western blotting, using the polyclonal antibody for STAT1. The positions of STAT1 α , STAT1 β and STAT1* are indicated.

A



B



during this process STAT1 α and β are converted to a shorter form, presumably by proteolytic cleavage.

STAT1 contains an ICE-like protease cleavage site

Because STAT1 α and β differ only at their C-terminus (see Introduction and Chapter Six), and are converted to a single product, it seemed likely that STAT1* is generated by a single cleavage event occurring near the C-terminus of the protein. By using a panel of antibodies raised against both the N- and C-terminal regions of the protein (Transduction Laboratories and Santa Cruz) it was confirmed that STAT1* lacks the C-terminal end of STAT1 α and β (data not shown). Since an ICE-like protease-mediated cleavage was strongly implicated by the above data, the C-terminal end of the human STAT1 protein sequence was inspected for an appropriate motif. No firm consensus has been derived for an ICE-like protease recognition site, although there is an absolute requirement for an aspartic acid residue. By comparison with the known cleavage sites of other defined substrates, such as DNA-dependent protein kinase (DNA-PK), poly-ADP-ribose polymerase (PARP) and the sterol response element binding proteins (SREBPs) (Figure 7.3), a potential cleavage site for ICE-like proteases is observed at the aspartic acid residue at position 694 which is not found in the corresponding region of the STAT family members, STATs 2 to 6 (Figure 7.3). Cleavage at this site in STAT1 would produce a species with a predicted molecular weight of 81kDa, in line with the observed size of STAT1*.

To verify that this sequence was indeed a target for cleavage, vectors were created capable of expressing wild-type STAT1 α and mutant forms in which the aspartic acid residue at position 694 was changed to alanine (D694/A) or glutamic acid (D694/E), or the glycine residue at amino acid 695 was changed to a stop codon (G695/STOP). These proteins were expressed in an N-terminally epitope-tagged form, using an epitope from the P and V proteins of simian virus 5 (SV5) (Hanke et al. 1992) (Figure 7.4). The expression plasmids for the epitope-tagged forms of STAT1 α were transfected into HeLaE cells using Lipofectamine, and the cells were primed and treated with dsRNA and cycloheximide for 3 hours. Western blot analysis of the whole cell extracts, using an anti-SV5-tag monoclonal antibody, is shown in Figure 7.4B.

When the epitope-tagged wild-type form of STAT1 α is expressed in HeLaE cells, a protein with mobility similar to that of the endogenous form of STAT1 α (91kDa) can be observed (Figure 7.4B) and interestingly, a small amount of a protein

Figure 7.3 STAT1 contains a potential cleavage site for an ICE-like protease.

Amino acid sequences of the C-terminal regions of STAT proteins are shown in the one letter code. The proteins are aligned with respect to the C-terminal signal-activated tyrosine residue (*). Amino acid residues in shaded boxes indicate identity with human STAT1. The aspartic acid at amino acid 694 in STAT1 (arrow) resides within a consensus motif for ICE-like proteases as indicated in the lower alignment with the identified cleavage sites of the known substrates PARP, DNA-PK, SREBP1a, SREBP2 (CPP32 substrates) Pro-IL-1 β and Pro-IGIF (ICE substrates) (Lazebnik et al. 1994, Song et al. 1996, Sleath et al. 1990, Howard et al. 1991, Gu et al. 1997, Wang et al. 1996).



*

YYSRPK	EAPEMELD	GPKGTGYIKTEL	STAT1
YYQ	EKVNIQERR..KYLK	STAT2
YC	..R	ESQEHPEADPGS	STAT3
YS	QPC	EVSRTE.RGDK..GYVPSVF	mSTAT4
YYT	..PVPC	..ESATLAKAVD..GYVKPQI	STAT5B
EAFR	SHYKPE	EQMGKDG...R	STAT6



CPP32	PMELD	GPKG	STAT1
substrates	GDEVD	GIDE	PARP
	GDEVD	NKVK	DNA-PK
	DSEPD	SEVF	SREBP1a
	KDEPD	SPPV	SREBP2
ICE	FFEAD	GPKQ	Pro-IL-1 β (minor)
substrates	AYVHD	AEVR	Pro-IL-1 β (major)
	DLES	DNFGR	Pro-IGIF

Figure 7.4 The aspartic acid residue at position 694 is required for STAT1* production

(A). pEFSTAT1 α SV5 was created by epitope tagging STAT1 α at the N-terminus in pEFSTAT1 α using oligonucleotides encoding the amino acids 95-108 (GKPIP α NPLLGLDST) of the P and V proteins of simian virus 5. The single codon changes to encode alanine or glutamic acid at aspartic acid 694 or to a stop codon at glycine 695 were introduced into pEFSTAT1 α by recombinant PCR, and verified by sequencing. This created the vectors pEFSTAT1 α (D694/A), pEFSTAT1 α (D694/E) and pEFSTAT1 α (G695/STOP), respectively. The amino acid sequences around the potential ICE-like protease site are shown for each protein.

B). HeLaE cells were transiently transfected, using lipofectamine, with pEFSTAT1 α SV5, pEFSTAT1 α (D694/A), pEFSTAT1 α (D694/E) and pEFSTAT1 α (G695/STOP). The cells were primed and uninduced (-) or induced (+) for 3 hours with dsRNA and cycloheximide, and the whole cell extracts were analysed by Western blotting, using an SV5 tag-specific monoclonal antibody (MAbSV5-P-k (Hanke et al. 1992) a kind gift from R. Randall, St. Andrews). The positions of reactive products running at 91 and 81kDa are indicated. This blot was stripped and reprobed with the polyclonal anti-STAT1 antibody (lower panel). The positions of STAT1 α , STAT1 β and STAT1* are indicated.

with similar mobility to STAT1* (81kDa) can also be observed in untreated cells. Some noticeable cell death was routinely caused by the Lipofectamine transfection procedure (data not shown), suggesting that a degree of apoptosis occurs in HeLaE cells during this treatment. When cells are treated with dsRNA and cycloheximide the conversion of the 91kDa to the 81kDa truncation form is much enhanced. The 81kDa form migrates with the same mobility as a protein produced by the expression of a form of STAT1 α truncated after amino acid 694 (G695/STOP), as expected if STAT1* is generated by cleavage at this site. This protein is unaffected by induction. In contrast to the wild type form, the D694/A and D694/E mutant forms of STAT1 α are not cleaved upon induction. Also, no constitutive cleavage is observed with these two mutants. Reprobing the Western blot with the STAT1 antibody shows that the endogenous STAT1 α and β are cleaved in each case (Figure 7.4C). These results confirm the presence of an ICE-like protease cleavage site at aspartic acid 694 in STAT1, and demonstrate that this site is cleaved in both STAT1 α and β during apoptosis.

