HEPATITIS B VIRUS AND THE CELLULAR RESPONSE TO INTERFERON

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To my wife, Claire.

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ABSTRACT

The effects of the hepatitis B virus (HBV) on the cellular response to interferon (IFN) have been studied. The four HBV genes have been isolated and examined for any effects on the IFN response pathway. The polymerase gene inhibited the effects of IFN and a deletion analysis showed that this inhibition was caused by the amino terminal domain (terminal protein) of the polymerase protein. The site of action of terminal protein has been studied: it reduced the response to IFNα by inhibiting the activation of the IFN inducible transactivating factor E. The effects of terminal protein on a number of other cellular signalling pathways have also been examined and terminal protein has been shown to inhibit cellular responses to IFNγ and double stranded RNA.

The clinical significance of terminal protein's inhibitory properties has been studied by examining its expression in liver biopsies taken from patients with chronic HBV infection. Overexpression of the terminal protein of HBV was associated with a failure to respond to IFN therapy. The mechanisms responsible for this increase in the expression of terminal protein have been investigated, but no single cause for the observed increase has been found. Inhibition of the cellular response to IFN by terminal protein may play a significant role in the pathogenesis of IFN resistant chronic HBV infection.

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LIST OF ABBREVIATIONS

Common abbreviations are as recommended by the Biochemical Journal. A number of others used frequently are listed below.

2-5A	2'5' oligoadenylate synthetase
bp	base- pairs
β-gal	β-galactosidase
dsRNA	double stranded RNA
CAT	Chloramphenicol acetyl transferase
DAG	Diacylglycerol
DTT	dithiothreitol
E4	Dulbeccos modified Eagle's medium
EDTA	ethylenediamine tetra acetic acid
gpt	guanine-phosphoribosyltransferase
HAT	hypoxanthine, aminopterin, thymidine
HEPES	N-2-hydroxyethylpiperazine - N-2 - ethanesulphonic acid
HBV	Hepatitis B virus
hr	hour (s)
IFN	Interferon
ICAM-I	Intercellular adhesion molecule I
ISRE	Interferon-stimulated-response-element
kb	kilobase(s)
LB	Luria broth
min(s)	minute(s)
MHC	Major histocompatability complex
NP40	Nonidet P40
OAC	Acetate anion, CH_3COO^-
PBSA	1% NaCl, 0.025% KCl, 0.14% Na_2HPO_4, 0.025% KH_2PO4
PKC	Protein kinase C

ABBREVIATIONS (continued)

PMSF	phenylmethylsulphonylfluoride
PIPES	1, 4- piperazidinediethanesulphonic acid
sec(s)	second(s)
SDS	sodium dodecyl sulphate
SSC	0.15 M NaCl, 0.015 M Na Citrate
TAE	40 mM Tris acetate,pH 7.5, 2mM EDTA
TBE	90 mM Tris borate, pH 8.3, 1 mM EDTA
TCA	Trichloroacetic acid
TE	10 mM Tris.HCl, 1 mM EDTA
TEMED	N,N,N',N',-tetramethylethylenediamine
TNF	Tumour necrosis factor
TP	Terminal protein domain of HBV polymerase
6TG	6-thioguanine
Tris	2- amino- 2 (hydroxymethyl)-1,3 propandiol

CHAPTER 1

INTRODUCTION

A) INTERFERONS

Interferons (IFN) are a group of related cytokines first identified by their ability to inhibit the growth of influenza virus (Isaacs and Lindenmann, 1957). In addition to their anti-viral properties IFNs modulate a number of cellular functions including cell growth and differentiation, and the immune response (reviewed in Pestka et al, 1987). IFNs are grouped into two types, I and II. Type I IFNs are induced by virus infection and include the α (leucocyte), β (fibroblast) and ω subspecies. There are at least 23 different human IFNα genes (Pestka et al,,1987), one human IFNβ and one human IFNω gene. IFNγ is the only Type II IFN found in humans and is induced by mitogenic or antigenic stimulation of T lymphocytes and natural killer cells (Pestka et al,,1987).

1) MECHANISM OF ACTION OF THE INTERFERONS

IFNs bind to species-specific cell surface receptors and induce the production of a large number of cellular proteins. Some of these proteins are induced by only one type of IFN and some are induced by both (Weil et al,, 1983). The induction of these proteins is necessary for the effects of IFN since, when new protein synthesis is inhibited, IFNs are unable to mount an effective anti-viral response (Taylor, 1964).

1.1) INDUCTION OF INTERFERON-INDUCIBLE GENES

1.1a) INTERFERON RECEPTORS

Types I and II IFNs bind to different cell surface receptors (Branca et al,, 1982). The receptor subunits which bind to human IFN have been

cloned and partially characterised (Aguet et al., 1988; Uze et al., 1990). The IFNγ binding subunit is a 95kD protein and, when its cDNA is transfected into mouse cells, the transfected cells bind human IFNγ but do not mount an anti-viral response (Aguet et al., 1988). This suggests that a species-specific secondary factor is required for IFNγ's activity.

Studies of the binding of different Type I IFNs suggest that they share a common receptor which binds all Type I IFN subtypes (Merlin et al., 1985). However mutant cells which bind and respond to IFNβ but not IFNα have been isolated (Pellegrini et al., 1989) suggesting that there may be more than one receptor for Type I IFNs, or that the binding site may consist of more than one subunit. When the cDNA encoding the Type I IFN receptor binding site is transfected into mouse cells the cells become sensitive to the anti-viral effects of some, but not all, human Type I IFNs (Uze et al., 1990). This suggests that an accessory factor is required for the activity of Type I IFNs and that the murine accessory factor is able to combine with the human receptor binding site and restore activity for only some subtypes. Those Type I IFNs which do not function in the transfected mouse cells are not bound by the cells, suggesting that this accessory molecule is also involved in Type I IFN binding. Hence both Type I and Type II IFNs bind to complex cell surface receptors which require at least two subunits for full activity.

1.1b) INTERFERON INDUCIBLE PROMOTERS

The binding of an IFN to a cell surface receptor induces the production of a large number of proteins. Some of these proteins have been isolated and identified, either by a direct analysis of proteins from IFN induced cells (McMahon and Kerr, 1983) or by differential screening of cDNA libraries from IFN treated and untreated cells (Friedman et al., 1984; Larner et al., 1984; Luster et al., 1988; Caplan and Gupta, 1988 ;

Fan et al., 1989). The functions of some of these IFN inducible genes are well characterised (see later) but many are of unknown function.

The isolation of IFN inducible proteins and cDNAs has allowed the corresponding genomic DNAs to be sequenced. Studies of the 5' regions of these genes have been performed to identify potential promoter sequences. Promoter sequences are DNA sequences which are found close to the transcriptional start sites of genes and which help to regulate their expression (reviewed in Maniatis et al., 1987). Expression of the majority of eukaryotic genes is controlled by changes in their rates of transcription by RNA polymerase II, and this is regulated by the interaction of RNA polymerase II with proteins bound to DNA (Ptashne, 1988). The DNA binding proteins that interact with RNA polymerase II bind to specific DNA sequences (promoter elements) that are usually clustered in the region immediately 5' of the site(s) of initiation of transcription. The promoter region usually contains one or more elements whose role is to allow basal transcription [eg the TATA motif and the CCAAT motif (Breathnach and Chambon, 1981; McKnight and Kingsbury, 1982)] as well as distinct DNA sequence motifs that confer inducible, temporal, or cell type specific control (reviewed in Maniatis et al., 1987). These specific motifs are usually interspersed on the DNA with the basal motifs and may act either to increase or to decrease gene transcription. Binding of transcriptional transactivators to the sequences within the gene promoter activates transcription by interacting, either directly or indirectly, with RNA polymerase II (reviewed in Ptashne, 1988; Ptashne and Gann, 1990). Other regulatory sequences can exist several hundreds or thousands of base pairs away from the gene and these are known as enhancers and function in either orientation. They are believed to act through protein-protein interaction between their binding factors and those bound at the promoter. The intervening DNA is postulated to loop out to allow this interaction (Muller et al., 1989).

Comparisons of the promoter sequences of some of the IFN inducible genes allowed the identification of a common region which may be the DNA target for the effects of IFN on transcription (Friedman and Stark, 1985). A more detailed analysis of this element has been achieved by cloning the promoters from IFN-inducible genes upstream of an heterologous reporter gene and analysing the effects of deletions and mutations on induction of the reporter gene by IFNα (Levy et al., 1986; Porter et al., 1987). These studies have identified a DNA element (called the IFN-Stimulated Regulatory Element - ISRE) which is present in the promoters of all IFNα inducible genes and is both necessary and sufficient to confer a response to IFNα on an heterologous gene. Although this short sequence confers IFNα inducibility it does not reproduce the level of induction seen with the native promoter, suggesting that either the context of the ISRE or other DNA elements are involved. Similar studies from genes that are induced by IFNγ have also been performed. These studies show that at least two distinct elements are involved in the induction of gene transcription by IFNγ. Some IFNγ inducible genes contain an ISRE which causes heterologous reporter genes to respond to IFNγ (Reid et al., 1989) but others (such as the MHC Class II genes) contain an alternative element which can also convey responsiveness to IFNγ (Boss and Strominger, 1986). Recent studies on the IFN inducible GBP gene have identified a third DNA element which can be activated by IFNγ (Decker et al., 1991) but this element has not yet been identified in any other IFNγ inducible gene (see Section 1.1d for a full discussion of promoters which are activated by IFNγ).

1.1c) INTERFERONα-INDUCIBLE TRANSACTIVATING FACTORS

The binding of IFNs to the cell surface has been investigated in some detail and the DNA elements which confer responsiveness to IFN

have been identified. Clearly some mechanism must exist whereby receptor occupation at the cell surface activates the IFN responsive DNA element within the nucleus. Studies in cells treated with IFN have shown that treatment with IFNα for a few minutes induces the appearance of an ISRE binding protein. This factor is called E or ISGF3 (Porter et al., 1987; Levy et al., 1988), the two are probably the same and for convenience this factor will subsequently be referred to as E. E is activated in the cytoplasm and then migrates to the nucleus (Dale et al., 1989). It appears when protein synthesis is inhibited and the appearance of E and the initiation of transcription are temporally similar (both can be detected after 5 minutes of IFN treatment) (Levy et al., 1988; Imam et al., 1990). Mutations in the ISRE which abolish the binding of E also abolish its ability to stimulate IFN inducible transcription (Kessler et al., 1988; Dale et al., 1989a) and purified E can stimulate ISRE dependant transcription in vitro (DE Levy and JE Darnell, Rockefeller University, New York - personnal communication). These experiments strongly suggest that IFN induced gene expression is triggered by activation of E in the cytoplasm and its migration to the nucleus where it binds to the ISRE and activates transcription.

Longer treatment of cells with IFN induces the formation of other proteins which bind to the ISRE. These proteins are dependent on new protein synthesis and the ISRE-protein complexes are variously known as M or ISGF2 (Porter et al., 1987; Levy et al., 1988). The nature of these complexes is not clear. The complex M contains at least 2 proteins (N. Rogers, Imperial Cancer Research Fund, London - personal communication), one of which is the protein IRF1, initially described as a transactivator for the IFNβ gene promoter (Miyamoto et al., 1988). The nature of the other protein(s) in this complex is unknown and the function of these secondary factors is still in some doubt. Since IFN induced gene transcription is followed by a decline in transcription and a

refractory state when the cells no longer respond to IFN (Larner et al., 1986), it has been suggested that these secondary factors act to inhibit transcription (Levy et al., 1988). However the complexes appear at a time when IFN inducible genes are still being actively transcribed and it seems more likely that these factors are secondary transactivators which function to increase gene transcription which has been initiated by the binding of E (Imam et al., 1990).

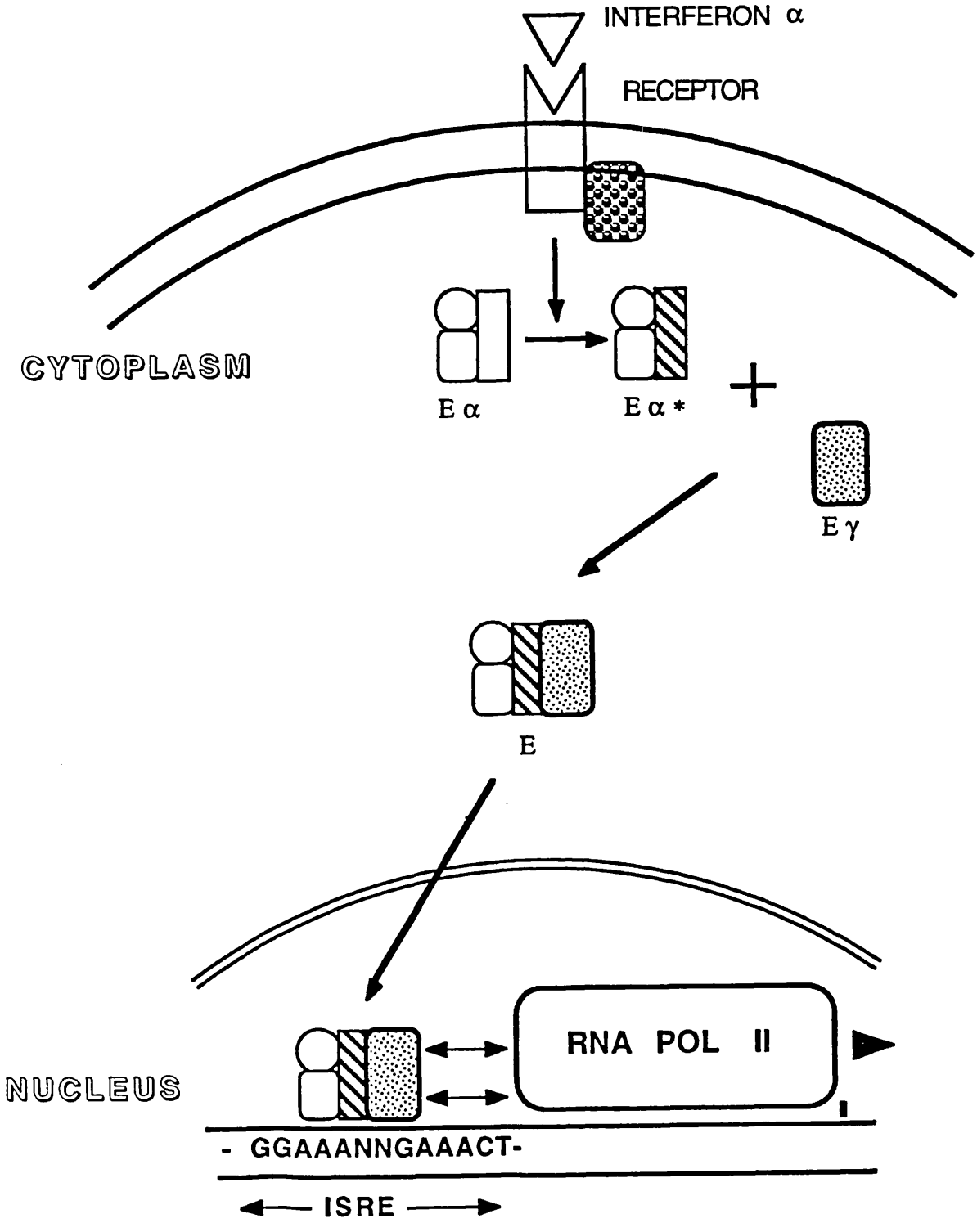
The activation of gene transcription following IFN treatment of cells is initiated by the activation of E. Studies using alkylating agents (Levy et al., 1989) and mutant cell lines (Bhandyopadhyay et al., 1990; McKendry et al., 1991) have shown that E contains at least two functional components - Eα, which is activated by IFN binding to the receptor and Eγ, which is present constitutively in the cytoplasm and whose concentration is increased by prior teatment with IFNγ. E has recently been purified (Kessler et al., 1991) and it contains at least four different protein subunits - three to make up Eα and one for Eγ. Presumably binding of IFN to the receptor activates one or more of the Eα subunits allowing it to form a complex with Eγ before migrating to the nucleus where it binds to the ISRE and activates transcription. Figure 1.1 outlines this mechanism.

The activation of E appears to be a crucial step in the pathway leading to the transcription of IFN inducible genes. It is not yet known how the binding of IFN to its receptor activates the Eα component of E. In general the binding of polypeptide ligands to their receptors activates distinct signal transduction pathways (reviewed in Pfeffer and Tan,1991). Three major groups of receptors are known: those possessing tyrosine kinase activity (such as platelet derived growth factor); ion channels which, upon ligand binding, allow the flux of specific ions (usually Ca^{2+}) through the membrane; and those which are coupled to enzymes which produce second messenger molecules such as cyclic nucleotides and

FIGURE 1.1

LIKELY MECHANISM FOR THE ACTIVATION OF E

IFNα binds to its receptor and activates at least one of the Eα subunits (hatched box). Active Eα then combines with Eγ to form complete E which is transported to the nucleus where it binds to the ISRE and activates transcription



diacylglycerol (DAG). There is no evidence to suggest that IFN binding to cells activates an ion channel (Larner et al., 1984) and tyrosine kinase activity has not been detected following IFN binding (Pfeffer and Tan, 1991). There is some evidence to suggest that cyclic nucleotides and DAG may be involved in the actions of IFN. The classical activation pathway of DAG involves hydrolysis of inositol phospholipid to form I-1,4,5-P_3 and DAG. The I-1,4,5-P_3 then liberates stored Ca^{2+} which can activate other signalling pathways. The DAG formed by the hydrolysis of inositol phospholipids can activate protein kinase C (PKC) which is then able to phosphorylate and activate a wide variety of cellular proteins (reviewed in Nishizuka, 1986). IFN does not appear to activate this classical pathway - IFN does not induce inositol phospholipid turnover (Pfeffer and Tan, 1991) and IFN treatment of cells is not accompanied by a release of Ca^{2+} from internal stores (Pfeffer et al., 1990). Active PKC can be assayed in cell extracts by its ability to phosphorylate an 80kD substrate, but in mouse cells treated with IFN this PKC substrate is not phosphorylated (Mehmet et al., 1987). Clearly, therefore, IFN does not activate the classical PKC pathway. However several groups have shown that IFN treatment of cells can induce an increase in DAG production (reviewed in Pfeffer and Tan, 1991) and treatment of cells with inhibitors of PKC (staurosporin or H-7) inhibits the actions of IFN (Pfeffer et al., 1990) suggesting that a PKC - like enzyme may be involved in IFN's actions. The enzyme PKC consists of several different isoforms and it is possible that IFN acts by activating a specific, non-classical, isoform. If IFN does act via a PKC mediated pathway then it is likely that the PKC isoform which is activated will phosphorylate a restricted range of proteins which include some of the proteins which constitute the E complex. There is some evidence that E is phosphorylated upon IFN treatment of cells (JE Darnell and C Schindler, Rockefeller University,

New York - personal communication) and it is possible that IFN acts by activating an enzyme, possibly a PKC isoform, which then phosphorylates and activates Eα. Clearly the available data are consistent with other possibilities - for example PKC- like enzymes may act to convert a precursor of Eα to a form which can then be activated by IFN binding to its receptor. Further work will be required to precisely identify the mechanism whereby E is activated.

1.1d) INTERFERONγ INDUCIBLE TRANSACTIVATING FACTORS

The factors activated when IFNγ binds to its receptor are not known. Genes which can be induced by IFNγ may contain one of two different promoters - the ISRE and the non-ISRE promoter associated with Class II MHC genes (see above, 1.1b). Studies with mutant cell lines (John et al., 1991) have identified a clone in which the induction of non-ISRE promoters by IFNγ is normal but the induction of some ISRE promoters by IFNγ is defective, indicating that these promoters use different IFNγ inducible transactivating factors. An ISRE binding factor known as G appears when cells are treated for several hours with IFNγ and it is possible that this factor is involved in the ISRE mediated response to IFNγ. Cell lines which do not respond to IFNγ lack G (Imam et al., 1990) suggesting that G may be involved in generating the ISRE mediated response to IFNγ, but this has not yet been confirmed. The factors which mediate the non-ISRE induction by IFNγ have not been firmly identified. Deletion analyses of the IFNγ inducible promoter of the HLA Class II gene, DRα, have identified two DNA sequences, called the X and Y boxes, which seem to be important for induction by IFNγ (reviewed in Kara and Glimcher, 1991). A number of factors which bind to these regions have been identified (Dorn et al., 1987) but their precise role is not yet known.

The promoter of the IFNγ inducible gene, GBP, contains an element (GAS) in addition to an ISRE. An IFNγ inducible factor, GAF, has been identified which binds to the GAS element and since the GAS DNA element can confer IFNγ inducibility on an heterologous promoter it is possible that this element and its associated binding protein play a role in the induction of this gene by IFNγ. However the nature of the GAF protein and its precise role in the IFNγ response pathway are unknown (Decker et al., 1991).

1.1e) MUTATIONAL ANALYSIS OF THE INTERFERON RESPONSE PATHWAY

A number of different experimental approaches have been used to study the IFNα response pathway. These include functional studies of the DNA promoter elements of IFN inducible genes and in vitro analyses of the factors which bind to these elements. Such studies have provided valuable information about the likely components of the IFN response pathway but these predominantly in vitro studies do not provide information about function in vivo. Functional studies can, however, be performed in mutant cells which lack elements in the IFN response pathway and cells which are defective in their response to IFN are potentially valuable tools for studying factors which are activated by IFN.

In order to isolate mutant cell lines which do not respond to IFN, a cell line containing an IFN- inducible selectable marker has been developed (Pellegrini et al., 1989). These cells (named 2fTGH) were derived from a strain of human fibrosarcoma cells (HT1080 cells) which lack the hypoxanthine-guanine phosphoribosyl-transferase gene (HPRT$^-$ cells) and they contain the IFN inducible promoter from the human 6-16 gene linked to the *Escherichia coli* guanine phosphoribosyl-transferase gene *(gpt)*. In the presence of IFNα gpt is produced and the cells die in

medium containing 6 thioguanine (6TG), because gpt converts 6TG to the toxic triphosphate. Hence clones of 2fTGH cells which do not respond to IFN can be selected by growth in 6TG plus IFN - only cells which do not respond to IFN survive in this medium.

The use of gpt as the IFN inducible marker in 2fTGH also allows the selection of revertant clones which have regained their response to IFN. If cellular purine synthesis is inhibited, by aminopterin, cells die unless they are provided with an alternative source of purines. Purines can be synthesised by an aminopterin insensitive salvage pathway in which hypoxanthine is converted to guanylic acid. This salvage pathway uses either the cellular HPRT enzyme or the bacterial gpt enzyme (reviewed in Fenwick, 1985). Since 2fTGH is derived from an HPRT $^-$ cell line, the salvage pathway is only able to function when gpt is produced. Thus if 2fTGH cells are grown in medium containing aminopterin and hypoxanthine they die unless the gpt enzyme is induced by IFN. Selection of 2fTGH cells in IFN plus hypoxanthine, aminopterin and thymidine medium (HAT) will therefore select for clones of cells which respond to IFN (note that thymidine is included in the selection because aminopterin also inhibits its production).

2fTGH cells have been mutagenised and clones which do not respond to IFN isolated (Pellegrini et al., 1989; John et al., 1991; McKendry et al., 1991). These studies have confirmed that Eγ is an essential component of the IFNα response pathway [cells that lack Eγ do not respond to IFNα (John et al., 1991)] and they have also shown that the IFN response pathway is much more complicated than previously believed. A mutant cell line which does not bind or respond to IFNα but does respond to IFNβ has been isolated, suggesting that there may be more than one receptor for Type I IFNs (Pellegrini et al., 1989). Other recently developed mutants have revealed surprising connections between the Type I and Type II IFN response pathways. A mutant cell line which

lacks active Eα has been cloned and as expected it does not respond to Type I IFNs. Surprisingly this clone does not respond to Type II IFNs and hence the two IFN response pathways must share common factors, perhaps one of the sub-units of Eα (McKendry et al., 1991). As the 2fTGH cell system contains a selectable marker which allows the selection of revertant clones it should be possible to complement these mutant clones and isolate genes which reverse the phenotype. Recent studies on one 2fTGH mutant that does not respond to IFNα have identifed a genomic DNA clone which can restore the response to IFN (S. Pellegrini, Pasteur Institute, Paris - personnal communication) showing that 2fTGH cells can be used to clone and characterise important elements in the IFN response pathway.

Although the 2fTGH system has been valuable in identifying unexpected elements in the IFN response pathways its use has been limited by the low frequency with which IFN resistant mutants have been isolated. The frequency of spontaneous IFN resistance in these cells is very low [>1 per 10^8 cells (Pellegrini et al., 1989)] and multiple rounds of mutagenesis are required to generate mutants at an acceptable frequency. Whilst the low frequency of IFN resistance in 2fTGH cells is a disadvantage in obtaining mutants in the IFN response pathway it does allow the cells to be used to identify factors which inhibit the response to IFN. Any factor which inhibits the cellular response to IFN should increase the frequency of IFN resistance in 2fTGH cells and hence these cells can be used to identify inhibitors of the IFN response pathway.

2) ANTI-VIRAL ACTIVITIES OF INTERFERON.

Some IFN inducible proteins have well defined activities against certain classes of virus. The 2'5' oligoadenylate synthetase enzyme

(2-5A) was one of the first IFN inducible proteins to be identified (McMahon and Kerr, 1983). After induction the enzyme is activated by double stranded RNA (dsRNA) whereupon it catalyses the synthesis of a family of 2',5'-linked oligonucleotides. These activate a ribonuclease (RNaseL) which destroys RNA (Williams and Silverman, 1984). dsRNA is not normally found in human cells but most RNA viruses either contain dsRNA in their genome or produce it during replication. This dependence on the presence of a viral component presumably ensures that RNaseL is only activated in virally infected cells. This may prevent its indiscriminate activation which might be harmful. The 2-5A system is potentially a valuable host defence against any virus which produces dsRNA. However, although cells expressing 2-5A constitutively are resistant to the effects of picornaviruses, they are not resistant to other viruses (Chebath et al,, 1987), suggesting that either 2-5A's anti-viral activities are relatively selective or that some viruses have evolved mechanisms to avoid its deleterious effects (see Section 5).

Type I IFNs also induce the production of a p68 protein kinase. This enzyme catalyses the phosphorylation of the α subunit of the protein synthesis initiation factor eIF-2 (Samuel, 1979). Phosphorylation of eIF-2α inhibits protein synthesis by preventing initiation of translation. Successful viral replication clearly requires protein synthesis and as IFN inhibits many viruses by preventing the synthesis of new viral proteins it is likely that the kinase plays a key role in the anti-viral activities of IFN. Direct evidence for this is, however, not yet available. Clearly indiscriminate kinase activity and inhibition of translation would have deleterious effects on cells and it is not surprising to find that the enzyme is only activated by dsRNA, which is normally only present in cells infected by RNA viruses. Hence 2-5A and protein kinase are potent anti-viral agents which are induced by IFN and are activated by viral dsRNA.

Genetic analyses of inbred strains of mice which are resistant to influenza virus infection led to the discovery of a gene (Mx1) which confers resistance to influenza infection (Lindenmann, 1962). Mx1 has been cloned and is an IFN inducible protein which inhibits the replication of influenza virus (Staeheli, 1986). The related human Mx gene has also been cloned and it inhibits the growth of vesicular stomatitis virus as well as the growth of the influenza virus (Pavlovic et al., 1990). The sites and modes of action of the Mx genes have not yet been determined.

The anti-viral functions of a few IFN inducible proteins have been identified and studied in detail. They are selectively anti-viral and each protein inhibits the growth of a small number of viruses by interfering with particular steps in the viral replication process. The wide ranging anti-viral activities of IFN are therefore not due to production of a single protein but rather to the production of a large number of selective anti-viral compounds. This multiplicity of viral inhibitors is probably advantageous. If IFN inhibits the replication of a virus in a number of different ways viral resistance to IFN is less likely to develop. Furthermore the different anti-viral properties of IFN inducible genes enables many different viruses to be effectively inhibited, even though they differ in structure and mode of replication. Presumably the many IFN-inducible genes identified genetically but not functionally will act to inhibit the growth of particular viral groups by inhibiting specific steps in the viral life cycle.

3) IMMUNOMODULATORY EFFECTS OF IFNS

Successful erradication of a virus from an infected host requires an immunological response which can eliminate infected cells (by cytotoxic T cell activity), neutralise circulating virions (by antibody production), and provide long term protection against re-infection (by generating

memory T cells). In addition to their direct anti-viral activities IFNs play a key role in generating this complex immunological response. Cytotoxic T cells are lymphocytes which are able to kill cells expressing viral proteins and this elimination of virally infected cells is an important mechanism of viral eradication (Taylor and Askonas, 1986). Cytotoxic T cells are only able to recognise viral proteins when they are associated with MHC Class I antigens (Zinkernagel and Doherty, 1974) and cells which do not express these MHC antigens (such as the murine teratoma cell line F9) are not lysed by cytotoxic T cells when they are infected by viruses (Zinkernagel and Oldstone, 1976). Semi quantitiative studies have shown that there is a direct relationship between the levels of MHC Class I expression on a cell surface and the susceptibility of the cell to T cell lysis (Kuppers et al., 1981). Hence the amount of MHC Class I antigen which is expressed determines how effectively virally infected cells are eliminated. MHC Class I antigen expression is increased by Types I and II IFNs and a number of studies have shown that IFN enhances the susceptibility of virally infected cells to T cell lysis (Blackman and Morris, 1985). Thus IFN enhances the destruction of virally infected cells by increasing the expression of MHC Class I antigens.

MHC Class I antigens do not present native viral proteins. The foreign proteins must first be processed in the cytoplasm to form protein fragments which are then transported into the endoplasmic reticulum where they combine with the MHC Class I proteins. The MHC protein-antigen complex is then transported to the membrane. Processing of viral antigens is initiated in the cytoplasm by a protein complex, the proteosome (Monaco and McDevitt, 1984), which contains multiple proteolytic enzymes, some of which have been cloned (Kelly et al., 1991). The proteosome generates peptide fragments which are transported into the endoplasmic reticulum by peptide transport proteins (Trowsdale et al., 1990). The proteosome and transporter enzymes

involved in antigenic processing are all inducible by IFN (Trowsdale et al, 1990). Hence IFNs assist in the development of an anti-viral immunological response by enhancing the processing and presentation of viral proteins to the immune system.

An essential step in the production of an immune response to a viral protein is the activation of T helper cells. These cells are induced by antigens in association with MHC Class II proteins (Schwartz, 1985) and studies on mouse peritoneal macrophages have shown that the level of expression of MHC Class II antigens correlates with the macrophage's ability to present antigen and activate T helper cells. IFNγ augments MHC Class II expression and treatment of cells with IFNγ increases their ability to stimulate helper T cells (Pober et al., 1983). Hence IFNγ enhances the immune response by increasing the presentation of viral antigens to immunoreactive T cells.

In addition to aiding in the presentation of viral peptides IFNs also enhance the immunological response by activating other cells of the immune system. IFNγ is released by activated lymphocytes, whereupon it activates a number of other cells directly (such as macrophages) and indirectly, by stimulating the release of other cytokines (reviewed in Trinchieri and Perussia, 1985).

4) ANTIGROWTH EFFECTS OF INTERFERON

IFNs inhibit the multiplication of some normal and some transformed cells (reviewed in Romeo et al., 1989). In some cells IFNs specifically inhibit the G1 to S transition (Balkwill and Taylor-Papadimitriou, 1978) and in others IFNs can inhibit cell proliferation without inhibiting overall cellular biosynthesis thus allowing the cells to continue to grow in volume and mass, but not to divide (Pfeffer et al., 1979). The antigrowth effects of IFN only partially overlap their antiviral

activities - in some cells the antigrowth effect requires higher and longer doses of IFN than does the antiviral effect (Cook et al., 1983).

The mechanism and physiological role of IFN's antigrowth effects is not yet clear. Presumably IFN exerts its effects on cell growth by altering the expression of a variety of intracellular growth regulatory factors (such as the proto-oncogenes) and a number of studies have shown that IFNs can cause a decrease in the expression of *c-myc* in some cell lines (reviewed in Clemens, 1985). For many cell lines it is not known whether this decrease in the expression of *c-myc* is the cause of IFNs' antigrowth effects or whether it is a consequence of other events. Studies on the murine cell line, 3T3, have shown that cells transfected with, and constitutively expressing *c-myc* are partially resistant to the anti-growth effects of IFNs, suggesting that for this cell line down regulation of *c-myc* is an important primary event in IFNs' anti-growth effects (reviewed in Kimchi, 1987). However for most cell lines the significance of IFNs' effects on the expression of proto-oncogenes is not yet clear.

The physiological role of IFN's antigrowth effects is also unknown. In some cell lines IFN is able to act as an autocrine growth regulator - treatment of macrophages with colony stimulating factor 1 causes an initial increase in growth, followed by a plateau. This plateau is associated with the production of IFN and can be abolished by treatment with anti-IFN antibodies (Moore et al., 1984). Hence in this situation IFN acts as an autocrine growth regulator, but other examples of this effect have not been identified.

5) INHIBITION OF INTERFERON'S EFFECTS BY VIRUSES

IFNs are potent anti-viral agents which prevent viral infections by inducing a specific anti-viral replication response and by stimulating the immune system. It is not surprising to find that some viruses have

evolved mechanisms for preventing IFN's effects. The p68 kinase and 2-5A enzyme are normally activated by viral dsRNA (see Section 2) but a number of viruses have evolved ways of preventing their activation. The reovirus, serotype 1, produces a protein, sigma 3, which is able to sequester dsRNA and thereby prevent activation of the dsRNA activated anti-viral proteins (Imani and Jacobs, 1988). The p68 kinase can also be specifically inactivated by some viral products. A number of adenoviral strains produce RNAs (the VA RNAs) which bind to p68 kinase but do not activate it. The binding of VA RNAs to the kinase prevents other dsRNAs from activating this enzyme and thus VA RNAs inhibit the kinase (Kitajewski et al., 1986). Other viruses inactivate the kinase by different mechanisms - in poliovirus infected cells there is proteolitic cleavage of the enzyme (Black et al, 1990) and in influenza infected cells an inhibitor of the kinase is activated (Katze et al, 1988). HIV inactivates the kinase by preventing its production - the HIV TAT protein down regulates the expression of the kinase (Roy et al., 1990). Hence a number of viruses have developed mechanisms to inactivate at least one of the IFN inducible anti-viral proteins.

Inhibition of the cellular inhibitors of viral replication is clearly of great advantage to a virus. However IFNs are also able to eradicate viral infections by activating an immunological response. The Epstein-Barr virus has developed a means of preventing this immunological enhancement - the BCRF1 protein of Epstein-Barr virus is homologous to, and capable of mimicking the activity of, interleukin 10 which inhibits the production of IFNγ in activated T cells (Hsu et al., 1990). Hence this virus interferes with another component of IFNs anti-viral repertoire - the activation of the immune system by IFNγ. Complete inhibition of all IFN's anti-viral activities would be a major survival advantage for a virus and it is not surprising to discover that some viruses have evolved such mechanisms. The E1A protein of the oncogenic adenoviruses

completely inhibits the cellular response to IFN by preventing the activation of the transactivating factor E (Ackrill et al., 1991). Recent work suggests that the Epstein-Barr virus may also be able to inhibit the cellular response to IFNs (Aman and von Gabain, 1990), although the mechanism of this effect is unknown. Hence many viruses have developed mechanisms to inhibit the anti-viral effects of IFN and thereby enhance their own survival.

