TRANSCRIPTIONAL REGULATION OF THE INTERFERON GENES

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DECLARATION.

The work reported in this thesis is my own and original work except where otherwise stated and has not been submitted for any other degree.

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SUMMARY.

Interferons (IFNs) are a class of regulatory cytokines capable of inducing a non-specific antiviral activity in target cells. Under normal conditions the synthesis of IFN is not usually detectable, but following exposure of cells to inducers such as virus or double stranded RNA, IFN is produced in large amounts. Expression of the IFN genes is tissue specific and comes about by the activation of transcription. Cis-acting virus responsive elements (VREs) responsible for transcriptional activation have been identified within the 5'-flanking sequences of the human IFN-α1 and -β genes and show considerable sequence similarity.

Although HeLa cells express IFN-β mRNA and protein following induction, no IFN-α transcripts are detectable. The tissue specific expression of the IFN genes in Hela cells was investigated by determining whether a hybrid reporter gene under the control of the virus responsive element of the human IFN-α1 gene (VREα1) was inducible. Evidence was obtained indicating that although the endogenous IFN-α1 gene is not expressed following virus induction, the reporter gene is. Consequently, it appears that the endogenous IFN-α1 gene in Hela cells is subject to some form of negative repression which the plasmid borne reporter gene is able to evade.

Tetramers of GAAAGT and AAGTGA, motifs present in the VREs of the IFN-α1 and -β genes respectively, have been shown to confer virus inducibility upon a heterologous promoter. Sequences of the type GAAANN are particularly frequent in the promoters of IFN and IFN-inducible genes and have been postulated to play a role in the induction of these genes. Tetramers of all such sequences present in VREα1 and in the promoter of the murine Mx gene were examined with regard to their inducibility when assayed in conjunction with a minimal promoter.

Three distinct types of hexamers were distinguished, all of which mediated virus inducibility, but differed in other respects. Type I oligonucleotides (NN= GT, GC, CT and CC) respond to IFNs, cause silencing and are transactivated by interferon regulatory factor-1 (IRF-1). Type II (NN= TG) and type III (NN= CG) oligonucleotides which neither respond to IFN or IRF-1 and do not silence were distinguished by the

criterion that only the type III oligonucleotide mediates constitutive transcription.

Using gel retardation analysis, the interaction of nuclear proteins with the tetrameric hexanucleotides was investigated. Such analysis revealed that the three types of oligonucleotide bind different nuclear proteins. Type I oligonucleotides all interact with the same proteins, which were competed by VREβ but by no other oligonucleotide tested. The type II oligonucleotide, GAAATG on the other hand has an affinity for different nuclear factors that also interact with the VREα1. One of these proteins was identified as the ubiquitous octamer factor OTF-1, while another was a novel protein, which while having affinity for both $(GAAATG)_4$ and VREα1 showed no affinity to any other element tested. This novel protein which interacts with VREα1 and $(GAAATG)_4$ at their common sequence GAAATGGAAA ('TG sequence'), was designated 'TG protein' and is a possible candidate for mediating the virus inducibility of these two elements, neither of which are transactivated by IRF-1.

Thus it appears that despite their similarity, the VRE elements of the IFN-α1 and -β genes contain different response elements which mediate their virus inducibility. The work of others has indicated that the IFN-β promoter uses at least two distinct virus responsive elements, a type I-like sequence (PRDI) and an NF-κB-binding sequence (PRDII). The IFN-α1 gene on the other hand uses a novel virus responsive element, the 'TG sequence' which is distinct from both PRDI and PRDII and may mediate virus inducibility through its interaction with the 'TG protein'. Thus, type I, type II and NF-κB elements represent three distinct terminal pathways mediating virus induction of the IFN genes.

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1. INTRODUCTION.

1.1 Viral Interference.

Viral interference, the ability of one virus to interfere with the replication of another, was first described in 1935 by Hoskins, who showed that monkeys infected with a neurotropic strain of yellow fever virus were protected against a viscerotropic strain of the same virus. Two years later, viral interference was shown to be a general phenomenon even occurring between immunologically unrelated viruses (Findlay and MacCullum, 1937) and in the absence of viral replication (reviewed in Schleringer, 1959).

1.2 Discovery of Interferon.

It was not until 1957 that Isaacs and Lindenmann discovered a molecule that mediated viral interference, which they termed interferon (IFN). In brief, they incubated heat-inactivated influenza virus with chicken chorioallantoic membrane and showed that the supernatant inhibited the replication of live virus in fresh tissue. They interpreted this finding by concluding that the membrane exposed to inactivated virus had released a factor (IFN) which conferred resistance to virus.

Subsequent work demonstrated that IFN was a proteinaceous factor produced transiently by virus infected cells, that does not directly inactivate virus or interfere with viral adsorption at the cell surface but induces a virus-resistant state in the surrounding cells (Isaacs et al., 1957; Lindenmann et al., 1959; Rubin, 1961; Taylor, 1964). Interferon-like molecules were soon found to be produced by a variety of cell types from different species (Henle et al., 1959; Ho and Enders, 1959a, 1959b; Tyrell, 1959), and it is now known that most viruses stimulate the production of IFN in virtually all vertebrate cells both in vivo and in tissue culture (Stewart, 1979).

1.3 Non-viral inducers of IFN.

The first report of a non-viral inducer of interferon showed that 'foreign' non-viral nucleic acids were capable of activating the synthesis of type I

IFN (Isaacs et al., 1963; Rotem et al., 1963). Subsequently, numerous type I IFN-inducing substances were identified, including mycoplasma (Rytel and Jones, 1966), bacteria (Younger and Stinebring, 1964), fungal extracts (Kleinschmidt et al., 1964), natural and synthetic double-stranded RNA (dsRNA)(Rotem et al., 1963; Field et al., 1967), endotoxins (Ho, 1964), polysaccharides (Claes et al., 1970), various low molecular weight compounds (Diederich et al., 1973; Meindl et al., 1976) and cytokines such as colony stimulating factor (CSF-1; Moore et al., 1984; Warren and Ralph, 1986) and interleukin-1 (IL-1; Van Damme et al., 1985). Type II IFN is induced by either antigens (Green et al., 1969) or mitogens (Wheelock, 1965).

1.4   Classification of IFNs.

IFNs were originally categorized into three distinct groups by virtue of their cellular origin; leukocyte IFN (mainly consisting of IFN-α), fibroblast IFN (mainly consisting of IFN-β) and immune IFN (the mitogen- or antigen-induced species of lymphocytes) (Gresser, 1961; Wheelock, 1965; Falcoff et al., 1972; Havell et al., 1975). Because of their similar physical and chemical properties, in particular their resistance to acid (pH 2) and heat (65 °C), leukocyte and fibroblast IFNs were grouped together as type I IFN, while immune IFN, which is sensitive to acid and to heat was designated as type II IFN.

The original IFN nomenclature based on the cellular origins of the IFN proved to be unsuitable, since many cell types produce a heterogeneous mix of IFNs. Leukocytes, for instance, secrete IFN-β as well as several related forms of IFN-α (Havell et al., 1975; Cavalieri et al., 1977; Allen and Fantes, 1980). IFN-α, -β and -γ were distinguished immunologically and were subsequently reclassified strictly on the basis of antigenic specificity, as IFN-α (leukocyte IFN), IFN-β (fibroblast IFN) and IFN-γ (immune IFN; Stewart et al., 1980). Upon cloning the individual IFN-α subtypes were recognized and designated IFN-α1, IFN-α2 etc.

1.5 Mechanisms of IFN induction of the antiviral state of the cell.

The action of IFNs are mediated through interactions with high affinity receptors in the cell membrane. Upon the binding of IFN, the IFN receptors are internalized and degraded (Aguet and Morgensen, 1984). IFN-α and -β appear to utilize a common receptor (Aguet and Morgensen, 1984; Zoon and Arnheiter, 1984) while IFN-γ utilizes a separate receptor (Branca and Baglioni, 1981; Orchanski et al., 1984; Merlin et al., 1985), for which the cDNA has recently been isolated (Aguet et al., 1988). It is unknown how the receptor/IFN complex mediates its effect to the nucleus, though evidence suggests internalization of type I IFN is not sufficient (Higashi and Sokawa, 1982; Huez et al., 1983; Arnheiter and Zoon, 1984) nor necessary (Ankel et al., 1973) to induce the antiviral state and that IFN may act before being internalized.

Establishment of the antiviral state by interferons requires RNA and protein synthesis (Lockhart, 1964; Taylor, 1964) and is accompanied by the synthesis of many new proteins (Weil et al., 1983). In most cells, IFN induces 15 to 20 different genes (Revel and Chebath, 1986), of which the majority are poorly characterized, as either encoding IFN-inducible proteins detected by two dimensional electrophoresis (Weil et al., 1983) or IFN-inducible cDNAs, coding proteins with no homology to any known protein (Friedman et al., 1984).

Some IFN-inducible proteins have been identified, such as class I and II histocompatibility antigens (Gresser, 1984; Rosa and Fellous, 1984), β-2 microglobulin (Wallach et al., 1982), guanylate binding proteins (Cheng et al., 1983), methallothionein II (Friedman et al., 1984) and thymosin β4 (Revel and Chebath, 1986; (derived from the 6-26 gene (Friedman et al., 1984)), though the role they play, if any in the establishment of the antiviral state is still unclear (Revel and Chebath, 1986). The function of three IFN-inducible proteins, 2'-5'-oligoadenylate synthetase, a dsRNA-dependent protein kinase and the Mx gene product have been described.

Protein synthesis in cell free extracts prepared from IFN-treated cells was found to be extremely sensitive to inhibition by dsRNA (Kerr et al., 1974). This phenomenon has been shown to be due to the activation of two interferon-induced, dsRNA-activated enzymes, 2'-5'-oligoadenylate

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synthetase and a dsRNA dependent protein kinase, which ultimately results in the inhibition of protein synthesis and consequently inhibition of viral growth (Baglioni, 1979).

Expression of 2'-5'-oligoadenylate (2-5A) synthetase is strongly induced by IFN, and requires the presence of dsRNA for activation. 2-5A synthetase converts ATP to oligonucleotides with the general structure ppp5'(2'p5'A)n (where n>2), commonly referred to as 2-5A (Kerr and Brown, 1978; Zilberstein et al., 1978). These oligonucleotides activate RNase L, an endogenous endonuclease which degrades both rRNA and mRNA, thereby inhibiting protein synthesis (Baglioni et al., 1979). After activation, RNase L, which is not induced directly by IFN (Silverman et al., 1982), cleaves RNA on the 3' side of UN sequences with a preference for UU and UA (Floyd-Smith et al., 1981; Wreschner et al., 1981).

2-5A is degraded to AMP and ATP by a second IFN-induced enzyme, 2'-5' phosphodiesterase. This enzyme may play a role in regulating the transient effect of 2-5A synthetase (Schmidt et al., 1979). In addition to its phosphodiesterase activity, it has been shown to remove the CCA terminus from tRNA and this may also result in inhibition of protein synthesis (Schmidt et al., 1979).

Although it is clear that the 2-5A synthetase system does not inhibit the replication of all viruses (Masters and Samuel, 1983; Munoz and Carrasco, 1983; Hersch et al., 1984), evidence for its contribution to the establishment of the antiviral state was obtained by demonstrating that in cells microinjected with 2-5A synthetase, growth of vesicular stomatitis virus (VSV) was effectively inhibited (Higashi and Sokawa, 1982). Furthermore, the extent of constitutive expression of 2-5A synthetase cDNA in chinese hamster ovary cells directly correlates with resistance against Mengo virus (Chebath et al., 1982). Surprisingly though, in the light of the results of Higashi and Sokawa (1982), constitutive expression of 2-5A synthetase was found to be insufficient to confer resistance against VSV to CHO cells (Chebath et al., 1982).

Treatment of cells with IFN results in the induction of the 48 and 68 kDa subunits of a 110 kDa protein kinase. In the presence of dsRNA, the 48 kDa subunit phosphorylates the 68 kDa component, activating an intrinsic protein kinase activity. This interferon-induced dsRNA-activated protein kinase phosphorylates other substrates including the α-subunit of the

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eukaryotic protein synthesis initiation factor 2 (eIF2α (Farrell et al., 1977)) and some histones (Roberts et al., 1976; Sen et al., 1978; Zilberstein et al., 1978).

Phosphorylation of the α-subunit of eIF2 results indirectly in the inhibition of protein synthesis by preventing the regeneration of eIF2·GTP eIF2 is needed for the formation of the methionyl-tRNA-40S ribosomal subunit complex required for the initiation of translation (Cooper and Forell, 1977; Lewis et al., 1978; De Benedetti et al., 1983). Following the formation of the methionyl-tRNA-40S complex the eIF2 is released in an inactive form bound to GDP. Normally the eIF2-associated GDP is exchanged with GTP to generate active eIF2·GTP. When phosphorylated the eIF2 becomes irreversibly bound to the GDP and thus cannot be recycled resulting in an inhibition of protein synthesis.

The role that the phosphorylation of histones by dsRNA-dependent protein kinase plays in the effects induced by IFN is not known (Baglioni et al., 1979); it has been postulated that it may effect transcription by altering the chromatin structure, however, evidence for such an effect is not available.

Both 2-5A synthetase and dsRNA-dependent protein kinase pathways are believed to be directed against viruses which produce dsRNA as part of their normal life cycle, which then activates the latent activity of these IFN-induced enzymes (Baglioni et al., 1979).

Currently, the murine Mx gene is one of the best studied of the IFN-inducible genes shown to play a role in the protection of cells against viral infection. Unlike the non-specific actions of the dsRNA-activated enzymes, the Mx gene product, a 72 kDa karyophilic protein induced by type I IFNs (Horisberger et al., 1983; Dreiding et al., 1985), specifically confers resistance against orthomyxoviruses (Lindenmann, 1964; Haller et al., 1979; Arnheiter et al., 1980; Arnheiter and Staeheli, 1983; Staeheli et al., 1984). It has been proposed that Mx acts by preventing transcription (Krug et al., 1985; Ransohoff et al., 1985) and/or translation (Meyer and Horisberger, 1984) of the viral mRNA. Evidence that the Mx gene product, in the absence of other IFN-mediated effects is directly responsible for viral resistance was obtained by constitutively expressing Mx cDNA in Mx⁻ cells and demonstrating that this conferred resistance against influenza virus, but not vesicular stomatitis virus (a rhabdovirus; Staeheli et al., 1986).

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Constitutive expression of Mx cDNA was also shown to confer resistance against influenza virus to non-murine cells (Noteborn et al., 1987).

Homologues of the murine Mx protein exist in humans (Horisberger and Hochkeppel, 1987), cattle (Horisberger, 1988), rats (Meier et al., 1988) and fish (Staeheli et al., 1989) and seem likely to confer resistance to orthomyxo or orthomyxo-like viruses. A second murine Mx gene (Mx2) has been identified whose hypothetical protein when aligned for the best fit with the murine Mx protein (Mx1) displays a 73% homology (higher in exons 3 to 8 (Staeheli and Sutcliffe, 1988; Aebi et al., 1989)). However, due to a frame shift mutation, it seems unlikely that this gene encodes a functional protein (Staeheli and Sutcliffe, 1988). Similar families of Mx-related proteins and mRNA, which probably arose from a common ancestral gene by duplication (Staeheli and Sutcliffe, 1988), exist in other species including humans (Aebi et al., 1989), cattle (Horisberger, 1988) and rats (Meier et al., 1988). A comparison of human and mouse Mx proteins show that the human MxA and murine Mx2 proteins are the most closely related, displaying 77% homology (Aebi et al., 1989). Surprisingly, the MxA gene product which is cytoplasmic not only confers resistance to influenza virus but also to vesicular stomatitis virus (Aebi et al., 1989; J. Pavlovic, unpublished results) suggesting that Mx-related genes may confer resistance against a wider variety of viruses than was at first thought.

1.6   Non-antiviral effects of IFN on the cell.

Beside their antiviral effects, both type I and II IFNs are now recognized as regulators of a variety of cellular functions, including proliferation, differentiation and various immunological reactions (De Maeyer and De Maeyer-Guignard, 1988). Since the first report of the antiproliferative effect of IFNs on mouse fibroblasts (Pauker et al., 1962), IFNs have been shown to inhibit the division of normal and transformed cells (Lindahl-Magnusson et al., 1972; Balkwill and Oliver, 1977; Balkwill et al., 1978; Gresser and Tovey, 1978; Van't Hull et al., 1978; Pfeffer et al., 1979).

Although the molecular mechanism of the antiproliferative effects of type I IFN is as yet unknown, the fact that IFN inhibits the expression of several proto-oncogene products implicated in cellular proliferation (including c-myc and c-fos; Giovanna et al., 1989) and that IFN is itself induced by

some of the same proteins (Giovanna et al., 1989) or other factors which stimulate them (Kelly et al., 1983; Greenberg and Ziff, 1984; Kruijer et al., 1984; Müller et al., 1984) suggests that IFN may be involved in the negative feed-back control of these proto-oncogenes. By negatively regulating the expression of factors required for cellular proliferation, IFN may induce an inhibitory effect on cell growth.

Type I IFN treatment of Daudi cells induces a cell surface protein, the expression of which correlates with the suppression of cell proliferation (Hillmann et al., 1987). This 17 kDa protein has been partially purified from membranes of induced Daudi cells and can inhibit the growth of Daudi, Namalwa and Hela cells. Although the function of this protein is unknown, it could potentially play a central role in the IFN induced inhibition of cell division.

Several immunological processes are influenced by IFN in both an inhibitory and stimulatory manner. For example, type I IFN severely inhibits the formation of antibody-producing cells (Braun and Levy, 1972) in contrast to IFN-γ which stimulates the generation of these cells (Nakamura et al., 1984). IFN-γ and to a lesser extent IFN-α suppresses monocyte migration (Thurnan et al., 1984), while type I IFN inhibits the maturation of monocytes to macrophages (Lee and Epstein, 1980) and suppresses delayed hypersensitivity reactions (DeMaeyer et al., 1975). In the presence of type I IFN there is a stimulation of several functions including phagocytosis by macrophages (Donahoe and Huang, 1976) cytotoxicity of sensitized lymphocytes (Einhorn et al., 1978) and expression of immunoglobulin receptors (Friedman et al., 1980). Both type I and type II IFNs stimulate the expression of histocompatability antigens (class I and class II (Gresser, 1984; Rosa and Fellous, 1984)) and natural killer cell activity (Gidlund et al., 1978; Djeu et al., 1978; Weigent et al., 1983).

Very low levels of constitutively expressed IFN-α transcripts are produced in the spleen, kidney, liver and peripheral blood leukocytes of healthy individuals (Tovey et al., 1987). This finding raises the possibility that the constitutive expression of IFN in vivo may play a role in the regulation of physiological processes such as immune recognition, differentiation, hematopoiesis and cell proliferation. Furthermore, human IFN has been detected in placental blood (Duc-Goiran et al., 1985) and amniotic fluid (Lebon et al., 1982), and it has been postulated that it may

play a role in fetal development or alternatively, help to maintain maternal tolerance.

Maternal recognition of pregnancy in sheep is dependent upon secretion of ovine trophoblast protein-1 (oTP-1) by the conceptus. The cDNA for this protein was cloned and the primary amino-acid sequence deduced from the nucleotide sequence (Imakawa et al., 1987). oTP-1 was found to display, throughout its length, striking amino-acid sequence homology with IFN-αs from a variety of species; the most extensive homology, 70.3%, is with bovine IFN-α_{II} (Imakawa et al., 1987). Purified oTP-1 has potent antiviral activity (10^7-10^8 units /mg) and is thus about as active as any known IFN (Pontzer et al., 1988; Roberts et al., 1989). Thus it appears that oTP-1 belongs to a subfamily of IFNs with a highly specialized function. cDNA clones corresponding to bovine trophoblast protein-1 (bTP-1) have been isolated and the inferred primary structure also indicates that it could be an IFN (Imakawa et al., 1989).

1.7   Cloning of the IFN cDNAs.

The development of recombinant DNA technology heralded a major breakthrough in the understanding of the IFN system, allowing the cloning of several distinct species of IFN cDNAs. Synthesis of IFN in E. coli and yeast enabled for the first time the isolation of substantial amounts of pure single IFN species, allowing their properties to be investigated unambiguously, in the absence of contaminating proteins. In addition, recombinant DNA technology allowed the chromosomal sequences of the IFN genes to be identified and the control of expression of these genes to be studied.

1.7.1   IFN-α cDNAs.

The first IFN cDNA clone to be isolated was that of IFN-α1 (Nagata et al., 1980a), by a process that relied on the ability of Xenopus oocytes to synthesize detectable amounts of IFN when microinjected with as little as 50ng of poly(A) RNA isolated from IFN producing cells (Reynolds et al., 1975). Poly(A) RNA was isolated from human leukocytes infected with Sendai virus and fractionated by sucrose gradient centrifugation. Fractions

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containing IFN mRNA, as detected by the oocyte assay were pooled. The approximately 10 fold enriched mRNA was then used as a template for the synthesis of double-stranded cDNA which was subsequently inserted into pBR322 and used to transform E. coli. The resulting transformants (~5000) were divided into pools of around 500 and rescreened in a hybridisation-translation assay (Harpold et al., 1978). The hybrid plasmids from each pool were denatured and irreversibly bound to filters which were then hybridized with virus-induced leukocyte mRNA. Hybridizing mRNA was recovered, injected into oocytes and homogenates were assayed for IFN activity. Positive pools were subdivided and reassayed until a single clone was identified. The cDNA insert of the isolated clone was shorter than expected (320 bp) and was thus used as a hybridization probe to search for additional clones with longer inserts. A plasmid was identified (Hif-2h) with a cDNA insert of 910 bp, corresponding to the expected length of a complete IFN cDNA. Confirmation that this clone did in fact encode for IFN was obtained by showing that it hybridized to IFN mRNA and that after expression in E. coli IFN activity was produced which could be neutralized by anti-IFN-α antibodies.