STAT1* is unable to support IFN signal transduction

Activation of STAT1 by α - and γ -IFN, by cytokines such as IL-6 and growth factors such as PDGF, involves a single tyrosine phosphorylation on amino acid 701. Once phosphorylated, STAT1 can homo- or heterodimerise to produce complexes capable of binding to DNA and activating transcription (see Chapter One and Chapter Six). Clearly, cleavage of STAT1 after amino acid 694 would produce a truncated form of the protein that cannot be phosphorylated on tyrosine 701, and thus should not be activated by α - or γ -IFN. To test this, the ability of STAT1* to rescue responses to α - and γ -IFN in U3 cells (which lack functional STAT1) was examined by cotransfecting the cells with a multimerised 9-27 gene (Reid et al. 1989) ISRE reporter construct, or pIRF[GAS]₂lucifer, respectively, and the wild-type and mutant forms of STAT1 α . In contrast to wild-type STAT1 α , the truncated form (G695/STOP) is completely unable to rescue either α -IFN (Figure 7.5A) or γ -IFN (Figure 7.5B) responses. Altering amino acid 694 from an aspartic acid to an alanine (D694/A) does not interfere with either signal transduction pathway (Figures 7.5A and 7.5B). These results demonstrate that the effect of the proteolytic cleavage of STAT1 during apoptosis would be to impair the ability of cells undergoing apoptosis to respond to a variety of cytokine- and growth factor-mediated signals, and possibly dsRNA-mediated signals, as discussed in Chapter Six (see below).

Figure 7.5 STAT1* is unable to sustain signal transduction responses to type I or type II IFN.

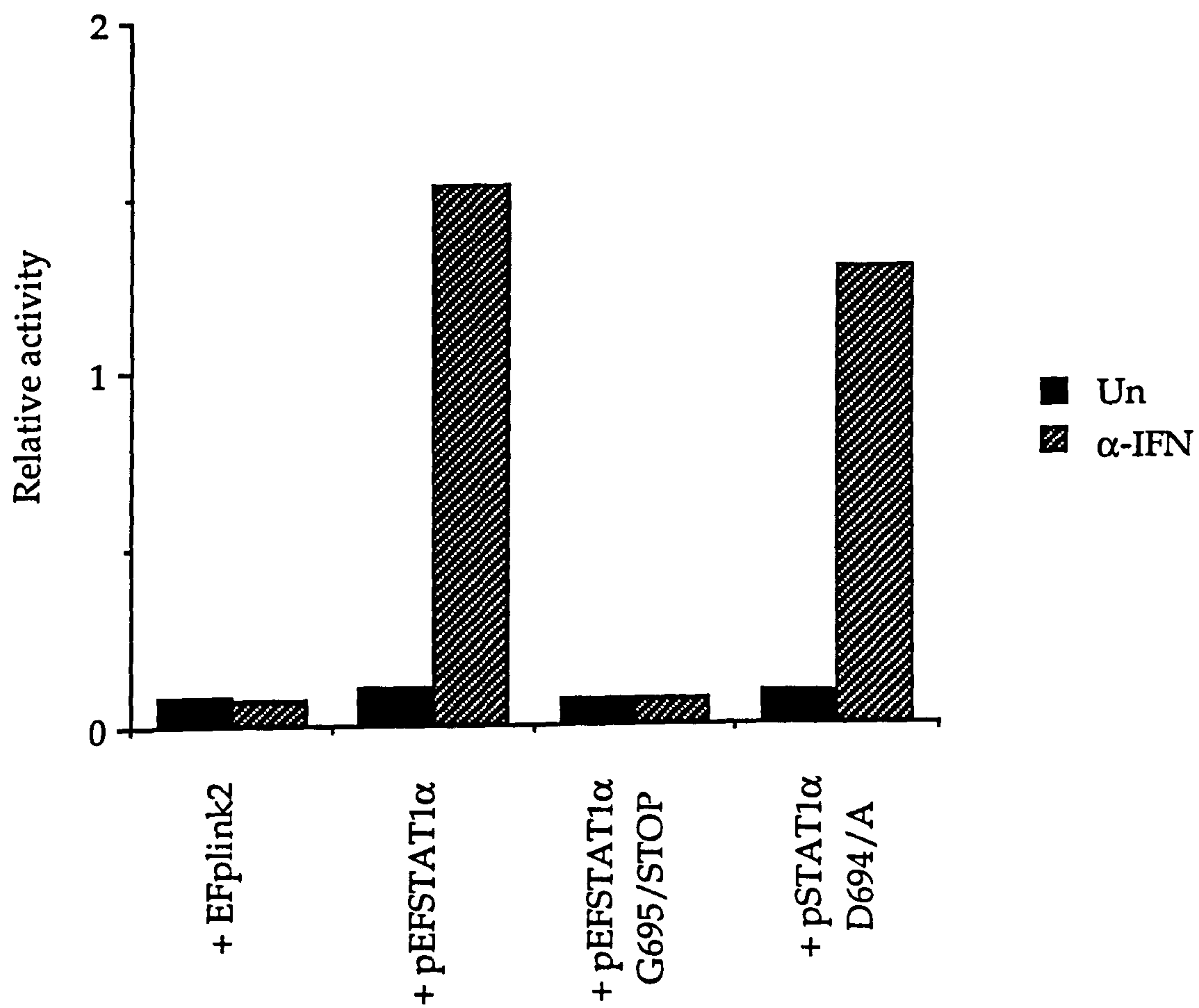
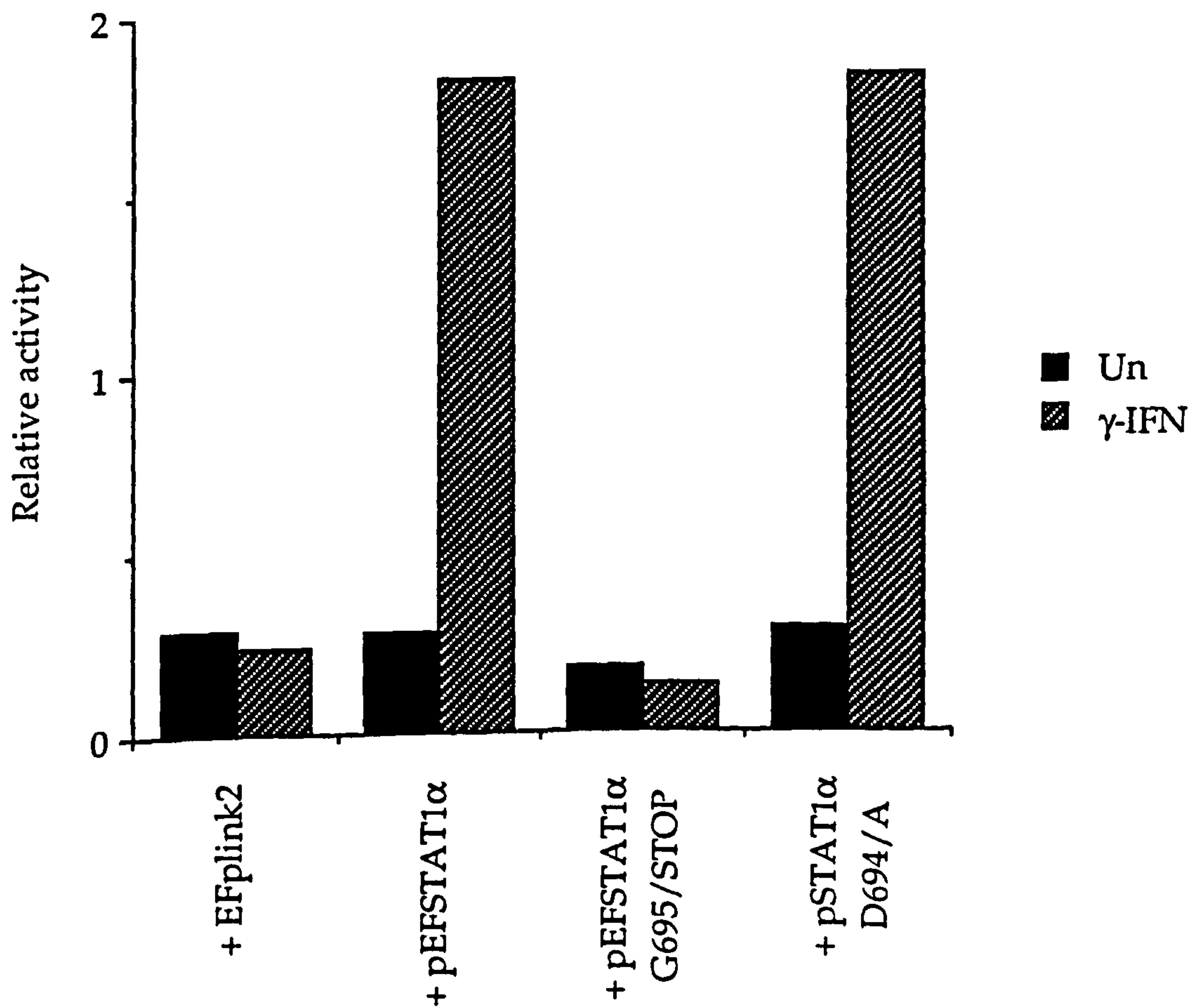
U3 cells were transiently transfected, using calcium phosphate precipitation, with pEFplink2 (vector), pEFSTAT1 α (wild-type), pEFSTAT1 α (G695/STOP) (G695/STOP), or pEFSTAT1 α (D694/A) (D694/A), pSV₂CAT and the reporter constructs:

- (A) p9-27[ISRE]₄tk Δ [-39]lucifer (4 copies of 9-27 gene core ISRE, (Reid et al. 1989), or
- (B) pIRF-1[GAS]₂tk Δ [-39]lucifer.

Extracts were made after:

- (A) 3 hours α -IFN treatment, or
- (B) 3 hours γ -IFN treatment,

and the relative gene expression was determined by normalising the luciferase values to the CAT values.

A**B**

Inhibitor studies suggest that STAT1 is cleaved by ICE and not CPP32

As ZVAD is a broad-specificity ICE-like protease inhibitor, it was not possible to determine which protease was responsible for the cleavage of STAT1. In an effort to distinguish between ICE- and CPP32-like activities, the more specific inhibitors YVAD-cmk and DEVD-CHO (Dubrez et al. 1996) were included in dsRNA and cycloheximide inductions of HeLaE cells, and the ability of these inhibitors to block the cleavage of STAT1 was assessed by Western analysis (Figure 7.6A). Although ZVAD is the most efficient inhibitor of STAT1* production, the ICE-specific inhibitor YVAD-cmk is also capable of blocking the cleavage at the higher concentrations, and this is mirrored by its ability to block the cell death observed in the absence of inhibitor, as judged by DNA laddering (Figure 7.6B). In contrast, the CPP32-specific inhibitor, DEVD-CHO, is incapable of blocking either the proteolysis of STAT1 or the cell death unless it was included at the highest concentrations, when it can be seen to inhibit both very slightly. It was, however, capable of blocking the cleavage of PARP by etoposide treatment (data not shown).

IRF-2 is cleaved by an ICE-like protease

It had been previously demonstrated that proteolysis is involved in both the activation of NF- κ B and the production of a post-induction repressor from full length IRF-2 during dsRNA induction (see Chapter One). The proteolytic degradation of I κ B following dsRNA treatment proceeds via a ubiquitin-mediated proteasomal pathway (Chen et al. 1995). It was shown by inhibitor studies, however, that the proteolysis of IRF-2 was mediated by a different mechanism to I κ B (Whiteside et al. 1994), and in light of the discovery of the activation of ICE-like proteases by dsRNA, the region of IRF-2 between amino acids 163 and 181, to which the site of proteolysis had been roughly mapped, was inspected for potential ICE-like protease sites. This inspection revealed the amino acid sequence glutamic acid-valine-aspartic acid-serine (EVDS), a good candidate for a cleavage site (see Figure 7.3).