B) HEPATITIS B VIRUS

1) STRUCTURE OF HEPATITIS B VIRUS

Hepatitis B virus (HBV) contains partially double stranded DNA encoding four overlapping open reading frames (Figure 1.2). Although its genome is DNA, HBV replicates by reverse transcription of an RNA intermediate (reviewed in Ganem and Varmus, 1987). In HBV infection three viral particles are produced. Dane particles (double-shelled 42 nm particles) represent the intact virion and are composed of a viral envelope (containing HBV surface proteins embedded in a host derived envelope) surrounding a viral nucleocapsid. The nucleocapsid consists of a basic phosphoprotein (HBV core protein) which surrounds the HBV DNA. The viral nucleocapsid particles can be released from the viral envelope by treatment with a mild detergent and such particles contain a polymerase activity that incorporates labelled dNTPs into the HBV genome (Kaplan et al., 1973). This polymerase activity is believed to be due to the presence of the viral polymerase within the virion but this has not been directly proven. In an HBV infection Dane particles are found along with rod like structures (20 nm in diameter and of variable length) and 20 nm spheres. These two latter particles are "dummy" virions, which contain only HBV surface proteins and are non-infectious. Their function may be to adsorb neutralising antibodies and thus protect the infectious particles from neutralising antibodies (Ganem and Varmus, 1987).

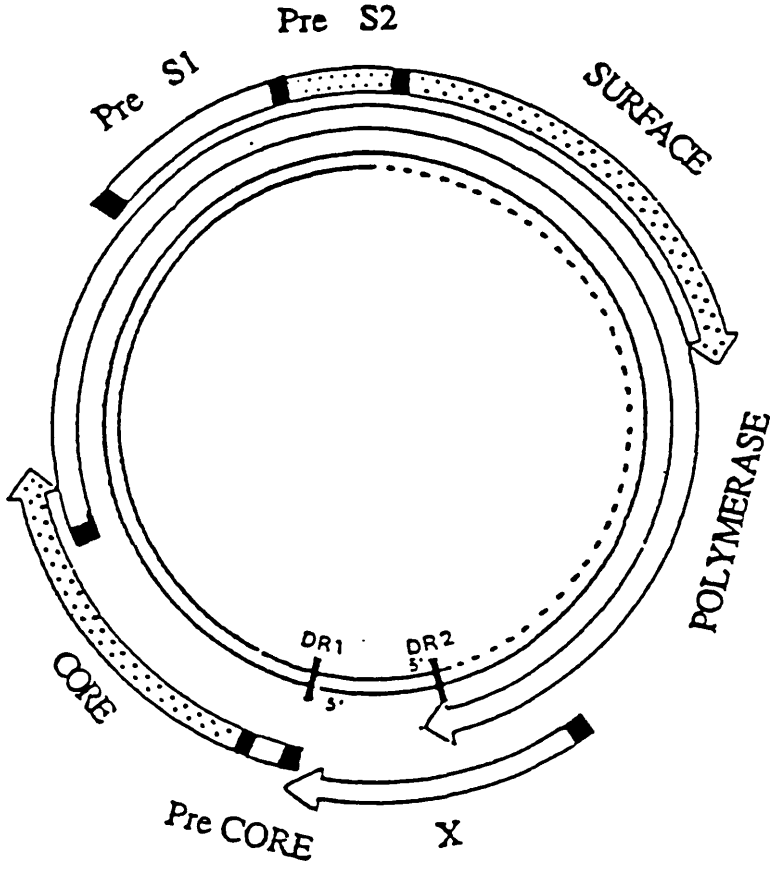
1.1) THE HEPATITIS B SURFACE PROTEIN

The HBV surface protein is encoded by the S gene, which contains three start codons. Each of these is used, producing three different surface proteins (PreS1, PreS2 and surface) which have an identical carboxy terminus but which differ in their amino termini (Heerman et al., 1984). The products of the HBV S gene form the viral envelope, which

FIGURE 1.2

Genomic organisation of HBV

Fine lines represent DNA, the HBV genes are shown as boxes with the start codons marked. (Derived from Galibert et al,, 1979)



contains equimolar amounts of each of the three proteins embedded in host derived lipid. The preS1 protein contains a viral attachment site which binds to hepatocytes and presumably allows viral entry into cells (Neurath et al., 1986). The site of attachment within the preS1 protein is, however, still not clear. The preS2 protein is highly immunogenic (Alberti et al., 1988) and is able to bind albumin polymers (Machida et al., 1983) but the physiological relevance of this is unknown. Surface proteins also give rise to the subviral particles which contain predominantly surface protein with small amounts of preS2 protein and host derived phospholipids (Heerman et al., 1984).

In addition to its structural functions the S gene also encodes a viral transactivator. Truncated preS1 proteins are able to transactivate a number of different cellular promoters (Kekule et al., 1990). Although these truncated proteins may not be produced during a typical viral infection, truncated HBV DNA, which might produce these proteins, is often found in integrated HBV sequences in patients with chronic HBV infections. It has been suggested that in chronic HBV infections these truncated proteins may be produced and play a role in the development of hepatocellular carcinoma (Kekule et al., 1990) which is often associated with chronic HBV infection.

1.2) THE HEPATITIS B CORE PROTEIN

The C gene of HBV encodes the nucleocapsid protein, core. The gene contains two start codons which are both used. When translation is initiated at the first, a 25kD protein (pre-core) is produced (Jean-Jean et al., 1989) and this protein contains an amino terminal signal peptide. This signal peptide causes the protein to enter the endoplasmic reticulum where it is degraded to form the immunogenic protein, e antigen, which

is secreted via the Golgi apparatus (Garcia et al., 1988; Ou et al., 1986) and expressed on the cell surface (Schlicht and Schaller, 1989) where it may act as a target for antibody mediated cell lysis. The function of the secreted protein (e antigen) is not clear but it may neutralise antibodies which would otherwise be available to bind to cells expressing core protein. The processed pre-core protein is not confined to the endoplasmic reticulum (Garcia et al., 1988). After entry into the endoplasmic reticulum the pre-core protein signal peptide is cleaved off and up to 70% of the pre-core protein re-enters the cytoplasm and is transported to the nucleus where its function is unknown (Ou et al., 1990). The site of the nuclear transport signal within the pre-core protein is controversial with reports of an amino terminal and a carboxy terminal nuclear localisation signal (Yeh et al., 1990; Ou et al., 1989).

Translation at the second start codon of the core gene gives rise to a 22kD protein (core) which binds to DNA and aggregates to form the viral nucleocapsid (Miyanohara et al., 1986). Mutants containing stop codons between the two core gene start signals have been identified and, as predicted, these pre-core mutants are infectious and give rise to an infection that does not produce circulating e antigen (Carman et al., 1989). Since the immunological response that eradicates HBV infected cells is directed, at least in part, against e antigen it is likely that during chronic HBV infection immunological selection pressure assists in the propagation of these e antigen minus viral mutants ie. viruses that have mutated so as not to produce e antigen have a survival advantage and thrive. Studies on the replication of viruses that contain this mutation have shown that they replicate less effectively than the wild-type virus (S Tong, INSERM, Lyon - personal communication) and this may be the reason why this mutation has not become the most prevalent viral strain. Recent studies (Omata et al.,1991; Liang et al., 1991) have shown that patients who develop fulminant HBV infections secrete viruses

which contain the pre-core mutation. It is not known whether these patients are infected with the mutant virus or whether the mutation develops during the infection. Nor is it clear why pre-core mutant viruses, which are replication incompetent and which do not produce e antigen, are associated with fulminant hepatitis. It has been suggested that the mutant viruses cause core protein to accumulate in the cytoplasm and that this causes cell death (Carman et al., 1991). Evidence to support this model is, however, not available.

The core gene of HBV may also encode an inhibitor of IFNβ production. Transfection experiments with constructs expressing the pre-core and core proteins show that these constructs can inhibit induction of the IFNβ promoter by dsRNA in Vero cells (Twu and Schloemer, 1989; Whitten et al., 1991). In macrophages and hepatoma cells Jochum et al.,(1990) have shown that core protein (and surface and X protein) can increase the rate of degradation of a variety of cytokine mRNAs, including that for IFNβ. The mechanism and physiological significance of this effect is unknown, but as patients with chronic HBV infection have reduced serum levels of IFN (see later) it is possible that core gene mediated inhibition of IFN production is of functional importance.

1.3) THE HEPATITIS B X PROTEIN

The small X open reading frame encodes a single protein, X. This is a transactivating factor that activates a number of viral and cellular promoters, including that of the HBV pre-genome (Unger and Shaul, 1990). A number of promoter elements which are transactivated by X have been defined. These include NfκB binding sites (Twu et al., 1989), AP-1 and AP-2 binding elements (Seto et al., 1990) and the C/EBP binding element in the HBV genome (Unger and Shaul, 1990). All of the

X activated DNA elements defined to-date are known to bind cellular proteins and attempts to demonstrate direct binding of X to these DNA elements have been unsuccessful (Seto et al., 1990). This suggests that X acts by interacting with cellular DNA binding proteins and it has been suggested that X increases the transcription of genes by increasing the interaction of cellular DNA binding factors with the transcriptional effector proteins (Seto et al., 1990). This mechanism of transactivation is used by another promiscuous viral transactivator, the E1A protein of the adenoviruses (reviewed in Martin et al., 1990). A number of studies have shown that if X protein is fused to a DNA binding protein the hybrid protein can transactivate the response element of the DNA binding domain, showing that X can activate transcriptional effector proteins (Unger and Shaul, 1990; Seto et al., 1990). An alternative possibility is that X enhances the association between DNA-binding proteins and DNA. X has been shown to alter the DNA binding specificity of the transactivating factors CREB and ATF-2 (Maguire et al., 1991), suggesting that X can alter the binding of transactivating proteins to DNA. Hence X probably activates transcription by interacting with cellular transactivators and it may alter either their affinity for DNA or their binding to the cellular transcriptional machinery.

X may also act by enzymatically altering cellular transactivating factors - there is some evidence to show that X can act as a protein kinase (Wu et al., 1990) but X has not yet been shown to phosphorylate known transactivators. It is probable that X acts in different ways to exert its effects on different promoter elements.

1.4) THE HEPATITIS B POLYMERASE

The POL gene of HBV encodes a large protein - polymerase. Polymerase has not yet been expressed in vitro in a functional form and

knowledge about its function has been derived from studies of its homology to other proteins and from functional experiments performed with viral or pro-viral particles. These particles contain small amounts of polymerase associated with HBV DNA and core proteins. Although the enzymatic properties of these particles are usually attributed to the presence of polymerase it is not clear whether the other components contribute to the observed activities.

Homology studies suggest that polymerase contains three domains (Toh et al., 1983; Khudyakov and Makhov, 1989) and this has been partially confirmed by mutational and functional analyses of the POL gene (Bavand et al., 1989; Radziwill et al., 1990). The amino terminal domain of polymerase is homologous to viral terminal proteins which bind to viral DNA. Studies in replicating viral particles from ducks infected with the duck HBV show that this domain (terminal protein) can form a covalent bond with DNA (Bosch et al., 1988), and it seems likely that the terminal protein from human HBV will also bind HBV DNA, perhaps assisting in the packaging of the DNA in virions. Terminal protein is also able to bind to the pregenomic RNA of HBV (Kochel et al., 1991). Since reverse transcription of RNA requires a primer (tRNA in the case of the well studied retroviral reverse transcriptases (Mizusawa et al., 1985) it is likely that the binding of terminal protein to the pre-genomic RNA primes reverse transcription by the reverse transcriptase that is encoded by the POL gene (Kochel et al., 1991).

Terminal protein is linked via a spacer arm, which is highly variable between different viral isolates, to two domains that are homologous to HIV reverse transcriptase and to RNaseH (Radziwill et al, 1990). Since HBV replicates via reverse transcription it is very likely that polymerase is a reverse transcriptase and there is some direct evidence for this (Offensperger et al., 1988; Bavand and Laub, 1988, Bavand et al., 1989). There is, as yet, no direct evidence to show that POL encodes an RNase,

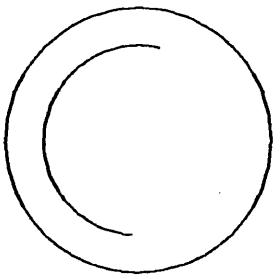
but studies with mutated POL genes strongly suggest that it does so (Radziwill et al., 1990). Mature HBV virions contain a DNA polymerase whose function is readily demonstrable (Robinson et al., 1974). It is presumed that this activity is contained within the polymerase protein but direct evidence for this is not available.

It is not clear what protein products are actually produced from the POL gene. In artificially infected cells, gel analysis of reverse transcriptase activity (presumably due to the polymerase protein) indicates that polymerase exists in two forms: a 90Kd form (the predicted size of the POL gene product) and a 70 kD form (Bavand and Laub, 1988; Bavand et al., 1989).This suggests that the gene may produce two protein species or that the protein may be post translationally modified to give rise to two proteins of different mobility. In woodchucks infected with woodchuck HBV, Feitelson et al.(1991) have used antibodies directed against the carboxy terminus of polymerase and have detected two polymerase proteins of different sizes, suggesting that polymerase does exist in at least two forms. In infected cells DNA polymerase activity (presumably from the polymerase protein) can be demonstrated in the cytoplasm where it is associated with pro-viral particles (Hirsch et al., 1990) but direct staining with antibodies against the amino terminus of polymerase show that it is nuclear (Goldin et al., 1990; H.Will, Max-Planck Institut, Munich - personal communication), perhaps suggesting that polymerase can partition into an active cytoplasmic form and an inactive nuclear form. It is not yet clear whether these two forms of polymerase, detected in two very different assay systems, are the same or different products of the POL gene. Preliminary observations (H. Will - personal communication) show that the amino terminus of polymerase contains a nuclear localisation signal and that the full length polymerase protein is rapidly degraded,from the carboxy terminus, to produce an amino terminal product that is relatively resistant to

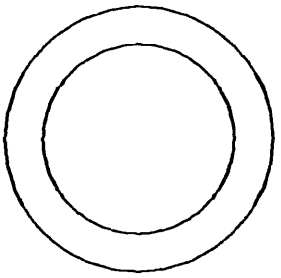
proteolysis (Bartenschlager et al., 1992). It therefore seems possible that the POL gene encodes a 90kD protein, which is enzymatically active and cytoplasmic, and that this is rapidly degraded to form other products, perhaps including isolated terminal protein which migrates to the nucleus where its function is obscure.

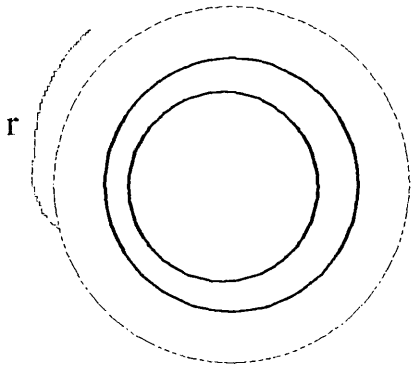
The HBV POL gene is homologous to the reverse transcriptases of retroviruses (see above). In addition to this sequence homology, HBV POL is similar to HIV reverse transcriptases in its genomic organisation. In retroviruses, sequences encoding the *pol* gene overlap the distal portion of the *gag* gene. Similarily the HBV POL gene overlaps the distal end of the core gene. In retroviruses both genes are represented in a single mRNA whose translation gives rise to a *gag-pol* fusion protein which is then processed by a protease encoded by the fusion protein. This retroviral fusion protein is generated from the out of frame *gag* and *pol* cistrons by ribosomal frameshifting during translation (Jacks and Varmus, 1985). Presumably this complex mechanism allows tight regulation of the production of retroviral polymerase and since indiscriminate reverse transcription might be deleterious to the host cell, the controlled production of reverse transcriptase may be of advantage to the virus. Although the genomic organisation of HBV suggests that polymerase might be produced by ribosomal frame shifting and proteolytic cleavage of a core-polymerase fusion protein (Craigen and Caskey, 1987) a number of experiments have shown that this mechanism is not used by HBV. The polymerase protein is formed by internal entry of ribosomes into a core-POL mRNA and no frameshifting occurs. HBV polymerase is formed as a single protein and is not derived from a polyprotein precursor (Jean-Jean et al., 1989a). Polymerase production in HBV therefore lacks the complex regulatory features which are found in retroviruses and it may be that overproduction of this potentially harmful protein is prevented by its rapid breakdown (see above).

REPLICATION OF HEPATITIS B VIRUS.



1 After entry into the cell the incomplete DNA is converted to a complete double-stranded DNA.

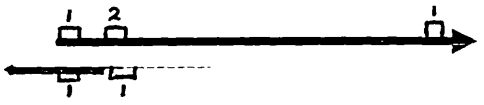




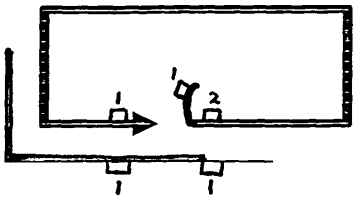
2 Cellular enzymes synthesise the pre-genomic RNA. Note that this RNA is overlength and contains a terminal redundancy - ˜r˜ .

3 The polymerase protein of HBV binds to the terminally redundant RNA and reverse transcribes DNA (the HBV minus strand). Note the direct repeat sequences - 1 and 2.

4 The RNA primer is degraded but the RNA complementary to the 3' direct repeat (DR1) persists.



5 The RNA complementary to the 3' DR1 DNA jumps to the 5' direct repeat (DR2) and acts as the primer for the synthesis of the plus strand DNA.



6 Circularisation of the DNA takes place, presumably by a plus strand transfer.

2) REPLICATION OF HEPATITIS B.

Following entry of HBV into an hepatocyte, viral replication is initiated by the formation of a complete double-stranded DNA. This involves filling in the single stranded region of the HBV genome and leads to the formation of covalently closed circular DNA, cccDNA (Mason et al., 1983). The "filling in" reaction is believed to be performed by the HBV polymerase protein and when it is complete the cccDNA serves as a template for production of an intermediate RNA (Summers and Mason, 1982). This pre-genomic RNA is longer than its parental DNA because it contains a terminal redundancy. The pre-genomic RNA acts as a template for the synthesis of viral genomic DNA which is produced by reverse transcription (Summers and Mason, 1982). The terminally redundant portion of the pre-genomic RNA acts as an initiation site for reverse transcription and terminal protein is believed to prime the reaction (see above) by binding to this region. Reverse transcription takes place in sub-viral particles that contain polymerase and core proteins as well as HBV RNA and DNA (Hirsch et al., 1990) and reverse transcription produces the minus DNA strand. This is then converted to a partially double-stranded DNA. The minus strand DNA contains two regions which are direct repeats (known as DR1 and DR2). After synthesis of the minus strand DNA the RNA primer is degraded (presumably by the RNaseH domain of the polymerase protein) but the RNA complementary to one of the direct repeats (DR1) is preserved (Loeb et al., 1991). This residual RNA is initially found at the 5' end of the minus strand, bound to DR1, but it subsequently jumps to bind to the homologous DR2 sequence, present at the 3' end of the DNA strand. This RNA, now bound to DR2, acts as a primer for the formation of the second DNA strand synthesis (Loeb et al., 1991), which is incomplete.

One strand of the partially double stranded DNA is then circularised, presumably by an intermolecular jump mediated by the repeated sequences. The viral DNA is then packaged and viral particles are formed and released.

During HBV replication viral proteins are produced from a variety of sub-genomic RNAs (reviewed in Ganem and Varmus, 1987). Transcription and production of these RNAs is regulated by viral promoters and enhancers, many of which have been identified. Some of the transactivating factors that bind to these DNA elements have also been identified and some are liver-specific factors (Antonucci and Rutter, 1989), explaining why HBV replication only occurs in hepatocytes. The X protein of HBV acts as a positive transactivator for some of these viral promoters (Spandau and Lee, 1988) but its precise role in regulating viral protein production is not yet clear.

3) HEPATITIS B ASSOCIATED MALIGNANCY

HBV is the main aetiological factor in human hepatocellular carcinoma (Beasley et al., 1981). It is still not clear how HBV infection causes malignant transformation as it does not possess a classical transforming gene. A number of different mechanisms of HBV carcinogenesis have been proposed. These include promiscuous transactivation of cellular proto-oncogenes by viral transactivators (either X or Pre S1) (Kim et al., 1991), insertional activation of proto-oncogenes during HBV integration into the genome (Wang et al., 1990) and induction of gene amplification (Hatada et al., 1988). It should be noted that HBV infection causes inflammation and cirrhosis and cirrhosis itself may increase the incidence of carcinoma [eg the incidence of hepatocellular carcinoma is increased in haemachromatosis (Finch and Finch, 1955)]. It seems likely that HBV is able to transform cells by a number

of different mechanisms and different mechanisms may dominate in different neoplasms.

4) PATHOLOGY OF HEPATITIS B INFECTION

Adult infection with HBV typically causes an acute self-limiting hepatitis (in 90% of cases) which is followed by viral clearance. A small number of patients (less than 2%) develop fulminant hepatitis and some (approximately 10%) do not experience any overt hepatitis after exposure but develop a chronic infection with sustained viraemia. Neonatal infection or infection in childhood usually causes a chronic infection (90%). Chronic HBV infection is associated with viral persistence and integration of HBV DNA into the human genome. It causes progressive liver damage leading eventually to cirrhosis and sometimes to hepatocellular carcinoma (Nielson et al., 1971).

The reasons why some patients develop chronic HBV infection are not clear. Since neonatal exposure usually causes chronic infection, the development of immunological tolerance to HBV antigens is a likely explanation for chronic neonatal infection. However patients with chronic neonatal infection can develop antibodies against HBV antigens when their immune system is appropriately stimulated (Su et al., 1991), suggesting that tolerance may not be responsible for neonatal chronicity. In adults the ability to respond to some HBV antigens (specifically the HBV surface proteins used in vaccines) is HLA linked (Craven et al., 1986), suggesting that some patients may be genetically unable to mount an effective immune response to HBV and hence might develop persistent infection. However some patients with chronic infection spontaneously eradicate the virus after many years and attempts to find a linkage between chronic HBV infection and immune phenotypes (HLA

typing) have not been successful (H. Thomas - personal communication). Immunological non-responsiveness to HBV antigens is thus an attractive possibility for the failure of some patients to eliminate HBV but the available data suggest that it is not the correct explanation.

In adults with chronic HBV infection there is evidence for a relative deficiency in the production of IFN: patients with chronic infections have reduced levels of circulating IFN (Ikeda et al., 1986); the production of IFNα by peripheral blood mononuclear cells is impaired (Kato et al., 1982) and the levels of 2-5A synthetase (an indirect assay for the production of IFN) are not elevated in peripheral blood cells from patients with chronic HBV infections (Poitrine et al., 1985). This IFN deficiency may be due to inhibition of IFN production by the core protein (see Chapter 1B, 1.2) and it is possible that the development of chronic infection in adults is due to this failure to produce IFN. Attempts to reverse the IFN deficiency with exogenous IFN have shown that viral replication in chronic HBV infection is often inhibited (Jacyna and Thomas, 1990) and in 50% of patients the virus is eradicated, suggesting that in some patients IFN deficiency may be responsible for chronicity. In neonatal HBV infection the concentration of IFN during infection can not be measured, but attempts to treat these patients with IFN have been disappointing. This may indicate that the mechanisms of chronicity in adult and neonatally acquired infection are different or that factors other than IFN deficiency are more important in neonatal infection.

4.1) MECHANISM OF VIRAL ERADICATION.

Eradication of HBV in an acute infection or in a chronic infection, after IFN therapy, probably occurs by the same mechanism. In both there is an increase in hepatic injury prior to viral clearance which is

associated with the production of antibodies directed against the nucleocapsid derivative, e antigen (Jacyna and Thomas, 1990). Patients who eradicate HBV also develop cytotoxic T cells directed against the HBV core protein (Mondelli et al., 1982). The association between the development of an immune response against HBV (both humoral and cell mediated) and viral clearance suggests that eradication of HBV is immunologically mediated, as is the case with a number of other viral infections (reviewed in Doherty and Zinkernagel, 1974). This has been tested directly in woodchucks : adult animals infected with woodchuck HBV develop an acute hepatitis which is followed by viral clearance. Treatment with the immunosuppressive drug cyclosporinA during the early stages of this infection causes a chronic infection, confirming that an immune response is required for successful eradication of HBV (Cote et al., 1991).

Hepatocytes normally express low levels of the antigen-presenting MHC Class I antigens (Montano et al., 1982; Harris and Gill, 1986). These cell surface proteins are essential for the presentation of viral antigens to the immune system (see Chapter 1A, 3) and cells which do not express MHC antigens are not lysed by cytotoxic T cells and may become chronically infected by non-cytopathic viruses (Joly et al., 1991). During IFN treatment hepatocyte expression of MHC Class I antigens is increased (Pignatelli et al., 1986; Chu et al., 1987) and it seems likely that IFN (produced naturally, during an acute infection, or administered therapeutically) may induce the immune response which eliminates HBV by enhancing hepatocyte expression of MHC antigens. This hypothesis is supported by observations in patients undergoing seroconversion: IFN increases the hepatic expression of HLA antigens and the liver is infiltrated by lymphocytes, particularily cytotoxic T cells and natural killer cells (Eggink et al., 1983, Pignatelli et al., 1986). IFN may also assist in viral elimination by directly inhibiting viral replication - tissue

culture studies show that IFN can inhibit the replication of HBV (Hayashi and Koike,1989; Tur-Kaspa et al., 1990).

Eradication of HBV requires the production of IFN and the subsequent development of an immunological response to HBV antigens. During an acute infection IFN is produced spontaneously and in some patients with chronic HBV infection therapeutic IFN can induce an exacerbation of the hepatitis and induce viral clearance. However some patients with chronic HBV infection do not respond to IFN treatment, suggesting that other factors are involved. One possible explanation for the failure of some patients to respond to IFN is that they produce a factor which inhibits the effects of IFN. Clinical studies have suggested that this is a likely explanation for the failure of IFN therapy as patients who do not respond to IFN therapy do not show the expected increase in hepatocyte HLA expression (Pignatelli et al., 1986). Tissue culture experiments with constructs containing the entire HBV genome have suggested that HBV might be able to inhibit the cellular response to IFN (Onji et al., 1990) and it has been suggested that a region within HBV, which is homologous to an IFN responsive element, might act to inhibit the response to IFN by quelching (Thomas et al., 1986). However other studies have suggested that HBV may be able to increase the cellular response to IFN (Zhou et al., 1990). The aims of this thesis were to determine whether HBV does inhibit the cellular response to IFN and to determine the mechanism of this inhibition.

MATERIALS AND METHODS

CHAPTER 2

1) CELLS

Adherent HeLa and HepG2 cells were obtained from Cell Services, Imperial Cancer Research Fund, London. 2fTGH cells were kindly supplied by Dr S. Pellegrini (Imperial Cancer Research Fund, London). All the cell lines were grown as monolayers in Dulbecco's modified Eagle medium (E4; Flow Laboratories) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Bocknek, Canada). The 2fTGH cell line was grown in the presence of hygromycin (40 μg/ml) to ensure that the 6-16/*gpt* construct was retained (Pellegrini et al., 1989). Cells were passaged upon reaching confluency by trypsinisation and replating at an appropriate dilution. Medium was removed and cells were detached by exposure to 0.01% trypsin for less than 1 min. Residual trypsin was inactivated by adding complete medium.

1.1) STORAGE OF CELLS

A semi-confluent plate of cells was trypsinised, suspended in 1.5 ml of a solution containing 50% (v/v) serum, 40% E4 and 10% dimethyl sulphoxide (FSA Laboratories) and cooled in ice in a single plastic vial. The cells were then frozen at -80°C and the following day they were stored in liquid nitrogen. To thaw cells, a vial was placed in a 37°C water bath and then added to 10 ml of pre-warmed growth medium in a 10-cm diameter tissue culture dish. The cells were allowed to settle for 4-5 hr, rinsed twice in medium and then incubated in fresh medium.

1.2) STABLE TRANSFECTION AND SELECTION OF CELLS

The method is a modification of that described by Graham and van der Eb (1973). Cells were seeded at 10^6 per 10-cm dish and the following day the medium was removed and replaced with 7 ml fresh medium. DNA for transfection was dissolved in 40 μl of sterile TE, pH 7.2 (10

mM Tris.HCl, 1mM EDTA) and mixed with 440 µl of 2 x HEBS (1% HEPES, 0.8% NaCl, 0.074% KCl, 0.025% Na_2HPO_4; pH adjusted to 7.05 with NaOH). 400 µl of CC mix (260 mM $CaCl_2$, 1mM Tris, pH 7.2, 0.1 mM EDTA) were added dropwise while air was bubbled through. The mixture was left for 20 min to allow a fine co-precipitate of $CaPO_4$ and DNA to form, and was then added to the cells. After 12 hr the medium was removed and the cells were washed twice in PBSA (1% NaCl, 0.025% KCl, 0.14% Na_2HPO_4, 0.025% KH_2PO_4) before their return to complete medium. After 24 hr cells were placed in medium for selection. To select transfected clones that did not respond to IFN cells were grown in 30 µM 6-thioguanine (6TG) plus IFN and an appropriate selectable marker drug [either neomycin (40 µg/ml) or puromycin (50 µg/ml) depending on the co-transfected marker DNA]. Selective medium was changed every four days, and after two to three weeks resistant colonies were visible. To select clones that did respond to IFN cells were grown in HAT medium (15 µg hypoxanthine, 0.2 µg aminopterin and 15 µg thymidine per ml) plus IFN.

1.3) TRANSIENT TRANSFECTION OF CELLS, CAT AND β-GALACTOSIDASE ASSAYS

HeLa cells were seeded at 10^5 per 3-cm well of a 6-well plate the day before transfection. DNA was precipitated with $CaPO_4$, as above, and left on the cells overnight. To analyse CAT activity the cells were washed twice in PBSA and then incubated in fresh medium (3ml) with or without added IFN. 48 hr later the cell cytoplasm was harvested by lysing the cells on the plate by adding 100 µl of NP40 lysis buffer (0.15 M NaCl, 10 mM Tris, pH 7.6, 1.5 mM $MgCl_2$ and 0.65% NP40). 30 µl of the cytoplasmic extract was then assayed for CAT activity by incubating it with unlabelled chloramphenicol and ^{14}C-acetyl-coenzymeA (Sleigh, 1986).

To analyse β-galactosidase activity cells were transfected and cytoplasmic extracts were prepared, as above. 100 µl of the extract was then incubated with 400 µl of LAC-Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) plus 100 µl of a solution of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (at a concentration of 4 mg/ml) until a pale yellow colour was visible. 250 µl of 2 M Na_2CO_3 was then added and the tubes were spun in a microfuge for 5 min. The supernatant was removed and the absorbtion at 440 nm determined against a control extract which contained only NP40 lysis buffer.

To stain cells for expression of β-galactosidase the cells were fixed by washing in 0.5% glutaraldehyde in PBSA (v/v) and then incubated in a solution of 5 mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ and 1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for 4 hr.

2) INTERFERONS

IFNα is a highly purified mixture of human subspecies obtained from Wellcome Research Laboratories, specific activity 10^8 IU/mg protein, (Allen et al, 1982) and was a gift of Dr K Fantes, Wellcome Research Laboratories. Recombinant human IFNβ was a gift of Triton Biosciences Inc. and recombinant human IFNγ (specific activity 2 x 10^7 IU/mg protein was a gift of Dr GR Adolf, Ernst-Boehringer-Institute fur Arzneimittel Forschung. Cells were treated with 500 IU/ml of IFN unless otherwise stated and all treatments with IFN α and β were for 6 hr unless stated to the contrary. IFNγ treatment was routinely for 18 hr.

2.1) RECEPTOR BINDING ASSAYS

This assay was carried out in triplicate. Doubling dilutions of ^{125}I-IFNγ (3(^{125}I) iodotyrosyl-IFNγ;) were added to six consecutive wells of a 96-well microtitre plate (from 400 to 12.5 IU/ml). The doubling

dilutions of radiolabelled IFNγ were repeated in the same number of wells but a 100 fold or greater excess of cold competitor IFNγ was added to these wells. The IFNs were diluted with E4, 10% serum 10 mM HEPES, pH 7.4. The volume per well was 100 µl. Cells removed from dishes with Versene were collected by centrifugation at 100 x g for 5 min. The cell pellet was resuspended in the E4-HEPES mix, as above, to a density of 5 x 10^6 cells per ml and 100 µl of the suspension (5 x 10^5 cells) was added to each prepared well. The mixture of cells and IFN was left on ice for 90 min and then washed three times in PBSA, 1% serum, 0.01% $CaCl_2$, 0.01% $MgCl_2$. The cells were pelleted between each wash by centrifugation at 500 x g for 2 min at 4 C. To carry out a Scatchard analysis (Scatchard, 1947) it was necessary to determine the total amount of radioactivity added to each well, the amount bound to the cells after washing and the amount remaining unbound and appropriate samples were assayed for ^{125} I in a gamma counter. The assay for IFNα receptors was performed as above using iodinated ^{125} I labelled IFNα, kindly prepared by Dr A Ackrill (ICRF), specific activity 1 x 10^7 IU/mg protein

3) ANALYSIS OF RNA

3.1) EXTRACTION OF RNA

All solutions, pipette tips and tubes used in preparing RNA were autoclaved before use and maintained free of RNase. The method used is a slight modification of that described in Maniatis et al., 1982. Cells were washed twice in ice cold PBSA and scraped from the plates using a rubber policeman. They were pelleted by centrifugation in a microfuge for 15 sec and then resuspended in 500 µl of NP40 lysis buffer, see above. Cells were incubated on ice for 5 min to lyse the cell membrane and the nucleii were pelleted by centrifugation in a microfuge at 4°C for

5 min. The cytoplasmic supernatant solution was adjusted to 10 mM EDTA, 0.5% SDS and then extracted twice with phenol/chloroform and once with chloroform. The RNA was precipitated by adding 0.1 volumes of 3 M NaOAc, pH 5.5, and 1 volume of propan-2-ol, incubated on dry ice for 20 min and centrifuged in a microfuge at 4°C for 15 min. The pellet was resuspended in distilled water and the concentration determined by measuring the absorbance at 260 nm (at this wavelength 1 mg of RNA per ml has an optical density of 40).

3.2) NORTHERN ANALYSIS OF RNA

This was carried out using a modification of the method of Seed (1982). Precipitated RNA (10 µg) was pelleted in a microfuge for 15 min and the pellet was then resuspended in 10 µl of sample buffer (50% deionised formamide, 2.2 M formaldehyde, 1 x MOPS running buffer (20 mM 3-N-morpholinopropanesulphonic acid). The RNA was denatured by heating it to 70 C for 10 min. Gel loading buffer (3% Ficoll, 0.05% bromophenol blue and 0.05% xylene cyanol and 50 µg/ml of ethidium bromide) was added and the sample was loaded into a 1.2% agarose slab gel made in 1 x MOPS running buffer, 10 mM EDTA, 50 mM NaOAc, pH 7.5, and 2.2 M formaldehyde. Electrophoresis was performed in 1 x MOPS running buffer until the bromophenol blue dye was close to the end of the gel.

The gel was then laid on top of a moist wick of 3MM paper fed from a reservoir of 20 x SSC (1 x SSC is 0.15 M NaCl, 0.15 M Na citrate, pH adjusted to 7.5 with NaOH). A nitrocellulose membrane (Millipore) the same size as the gel was first wetted in water and then placed on top of the gel. A stack of 3MM paper and paper towels (the same size as the gel) was placed on top of the filter, weighted down and left overnight. The paper towels cause the 20 x SSC to be drawn up through the gel, transferring the RNA onto the nitrocellulose filter. The

filter was then baked for 1-2 hr at 80°C. The ethidium bromide in the gel loading buffer allows visualisation, on a UV transilluminator, of 28S and 18S ribosomal RNA transferred to the filter. This allows the success of the procedure to be monitored and, as the molecular weights of 28S and 18S ribosomal RNA are known (4.7 kb and 1.9 kb respectively), the molecular weights of the mRNA of interest can be estimated.