Nucleotide sequence analysis of the cDNA insert of Hif-2h (IFN-α1) enabled the prediction of the amino acid sequence of its open reading frame (Mantei et al., 1980) and comparison with the amino terminal sequence of lymphoblastoid IFN, which had recently been determined by Zoon et al. (1980). Surprisingly, the two IFNs sequences differed in 5 out of 20 positions, arousing the suspicion that more than one type of IFN-α might exist.

Using the IFN-α1 cDNA as a probe, a second IFN producing clone was identified (IFN-α2) which in addition to displaying a different target specificity to IFN-α1, differed in 17% of its amino acids and in 5 out of 33 of the amino terminal residues of lymphoblastoid IFN (Streuli et al., 1980). It therefore became clear that at least 3 different forms of IFN-α existed and that the multiple peaks of IFN-α activity separated by HPLC (Rubinstein et al., 1979) were potentially the products of multiple genes and not one form of IFN-α differing in its degree of glycosylation. Goeddel et al. (1981) estimated from Southern analysis that at least 10 IFN-α genes existed and using an IFN-α cDNA as a probe identified 8 distinct IFN cDNAs. Isolation of chromosomal clones revealed that at least 24 IFN-α genes existed of which 15 are potentially active and 9 encode pseudogenes (Nagata et al.,

1980b; Brack et al., 1981; Henco et al., 1985; Weissmann and Weber, 1986). Multiple forms of IFN-α were also detected in many vertebrates including mouse (Shaw et al., 1983; Kelly and Pitha, 1985; Zwarthoff et al., 1985), cattle (Capon et al., 1985), rat (Dijkema et al., 1984), and monkey (Pestka, 1986).

1.7.2   IFN-β cDNA.

The first successful cloning of an IFN-β cDNA was reported by Taniguchi et al. (1979) who used a two step approach of differential hybridization (St. John and Davis, 1979; Hoeijmakers et al., 1980) followed by hybridization-translation (Harpold et al., 1978) to identify single IFN-β clones. Double-stranded cDNA was prepared from 12S poly(A) RNA isolated from poly rI-rC-induced fibroblasts enriched for IFN mRNA and clones were screened with radioactive cDNA prepared from induced and uninduced libraries respectively. Colonies hybridizing with the induced but not the uninduced cDNA were selected for further analysis. A hybridization-translation assay, similar to that employed in the cloning of the IFN-α1 cDNA (see above) was then used to identify the IFN-β clones amongst those selected by differential hybridization, enabling individual IFN-β clones to be identified. Confirmation that the isolated cDNA was in fact that of IFN-β was obtained (Taniguchi et al., 1980a) when the predicted amino acid sequence was found to agree with the amino terminal sequence of IFN-β obtained by Knight et al. (1980). Additional evidence that this clone was an IFN-β cDNA was presented when it was demonstrated that the cDNA when expressed in E. coli produced a polypeptide with IFN activity (Taniguchi et al., 1980b). Two independent isolations of IFN-β cDNAs were subsequently made which in addition to possessing an identical nucleotide sequences to the clone of Taniguchi, produced biologically active IFN when expressed in E. coli (Derynck et al., 1980a, 1980b; Goeddel et al., 1980; Taniguchi et al., 1980c). Evidence obtained from Southern analysis indicated indicated that unlike IFN-α, IFN-β was not a member of a multi-gene family (Goeddel et al., 1981; Wilson et al.,1981).

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1.7.3   IFN-γ cDNA.

Two IFN-γ cDNA clones were independently isolated at about the same time utilizing a differential hybridization assay (Gray et al., 1982) or the hybridization-translation approach (Devos et al., 1982). Nucleotide sequence analysis showed that the two cDNAs were almost identical and that when expressed in E. coli or monkey cells produced IFN activity that was acid labile and neutralized by anti-IFN-γ antibodies but not those against IFN-α or -β. The identification and cloning of a single IFN-γ chromosomal gene, Southern analysis (Gray et al., 1982; Gray and Goeddel, 1983; Taya et al., 1982) and the existence of a single homologous IFN-γ mRNA in lymphocyte and spleen cells (Derynck et al., 1982) all indicate that a single IFN-γ gene exists in man.

1.8   Structure of the IFN genes.

1.8.1   IFN-α genes.

The large number of IFN-α genes isolated to date can be divided into two subfamilies; subfamily IFN-α_I which consists of at least 15 genes of which 14 are thought to be functional and subfamily IFN-α_{II} with 6 genes of which one is functional (reviewed in Weissmann and Weber, 1986). The genes of IFN-α_I subfamily diverge from each other by less than 13% in replacement sites and 35% in silent sites, whereas the IFN-α_{II}1 gene diverges by at least 28% and 62% in replacement and silent sites, from all members of the IFN-α_I family (Henco et al., 1985). All the IFN-α genes lack introns, encode leader sequences of 23 amino acids (with one exception which may have a leader of 16 amino acids) which are cleaved off during maturation to produce active IFN molecules of usually 166 amino acids (human IFN-α2 is 165 and human IFN-α_{II}1 172 residues long), and have 5´-non-translated regions of 67 to 69 nucleotides (nt) and 3´-non-translated regions of between 240 and 440 nt (reviewed in Weissmann and Weber, 1986). A comparison of the amino acid sequence of all known IFN-α loci reveals regions of strong conservation, particularly amino acids 135 to 151, which in addition to being conserved between all human IFN-α proteins are conserved in other species, as well as in IFN-β (see Figure 1 in Weissmann and Weber, 1986). All known human IFN-α genes lie on

chromosome 9 (Owerbach et al., 1981; Shows et al., 1982) and several are within a few kilobases of each other (Brack et al., 1981), while others are less closely linked.

1.8.2   IFN-β gene.

The human IFN-β is a single copy gene (Goeddel et al., 1981; Wilson et al., 1983) which encodes a preprotein of 187 amino acids from which a signal peptide of 21 amino acids is cleaved off upon maturation (Derynck et al., 1980a; Goeddel et al., 1980). Its 5'-non-translated sequence is between 73 and 76 nt in length and the 3'-non-translated region comprises of approximately 200 nt. As the IFN-α genes, the IFN-β gene contains no introns (Lawn et al., 1981; Ohno and Taniguchi, 1981) lies on chromosome 9 (Meager et al., 1979; Owerbach et al., 1981; Shows et al., 1982) and is closely linked to the IFN-α genes (Ohlsson et al., 1985). A single IFN-β gene has been shown to exist in all vertebrates tested, except for ungulates, which possess several IFN-β genes (Wilson et al., 1983; Leung et al., 1984).

1.8.3   IFN-γ gene.

A single IFN-γ gene exists in man, which in contrast to the type I IFN genes contains 3 introns (Gray and Goeddel, 1983; Taya et al., 1982) and is located on chromosome 12 (Nayor et al., 1983). The gene encodes a product of 166 amino acids from which a 20 amino acid signal peptide is cleaved to form the mature product of 146 amino acids (Gray et al., 1982; Devos et al., 1982). It has 127 and 584 nucleotide 5'- and 3'-non-translated sequences, respectively. Two species of IFN-γ exist, of 20 and 25 kDa (Yip et al., 1982), this heterogeneity is ascribed to varying degrees of glycosylation (Kelker et al., 1984). The IFN-γ genes of mouse (Gray and Goeddel, 1983), rat (Dijkema et al., 1985) and cattle (Derynck, 1983) have been cloned and all species examined contain a single IFN-γ gene with introns in positions corresponding to those of the human gene.

1.9   Evolution of the IFN genes.

The similarity between the IFN-α and -β genes (45% at the nucleotide and 29% at the amino acid level (Taniguchi et al., 1980d)) indicates that in all probability they diverged from a common ancestor. It is assumed that sequence divergence between two homologous genes in different species is proportional to the time elapsed since the separation of the species. Based on this assumption it should be possible to estimate the divergence of the IFN-α and -β genes within a species, although the calculation is complicated by the unknown frequency of gene rectification (as a result of gene conversion and recombination), allowing only the minimum time since the gene duplication occurred to be estimated. Weissmann and Weber (1986) calculated that the IFN-α and -β genes probably diverged some 200-300 million or more years ago.

The relationship of the IFN-γ gene to the IFN-α and -β genes is not easy to gauge. Although no significant homologies are apparent at the nucleotide level, appropriate alignment suggests there may be conservation of several amino acid clusters and single residues between the various IFN species (DeGrando et al., 1982; Epstein, 1982; Gray and Goeddel, 1983). Whether these blocks of homology are significant (ie. evidence for a common ancestor) or the result of sequence constraint dictated by the convergent evolution of the IFN proteins has not been determined. If the IFN-γ gene is derived from the same ancestor as IFNs -α and -β then it must have branched off much earlier than the -α and -β genes.

All mammalian species examined possess a large IFN-α gene family, hence it was probably established some time prior to the divergence of mammals, 85 million years ago (Weissmann et al., 1982). The divergence of the individual IFN-α genes indicates that their origin is far more recent (23 million years ago (Miyata and Hayashida, 1982)) but as discussed above this should be taken only as a minimum indication of the divergence of these genes. Two possible explanations have been proposed to explain the origins of multiple gene families, either sequential duplication of the genes, spread over a long evolutionary period (Ohno, 1970) or a `big bang' event resulting in the duplication of multiple copies of the same gene in germ cells during a single generation, such events have been shown to occur in somatic cells (Edlund and Normack, 1981). Either of these events

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could explain the origin of the IFN-α gene family. Once duplicated, the individual members would begin to diverge by acquiring deletions, insertions and substitutions resulting in the heterogeneity now seen in the IFN-α gene products.

Presumably the IFN-β gene family of ungulates arose by similar events. As the pig, horse and cow all possess multi-member IFN-β gene families and other mammals do not, the amplification of the -β gene probably occurred some time between the radiation of mammals and the divergence of the ungulates ie. between 55 and 85 million years ago.

1.10   IFN gene expression is under differential control.

Hiscott et al. (1984a) studied the expression of several IFN genes using the S1 assay, under conditions that allowed for the specific and quantitative detection of the individual IFN gene transcripts. They showed that the IFN-α genes and the IFN-β gene are transcribed in a differential, cell specific manner. In different cell lines the ratio of different IFN transcripts are not uniform, for example leukocytes express twice as much IFN-α1 as IFN-α2 or -β whereas in Namalwa cells IFN-β was expressed at levels 3 and 5 times higher than that of IFN-α1 and -α2 respectively. Some of the other IFN-α subtypes are also subject to differential expression, but generally the differences in expression are less pronounced. The ratios of the the individual IFN-α mRNAs were shown to vary considerably within a particular cell type, in general, IFN-α1, -α2 and -α4 represented the major species, while IFN-α5, -α7, -α8 and -α14 transcripts were present at 5 to 20 fold lower levels (Hiscott et al., 1984a, 1984b). The use of various induction protocols did not significantly affect the proportion of IFN transcripts and surprisingly the number of IFN transcripts did not always correlate with IFN activity.

In order to investigate the potential role the various α-IFNs play during differentiation, the transcript levels of several species of IFN in peripheral blood leukocytes of heathy and leukemic individuals after induction by Sendai virus was measured (Hiscott et al., 1984b; Streuli, 1986). Leukemic leukocytes are arrested at different stages of hematopoietic differentiation (Klein, 1984) and thereby offer a system in which the expression of the IFN genes can be studied at various stages of differentiation. Certain leukemic

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leukocytes not belonging to the monocyte/macrophage lineage, showed striking differences in the proportion of the individual IFN-α mRNA species they produced when compared to each other and to normal lymphocytes (Streuli, 1986). This observation lends weight to the idea that the individual IFN subtypes may play distinct roles in the regulation of hematopoietic differentiation and/or cell growth.

1.11 The individual IFN-α subtypes may have different functions.

The multiplicity of IFN-α genes and the fact that certain of the IFN-α subtypes are differentially rather than coordinately controlled raises the question of whether the individual IFN-α species preferentially carry out different functions. It has been well established that the individual IFN subtypes display distinct differences in their target specificity when tested on cell lines from different species (Desmyter and Stewart, 1976; Lin et al., 1978; Streuli et al., 1980; Streuli et al., 1981; Weck et al., 1981a, 1981b; Yelverton et al., 1981) and even on cell lines from the same species (Weck et al., 1981a; Goren et al., 1983). Furthermore, the degree of stimulation by different IFN-α species of the various activities such as antiviral activity, natural killer cell activation, antiproliferative activity and 2-5A synthetase induction etc., differs between cell lines (Rehberg et al., 1982; Goren et al., 1983) and even within the same cell type (Weck et al., 1981a). These findings imply that the different forms of IFN-α may have different functions. It has been suggested that this may come about if cells have more than one type of IFN-α receptor to which the the IFN-α species have varying affinities and if different cells lines have different proportions of these receptors (Weissmann and Weber, 1986).

1.12 Control of IFN gene expression.

By stably transfecting mouse L929 cells with the human IFN-α1 gene, Mantei and Weissmann (1982) demonstrated that it was possible to obtain correctly initiated human IFN-α1 mRNA, upon induction with Newcastle disease virus (NDV). The kinetics were similar to those seen for the endogenous IFN genes. Similar work by a number of other labs using a

variety of vector systems and cell lines gave similar results with the human IFN-β gene (Canaani and Berg, 1982; Hauser et al., 1982; Ohno and Taniguchi, 1982; Zinn et al., 1982; Mitrani-Rosenbaum et al., 1983). Transfer of the IFN genes into foreign cells provided a system in which genes modified by DNA reconstruction or site-directed mutagenesis could be tested in vivo, in the quest to identify the cis-acting sequences required for IFN gene regulation. Furthermore, the observation that relatively short chromosomal fragments encoding the IFN genes contained sufficient information for the accurate regulation of these genes in foreign cells gave the first indication of the location of these cis-acting DNA sequences.

1.13 The 5'-flanking sequences of the IFN genes mediate viral induction of transcription.

The steady state level of an mRNA in the cell is determined by the rate of synthesis and degradation of the transcript. Thus, accumulation of IFN mRNA following induction could arise by either the stabilization of rapidly turning over mRNA or the activation of transcription from previously silent genes or a combination of both. To clarify this issue, Weidle and Weissmann (1983) introduced into L929 cells a hybrid gene in which the 5'-flanking sequence derived from the IFN-α1 gene was fused to the rabbit β-globin transcription unit. Correctly initiated and spliced β-globin transcripts were detected only after infection with NDV, with kinetics similar to those described for the endogenous IFN mRNAs. Transcription from a construct with the β-globin promoter linked to the IFN-α1 transcription unit, or from the complete β-globin gene was constitutive and upon NDV induction a decrease in the transcript levels was observed (Weidle and Weissmann, 1983). A similar approach with the human IFN-β gene illustrating that a hybrid gene consisting of the 5'-flanking region of IFN-β gene linked to a thymidine kinase transcriptional unit was also virus inducible (Ohno and Taniguchi, 1983), demonstrating that activation of transcription of the IFN-β as well as the IFN-α gene is mediated by elements located in the 5' regions of these genes.

Raj and Pitha (1983) studied the induction of the endogenous IFN-β gene using nuclear run-on experiments and concluded that the accumulation of IFN-β transcripts following induction is the result of both activation of transcription as well as stabilization of the transcripts. The role

of transcript stabilization was confirmed by Nir et al. (1984) who used a truncated IFN-β gene (retaining 5' sequences to position -40) which synthesized IFN-β RNA at a constant rate whether the cell was induced or not, and showed that upon induction, stabilization of IFN-β mRNA resulted in an accumulation of IFN-β transcripts in the cell. In contrast, Whittemore and Maniatis (1990) found no evidence that mRNA stability was regulated during viral induction of the IFN-β gene. Instead they showed that IFN-β mRNA decayed at a constant rate throughout induction and that the post-induction decrease in transcript levels is due mainly to a decrease in the rate of transcription.

Shaw and Kamen (1986) noticed that many transiently expressed genes (including both type I and II IFNs) contain AU-rich sequences in their 3' untranslated regions. These authors showed that the 3' non-coding regions of the GM-CSF when joined to the β-globin mRNA mediated a rapid and selective degradation of this RNA and postulated that the function of this AU-rich sequence may be to direct mRNA degradation. The IFN-β transcripts were found to contain such 3' AU-rich destablizing sequences, in addition to 5' destablizing sequences. Both of these destablizing sequences were shown to be associated with small increases in the rate of deadenylation of IFN-β mRNA (Whittemore and Maniatis, 1990). Deletion of the AU-rich region of the IFN-β gene did not lead to an increase in mRNA stability indicating that these sequences do not function in induction but rather to decrease the transcript level post-induction.

1.14 Localization of the DNA sequences required for the regulation of induced expression of the IFN-α and -β genes.

1.14.1 The IFN-α1 gene.

Several groups have identified DNA sequences responsible for induced transcription of the IFN genes by generating various deletions in the 5'-flanking regions of the IFN-α1 and -β genes and expressing the resulting hybrids in various host cell lines. Ragg and Weissmann (1983) located the 5' boundary of the region required for inducible transcription of the human IFN-α1 gene in L929 cells between positions -117 and -74 with respect to the cap site. The 3' boundary of this region was subsequently localized by

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joining segments of the IFN-α promoter extending from position -675 to several points between -22 and -117 to a truncated β-globin gene and testing for virus induced activation of transcription (Ryals et al., 1985). Induction was found to be maximal when the 3' end point was -64, and there was a stepwise loss of activity as the truncations extended further upstream until at position -86 inducibility was completely lost. The 5' boundary was further mapped by constructing 5' deletions of a hybrid IFN-α promoter which extended to position -64 on the 3' side. Deletions extending to position -109 left the promoter fully inducible while further truncations reduced the inducibility stepwise until at position -79 it was completely abolished (Ryals et al., 1985). Thus, a 46 base pair (bp) sequence located between positions -109 and -64 was shown to be required for the maximal induction of the IFN-α1 gene while a segment of only 22 bp (positions -85 to -64) was shown to retain marginal virus inducibility (Figure 1).

Subsequent work by Kuhl et al. (1987) showed that sub-sequences of the 46 bp virus-responsive element of the IFN-α1 gene (VREα1) were themselves virus inducible when multimerized. VREα1 consists of essentially two 19 bp repeats, repA and repB (see Figure 1) and it was found that tetramers of repA (tetra-repA) or of the hexanucleotide GAAAGT, which is present in repB, were both able to confer virus inducibility on a reporter gene. In addition, both tetra-repA and $(GAAAGT)_4$, but surprisingly not VREα1, when inserted between an enhancer and a TATA box, caused suppression of the constitutive activation of transcription elicited by the enhancer. This suppression was fully abrogated by virus induction and was therefore designated 'reversible silencing' (Kuhl et at., 1987). This result and the observations by Ryals et al. (1985), that there was a stepwise drop in inducibility as VREα1 was truncated, implies that multiple sequence motifs (which when multimerized are themselves virus inducible) contained in VREα1 may function cooperatively to contribute to induction.

1.14.2   The IFN-β gene.

Several groups identified the DNA sequences required for the induction of the human IFN-β gene and while they all essentially agreed on the 3' boundary, results for the 5' boundary differed from group to group. Fujita et al. (1985) constructed various IFN-β deletions and introduced them into

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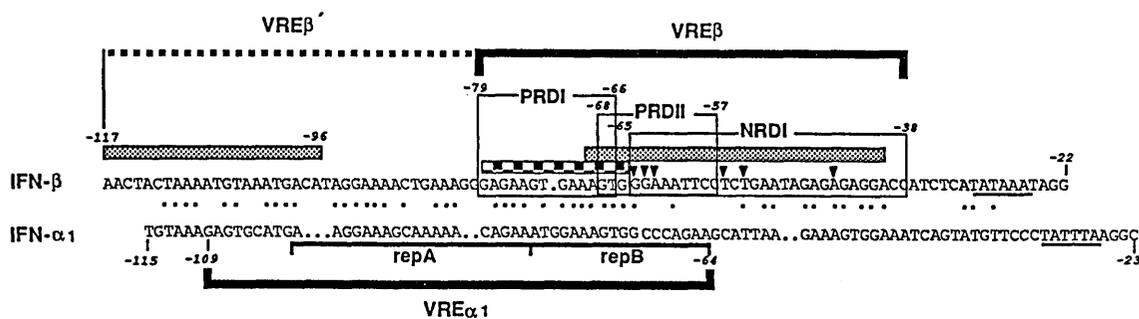
Figure 1 Comparison of the IFN-α1 and IFN-β promoter regions
(adapted from Kuhl et al., 1987).

Numbering is from the cap site of the human IFN-β (Fujita et al., 1985) and the IFN-α1 gene (Ryals et al., 1985), respectively. VREα1 (Ryals et al., 1985; Kuhl et al., 1987) and VREβ (Goodbourn et al., 1985) are indicated by horizontal brackets. Sequences 5' to VREβ necessary for optimal induction in the experimental system of Fujita et al. (1985) are indicated by dotted lines. Boxes above the sequences indicate protected regions in the IFN-β promoter (stippled, before induction; vertical stripes, after induction, as identified by DNaseI genomic footprinting (Zinn and Maniatis, 1986). The two positive regulatory domains, PRDI and PRDII, and the negative regulatory domain, NRDI, proposed by Goodbourn and Maniatis (1988) are indicated by open boxes. Arrow heads indicate point mutations in NRDI that lead to derepression of uninduced transcription (Goodbourn and Maniatis, 1988).

mouse L929 cells. Expression of the mutant genes was measured at both the RNA and protein levels, after viral, poly rI-rC or mock induction. In their expression system, Fujita et al. (1985) was demonstrated that the upstream boundary required to support maximal induction of the IFN-β gene was between position -117 and -105 relative to the cap site and that sequences downstream of position -40 play no role in viral induction of this gene. The sequence requirements for virus and poly rI-rC induction were found to be essentially the same. The results were confirmed using a transient expression system (Fujita et al., 1987). Similar results were obtained by Dinter et al. (1983), who, using permanently transformed murine cells, mapped the 5' boundary of the virus responsive element of the IFN-β gene to between positions -107 and -68.