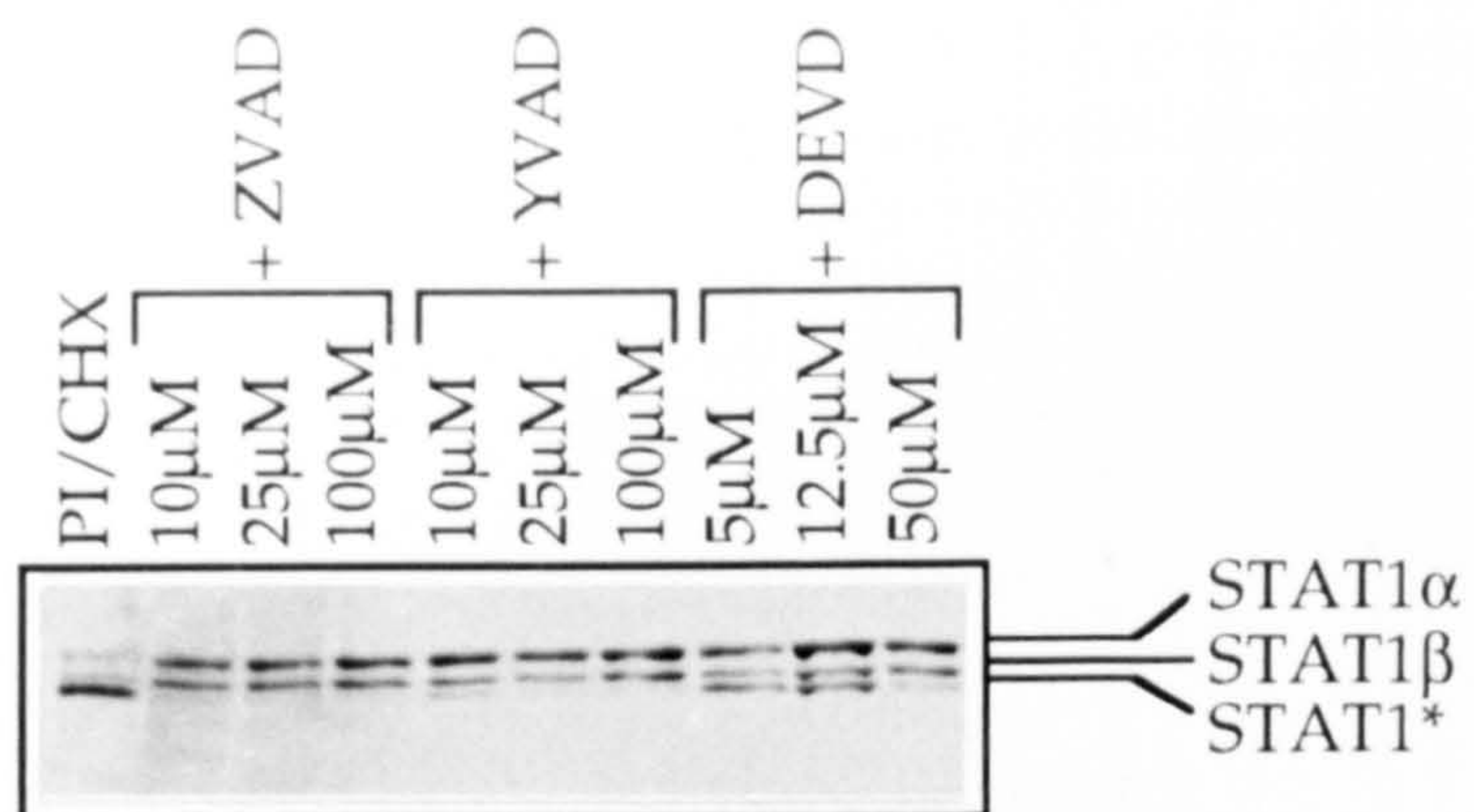
SV5 epitope-tagged forms of full length IRF-2 and the Δ 163 C-terminal truncation product of IRF-2, which had been demonstrated to migrate similarly to IRF-2* (Whiteside et al. 1994), were introduced into HeLaE cells by Lipofectamine transfection, and the primed cells were induced with dsRNA and cycloheximide for 2 and 4 hours, with and without the inclusion of ZVAD. Western analysis of the whole cell extracts from this induction (Figure 7.7B) shows that, as was the case in Figure 7.4, there appears to be considerable proteolysis in the absence of induction

Figure 7.6 The effect of inhibitors of ICE-like proteases on STAT1* production

(A) Primed HeLaE cells were treated for 3 hours with dsRNA and cycloheximide, in the presence of varying concentrations of the ICE-like protease inhibitors ZVAD, YVAD-cmk and DEVD-CHO, as indicated. The cells were harvested and the nuclear extracts were analysed for STAT1 by Western blotting using the STAT1 polyclonal antibody. The positions of STAT1 α , STAT1 β and STAT1* are indicated to the right of the panel.

(B). Nucleosomal DNA was isolated from the cytoplasm extract of the cells harvested in A, and 1 μ g of this was end-labelled with of $\alpha^{32}\text{P}$ -dATP and electrophoresed on a 1.8% agarose gel. The autoradiograph of the gel is shown.