3.3) PREPARATION OF cDNA PROBES

The DNAs used as templates have been previously described: 6-16, 1-8, 2'5'-oligoadenylate synthetase (2-5A), actin (Kelly et al., 1985); HLA DRα (Lee et al., 1982); HLA8ABC5 - MHC Class I (Trowsdale et al., 1984); Ring 4 (Trowsdale et al., 1990). Radiolabelled probes were made by random priming DNA using a modification of the method of Feinberg and Vogelstein (1983). DNA (50 -100 ng) in 2 µl of TE, pH7.2, was added to 10 µl of HTM buffer (0.5 M HEPES pH 6.6, 0.125 M Tris.HCl, pH 8.0, 12.5 mM $MgCl_2$, 20 mM β-mercaptoethanol) and 2 µl of random oligonucleotide primers (Pharmacia; 90 OD units/ml). This mixture was heated at 90 C for two min to denature the DNA and then returned to ice. 50 µCi of α^{32}P- labelled dCTP (7,000 Ci/mmol) and dATP, TTP, dGTP were added (each at 60 µM). The reaction was started by adding 1-5 units of the Klenow fragment of DNA polymerase I (Amersham) and incubated at 37 C for 2 hr. The probe was separated from the unincorporated nucleotides by centrifugation for 10 sec in a microfuge through fine Sephadex G-50 packed in a Costar Spin-X column (1 ml bed volume). Probes synthesised in this way usually had specific activities of approximately 10^9 cpm per µg of input DNA.

3.4) HYBRIDISATION OF NORTHERN FILTERS

The filter was wetted in 6 x SSC and prehybridised by sealing in a plastic bag with 5-10 ml of 50% deionised formamide, 5 x SSC, 5 x Denhardt's reagent (0.02% (w/v) each of bovine serum albumin, Ficoll

and polyvinylpyrrolidone), 0.1% SDS, 100 µg/ml sheared and denatured herring testis DNA. The filter was rocked in this solution for at least 2 hr at 42°C. Hybridisation was carried out under the same conditions with the addition of 2 x 10^6 cpm of denatured random-primed probe per ml of solution. After 16 hr the filter was washed once in 2 x SSC, 0.1% SDS, then twice in 0.2 x SSC, 0.1% SDS. Washing was carried out at 60°C and each wash was for 30 min. The filter was then blotted dry, wrapped in saran wrap and assayed by autoradiography.

To remove bound probe from the filter the filter was heated at 98°C for 5 min in a solution of 0.02% sodium pyrophosphate, 1% SDS and 1mM Tris.HCl.

3.5) RNASE PROTECTION ASSAYS

The method is adapted from Sambrook et al (1989). The 6-16, 9-27 and actin DNAs used as templates have been described previously (Ackrill et al., 1991a). The probes protect fragments of 190,160 and 130 bp respectively. The template for IFNβ yields a protected fragment of 270 bp (Zinn et al., 1983) and the GBP and p68 kinase probes (Decker et al., 1989; Meurs et al., 1990) protect fragments of 130 and 300 bp, respectively.

The template probes were in pGEM vectors and radiolabelled probes were made by in vitro transcription of linearised DNA templates using SP6 RNA polymerase. The DNA template was linearised at the 3' end of the desired probe by digestion with an appropriate enzyme, extracted twice with phenol/chloroform and precipitated with ethanol. Labelling was at 37°C and each reaction mixture contained 10 µl of α^{32}P - UTP (110 µCi at 400 Ci/mmol), 4 µl of 5 x buffer (200 mM Tris.HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine), 3 µl of 10 mM rATP, rGTP, rCTP, 1 µl of 200mM dithiothreitol (DTT), 1 µl of RNasin (RNase-inhibitor, 60 units/ml, Boehringer Mannheim), 5 units of SP6 RNA polymerase and 1

µg of prepared template DNA. The reaction mixture was incubated for 2 hr before adding 1 µl of RNase free DNase (Boehringer Mannheim) for a further 10 min. The volume was increased by adding 80 µl of TES (10 mM Tris.HCl, pH7.4, 1 mM EDTA and 0.1% SDS) and the mixture was extracted with phenol/chloroform. The probe and 20 µg of tRNA (Boehringer Mannheim) were co-precipitated with ethanol (3 volumes) and 2.5 M NH_4OAc. The probe was resuspended in TES, reprecipitated and stored at -80°C.

RNA was mapped as follows. 10 µg of RNA was suspended in 24 µl of deionised formamide and mixed with 3 x 10^5 cpm of each probe and 3 µl of hybridisation mix (400 mM PIPES, pH 6.4, 4 M NaCl, 10 mM EDTA, pH 8.0). The volume was adjusted to 30 µl by adding TES. The mix was heated to 85°C for 10 min to denature the RNA and then incubated at 47°C overnight to allow complementary RNAs to anneal to the probe. A solution (350 µl) of RNase A (40 µg/ml) and RNase T1 (2µg/ml) in 10 mM Tris.HCl plus 5 mM EDTA plus 300 mM NaCl was added and incubated at 37°C for 30 min to destroy single stranded RNAs. The RNases were then removed by incubating the reaction at 37°C for a further 15 min with 20 µg of Proteinase K (Boehringer Mannheim) and 20 µl of 10% SDS followed by extraction with phenol. The protected fragments were co-precipitated with 20 µg of tRNA by adding an equal volume of propan-2-ol and then dissolved in 5 µl of formamide/dye mix (9 volumes of formamide: 1 volume of gel loading buffer) and separated by electrophoresis in a 6% denaturing gel.

The denaturing gels were made from a solution of 40% acrylamide (38% acrylamide; 2% bis-acrylamide - Biorad) altered to the desired percentage in 1 x TBE (90 mM Tris-borate, pH8.3, 1 mM EDTA) and 7 M urea. 175 µl of fresh 10% (w/v) ammonium persulphate and 50 µl of N,N,N',N', tetramethylethylenediamine (TEMED) were added to a gel

solution of 50 ml to catalyse polymerisation. The gel was formed between 20 x 40 glass plates separated by 0.4 mm spacers. Electrophoresis was in 1 x TBE at a constant 40 Watts and the gel was pre-run for 1 hr. Radiolabelled DNA markers were prepared by random priming (see northern probe preparation) an Msp1 digest of pBR322 DNA and an aliquot of these markers were electrophoresed in parallel with the RNA samples.

4) BAND SHIFT ASSAYS

4.1) PREPARATION OF WHOLE CELL EXTRACTS

The method used was a modification of that of Zimarino and Wu (1987). Frozen cell pellets were thawed in three volumes of extraction buffer [10 mM HEPES, pH7.9, 0.4 M NaCl,1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol and 0.5 mM phenylmethanesulphononylfluoride (PMSF)]. The lysate was centrifuged at 100,000 x g for 15 min and the supernatant was used. The concentration of protein was determined by diluting samples 1:1000 in Bradford's reagent (Biorad - Bradford, 1976), incubating them at room temperature for 10 min and determining the absorbance at 595 nm. Concentrations were determined by comparison with known concentrations of bovine serum albumin. The protein concentration of samples prepared in this way were usually 6 to 7 mg/ml.

4.2) PREPARATION OF PROBES

Oligonucleotides synthesised by Dr I Goldsmith (ICRF) were supplied as precipitates under ethanol and were resuspended in TE (pH,7.5) before use. The 9-27 39-mer probe contains the 9-27 ISRE and flanking sequences (TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAATACA). Single stranded HPLC purified oligonucleotides (200

ng per reaction) were end- labelled using 10 units of T4 phage polynucleotide kinase. The reaction was carried out at 37°C for 30 min in 70 mM Tris.HCL, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT. The phosphate donor was 100 µCi γ ^{32}P- labelled ATP (5000 Ci/mmol). NaCl was then added to a concentration of 75 mM and a slight excess of unlabelled oligonucleotide, representing the complementary strand was added. The mixture was heated to 70°C and allowed to cool slowly over several hours to allow the strands to anneal. The labelled probe was separated from unincorporated nucleotides by spinning the mixture through a fine G-50 Sephadex column with a 1ml bed volume.

4.3) BINDING REACTIONS

The method used is a modification of that described by Zimarino and Wu (1987). 5 µl of cell extract (6-7 mg protein / ml) was diluted with 5 µl of extraction buffer (see above) without NaCl and preincubated with 1.5 µl of poly (dI.dC)-poly(dI.dC) (5 mg/ml, Pharmacia) for 10 min. Additional components were added in 10 µl total volume to final concentrations of 0.5 mg/ml yeast tRNA, 0.5 mg/ml random pentanucleotides (pdN5, Pharmacia), 0.25 mg/ml sheared E. Coli DNA, 2 mg/ml bovine serum albumin, 4% Ficoll and 1 ng end labelled probe (30,000 cpm)/10 µl. After incubation for 15 min, DNA-protein complexes were separarted by electrophoresis in 6% polyacrylamide gels in 0.5 x TBE. Binding reactions were carried out at room temperature and the final concentration of NaCl was 93 mM. In complementation bandshift assays 2.5 µl of each starting extract were mixed for 10 min at room temperature before the DNA probe was added.

4.4) POLYACRYLAMIDE GEL ELECTROPHORESIS

The gels were made from a solution of 30% acrylamide: 0.8% bis-acrylamide (Biorad) by diluting 1:5 in 0.5 x TBE. Polymerisation was catalysed by adding 300 µl of 10% (v/v) ammonium persulphate and 50

µl TEMED. Electrophoresis was performed in 0.5 x TBE.

5) ANALYSIS OF PROTEINS.

5.1) SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

The method used was that of Laemmeli (1970). The running gel was 0.37 M Tris.HCl, pH 8.8, 0.1% SDS, and acrylamide [29.25 acrylamide: 0.75 bis-acrylamide (Biorad)] at varying final concentrations, usually 10% or 12%. Polymerisation was catalysed by adding 25 µl of TEMED and 100 µl of 10% (v/v) ammonium persulphate to 30 ml of the gel solution. The solution was poured between two 10 cm x 10 cm glass plates separated by 0.8 cm spacers. The stacking gel was 125 mM Tris.HCl, pH6.8, 0.1% SDS, 5% acrylamide and 10 ml of this solution was polymerised by the addition of 10 µl of TEMED and 100 µl of ammonium persulphate and layered on top of the running gel.

Cell extracts were prepared for electrophoresis by resuspending cell pellets in an equal volume of 2 x loading buffer (125 mM Tris.HCl, pH6.8, 4% SDS, 20% glycerol (v/v), 1.6 M β-mercaptoethanol and 0.2% bromophenol blue) and sonicating for 3 min to shear the DNA. Samples were boiled for 2 min prior to loading and then electrophoresed in 25 mM Tris.HCl, pH 8.3, 192 mM glycine, 0.1% SDS at 50 mA for 4 hours. Rainbow protein molecular weight markers or radiolabelled molecular weight markers (both from Amersham) were run in parallel.

5.2) WESTERN TRANSFER OF PROTEINS.

The method used is adapted from Towbin et al (1979). Protein was transferred onto nitrocellulose (Millipore) in 20% methanol, 192 mM glycine, 25 mM Tris base using a Biorad dry blotting device for 3 hr. The filter was incubated overnight in PBSA plus 5% (w/v) milk powder (Marvel) and then incubated with primary antibody for periods ranging

SUMMARY OF ANTIBODIES DIRECTED AGAINST HBV TERMINAL PROTEIN AND THEIR USAGE.

ANTIBODY	ANTIGEN	ORIGIN	USAGE	
			Direct cell staining	Western blotting
M	Terminal protein*	Rabbit (Polyclonal)	+	-
W	Peptide (aa 30-50)	Mouse (monoclonal)	+	+
C	Peptide (aa 1-40)	Guinea pig (Polyclonal)	-	+

+ denotes successful usage, - indicates that this antibody does not work in this assay.

* full length terminal protein was produced as a fusion protein in bacteria - see Goldin et al. (1990) for details. Peptides were manufactured in vitro, the numbers indicated in brackets (aa) refer to the positions of the amino acids in polymerase- the first methionine of polymerase is designated 1.

from 2 to 16 hr at 4°C. The primary antibodies were used at dilutions ranging from 1:50 to 1:500 and each dilution was used with different concentrations of Tween-20, ranging from 0.1% to 5%. Filters were incubated with primary antibody in a sealed bag (total volume 5-10 ml) and 3 different anti-polymerase antibodies were used: antibody M is a polyclonal rabbit antibody raised against a bacterial fusion protein containing the terminal protein domain of HBV polymerase[(kindly supplied by Dr M McGarvey, St Mary's Hospital, London (Goldin et al, 1990)]; antibody W is a murine monoclonal raised against amino acids 30-50 of HBV polymerase (kindly supplied by Dr H Will, Max-Planck-Institut, Munich) and antibody C is polyclonal guinea pig antiserum raised against amino acids 0-40 of HBV polymerase (kindly supplied by Dr B Clarke, Wellcome Institute for Research, London). After incubation with primary antibody the filter was washed three times in 0.1% Tween-20 in PBSA and then incubated with the secondary antibody at dilutions ranging from 1:1000 to 1:50,000 in PBSA, 5% Marvel and 0.1% Tween-20. The secondary antibodies were all conjugated with peroxidase and were raised against the species of the primary antibody. After incubation with the secondary antibody for 1 hour the filters were washed 3 times in PBSA, 0.1% Tween-20 and washed once in PBSA and varying concentrations of Tween-20 ranging from 0.1% to 5%. All the washes were performed at room temperature for 15 min. Binding of the peroxidase-conjugated antibody was visualised by incubating the filter with a substrate for peroxidase, diaminobenzidine, which yields a brown product. Six mg of diaminobenzidine tetrachloride was dissolved in 10 ml PBSA and 100 µl of a 3% solution of H_2O_2 was added. After the brown colour developed the reaction was stopped by washing in water. Bound antibody was also detected using the chemiluminescence technique (Amersham) in which the filter was immersed in the reagents, as supplied by Amersham, for one min and

then blotted dry before rapidly exposing it to X-ray film. In this technique, peroxidase acts on a substrate to generate flashes of light which can be detected by autoradiography.

5.3) PROTEIN LABELLING AND IMMUNOPRECIPITATION

Cells from a semi-confluent 10-cm plate were grown for 1 hr in E4 medium lacking methionine. The cells were then grown in E4 medium, minus methionine, containing ^{35}S methionine (600-1000 Ci/mmol) at 100 µCi/ml. The cells were harvested 4 hr later, washed twice in PBSA, lysed in 1 ml lysis buffer (50 mM Tris.HCl, pH 7.4, 1% NP40, 5 mM EGTA, 150 mM NaCl and 50 µg/ml PMSF) and spun for 1 min in a microfuge. The supernatant solution (500 µl) was mixed with antisera (10 µl to 50 µl) and the mixture tumbled for 12 hr at 4°C. 20 µl of protein A sepharose (1:1 suspension in PBSA) was added and the mixture tumbled for a further 1 hr. The sepharose beads were pelleted in a microfuge and washed 5 times in lysis buffer. The beads were then taken up in an equal volume of sample buffer (see above), incubated at 95°C for 5 min and spun down. The supernatant solution was run in a polyacrylamide gel which was then dried and analysed by autoradiography.

5.4) DIRECT STAINING OF CELLS AND LIVER BIOPSIES.

Cells were prepared for staining by growing them on sterile glass cover slips for 24 hr. The cover slips were rinsed in PBSA and cells were fixed by immersing them in acetone for 30 sec. Liver biopsy specimens, prepared by dewaxing in xylene, were dehydrated in decreasing concentrations of alcohol / water [final concentration of alcohol was 60% (v/v)]. Endogenous peroxidase was blocked by incubating the samples in 1% H_2O_2 in methanol for 15 min. The sections and cells were washed in PBSA before incubation with the primary antibody. Primary antibodies were diluted in PBSA containing 20% (v/v) normal swine serum and all HBV related antibodies were used at a dilution of 1:400. Antibodies

against core and surface proteins (Goldin et al., 1990) were kindly supplied by Dr J. Waters, (St Mary's Hospital, London) and the anti-polymerase serum was antibody M, described above. Primary antibodies were added to the specimens for 1 hr, the slides and cover slips were washed in PBSA and then incubated with second antibody. To detect bound murine or rabbit anti HBV antibodies, biotinylated rabbit anti-mouse or biotinylated swine anti-rabbit antibodies were used at a dilution of 1:300 for 30 min before incubating the sections with peroxidase conjugated avidin/biotin complex (supplied by Dako and used as recommended) for a further 30 min. The sections were then washed and bound antibody visualised by incubating the slides with diaminobenzidine, as described above.

To doubly stain biopsies, HBV proteins were first detected as above and the slides were then incubated with anti- β_2-microglobulin antibodies at a dilution of 1:100 for 12 hr. When the anti-HBV antibody was murine (surface) rabbit anti-β_2-microglobulin antibodies were used and when the primary antibody was derived from a rabbit (polymerase) murine anti-β_2-microglobulin antibodies (Sigma) were used. Bound antibody was detected by incubating with an alkaline phosphatase conjugated antibody directed against the appropriate species at a dilution of 1:500 for 1 hr. After washing, the slides were incubated with APAAP reagent (Dako) as recommended and the alkaline phosphatase visualised by incubation with Fast Red (10 μg in 10 ml PBSA with 2 μg of levamisole) for 5 min. Slides and cells were counterstained by immersion in haematoxilin. To quantitate the expression of the various proteins in liver biopsy specimens the biopsies were examined and the number of cells expressing the various proteins were counted. Slides were examined without prior knowledge of the patients details and at least 1000 cells per slide were examined.

5.5) ADSORBTION OF ANTI-POLYMERASE SERUM

In an attempt to reduce binding to cellular proteins antibody M was passed through a column (final bed volume 1 ml) containing activated sepharose (Sigma) cross linked to 200 mg of protein, derived from 2fTGH cells by lysis in NP40 lysis buffer (cross linking was performed in accordance with the manufacturers instructions). Antibody which was eluted from the column with NP40 lysis buffer was used for a number of Western blot and immunoprecipitation experiments.

5.6) DETECTION OF REVERSE TRANSCRIPTASE ACTIVITY

The method used was modified from Offensperger et al.(1988). Cytoplasmic cell extracts were prepared as described (see Preparation of whole cell extracts) and 10 µl of extract was added to a mix containing final concentrations of 1.5 mM $MgCl_2$, 150 mM NaCl supplemented with 1 µCi of α^{32}P-labelled dTTP . A reverse transcriptase substrate [poly (rA)-$(dT)_{12}$ (Pharmacia)] was added at a final concentration of 100 µg/ml and the mixture incubated at 37°C for varying times. 50 µg of calf thymus DNA, 0.5% sodium pyrophosphate, and 1ml 10% trichloroacetic acid (TCA) were added and samples were placed on ice for 30 min. TCA- precipitable counts were collected on glass fibre filters, washed three times in 10% TCA and then measured.

6) BACTERIAL STRAINS.

The E.Coli strain DH5 was used for growing plasmids (sup E44, hsd17, recA1, endA1, gyrA96, thi-1, relA1: Sambrook et al 1989). E.Coli were grown in liquid culture by vigorous shaking overnight at 37°C in Luria Broth (LB; 1% Bacto-trypone, 1% NaCl, 0.5% Bacto-yeast extract). The plasmids carried the β-lactamase gene which encodes resistance to ampicillin. E Coli transformed by these plasmids were grown in LB

containing 50 µg/ml ampicillin or on petri plates with 1.5% bact-agar (L-agar) and antibiotics.

6.1) TRANSFORMATION OF E.COLI

6.1a) PREPARATION OF COMPETENT BACTERIA.

The method of Hanahan (1983) was used. An overnight culture of E.coli, grown in SOB medium (2% bacto-pyrone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) was innoculated at 1:500 dilution in fresh SOB and grown until the culture reached log-phase (absorbance at 550 nm approximately 0.3). The flask was chilled on ice and the bacteria harvested by centrifugation at 1000 x g at 4°C and resuspended in 0.33 of the original volume with ice cold FB1 solution (100 mM RbCl, 50mM $MnCl_2.4H_20$, 30 mM KOAc, pH 6.2, 10 mM $CaCl_2.2H_20$, 5% glycerol, pH adjusted to 5.8 with acetic acid and filter sterilised). The bacteria were incubated on ice for 15 min, centrifuged as before and resuspended in 0.08 volumes of ice cold FB2 (10 mM MOPS pH6.8, 10 mM RbCl, 75 mM $CaCl_2,2H_20$, 15% glycerol; pH adjusted to 6.8 with NaOH and filter sterilised). After incubation on ice for a further 15 min the bacteria were dispensed in 100 µl aliquots, flash frozen in a dry ice/ethanol bath and stored at -80°C.

6.1b) TRANSFORMATION OF E.COLI WITH PLASMIDS

Aliquots of frozen competent bacteria were recovered by thawing on ice. Approximately 10 ng of DNA was added, mixed gently and incubated on ice for 30 min. Uptake of DNA was furter facilitated by a heat shock at 42°C for 90 sec. The bacteria were returned to ice before adding 500 µl of LB followed by incubation at 37°C for 1 hr. 200 µl of the mix of bacteria was plated on L-agar containing ampicillin (50 µg/ml), allowed to dry and incubated at 37°C for 16 hr.

6.2) PURIFICATION OF PLASMIDS FROM BACTERIA

6.2a) LARGE SCALE PURIFICATION

A modification of the method of Birnboim and Doly (1979) was used. A 500 ml overnight culture of E.Coli was centrifuged at 4,000 x g and the pellet resuspended in 10 ml solution 1 (25 mM Tris.HCl, pH 8.0, 10 mM EDTA). After incubation for 5 min at room temperature 20 ml of freshly prepared solution 2 was added (0.2 M NaOH, 1% SDS) and mixed by inverting the tube three times. After 5 min on ice, 15 ml of ice-cold solution 3 (prepared by mixing 60 ml KOAc with 11.5 ml glacial acetic acid and 28.5 ml water) was added and mixed thoroughly. After 15 min the mixture was spun at 25,000 x g. Cell DNA and other debris pelleted to the bottom of the tube while the plasmid remained in solution. Plasmid DNA was precipitated from the supernatant solution at room temperature by adding an equal volume of propan-2-ol and was then recovered by centrifugation. The DNA pellet was dried in a desiccator and resuspended in TE, pH 7.2. CsCl and ethidium bromide were added to final concentrations of 0.9 g/ml and 200μg/ml respectively and the solution was heat sealed into 4 ml sealable tubes (Beckman). After centrifugation for 4hr at 400,000 x g supercoiled plasmid DNA, present as a discrete band near the middle of the tube, was removed with a needle and syringe and the ethidium bromide extracted with water-saturated butan-1-ol. Plasmid DNA was precipitated with ethanol, washed in 70% ethanol and water (v/v), dried and resuspended in water. The concentration was determined by measuring the absorbance at 260nm (50 mg of DNA in 1 ml has an absorbance at 260 nm of 1).

6.2b) SMALL SCALE PLASMID PURIFICATION

A 1.5 ml overnight culture of bacteria was centrifuged for 10 sec in a microfuge and the bacterial pellet resuspended in 100 μl of solution 1. After 5 min at room temperature 200 μl of solution 2 was added and

mixed by inverting the tube. After 5 min on ice 150 µl of ice-cold solution 3 was added and mixed thoroughly. After a further 5 min on ice the tube was centrifuged in a microfuge for 5 min at 4°C. The supernatant solution was transferred to a fresh tube and the remaining proteins were extracted with phenol/ chloroform. Plasmid DNA was precipitated by adding equal volumes of ethanol and recovered by centrifugation. The DNA pellet was suspended in 50 µl of TE containing 10 mg/ml DNase free RNase (Sigma) to digest contaminating RNA.

7) MANIPULATION OF DNA.

7.1) RESTRICTION DIGESTION OF DNA.

Enzymes were purchased from New England Biolabs and digestion of DNA was carried out under the conditions recommended by the manufacturers. DNA was routinely digested at a concentration of 50 ng/µl and precipitated between digestions that required significantly different conditions. Restriction digests were analysed by agarose gel electrophoresis.

7.2) AGAROSE GEL ELECTROPHORESIS

Agarose (Gibco) was dissolved in 1 x TAE (40 mM Tris-acetate, pH 7.5, 2 mM EDTA). The concentration of agarose used depended on the size of the expected fragments, and was normally 0.8%. Ethidium bromide at a final concentration of 20 µg/ml was added to the running buffer, allowing visualisation of the DNA on a UV transilluminator. Molecular weight markers (Bethseda Research Laboratories) were electrophoresed in parallel to allow size determination.

7.3) LIGATION OF DNA

Digested vector DNA was incubated with 1 unit calf intestinal phosphatase (Boehringer Mannheim) at 37°C for 30 mins. The enzyme was removed by immediate electrophoresis on an agarose gel. Removal of the phosphatase groups from the vector prevents it recircularising in

subsequent ligation reactions. When it was neccessary to remove recessed ends of DNA fragments, created after enzyme digestion, the DNA overhangs were filled in with the Klenow fragment of DNA polymerase I. Reactions were at 37°C for 30 min and used approximately 1 µg of DNA with 10 x Klenow buffer (200 mM Tris.HCl, pH 7.5, 50 mM $MgCl_2$, 2mM each of ATP, TTP, CTP, GTP, 100 mM β-mercaptoetanol). The reaction was stopped by incubation at 65°C for 15 minutes and the DNA extracted with phenol/chloroform and precipitated with ethanol. DNA fragments were purified from agarose gels using a Stratagene "Geneclean" kit.

In ligation reactions, mixtures of approximately 100 ng of phosphatased vector and a three fold molar excess of fragment were suspended in ligase buffer (50 mM Tris.HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM ATP, 20 mM DTT) with 40 units of T4 DNA ligase. The total volume was 10 µl and the reactions were incubated at room temperature overnight.

7.4) SEQUENCING OF DNA

The method used was adapted from that of Sanger et al (1977). Plasmid DNA was sequenced enzymatically by the dideoxy chain-termination procedure using the USB Sequenase kit as described by the manufacturer.

8) DNA CONSTRUCTS

8.1) HBV GENE CONSTRUCTS

DNA constructs were made from a monomer of HBV subtype *ayw* (kindly supplied by Dr P. Karyannis, St Mary's Hospital, London) and appropriate fragments were sub-cloned into the polylinker in the vector pJ3ω (Morgenstern and Land, 1990). The 0.8 kb *Fsp 1-Ssp 1* (core) and 2

kb *EcoRI-Bgl 11* (surface) fragments were cloned into the *Sma 1* and *EcoRI- Bgl 11* sites respectively (Figure 2.1). A polymerase insert was constructed as shown in Figure 2.2. HBV was excised from the vector and recircularised. The 2.5 kb *BspM11- HgiA1* fragment was excised and linked to a synthetic oligomer designed to complete the open reading frame and to include a new *Cla 1* site adjacent to the ATG of the polymerase gene. The recircularised fragment was cut with *Cla 1* and cloned into the *Cla 1* site of pJ3ω in both the forward and reverse orientations. The X insert was the *Nco 1-Alu 1* fragment of HBV *adr4* excised and cloned into a vector to add a *Stu 1* and *Bgl 11* site to the 5' and 3' ends respectively (kindly supplied by Dr I. Saito, National Institute of Health, Tokyo). The *Stu 1 - Bgl 11* fragment was excised and cloned into the *Sma 1 - Bgl 11* site in pJ3ω. The constructs pJ3-TP and pJ3-POL-COOH were made by excising the *Ban 11 - Kpn 1* or *BspM11 - Ban 11* fragments from pJ3POL and recircularising the construct after blunting the ends.

Mutant HBV genomes were prepared, from a wild-type HBV genome, by polymerase chain reaction using primers which contained the requisite base changes. One primer commenced at the unique *Fsp1* site in HBV (adjacent to the pre-core ATG) and extended through the mutated region and the second primer commenced 200 bp upstream from the POL ATG. Polymerase chain reaction DNA synthesis was performed using reagents supplied by Amersham, in accordance with the manufacturers recommendations, and the DNA produced in this way was cut with *BspM11* (which cuts a unique site adjacent to the second primer) and the resulting *Fsp1* to *BspM11* mutated DNA fragment subcloned into appropriate constructs.

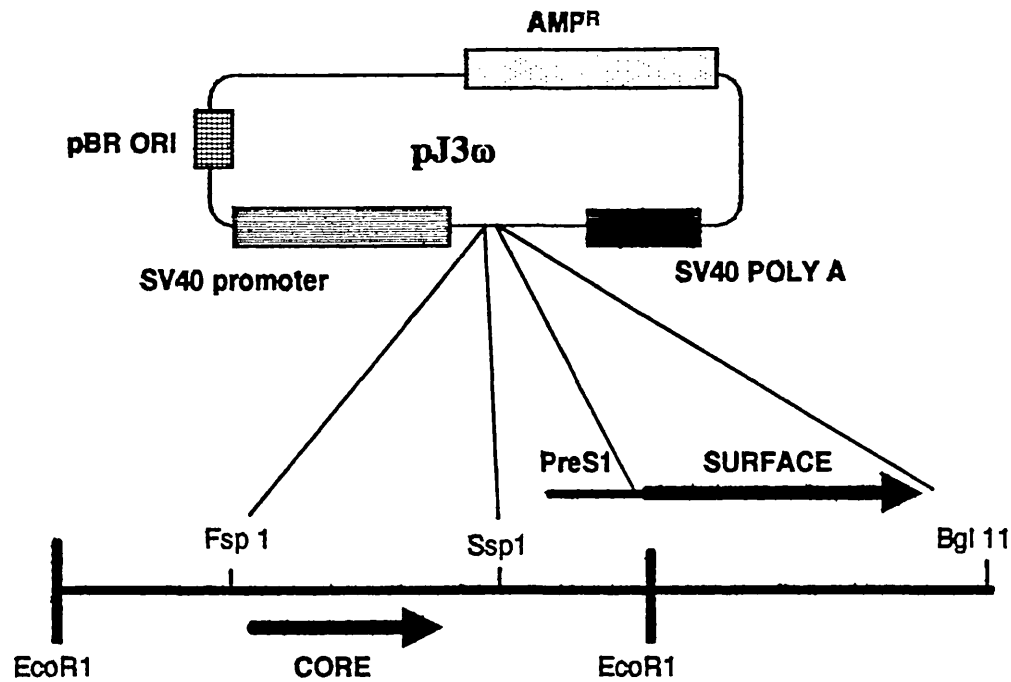
8.2) EBNA-1 and EBO constructs

These were kindly supplied by Dr D.Goeddell (Genentech) and they

are diagrammed overleaf (Figure 2.3). p220.2 ori contains the gene encoding EBNA-1 and pSW8 contains the EBV virus origin of replication (oriP) and a cloning site (*Sfi1*) adjacent to the cytomegalovirus promoter (CMV). cDNA libraries were cloned into the *Sfi1* site.

FIGURE 2.1

Subcloning HBV surface and core genes into pJ3ω



Linearised HBV dimer (incomplete) showing relevant enzyme sites and genes.

FIGURE 2.2

Sub-cloning of HBV polymerase into pJ3ω.

See text for details

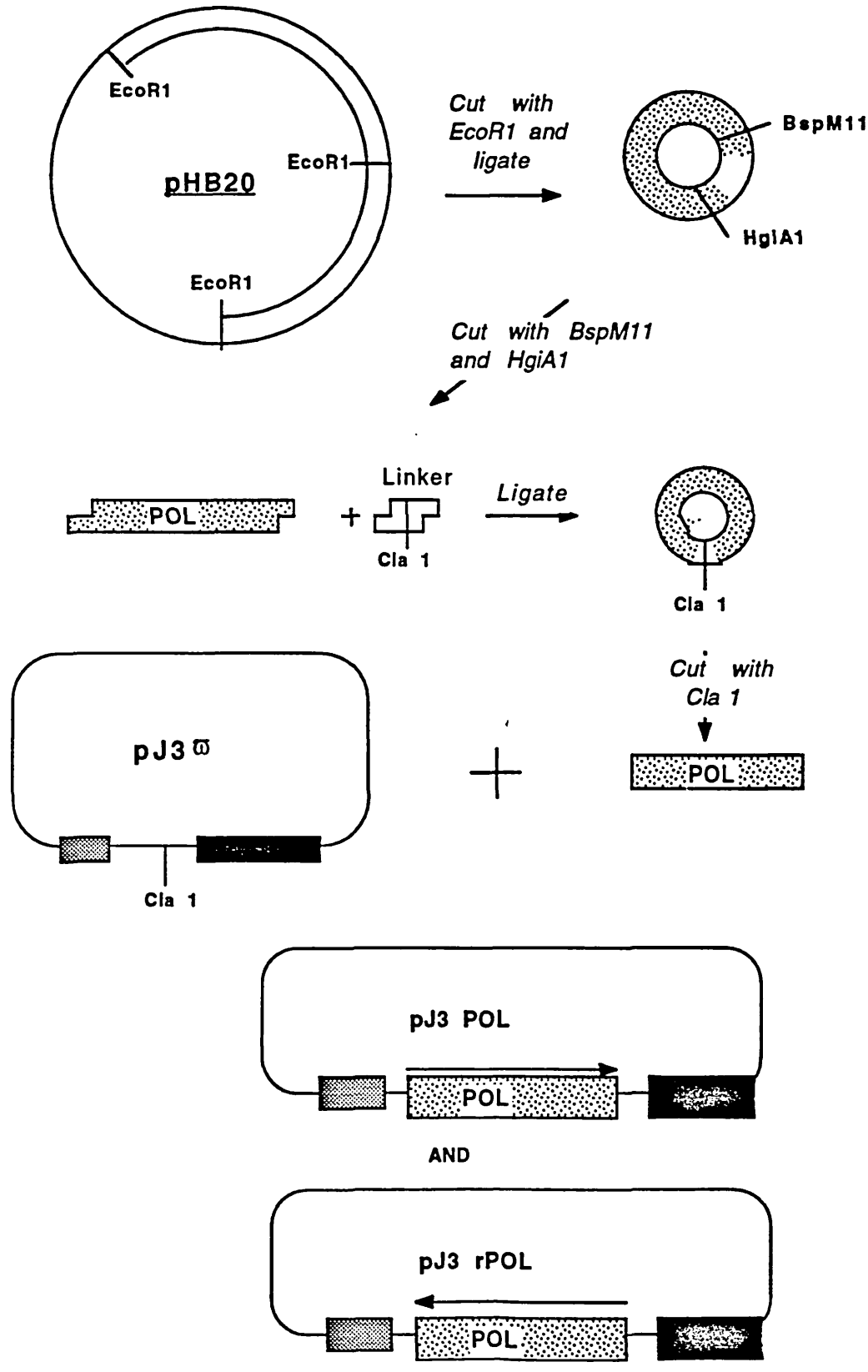
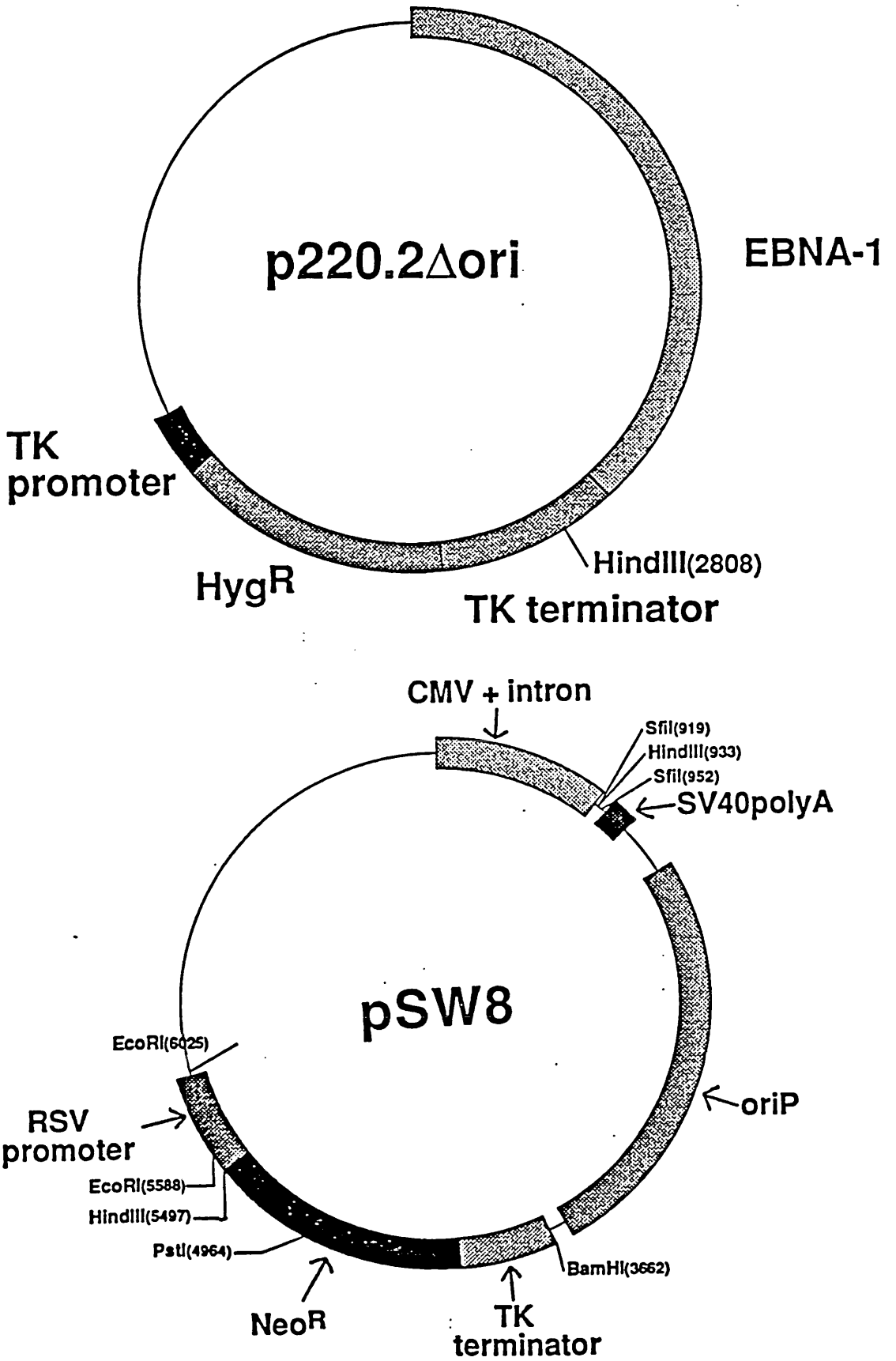


FIGURE 2.3

Diagram of EBNA-1 and EBO containing constructs



METHODS APPENDIX

A) Materials

Commonly used reagents were obtained from the suppliers listed below. Exceptions are noted in the text.