Maniatis and coworkers (Zinn et al., 1983; Goodbourn et al., 1985), using murine C127 cells transformed by bovine papilloma virus-derived vectors carrying various IFN-β deletions, concluded that the 5' boundary for induction lies between positions -79 and -75 (numbering according to Fujita et al., 1985). By substituting the IFN-β TATA box region (-39 to +74) with the corresponding region from the HSV TK gene (-39 to +57), they showed that sequences downstream of -40 play no role in the induction of the IFN-β gene. Consequently these authors concluded that the virus responsive element of the human IFN-β gene (VREβ) extends from position -39 to -79. Using a different host-vector system these authors obtained essentially the same results (cited in Taniguchi, 1988). However, they observed that in HeLa cells the sequence requirement for maximal induction of the IFN-β gene was similar to that described by Fujita et al. (1985). Furthermore, in a transient expression system using C127 cells, Goodbourn et al. (1985) found that although a deletion to position -79 did not abolish virus inducibility, the presence of additional sequences up to position -109 resulted in a 4 fold increase in induced expression.

The different sequence requirements for the maximal induction of the human IFN-β gene reported by the various groups could be the result of the different assay systems used. Different cell lines may contain varying amounts of factor required for the transcriptional control. In a cell line with a high factor concentration, the sequence requirement may be less stringent than in a cell line that contains lower amounts of the factor. It is also conceivable that the expression of the mutant IFN-β genes may be

influenced by the vector, if for example the vector contains sequences that can substitute an activity normally provided by the gene promoter, then sequences which are normally essential could become dispensable.

In an attempt to further dissect the region involved in viral activation of the IFN-β gene, a series of deletions of the VREβ were analyzed (Goodbourn et al., 1986). This study indicated that the VREβ consists of at least two functional elements, a constitutive transcriptional domain (-79 to -57) whose activity is controlled by an adjacent negative regulatory sequence (-55 to -36).

The active domains of VREβ were more accurately located and characterized by studying the effect of a large number of single point mutations in the VREβ had on the relative transcription levels in both uninduced and induced cells, in addition to the relative induction ratio (Goodbourn and Maniatis, 1988). It was possible to distinguish three distinct domains in VREβ, two genetically distinct positive regulatory domains (PRDI and PRDII) and an overlapping negative regulatory domain (NRDI (Figure 1)). Certain point mutations within NRDI resulted in increased transcription in uninduced cells, but had little effect in induced cells. Such mutations mapped between positions -39 and -55; mutations upstream of -55 overlapped with PRDII, so that the 5' boundary of NRDI could not be determined, although mutations with a similar phenotype were observed at nucleotides -65, -64 and -62, indicating that NRDI may extend as far upstream as nucleotide -65.

Mutations in the region -79 to -66 (PRDI) generally resulted in a decrease in both induced and uninduced expression and in some cases in a lower degree of induction, indicating that PRDI is potentially involved in the induction of transcription mediated by VREβ. PRDII (-68 to -57) differed from PRDI in that mutations in this region resulted in a reduction of expression in induced cells only. Presumably in the uninduced state no loss of activity was observed as this region is repressed by the adjacent NRDI.

Fan and Maniatis (1989) showed that neither PRDI nor PRDII display any detectable transcriptional activity as single copies but that any pairwise combination of these elements act synergistically as virus-inducible sequences. Furthermore PRDI is inducible by both type I and type II IFNs and capable of silencing the SV40 enhancer. PRDII however, is not

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inducible by IFN and shows no silencing activity (Fan and Maniatis, 1989). Both these elements displayed significant cell type-specific differences (Fan and Maniatis, 1989) that may partly explain the different sequence requirements identified as being necessary for the maximal induction of the β-IFN gene (Dinter et al., 1983; Zinn et al., 1983; Fujita et al., 1985; Goodbourn et al., 1985; Fujita et al., 1987).

Within the longer virus responsive element (VREβ'; -117 to -39), identified by Fujita et al. (1985) are seven copies of a hexamer motif with the consensus sequence $AA^A/_G^T/_GGA$ (see Figure 1). These authors postulated that these hexamers were responsible for the virus induction of the IFN-β gene, correlating the loss of one or more of these elements with the stepwise reduction in inducibility of the truncated gene (Fujita et al., 1985; Fujita et al., 1987). Multimers of several of these hexanucleotides were shown to mediate virus-induced activation of transcription (Fujita et al., 1987). The most active of these hexamers, AAGTGA, when multimerized, contributed incrementally to the to the inducibility of a hybrid gene in an orientation and distance independent manner, although as a single copy it displayed no activity.

1.15 Nuclear factors specifically interact with IFN-β promoter elements.

Using gel retardation analysis and DNase I footprinting, Keller and Maniatis (1988) identified three distinct VREβ binding factors in vitro. Two of these were detected in both induced and uninduced nuclear extracts (one binding PRDI (PRDI-BFc) the other PRDII (PRDII-BF) while the third, binding to PRDI, was detected only in extracts from induced cells (PRDI-BFi). The DNA/protein interactions of the two PRDI-binding factors were indistinguishable. PRDI-BFi which migrates faster than PRDI-BFc may be a proteolytic digestion product of PRDI-BFc or an entirely different protein of lower molecular weight. Using the VREβ point mutants generated by Goodbourn and Maniatis (1988) it was shown that there was a correlation between the in vivo activities of these mutants and their ability to bind these factors in vitro (Keller and Maniatis, 1988). Furthermore, genomic DNase I footprinting of the human IFN-β 5'-flanking region indicated that in the uninduced state a factor bound to NRDI which upon

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induction dissociated and is replaced by a second factor binding to PRDI (Zinn and Maniatis, 1986).

Fujita et al. (1988) used gel retardation and DNase I footprinting analysis to characterize a nuclear factor designated IFN Regulatory Factor-1 (IRF-1) that specifically bound to both tetramers of AAGTGA and the VREβ' (this activity was later demonstrated to be the result of two distinct factors, IRF-1 and IRF-2; see below and Harada et al., 1989). Competition experiments indicated that IRF-1 had highest affinity for tetrameric AAGTGA (~10 and 80 fold higher than for VREβ' and VREα1, respectively) and that the affinity of IRF-1 for VREβ' and VREβ' mutants correlated with the ability of these sequences to mediate virus inducibility in vivo.

1.16 Identification and characterization of VREβ binding factors.

Miyamoto et al. (1988), using the 'ligand blotting technique' (Singh et al., 1988), isolated a cDNA encoding a nuclear factor which specifically bound to $(AAGTGA)_4$. The DNA binding properties of this factor, expressed in E. coli were similar to those of what was first thought to be murine IRF-1 (but was in fact later recognized to be IRF-2; Fujita et al., 1988; Harada et al., 1989). The authors designated this cDNA as IRF-1. It was shown subsequently that high level expression of the IRF-1 cDNA in transfected COS cells resulted in induction of the endogenous IFN-α and β genes (Fujita et al., 1989a). Since IRF-1 is itself virus (and IFN) inducible (Miyamoto et al., 1988), it seems likely that IRF-1 is directly involved in virus induction of the IFN genes (Harada et al., 1989).

Using IRF-1 cDNA as a probe, a cDNA coding a second factor (IRF-2) was identified which displayed 62% homology in its deduced amino acid sequence with the N-terminal region of IRF-1. The C-terminal portions of these two proteins were only 25% homologous (Harada et al., 1989). The DNA binding properties of IRF-2 produced in E.coli when compared to IRF-1 were found to be virtually indistinguishable, suggesting that these factors bind the same sequences. It was subsequently shown (Harada et al., 1989) that IRF-2 was responsible for the 'IRF-1' activity detected in uninduced L929 cells by Fujita et al. (1988).

IRF-2, as IRF-1 is inducible by both virus and IFN, albeit with slightly different kinetics (Harada et al., 1989). However only the IRF-1 cDNA was able to transactivate the IFN genes upon transfection of COS cells. Cotransfection of IRF-2 together with IRF-1 did not significantly affect IFN induction; IRF-2 does not seem to function as a repressor in the classical sense. The physiological role of IRF-2 is thus unclear and Harada et al. (1989) have postulated that it may act as a place holder on the DNA to ensure that sequence elements required for activation of the IFN genes by IRF-1 are prevented from binding non-specific DNA binding proteins that might interfere in transcription, thus keeping the gene in a state readily accessible for transcription.

Multimers of AAGTGA contain sequences similar to that of PRDI (see below) and therefore it seems likely that PRDI and multimers of AAGTGA interact with the same DNA binding factors. Even so, the relationship of PRDI-BFc and -BFi with IRF-1 and -2 is not as yet clear. The homology shared by PRDI and the consensus sequence of the IFN-responsive element (see below) suggests that the pathways of virus and IFN induction may overlap, with IRF-1 and -2 playing a role in both. The observation that a number of IFN-inducible genes are inducible by virus or dsRNA further supports this idea (Tiwari et al., 1987; Wathelet et al., 1987; Hug et al., 1988).

GAGAAGTGAAAGTG   PRDI

AAGt gaAAGTGAAAGTGA   (AAGTGA)$_3$

a/Gga AANNGAAAG/cTt/c   IFN-inducible consensus sequence
                                         (Hug et al., 1988)

Clark and Hay (1989) purified a factor EBP1 which binds a sequence present in the SV40 enhancer and noted, as did Hiscott et al. (1989), a strong sequence similarity between the PRDII, the EBP1 binding site in the SV40 enhancer, the NF-κB binding site in the κ light chain enhancer and the H2TF1 binding site in the mouse class I histocompatability gene enhancer. EBP1 specifically recognized these sequence motifs (Clark and Hay, 1989) and it was subsequently shown that upon induction with either virus or dsRNA, a factor indistinguishable from NF-κB bound to PRDII, and that PRDII and NF-κB binding sites were interchangeable (Fujita et al.,

1989b; Lenardo et al., 1989; Visvanathan and Goodbourn, 1989). Furthermore, point mutations within PRDII that reduce IFN-β induction in vivo correlate with a decrease in the affinity that NF-κB has for PRDII in vitro (Lenardo et al., 1989; Visvanathan and Goodbourn, 1989).

Mature B cells display significant constitutive NF-κB activity (Sen and Baltimore, 1986a; Atchison and Perry, 1987; Lenardo et al., 1987). In other cell lines, NF-κB can be activated by lipopolysaccharides (LPS), phorbol esters (such as phorbol myristate acetate (PMA)), virus and dsRNA (Sen and Baltimore, 1986b; Fujita et al., 1989b; Lenardo et al., 1989; Visvanathan and Goodbourn, 1989). In non-B and pre-B cells, NF-κB is associated with a cytoplasmic inhibitor (I-κB); upon induction, this complex dissociates to release active NF-κB which is translocated to the nucleus and activates transcription through interactions with its binding sites located in the promoters of a variety of genes (Baeuerle and Baltimore, 1988a, 1988b). Release of NF-κB from I-κB is insufficient to activate transcription of the IFN-β gene, as shown by the fact that although LPS and PMA greatly stimulate NF-κB binding, they do not cause induction of the IFN-β gene (Lenardo et al., 1989). Although it seems likely that IFN-β gene expression is at least in part regulated by the binding of NF-κB to PRDII, it appears that a second signal, mediated by either virus or dsRNA but not LPS or PMA, is required; either the release of repression mediated by NRDI and/or the presence of IRF-1.

1.17   Outline of the work described in this thesis.

When I started this work it was well established that the induction of the IFN genes was the result of the activation of transcription (Ohno and Taniguchi, 1983; Raj and Pitha, 1983; Weidle and Weissmann, 1983) and that short 5'-flanking sequences (the VRE elements) were responsible for mediating this effect on the IFN genes (Dinter et al., 1983; Zinn et al., 1983; Fujita et al., 1985; Goodbourn et al., 1985; Ryals et al., 1985). Although the virus responsive element of the IFN-α1 gene had been identified (Ryals et al., 1985), nothing was known about factors interacting with this region that mediates virus inducibility upon the IFN-α1 gene. The aim of this work was to characterize the sequences contained within the VREα1 with regard to their transcriptional activity and factor binding potential, in the belief that

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such an approach may ultimately lead to the eventual elucidation of the molecular mechanism of induction of the IFN genes.

Tetramers of GAAAGT or AAGTGA, motifs present in the IFN-α1 and -β promoters respectively, confer virus inducibility upon a minimal promoter (Fujita et al., 1987; Kuhl et al., 1987). Furthermore, sequences of the type GAAANN are inordinately frequent in the promoters of the IFN and IFN-inducible genes and have been postulated to play a role in the induction of these genes (Hug et al., 1988). Consequently a study of the in vivo and factor-binding activities of tetramers of all the GAAANN variants present in the VREα1 and Mx promoters was made in the hope that an investigation of these elements would lead to a better understanding of the induction of the IFN genes. Three distinct types of $(GAAANN)_4$ sequences, all of which mediated virus inducibility were identified and found to bind different nuclear proteins as judged by gel retardation analysis, indicating that potentially more than one pathway is be involved in the virus induction of the IFN and IFN-inducible genes.

In addition preliminary studies were undertaken to investigate the control of the differential regulation of the IFN-α1 gene in Hela cells. The expression of the endogenous IFN-α1 gene and a transiently introduced plasmid-borne reporter gene under the control IFN promoter elements was studied and evidence obtained suggesting that the endogenous IFN-α1 gene in Hela cells is under negative control, which the reporter gene is some how able to evade.

2.   RESULTS.

2.1   Control of IFN-α1 expression in Hela cells.

Under normal conditions cells produce little or no IFN but upon infection by virus the IFN genes are expressed differentially to produce a mixture of IFN-α and/or IFN-β mRNA and protein. (Stewart, 1979; Lengyel, 1982). Hiscott et al. (1984a) showed that Hela cells treated with NDV expressed exclusively IFN-β mRNA; no IFN-α transcripts were detected. In order to investigate the inability of HeLa cells to express the IFN-α genes following viral infection, HeLa cells were transiently transfected with plasmids containing a rabbit β-globin gene under the control of either a minimal β-globin promoter (ID1, Figure 2) or a hybrid human IFN-α1/β-globin promoter, which contained two copies of VREα1 (13P, Figure 2).

The rabbit β-globin gene when truncated to position -56 (such that it retains the TATA box but no other functional elements) is neither constitutively active nor virus inducible (Dierks et al., 1983; Ryals et al., 1985). When placed at position -56 of the β-globin promoter, two copies of the 46-bp VREα1 do not affect its constitutive expression, however they do confer virus inducibility on the downstream β-globin promoter in L929 cells (Kuhl et al., 1987). The use of such a gene made it possible to ascertain whether the IFN-α1 promoter sequences identified as being sufficient to mediate full induction by virus were functional in Hela cells or whether the block in transcription of the endogenous IFN-α genes was occurring at some other point.

2.1.1   An exogenous human IFN-α1 promoter fragment, but not the endogenous IFN-α1 gene is virus inducible in Hela cells.

Hela cells were transiently transfected with the test plasmids, using the calcium phosphate method (Wigler et al., 1978). 48 hours after transfection the cells were either NDV or mock induced and 8 hours later total RNA was isolated. The amount of β-globin and human IFN-α1 mRNA in the transfected cells was determined by S1 nuclease analysis using appropriate 5′-^{32}P-labeled complementary probes.

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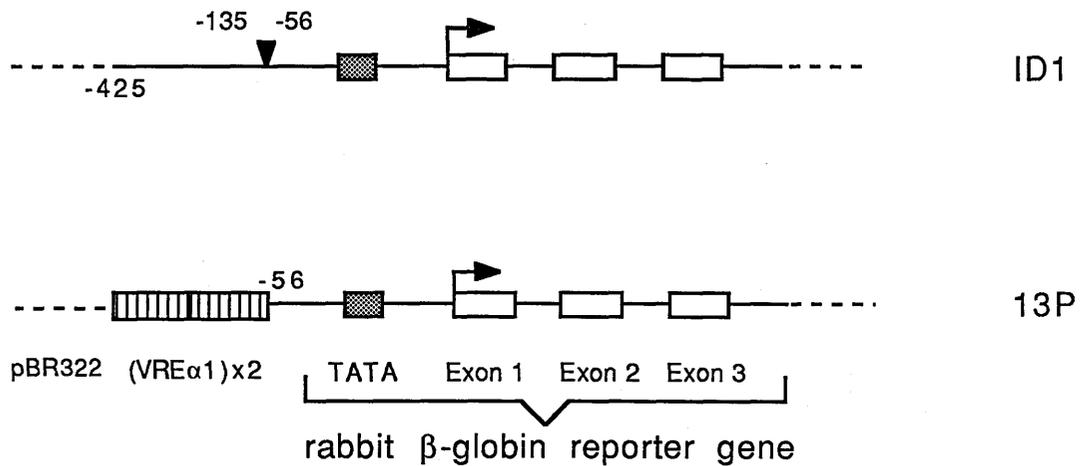
Figure 2   Map of the rabbit β-globin expression plasmids used to transfect HeLa cells.

Both ID1 and 13P are described in detail elsewhere (Ryals et al., 1985 and Kuhl et al., 1987). In brief, rabbit β–globin sequences from -425 to +1646, which contain a deletion from -135 to -56 were cloned into pBR2670 (a derivative of pBR322 lacking the sequences between positions 656 and 2346) to generate ID1. 13P was created by cloning β-globin sequences from -56 to +1646 downstream of two copies of the VREα1 (human IFN-α1 gene -109 to -64) in pBR2670.

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β-globin transcripts were assayed by S1 analysis using as a probe a 5′-^{32}P-labeled 452-bp PstI-BamHI fragment of the β-globin gene from which the first intron had been excised (Weber et al., 1981). Correctly initiated β-globin transcripts gave a signal of 353 nt (Figure 3 and Ryals et al., 1985).

In the absence of viral induction, no signals were detected with any of the constructs (Figure 5A). Upon induction by virus, a signal of 353 nt was observed with RNA isolated from Hela cells transfected with 13P, indicative of correctly initiated and spliced β-globin transcripts (Figure 3; Ryals et al., 1985). No β-globin mRNA was detected in the RNA isolated from ID1 or mock-transfected cells after induction by virus.

To confirm that the endogenous IFN-α1 gene was not expressed in Hela cells, even after induction by virus, as previously observed by Hiscott et al. (1984a), samples of RNA isolated from 13P transfected cells (NDV and mock induced) were assayed for IFN-α1 specific mRNA by S1 mapping. Using a 5′-^{32}P-labeled 360-bp EcoRI-RsaI fragment of the human IFN-α1 gene as probe (Figure 4), the 3′-ends of IFN-α1 transcripts were S1 mapped. A probe complementary to sequences in the 3′-non-translated region of the IFN-α1 transcripts was chosen as this region displays the greatest sequence divergence with the other IFN-α transcripts (Henco et al., 1985) and as such does not cross-hybridize with transcripts derived from any of the other IFN-α genes (Streuli, 1986). Due to the heterogeneity of the 3′ end of the IFN-α1 mRNA caused by multiple polyadenylation sites, protected fragments of 264, 314 and 360 nt would be indicative of human IFN-α1 mRNA (Figure 4; Mantei and Weissmann, 1982). None of these predicted IFN-α1 specific S1-resistant fragments were detected (Figure 5B). Although 0.1 fmol (equivalent to ~60 strands/cell) of an SP6 IFN-α1 RNA transcript (gift from M. Streuli) was readily detected after exposure of the gel to film at -70°C with an intensifying screen for 36 hours, no signals were detected from the Hela cell RNA even after exposure for 30 days (data not shown).

This result shows that while IFN-α1 promoter elements are virus inducible in Hela cells when introduced on a transiently transfected plasmid, the endogenous IFN-α1 gene in the same cells is not expressed. This finding implies that the differential expression of the IFN genes in Hela cells is, at least in part, due to some form of negative regulation of the
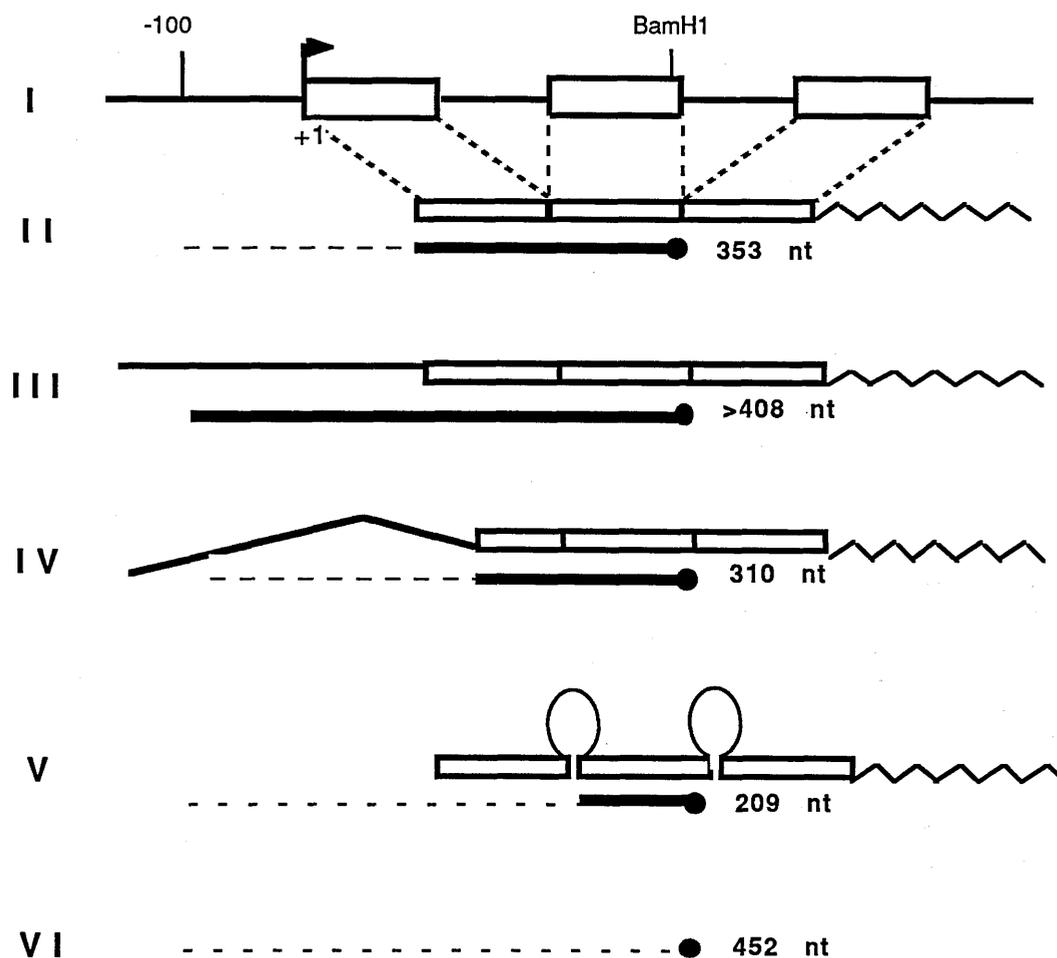
Figure 3   Schematic representation of the S1 mapping of β-globin transcripts (adapted from Kuhl et al., 1987).