A



B

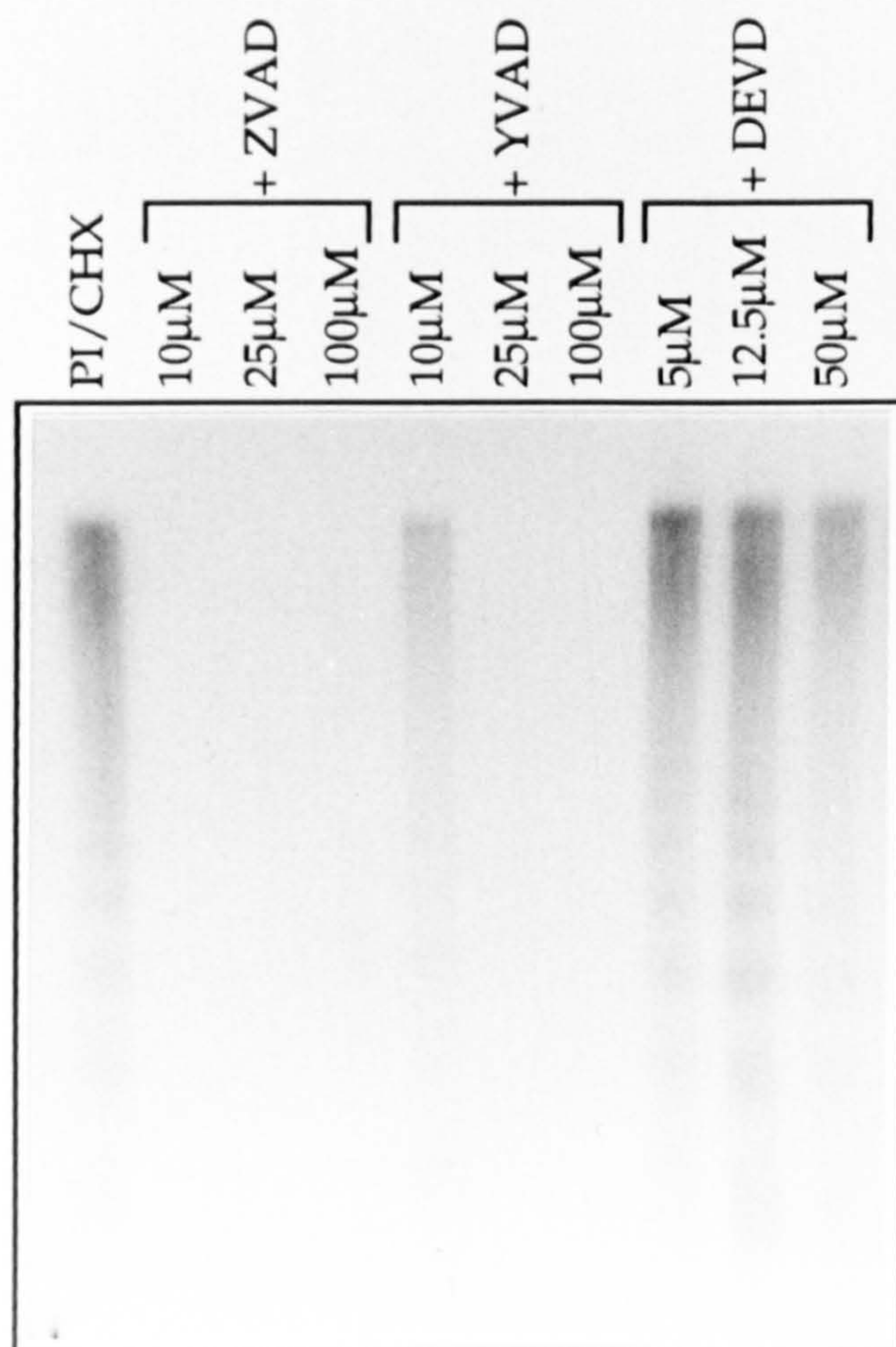


Figure 7.7 IRF-2 is cleaved by an ICE-like protease

(A). The amino acid sequence of human IRF-2 between residues 163 and 182 is shown in the one letter code. The potential ICE-like protease site EVDS is underlined.

(B). A cDNA for IRF-2 was inserted into pEFSV5plink2 to create pEFIRF-2SV5, capable of expressing N-terminally SV5 epitope-tagged IRF-2. This vector was transiently transfected, using Lipofectamine, into HeLaE cells. The cells were primed, and uninduced or induced with dsRNA for 2 or 4 hours (PI/CHX 2, 4hrs) as indicated, in the absence or presence of 100 μ M ZVAD. Whole cell extracts were prepared and analysed by Western blotting, using the anti-SV5-tag monoclonal antibody described in Figure 7.4B. An untransfected primed HeLaE cell extract was included (left hand lane) as was an extract from cells transfected with pEFIRF-2 Δ 163 (right hand lane), which expresses an SV5 epitope-tagged form of IRF-2 Δ 163, a size marker for the breakdown product of IRF-2 observed during induction, IRF-2*. The positions of the antibody reactive IRF-2 and IRF-2* are indicated to the side of the panel.

leading to the formation of a breakdown product which migrates with very similar mobility to the $\Delta 163$ form of IRF-2. This constitutive proteolysis is again presumably caused by the Lipofectamine transfection method, but in this case induction does not seem capable of enhancing this. However, the inclusion of ZVAD in the induction medium completely blocks this proteolysis, indicating that the production of IRF-2* is caused by an ICE-like protease which is likely to cleave the full length protein after the aspartic acid residue at position 170.

ZVAD superinduces β -IFN induction

The discovery that an ICE-like protease(s) is activated by dsRNA treatment of HeLaE cells, resulting in the cleavage of at least two factors that have been implicated in the regulation of β -IFN induction, made it of considerable interest to discover the contribution of ICE-like proteases to the extent of induction. Figure 7.8 shows the results of experiments to investigate the effect of ZVAD on the induction of the β -IFN gene in HeLaE cells. In Figure 7.8A HeLaE cells were induced for three hours with dsRNA, with and without cycloheximide, and with and without the inclusion of ZVAD in the induction medium, and the RNA from these cells was analysed by RNase mapping. In the presence of ZVAD a dramatic superinduction of β -IFN mRNA is observed (lanes 10-12). Figure 7.8B demonstrates that in contrast to the superinduction achieved by the inclusion of ZVAD in the induction medium, the serine protease inhibitor, TPCK, completely blocks β -IFN induction, presumably as a consequence of its ability to inhibit NF- κ B induction (Mellits et al. 1993).

Discussion

The experiments performed in this Chapter have demonstrated that treatment of HeLaE cells with dsRNA activates apoptosis, as judged by the breakdown of the chromatin into nucleosomes, and that this can be enhanced by the addition of cycloheximide. As a consequence of apoptosis an ICE-like protease(s) is activated and two new substrates for cleavage by this class of protease have been defined, namely STAT1 and IRF-2. The cleavage of STAT1 during apoptosis is clearly a distinct phenomenon from that in which γ -IFN-activated STAT1 is cleaved by the ubiquitin-dependent proteasome pathway (Kim and Maniatis 1996).