Chemicals and drugs - Sigma or BDH

Antibodies - Dako

Restriction enzymes - New England Biolabs

Radiochemicals - Amersham International

RESULTS

CHAPTER 3

INHIBITION OF THE CELLULAR RESPONSE TO INTERFERON BY HEPATITIS B.

1) EFFECTS OF THE HEPATITIS B GENOME.

To examine the effects of HBV on the cellular response to IFN, cell lines transfected with HBV were studied. The cell line 2A9 (kindly supplied by Dr Izumu Saito, University of Tokyo, Japan) is a HeLa cell derivative containing multiple copies of the HBV genome (subtype *ayw*) in a head-to-tail array. 2A9 cells express the HBV core, surface and X proteins (I. Saito - personal communication). The IFN response of these cells was examined by treating them with IFNα and examining the induction of IFN-inducible RNAs by northern blotting. Fig 3.1 shows the result for the 6-16 gene. There was no decrease in its induction but a slight increase in constitutive expression was seen. To determine whether this constitutive expression was associated with changes in transactivating factors that bind to the ISRE the binding of these factors was studied in a band shift assay (Figure 3.1C). There was no difference in the factors which bound to the ISRE when cells transfected with HBV, and expressing 6-16 constitutively, were compared with control cells. The mechanism responsible for the constitutive expression of the 6-16 gene is therefore not clear.

The X protein of HBV transactivates a number of cellular promoters (See Chapter 1B, 1.3) including the IFNβ gene promoter (Twu and Schloemer, 1987) and the increase in 6-16 expression in 2A9 cells may be due to X activating the 6-16 promoter - either directly or via the induction of IFNβ. As no changes in the IFN-inducible ISRE binding factors were seen in 2A9 cells it is unlikely that the increase in 6-16 expression is due to IFNβ production. Since our aim was to identify

FIGURE 3.1

Effects of IFNα on the expression of the 6-16 gene in HeLa cells transfected with genomic HBV DNA.

A) Northern blot of parental HeLa cells and transfected cells (2A9), untreated (-) and after treatment with IFNα. The blot was probed with a cDNA probe from the 6-16 gene.

B) Photograph of the nitrocellulose membrane shown in A illuminated with UV light.

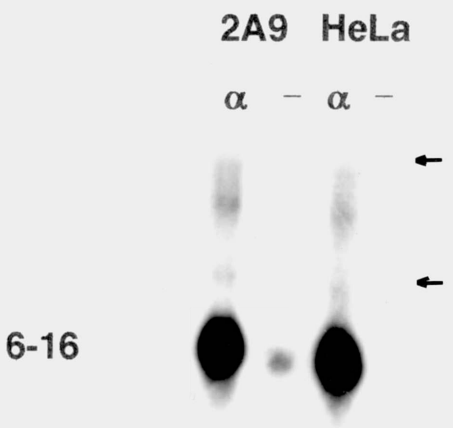
C) Band shift assay, using the 9-27 ISRE probe, in 2A9 and control cells. The IFN inducible factors that are known to bind to the ISRE (E and M) are indicated.

Notes

The positions of the ribosomal RNAs are indicated by arrows.
The 6-16 mRNA is an IFN inducible mRNA, used here as an indicator of the cell's ability to respond to IFN.

FIGURE 3.1

A



B

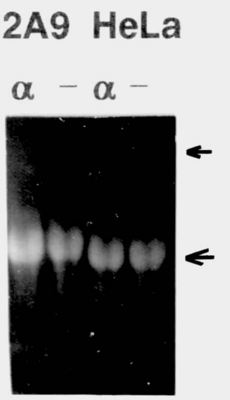


FIGURE 3.1

C

inhibitory factors within HBV the mechanism of this transactivating effect was not studied further.

The failure of 2A9 cells to show any decreased response to IFNα may be due to a failure of this clone to produce the putative inhibitor in an appropriate amount. Other HBV transfectants were therefore studied. In order to examine large numbers of transfected cells we used the cell line 2fTGH (see Chapter 1A, 1.1e) which contains the *gpt* gene under the control of the IFN-inducible 6-16 promoter (Pellegrini et al., 1989). In the presence of IFNα gpt is expressed and the cells die in medium containing 6TG (gpt converts 6TG to the toxic triphosphate). Cells that do not respond to IFNα survive in medium containing 6TG plus IFN and can be selected. Since the frequency of IFNα resistance in 2fTGH is very low [<1 in 10^8 cells (Pellegrini et al., 1989)] these cells can be used to identify inhibitors of the IFN response pathway even if they inhibit the response in only a small proportion of transfected cells.

2fTGH cells (4 x 10^5) were transfected with a construct containing an HBV dimer, subtype *ayw*, subcloned into the *EcoRI* site of a pBR based vector (kindly supplied by Dr P.Karyannis, St Mary's Hospital, London) and a neomycin resistance plasmid. As a control, cells were transfected with the drug resistance plasmid and the vector alone. Successful transfectants were selected in G418, and 8 HBV transfectants were ring cloned and examined. Expression of HBV RNA was assessed by probing northern blots with a genomic HBV probe. HBV specific RNAs were detected in two clones (Figure 3.2A shows the expression of HBV RNA in one of these clones, H1) and the IFN response of these HBV expressing clones was analysed. Figure 3.2C shows a typical result from the clone, H1. The induction of the 6-16 mRNA was normal. Furthermore there was no evidence of any increase in the constitutive expression of the 6-16 gene in these clones, unlike in 2A9 cells. This

FIGURE 3.2

Expression of HBV and response to IFNα of 2fTGH cells transfected with genomic HBV.

A) Northern blot from 2A9 cells (which are known to express HBV mRNA), parental 2fTGH cells and cells transfected with HBV (H1), probed with genomic HBV DNA. Note that the pattern of HBV RNA expression differs in the two HBV transfected cell lines, probably due to different interactions between cell-specific factors and HBV promoters.

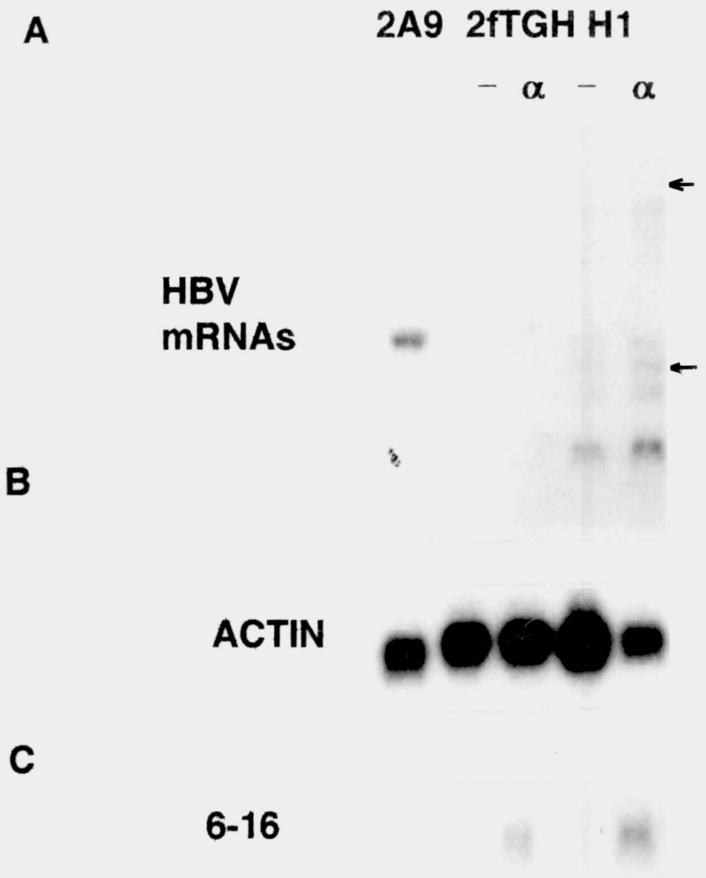
B) The same membrane probed with an actin probe.

C) The same membrane probed with a 6-16 cDNA probe.

Notes

The positions of the ribosomal RNAs are indicated by arrows.

FIGURE 3.2



may be because they do not produce adequate amounts of the putative transactivating factor (probably X protein), but this was not investigated further.

Direct analysis of a small number of HBV transfectants showed that a minority expressed HBV RNA (2 out of 8) and that these clones responded normally to IFNα. To examine a large number of transfectants for any decrease in their response to IFN, the remaining HBV transfected clones (80) were pooled, grown to a density of approximately 8 x 10^5 cells in a 10-cm diameter dish, and then selected in 6TG plus IFNα for three weeks. As a control, cells transfected with the HBV vector alone were grown in similar conditions. No colonies grew from either pool of transfectants, indicating that no cells were able to inhibit the response to IFNα. Hence expression of HBV RNA occurs in a minority of 2fTGH transfectants and none of the transfectants studied showed evidence of any decrease in their response to IFNα, either by a direct analysis or by selection in 6TG plus IFN.

2) EFFECTS OF INDIVIDUAL HEPATITIS B VIRAL GENES.

The failure to detect any inhibitory effect of genomic HBV in transfected cells may be due either to its inability to inhibit the activation of IFN-inducible RNAs, or to a failure of the cells to produce appropriate amounts of the putative inhibitor. The experiments described so far were performed with a dimeric HBV construct which uses native viral enhancers and promoters to regulate express - ion of its genes. These viral regulators are tissue specific (Antonucci and Rutter, 1989; Honigwachs et al, 1989) and, in cells other than hepatocytes, HBV proteins are poorly expressed. To obtain high level expression of each HBV protein, experiments using the individual HBV genes, under the control of the simian virus 40 (SV40) promoter were performed.

2.1) ANALYSIS OF TRANSIENTLY TRANSFECTED CELLS

The four HBV open reading frames were subcloned into the eukaryotic expression vector pJ3ω (See Chapter 2, 8.1). To assess the effect of each open reading frame on the response to IFNα, a transient expression assay system was used. Each construct was transfected into HeLa cells, along with an IFN inducible reporter construct containing 1040 bp from the upstream region of the 6-16 gene placed next to the bacterial CAT gene (Porter et al., 1987). This construct produces large amounts of CAT when cells transfected with it are treated with IFNα and it is also inducible (although to a much smaller degree) by IFNγ (Porter et al., 1987). After transfection cells were treated with IFNα or IFNγ for 48 hours and CAT activity was assayed. Table 3.1 summarises the results. Co-transfection with a construct expressing the HBV core gene did not affect the response to IFN. The constructs containing the surface and X genes caused a slight increase in both constitutive and induced activity, confirming previous reports that they can act as promiscuous cellular transactivators (Zhou et al., 1990; Hu et al., 1990). The POL gene construct inhibited responses to both IFNα and IFNγ. This inhibition was due to the polymerase protein since a construct containing the POL gene in the non-coding orientation (rPOL) was not inhibitory. When the CAT gene was regulated by a different promoter (SV40), no inhibition was seen after co-transfection with the POL construct (Table 3.2), indicating that polymerase selectively inhibits the IFN inducible 6-16 promoter. The effect of the POL gene construct was concentration dependent (Table 3.1) - when the ratio of POL DNA to reporter DNA was reduced the inhibition decreased. This may explain why other groups who have examined the effects of polymerase on IFN inducible promoters have not reported any inhibitory effects (Zhou et al., 1990).

To confirm that the HBV constructs used in the transient assays

TABLE 3.1

Effects of individual HBV open reading frames on expression of an ISRE-CAT construct in IFN treated cells.

TEST CONSTRUCT	CAT ACTIVITY cpm			FOLD INDUCTION WITH	FOLD INDUCTION WITH
	No IFN	IFNα	IFNγ	IFNα	IFNγ
Vector alone	86	5988	538	**77** (70-83)	**6** (5.5-6.5)
Core	114	4532	ND	**50** (40-85)	ND
Surface	381	43126	ND	**80** (57-113)	ND
X	568	67264	ND	**85** (74-118)	ND
POL	49	945	76	**23** (19-26)	**1.8** (1.6-1.9)
rPOL	76	4091	286	**55** (50-70)	**4** (3.5-6.5)
Vector alone*	63	8747	418	**100** (70-130)	**6** (4-8)
POL*	56	1723	158	**40** (30-49)	**2.6** (2.0-3.2)

Cells were transfected with an HBV reporter construct in the molar ratio 15:1, except for those marked * when the ratio was 7:1.Each experiment was performed in duplicate four times.CAT activity data is from a typical experiment and fold induction data are means from four experiments; ranges are in parentheses.

TABLE 3.2

Effects of HBV POL gene constructs on expression of an SV40-CAT construct.

TEST CONSTRUCT	CAT ACTIVITY cpm
POL	33607
rPOL	41640

Cells were transfected with an HBV construct and a reporter construct in the ratio 15:1. Results are means of three experiments.

TABLE 3.3

Expression of HBV proteins in HeLa cells transiently transfected with various HBV constructs.

	ANTIBODY DIRECTED AGAINST			
CONSTRUCT	CORE	SURFACE	X	POLYMERASE
pJ3-CORE	+	-	-	-
pJ3-SURFACE	-	+	-	-
pJ3-X	-	-	+	-
pJ3-POL	-	-	-	+
pJ3-rPOL	-	-	-	-

HeLa cells were transfected with the appropriate construct and 48 hours after transfection cells were stained with the antibodies listed above [antibodies against core and surface proteins were kindly supplied by Dr J Waters (St Mary's Hospital) and those against X and polymerase were supplied by Dr M McGarvey (St Mary's Hospital)]. + indicates that bound antibody was detected (approximately 20% of the cells stained positively in each case), - denotes no staining.

expressed the appropriate HBV protein, HeLa cells transfected with each of the constructs were stained with a panel of antibodies directed against the different HBV proteins. The results are tabulated in Table 3.3. Cells transfected with the HBV constructs stained only with the appropriate antisera, confirming that these constructs express the requisite HBV proteins.

2.2) ANALYSIS OF STABLY TRANSFECTED CELLS

To confirm the results from transient transfections and to examine the effects of polymerase on natural promoters, attempts were made to isolate stable cell lines expressing polymerase. 2fTGH cells (4×10^5) were transfected with the POL gene construct and a neomycin resistance plasmid. Successful transfectants (120) were selected in G418, 8 colonies were ring cloned and their response to IFN studied. As shown in Figure 3.3A one of the clones, A2, appeared to have a slight decrease in its response to IFNα, when the induction of the 6-16 gene was analysed by northern blotting. Since northern blotting is a relatively insensitive technique, the same mRNA was analysed using the more sensitive RNase protection technique. Figure 3.3B shows that using this assay a clear decrease in the response to IFNα was seen. Clone A2 grew slowly and during prolonged culture (3 weeks) its growth rate increased. After 3 weeks in culture the response of A2 to IFNα was again tested (both by northern blotting and RNase protection) and at this time its response was normal (Figure 3.3C). Other groups have reported that it is difficult to express polymerase in stably transfected cell lines (Bavand and Laub, 1988) and it is possible that shortly after transfection, A2 expressed HBV polymerase, which inhibited the response to IFN, and that with prolonged passage expression declined and hence the response to IFN returned to

FIGURE 3.3

Effects of IFNα on 2fTGH cells transfected with the HBV POL gene.

A) Northern blot of untreated and IFNα treated parental 2fTGH cells and two POL transfectants (A1 and A2). RNA was extracted from the cells approximately three weeks after transfection. The blot was probed with cDNA probes for actin and 6-16.

B) Same RNA as in A, analysed by an RNase protection assay with probes for the 6-16, 9-27 and actin mRNAs.

C) Northern blot of untreated and IFNα treated 2fTGH cells and A2 cells grown for a further 3 weeks.

Notes

The positions of the ribosomal RNAs are indicated by arrows.

The radioactive RNase protection probes should yield protected fragments of 190, 160 and 130 bp for 6-16, 9-27 and actin mRNAs respectively. The doublets for the 6-16 and 9-27 signals reflect the major and minor transcription start sites for these genes (discussed in Ackrill et al., 1991).

FIGURE 3.3


A
2fTGH
A1
A2
– α – α – α
ACTIN
6-16
B
2fTGH
A2
– α – α
MARKERS – BP
6-16
190
9-27
160
143
ACTIN
123
C
2fTGH
A2
– α – α
ACTIN
6-16

normal. However insufficient RNA and protein was extracted from the cells at an early stage to test this directly. An alternative possibility is that the decrease in the response to IFN in clone A2 was due to clonal variation in the IFN response of 2fTGH cells, and was not related to transfection with HBV polymerase. However 2fTGH cells are themselves derived from a single clone and it is unlikely that cells from the same clone will show significant variation in their response to IFN. Clonal variation can not explain why A2 cells regained their response to IFN, and it is more likely that the observed reduction in the response to IFN was due to expression of polymerase.

Direct analysis of cells transfected with HBV polymerase did not show evidence of any sustained IFN inhibitory activity. A large number of POL-transfected cells were therefore screened for failure to respond to IFNα, by selection in 6TG plus IFNα. 2fTGH cells (2.4×10^6) were transfected with the POL construct and a drug resistance plasmid (either neomycin or puromycin) in two pools. One pool was selected in the appropriate drug to assess transfection efficiency and the other was selected in 6TG, IFNα and drug, to determine the frequency of IFN resistance.The other HBV gene constructs were used as controls. Table 3.4 summarises the results from a number of transfections. No clones that grew in 6TG and IFN were identified in cells transfected with the surface and X constructs. Two clones were obtained after transfection with the core and rPOL constructs and 18 clones were isolated from 1060 POL transfectants. Some of these colonies were grown in the absence of selection for three weeks and their response to IFNα tested by analysing the induction of the 6-16 gene,by northern blotting. Table 3.5 shows the results. Both of the clones transfected with core and rPOL responded normally, as did three of the POL transfectants, indicating that their growth in 6TG plus IFN was not due to inhibition of the IFN response.

TABLE 3.4
Frequency of survival in 6TG plus IFNα in stably transfected 2fTGH cells

Test Construct	NUMBER OF CLONES Transfected	Surviving in 6TG+IFN
rPOL	880	1
Surface	180	0
X	300	0
Core	303	1
POL	1060	18

TABLE 3.5
Response to IFNα of clones surviving selection in 6TG plus IFNα

Clone	Derivation (transfected DNA)	Induction of 6-16 mRNA
C1	Core	Normal
R1	rPOL	Normal
P1	POL	Normal
P2	POL	Normal
P3	POL	Decreased
P4	POL	Decreased
P5	POL	Normal
P6	POL	Decreased

Their survival was probably due to loss of the 6-16 *gpt* construct, which has been shown to give rise to non-IFN resistant colonies (Pellegrini et al., 1989). Three POL transfected clones showed a reduced response to IFNα and two of these were examined further. Both showed a similar response in subsequent experiments and, to avoid repetition, the results from one (POLA+) are described.

2.2a) RESPONSE TO INTERFERON OF POLYMERASE TRANSFECTED CELLS

The response to IFN of POLA+ cells was tested by treating cells with IFN (either IFNγ or IFNα) and analysing the induction of a number of IFN inducible genes by northern blotting or RNase protection assays (Figure 3.4). For all genes tested the response to IFNα in polymerase-transfected cells (both POLA+ and the second POL transfected clone, POLB+) was reduced. Densitometric analyses of the northern blots and RNase protection experiments showed that the response to IFNα was reduced by approximately 70%. There was no change in the response to IFNγ but polymerase transfected cells also had a reduced response to IFNβ (Figure 3.5). The non-IFN inducible genes, γ-actin and glyceraldehyde-3- phosphate dehydrogenase (GAP-DH) were expressed normally in POLA+ cells (Figure 3.4B). Hence polymerase inhibits the actions of Type I IFNs but not Type II IFNs and it does not alter the expression of genes that are not induced by IFN. The inhibitory effect of polymerase could not be overcome by prolonged treatment of the cells with IFNα (Figure 3.6), indicating that polymerase inhibited the response to IFN rather than delaying its onset of action.

FIGURE 3.4

Induction of IFN inducible mRNAs in 2fTGH cells and POLA+ cells.

Cells were treated with IFN and cellular RNAs were examined as described.

A) RNase protection assay with probes directed against the 6-16 and 9-27 RNAs.

B) Northern blot probed with cDNAs for the 1-8 gene famil y,actin and GAP-DH. The membrane was stripped between each analysis.

C) Northern blot probed simultaneously with cDNAs for the HLA Class I and RING 4 genes.

D) Photograph of the membrane used in C illuminated with uv light.

FIGURE 3.4

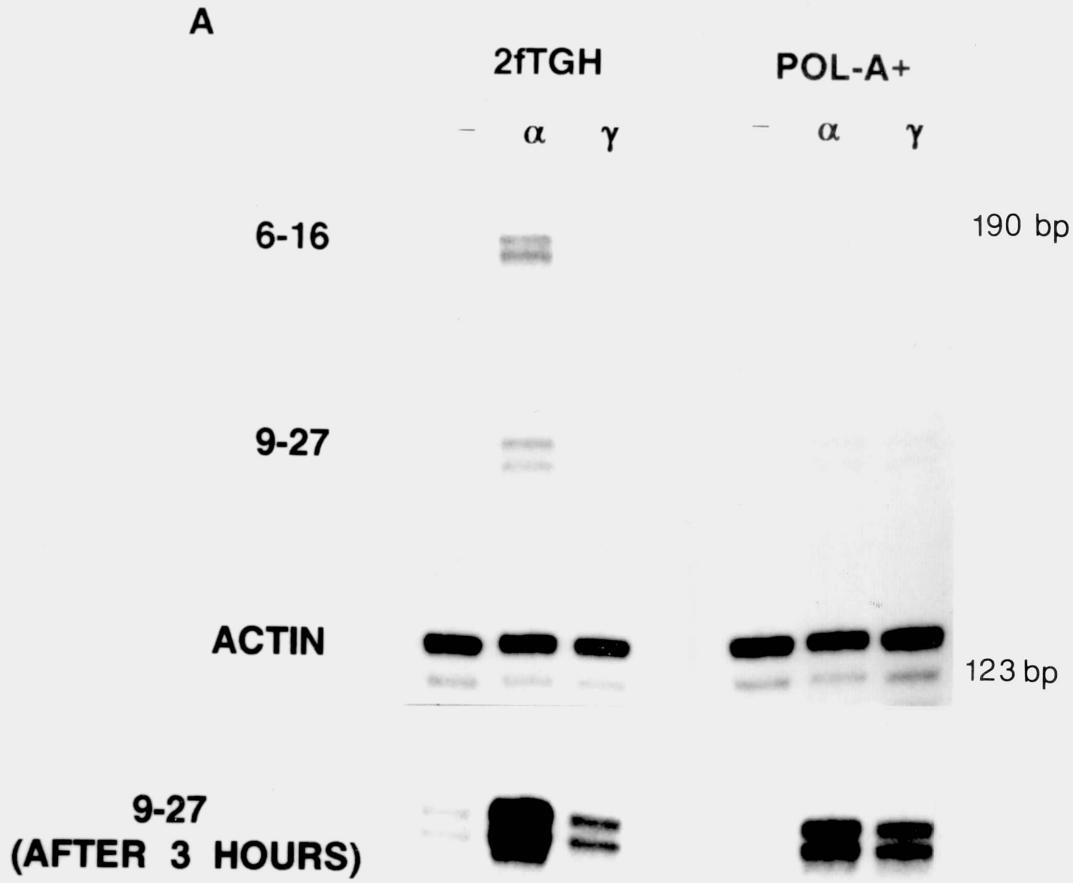
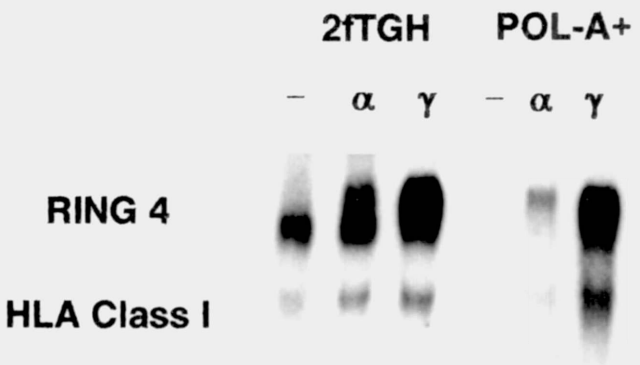

A
2fTGH
POL-A+
– α γ
– α γ
6-16
190 bp
9-27
ACTIN
123 bp
9-27
(AFTER 3 HOURS)
B
POLA+ 2fTGH
– α – α
1-8
ACTIN
GAP-DH

FIGURE 3.4
(Continued)

C



D

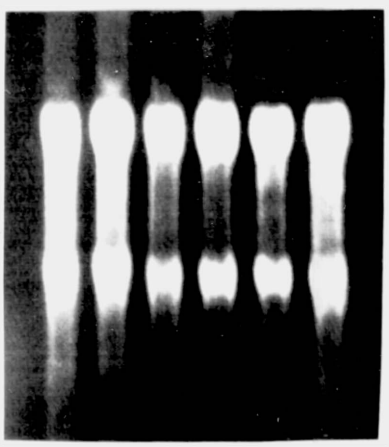


FIGURE 3.5

Effects of IFNs α, β and γ on 2fTGH cells.

Cells were treated with IFN and RNA was then analysed in an RNase protection assay using the probes for the 6-16, 9-27 and actin mRNAs.

FIGURE 3.6

Responses to different durations of IFNα treatment in 2fTGH and POLA+ cells.

Cells were treated for the times shown and RNA was then analysed using an RNase protection assay.

FIGURE 3.5

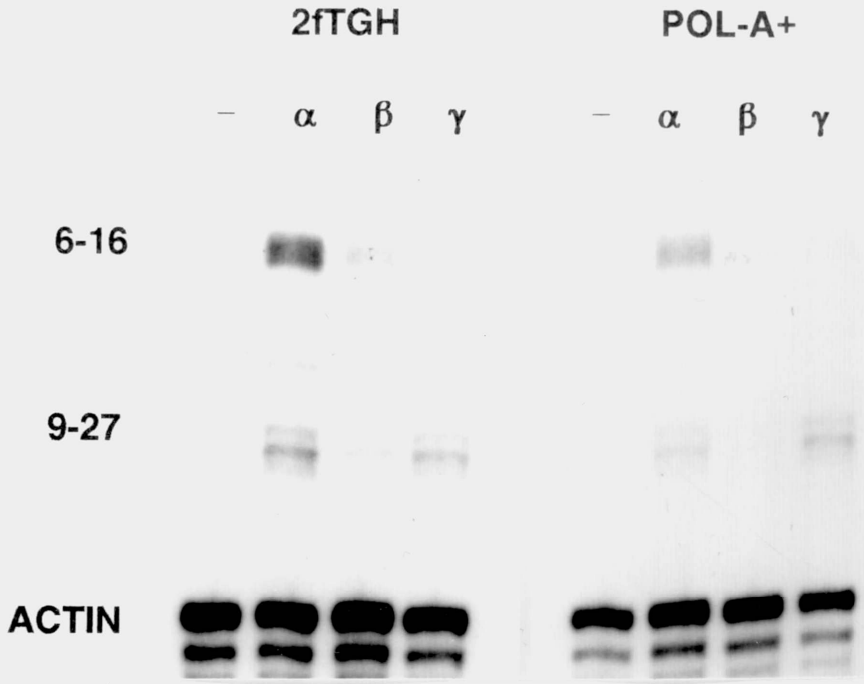
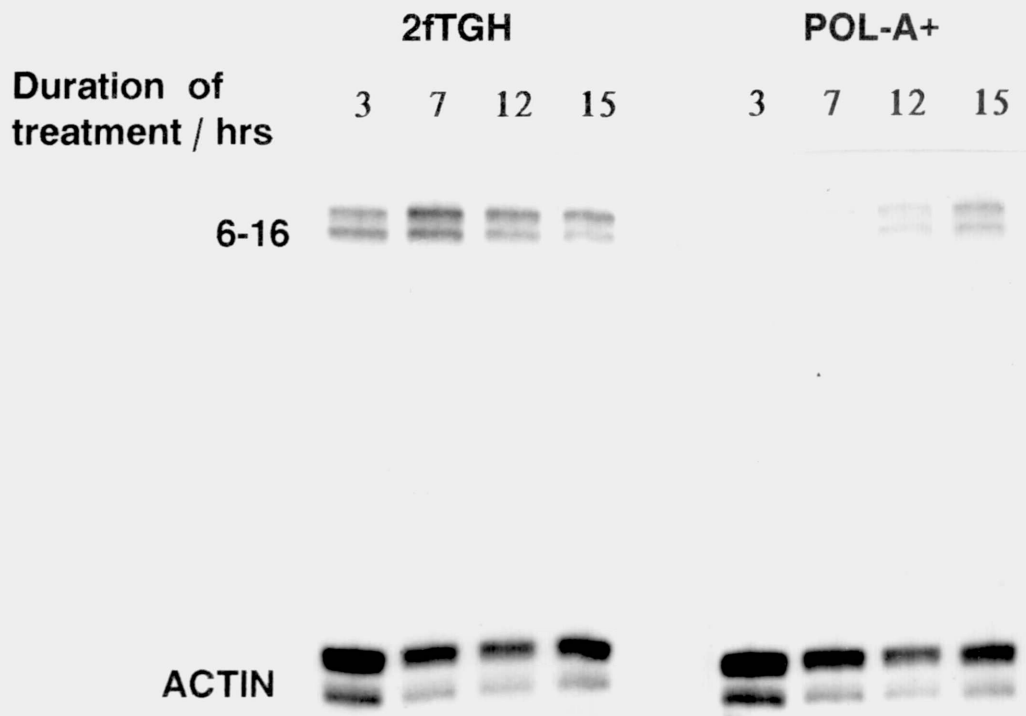


FIGURE 3.6



2.2b) EFFECT OF POLYMERASE INHIBITORS ON THE INHIBITORY EFFECT OF POLYMERASE

The retroviral inhibitor, 5-azacytidine, and the drug suramin have both been reported to inhibit the activity of HBV polymerase (Offensperger et al, 1988; Ueda et al, 1988). To determine whether these drugs could reverse the IFN inhibitory effects of polymerase, their effects on the response of POLA+ cells to IFNα were studied. POLA+ cells were treated with 5-azacytidine at a concentration of 10 μg/ml and suramin (20 μg/ml) for 12 hours and then treated with IFN (Figure 3.7A). Azacytidine did not reverse the inhibitory effects of polymerase, suggesting that inhibition of the enzymatic activity of polymerase does not inhibit its effects on the cellular response to IFN. Since subsequent experiments indicated that the reverse transcriptase activity of polymerase was not necessary for its inhibitory properties (see Chapter 4) other reverse transcriptase inhibitors were not investigated.

The effects of suramin on the IFN response of POLA+ cells was surprising - it completely inhibited the response to IFNα. To determine whether suramin could also inhibit the response to IFNα in cells which did not express polymerase 2fTGH cells were treated with this drug at two different concentrations (2 μg/ml and 20 μg/ml). Figure 3.7B shows the result. Suramin inhibited the response to IFNα at both concentrations. Since our aim was to investigate the effects of HBV on the cellular response to IFN this effect was not studied further.