(I) Rabbit β-globin gene: empty boxes, exons; lines between two boxes, introns. (II) Test RNA hybridized to the 5'-^{32}P-labeled (•) probe. (III) Hybrid between probe and correctly spliced transcripts initiated upstream of promoter; the length of the protected probe depends on the position of the 5' end point of the globin promoter segment. (IV) Hybrid between probe and transcripts which originated upstream of the promoter and were spliced to position +48 of the first globin exon (Ryals et al., 1987). (V) Hybrid of probe and unspliced RNA. (VI) The probe was a PstI-BamHI 452-bp fragment, 5'-^{32}P-labeled at the BamHI site, derived from a β-globin genomic DNA fragment from which the small intron had been deleted (Weber et al., 1981). In all cases the fragment of the probe protected after S1 digestion is shown by thick lines.
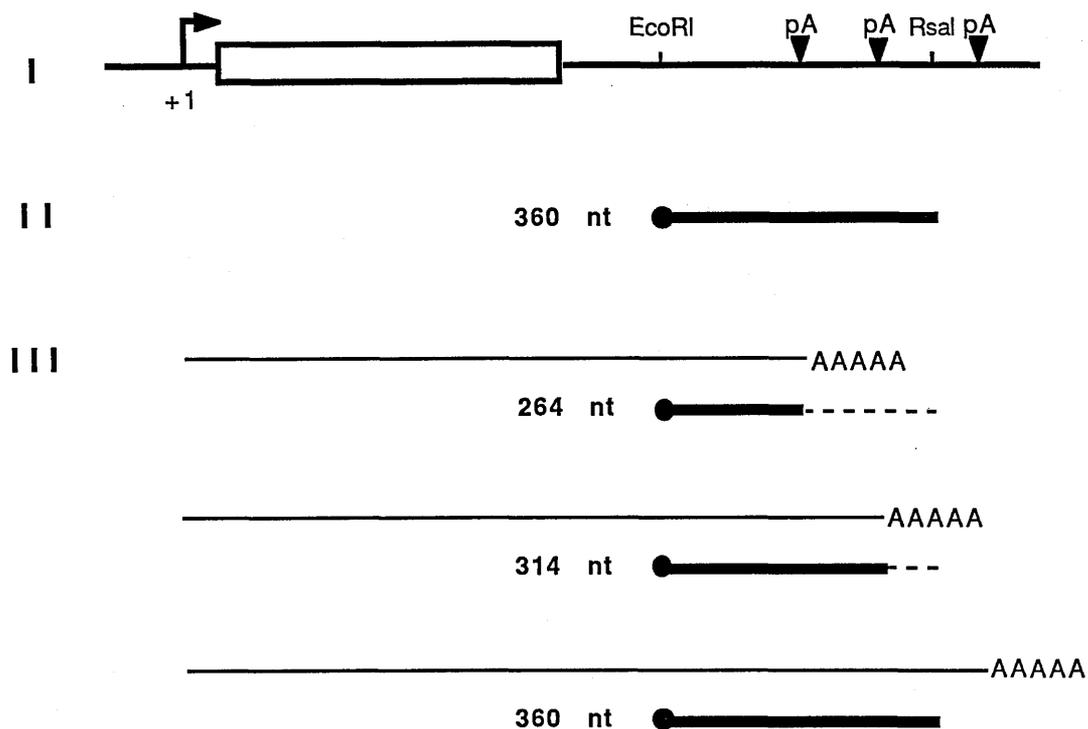
Figure 4 Schematic representation of 3'-S1 mapping of the endogenous IFN-α1 transcripts.

(I) IFN-α1 gene: coding region boxed; pA, polyadenylation sites. (II) The probe was an EcoRI-RsaI 360-bp fragment, 5'-^{32}P-labeled (•) at the EcoRI site, derived from a human IFN-α1 genomic DNA fragment. (III) Test RNA hybridizing to the 5'-^{32}P-labeled probe would result in protected fragments of the indicated size, depending on the polyadenylation site utilized. In all cases the fragment of the probe protected after S1 digestion is shown by thick lines.
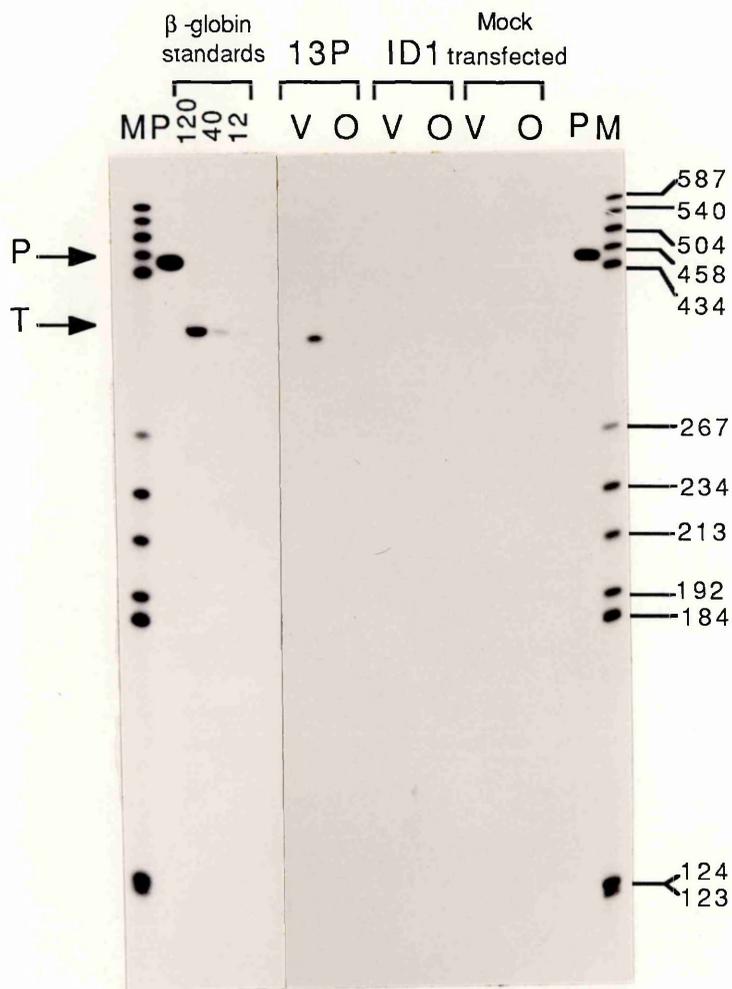
Figure 5A　　An exogenous human IFN-α1 promoter fragment, but not the endogenous gene, is virus inducible in Hela cells.

Hela cells were NDV or mock induced 48 hours after transfection with 10 μg of test plasmid (or with H_2O; mock transfection). Total RNA was recovered after 8 hours induction and 16 μg (12.5 μg in the case of ID1, mock induced) were S1 mapped using either an IFN-α1 or β-globin 5′-^{32}P-labeled probe.

(A) Autoradiogram of S1 mapping a using β-globin probe, after 60 hours at -70 °C with an intensifying screen. M, pBR322 digested with Bspl and 5′-^{32}P-labeled; P, probe; V, NDV induction; O, mock induction; β-globin standards, β-globin RNA (in pg); T, test signal.

32
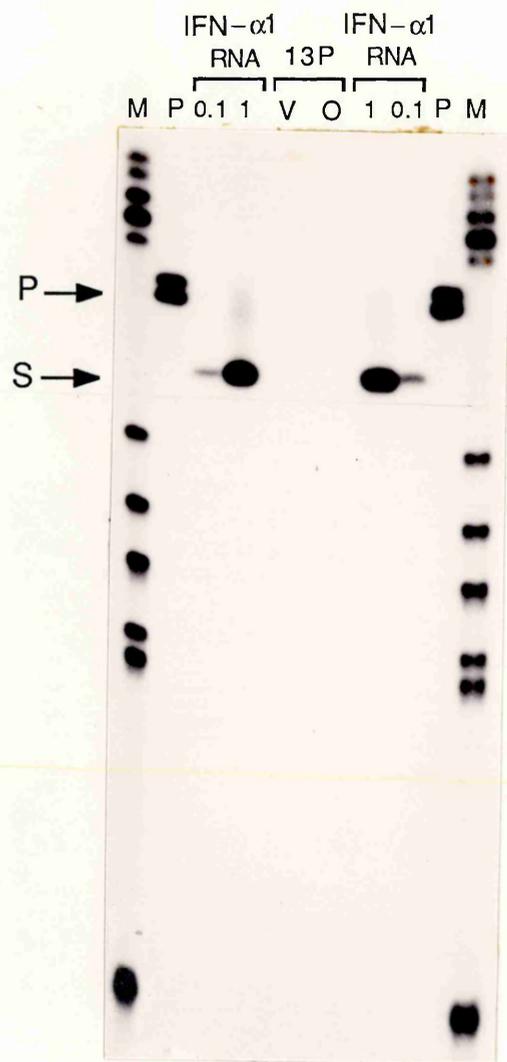
Figure 5B   An exogenous human IFN-α1 promoter fragment, but not the endogenous gene, is virus inducible in Hela cells.

(B) Autoradiogram of S1 mapping using human a IFN-α1 probe, after 36 hours at -70 °C with an intensifying screen.
Experimental procedures and abbreviations as in Figure 5A. IFN-α1 RNA, SP6 transcripts of human IFN-α1 gene (in fmol); S, signal obtained from SP6 IFN-α1 transcript (Streuli, 1986).

endogenous IFN-α1 gene, which the plasmid-borne IFN-α1/β-globin hybrid gene is not subject to.

A variety of mechanisms can be proposed to explain such a repression of the IFN-α1 gene (see Section 3.1). However, inactivation of the endogenous gene due to a deletion or rearrangement cannot be ruled out as an explanation for this phenomenon.

2.2   Tetrameric oligonucleotides of the type GAAANN mediate different patterns of inducibility by virus and IFN.

A large number of virus and IFN-inducible genes have been isolated from a variety of organisms (Revel and Chebath, 1986; Weissmann and Weber, 1986; and references therein), and their 5'-flanking regions have been shown to be required for inducibility (Friedman and Stark, 1985; and references therein). Sequences of the type GAAANN are inordinately frequent within these promoters (Table 1) and have been postulated to play a central role in the induction of these genes (Hug et al., 1988). Tetramers of AAGTGA and GAAAGT (a permutation of AAGTGA) confer virus inducibility on a downstream reporter gene (Fujita et al., 1987; Kuhl et al., 1987). In this study, tetramers of all the GAAANN variants found in the VREα1 and in the Mx promoter (with the exception of GAAAAA; Figure 6), were examined with regard to their transcriptional activities in vivo.

The effect on transcription of oligonucleotides of the type $(GAAANN)_4$ (abbreviated NN_4; for example GT_4 for $(GAAAGT)_4$) and of natural IFN promoter fragments was determined by measuring the steady state level of stable reporter transcripts using quantitative S1 analysis. The oligonucleotides to be tested were synthesized chemically with ClaI and HindIII-compatible overhanging ends, to allow their cloning between the ClaI and HindIII sites 56 bp upstream of the β-globin cap site, as shown in Figure 7.

The oligonucleotides were cloned into β-globin expression vectors, with and without the SV40 enhancer. When present, the SV40 enhancer was 1291 bp upstream of the site into which the the oligonucleotides were inserted, separated by an `inert´ DNA fragment derived from the 5´ flanking

Gene	Sequence	Position	Reference
Human IFN-α1 [a]	GAAACA	-135 to -130	Nagata et al.
	GAAAGC	-96 to -95	(1980b)
	GAAATG	-85 to -80	
	GAAAGT	-79 to -74	
	GAAAGT	-56 to -51	
	GAAATC	-49 to -44	
Murine IFN-α4 [a]	GAAAGT	-97 to -92	Raj et al.
	GAAAAG	-91 to -87	(1989)
	GAAAGC	-79 to -74	
Human IFN-β [a]	GAAACT	-117 to -112	Ohno and Taniguchi
	GAAAAC	-90 to -85	(1981)
	GAAAGG	-83 to -78	
	GAAAGT	-70 to -65	
	GAAATT	-62 to -57	
	GAAAGG	-6 to -1	
Murine Mx [b]	GAAACT [d]	-126 to -131	Hug et al.
	GAAACG [d]	-121 to -126	(1988)
	GAAAGG [d]	-109 to -114	
	GAAAAA [d]	-67 to -72	
	GAAACC [d]	-59 to -64	
Murine IRF-1 [b]	GAAATC [d]	-122 to -127	Miyamoto et al.
	GAAATG	-118 to -113	(1988)
	GAAATC [d]	-21 to -26	
Human IFI-54K [b]	GAAAAA	-136 to -131	Wathelet et al.
	GAAAAA	-129 to -124	(1988)
	GAAATT [d]	-107 to -113	
	GAAAGT [d]	-100 to -105	
	GAAACT [d]	-94 to -99	
	GAAAGT [d]	-88 to -93	
	GAAACA	-64 to -59	
Human IFI-56K [b]	GAAAGT [d]	-133 to -138	Wathelet et al.
	GAAAGG [d]	-107 to -112	(1987)
	GAAACC [d]	-100 to -105	
	GAAACC [d]	-94 to -99	
Human 2-5 A synthetase [c]	GAAAGG	-147 to -142	Wathelet et al.
	GAAACT [d]	-100 to -105	(1987)
	GAAATT	-76 to -71	
	GAAAGT	-34 to -29	
Human 6-16 [c]	GAAACT	-145 to -140	Porter et al.
	GAAAAT	-110 to -105	(1988)
	GAAACT	-104 to -99	
	GAAATA	-88 to -83	
	GAAACT	-82 to -77	

[a] virus inducible genes   [b] virus and IFN inducible genes
[c] IFN inducible genes   [d] antisense strand

Table 1.   Occurrence of GAAANN sequences within the 150-bp
preceding the cap site of virus and IFN inducible
genes.

IFN-α1

VREα1

TGTAAAGAGTGCATGAAGGAAAGCAAAACAGAAATGGAAAGTGGCCCAGAAGCATTAAGAAAGTGGAAATCAGTATGTTCCCTATTTAAGGC

CTTTCG   CTTTACCTTTCA   CTTTGA CTTTAG

-115 -109   -64   -32

Mx

CAGGGTGAGTTTCGTTTCTGAGCTCCTTCATATGTGGTGGGGTGTGGGCACAGCAAGCTCAGGCTTTTCACGGTTCAATTCTCCTCTGG

TCAAAGCAAAG   GGAAAG   AAAAAG CCAAAG

-138   -100   -46

AAGGGCCCGAGACCCAGCACTATTTAAAGAG

-27
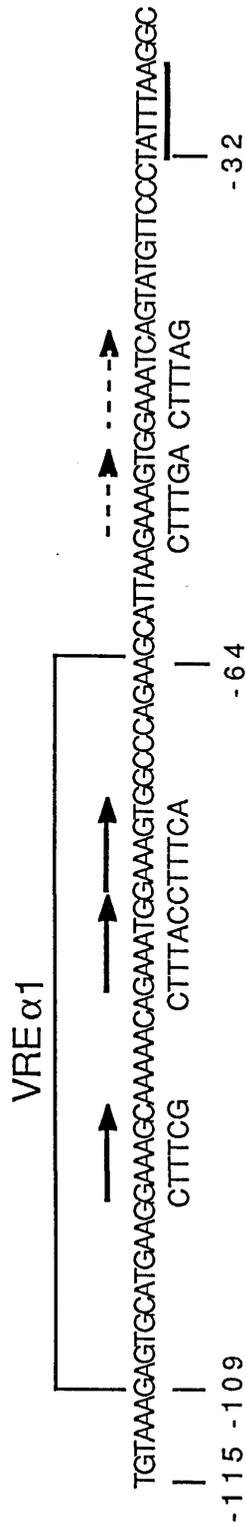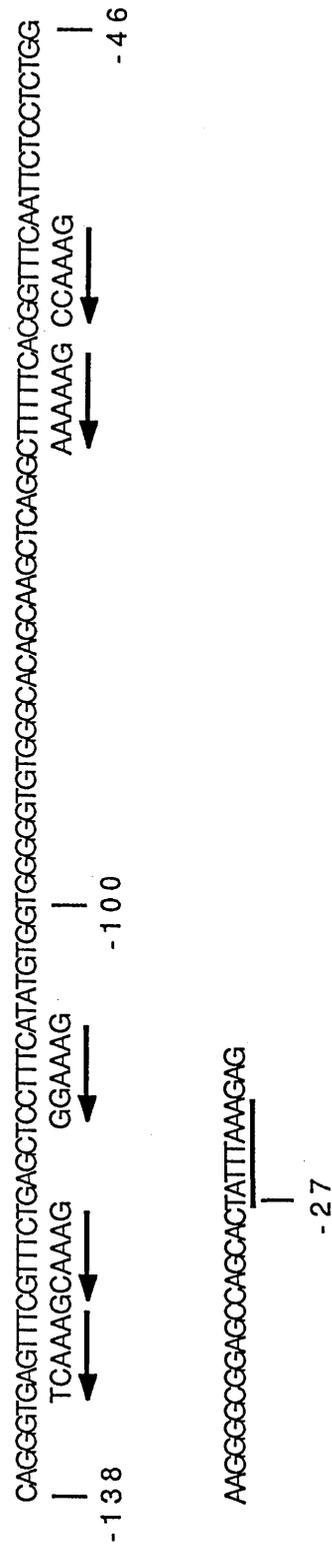
Figure 6   Sequences of the type GAAANN are inordinately frequent in the IFN-α1 and Mx promoters.

Numbering is from the cap site of the human IFN-α1 (Ryals et al., 1985) and murine Mx genes (Hug et al., 1988), the VREα1 was described by Kuhl et al. (1987). The TATA boxes of both genes are underlined, solid arrows indicate hexanucleotides of the type GAAANN present in VREα1 and the Mx promoter, and dotted arrows hexanucleotides present in the IFN-α1 promoter, but outside the VREα1.

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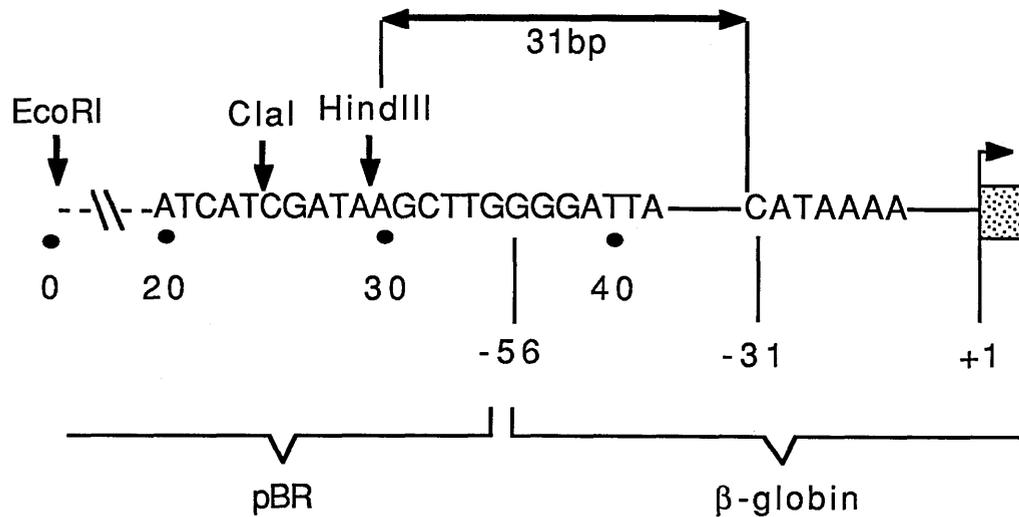
Figure 7   Structure of the region into which promoter elements were inserted.

When required, the SV40 enhancer 72 bp repeat was inserted into the EcoRI site, a 1291 bp spacer between the XhoI site in the linker at the 3′ end of the SV40 enhancer and the ClaI site, and promoter elements between the ClaI and HindIII sites.

region of the β-globin gene (Kuhl et al., 1987). Several intrinsic properties of the oligonucleotides were examined using these constructs; (1) constitutive transcriptional activity, (2) inducibility by virus and (3) inducibility by IFN-γ.

2.2.1   The assay system.

The rabbit β-globin gene functioned as a reporter, with test sequences cloned at position -56 relative to the cap site (see Figure 7), while a thymidine kinase/glucocorticoid receptor gene under the control of the cytomegalovirus (CMV) promoter/enhancer served as an inducible reference plasmid (pSTC 407-556; Severne et al., 1988). Murine L929 cells were transiently transfected with a mixture of the test and reference plasmid, in addition to either pIRF-L, an IRF-1 expression plasmid (Miyamoto et al., 1988) or the 'empty' expression vector, CDM8 (Seed, 1988). Forty eight hours after transfection the cells were either NDV, IFN or mock induced, and 8 hours later total RNA was isolated.