The most effective protease inhibitor used in these studies is ZVAD, which is a general ICE-like protease inhibitor (Fearnhead et al. 1995), and it has yet to be determined which specific member(s) of the ICE-like protease family is involved in generating STAT1* and IRF-2*. The relative inhibitory activities of YVAD-cmk and

Figure 7.8 ZVAD can superinduce the β -IFN gene

(A). HeLaE cells were treated as follows:

uninduced (Un, lanes 1 and 7)

induced with dsRNA for 3 hours (I, lanes 2 and 8)

induced in the presence of cycloheximide for 3 hours (I/CHX, lanes 3 and 9)

primed, induced for 3 hours (PI, lanes 4 and 10)

primed, induced in the presence of cycloheximide for 2 hours (PI/CHX 2hrs, lanes 5 and 11)

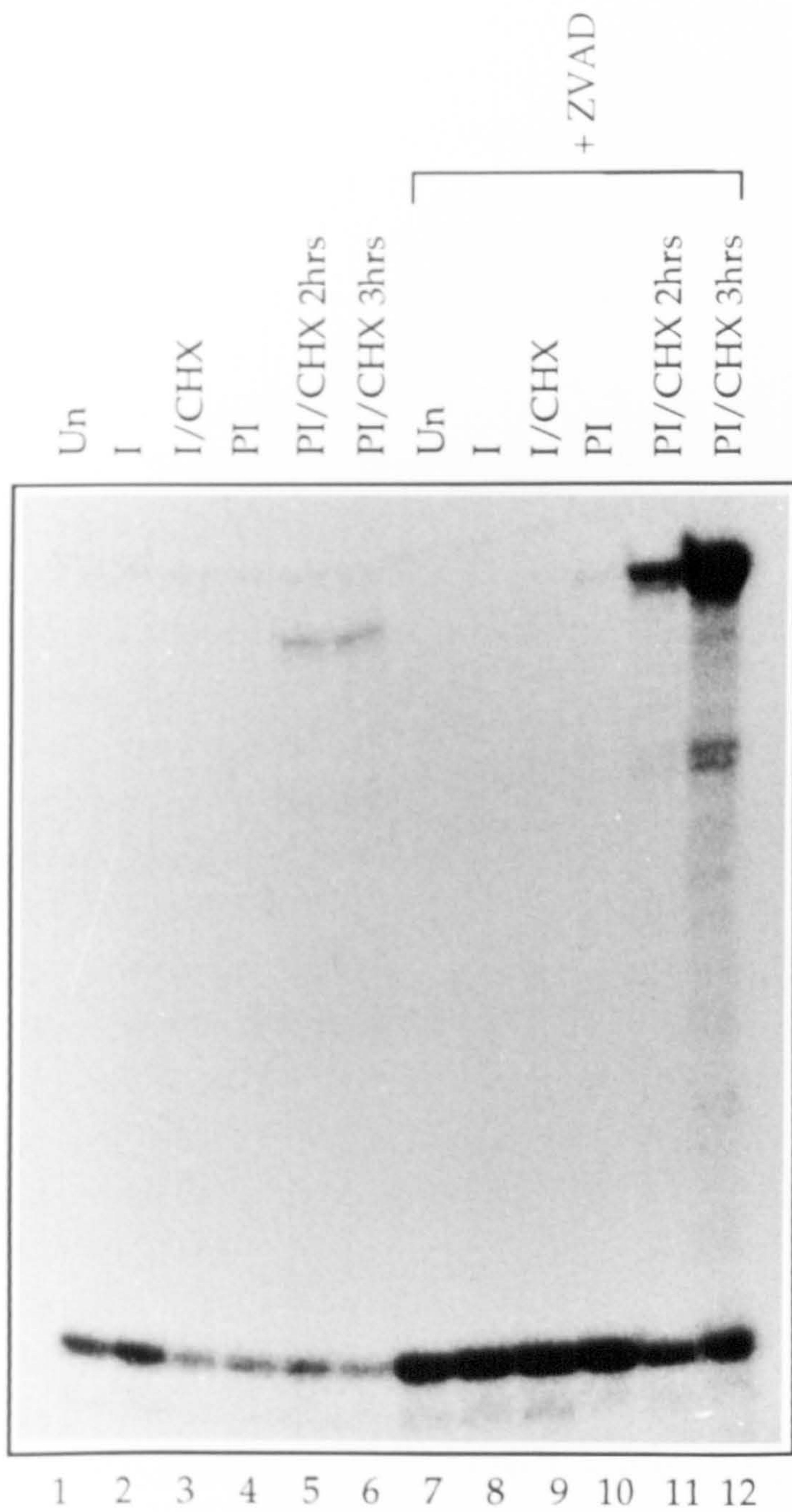
primed, induced in the presence of cycloheximide for 3 hours (PI/CHX 3hrs, lanes 6 and 12)

in the absence (lanes 1-6) or presence (lanes 7-12) of 100 μ M ZVAD.