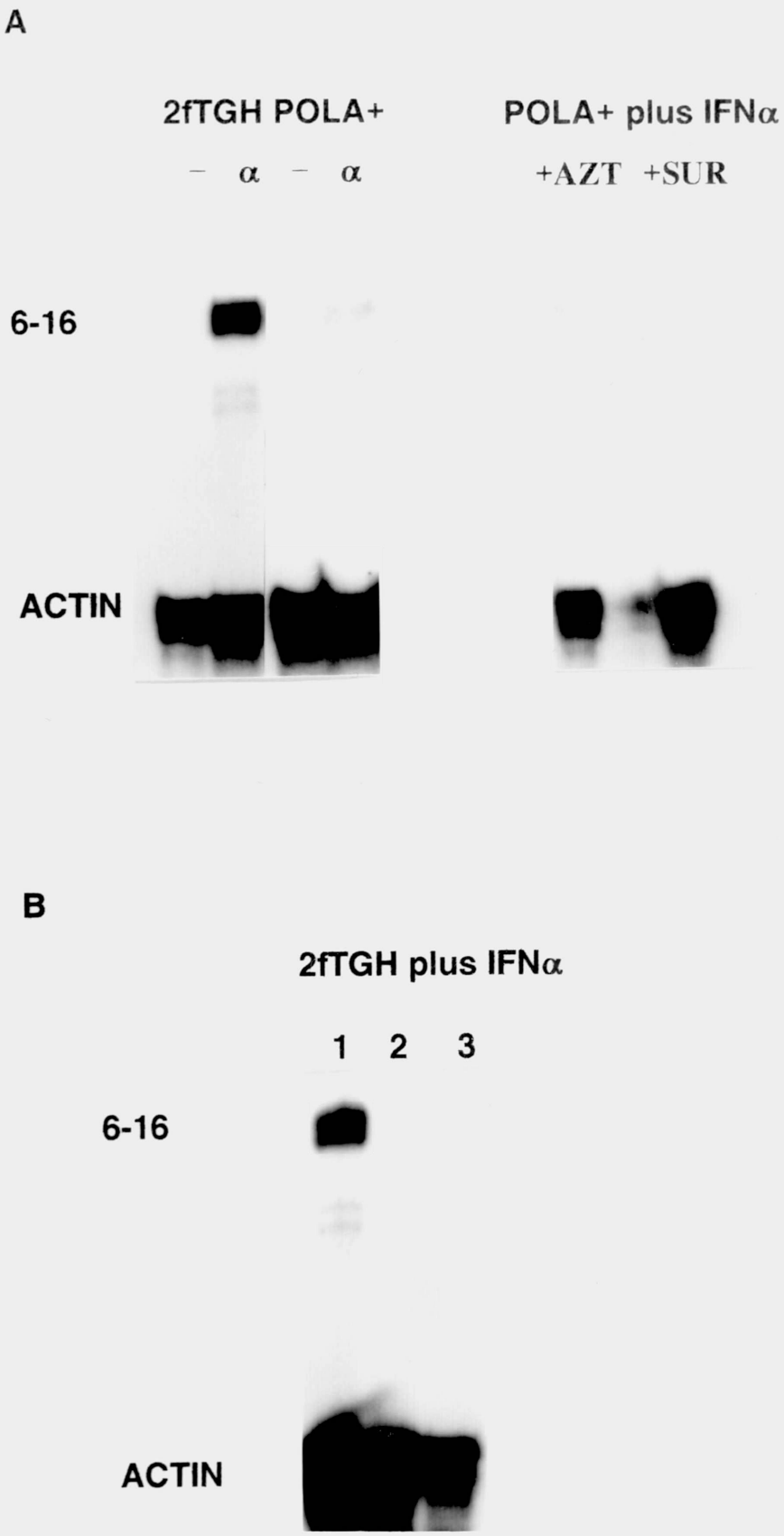
FIGURE 3.7

Effect of 5-azacytidine or suramin on the response to IFN

A) POLA+ cells were treated with azacytidine (AZT) or suramin (SUR) for 12 hr and then challenged with IFNα for 6 hr (in the presence of the appropriate drug). Cellular RNA was then assayed by RNase protection. As a control 2fTGH and POLA+ cells were treated with IFNα alone and their response is shown on the left.

B) 2fTGH cells were treated with suramin (2 or 20 μg/ml) for 12 hr and then tested with IFNα. Response was assessed by RNase protection using a probe for the 6-16 mRNA.The left lane (1) is a positive control RNA from untreated cells and the two right lanes are from cells treated with suramin 2 μg/ml (2) or 20 μg/ml (3).

FIGURE 3.7


A
2fTGH POLA+
POLA+ plus IFNα
– α – α
+AZT +SUR
6-16
ACTIN
B
2fTGH plus IFNα
1 2 3
6-16
ACTIN

2.2c) EXPRESSION OF POLYMERASE

To confirm that POLA+ cells expressed the polymerase protein a number of assays were used. Three different antibodies against different regions of polymerase were available (see Chapter 2, 5.2) and each antibody was tested in Western blot and immunoprecipitation assays of proteins from POLA+ cells. Western blot assays of proteins from liver biopsies of HBV infected patients have been performed with these antibodies and none successfully identified polymerase (M. McGarvey and H. Wills - personal communication), suggesting that they are unable to detect small amounts of polymerase protein in liver tissue. Western blots from POLA+ and 2fTGH cells were prepared and incubated with each of the antibodies using a wide range of conditions. Figure 3.8A shows a typical result obtained using antibody M. The expected size of the polymerase protein is 90 kD (Bavand and Laub, 1988) and all the antibodies bound to proteins of approximately this size in control cell extracts. Attempts to prevent binding to these cellular proteins by increasing the stringency of the washing conditions, from 0.1% Tween-20 to 5% Tween 20, were tried without success. A variety of different antibody concentrations were also tried (ranging from 1:50 to 1:500) and attempts were made to adsorb cross reacting antibodies by passing the antisera through a column containing proteins from 2fTGH cells (see Chapter 2, 5.5). These manipulations did not allow polymerase protein to be identified. The antibodies were also used to immunoprecipitate radiolabelled cellular extracts (Figure 3.8B) and, again, background binding to cellular proteins prevented detection of polymerase. Since these techniques did not allow detection of polymerase protein other approaches were tried.

FIGURE 3.8

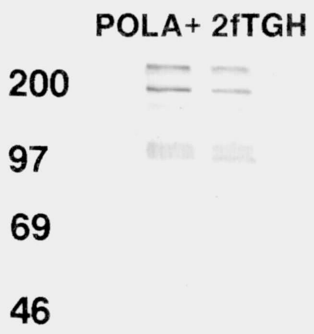
Attempts to detect HBV polymerase protein in POLA+ cells.

A) Western blot of proteins from 2fTGH and POLA+ cells . Proteins were separated on a 12% acrylamide gel, transferred to nitrocellulose and probed with antibody M at a concentration of 1:200. Bound antibody was visualised with diaminobenzidine. Rainbow markers were used to assess the size of the proteins and they are indicated on the left.

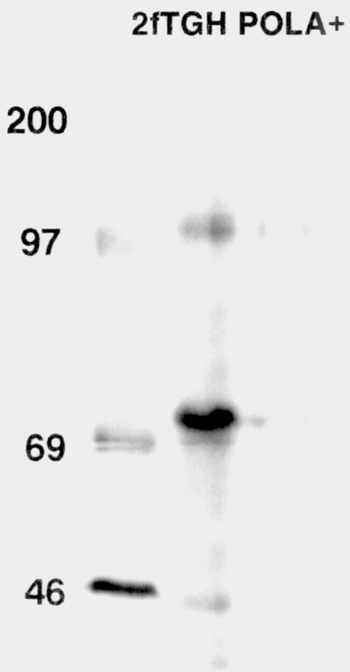
B) Immunoprecipitation analysis of radiolabelled proteins from 2fTGH and POLA+ cells using antibody W. Bound proteins were separated on a 10% acrylamide gel. ^{14}C radiolabelled markers are shown on the left.

FIGURE 3.8

A



B

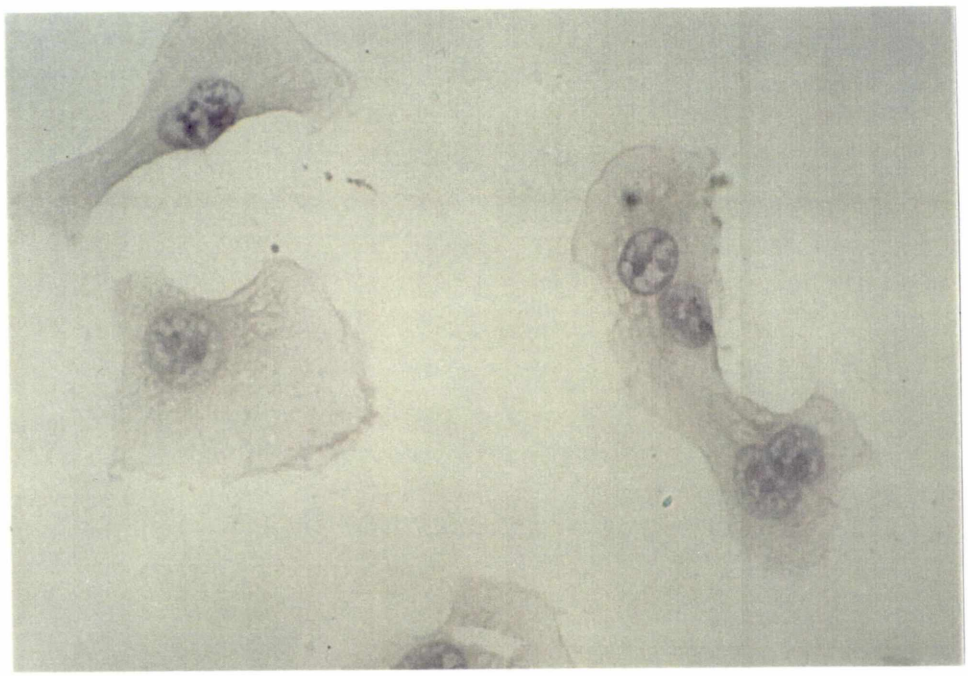


Antibody M has been used successfully to identify polymerase by direct staining of liver biopsies from patients with chronic HBV infection (Goldin et al., 1990). This antibody was used to stain POLA+ cells (Figure 3.9) which were positive, but 2fTGH cells were not, suggesting that POLA+ cells express the polymerase protein. To confirm this a functional assay was used. The polymerase protein contains a reverse transcriptase whose activity can be assayed in vitro by measuring the incorporation of radiolabelled dATP into DNA produced from an RNA primer (Offensperger et al., 1988). Cell extracts were assayed for this activity using the conditions described by Offensperger et al. The assay was performed three times and Figure 3.10 shows a typical result: there was a slight increase in incorporation of ATP in extracts from POLA+ cells. To confirm that this slight increase was due to genuine DNA synthesis a portion of the assay material was analysed by gel electrophoresis. This assay (Figure 3.10) confirmed that POLA+ cell extracts contain an enzyme which can synthesise DNA from an RNA template - a reverse transcriptase - although the activity of the enzyme under the conditions used was small. Hence POLA+ cells express polymerase protein which can be detected both in functional assays and by direct staining of the cells.

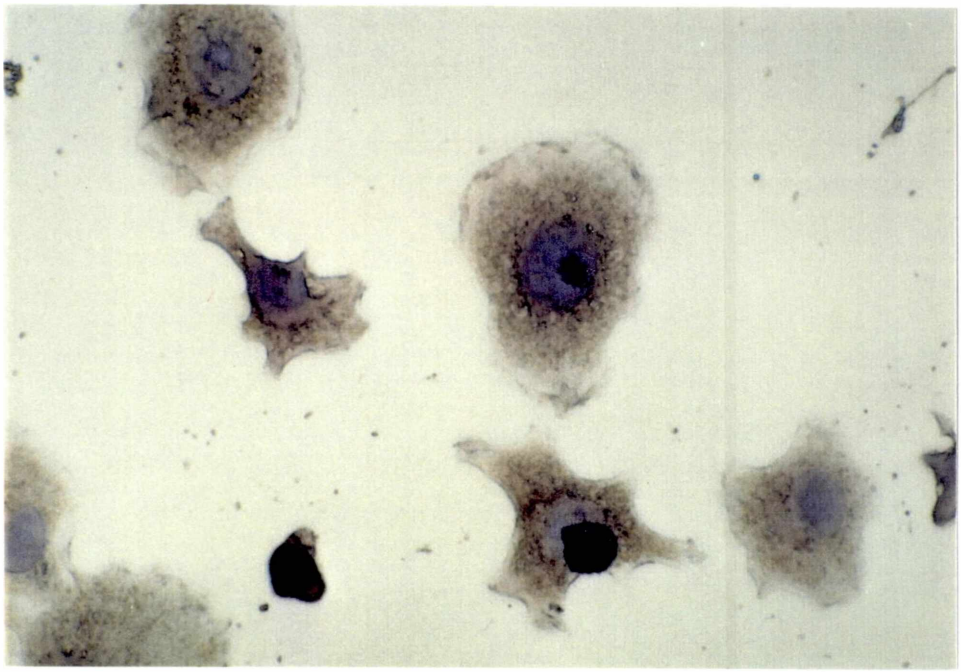
POLA + cells grew very slowly (the growth curve for 2fTGH and POLA+ cells is shown in Figure 3.11) and direct examination of the tissue culture plates showed that many of the cells detached from the plate. This suggests that POLA+ cells are sick. When the selection for IFN resistance (6TG plus IFN) was removed for three weeks the rate of growth of the cells returned to normal and examination of the tissue culture plates showed that the cells were growing normally. At this time the response to IFNα was normal, and the cells did not express polymerase protein (assayed by direct staining). It is likely that

FIGURE 3.9

Direct staining of 2fTGH and POLA+ cells with antibody M



2fTGH



POLA+

FIGURE 3.10

A) Reverse transcriptase activity in POLA+ cells.

Cell extracts were incubated with an RNA template and radiolabelled dATP. TCA-precipitable counts were measured at various times.

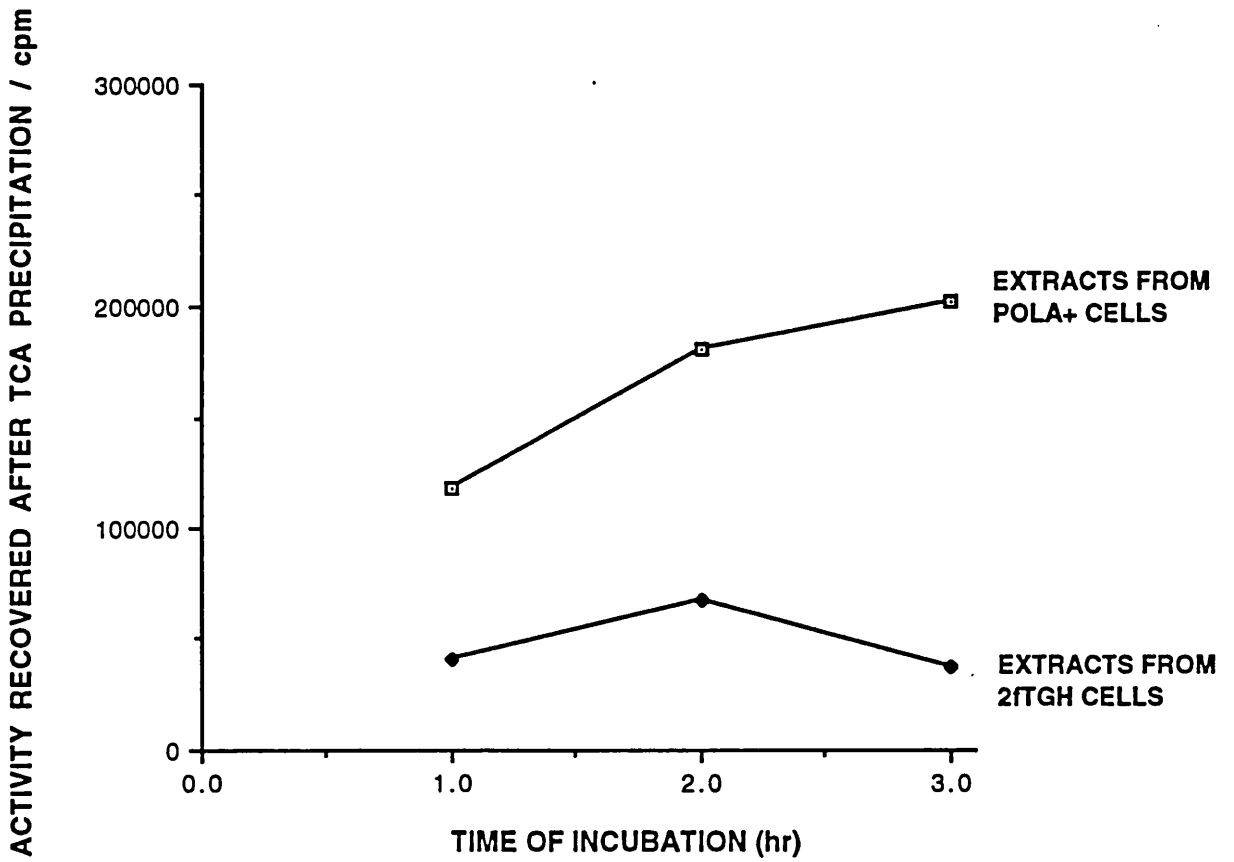


FIGURE 3.10(Continued)

B) Gel electrophoresis of the product of a reverse transcription assay.

2 µl of a reaction mix, after a 3 hour incubation, were electrophoresed in a 6% acrylamide gel and the dried gel autoradiographed. Size markers, from a *Msp 1* digest of pBR are on the left and the final right lane is from a reaction mix without any added cell extract.

FIGURE 3.10

B

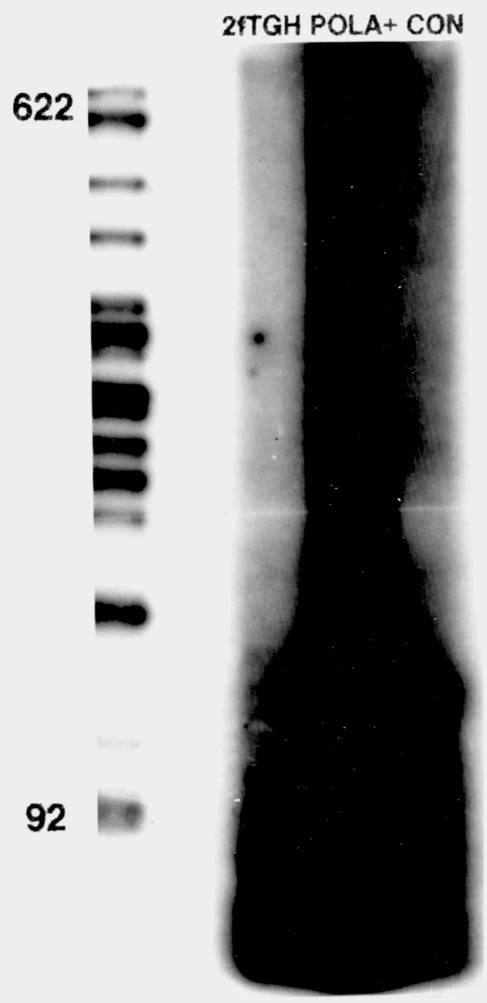
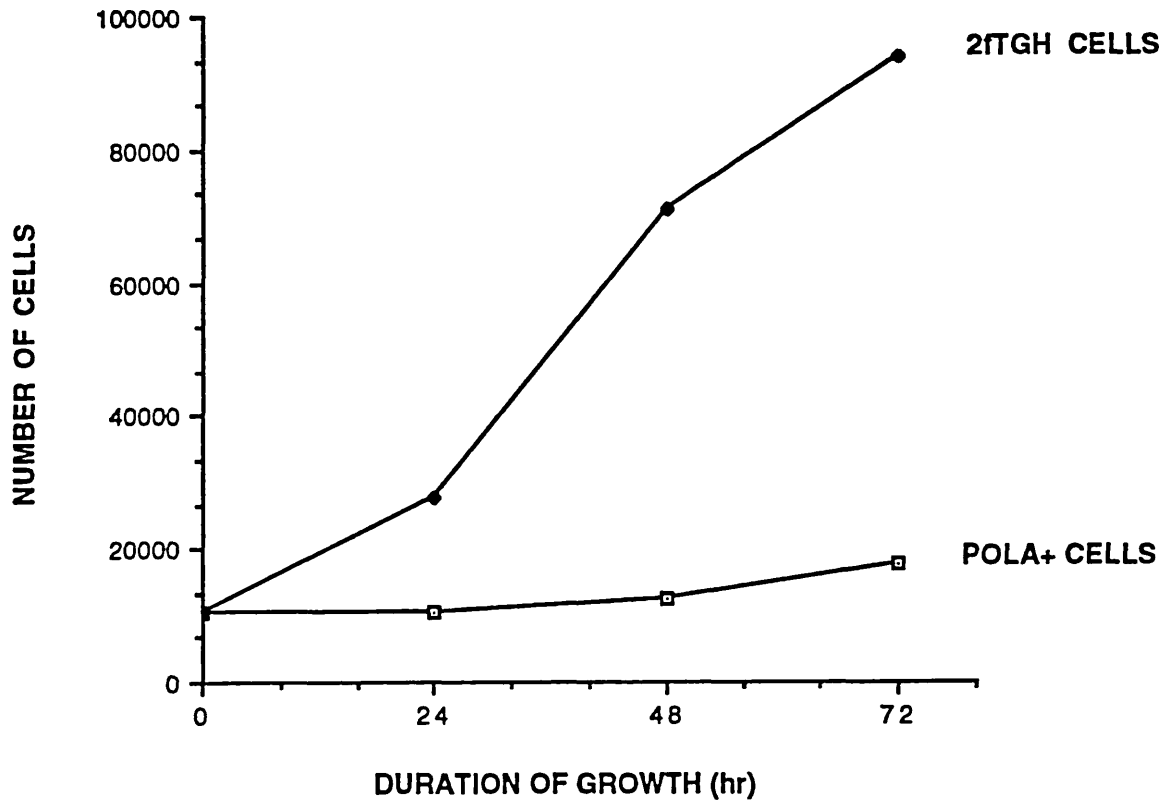


FIGURE 3.11

Growth curve for POLA+ and 2fTGH cells.

Cells were plated out at equal density and the number of cells counted every 24 hours by detaching the cells and counting with a Coulter counter.



polymerase protein is toxic to the cells and that expression is rapidly lost in the absence of any positive selection. Cells transfected with polymerase and selected for IFN resistance can express polymerase stably, but only if the selection is maintained.

CHAPTER 4
ANALYSIS OF POLYMERASE DOMAINS

Cells expressing HBV polymerase show a reduced response to IFNα. However these cells are sick and the expression of polymerase is quickly lost if the selection for IFN resistance is withdrawn. It is possible that the observed reduction in the IFN response is due to the toxicity of polymerase rather than to a specific effect on the IFN response pathway. To investigate this, experiments were performed using the different domains of polymerase to see if the IFN inhibitory properties of polymerase could be dissociated from its toxic effects.

The full length polymerase protein (Figure 4.1) contains an amino terminal domain (terminal protein) which is linked by a spacer arm to a carboxy terminus that contains the enzymatic domains (Bartenschlager and Schaller, 1988; Toh et al, 1983). To determine which domain is responsible for inhibiting the IFN response two constructs were made (see Chapter 2, 8.1). pJ3-TP contains the region encoding the amino terminal 270 amino acids of polymerase and pJ3-POL.COOH contains the enzymatic region from polymerase (Fig 4.1). The effects of these two constructs on the response to IFN were assayed.

1) EFFECTS OF POLYMERASE DOMAINS IN TRANSIENT ASSAYS

The effects of the two polymerase fragments on the induction of a 6-16 promoter-CAT construct were studied by transfecting each of the two constructs into HeLa cells along with an IFN inducible reporter construct (see Chapter 3, 2.1). Table 4.1 shows the results. pJ3-POL.COOH did not affect the response to IFN, but the construct encoding terminal protein (TP) inhibited responses to both IFNα and IFNγ. The inhibitory effect of pJ3-TP was concentration dependent:

FIGURE 4.1

Diagram of polymerase domains and constructs

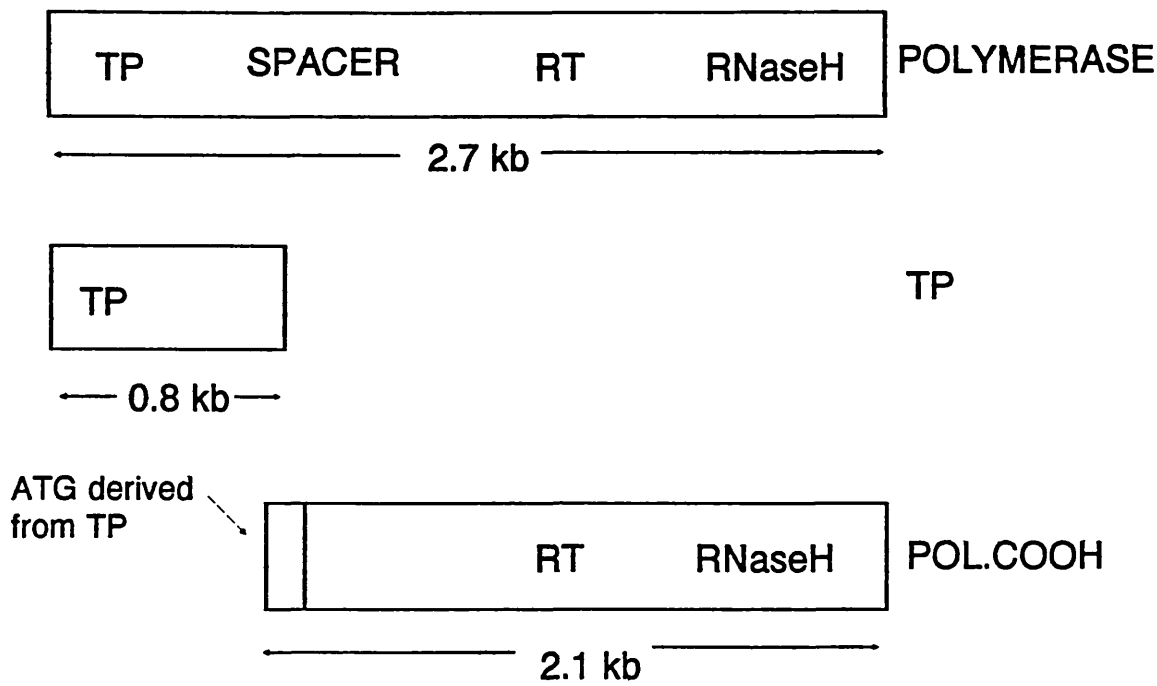


TABLE 4.1

Effects of different polymerase domains on transient expression of an ISRE-CAT construct in IFN treated cells.

TEST CONSTRUCT	CAT ACTIVITY cpm			FOLD INDUCTION WITH	FOLD INDUCTION WITH
	No IFN	IFNα	IFNγ	IFNα	IFNγ
Vector alone	86	5988	538	**77** (70-83)	**6** (5.5-6.5)
pJ3-TP	50	537	75	**8** (7-10)	**1.5** (1.0-1.7)
pJ3 POL-COOH	81	5249	320	**65** (30-88)	**4** (3-4.5)
pJ3-TP*	61	811	143	**27**(11-30)	**1.7** (1.5-2)

Cells were transfected with an HBV domain plus a reporter construct in the molar ratio 15:1, except for those marked * when the ratio was 7:1.Cat activity is the result from a typical experiment and fold induction data are the means from three experiments; ranges are in parentheses.

small amounts were not inhibitory, but large amounts reduced the response.

2) EFFECTS OF POLYMERASE DOMAINS IN STABLY TRANSFECTED CELLS.

To confirm the results from the transient experiments, 2fTGH cells were transfected with both of the POL derived constructs and the transfected cells were selected for successful transfection and resistance to IFN. Table 4.2 summarises the results. Successful transfectants were isolated only rarely from cells transfected with pJ3-POL.COOH, suggesting that this region of POL may be responsible for the toxic effects of polymerase. Cells transfected with the construct encoding TP were obtained readily and approximately 1 in 30 of the transfected cells grew in medium which selects for IFN resistance (6TG plus IFN). Three of the TP transfected IFN resistant clones were analysed for their ability to respond to IFN, by a northern blot analysis of the induction of the 6-16 gene. All three failed to repond to IFNα (Table 4.2). Two of the clones (TP-A and TP-B) were analysed further. Both showed an identical response in all of the assays which were performed and the results for one clone (TP-A) are presented. TP-A cells did not respond to IFNs α, β or γ (Figure 4.2). The inhibition of the IFN response pathway could not be overcome by increasing the duration of IFNα treatment nor by increasing the concentration of IFNα (Figure 4.3).

TABLE 4.2
Frequency of survival in 6TG plus IFN of 2fTGH cells transfected with different polymerase domains

	NUMBER OF CLONES		
TEST CONSTRUCT	TRANSFECTED	SURVIVING IN 6TG/IFN	INSENSITIVE TO IFNα
pJ3-TP	201	6	3/3
pJ-POL-COOH	20	0	

1.6×10^6 cells were transfected with each of the HBV constructs and a puromycin resistance plasmid. Transfected clones are the number of puromycin-resistant colonies. Three colonies which survived in 6TG plus IFN were tested for their response to IFNα by northern blotting. The number of clones that did not respond is shown in cloumn 4.

FIGURE 4.2

Response to IFN α,β and γ of 2fTGH and TP-A cells.

A) RNase protection assay of cells treated with IFNα and γ, analysed with probes for the 6-16 and 9-27 m RNAs.

B) RNase protection assay of cells treated with IFNβ, analysed with a probe for the 6-16 mRNA.

FIGURE 4.2

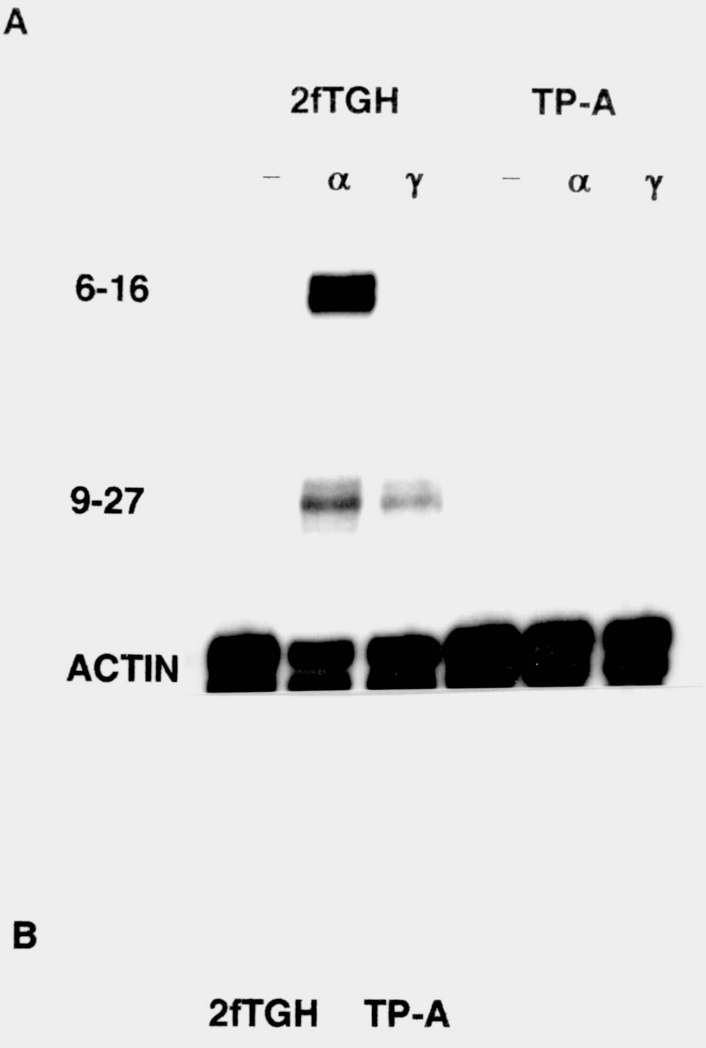


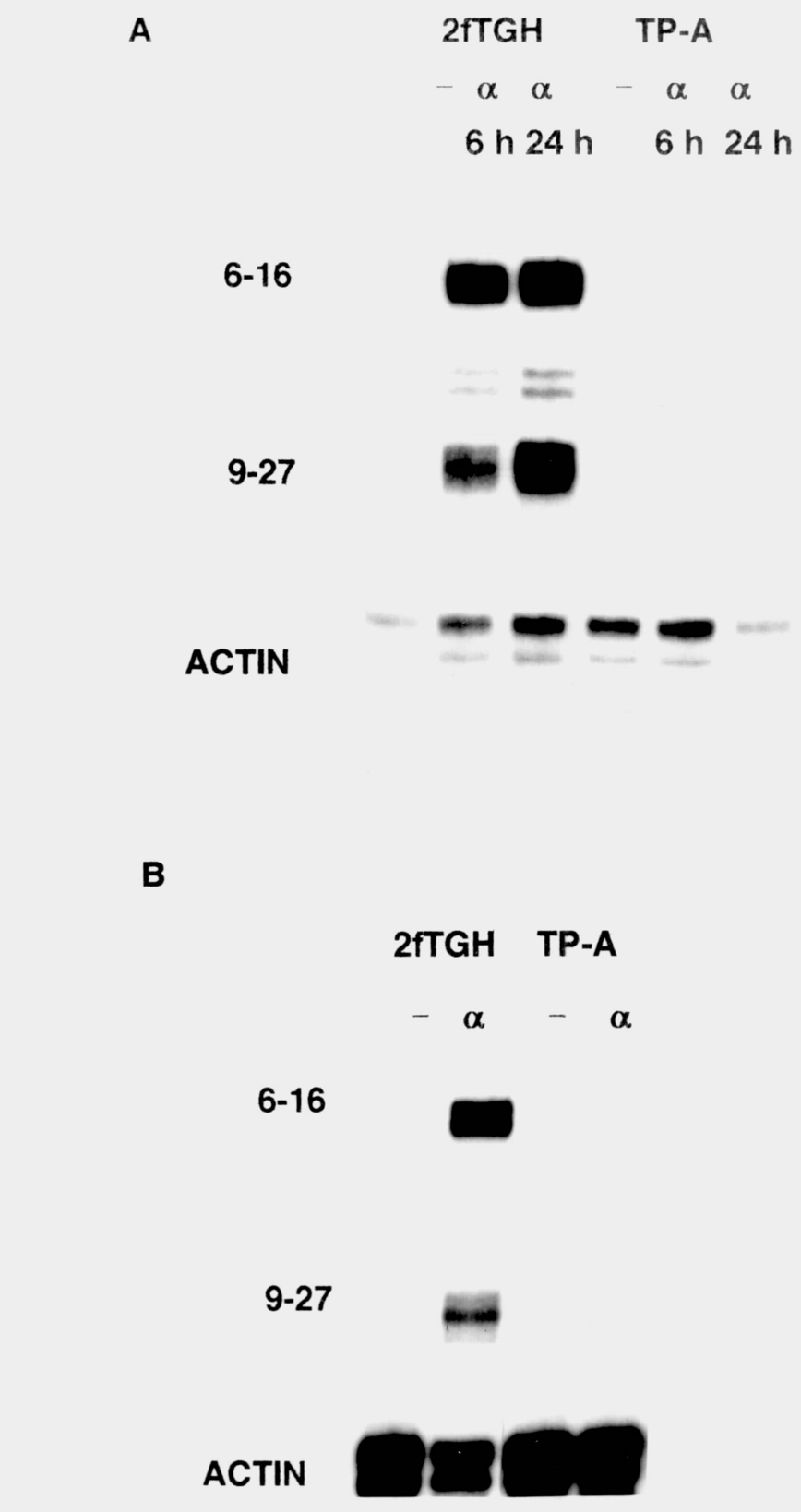
FIGURE 4.3

Response of 2fTGH and TP-A cells to varying concentrations and times of treatment with IFNα.

A) Effect of 24 hr treatment with IFNα on induction of the 6-16 and 9-27 gene, analysed by RNase protection.

B) Effect of 10,000 IU/ml of IFNα on induction of the 6-16 and 9-27 genes, analysed by RNase protection.

FIGURE 4.3


A
2fTGH
TP-A
– α α – α α
6 h 24 h 6 h 24 h
6-16
9-27
ACTIN
B
2fTGH TP-A
– α – α
6-16
9-27
ACTIN

IFNα activates genes by a single pathway but IFNγ uses at least two separate pathways (see Chapter 1A, 1.1d). The failure of the 9-27 gene to respond to IFNγ in TP-A cells shows that the IFNγ-ISRE pathway is inhibited. To determine whether the non-ISRE IFNγ response pathway is also inhibited by TP, the induction of the DRα mRNA (an HLA Class II gene that is activated via a non-ISRE pathway) was studied (Figure 4.4). No induction was seen showing that TP can inhibit both of the well defined IFNγ activation pathways. The GBP gene may use a unique pathway for its induction by IFNγ (See Chapter 1A, 1.1d) and to determine whether this activation pathway was also inhibited by TP the induction of GBP was examined (Figure 4.4D), again TP inhibited the response. A number of other IFN inducible genes were were also tested, and none responded to either IFNα or IFNγ (Figure 4.4) showing that TP can completely abolish the cells ability to respond to all types of IFN.