Test β-globin transcripts were assayed as in Section 2.1.1. Transcripts derived from the internal reference plasmid were detected using a probe which generated a signal of 148 nt (Figure 8). Transcription of the reference, under the control of the CMV promoter/enhancer was also virus inducible, thus allowing corrections to be made for the efficiency of virus induction, as well as RNA recovery and transfection.

Radioactivity in the test and reference signals was determined by Cerenkov counting. Test signals were normalized relative to the corresponding reference signals and quantitated relative to signals given by known amounts of β-globin mRNA and the number of strands per cell was determined as in Kuhl et al. (1987).

2.2.2   Tetrameric oligonucleotides of the type (GAAANN)$_4$ mediate different patterns of transcriptional activity.

A 'minimal promoter' which retains a TATA box but no additional upstream elements, is essentially silent and requires the presence of
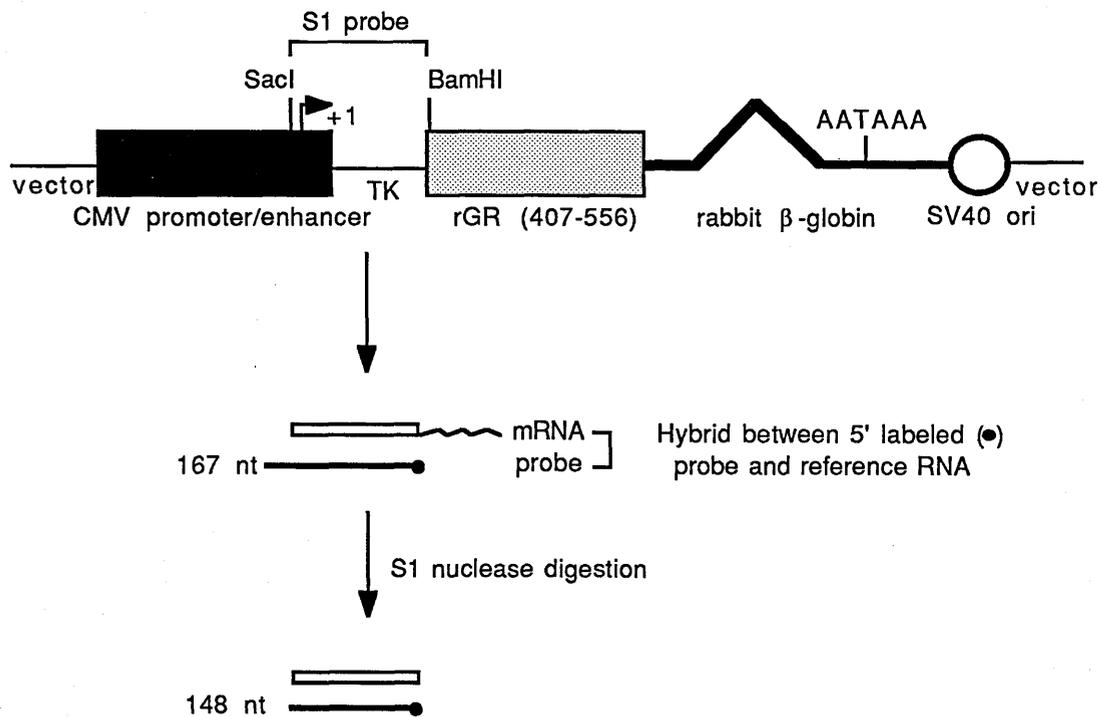
Figure 8   Schematic representation of S1 mapping of the internal reference gene.

pSTC407-556 (Severne et al., 1988) served as an inducible internal reference, whose transcripts were S1 mapped using a probe prepared by 5′-labeling at the BamHI site and cleaving with SacI.

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additional elements for activity, such as a CAAT box (Dierks et al., 1983), a heat shock element (Bienz and Pelham, 1986) or a tetrameric repeat of repA (Kuhl et al., 1987). In this context, tetrameric GAAANN elements were assayed for their ability to mediate constitutive transcription and confer inducibility by virus and IFN-γ on the β-globin gene.

2.2.3 The tetrameric oligonucleotides mediate inducibility by virus.

All of the tetramerized hexanucleotides tested, as well as both VREα1 and VREβ, mediated induction by virus, though the transcript level and degree of inducibility varied greatly between the various elements. In the absence of the enhancer (Figure 9; V, column no IRF), CC_4 exhibited the highest degree of inducibility, >58 fold, about 7 times greater that that of the next highest, GT_4. The least inducible was TG_4, for which the level of induction in the absence of the enhancer was so weak that it was not possible to quantitate, although inspection of the autoradiograph revealed weak virus induction.

2.2.4 The upstream SV40 enhancer and oligonucleotides interact through enhancer coupling to potentiate transcription.

When placed 1300 bp upstream of the TATA box of a minimal promoter, the SV40 enhancer is unable to activate transcription in the absence of an appropriate upstream element placed close to the TATA box (Pelham, 1982; Hen et al., 1982; Treisman and Maniatis, 1985; Kuhl et al., 1987). The ability of an upstream enhancer to exercise its effect upon a minimal promoter was termed enhancer coupling, and is expressed as the ratio of transcript level obtained in the presence of an enhancer at distance (Figure 10) to that obtained with the oligonucleotide alone (Figure 9), in either the uninduced or induced state.

In the absence of induction, only CG_4, VREα1 and VREβ showed enhancer coupling (because these were the only elements mediating some degree of constitutive transcription; Figure 11). Upon viral induction, all of
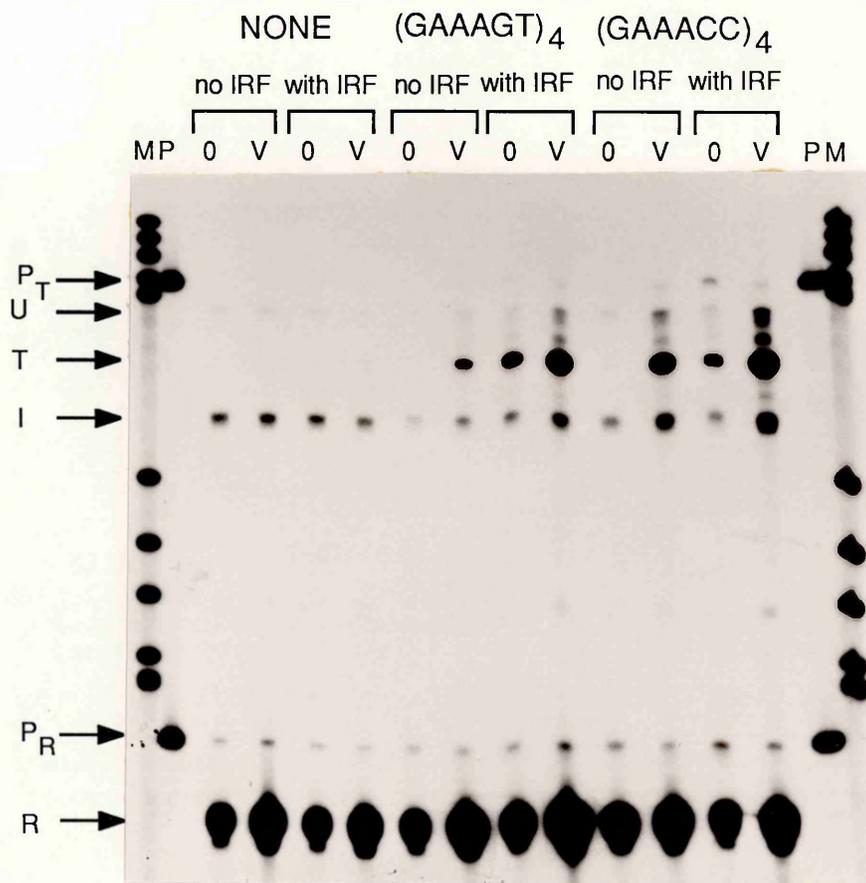
Figure 9A   Activation of transcription by NDV and/or cotransfection
with IRF-1 expression plasmid mediated by various
oligonucleotides (without SV40 enhancer).

Mouse L929 cells were transfected with 7 µg each of the test and reference plasmids, and
with 7 µg of either the IRF-1 expression plasmid pIRF-L ('with IRF') or the corresponding
'empty' expression vector CDM8 ('no IRF'). Induction with NDV or mock induction were
carried out 48 hours after transfection. Total RNA was recovered 8 hours after beginning of
induction and 50 µg were S1 mapped simultaneously with the probes for the reporter and
the internal control.

(A)-(C)   Autoradiograms of S1 mapping after 5 days at -70°C with an intensifying screen. 0,
mock induction; V, NDV induction; globin standards, globin RNA (in pg); M, pBR322
digested with BspI and 5′-^{32}P-labeled; I, aberrantly spliced readthrough β-globin transcripts;
P, probes; P_R, undigested reference probe; P_T, undigested test probe; R, signal from the
reference gene; T, test signal; U, readthrough β-globin transcripts (see Fig.1D, Kuhl et al.,
1987). Results summarized in Figure 9D.

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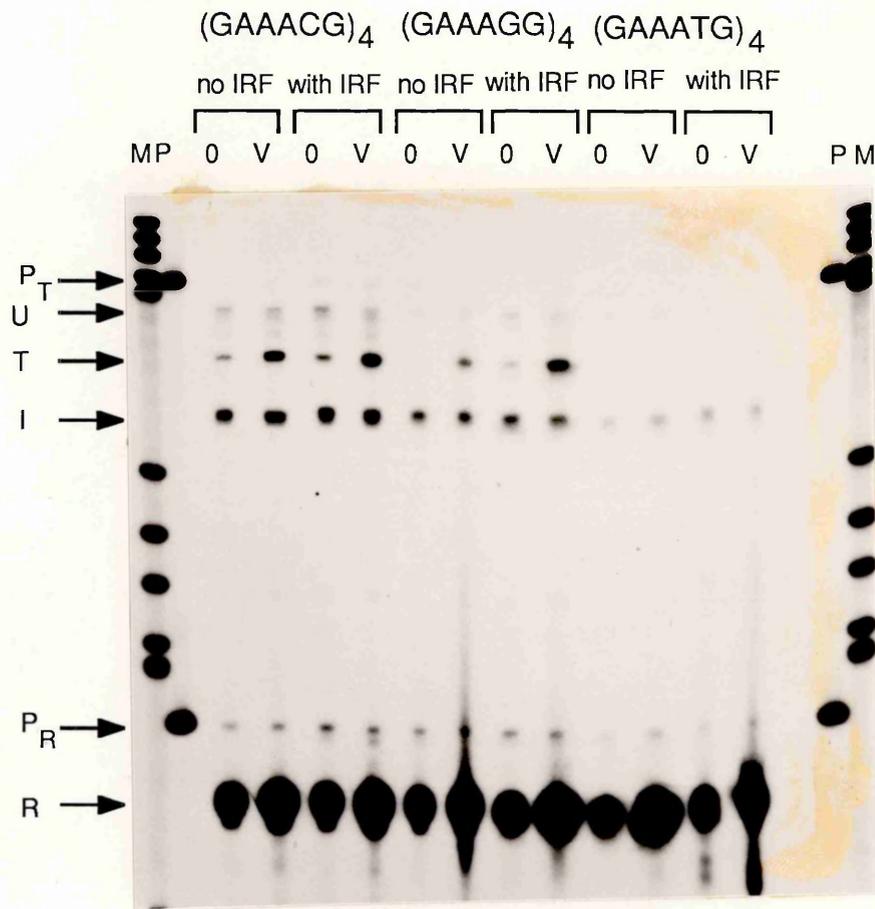
Figure 9B   Activation of transcription by NDV and/or cotransfection
with IRF-1 expression plasmid mediated by various
oligonucleotides (without SV40 enhancer).

Autoradiogram of S1 mapping after 5 days at -70 °C with an intensifying screen.
Experimental procedures and abbreviations as in Figure 9A. Results summarized in Figure
9D.
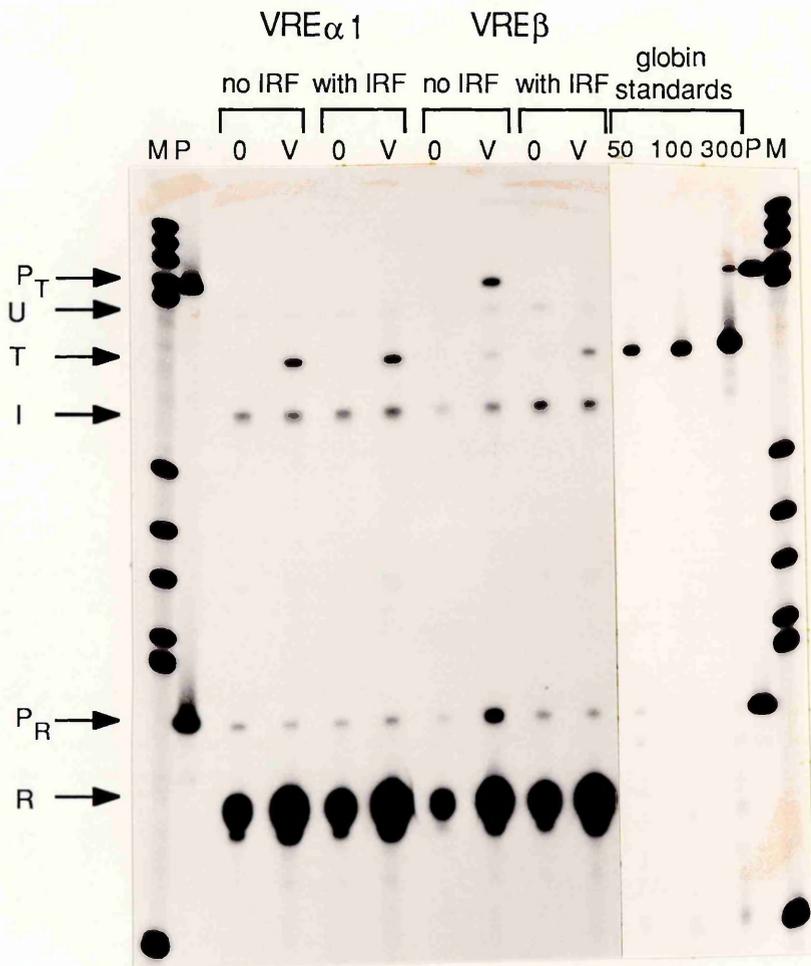
Figure 9C   Activation of transcription by NDV and/or cotransfection
with IRF-1 expression plasmid mediated by various
oligonucleotides (without SV40 enhancer).

Autoradiogram of S1 mapping after 5 days at -70 °C with an intensifying screen.
Experimental procedures and abbreviations as in Figure 9A. Results summarized in Figure
9D.
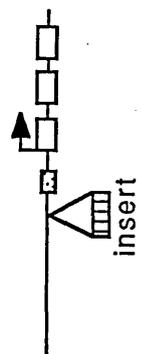
TRANSCRIPT LEVEL
(strands per cell)

INDUCTION

insert	no IRF		with IRF		NDV	IRF	IRF+NDV
	MOCK	NDV	MOCK	NDV			
24 bp pBR spacer	<2.5	<2.5	<2.5	<2.5	-	-	-
(GAAAGT)$_4$	<2.5	20	25	70	>8	>10	3.5
(GAAACC)$_4$	<2.5	145	32.5	215	>58	,>13	1.5
(GAAATG)$_4$	<2.5	<2.5 *	<2.5	<2.5 *	-	-	-
(GAAACG)$_4$	5.5	27.5	7.5	25	5	1.4	0.9
(GAAAGG)$_4$	<2.5	12.5	3	35	>5	>1.2	2.8
VREα1	<2.5	25	<2.5	20	>10	-	0.8
VREβ	<2.5	8	<2.5	10	>3	-	1.3

Figure 9D Activation of transcription by NDV and/or cotransfection with IRF-1 expression plasmid mediated by various oligonucleotides (without SV40 enhancer).

Summary of results from the S1 mapping experiments depicted in Figures 9A-C. Transcript levels were calculated as described in Kuhl et al. (1987), values are given in strands per cell; in cases where the test signal was not significantly above background, the value is given by "<2.5 strands per cell", as 2.5 strands per cell would have given a significant value. Experimental procedures were as outlined in the legend to Figure 9A. Hatched box ("insert"), the oligonucleotide to be tested; dotted box, TATA sequence; white boxes, β-globin exons. * , NDV (but not mock) induced cells gave a distinct signal both in the presence or absence of IRF-1 cotransfection, as estimated visually, but the radioactivity was not significantly above background.

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Figure 10A   Induction of transcription by NDV, IFN-γ and/or by cotransfection with IRF-1 expression plasmid mediated by different oligonucleotides (with SV40 enhancer at a distance).

Autoradiogram of S1 mapping after 15 hours at -70 °C with an intensifying screen. Experimental procedures and abbreviations as in Figure 9A. Induction with rat IFN-γ (1000 units/ml) was carried out 48 hours after transfection. γ, IFN-γ induction. Results summarized in Figure 10G.

Figure 10B   Induction of transcription by NDV, IFN-γ and/or by
cotransfection with IRF-1 expression plasmid mediated
by different oligonucleotides (with SV40 enhancer at a
distance).

Autoradiogram of S1 mapping after 15 hours at -70 °C with an intensifying screen.
Experimental procedures and abbreviations as in Figure 10A. Results summarized in Figure
10G.
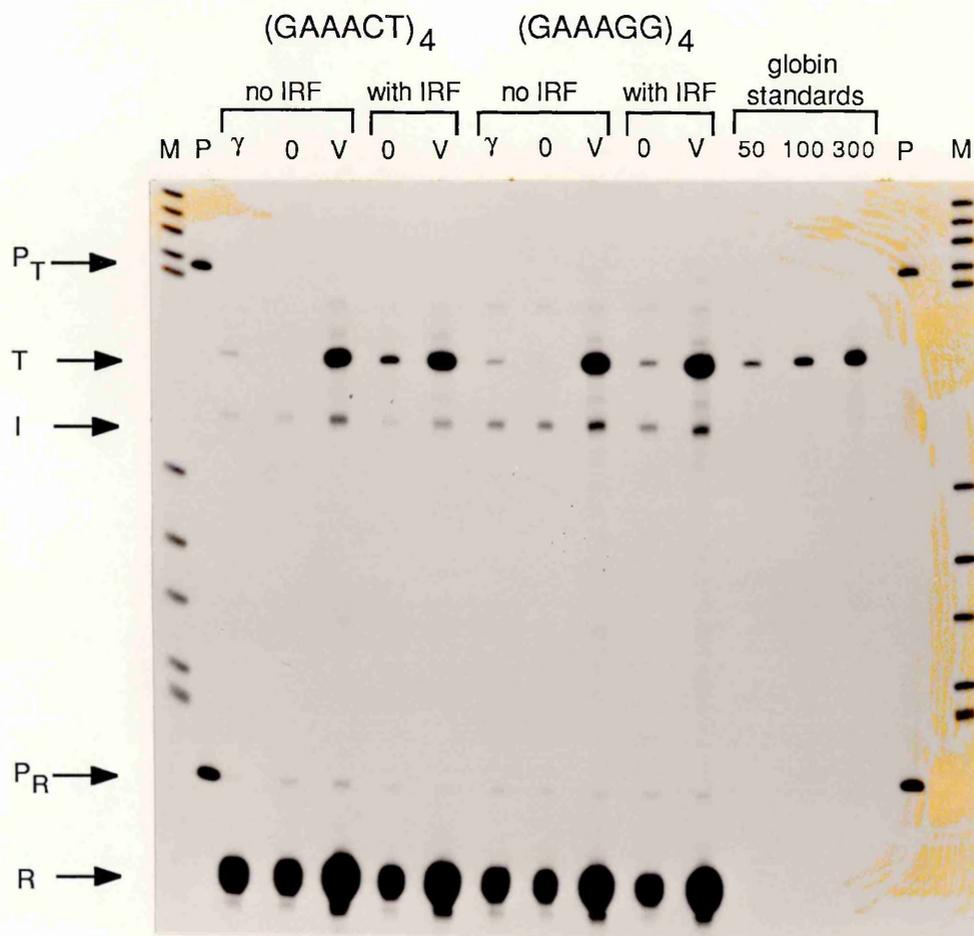
Figure 10C   Induction of transcription by NDV, IFN-γ and/or by cotransfection with IRF-1 expression plasmid mediated by different oligonucleotides (with SV40 enhancer at a distance).

Autoradiogram of S1 mapping after 15 hours at -70 °C with an intensifying screen. Experimental procedures and abbreviations as in Figure 10A. Results summarized in Figure 10G.
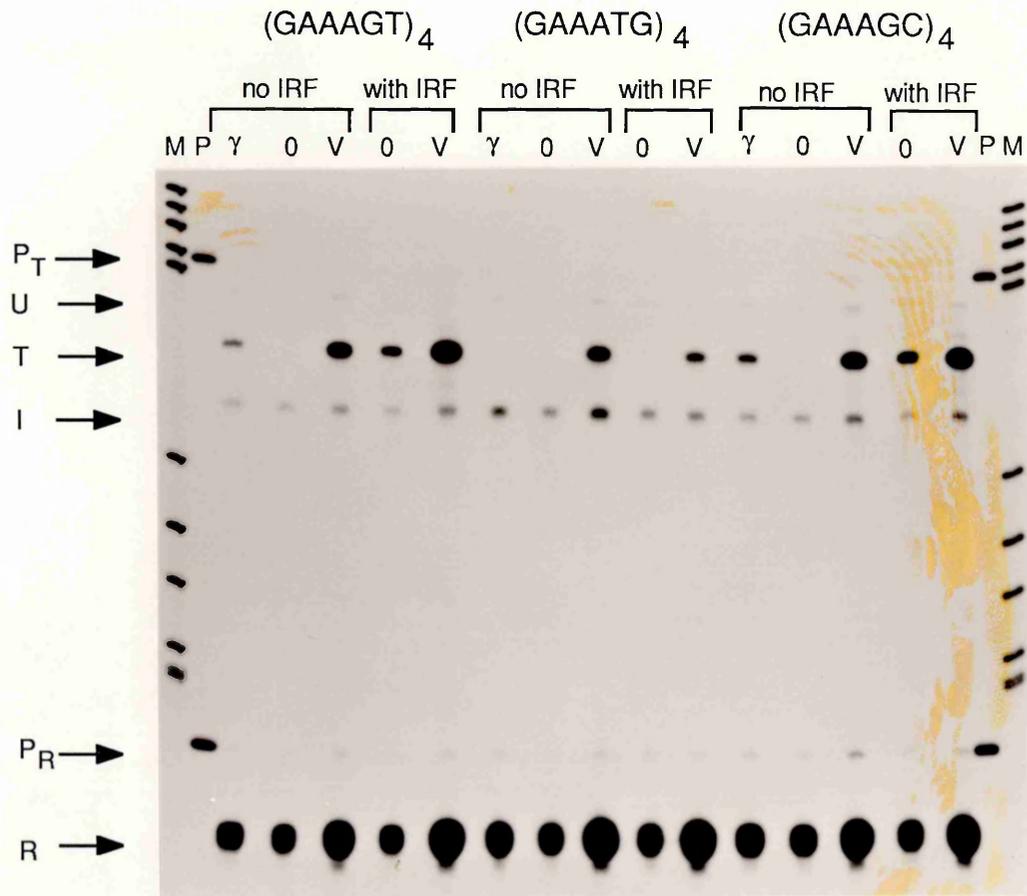
Figure 10D   Induction of transcription by NDV, IFN-γ and/or by cotransfection with IRF-1 expression plasmid mediated by different oligonucleotides (with SV40 enhancer at a distance).