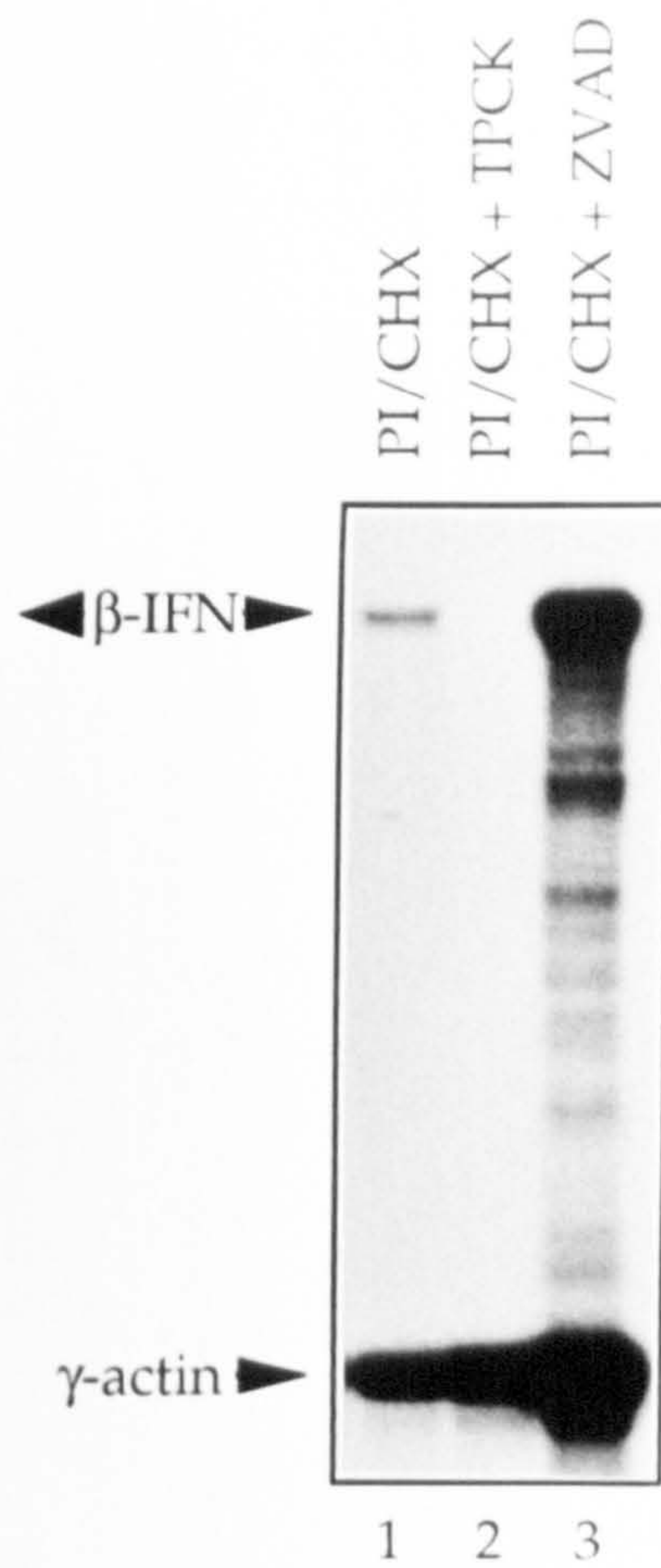
(B). Primed HeLaE cells were treated for 3 hours with dsRNA and cycloheximide (PI/CHX, lane 1) and with the addition of 100 μ M TPCK (PI/CHX + TPCK, lane 2) or 100 μ M ZVAD (PI/CHX + ZVAD, lane 3)

In both experiments, cells were harvested and RNA was prepared and analysed by RNase mapping, using probes for β -IFN and γ -actin. The positions of the protected fragments are indicated.

A



B



DEVD-CHO suggest that the protease involved in the cleavage of STAT1 is more likely to be, or be activated by, ICE than CPP32 (Figure 7.6). In support of this, it can be seen from Figure 7.3 that although the cleavage site in STAT1 has a number of similarities to the cleavage sites in proteins that are known to be substrates for CPP32, it is most similar to a minor ICE-specific cleavage site in pro-IL-1 β , which is not cleaved by CPP32 (Nicholson et al. 1995). ICE appears to be rather promiscuous in its preferences, since the major and minor pro-IL-1 β cleavage sites (Sleath et al. 1990, Howard et al. 1991) and a recently identified site in pro-interferon- γ inducing factor (pro-IGIF) (Gu et al. 1997) show little similarity. Neither CPP32 nor ICE can cleave nuclear lamins, and a third member of the ICE-like protease family (Mch2) is required for this function (Orth et al. 1996). The cleavage sites in these proteins are unknown.

These observations indicate that different members of the ICE-like protease family have distinct specificities, although no general rules have yet been established. It is interesting that STAT1 does not appear to be cleaved at amino acid 143 (data not shown), despite having an ELDS motif here which is clearly very similar to the proposed site in IRF-2. Although the ELDS motif may not be an ICE-like protease recognition sequence, it is equally possible that cleavage by ICE-like proteases is restricted by the domain structure of some substrates with the result that the recognition sequence is inaccessible. Etoposide treatment may activate apoptosis by a different mechanism to dsRNA, because this agent reproducibly leads to the loss of STAT1 β in preference to STAT1 α (Figure 7.2B and data not shown). Etoposide-mediated apoptosis is thought to proceed via a CPP32-dependent, DEVD-CHO sensitive pathway (Dubrez et al. 1996), which may suggest that the EVDG site in STAT1 is a substrate for a protease in this pathway in the context of STAT1 β but not STAT1 α . Alternatively, the long-term treatment required to achieve significant apoptosis with etoposide may cause a down-regulation of STAT1 β by another mechanism, for example by altering the ratio of splicing of the STAT1 transcript in favour of STAT1 α . This remains to be determined.

The C-terminal cleavage product of STAT1 has lost the tyrosine residue at position 701 and thus cannot be activated by α - or γ -IFN as shown here, or presumably by the other ligands that activate by promoting this phosphorylation. Cleavage of STAT1 would result in the shut off during apoptosis of JAK/STAT pathways in which STAT1 is necessary for signal transduction. If the truncated form of STAT1 is able to act as a dominant negative form of the molecule, as has been reported for an artificially derived truncation product of STAT5 (Mui et al. 1996), it

is possible that the cleavage could also inactivate other JAK/STAT pathways which involve STAT1, although preliminary observations of the role of the G695/STOP mutant in HeLaE cells suggests do not provide evidence for this, at least in the case of IFN induction.

To date, none of the specific cleavages by ICE-like proteases has been demonstrated to be pivotal in the induction of apoptosis, and it is probable that cell death is brought about by an accumulation of protein damage (Martin and Green 1995, Fraser and Evan 1996, Takahashi and Earnshaw 1996). The cleavage of DNA-PK, PARP and nuclear lamins is probably involved in the mechanism by which the cell nucleus is broken down, but the reason for cleavage of the other identified ICE-like protease substrates in apoptosis is unclear. It is interesting to speculate as to what the physiological relevance of the cleavage of STAT1 in response to apoptotic signals would be. It has previously been observed that many cell types, including fibroblast (Harrington et al. 1994) and haematopoietic cells (Lotem and Sachs 1996), can undergo apoptosis as a result of becoming depleted of growth factors or cytokines and can be rescued by the addition of defined survival factors, which include the STAT1 activators PDGF (Harrington et al. 1994), CSF (Williams et al. 1990), and γ -IFN (Lotem and Sachs 1995). One consequence of apoptosis, therefore would be to downregulate the ability of a cell to respond to such factors. However, since multiple growth factors can protect cells against apoptosis, it is probably unlikely that STAT1 cleavage alone would be sufficient to render cells insensitive to protection by survival factors. In support of this theory, preliminary experiments indicate that overexpression of the uncleavable forms of STAT1 in HeLaE cells cannot protect them from dsRNA-induced apoptosis (data not shown). It should also be remembered that STAT1-negative cells (U3) and STAT1-knockout animals (Durbin et al. 1996, Meraz et al. 1996) are perfectly viable. Nevertheless, inactivation of STAT1 function may be an example of a class of modifications that ensures that the execution phase of apoptosis is irreversible. Further study of the contribution of STAT1 towards survival in γ -IFN-dependent cell lines is warranted.