FIGURE 4.4

Effects of IFN treatment on the induction of a number of IFN inducible genes in 2fTGH and TP-A cells.

A) Induction of DRα

B) Same membrane as in A), stripped and re- probed with cDNAs for the HLA Class I, 1-8 and actin genes.

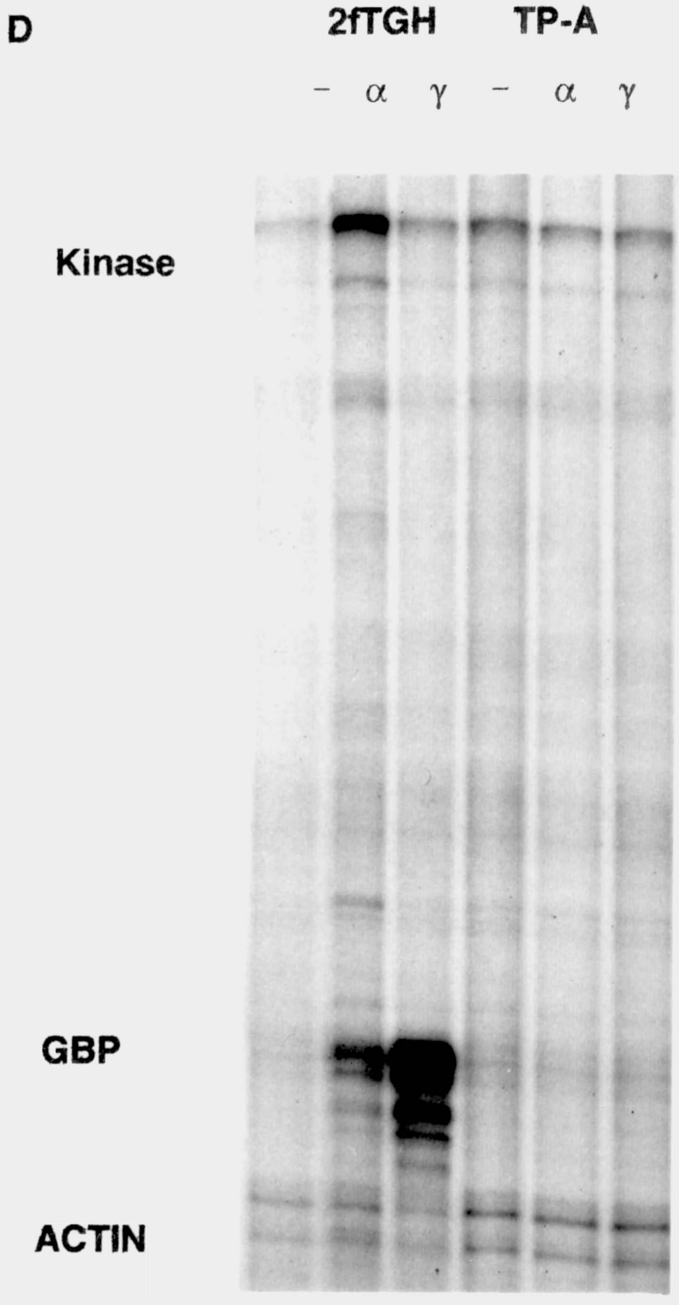
C) Northern blot probed with a cDNA for the RING 4 gene.

D) RNase protection analysed with probes for the p68 kinase and GBP mRNAs.

FIGURE 4.4


A
2fTGH
TP-A
– α γ – α γ
DR α
B
2fTGH
TP-A
– α γ – α γ
HLA Class 1
1-8
ACTIN
C
2fTGH
TP-A
– α γ – α γ
RING 4

FIGURE 4.4
(Continued)



3) EXPRESSION OF TERMINAL PROTEIN.

To confirm that IFN resistant cells transfected with pJ3-TP expressed TP the cells were stained with an antibody directed against terminal protein (antibody M - see Chapter 2, 5). TP-A cells stained with the antibody whereas control cells, 2fTGH, did not (Figure 4.5A). The expression of TP was also examined by western blotting, (Figure 4.5B). A band of the predicted size for terminal protein was consistently detected in TP-A cells and was absent in the parental cells, confirming that these IFN resistant cells express the HBV terminal protein.

4) ANALYSIS OF THE ACTIVE SITE WITHIN TERMINAL PROTEIN

To identify the region within TP responsible for its inhibitory properties a deletion analysis was performed. The construct pJ3-TP was used to make three new constructs containing smaller fragments of TP. pJ3-TP.X contains 520 nucleotides of the POL gene commencing at the natural ATG and constructs pJ3-TP.E and pJ3-TP.B contain 360 and 120 nucleotides respectively. Each construct was transfected into 2fTGH cells and successful transfectants (approximately 200 for each construct) were selected for IFN resistance in 6TG plus IFN. No IFN resistant clones were obtained. The transfected cells were stained with the antibody directed against TP (antibody M). No positive staining was seen, suggesting either that these constructs do not produce protein in stably transfected cells, or that this antisera is unable to bind to truncated TP. The failure to detect expression of the truncated terminal proteins makes it impossible to interpret the results of these experiments, as it is possible that no viral protein was expressed in the transfected cells. This approach to analysing the active site within TP was therefore abandoned (See Chapter 7 for a discussion of alternative approaches to determining the active domain within TP).

FIGURE 4.5

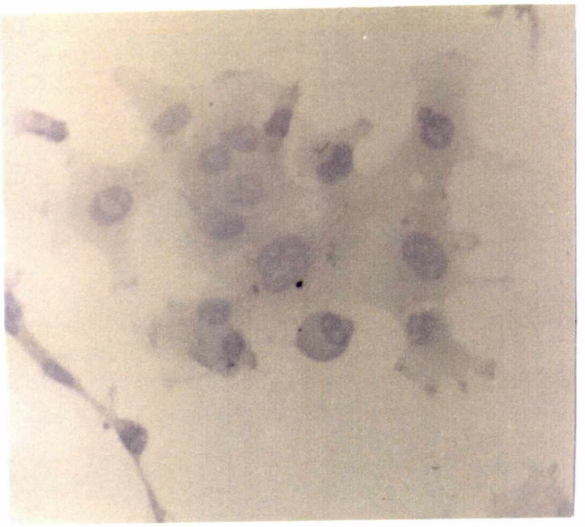
Detection of TP

A) Expression of TP, detected by direct staining of cells. Cells were treated with an antibody directed against TP and bound antibody was visualised with diaminobenzidine, which stains bound antibody brown.

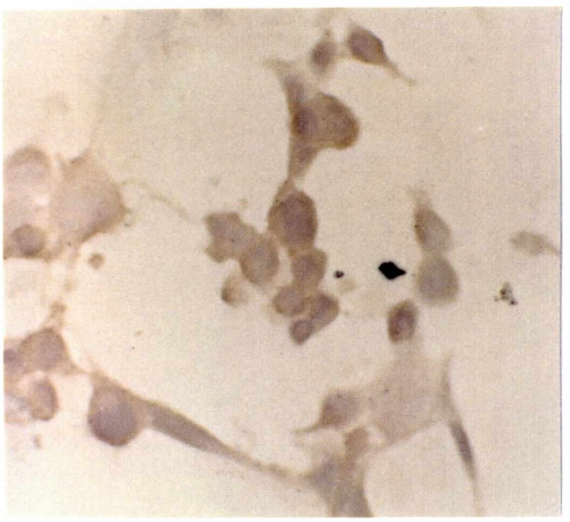
B) Expression of TP detected by Western blotting. Western blots were probed with a monoclonal antibody directed against TP (antibody W at a concentration of 1:100) and bound antibody was visualised using a chemiluminescence technique. The predicted size of TP is 22 kD and a band of the appropriate size is visible in TP-A cells but not in the parental controls.

FIGURE 4.5

A



2fTGH



TP-A

FIGURE 4.5
(Continued)

B

5) EFFECTS OF TERMINAL PROTEIN ON OTHER INDUCTION PATHWAYS.

Double stranded RNA (dsRNA) induces expression of the IFNβ gene (Havell et al., 1978) and the 6-16 gene. Induction of the 6-16 gene is both direct and via the induction of IFN (Wathelet et al., 1989). Since patients with chronic HBV infection have low levels of circulating IFN it is likely that induction of IFN is inhibited by the virus. To determine whether TP can inhibit the production of IFN, TP-A cells were treated with dsRNA and the induction of IFNβ and 6-16 assayed by RNase protection, Figure 4.6. dsRNA did not induce expression of either IFNβ or 6-16 in cells expressing TP, showing that the activation pathway used by dsRNA is also inhibited by TP. 2fTGH cells are derived from human fibroblasts and do not produce IFNα (R.McKendry, personal communication). It was therefore not possible to examine the effects of TP on the induction of other Type I IFNs.

A number of cytokines other than IFNs have been reported to be absent in patients with chronic HBV infection (Muller and Zielinski, 1990). The effects of TP on other cytokine pathways were therefore examined. Tumour necrosis factor α (TNF) is able to induce both the HLA Class I genes and intercellular adhesion molecule I (ICAM-I), (reviewed in Springer, 1990). The effects of TNF on both of these genes was studied (Figure 4.7). Induction of ICAM-1 was normal in cells expressing TP but TNF was not able to induce HLA Class I mRNA. This suggests that TNF uses at least two activation pathways: one, which activates ICAM-I is functional in the presence of TP, the other, which activates HLA Class I, is defective. It is possible that the defective pathway acts via IFN, since IFN is known to be induced by TNF (Springer, 1990).

A number of other cytokines were also tested. IL-6 and IL-1 are both reported to activate HLA Class I gene expression (Springer, 1990). However in the parental 2fTGH cell line neither of these cytokines induced expression of this mRNA so it was not possible to examine the effects of TP on their activation pathways in these cells.

FIGURE 4.6

Effects of TP on induction of genes by double stranded RNA

2fTGH and TP-A cells were treated with dsRNA for 3 hr and the induction of the 6-16 gene (left panel) and IFNβ gene (right panel) were analysed by RNase protection.

FIGURE 4.6

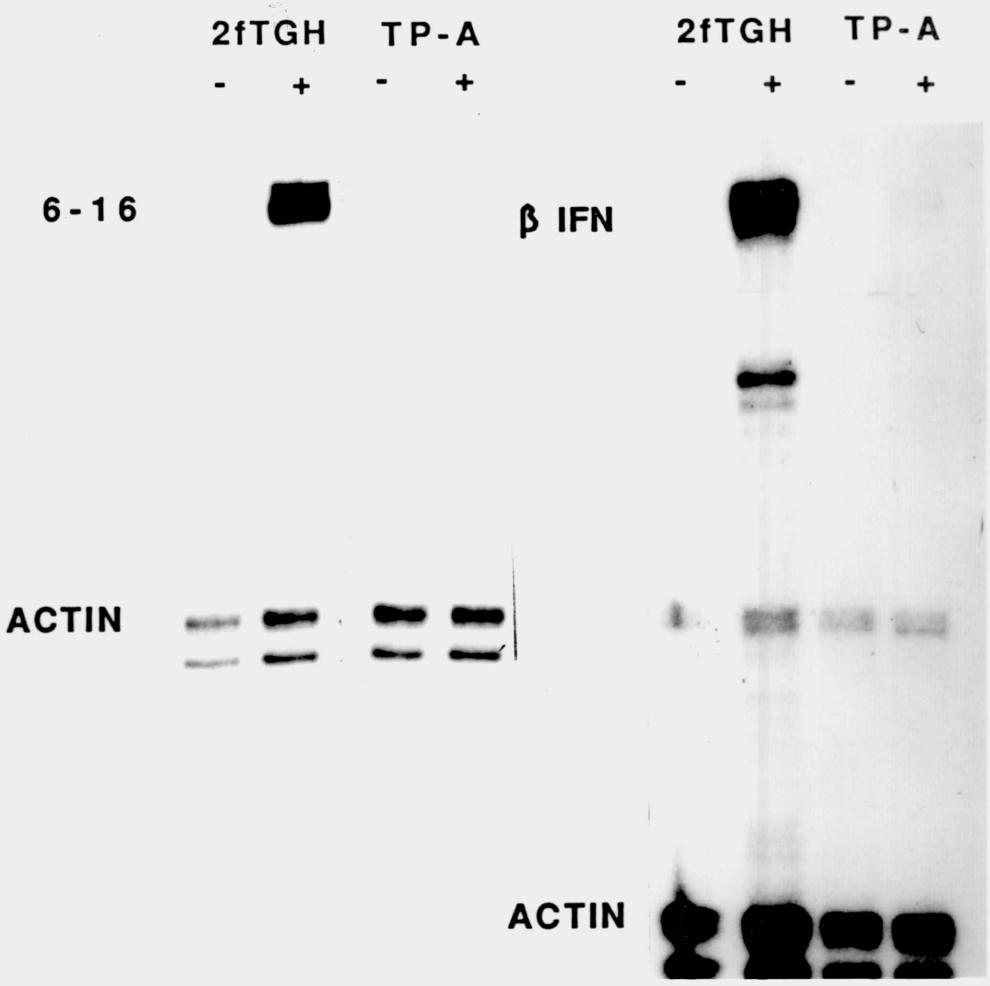
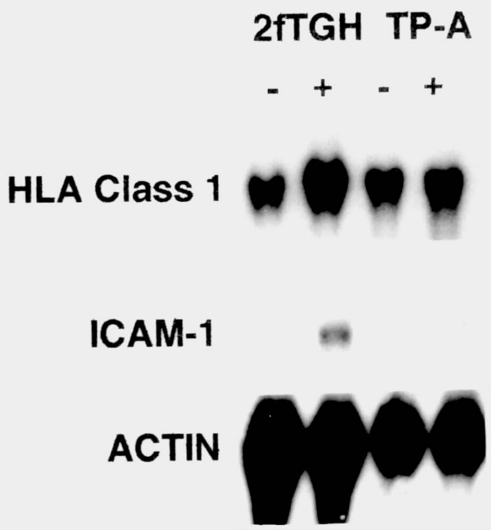


FIGURE 4.7

RESPONSE TO TNF of 2fTGH and TP-A cells

Cells were treated with 10 ng/ml of TNFα and RNA was prepared 24 hr later. Northern blots were then probed with appropriate cDNAs. The upper panel shows the results for HLA Class I mRNA and the lower panels are results from the same membrane probed with cDNAs encoding actin and ICAM-1. The reduced induction of ICAM-1 in TP-A cells treated with TNF may be due to the relative underloading of these lanes, or to the impaired response to IFN (presumed to be induced by TNF) in these cells.

FIGURE 4.7



CHAPTER 5

SITE OF ACTION OF TERMINAL PROTEIN

1) INHIBITION OF THE RESPONSE TO INTERFERONα

IFNα exerts its effects on gene transcription by binding to a cell surface receptor and activating the transactivating factor, E. E contains two subunits: Eα is activated by receptor bound IFN and then combines with Eγ, present constitutively in the cytoplasm, to form active E. Active E then migrates to the nucleus where it binds to a DNA element present in the promoter of IFN inducible genes (the ISRE) and activates gene transcription (see Chapter 1A, 1, for a full discussion of the mechanism of IFN activated transcription). The effects of TP on the different elements in the IFNα activation cascade were studied.

The number of IFNα receptors and their affinities for IFN were studied using the method of Scatchard (Scatchard, 1949), Figure 5.1. TP-A cells expressed IFNα receptors of the same affinity as the parental cells (determined by the slope of the Scatchard plot) but there was a slight decrease in the number of IFNα receptors in TP-A cells (determined from the intercept on the x axis). However this small decrease can not be sufficient to account for the complete absence of the IFN response in these cells.

The effect of TP on the activation of E was studied by band shift assays (Figure 5.2). Lanes 1-4 show that E was not activated in cells expressing TP. In control cells treatment with IFNα for 2 hr induced the activation of E (lanes 1 and 2), but treatment of TP-A cells did not (lanes 3 and 4). When cell extracts containing active E were incubated with extracts that contained TP, E could still be detected (lane 5), suggesting that TP did not inhibit the binding of preformed E to the ISRE. TP therefore inhibits the formation of active E.

To determine whether TP blocks the activation or function of Eα or

FIGURE 5.1

Scatchard plot performed with IFNα

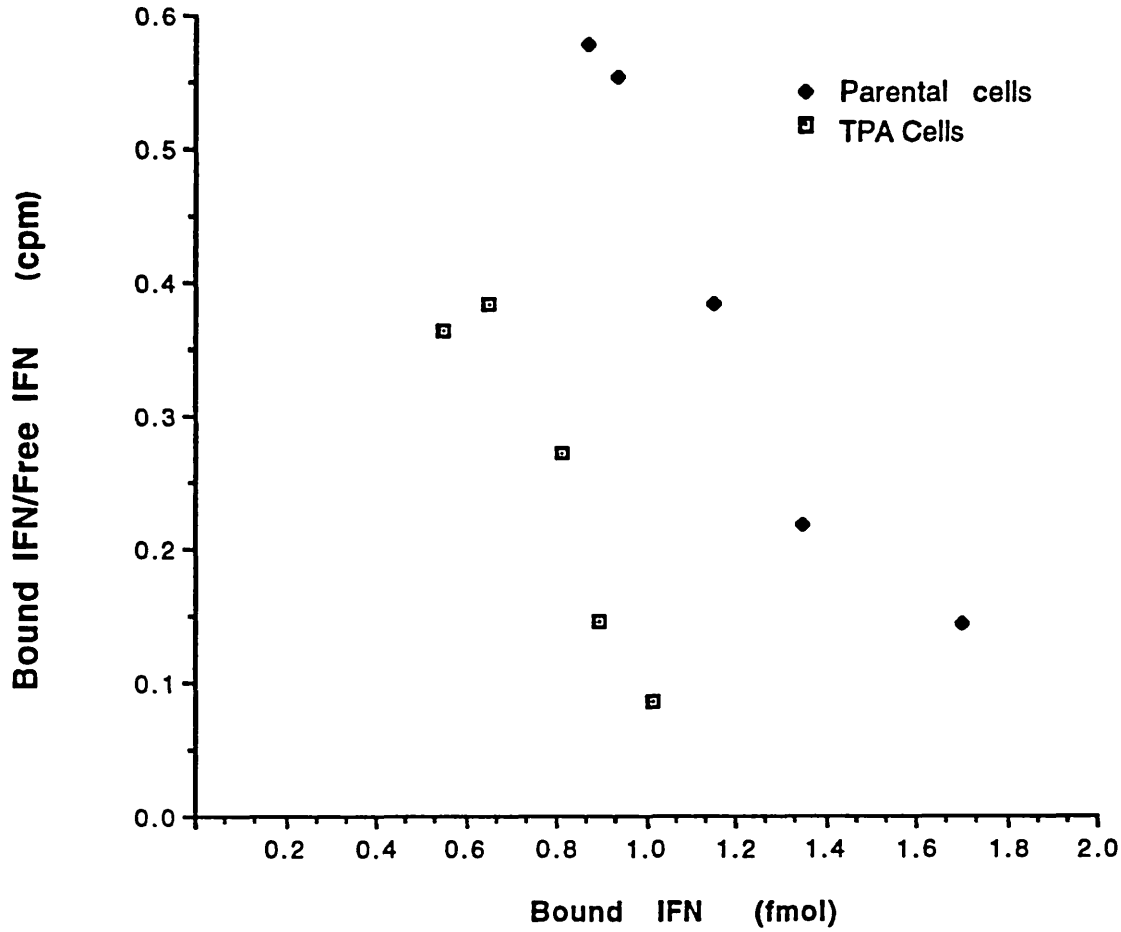


FIGURE 5.2

Band shift analysis of extracts of TP-A and control cells after treatment with IFN.

The POL-A- cells used as controls are revertants of POL-A+ cells that no longer express polymerase. Similar results were obtained with 2fTGH cells.

Lanes 1-4 : POL-A$^-$ and TP-A cells untreated (-) or treated with IFNα, showing the absence of E in IFN treated TP-A cells.

Lane 5: Mixed extract from control cells treated with IFNα (lane 2) and untreated TP-A cells (lane 3) showing that TP does not inhibit the binding of pre-formed E.

Lane 6: Mutant cells (U2) treated with IFNα,showing no induction of E.

Lane 7: Mixed extracts from POL-A$^-$ cells treated with IFNγ (containing an induced level of Eγ) and U2 cells treated with IFNα (containing Eα), showing formation of E by complementation. (For controls see lanes 6 and 9).

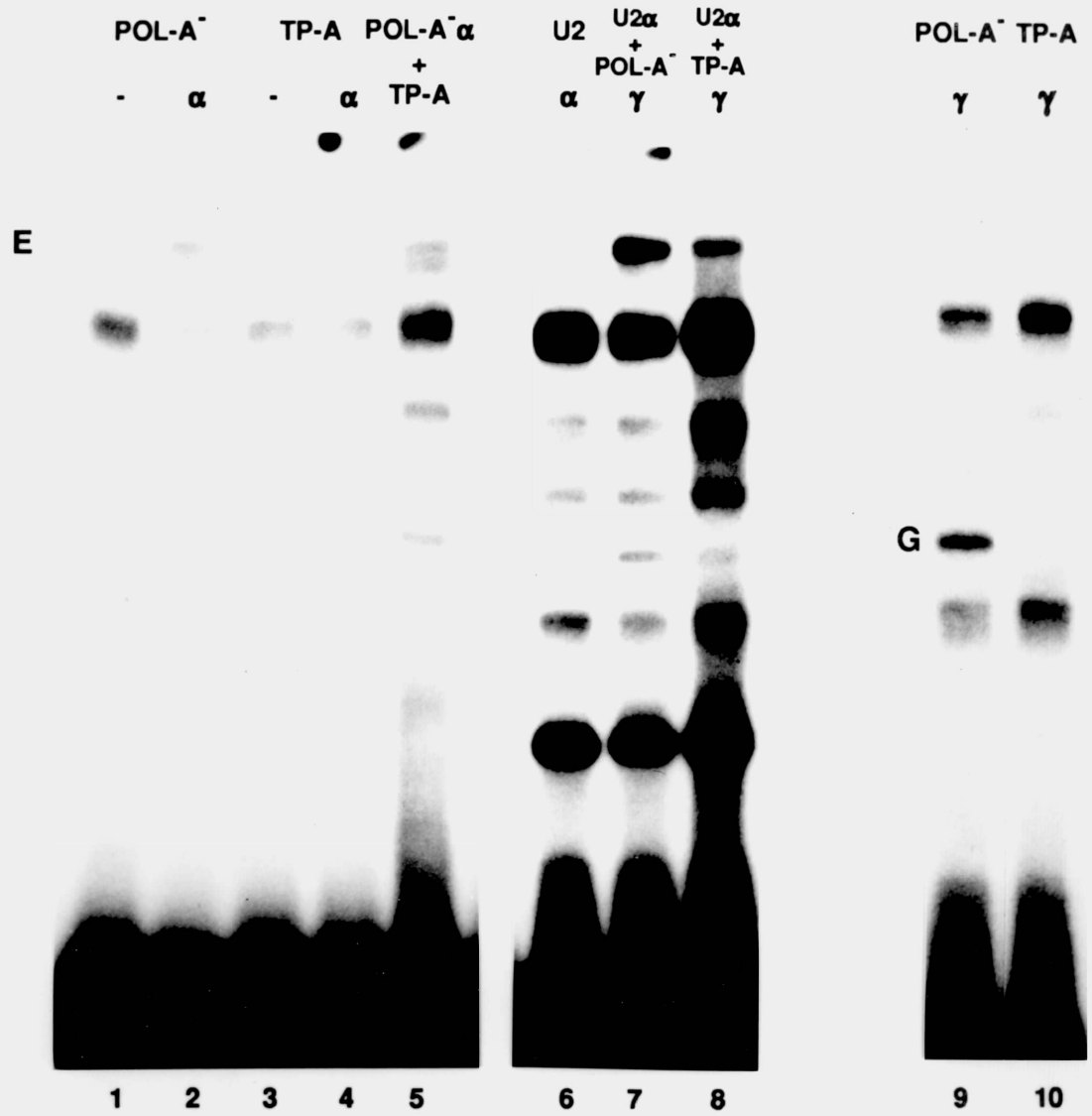
Lane 8: Mixed extract from TP-A cells treated with IFNγ (containing an uninduced level of Eγ) and U2 cells treated with IFNa (containing Eα), showing formation of E by complementation, but at a lower level than in lane 7, presumably because IFNγ does not induce Eγ in TP-A cells). For controls see lanes 6 and 10.

Lanes 9 and 10: POL-A$^-$ and TP-A cells after treatment with IFNγ, showing the induction of G in control cells but not in cells expressing TP.

Notes

The band shift assay was performed using the standard conditions first described by Dale et al. (1989a). See Methods for a full description of the assay conditions. Under the conditions used the protein complex (shifted band) defined as E has been shown to bind specifically to the ISRE and is only present in cells treated with IFNα. The band G also binds specifically to the ISRE and is only found after treatment of the cells with IFNγ.

FIGURE 5.2


POL-A⁻
TP-A
POL-A⁻α + TP-A
U2
U2α + POL-A⁻
U2α + TP-A
POL-A⁻
TP-A
-
α
-
α
α
γ
γ
γ
γ
E
G
1
2
3
4
5
6
7
8
9
10

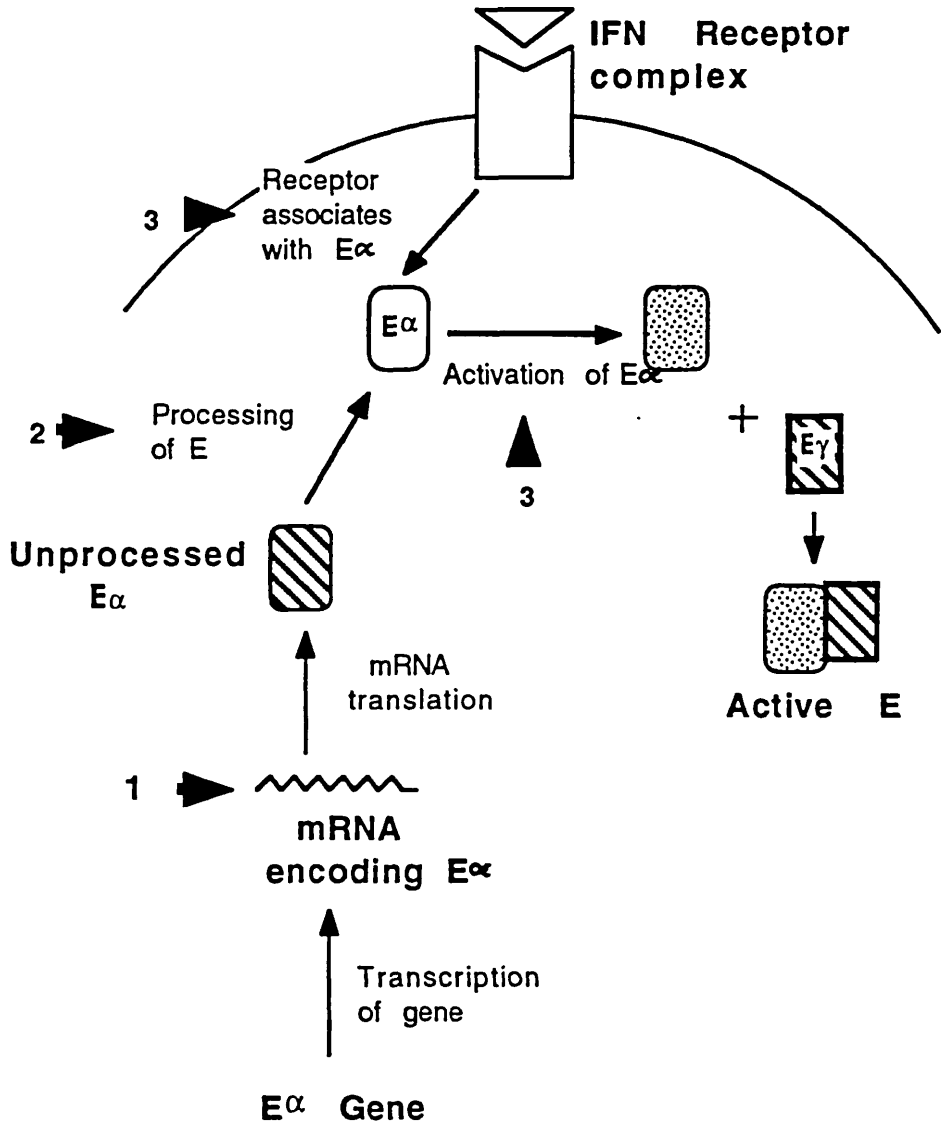
Eγ a series of complementation assays were performed. The mutant cell line U2 contains Eα but not Eγ (John et al., 1991) and hence does not produce E when treated with IFNα (Fig 5.2, lane 6). Addition of a cellular extract containing Eγ to an extract from U2 cells causes E to appear (lane 7), showing that IFN treated U2 cells are defective in Eγ, but contain active Eα. When cellular extracts from IFNα treated U2 cells (containing active Eα) were incubated with extracts from cells containing TP, E was formed, indicating that TP-A cells contain Eγ but that Eα is either absent or can not be activated.

TP may inhibit the formation of active Eα at a number of different sites, illustrated in Figure 5.3. It may prevent the production of unprocessed Eα by binding to the mRNA (or possibly the DNA) encoding one of the Eα proteins (1). It may block the processing of the precursor to Eα by binding to one or more of the unprocessed Eα proteins (2). Alternatively TP might inhibit the conversion of latent Eα to active Eα either by interfering with the element that conveys the signal for Eα activation from the IFN receptor to latent Eα (3), or by binding directly to one or more of the latent Eα sub-units. To determine the precise site of action of TP a genetic complementation analysis was performed.

FIGURE 5.3

Possible sites of action of TP

See text for details



1.1) GENETIC ANALYSIS OF THE EFFECTS OF TERMINAL PROTEIN

The inhibitory effect of TP is probably concentration dependent, since large amounts of constructs encoding TP are required to inhibit the response to IFN in transient assays (see Chapter 4). This suggests that TP interacts with its target factor stoichiometrically. If this is so then overexpression of the target factor should reverse the inhibitory effects of TP. TP-A cells contain a selectable marker (the *gpt* gene under the control of an IFN inducible promoter) that can be used to select for cells that respond to IFN by growth in HAT medium plus IFN (see Chapter 1A, 1.1e for a detailed discussion of this selection). If overexpression of the target of TP (be it a DNA, RNA or protein) is achieved in TP-A cells, they should recover their response to IFN and grow in HAT plus IFN medium. To determine whether complementation of TP-A cells was feasible the reversion frequency was determined, to identify the frequency with which TP-A cells regain their response to IFN. TP-A cells (5×10^6) were plated at a density of 5×10^5 cells per 10- cm plate and grown for three weeks in HAT plus IFN medium. No revertant colonies grew, showing that the reversion frequency is low. Experiments were therefore initiated in which cDNA libraries were transfected into TP-A cells and revertants that responded to IFNα were selected in HAT plus IFNα medium.

The successful reversion of the inhibition of the IFN response by TP presumably requires high level expression of the target factor. High level expression of transfected cDNAs can be achieved by transfecting EBNA-1 expressing cells with constructs containing a cDNA and the Epstein Barr virus (EBV) origin of replication (EBO). This transfection system was first described by Margolskee et al.(1988), and it has a number of advantages over conventional systems. The presence of

EBNA-1 in cells allows high frequency transfection with vectors containing EBO and the transfected DNA is maintained episomally, allowing its rapid recovery. Large amounts of episomal DNA can be produced in cells, with high level expression of cDNAs contained within the vector. The EBV based vector system provides an opportunity to express cDNA libraries at high levels in TP-A cells and to clone any gene(s) that can restore their response to IFNα, by selection in HAT plus IFN medium.

To obtain cells which express the EBNA-1 protein TP-A cells (4×10^5) were transfected with 20 μg of a construct containing the EBNA-1 gene under the control of the cytomegalovirus early promoter (kindly supplied by Dr D. Goeddell - see Chapter 2, 8.2, for a description of the EBNA-1 construct) and 2 μg of a construct expressing neomycin. Successful transfectants (20) were selected in G418. To determine which transfected clones expressed high levels of EBNA-1 protein a functional assay was used. Cells expressing EBNA-1 should have a high transfection efficiency for vectors containing EBO. EBNA-1 transfected TP-A cells (10^4) were tested for an increase in their transfection efficiency with 2 μg of a construct containing EBO and the β-galactosidase gene. Four days after transfection the cells were stained with a substrate for β-galactosidase to assess its expression (Table 5.1). Two clones showed an increase in the number of cells expressing β-galactosidase, when compared to parental TP-A cells, suggesting that their transfection efficiency had increased, presumably due to expression of the EBNA-1 protein. One of these clones (TP-A.E5) had a transfection efficiency of approximately 1%, as judged by the number of cells stained, and this clone was used for subsequent cDNA transfections.

Two cDNA libraries in an EBO containing vector were kindly supplied by Dr D Goedell. The libraries were derived from human neutrophils and one contained 5×10^5 clones and the other 1×10^5

TABLE 5.1

Expression of β-galactosidase in TP-A cells transfected with EBNA-1.

TP-A cells were transfected with an EBNA-1 expressing plasmid and the resulting clones were transfected with a construct containing the LAC-Z gene (which encodes the β-galactosidase protein) and the Epstien-Barr virus origin of replication (EBO). Three days after transfection expression of β-galactosidase was determined by staining the cells. The results for parental cells and 6 transfectants are shown.

CLONE	Number of cells staining per 1000 cells
TP-A	15
TP-A.E1	14
TP-A.E2	45
TP-A.E3	12
TP-A.E4	17
TP-A.E5	95
TP-A.E6	17

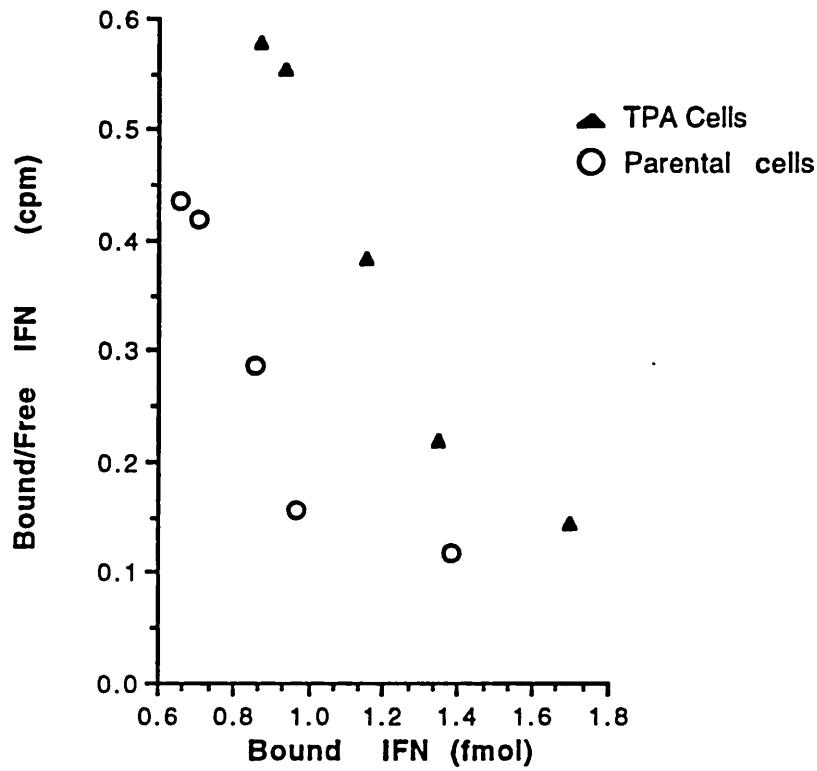
clones. Twenty μg of DNA from each of these libraries was transfected into 5 x 10^7 TP-A.E5 cells. Assuming a transfection efficiency of 1% (estimated from the number of cells expressing β-galactosidase in the experiment described above) approximately 5 x 10^5 cells were successfully transfected. Experiments on EBNA-1 expressing 2fTGH cells transfected with these libraries have shown that each transfected cell contains multiple copies of the transfected plasmid (R McKendry, ICRF - personal communication). It is therefore likely that TP-A.E5 cells were transfected with several copies of the cDNA libraries (5 x 10^5 cells were each transfected with several cDNAs from a library containing no more than 5 x 10^5 clones). Twenty-four hours after transfection the cells were selected in HAT plus IFNα medium for three weeks, to isolate any clones which had regained their response to IFN. No IFN responsive clones were identified. The failure to reverse the phenotype of the TP-A cells may be due to an error in our original assumption (ie TP may not act stoichiometrically) or the libraries used may not contain the appropriate cDNA. Further experiments will be required to evaluate this issue further.