Autoradiogram of S1 mapping after 15 hours at -70 °C with an intensifying screen. Experimental procedures and abbreviations as in Figure 10A. Results summarized in Figure 10G.
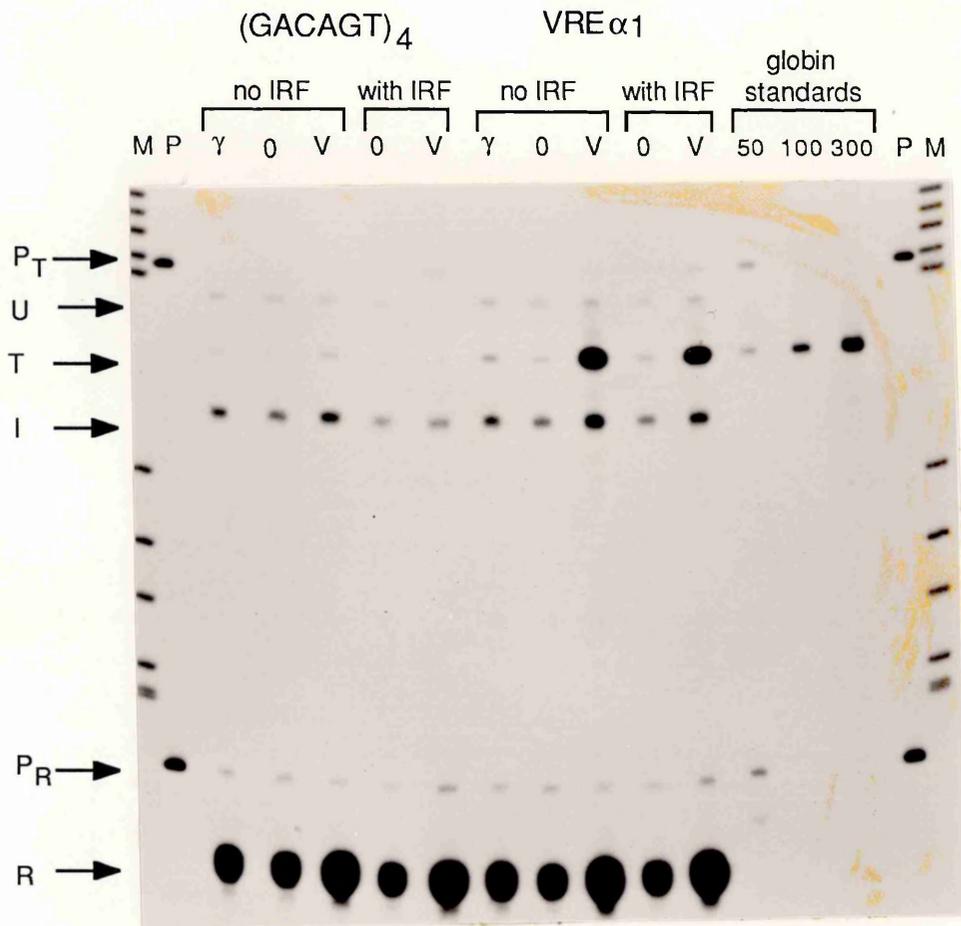
Figure 10E   Induction of transcription by NDV, IFN-γ and/or by cotransfection with IRF-1 expression plasmid mediated by different oligonucleotides (with SV40 enhancer at a distance).

Autoradiogram of S1 mapping after 15 hours at -70 °C with an intensifying screen. Experimental procedures and abbreviations as in Figure 10A. Results summarized in Figure 10G.
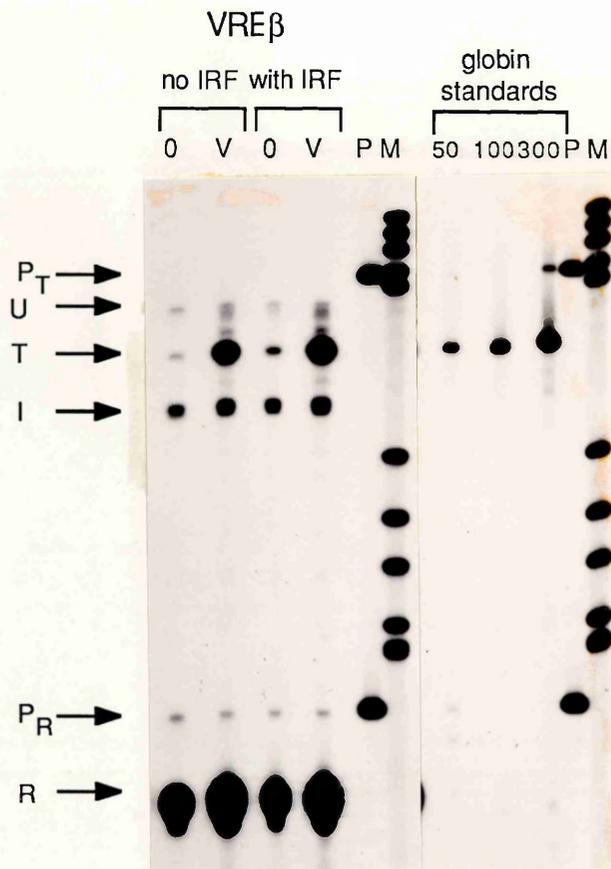
Figure 10F Induction of transcription by NDV, IFN-γ and/or by cotransfection with IRF-1 expression plasmid mediated by different oligonucleotides (with SV40 enhancer at a distance).

Autoradiogram of S1 mapping after 5 days at -70 °C with an intensifying screen (from a separate experiment to that shown in Figures 10A-E). Experimental procedures and abbreviations as in Figure 10A. Results summarized in Figure 10G.

TRANSCRIPT LEVEL
(strands per cell)

INDUCTION

| insert	no IRF MOCK	IFN-γ	NDV	with IRF MOCK	NDV	IFN-γ	NDV	IRF	IRF+ NDV
NONE	<2.5	<2.5	<2.5	<2.5	<2.5	-	-	-	-
(GAAAGT)₄ +	<2.5	45	310	100	575	>18	>124	>40	1.8
(GAAAGC)₄	<2.5	70	385	135	610	>28	>154	>54	1.6
(GAAACT)₄	<2.5	8.5	235	50	355	>3.4	>94	>20	1.5
(GAAACC)₄	<2.5	25	345	70	680	>10	>138	>28	2
(GAAATG)₄	<2.5	<2.5	175	<2.5	80	-	>70	-	0.5
(GAAACG)₄	50	60	415	60	455	1.2	8.3	1.2	1.1
(GAAAGG)₄	<2.5	18.5	530	22.5	695	>7.4	>212	>9	1.3
(GACAGT)₄	4	5	11	2.5	8	1.2	2.8	0.6	0.7
VREα1	11	18	445	14.5	270	1.6	40	1.3	0.6
VREβ *	4.5	ND	175	12	245	ND	39	2.7	1.4

Figure 10G   Induction of transcription by NDV, IFN-γ and/or by cotransfection with IRF-1 expression plasmid mediated by different oligonucleotides (with SV40 enhancer at a distance).

Summary of the S1 mapping experiments depicted in Figures 10A-F. Transcript levels were calculated as described in Kuhl et al. (1987). Experimental procedures were as outlined in the legend to Figure 10A and abbreviations as in Figure 9D. All constructions contained the SV40 enhancer (black box) ("Enh") 1291 bp upstream of the oligonucleotide ("insert") to be tested. +, average of 2 parallel transfections. * , average of 2 parallel transfections, from a separate experiment.

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INSERT	UNINDUCED	INDUCED
`inert´ spacer	-	-
$(GAAAGT)_4$	-	16
$(GAAAGC)_4$	ND	ND
$(GAAACT)_4$	ND	ND
$(GAAACC)_4$	-	2.4
$(GAAATG)_4$	-	>70
$(GAAACG)_4$	9	15
$(GAAAGG)_4$	-	42
$VRE\alpha1$	>4.4	18
$VRE\beta$	>2	22

Figure 11. The upstream SV40 enhancer and oligonucleotides interact through enhancer coupling to potentiate transcription.

Enhancer coupling is the ratio of transcripts given by an oligonucleotide preceded by an enhancer at a distance (Figure 10) to that given by the oligonucleotide alone (Figure 9). 'ND', not determined; –, not measurable.

the oligonucleotides displayed some degree of enhancer coupling. Tetrameric TG_4 exhibited the highest degree of induced enhancer coupling, >70 fold. GG_4 the next highest, displayed a 42 fold stimulation of transcription in the presence of the enhancer, $VRE\alpha1$ and $VRE\beta$ approximately 20 fold, CG_4 and GT_4, 15 fold and CC_4, 2.4 fold. The low degree of induced enhancer coupling seen with CC_4 was detectably higher than that of the 'inert' spacer control, for which no enhancer coupling was observed.

2.2.5 GT_4, GC_4, CT_4, CC_4 and GG_4 are all inducible by IFN-γ.

In addition to being inducible by virus, the majority of the tetrameric hexanucleotides tested were found to be inducible with IFN-γ (Figure 10; γ, column no IRF), albeit at a reduced level. In the presence of the upstream enhancer, all of the hexanucleotide repeats with the exception of TG_4 and CG_4 displayed inducibility in response to IFN-γ, at levels between >3 and >28 fold. Of the natural IFN promoter segments, only $VRE\alpha1$ was tested and transcription from this element was found to be essentially unaffected in the presence of IFN-γ.

2.2.6   Only CG_4 displays constitutive activity.

With the exception of CG_4, all of the tetrameric hexanucleotides tested lack constitutive transcriptional activity, both in the presence and absence of the SV40 enhancer (Figure 10 and 9; 0, column no IRF). Constitutive transcription mediated by the CG_4 oligonucleotide in the absence of the upstream SV40 enhancer is marginally (>2 fold) above the background levels; the presence of the enhancer stimulated transcription 9 fold.

Although the increase observed in transcription in the presence of the enhancer can be attributed, in part, to the 2 fold stimulation obtained when the SV40 enhancer is 1300 bp upstream of the minimal promoter, (Kuhl et al., 1987), this does not completely account for the activation. It is possible that some part of this increase is due to enhancer coupling, as discussed in Section 2.2.4.

2.2.7 GT_4, GC_4, CT_4, CC_4, and GG_4 all mediate activation by IRF-1.

Taniguchi and co-workers identified and cloned the cDNA encoding Interferon Regulatory Factor 1 (IRF-1), the level of which is increased after virus induction and which has been implicated as having a role in the induction of the IFN genes (Fujita et al., 1988; Miyamoto et al., 1988). Introduction of an IRF-1 expression plasmid by transient transfection into cos cells leads to the expression of the previously silent endogenous IFN-α and β genes, in the absence of viral stimulation (Fujita et al., 1989a).

IRF-1 binds to $(AAGTGA)_4$ (a permutation of GT_4; Fujita et al., 1988) and it was of course of great interest to determine which, if any, of the virus inducible $(GAAANN)_4$ elements could be activated by IRF-1. To this end, an expression plasmid containing the IRF-1 gene under the control of the CMV promoter/enhancer (pIRF-L; Miyamoto et al., 1988) was co-transfected with a reporter plasmid containing a tetrameric oligonucleotide preceded by the SV40 enhancer at a distance.

Cotransfection with pIRF-L stimulated the transcription of GT_4, GC_4, CT_4 and CC_4 over 20 fold, to levels approximately 3 to 5 times lower than those obtained by virus induction alone (Figure 10; 0, column with IRF). Of the remaining tetrameric hexanucleotides tested, only GG_4 showed any significant activation by IRF-1 (>9 fold). CG_4 and TG_4 were unaffected by cotransfection with pIRF-L, although both were efficiently induced by virus.

Cotransfection with IRF-1 stimulated transcription from VREβ only 2.7 fold and VREα1 not at all. The failure of pIRF-L to transactivate VREα1 was surprising in view of the findings of Fujita et al. (1989a) that both the endogenous IFN-α and -β genes were activated by the transfection of an IRF-1 expression plasmid into cos cells. The apparent discrepancy between these results may be the consequence of pIRF-L replicating to a high copy number in cos cells (which contain the SV40 T antigen) but not in L929 cells (which lack the SV40 T antigen; Lusky and Botchan, 1981). Amplification of the IRF-1 expression plasmid in cos cells could result in more efficient expression of IRF-1, such that it reaches a level sufficient to activate transcription.

Cotransfection of L929 cells with pIRF-L and plasmids containing GT_4, GC_4, CT_4 or CC_4, followed by virus induction (Figure 10; V, column with IRF) led to a 1.5 to 2 fold stimulation of transcription above that obtained following virus induction alone (Figure 10; V, column no IRF). Virus induction of cells cotransfected with pIRF-L and plasmids containing either CG_4 and TG_4, (Figure 10; V, column with IRF) gave transcript levels equal to or lower than those obtained with NDV induction alone (Figure 10; V, column no IRF).

The results obtained in the absence of an enhancer were in general agreement with those obtained in the presence of an enhancer, although as expected the transcription levels were lower (Figure 9; V, column with IRF). However, for GT_4, IRF-1 stimulation of transcription was greater than that obtained after induction by virus. In the absence of an enhancer, transcript levels derived from the GT_4 element were 1.25 fold higher after IRF-1 transactivation (Figure 9; 0, column with IRF) than after virus induction (Figure 9; V, column no IRF). This was in contrast to the finding when the SV40 enhancer was present, in which case 3 times more β-globin transcripts were detected after induction by virus (Figure 10; V, column no IRF) than after transactivation by IRF-1 (Figure 10; 0, column with IRF). Unlike GT_4, all other oligonucleotides tested exhibited higher levels of transcription after induction by virus (Figure 9; V, column no IRF) than after transactivation by IRF-1 (Figure 9; 0, column with IRF).

When pIRF-L and GT_4 plasmids were cotransfected and the cells treated with virus (Figure 9; V, column with IRF), stimulation of transcription was 3.5 fold higher than with virus induction alone and resulted in a transcript level higher than the sum of the transcripts obtained after induction by virus (Figure 9; V, column no IRF) and transactivation by IRF-1 (Figure 9; 0, column with IRF). Similar, though less pronounced, IRF-1-mediated stimulation of virus-induced transcription was seen with GC_4, CT_4, CC_4 and GG_4 both in the presence and absence of the enhancer (when tested).

The apparent synergistic stimulation of virus-induced transcription mediated by cotransfection of pIRF-L can probably be attributed to increased expression of IRF-1, brought about by virtue of the virus inducibility of the CMV promoter/enhancer (Kuhl et al., 1987) which directs expression of pIRF-L.

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Thus, it appears that while all of the NN_4 elements tested and both VREα1 and VREβ are virus-inducible, only some are activated by IRF-1 (GT_4, GC_4, CT_4, CC_4, GG_4 and VREβ). This indicates that a pathway(s) other than that involving IRF-1 may be involved in the viral induction of elements not activated by IRF-1 (TG_4, CG_4 and VREα1). Thus, the observation that VREβ is weakly stimulated in the presence of IRF-1 while the transcriptional activity of VREα1 is unaffected indicates that these two genes may be induced by different pathways.

2.2.8   Tetrameric oligonucleotides mediating inducibility by virus are of distinct types.

The $(GAAANN)_4$ elements were categorized into three groups based on the effect they had on transcription:

Type I oligonucleotides; Oligonucleotides of this type show no constitutive expression, are strongly inducible by virus, display a moderate induction by IFN-γ and IRF-1. This group includes GT_4, GC_4, CT_4 and CC_4 (see Table 2)

Type II oligonucleotides; The sole representative of this group identified to date is TG_4, which differs from those of type I by the criterion that it fails to respond to both IFN-γ and IRF-1. As type I, the type II oligonucleotide displays no constitutive expression and is inducible by virus.

Type III oligonucleotides; The distinguishing characteristic of type III oligonucleotides, which are represented by CG_4, is their high constitutive expression. Type III oligonucleotides are moderately inducible by virus but display no inducibility by IFN-γ or IRF-1.

The behavior of GG_4 was found to be intermediate, in that in some experiments it displayed weak constitutive transcription, a characteristic associated with type III oligonucleotides, while at the same time was inducible by IFN-γ and IRF-1, indicating that it shared properties with type I oligonucleotides.

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	constitutive activity	virus induction	IFN induction	IRF-1 response
Type I				
(GAAAGT)$_4$	−	+	+	+
(GAAAGC)$_4$	−	+	+	+
(GAAACC)$_4$	−	+	+	+
(GAAACT)$_4$	−	+	+	+
Type II				
(GAAATG)$_4$	−	+	−	−
Type III				
(GAAACG)$_4$	+	+	−	−
Type I/Type III				
(GAAAGG)$_4$	+/−	+	+	+
Virus Responsive Elements				
VREα1	−	+	−	−
VREβ	−	+	−	(+)

Table 2   Properties of tetrameric hexanucleotides of the type (GAAANN)$_4$ and of virus responsive elements.

2.3   Interactions between nuclear proteins and the (GAAAGT)$_4$ and VREα1 motifs.

The binding of nuclear factors from virus-induced and uninduced Namalwa cells to GT$_4$ and VREα1 was studied using gel retardation analysis (Fried and Crothers, 1981; Garner and Revzin, 1981). To determine the specificity of the protein-oligonucleotide interactions, competition experiments were performed by mixing labeled oligonucleotide with various amounts of an unlabeled one prior to incubation with nuclear extract. The resulting complexes were resolved by electrophoresis through non-denaturing polyacrylamide gels and visualized by autoradiography.

2.3.1   Evidence for a common factor(s) binding to type I tetrameric oligonucleotides.

Incubation of 5'-^{32}P-labeled GT$_4$ with uninduced Namalwa nuclear extract resulted in the formation of 3 retarded complexes (Figure 12). Competition with an excess of unlabeled competitor oligonucleotide revealed that only type I oligonucleotides (namely GT$_4$, GC$_4$, CT$_4$ and CC$_4$) efficiently displaced the 3 retarded bands. At a 25 fold molar excess of the type I oligonucleotides a reduction of approximately 50% in the intensities of the retarded bands was obtained (as judged by inspection of the autoradiogram) and at a 625 fold molar excess the retarded bands had essentially disappeared (Figure 12).

Of the other oligonucleotides tested, only VREβ competed significantly. Approximately a 5 fold higher molar excess of VREβ competitor was required to compete the GT$_4$ band shift to an equivalent degree as of the type I oligonucleotide competitors (Figure 12). None of the other oligonucleotides tested (VREα1, CG$_4$, GG$_4$, TG$_4$ or (GACAGT)$_4$) competed the GT$_4$ band shift efficiently. Generally, levels of competition at least 100 times higher than those required with type I oligonucleotides were needed to obtain similar levels of competition (Figure 12).