Inclusion of ZVAD in the induction mix causes a marked superinduction of β -IFN mRNA, even above the already superinduced levels caused by cycloheximide. It has been observed that γ -actin mRNA is more stable than β -IFN mRNA during dsRNA and cycloheximide inductions (S. Goodbourn, pers. comm.), and a trivial explanation for the superinduction caused by ZVAD is that it is a consequence of its ability to keep the cells alive, and thus protect the β -IFN mRNA from degradation. However, given the potential positive and negative roles played by STAT1, and IRF-

2*, respectively, in β -IFN induction, it is also possible that ZVAD, by inhibiting an ICE-like protease(s), can superinduce the β -IFN gene by stabilising STAT1, or blocking the production of IRF-2*, or both.

This may in fact indicate the relevance of both STAT1 and IRF-2 cleavage during apoptosis. If viral infection activates apoptosis, the infected cells will be cleared by phagocytosis thus preventing the exposure of neighbouring cells to newly synthesised virions. In such a circumstance IFN synthesis would be unnecessary for the prevention of viral infection, and the downregulation of β -IFN induction by the cleavage and inactivation of STAT1 and the formation of a post-induction repressor, IRF-2*, would ensure that neighbouring cells were exposed to minimal amounts of IFN. However, if viral infection does not lead to apoptosis, for example if the infected cell type lacks a component of the apoptotic pathway or the virus encodes an inhibitor of ICE-like proteases, then IFN synthesis would be desirable to limit the spread of infection. In this regard, it is interesting that MG63 cells, which are much more efficient producers of β -IFN in response to dsRNA treatment than HeLaE cells, undergo apoptosis much less readily during induction (data not shown).

It may be possible to reproduce the superinduction observed by the inclusion of ZVAD in the induction mix by overexpressing the uncleavable forms of STAT1 in HeLaE cells. Similar experiments involving the overexpression of uncleavable forms of IRF-2 may not be as successful, because the endogenous IRF-2 would still be cleaved, and because of its much more stable DNA-binding properties, IRF-2* may not be competed out by the mutant forms of the protein. However, cell lines derived from IRF-2 "knockout" mice may become available for study, allowing the analysis of the cleaved and uncleavable forms of IRF-2 in induction. In support of a role for IRF-2* in post-induction repression of the β -IFN promoter, it was noted that the induction of the β -IFN gene is not only intensified in the presence of ZVAD, but is prolonged as well (data not shown).

The induction of apoptosis by dsRNA is consistent with previous observations that infection of cells by certain viruses also leads to apoptosis (Takizawa et al. 1993). It would appear that the proximal inducer of apoptosis during infection is, like the requirement for the induction of β -IFN, dsRNA, presumably formed during viral transcription or replication. The mechanism by which this proceeds is unclear, but as for β -IFN induction, there is evidence to implicate PKR in this process.

Several studies have demonstrated a role for PKR in apoptosis. It has been shown that the overexpression of PKR in HeLa cells (Lee and Esteban 1994) and

U937 cells (Yeung et al. 1996) can cause apoptosis, and that antisense and dominant interfering mutant expression experiments with PKR constructs have blocked apoptosis in a number of systems, including the induction of apoptosis in HeLa cells by dsRNA (Takizawa et al. 1996, Yeung et al. 1996). The apoptotic activity of IRF-1 may well be a consequence of its ability to induce PKR, as well as ICE (Tanaka et al. 1994, Tamura et al. 1995) (Kirchhoff et al. 1995) and a recent report has shown that dsRNA-induced apoptosis is defective in MEFs from PKR "knockout" mice (Der et al. 1997). Many of these studies require the incubation of the cells with the inducer of apoptosis for 18 hours or more, and the involvement of PKR may reflect the requirement for signal transduction of the dsRNA signal and expression of inducible "death genes". For example, it has been reported that dsRNA treatment can upregulate the synthesis of Fas ligand via the activation of the transcription factor NFIL-6 (Wada et al. 1995), and that this can be blocked by dominant interfering mutants of PKR (Takizawa et al. 1996). However, it is clear from the experiments in this Chapter that in HeLaE cells, like the induction of β -IFN, dsRNA can induce apoptosis in a rapid and protein synthesis-independent manner, and it would be illuminating to assess the role of PKR in this system.

Finally, it may be possible that dsRNA can activate ICE-like proteases without the concomitant activation of apoptosis. IRF-2* has also been observed in MG63 cells (Palombella and Maniatis 1992) under conditions in which it has not been able to observe significant cell death or STAT1 cleavage (data not shown), and the majority of cell lines in which β -IFN induction has been studied show normal post-induction repression kinetics but do not appear to be killed during induction. It has been reported that IRF-2 contains a latent activation domain, and also that IRF-2 can function as a transcriptional activator of the histone H4 gene FO108 (see Chapter One). Although no evidence for naturally occurring, C-terminally-truncated, transcriptionally active IRF-2 was presented in either report, a 40kDa IRF-2 breakdown product containing the N-terminus has been observed during dsRNA and cycloheximide induction of HeLaE cells (S. Whiteside, PhD. Thesis, London University, 1992), whose appearance precedes that of IRF-2*. This 40kDa product should contain the transactivation domain, which has been mapped to between amino acids 163 and 241. There are potential ICE-like cleavage sites in IRF-2 at amino acids 210 and 242, which if cleaved would leave a residual transcriptionally active protein of around 40kDa. As it has been suggested that ICE-like proteases are activated sequentially, it is intriguing to speculate that sequential cleavages of IRF-2 could generate first an activator and secondly a repressor of β -IFN transcription, and

that such proteases can be active in normally growing cells without driving the cells into an apoptotic pathway. Presumably, the formation of mature protein from pre-IL-1 β and pre-IGIF by ICE-like protease cleavage can also take place under non-apoptotic conditions. These are important areas for further study of the role of ICE-like proteases in the induction of β -IFN.

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