2) INHIBITION OF THE RESPONSE TO INTERFERONγ

The IFNγ activation pathway is poorly understood (See Chapter 1A, 1.1d). IFNγ binds to a cell surface receptor and activates an ISRE binding protein known as G. To investigate the effects of TP on the IFNγ activation pathway the IFNγ receptors were studied using the method of Scatchard (Figure 5.4). The affinity of the IFNγ receptors and their number was not decreased in TP-A cells, showing that TP does not inhibit the binding of IFNγ to its receptor. The effects of TP on the induction of the IFNγ inducible factor, G, were studied using a band shift procedure. Figure 5.2 (Lanes 9 and 10) shows the result. The transactivating factor G was not activated in TP-A cells. Hence TP may inhibit the response to IFNγ by blocking the activation of the transactivating factor, G.

FIGURE 5.4

Scatchard plot performed with IFNγ



3) SEQUENCE HOMOLOGY BETWEEN TERMINAL PROTEIN AND OTHER PROTEINS

The only known functions of TP are to bind to the pregenomic HBV RNA and to the HBV DNA (Chapter 1,2.4). The functions of a large number of proteins are known and it is possible that information about the inhibitory effects of TP might be gained by analysing its relationship to proteins of known function. The protein sequence database PIR was therefore searched using the amino acid sequence of TP as the query sequence. No proteins showed close homology to TP (> 5% homology). However two proteins, Delta antigen and ISG15 showed some homology to a region within TP (Figure 5.5) and these two proteins were therefore examined further. (Experiments on Delta antigen and ISG 15 were performed in collaboration with Dr Alistair McNair, ICRF).

Delta antigen is the sole protein produced by the RNA virus, Delta, which causes hepatitis but requires HBV as a helper virus to form infectious virions (Gowans et al, 1988). Patients with chronic hepatitis who are infected with Delta virus do not respond to IFNα therapy [neither the HBV nor the Delta virus infection is eradicated by IFN (Porres et al., 1989)]. It is probable that their failure to respond to treatment is related to the inhibitory effects of the HBV virus but it is also possible that Delta virus itself can inhibit the response to IFN. To test this, experiments were conducted using transient assay systems and 2fTGH transfections with DNA encoding Delta antigen. These experiments (Dr Alistair McNair - personal communication) have not demonstrated any inhibitory effects of Delta antigen on the cellular response to IFN.

ISG15 is an IFN inducible protein whose intracellular concentration rises rapidly after IFN treatment. Since IFNs effects are transient and are followed by a period when the cells are refractory to further treatment with IFN it is possible that a natural inhibitor of IFN acts to

FIGURE 5.5

Proteins homologous to TP

The amino acid sequence (one letter code) is shown with the position of the amino acids within the protein (the first amino acid is designated 1)

```
TP (aa 33-53)          PLEEELPRLADEGLN-------RVAED
                          ||||  ||   |         ||
DELTA (aa 125-146)     EEEEELRRLTEEDERRERRVAG

TP (aa 30-47)          EAGPLEEELPRLADEGLN
                       |   |||   ||       ||
ISG15 (aa 127-143)     EGKPLEDQLPLGEY--GLK
```

downregulate the cells response to further IFN. This hypothesis is confirmed by experiments (Larner et al., 1986) which show that the IFN inducible refractory period can be abolished by inhibitors of protein synthesis, suggesting that production of a protein is required for the development of a refractory state. To determine whether ISG15 is a natural inhibitor of the IFN response pathway experiments with a construct expressing ISG15 were initiated. These experiments (Dr A McNair - personnal communication) suggest that ISG15 does not inhibit the cellular response to IFN.

CHAPTER 6
EFFECTS OF TERMINAL PROTEIN IN HBV INFECTIONS

In tissue culture systems using non-liver cell lines TP* can inhibit the cellular response to IFN. To determine the physiological relevance of this inhibition, experiments were performed in patients with chronic HBV infection.

1)STAINING FOR HBV PROTEINS AND β_2 - MICROGLOBULIN.

To examine the effects of terminal protein on the expression of IFN inducible genes in patients the cell surface protein β_2-microglobulin (β_2m) was used. This protein is a component of the MHC Class I molecule that can be induced by IFN. Previous studies have shown that β_2m can not be detected in liver biopsies from patients who are not receiving IFN therapy (Nagafuchi and Scheuer, 1986). However, in biopsies from patients who are receiving treatment with IFN, expression of β_2m is increased (Pignatelli et al, 1986) and can be detected. Hence expression of β_2m can be used to determine whether an individual hepatocyte has responded to IFN.

Liver biopsies from four patients with chronic HBV infection, who had never received IFN therapy, were stained with both of the antibodies directed against β_2m (see Chapter 2, 5.4). Infiltrating lymphocytes, which express this protein constitutively, stained positively (Fig 6.1A) but β_2m was not detected on the hepatocytes, confirming that it can not be detected in liver cells from patients who are not receiving IFN therapy.

* TP refers specifically to the amino terminal 270 amino acids of HBV polymerase (encoded by the construct pJ3-TP). The amino terminal region of polymerase detected in liver biopsies contains an unknown number of amino acids and is hence referred to as terminal protein rather than TP.

FIGURE 6.1

Expression of β_2m in liver biopsies.

A) Biopsy from a patient who had not received IFN therapy. There is no staining of the hepatocytes, but lymphocytes that express constitutive β_2m stain positively (black arrow).

B) Biopsy from a patient who was receiving IFN therapy. Many hepatocytes express β_2m (black arrow), but those expressing terminal protein (white arrow) do not.

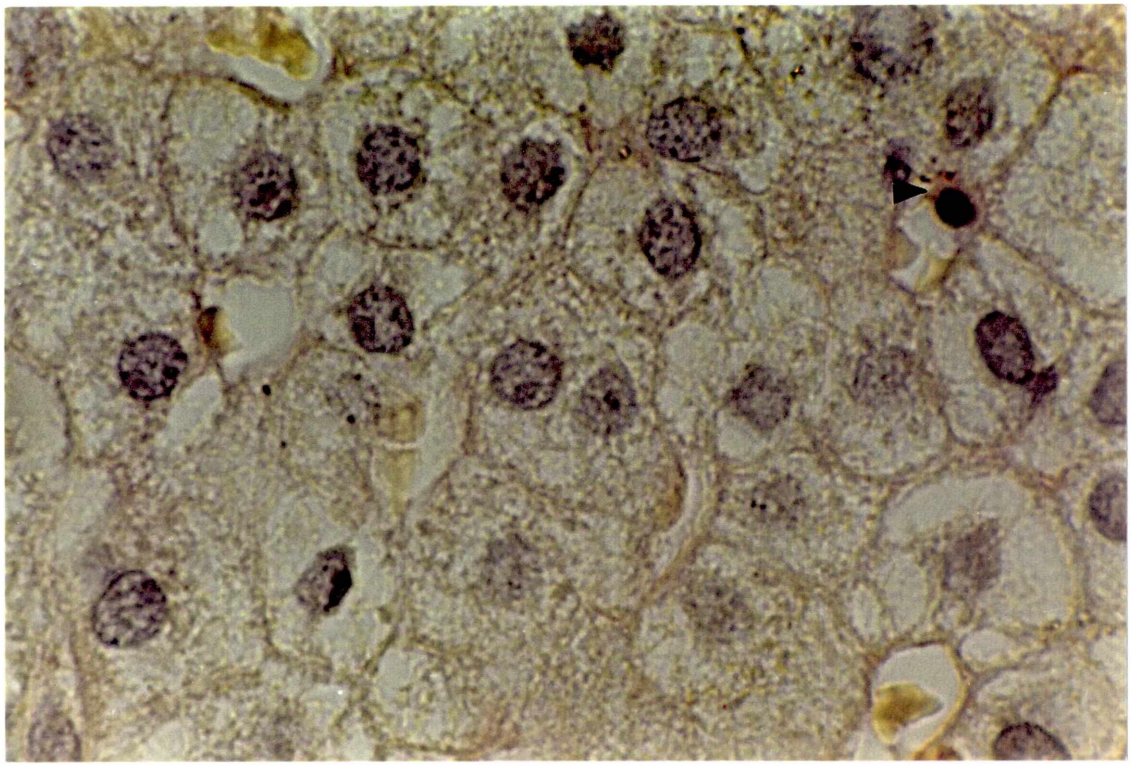
C) As B, photographed under oil.

Notes

The biopsy was stained with anti β_2 microglobulin antibodies (Sigma) and the antibody directed against terminal protein, antibody M.

FIGURE 6.1

A



B

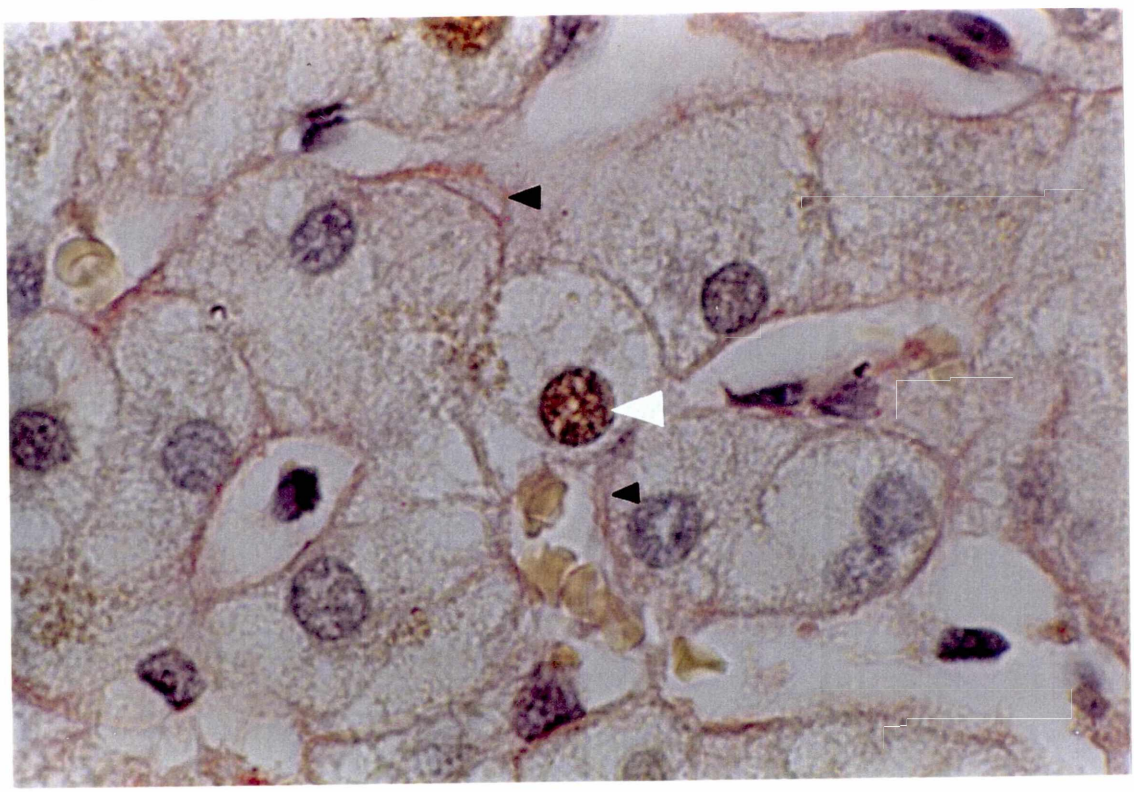
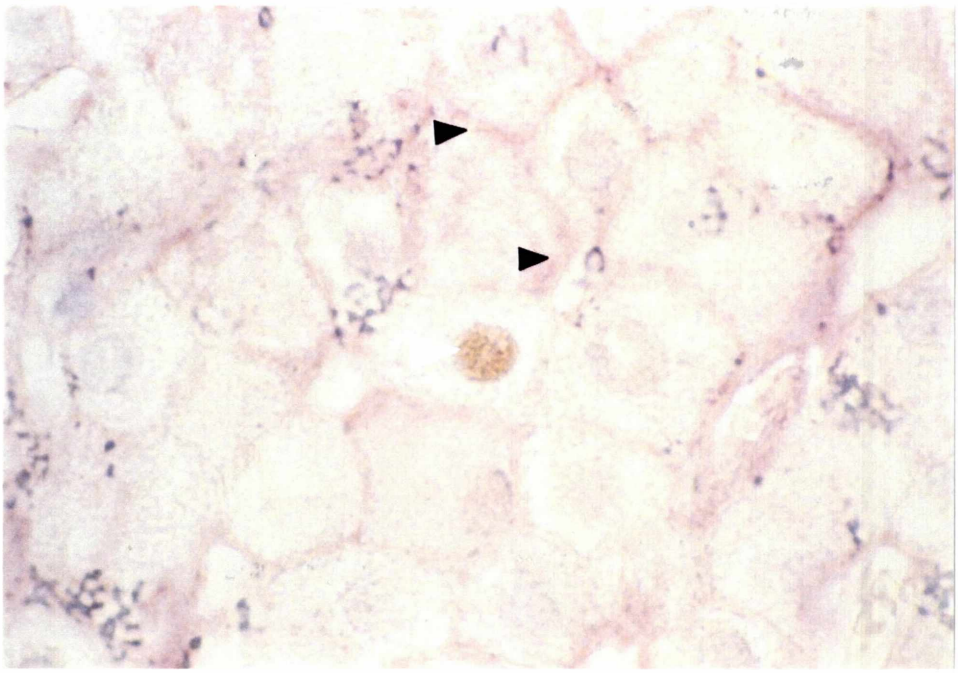


FIGURE 6.1 (Continued)

C

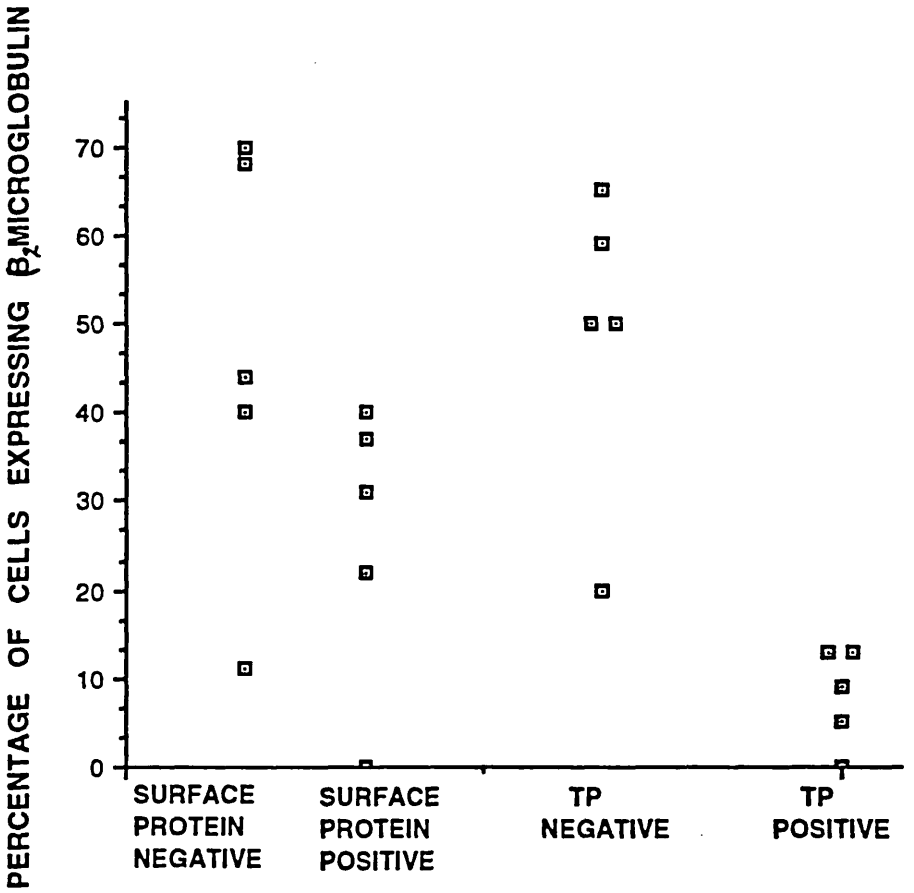


Liver biopsies taken whilst the patient was receiving IFN therapy were available from five patients (mean age 32 yrs, two were female). All were treated unsuccessfully with lymphoblastoid interferon (10Mu/m^2 three times a week for 12 weeks). Liver biopsies were performed at times ranging from 1 day to 2 months after starting treatment. Biopsies were stained with antibodies directed against β_2m and in these specimens (Figure 6.1 B and C) staining of the cytoplasm and membranes of hepatocytes was seen, indicating that IFN increases the expression of β_2m. Detection of β_2m on the surface of an hepatocyte was regarded as evidence that that cell had responded to IFN. The biopsies were doubly stained with antibodies against terminal protein and surface protein, as well as with the antibodies against β_2m, and the percentage of cells expressing the various proteins was determined by counting at least 1000 cells from each biopsy (Figure 6.2). The antibodies against terminal protein and surface proteins are from different species and therefore a separate antibody was used to stain for β_2m in each case (see Chapter 2, 5.4). There was no significant difference in the number of uninfected cells expressing β_2m with the two antibodies, suggesting that they are of comparable sensitivity. The percentage of cells that expressed surface protein and responded to IFN (median = 32) was significantly less than the percentage of uninfected cells that responded (median = 44 $p < 0.05$, Wilcoxon rank sum analysis for unpaired non-parametric variables), indicating that infection of cells with HBV may inhibit their response to IFN. Cells which express terminal protein in the cell nucleus rarely co-express β_2m (median = 9%), ie they do not usually respond to IFN. The percentage of cells expressing nuclear terminal protein and β_2m was significantly lower than the percentage of cells expressing surface protein and β_2m, suggesting that whilst cells expressing surface protein sometimes respond to IFN, cells expressing terminal protein respond only rarely, suggesting that in patients terminal protein may inhibit the cellular

FIGURE 6.2

Co-expression of HBV surface protein and nuclear terminal protein and β_2m.

Liver biopsies from patients receiving IFN were stained with an antibody against β_2m. Cells which were stained on all or all but one of the visible cell edges were classed as β_2m positive. Cells which showed diffuse cytoplasmic staining (surface) or nuclear staining (terminal protein) were classed as positive for that HBV protein. A few cells stained positively in the cytoplasm with the antibody directed against TP, but the number of such cells was small and too few cells were observed to allow any meaningful analysis.



response to IFN. The finding that some cells which express terminal protein do respond to IFN may be due to the concentration dependence of terminal protein's inhibitory properties (see Chapters 4 and 5). Since large amounts of a construct expressing TP are required to inhibit the response to IFN in transient assay systems it is probable that large amounts of TP are required to inhibit the cellular response to IFN. In patients some cells that express detectable terminal protein may not express enough to inhibit the IFN response.

2) EXPRESSION OF HBV PROTEINS AND RESPONSE TO INTERFERON THERAPY

To determine whether the expression of terminal protein was different in patients with IFN-resistant HBV infections, we examined pre-treatment liver biopsies taken from patients about to undergo therapy. Of 28 patients studied, 15 responded to therapy (ie lost HBeAg and developed anti- HBe antibodies) and 13 did not. There was no significant difference in age, duration of infection and extent of liver damage (assessed by measuring the degree of hepatic inflammation using the Knoedel scoring system (Knoedel et al., 1983) between responders and non-responders (Table 6.1).

The pre-treatment expression of HBV proteins in the two groups of patients is shown in Figure 6.3. There was a significant increase in the percentage of cells expressing nuclear terminal protein in non-responders (median = 16%) compared to responders (median = 8%, $p<0.05$, Wilcoxon rank sum analysis for paired non-parametric variables) suggesting that overexpression of this protein is associated with failure of IFN treatment. There was a slight increase in the number of cells expressing nucleocapsid protein in non-responders (median = 21%) compared to responders (median = 14%) but this was not statistically

significant ($p>0.05$). Expression of surface protein was similar in the two groups of patients (median in non-responders = 11%, median in responders = 15%, $p>0.05$). Hence, high level expression of terminal protein is associated with failure to respond to IFN treatment.

TABLE 6.1

Patient details for patients whose liver biopsies were examined before and after IFN therapy.

	Non-responders	Responders
Female	3	4
Male	10	11
Mean age (yrs)	39	37
Mean duration of infection (months)	55	48
Mean Knoedell score	7.1	7.2

FIGURE 6.3

Expression of HBV proteins in patients who did or did not respond to IFN therapy

Results are expressed as bar charts, with the percentage of cells expressing each HBV protein shown on the x axis and the number of patients shown on the y axis.

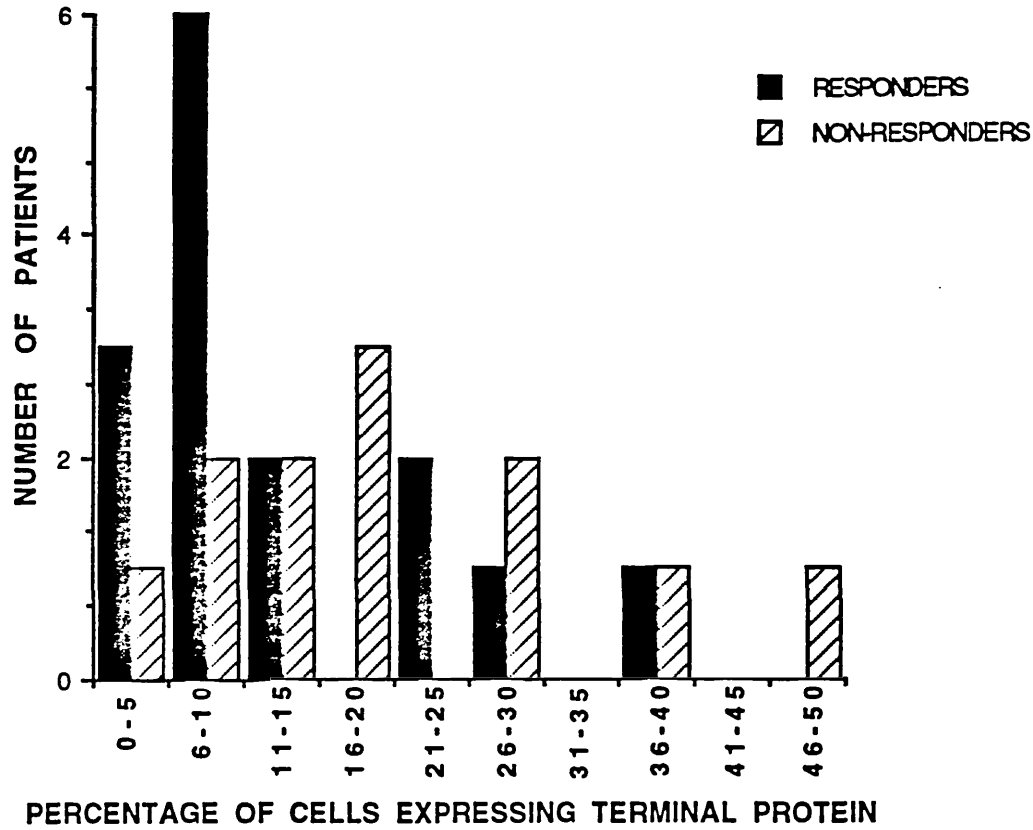
A: expression of nuclear terminal protein

B : expression of nucleocapsid protein

C : expression of surface protein

FIGURE 6.3

A


RESPONDERS
NON-RESPONDERS
NUMBER OF PATIENTS
0
2
4
6
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
PERCENTAGE OF CELLS EXPRESSING TERMINAL PROTEIN

B

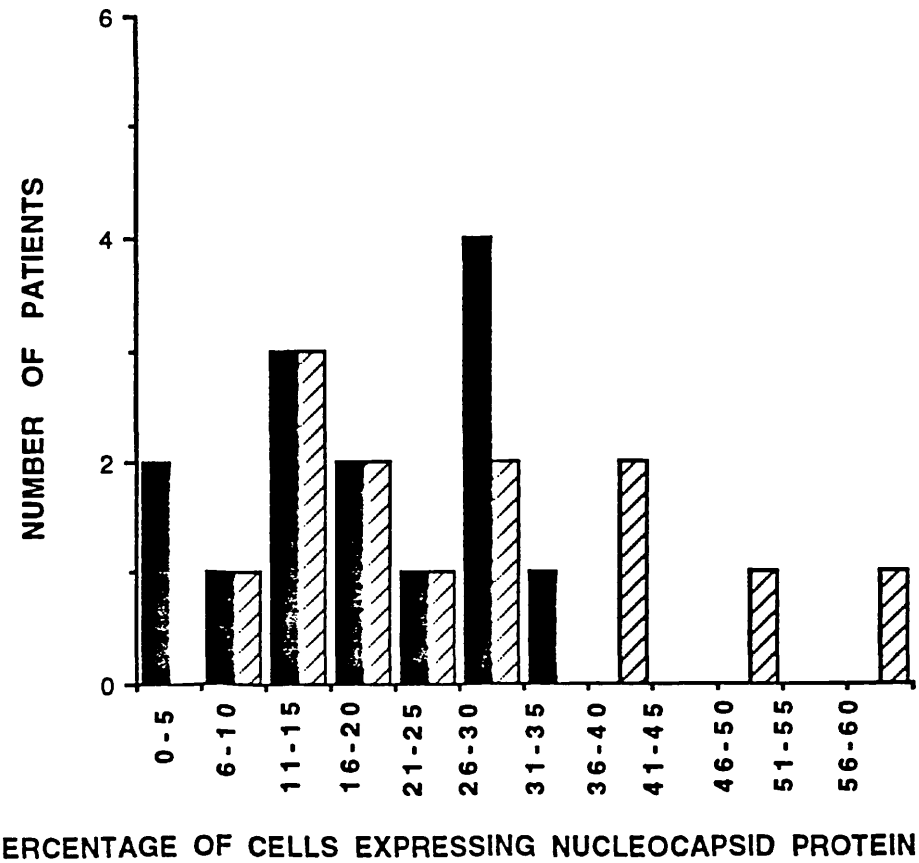
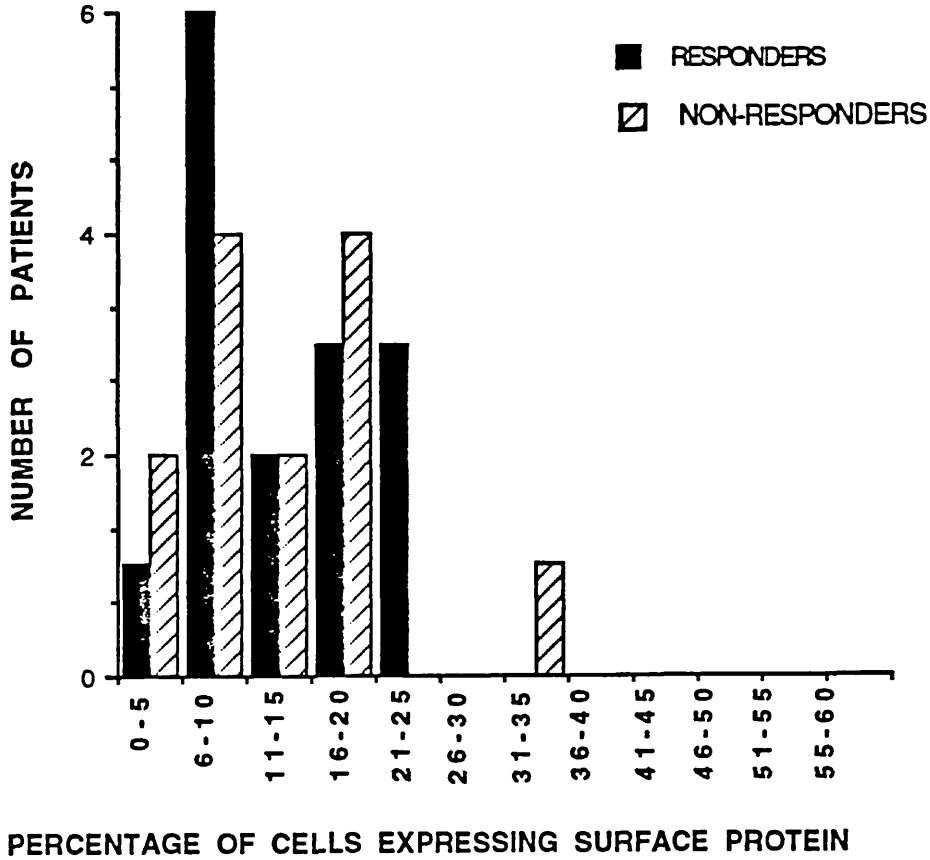

NUMBER OF PATIENTS
0
2
4
6
0-5
6-10
11-15
16-20
21-25
26-30
31-35
36-40
41-45
46-50
51-55
56-60
PERCENTAGE OF CELLS EXPRESSING NUCLEOCAPSID PROTEIN

FIGURE 6.3 (Continued)

C



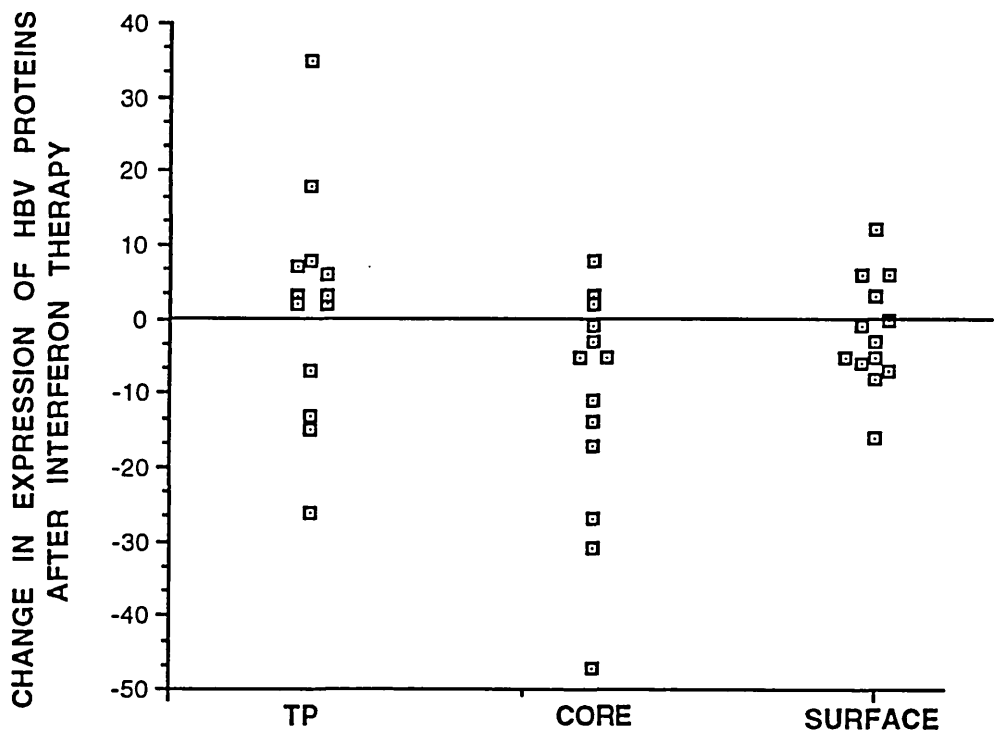
3) EFFECTS OF INTERFERON THERAPY ON EXPRESSION OF HEPATITIS B PROTEINS

The effects of IFN therapy on expression of HBV proteins was studied by examining post treatment liver biopsies from the 13 patients who did not respond to treatment (Figure 6.4). IFN therapy did not significantly alter the number of cells expressing terminal protein (median = 16% before treatment, median = 19% after treatment $p > 0.05$) but there was a significant decrease in the number of cells expressing the nucleocapsid protein (median before therapy = 24%, median after therapy = 12%, $p < 0.05$). There was no significant change in the number of cells which expressed surface protein after IFN therapy (median = 11% pretreatment, median = 8% post-treatment). These results suggest that IFN treatment is able to eliminate some of the cells expressing the nucleocapsid protein but not cells expressing nuclear terminal protein, suggesting that cells expressing nuclear terminal protein are resistant to the effects of IFN. It was interesting that cells expressing surface protein were not eliminated by IFN therapy. Failure to eliminate these cells may be related to failure of immunocytes to be sensitised to this protein, as previously suggested (Sampliner et al., 1979).

FIGURE 6.4

Changes in the expression of HBV proteins after IFN therapy

Values are expressed as the percentages of cells expressing the protein before treatment minus the percentage of cells expressing the protein after therapy



4) MECHANISM OF OVER-EXPRESSION OF TERMINAL PROTEIN

4.1) VIRAL MUTANTS

The factors that regulate production of HBV polymerase are poorly understood. In an acute infection the pregenomic HBV RNA may be used both as a template for reverse transcription and to synthesise polymerase and core proteins. This pregenomic RNA contains several potential translational start codons (the DNA sequence - ATG). This sequence motif can be used either to initiate translation of a mRNA and/or it can be used to encode the amino acid methionine. The factors which determine whether a particular ATG is used as a translational start site or as a coding sequence are complex (reviewed in Kozak, 1991). In general ATG sequences which are associated with a specific DNA sequence, known as a Kozak consensus sequence, are used for translation and those which are not associated with this sequence are not. However a number of other factors, such as the length and sequence of the RNA preceeding the ATG also influence translational usage. The first ATG (ie the ATG nearest the 5' end of the RNA) of the pregenomic HBV RNA is associated with a Kozak consensus sequence and hence this ATG (which produces pre-core proteins) may be preferentially used by ribosomes (Ou et al, 1990). The other ATGs on this mRNA (which initiate translation of the core and polymerase genes) are not associated with Kozak sequences and hence are translated relatively poorly. One likely region for mutations that might affect formation of polymerase and terminal protein is near these ATGs. Mutations in the sequences surrounding the pregenomic mRNA ATGs might significantly alter the translation of the pregenomic RNA.

The DNA sequence surrounding the first (precore) ATG of the

pregenomic mRNA were examined to see if there were any consistent changes in patients who did not respond to IFN. Sequence data was obtained by polymerase chain reaction amplification of HBV DNA extracted from pre-treatment serum samples from 10 patients who subsequently received IFNα therapy (the primers used to amplify and sequence the DNA have been described previously, Carman et al, 1989). Six of these patients responded to IFN and four did not (sequence data from Dr WC Carman, Glasgow University).The sequences are shown in Figure 6.5. All patients who responded to IFN therapy had wild-type sequence but three of the four non-responders had consis tent changes around the first ATG that disrupt the Kozak sequence and are predicted to decrease usage of the first ATG. They therefore might increase usage of later pregenomic ATGs, and may decrease the production of pre-core and core proteins and increase production of polymerase.