In conclusion, these findings imply that the type I oligonucleotides bind one or more common factor(s) with more or less equal affinity. With the exception of VREβ, which has a weak affinity for these factors, none of the other elements tested, in particular VREα1, appeared to specifically bind
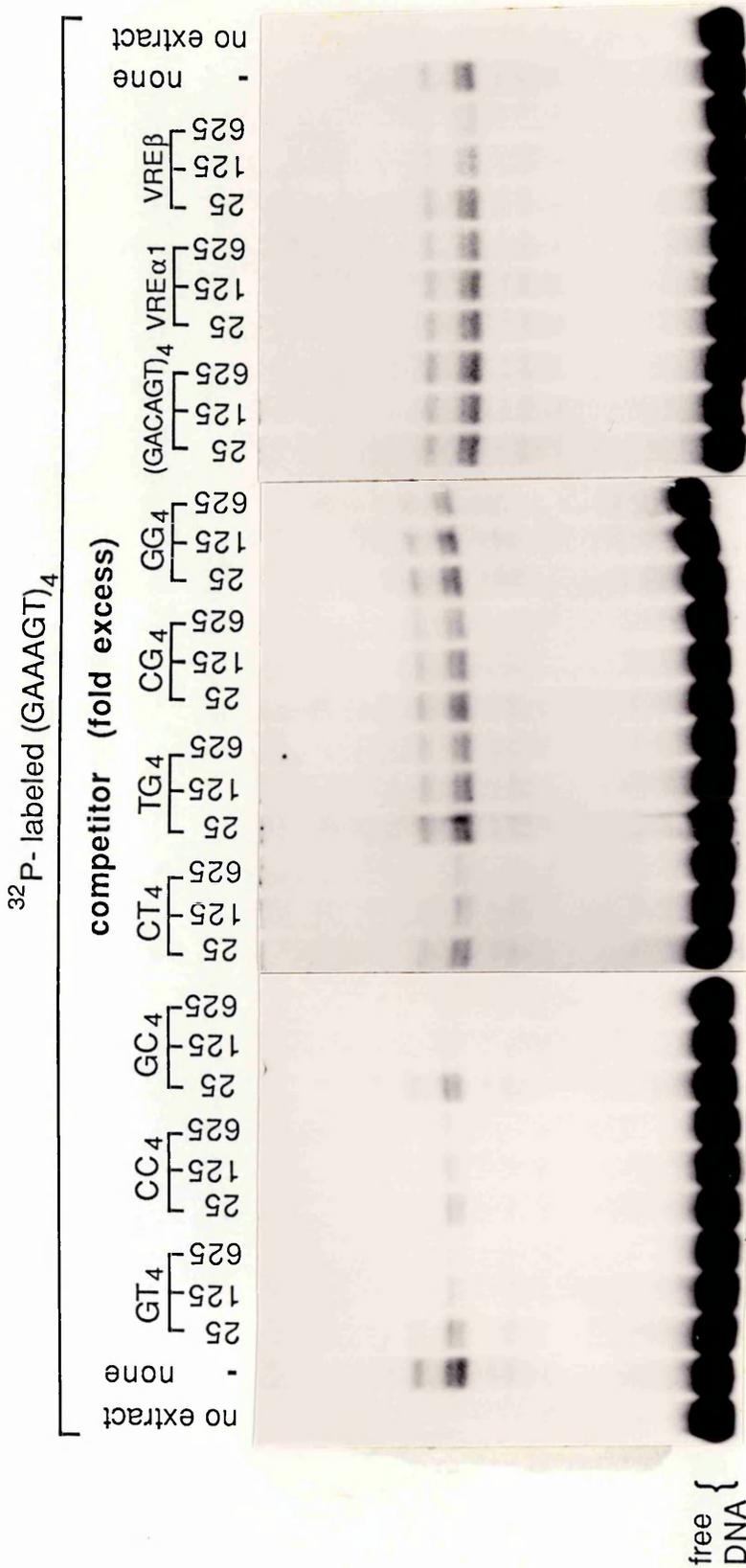
Figure 12   A common factor(s) binds to all type I tetrameric oligonucleotides and VREβ but not to types II, III or VREα1.

Autoradiogram of gel retardation competition experiment using labeled GT₄ after 48 hours at -70 °C with an intensifying screen.

Gel retardation experiments were performed by incubating 5'-^{32}P-labeled GT₄ (3.75 fmol) with 2 μg calf thymus DNA as a non specific competitor and nuclear extract (6 μg protein) from uninduced Namalwa cells, and analyzed as described in the Experimental Procedures. In the competition experiments, the 5'-^{32}P-labeled GT₄ was mixed with the indicated molar excess of unlabeled oligonucleotide prior to the addition of nuclear extract.

the type I oligonucleotide binding factor(s). It is not known which, if any of the GT_4 retarded bands detected using Namalwa nuclear extract corresponds to IRF-1 or -2.

2.3.2   VREα1 binding factors have affinity for TG_4 but for none of the other NN_4 elements.

At least 3 DNA/protein complexes were obtained when ^{32}P-labeled VREα1 was incubated with uninduced Namalwa extract (Figure 13). Competition experiments similar to those done with GT_4 were performed to ascertain which if any of the factors binding to VREα1 also had affinity for other oligonucleotides. Self competition in the VREα1 band shift required a substantially higher molar excess than did self competition in the GT_4 band shift. At a 625 fold molar excess of VREα1, approximately 50% competition of all the VREα1 bands was obtained (Figure 13), a level of competition achieved at a 25 fold molar excess of GT_4 in the GT_4 band shift (Figure 12). The high levels of VREα1 required to compete the VREα1-retarded bands perhaps reflects the abundance of the factor(s) involved in these interactions or that it the factor(s) displays a low affinity for VREα1 (relative to the GT_4 binding factors to GT_4).

TG_4 and VREβ competed all 3 of the VREα1 retarded bands, but at varying efficiencies (Figure 13). The fastest migrating of the 3 retarded bands ('TG protein'; see Section 2.3.4) was competed about 2 to 5 and 50 fold less well by TG_4 and VREβ respectively, than by an equivalent amount of VREα1 (for confirmation of this result see Section 2.3.4). In contrast, the intermediate band was competed better by VREβ than TG_4, requiring about 5 times more VREβ and 10 times more TG_4 to obtain the same effect as VREα1. TG_4 competed the slowest migrating band with a similar efficiency as VREα1; VREβ on the other hand displayed approximately a 5 fold lower efficiency.

Almost no competition of the 3 VREα1 retarded bands was observed with any of the other NN_4 elements, except for a general reduction in the intensities of the bands observed with all the tetrameric hexanucleotides at a 15 625 fold molar excess (Figure 13).

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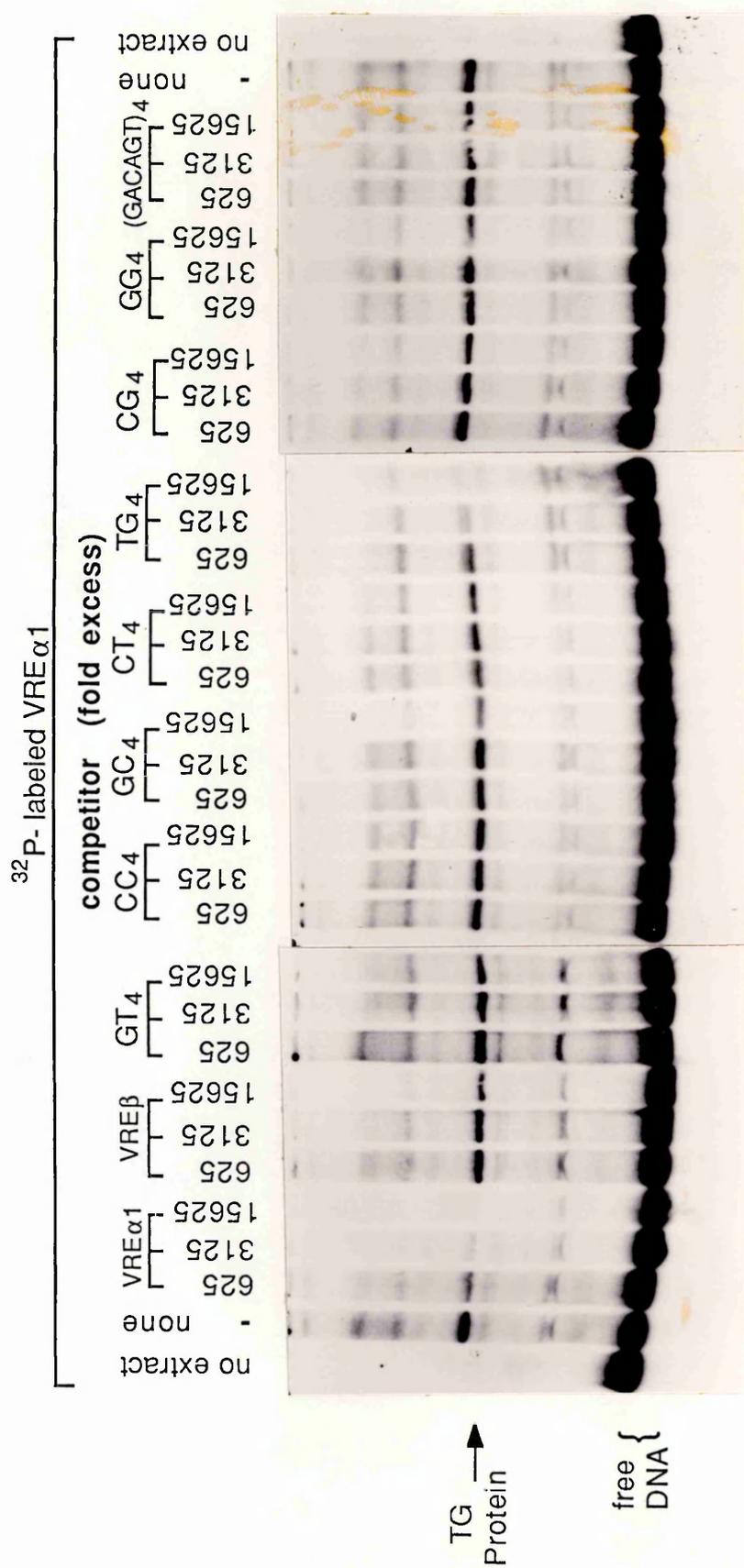
Figure 13 VREα1 binding factors show affinity for TG₄, but for none.of the other tetrameric hexanucleotides.

Autoradiogram of gel retardation competition experiment using 5'-32P-labeled VREα1 after 32 hours at -70°C with an intensifying screen. Gel retardation experiments were performed as described in Figure 12, except that 5'-32P-labeled VREα1 (3.75 fmol) was incubated with 0.5 µg of calf thymus DNA as non specific carrier.

It appears from these results that perhaps as many as 3 different factors are capable of binding to the VREα1, 2 of which (the proteins involved in the formation of the fastest ('TG protein'; see section 2.3.4) and slowest migrating complexes) show a similar affinity for TG_4 and VREα1, a weaker affinity for VREβ but no affinity for any of the other oligonucleotides. The third factor (involved in the formation of the complex with intermediate mobility) shows a roughly equal, though weak affinity for TG_4 and VREβ.

2.3.3 Tetrameric oligonucleotides of Type I, II and III give distinct gel retardation patterns.

Subsequent gel retardation analysis performed by P. Gallant (1899) showed that the other type I oligonucleotides GC_4, CT_4 and CC_4 all generated the same pattern of 3 bands as GT_4 (see Figure 12). The type II oligonucleotide TG_4 gave a major band which did not line up with any given by the other NN_4 oligonucleotides, and two weaker bands. Type III oligonucleotide CG_4 also generated 3 bands, of which only one had the same mobility as seen in the type I pattern. GG_4 gave a pattern similar to that of CG_4.

Gel retardation competition experiments were performed (by P. Gallant) with various unlabeled oligonucleotides and the radioactivities in selected bands were quantitated (Gallant, 1989). In summary: (1) the three bands generated by any type I oligonucleotide were displaced efficiently by any member of the same type. Other oligonucleotides, such as CG_4, GG_4, TG_4, AA_4, or the 'mutant' $(GACAGT)_4$ competed only at 100 to 1000 fold higher levels; VREβ was a poor and VREα1 a very poor competitor. (2) With labeled type II oligonucleotide TG_4, the bands were competed efficiently by TG_4 and VREα1 but not by any other tetrameric oligonucleotide. (3) Type III oligonucleotide CG_4 was competed 30-100 fold less well by GG_4 than by itself, even worse by TG_4 and not by any other oligonucleotide. (4) GG_4 gave a pattern similar to that of CG_4, was competed efficiently by CG_4, less well by TG_4 and not by any other oligonucleotides. Thus, the 3 types of oligonucleotides mediating virus inducibility formed different, characteristic complexes.

2.3.4 Type II oligonucleotide TG$_4$ and VREα1 interact with a presumably novel 'TG protein'.

Further analysis of proteins interacting with TG$_4$, performed by P. Gallant and D. Maguire, showed that of the 3 retarded bands one is due to the ubiquitous octamer factor, OTF-1 (see discussion) while another was competed by all competitor DNA oligonucleotides tested and was thus considered to be unspecific. The retarded band with highest mobility was competed by TG$_4$ and VREα1 only. The protein giving rise to this interaction was designated 'TG protein'.

Using labeled VREα1 and partially purified TG protein (from A. Sailer), a band shift competition experiment was performed to determine the specificity of TG protein binding to VREα1 (Figure 14). TG protein binding was competed efficiently by VREα1, about 1.8 times less well by TG$_4$ and 30 and >50 times less well by VREβ and GT$_4$, respectively. Thus TG protein binds specifically to VREα1 and TG$_4$ which share the sequence GAAATGGAAA (positions -75 to -84 of IFN-α1 promoter)

In order to determine whether the TG protein forms similar contacts to sequences within TG$_4$ and VREα1, methylation interference experiments were performed (in collaboration with D. Maguire). Methylation interference is based on the fact that methylated guanine or adenine residues can interfere with the binding of a protein to its binding site (Siebenlist and Gilbert, 1980). Thus, a comparison of the distribution of methylated residues in DNA following separation of free and bound DNA after gel retardation analysis indicates which guanine and adenine residues cannot be modified without interfering with the formation of a DNA/protein complex.

^{32}P-labeled TG$_4$ and VREα1 were partially methylated and used in a scaled-up binding reaction with partially purified TG protein. A 625-fold excess of unlabeled GT$_4$ (shown previously to be sufficient to compete binding to residual IRF-1 or IRF-2 by more than 90%) was added. The DNA in the protein-DNA complex (bound) and the free DNA were recovered after gel electrophoresis, a G>A cleavage reaction was performed, and the product analyzed on a denaturing polyacrylamide gel.

Figure 15 shows that the methylation of neighboring guanine residues (doubly underlined) in the sequence common to both oligonucleotides,

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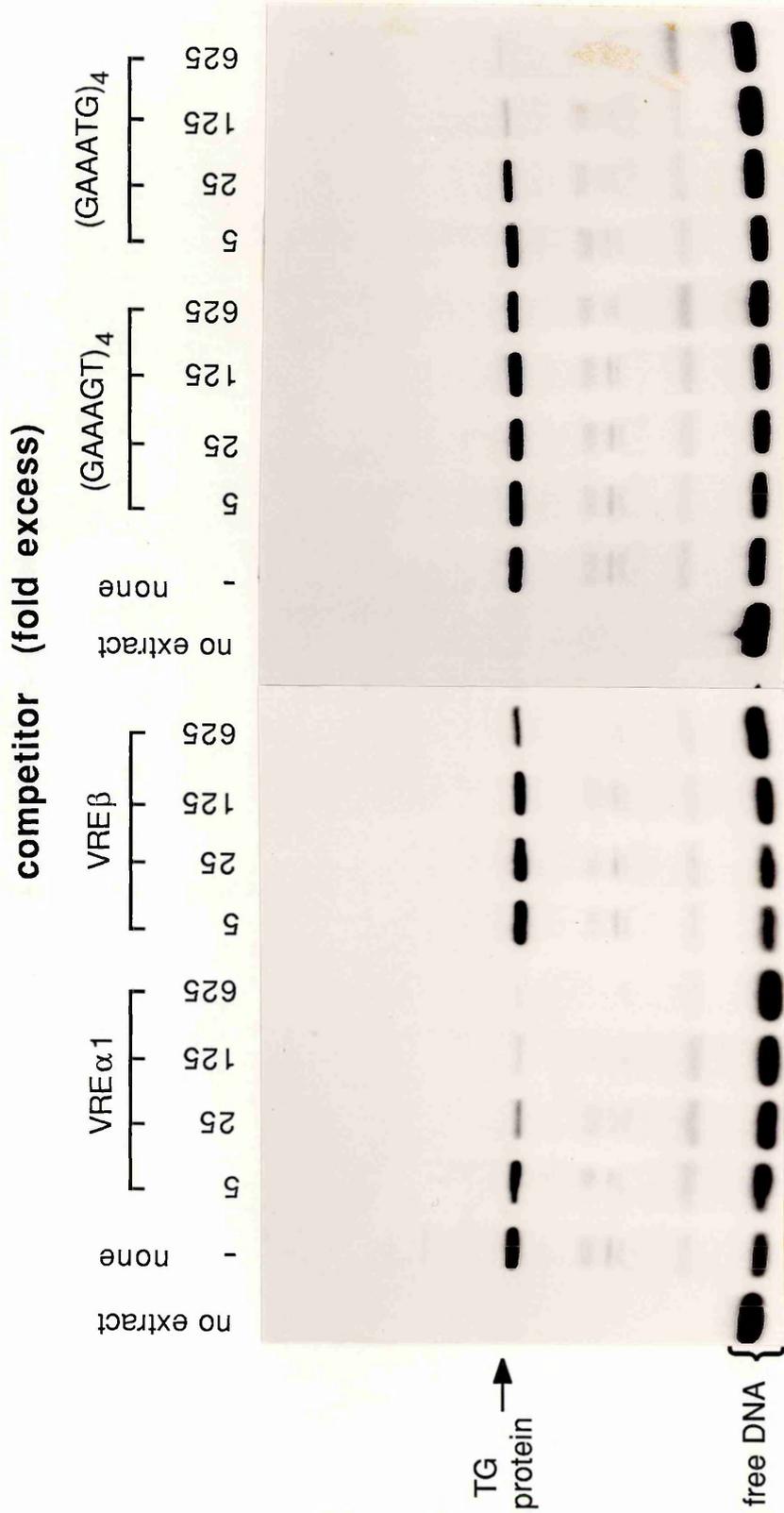
Figure 14 Gel retardation competition: TG protein binds specifically to VREα1 and TG₄ but not VREβ or GT₄.

Autoradiogram of gel retardation competition experiment using 5'-³²P-labeled VREα1 after 8 hours at -70°C with an intensifying screen. Gel retardation experiments were performed as described in Figure 12, except that 5'-³²P-labeled VREα1 (3.75 fmol) was incubated with 80 ng of poly dI-dC as non specific carrier DNA and 0.5 µl partially purified (step I) TG protein and analyzed as described in Experimental Procedures. The 5'-³²P-labeled VREα1 was mixed with the indicated molar excess of unlabeled oligonucleotide prior to the addition of TG protein. For quantitation, the radioactivity in the retarded band was determined by Cerenkov counting bands cut from the dried gel. The 100% value was 1250 cpm.
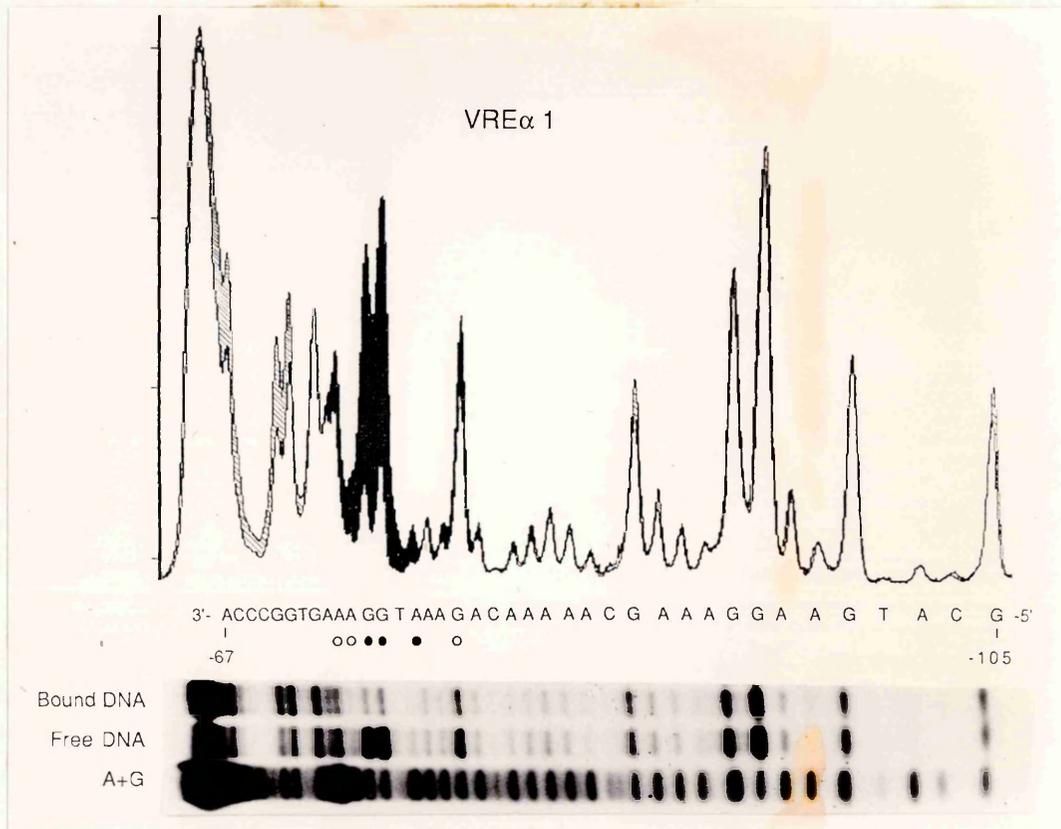
Figure 15A TG protein makes similar contacts with the TG sequence of VREα1 and TG$_4$.

The methylated, labeled oligonucleotides were incubated with partially purified TG protein (TG$_4$ step I and VREα1 with step II preparation; see Experimental Procedures) in the presence of a 625 fold excess of unlabeled GT$_4$, and bound and free oligonucleotides isolated after separation by gel retardation. After alkaline cleavage, the products were separated on sequencing gels and visualized by autoradiography. The lanes were scanned with a Molecular Dynamics 300A Computing Densitometer and the profiles pertaining to free and bound products superimposed. The black areas correspond to regions where the bands of free product are more intense than those of the bound product and indicate interference by methylation; the hatched areas show where the bound product had stronger bands than the free product.