To test this hypothesis two constructs were made. Each contained the HBV enhancer and the core promoter region. The core gene is intact in both constructs, but 120 bp from the POL ATG the LAC Z gene, encoding β-galactosidase, was cloned in-frame with the POL open reading frame. Hence production of polymerase could be assessed by examining production of β-galactosidase. The constructs were made with and without the mutations observed in patients and each was transfected into HepG2 cells along with an SV40 CAT construct (as a transfection control). Two days after transfection expression of β-galactosidase (equivalent to polymerase) was assessed enzymatically. There was no significant change in β-gal production with the mutant promoters, (Figure 6.6) suggesting that either these mutations do not increase production of the polymerase protein or that the assay system used was not sufficiently sensitive to detect small changes in polymerase production.

FIGURE 6.5

Pre-core flanking sequences in patients who did or did not respond to IFN.

The ATG which acts as the start signal for precore translation is underlined.

Wild type sequence	CAGCACCATGCAACT
Kozak consensus	GCCACCATGG
Responder patients (6)	CAGCACCATGCAACT
Non-responders (1)	CAGCACCATGCAACT
Non-responders (3)	CA TCATCATGCAACT

FIGURE 6.6

Analysis of Kozak sequence mutations.

A) Schematic diagram of test constructs

The HBV regions illustrated were cloned into a pBR based vector as shown. (Numbers in brackets are nucleotides in genomic HBV with the EcoR1 site equal to 1). The core promoter was either wild type or contained the mutations previously described.

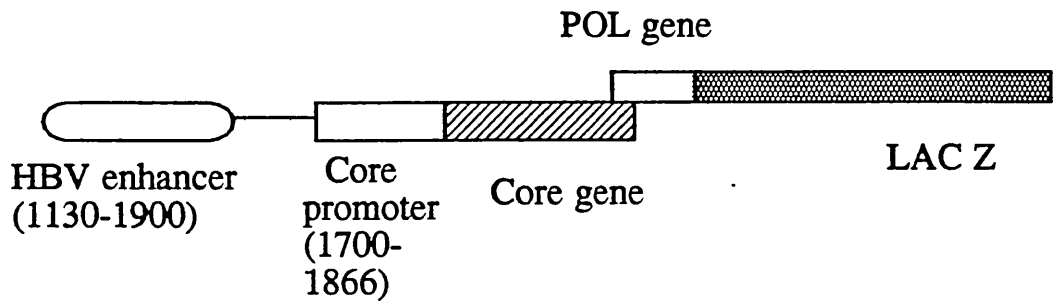
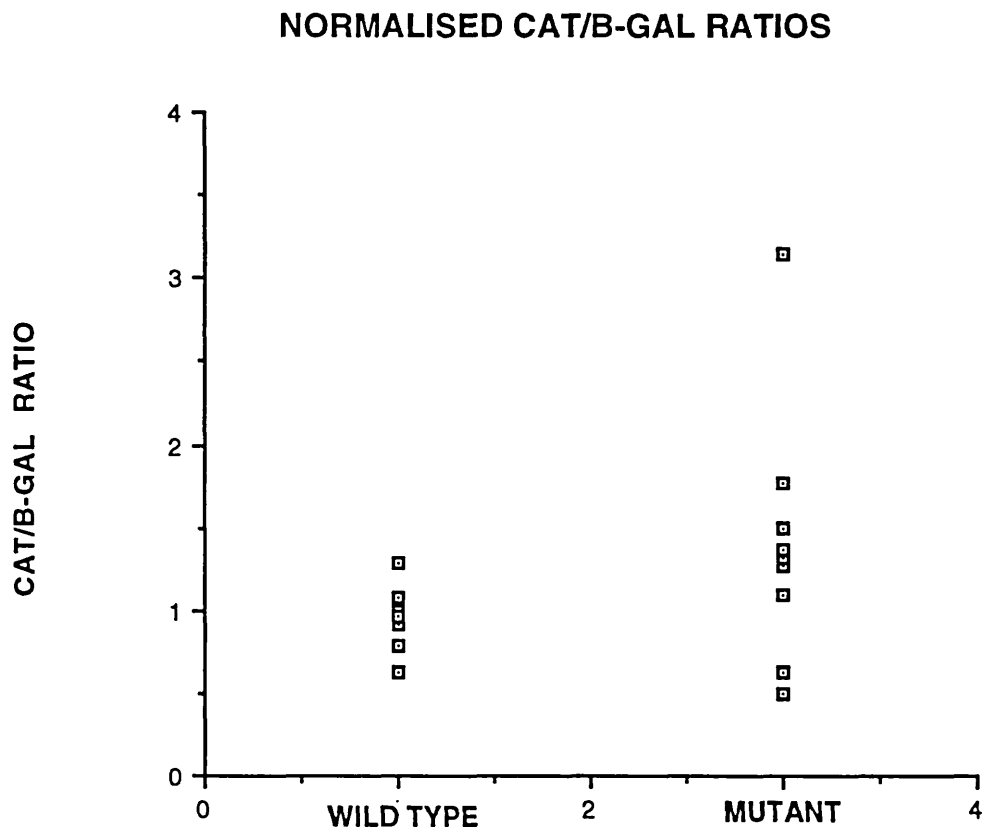


FIGURE 6.6 (Continued)

B) Production of β-galactosidase after transfection of test constructs into HepG2 cells.

Results are expressed as the ratio of β-galactosidase : CAT activity and each point is the average from two simultaneous experiments.



Studies to identify mutant viruses which might give rise to the phenotype observed in patients who do not respond to IFN (ie overproduce terminal protein) have therefore been unsuccessful. However to-date only one region of HBV has been investigated and there are a number of other regions within HBV where mutations might alter the production of polymerase. Further work will be required to address this possibility.

4.2) INTEGRATION OF HBV AND PRODUCTION OF TERMINAL PROTEIN

An alternative mechanism where terminal protein could be overproduced in patients with IFN-resistant HBV infection is by integration of HBV DNA into the host genome so as to cause overproduction of terminal protein. To determine whether HBV integration could generate terminal protein the published sequences of integrated HBV DNA were examined. The EMBL DNA Sequence Database was searched for sequences of integrated HBV and 16 were found. These sequences, and the HBV POL gene are shown schematically in Figure 6.7. In many (7/16) of these integrated sequences the polymerase ATG was intact and the coding sequence for terminal protein was present. Hence when HBV DNA integrates in the genome active terminal protein might be produced.

In patients with chronic HBV infection we have found that overproduction of terminal protein is associated with a failure to respond to IFN therapy. Attempts to determine the molecular basis for this increased production have been inconclusive.

FIGURE 6.7

Sequences of integrated HBV sequences

The upper panel shows linearised HBV with the direct repeat (DR) sequences marked. The POL open reading frame is also shown.The lower panels show schematically the integrated HBV sequences. The bold lines are HBV sequence (aligned with the upper panel) and the fine lines are regions of genomic DNA which join the HBV sequences.

A) Sequence from Ziemer et al.,1985.

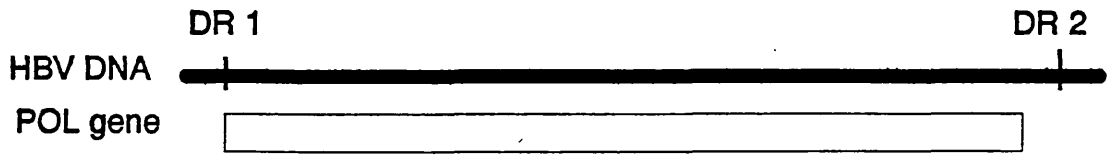
B) and C) Data from Yaginuma et al,1987.

D) Data from Hino et al, *1987*

E) Data from Matsumoto et al, 1988.

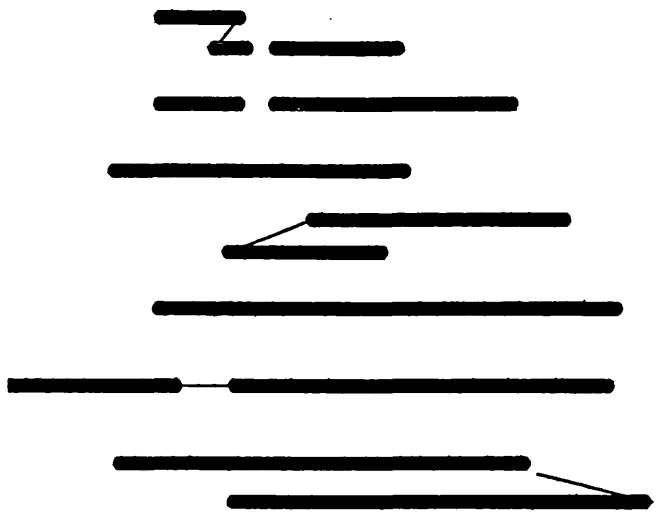
All the sequences are from hepatocellular carcinomas, except for C) which is from a patient with chronic HBV infection.

HBV DNA

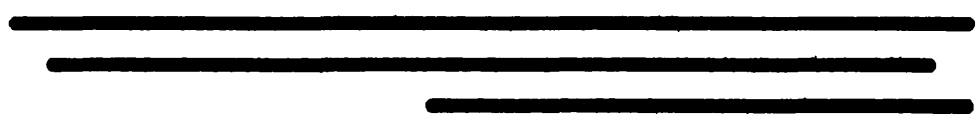


INTEGRATED SEQUENCES

A) Alexander cell line



B)Tissue sample 1707



C)Tissue sample CAH

D) Tissue samples C3 and C4

E) HCC Tissue samples

CHAPTER 7

DISCUSSION

Clinical studies on patients with chronic HBV infection show that up to 50% can be cured by IFN therapy (Jacyna and Thomas, 1990). The reasons why some patients do not respond to treatment are poorly understood. Clinical studies on patients who do not respond to therapy have shown that they do not show the expected increase in hepatic expression of HLA Class I proteins (Pignatelli et al, 1986), suggesting that they may be unable to respond to IFN. One possible explanation for this is that HBV contains a factor that inhibits the response to IFN. Viral inhibitors of the cellular response to IFN have been described elsewhere (Ackrill et al, 1991) and HBV is also known to impair the production of IFN (Twu and Schloemer, 1988). It is therefore likely that HBV contains an inhibitor of the IFN response pathway and this possibility has been investigated.

1) EFFECTS OF GENOMIC HBV AND POLYMERASE

Studies on cell lines transfected with the entire HBV genome did not show any evidence of a failure to respond to IFN. However HBV proteins are poorly expressed in non-liver cell lines and it is likely that the failure to detect any inhibitory effects of the whole HBV genome is due to low expression rather than to a failure of genomic HBV to inhibit the IFN response. This problem could be overcome by transfecting genomic HBV into hepatocellular carcinoma cell lines where liver specific factors should be available to facilitate expression of the HBV genes. However other groups have found that even in these cells it is difficult to obtain stable expression of all the HBV proteins (B. Clarke -

personal communication). To avoid these difficulties we used non-liver cell lines and sub-cloned the individual HBV genes into eukaryotic expression vectors which express the cloned genes in human cell lines. In this way we tried to ensure that the HBV genes under examination were expressed at a high level. One problem with this approach is that it is impossible to study interactions between HBV proteins that might influence their effects on the IFN response pathway. To overcome this, non-liver cell lines were used to identify potential inhibitors of the IFN response pathway and the effects of these proteins were then studied in the most relevant context - patients with chronic HBV infection.

Transient assay systems are an experimentally convenient way of screening potential inhibitors of the IFN response pathway. Using these assays it has been shown that the polymerase protein of HBV can inhibit the cellular response to IFN. However in transient assay systems neither the reporter DNA nor the test construct are integrated into genomic DNA and the transfected DNA may be expressed at non-physiological levels.. It is possible that under these conditions artefacts may arise that do not represent the normal situation, when the reporter gene is integrated in the genome. To overcome this problem and to analyse the site of action of polymerase cell lines stably expressing the polymerase protein were developed.

Initial attempts to isolate polymerase-expressing stable cell lines which did not respond to IFN were only partially successful - one clone was obtained which showed an unstable decrease in its response to IFN, but expression of polymerase could not be confirmed in this clone. The most likely explanation for the failure to isolate IFN resistant clones from cells transfected with polymerase is that the toxicity of polymerase prevented its expression. This hypothesis is in accordance with the experience of others who have been unable to develop cell lines stably

expressing the polymerase protein (Bavand and Laub, 1989, B. Clarke - personnal communication). It is perhaps not surprising that expression of polymerase is difficult to achieve. Polymerase contains an RNase domain and a reverse transcriptase domain. Unregulated expression of these elements may be harmful to cells and a construct expressing these domains has a greatly reduced transfection efficiency (about 10 fold lower than those of other DNA constructs), suggesting that these polymerase domains are indeed toxic. It is likely that the carboxy terminus of polymerase can not normally be expressed in cell lines.

To determine whether polymerase can inhibit the response to IFN of endogenous genes and to derive cell lines stably expressing this protein the cell line 2fTGH was used. These cells contain an IFN inducible toxin and have two advantages : they can be used to screen a large number of clones for those that do not respond to IFN and they can be used to apply a direct selection pressure for any protein which inhibits the IFN response pathway. We have used these cells to screen a large number of cells transfected with HBV polymerase and we find that 1 in 50 transfectants do not respond to IFN. Since the frequency of IFN resistance in polymerase transfectants is much greater than in cells transfected with other constructs it is likely that polymerase can inhibit the IFN response of endogenous genes. Polymerase expression in 2fTGH cells can be maintained by growing the cells in medium which selects for IFN resistant clones (6TG plus IFN). If this positive selection pressure is withdrawn polymerase expression is rapidly lost and the response to IFN returns. Hence the inhibition of the cellular response to IFN is only present when 2fTGH cells express polymerase, confirming that the expression of polymerase is responsible for the inhibition of the cellular response to IFN.

An alternative explanation for these results is that polymerase can

only be expressed in cells which do not respond to IFN. In this scenario 2fTGH cells are presumed to lose their response to IFN and then become permissive for polymerase expression. This explanation is extremely unlikely as the frequency of IFN resistance in 2fTGH cells is low (Pellegrini et al., 1989) and the frequency with which IFN resistant polymerase expressing cells are found is some 10^7 times greater than the frequency of IFN resistant clones. It is therefore likely that polymerase inhibits the response to IFN and that this inhibition allows cells expressing it to be selected.

The frequency of IFN resistant clones in 2fTGH cells transfected with polymerase is low - only 1 in 50 of the transfected cells survive the selection for IFN resistance. This low frequency may be because the effects of polymerase on the IFN response pathway are concentration dependent and only cells that express large amounts of polymerase can inhibit the IFN response. Since polymerase may be toxic when it is expressed at very high levels it seems likely that only a very small proportion of transfected cells will express the requisite amount of polymerase - cells that express too much polymerase will die as a result of the toxic properties of polymerase and cells that express too little will not inhibit the response to IFN and hence will not survive the selection. The hypothesis that polymerase must be expressed in large amounts to inhibit the response to IFN is confirmed by experiments in cells transiently transfected with polymerase- expressing constructs: large amounts of polymerase-encoding DNA are required to inhibit the response to IFN in these assays. Since large amounts of polymerase DNA are required to inhibit the response to IFN we presume that large amounts of the protein are required. However it is possible that the polymerase-encoding DNA is poorly translated and/or poorly processed and hence only small amounts of protein are required but large amounts

of DNA are needed to produce this protein.

In transient cell systems,polymerase inhibits responses to both IFNα and IFNγ. In cells stably transfected with polymerase only the response to IFNα is inhibited and the inhibition is incomplete. This is probably because the transfected cells do not express sufficient polymerase to completely inhibit the response to IFNα and do not express enough to affect the response to IFNγ. We presume that expression of sufficient polymerase to inhibit the response to IFNγ is toxic to these cells and hence clones which do not respond to IFNγ can not be selected.

2)EFFECTS OF TERMINAL PROTEIN

The toxicity of HBV polymerase makes it difficult to analyse its effects on the IFN response pathway. These difficulties have been overcome by a deletion analysis of polymerase, which have shown that the terminal protein domain is responsible for the inhibitory effects. Terminal protein is not toxic to cells (as determined by the normal growth characteristics of cells stably expressing this protein) and can be readily expressed in stable transfectants. The frequency of IFN resistance in 2fTGH cells transfected with a construct expressing terminal protein is higher than the frequency of resistance in polymerase transfectants but is still relatively low -1 in 30 cells transfected with terminal protein are resistant to the effects of IFN. The relatively low frequency with which IFN resistant clones are isolated from cells transfected with terminal protein may be because large amounts of this protein are required to inhibit the response to IFN and that high level expression is not readily achieved. It would be interesting to observe the concentration dependence of terminal protein's effects in stable transfections but at present antibodies which can be used in quantitative

assays are not available.

3) MECHANISM OF ACTION OF TERMINAL PROTEIN AND POLYMERASE

There is no direct evidence that polymerase and terminal protein act on the IFN response pathway in the same way. However the features of both are similar - both appear to act in a concentration dependent fashion and for both large amounts of DNA are required to inhibit the response to IFN. Since the carboxy terminal region of polymerase does not possess any IFN inhibitory properties it is likely that both polymerase and terminal protein affect the IFN response pathway in the same way.

The mechanism by which terminal protein exerts its effects on the IFN response pathway remains unclear. Terminal protein inhibits a number of different response pathways (both the ISRE and non-ISRE IFN response pathways are inhibited as is the induction pathway used by dsRNA) but all the affected pathways are IFN related. Terminal protein may interfere with each pathway differently or it may act on a common element in these related pathways. Since polymerase can inhibit the response to IFNα without inhibiting the response to IFNγ it is unlikely that polymerase/terminal protein acts on a single common factor and it is more probable that polymerase/terminal protein interacts with different elements in each pathway, although the elements may be related. An alternative possibility is that polymerase and terminal protein act on an element that is common to all of the pathways but that in the IFNγ response pathway the element is poorly accessed by polymerase but is readily accessed by the smaller terminal protein.

The inhibition of the IFNα response pathway has been studied in

most detail. Terminal protein acts by preventing the formation of an essential transactivating factor, active Eα. The process by which Eα is activated in normal cells is not yet understood. Preliminary data (J. Darnell, C Schindler - personal communication) suggests that Eα is phosphorylated when IFNα binds to its receptor and it is most probable that this phosphorylation activates it, probably allowing it to bind to Eγ and form the transactivating factor E. Terminal protein might act by preventing the synthesis of one or more of the protein sub-units of Eα. However recent work (Dr A McNair, GRF - unpublished) using antisera directed against the protein sub-units of Eα has shown that cells expressing terminal protein contain normal amounts of all three of the Eα sub-units. Since the protein sub-units of Eα are present in cells expressing terminal protein, terminal protein must act by inhibiting the conversion of Eα from an inactive to an active form.

There are a number of different ways in which terminal protein might inhibit the formation of active Eα. The Eα proteins may require modification (perhaps by phosphorylation) before they can be activated and terminal protein might inhibit this, either by binding directly to one or more of the unprocessed Eα subunits or by interacting with the modifying enzyme. Alternatively terminal protein might interact with the modified, inactive Eα sub-units preventing their subsequent activation by IFN. Terminal protein might also interact with the enzyme(s) which activate the latent, modified Eα subunits.

It is not known how terminal protein inhibits cellular responses to IFNγ and dsRNA. There is some evidence for the involvement of Eα in the IFNγ induction pathway (some of the Eα subunits are phosphorylated when cells are treated with IFNγ - J. Darnell - personal communication) but Eα is unlikely to be involved in the dsRNA induction pathway. Terminal protein may interact with different factors to inhibit

cellular responses to these agonists or terminal protein may interact with an enzyme which is common to all of the pathways. Since these different pathways can specifically activate different genes it seems unlikely that they share a common activating enzyme, but they may share a common priming enzyme which converts latent transactivating factors to a form which can be activated by an appropriate stimulus. Terminal protein may act by inactivating an enzyme which primes the transactivating factors (a sub-unit of Eα in the case of the IFNα pathway). However alternative possibilities do exist - for example the enzymes which activate the transactivating factors in the pathways that are inhibited by terminal protein may be different (allowing specificity), but homologous, allowing inhibition by a single protein. At present there is insufficient data to determine precisely where terminal protein acts but the recent cloning of Eα should permit a full understanding of terminal protein's effects in the near future.

The active regions within terminal protein are unknown. Attempts to define an active site failed because expression of truncated products of terminal protein could not be assessed. This problem might be overcome by the development of better antibodies which are known to recognise fragments of terminal protein or it may be possible to introduce point mutations into terminal protein and examine the effects of these on its IFN inhibitory properties. An alternative strategy is to await the identification of the target enzyme with which terminal protein interacts and use an *in vitro* analysis of the effects of terminal protein derived peptides on this enzymes activity.

4) ROLE OF TERMINAL PROTEIN IN HBV INFECTIONS

4.1) PRODUCTION OF TERMINAL PROTEIN

The intact HBV polymerase protein can inhibit the cellular response to IFN in tissue culture systems. However in an infected hepatocyte polymerase is known to associate with the nucleocapsid protein and the pregenomic RNA, forming encapsidation complexes (Hirsch et al, 1990). Presumably this sequestration of polymerase will prevent it from being available to inhibit the cellular response to IFN. It therefore seems unlikely that intact polymerase acts as an inhibitor of IFN in patients, but the terminal protein domain may be available to inhibit the response. However terminal protein is a part of the polymerase protein and it is questionable whether it exists in a free form.

Little is known about the production, distribution and function of the products of the POL gene in infected hepatocytes. This is chiefly due to problems in developing good quality antibodies against polymerase: to-date it has not been possible to directly assay the products of the POL gene in hepatocytes, either from infected patients or in tissue culture systems. This problem has been partially overcome by Bartenschlager et al (1992) who introduced phosphorylation sites into the POL gene and transfected these variants into hepatocytes using a vaccinia based system. Using a combination of poor quality antibodies and phosphorylation of the novel phosphorylation site with radiolabelled phosphates, they have been able to identify POL gene products in hepatocytes. They find that the full length polymerase protein has a very short half-life and is rapidly degraded to form free terminal protein, which is relatively stable and which migrates to the nucleus. An obvious difficulty in interpreting these experiments is that the variant proteins may not behave in the same way as the natural protein. However their observations confirm the

impression from other experiments which have sugested that polymerase is unstable and it seems probable that polymerase is rapidly degraded in hepatocytes (Schaller et al,1988). Other groups (H Will - personnal communication) have found that the terminal protein domain of polymerase contains a nuclear localisation signal and using antibodies directed against terminal protein this group has identified terminal protein in the nucleus of infected hepatocytes. These experiments indicate that a mechanism exists which might produce free, nuclear, terminal protein in patients with HBV infections. We have used antibodies directed against terminal protein to examine the distribution of terminal protein in patients with chronic HBV infections. We find that antibodies directed against terminal protein bind to nuclear proteins and, rarely, to cytoplasmic proteins. These experiments suggest that in chronically infected hepatocytes free terminal protein does exist and is localised to the nucleus but clearly further experiments will be required to confirm this model of polymerase processing.

4.2) EFFECTS OF NUCLEAR TERMINAL PROTEIN

Studies on patients with chronic HBV infection show that cells which express nuclear terminal protein do not respond to IFN, as judged by expression of the IFN inducible protein β_2m, confirming that in patients terminal protein might act as an inhibitor of the IFN response pathway. If terminal protein does inhibit the IFN response pathway then IFN treatment should not eliminate cells which express it. This has been tested and we have found that IFN therapy does not eliminate cells expressing this protein. Alternative explanations for this observation do exist - terminal protein may not be recognised by immunocytes or may not be expressed on the cell surface, but the data are consistent with the hypothesis that terminal protein acts to inhibit the IFN response pathway in patients.

In patients with chronic HBV infection expression of nuclear terminal protein is associated with a failure to respond to IFN. However observations on the site of action of terminal protein suggest that it acts in the cytoplasm. The most likely explanation for this discrepancy is that some nuclear terminal protein diffuses from the nucleus and is available in the cytoplasm where it inhibits the IFN response pathway. If this cytoplasmic terminal protein is of low abundance then it may not be detectable by antibody staining techniques. Although we have not been able to demonstrate cytoplasmic terminal protein in liver biopsies from patients, cells transfected with a construct that expresses terminal protein did show evidence of both nuclear and cytoplasmic staining, suggesting that terminal protein may be able to partition into both cellular compartments.

If nuclear terminal protein does inhibit the response to IFN then it is reasonable to question why patients with acute HBV infection do not develop IFN resistant disease. The most likely explanation for this is that in an acute infection nuclear terminal protein is not formed - we predict that the polymerase which is formed in an acute infection will be encapsidated and not available to be degraded and inhibit the IFN response. It would be interesting to test this hypothesis directly but tissue samples from patients with acute HBV infection are not available - it is deemed unethical to expose patients to the risk of a liver biopsy for purely research purposes.

The finding that some patients with chronic HBV infection do respond to IFN suggests that terminal protein's anti IFN effects are not always present. There are three likely explanations for this - viral variants may exist which are ineffective inhibitors of the IFN response or host variation in terminal protein's target protein may render some patients resistant to its IFN inhibitory effects. An alternative possibility is that the

concentration of terminal protein is variable and in some patients insufficient terminal protein is expressed to inhibit the response to IFN. It is not possible to test this hypothesis directly as quantitative tests for terminal protein are not available. However a semi-quantitiative analysis of nuclear terminal protein has been performed by counting the number of cells which express it. Using this assay we have found that there is a significant increase in the expression of terminal protein in patients who do not respond to IFN, although the difference between non-responder and responder patients was small. This suggests that one of the factors which determines the response to IFN is the level of expression of terminal protein, although other factors, such as the activity of the host immune system may also be involved.

Attempts to determine the factors which might influence the expression of terminal protein have been only partially successful. One likely mechanism which could cause significant variation in the expression of terminal protein is the integration site of HBV DNA. If the HBV genome integrates close to an active promoter and if the POL gene ATG is intact then large amounts of polymerase, and its degradation product (terminal protein), will be produced. The sequences of integrated HBV have been examined and in many the POL gene and its ATG are preserved, showing that this is a possible explanation for the observed variation in terminal protein expression. Caselmann et al.(1989) have examined the integration of HBV in patients before and after IFN therapy. They report that prior to therapy there are multiple integration sites (Southern blots from pre-treatment hepatocyte DNA show a smear when probed with HBV DNA) but after therapy the number of HBV integrations decreases dramatically. (Southern blots from post- treatment hepatocytes show a small number of distinct bands when probed with HBV DNA). These results suggest that IFN therapy may eliminate many

cells containing integrated HBV DNA but a small number of clones survive. This clonal selection of hepatocytes after IFN therapy is compatable with the hypothesis that it is integrated HBV DNA which causes IFN resistant HBV infections - ie IFN therapy eradicates hepatocytes in which integrated HBV expresses low levels of terminal protein but cells in which integration causes the expression of large amounts of terminal protein survive and multiply.

An alternative mechanism which might increase the production of terminal protein is mutation within the virus. HBV is a highly variable virus (Miller at al., 1990) and any mutations which increase the production of terminal protein might convey a significant survival advantage. Mutation at a large number of sites could alter the production of terminal protein - mutations which alter the stability of the full length polymerase protein may alter the production of terminal protein as might changes in the POL regulatory elements. Although mutations within the POL gene are an attractive site for mutations that could increase the production of terminal protein, mutations within the gene might also impair the function of polymerase and render the virus replication incompetent. However Blum et al (1991) have shown that missense mutations within the POL gene that terminate HBV replication can be complemented by wild-type virus. Hence it is possible that within a cell two viral strains might co-exist - one, with a defective POL gene could overproduce terminal protein and the other could allow viral replication. Tran et al.(1991) have detected a frame shift mutation in a patient that leads to the production of a terminal protein-surface protein fusion product. This mutation developed after IFN therapy and it is possible that such mutations may be responsible for the overproduction of terminal protein which we have observed. Hence mutations within the POL gene and trans complementation of replication incompetent viruses are

possible mechanisms for the overproduction of terminal protein.

Another possible cause for an increase in the expression of terminal protein is mutations within the POL gene regulatory elements. We have studied one such site - the precore ATG. Changes which are predicted to increase the production of polymerase were found in three out of four patients who did not respond to IFN therapy strongly suggesting that these mutant viruses are able to avoid the anti-viral effects of IFN. However we have been unable to confirm that these mutations do, in fact, increase the production of polymerase. It is possible that the assay system which was used to examine the effects of these mutations was insufficiently sensitive and further experiments will be required to exclude the possibility that these mutations are of functional significance.

Although viral mutants are one possible explanation for changes in terminal protein's expression and IFN resistant HBV infection there is, as yet, no evidence to suggest that mutant viruses do consistently give rise to IFN resistant disease. Indeed the only study which has examined this possibility (Miller et al., 1990) suggested that mutant viruses were not responsible for chronic HBV infections in woodchucks. At present there is insufficient data to reach any conclusions regarding the mechanism of high level expression of terminal protein - a number of possible mechanisms exist and it is not yet clear which, if any, are present in patients with chronic, IFN resistant HBV infections.

CONCLUSION

The hypothesis that HBV encodes an IFN inhibitor has been investigated. The POL gene product of HBV inhibits the cellular response to IFN and this inhibition is due to the terminal protein domain of the polymerase protein. Terminal protein inhibits the response to IFN by preventing the activation of the transactivating factor, E. In patients with chronic HBV infection overexpression of terminal protein is associated with failure to respond to IFN therapy, suggesting that IFN resistant chronic HBV infection may be caused by overproduction of this viral inhibitor of the IFN response pathway.

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ADDENDUM

1) INTERFERON TREATMENT AND HEPATOCELLULAR CARCINOMA.

Chronic infection with HBV is a major cause of hepatocellular carcinoma (Beasley et al., 1981). The mechanism(s) whereby chronic infection with HBV causes malignant transformation is unknown. Almost all HBV related hepatocellular carcinomas studied to-date contain integrated viral DNA (Ganem, 1990) indicating that integration of viral DNA precedes or accompanies the transforming event.

In woodchucks infected with woodchuck HBV (which is closely related to human HBV) hepatocellular carcinoma develops after a few years of chronic infection. Recent work (Fourel et al., 1990) has shown that the virus integrates into woodchuck chromosomal DNA adjacent to the *myc* gene in 20% of woodchuck HBV related hepatocellular carcinomas. This integration causes deregulation of the *myc* gene, which is presumed to cause cellular proliferation and malignant transformation. In humans integration of HBV has been observed within proto-oncogenes (Wang et al., 1990), but the frequency with which this occurs is much lower than in woodchucks (Ganem, 1990), suggesting that insertional activation of oncogenes is not a common cause of human HBV associated carcinoma.

HBV contains two promiscuous transactivators (the X and the preS1 proteins - see Chapter 1) and it is possible that these transactivate cellular proto-oncogenes (Kim et al., 1991). However the preS1 protein is not normally produced during HBV infection and it can only be formed from integrated HBV sequences (Kekule et al., 1990). The X protein of HBV is produced during HBV replication but only in very small amounts, which may not be sufficient to activate cellular proto-oncogenes (Kaneko and Miller, 1988). However integrated DNA encoding X protein is tumorigenic in transgenic mice (Kim et al., 1991), suggesting that

integration of HBV DNA may permit X protein to be produced in large amounts. Hence current models of HBV related carcinoma depend upon integration of HBV DNA as a crucial step in malignant transformation.

IFN treatment of patients with chronic HBV infection eliminates replicating virions in up to 50% of patients (Jacyna and Thomas, 1990). However many patients who are clinically cured continue to produce viral proteins - usually the HBV surface protein (Jacyna and Thomas, 1990). Integrated, rearranged HBV sequences which only produce the surface protein have been identified (Ziemer et al.,1985) and it is believed that production of surface protein after IFN therapy is due to persistence of integrated viral DNA. Since integration of HBV DNA is a crucial step in the development of hepatocellular carcinoma it is possible that successful IFN treatment will not remove the risk of malignant transformation. The role of terminal protein in the development of hepatocellular carcinoma is unknown. However one likely explanation for the observed overproduction of terminal protein in patients is integration of HBV DNA (see Chapter 6). If this is the case then it seems likely that those hepatocytes that contain integrated HBV sequences, producing terminal protein, will not be eradicated by therapy. These cells may persist after IFN treatment and may subsequently give rise to hepatocellular carcinoma. Hence IFN treatment of patients with chronic HBV infections may not reduce the risk of developing carcinoma and it is possible that, by increasing the proportion of cells that contain integrated HBV DNA, therapy with IFN may actually increase the risk of developing hepatocellular carcinoma. To-date insufficient patients have been subjected to prolonged follow-up after IFN therapy to determine whether this hypothetical risk is real.

2) CHRONOLGICAL ORDER OF EXPERIMENTS

The experiments detailed in this thesis have not been described in chronological order. The order in which experiments were performed is outlined below.

2.1) INITIAL EXPERIMENTS.

Clinical studies suggest that patients with chronic HBV infections may be unable to respond to IFN. To determine whether this is due to inhibition of the cellular response to IFN by HBV, cell lines transfected with genomic HBV DNA were studied (Chapter 3). These experiments showed that cells containing HBV DNA responded normally to IFN.

2.2) CONFIRMATION THAT HBV CAN INHIBIT THE CELLULAR RESPONSE TO IFN IN PATIENTS.

The finding that cells containing genomic HBV DNA respond to IFN led to speculation that HBV may be unable to inhibit the IFN response pathway. The clinical observations that suggested that HBV may inhibit the response to IFN were therefore re-examined. Pignatelli et al. (1986) examined the expression of IFN inducible HLA Class I antigens on the surface of hepatocytes in patients receiving IFN therapy. They reported that patients who did not respond to IFN therapy did not show any increase in the expression of HLA antigens, whereas an increase was seen in patients who did respond to treatment. The authors concluded that HBV could inhibit the cell's ability to respond to IFN. An alternative explanation for these results is that in some patients hepatocytes are unable to respond to exogenous IFN - i.e. the observed decrease in the response to IFN was due to variation in the patients rather than to inhibition by the virus. To determine whether the failure to respond to IFN was related to infection with HBV, liver biopsies from patients receiving IFN were doubly stained with antibodies directed

against an IFN inducible protein, β_2 microglobulin (β_2m), and an HBV protein - surface antigen. If the failure to respond to IFN was due to the non-responsiveness of hepatocytes to IFN then all cells in the biopsy should not express β_2m. However if the failure to respond to IFN was related to infection with HBV then only those cells expressing HBV proteins should fail to respond to IFN. These experiments (described in Chapter 6) showed that in patients who did not respond to IFN therapy uninfected cells were able to respond to IFN but cells expressing HBV surface protein often failed to do so i.e. they did not express β_2m. These experiments persuaded us to continue to search for a viral inhibitor of the IFN response pathway.

2.3) ANALYSIS OF INDIVIDUAL HBV GENES

The four HBV genes were individually examined to see if any could inhibit the cellular response to IFN. These experiments (described in Chapters 3 to 5) showed that the terminal protein domain of the polymerase protein could inhibit the cellular response to IFN. To determine whether this inhibitory effect also occured in patients liver biopsies taken from patients with chronic IFN- resistant HBV infections were examined, using antibodies directed against the terminal protein domain of polymerase. These experiments, described in Chapter 6, indicated that overexpression of the terminal protein domain of polymerase was associated with a failure to respond to IFN therapy, suggesting that inhibition of the cellular response to IFN by terminal protein may be of clinical significance.

ADDENDUM

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