(A) Autoradiogram and densitometric scanning of methylation interference performed with 5'-^{32}P-labeled VREα1.
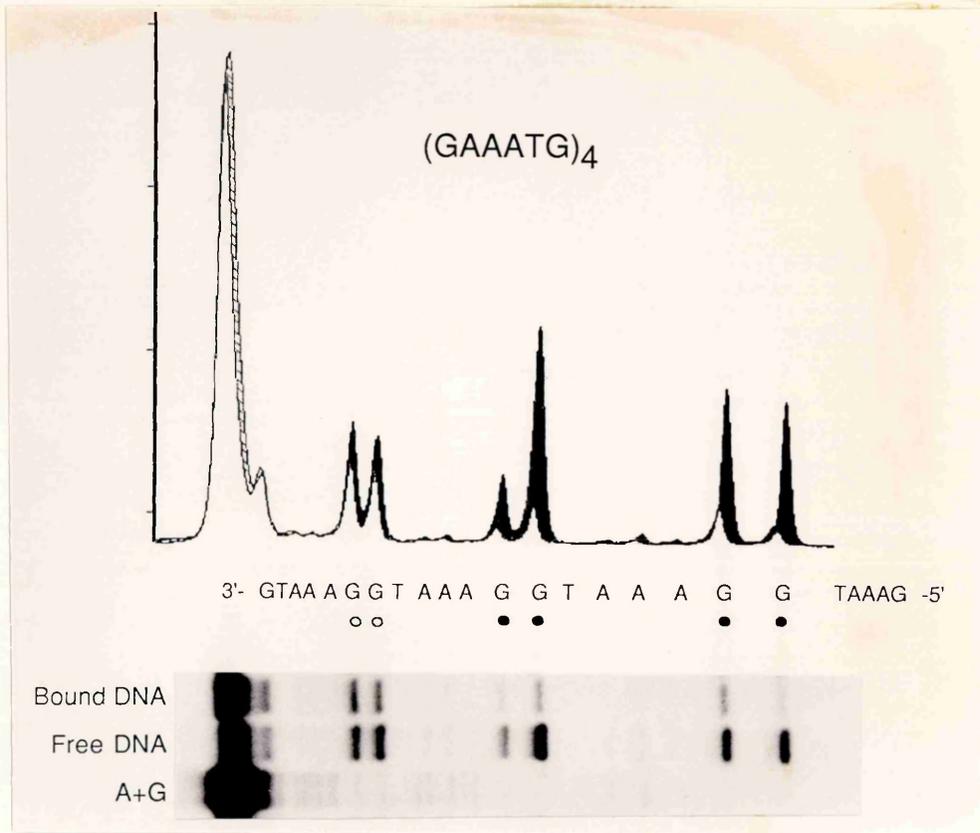
Figure 15B   TG protein makes similar contacts with the TG sequence of VREα1 and TG$_4$.

Autoradiogram and densitometric scanning of methylation interference performed with 5'-^{32}P-labeled TG$_4$. Experimental procedures as in Figure 15A.

...$\underset{\sim}{G}$AAAT$\underset{\approx}{GG}$AAA..., strongly interferes with the binding of the TG protein. In both cases weaker interference with binding was also observed when the preceding G was methylated (wavy underline). Consequently, it appears that the TG protein makes similar interactions with the sequence common to both TG$_4$ and VREα1.

2.3.5 Extracts prepared from virus-induced cells give the same band shift pattern as those prepared from uninduced cells.

Nuclear extracts prepared from Namalwa cells 15 min, 45 min, 2.25 hr, 4.5 hr or 9.5 hr after induction with NDV gave essentially the same pattern of retarded bands with either GT$_4$ or VREα1 as extracts derived from uninduced Namalwa cells (data not shown).

2.4 Determination of the sequences required for virus induction of the IFN-α1 promoter in Namalwa cells.

The inability to detect differences between induced and uninduced Namalwa cell nuclear extracts in the band shift assay was somewhat unexpected in view of the observation of specific, inducible complexes with the IFN-β regulatory elements (Keller and Maniatis, 1988). A possible explanation for this was that perhaps the elements of the human IFN-α1 gene identified as being important for virus induction in murine L929 cells (Ragg and Weissmann, 1983; Ryals et al., 1985; Kuhl et al., 1987) are not necessarily the same as those required for the virus induction of the IFN-α1 gene in Namalwa cells. To address this question, Namalwa cells were transiently transfected with β-globin expression plasmids into which had been inserted various IFN-α1 promoter fragments (see Figure 16C).

Namalwa cells were transiently transfected with a mixture of test and reference plasmids. The reference plasmid contained the β-globin gene under the control of VREα1 with a small substitution in the first exon which gives rise to imperfect hybridization between the probe and reference RNA and thus results in the correspondingly shorter S1 digestion product of 218 nt (see Figure 1D in Kuhl et al., 1987). 48 hours later the cells were

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either NDV or mock induced and total RNA was isolated after a further 8 hours. Test and reference transcripts were assayed by S1 analysis as in Section 2.1.1. Test signals were normalized relative to the corresponding reference signals, to compensate for variations in the efficiencies of transfection, induction and RNA recovery.

2.4.1   VREα1 mediates inducibility by virus in Namalwa cells.

A single copy of VREα1, when joined to position -56 of the β-globin gene conferred weak virus inducibility (>4.4 fold, 11 strands per cell; Figure 16) upon the β-globin reporter gene in Namalwa cells. Dimerization of the VREα1 dramatically increased the inducibility to >94 fold (235 strands per cell; Figure 16). Longer IFN-α1 promoter fragments (-141 to -41, -22 or -6 and -675 to -6) were all found to be less active than the a single copy of the VREα1, giving inducibilities of between >3.2 and >2.2 fold and 5 between 8 strands per cell (Figure 16).

These results observed with Namalwa cells were almost identical to those previously obtained in a similar assay system using murine L929 cells (Kuhl et al., 1987; Büeler, 1988; Näf, 1989), suggesting that the mechanisms that govern virus inducibility in L929 cells are similar to those in Namalwa cells.

A promoter consisting of GT_4 supported by the SV40 enhancer 28 bp upstream (enhancer close-by) was also inducible in Namalwa cells and gave the highest level of induced transcripts of any of the constructs tested (350 strands per cell), 1.5 times more than dimerized VREα1 (Figure 16). However, because of the high constitutive expression of the GT_4 construct due to the close-by enhancer (60 strands per cell), the level of inducibility was lower (Figure 16).

The only discrepancy between the results obtained in Namalwa and L929 cells was seen with the GT_4 construct, which in L929 cells was silent in the absence of viral induction (<2.5 strands per cell; Näf, 1989 [1]), but in Namalwa cells showed strong constitutive activity. The constitutive activity of the GT_4 construct in Namalwa cells may be due to the proximity of the

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[1]   The number of strands per cell calculated in Näf (1989) was over-estimated by a factor of 2 and this has been corrected for in the values quoted in this work.
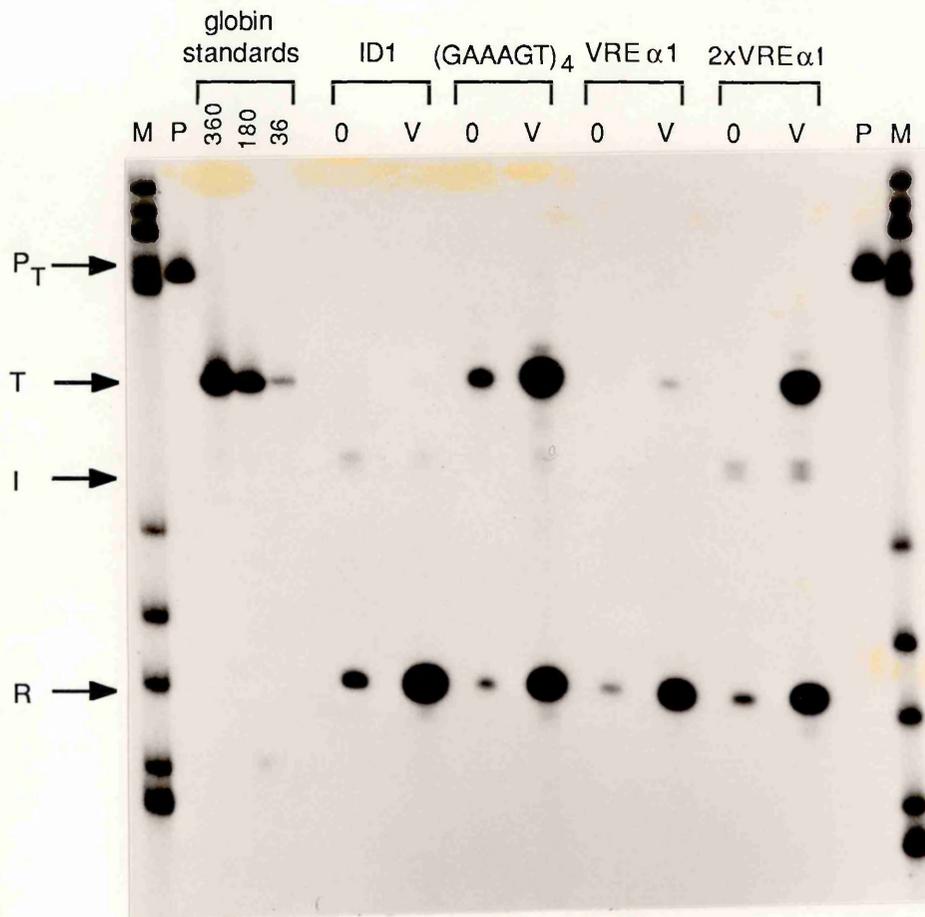
Figure 16A   VREα1 mediates inducibility by virus in
                   Namalwa cells.

(A)-(B)   Autoradiogram of S1 mapping after 38 hours at -70 °C with an intensifying screen.
Namalwa cells were transfected with 2.5 µg of each of the test and reference plasmids and
48 hours later either mock or NDV induced. Total RNA was recovered after a further 8 hours
and 50 µg were S1 mapped. Abbreviations as in Figure 9A; GT_4, plasmid 61P; VREα1;
plasmid 12P; 2xVREα1, plasmid 13P. Schematic representation of the plasmids used in
this experiment and a summary of the results can be found in Figure 16C.
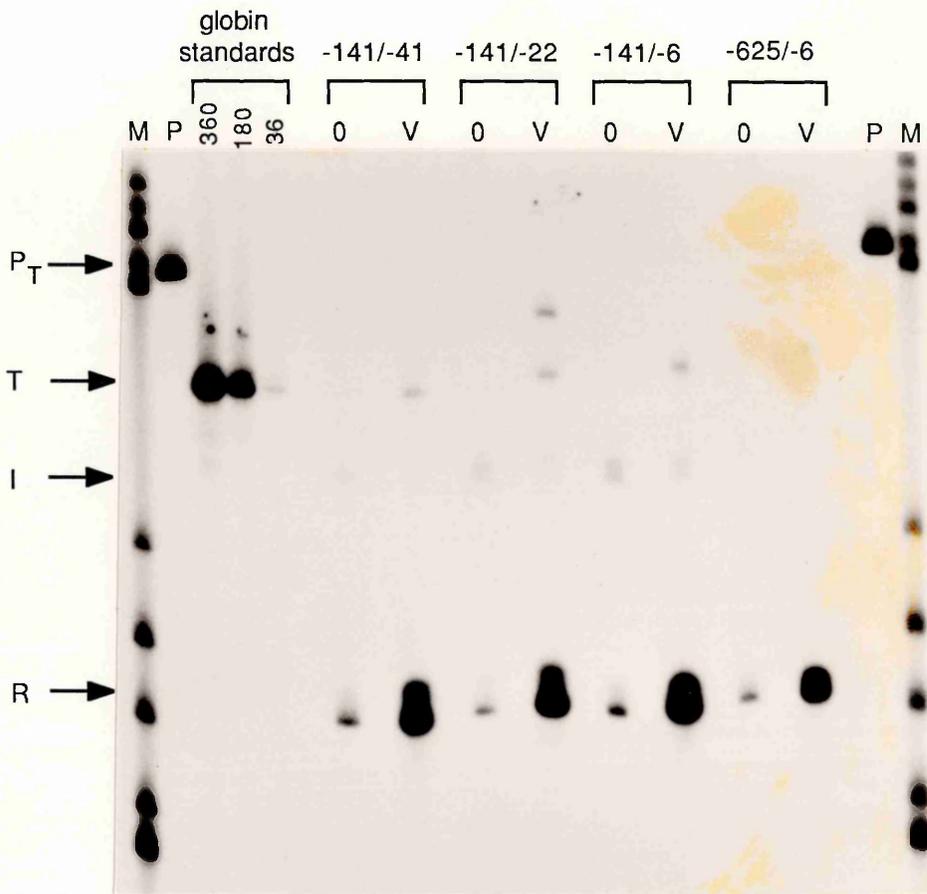
Figure 16B   VREα1 mediates inducibility by virus in
Namalwa cells.

Autoradiogram of S1 mapping after 38 hours at -70 °C with an intensifying screen.
Experimental procedures as in Figure 16A. Abbreviations as in Figure 9A; -141/-41, plasmid
CF2/42P; -141/-22; plasmid CF1/42P; -141/-6, plasmid RβGα-141/-6; -625/-6, plasmid pIG.
Schematic representation of the plasmids used in this experiment and a summary of the
results can be found in Figure 16C.

TRANSCRIPT LEVEL
(strands per cell)

	Uninduced	Induced	Inducibility

ID1

-135 -56

< 2.5   < 2.5   —

61P

GT$_4$

60   350   5.8 fold

12P

-109 -64

VREα1

< 2.5   11   >4.4 fold

13P

2x VRE α1

< 2.5   235   >94 fold

CF2/42P

-141 -41

< 2.5   6.5   >2.6 fold

CF1/42P

-141 -22

< 2.5   8   >3.2 fold

R βG α-141/-6

-141 -6

< 2.5   5   >2 fold

pIG

-675 -6

< 2.5   7   >2.8 fold

GT$_4$

TATA   Exon 1   Exon 2   Exon3

pBR   SV40   (GAAAGT)$_4$ VREα1   IFN-α1
    enhancer   promoter

Rabbit β- Globin gene
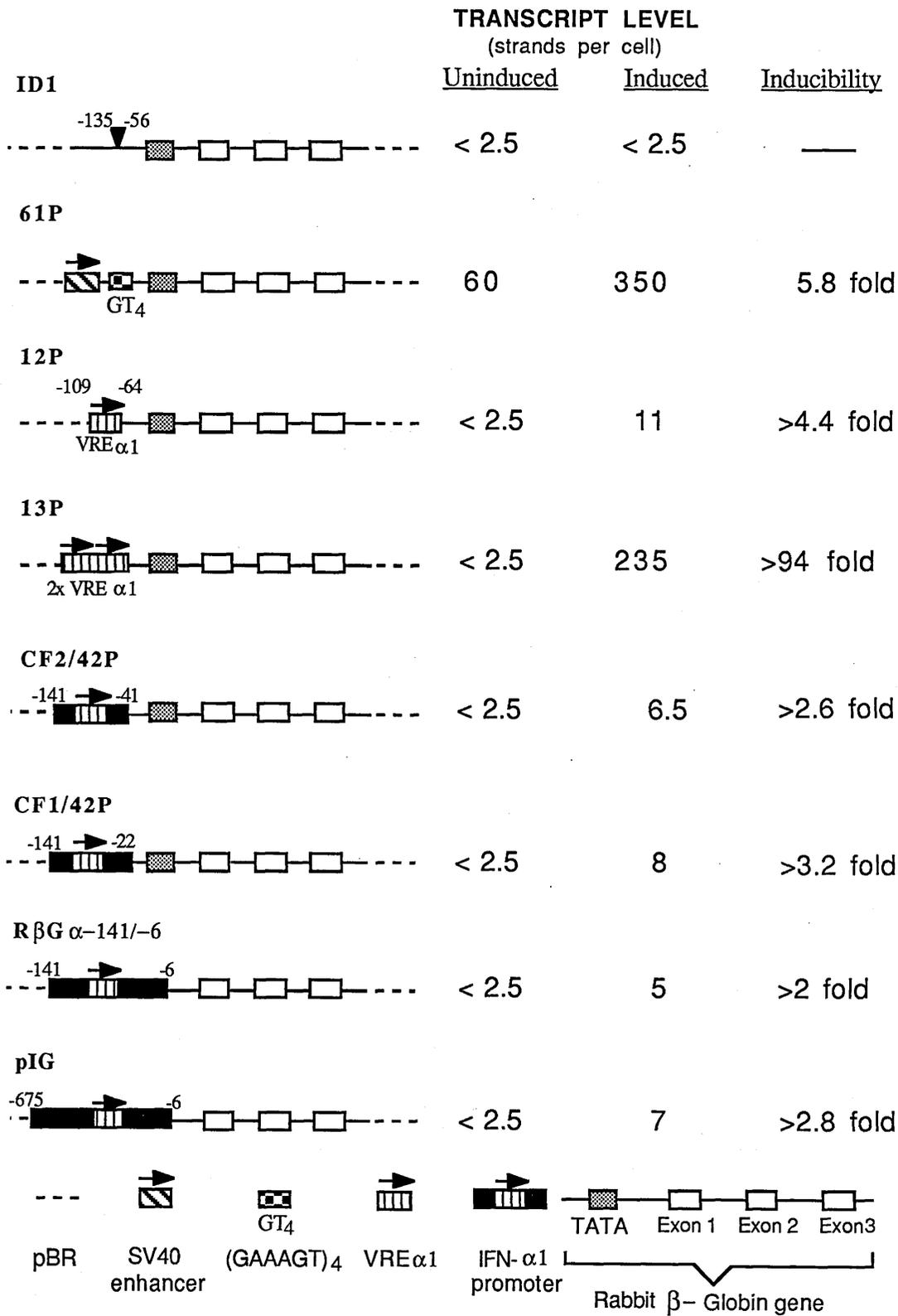
Figure 16C   VREα1 mediates inducibility by virus in
Namalwa cells.

Summary of the S1 mapping experiments depicted in Figures 16A-B. Transcript levels were calculated as described in Kuhl et al. (1987). Experimental procedures as in Figure 16A.

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enhancer to the TATA box. A level of 45 strands per cell was found with H-ES in L929 cells (Näf, 1989 [1]), a plasmid in which the GT_4 element has been replaced with an inert spacer. In L929 cells, the GT_4 element reduces the level of constitutive transcription by >20 fold (Näf, 1989) as a consequence of reversible suppression of the constitutive activity of the SV40 enhancer ('silencing'; Kuhl et al., 1987).

The differences in the constitutive activity displayed by GT_4 with the enhancer close by in Namalwa and L929 cells could be due to either the absence of silencing in Namalwa cells, an increased activity of the SV40 enhancer when placed 82 bp upstream of the of the TATA box in Namalwa cells as compared to L929 cells or a combination of both. Since the effect the SV40 enhancer has on constitutive transcription in Namalwa cells when in this position is not known, it is not possible to differentiate between these two possibilities.

3. DISCUSSION.

The IFN genes provide a particularly interesting, if complex model for the study of the regulation of transcription, being expressed in an inducible and tissue-specific manner. In addition, the individual IFN gene transcripts are subject to differential rather than coordinate regulation.

3.1 Control of the differential expression of the IFN genes.

Hiscott et al. (1984a, 1984b) suggested that the differences in the levels of the individual IFN transcripts in different cell types could be a result of a number of mechanisms: (1) there could be several pathways of induction, one of which would be prevalent in a particular cell type, (2) there could be cell specific differences in the degradation rate or translation of individual IFN mRNAs or (3) the induction of the individual IFN genes could as a consequence of tissue-specificity or cellular differentiation be different in different cell types.

3.1.1 Is the differential expression of the IFN genes the result of several alternative pathways?

The existence of several pathways, each of which results in the induction of a different set of the IFN genes, could explain the differential expression of the IFN genes. As different induction protocols do not significantly affect the proportion of IFN mRNA produced by a particular cell line (Hiscott et al., 1984a), the existence of several pathways seems unlikely.

3.1.2 The role of messenger stability and translational efficiency in the differential expression of the IFNs.

Messenger stability and the rate of translation have been implicated in influencing the production of active IFN-β following induction (see Section 1.13). The observation that that IFN-β production does not always correlate with mRNA levels indicates that translation may contribute to the differential synthesis of the IFNs (Hiscott et al., 1984a).

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Although messenger stability and the efficiency of translation may contribute in part to the level of IFN-β synthesis in the cell, there is no evidence that similar mechanisms are involved in the differential expression of the α-IFNs (see Section 1.10). Consequently, it seems unlikely that such mechanisms can be used to explain the differential synthesis of the α-IFN genes.

3.1.3   What role does development play in the differential expression of the IFN genes?

The differential expression of the IFN genes may be explained if in different cell types the individual IFN genes are more or less susceptible to induction as a consequence of developmental control. The effect of differentiation on the regulation of the IFN genes has been demonstrated by Hiscott et al. (1984b) and Streuli (1986) who, using leukemic leukocytes arrested at various stages of hematopoietic differentiation, showed there to be remarkable differences in the proportion of the individual IFN mRNAs produced (see Section 1.10). Further evidence of the effect of differentiation on IFN gene expression comes from studying the differentiation of pluripotential embryonic carcinoma (EC) cells. EC cells do not produce IFN in response to induction with either virus or poly rI-rC, nor are they sensitive to its effects (Burke et al., 1978). However, upon differentiation, the EC cells acquire the ability to both produce and respond to IFN (Burke et al., 1978; Francis and Lehman, 1989).

Differentiation of cells is accompanied by a variety of epigenetic changes which include alterations in the pattern and level of DNA methylation and in the overall chromatin structure. Such changes in genome structure are known to influence gene expression (for review see Gross and Garrard, 1988) and may directly or indirectly explain the tissue-specific expression patterns of the IFN genes.

a)   Methylation of DNA.

Methylation of the genome is widespread in vertebrates where 60-90% of CpGs are methylated in a pattern inherited from one generation to

another in a tissue and species-specific manner (Waalwijk and Flavell, 1978; Mandel and Chambon, 1979; McGhee and Ginder, 1979). Evidence from the study of a large number of individual tissue-specific genes revealed a correlation between the expression of these genes with the undermethylation of their sequences (Mandel and Chambon, 1979; Shen and Maniatis, 1980; Bird et al., 1981; Ott et al., 1982; Grainger et al., 1983). Typically these genes are highly methylated in non-expressing cell types but are considerably undermethylated (particularly in the CpG sites located in the 5'-flanking sequences of these genes) in tissues in which they are expressed (Kruczek and Doerfler, 1982; Busslinger et al., 1983; Bird, 1984). Furthermore, Busslinger et al. (1983) showed that in the case of the human γ-globin gene hypermethylation is sufficient to repress expression. In fibroblasts, the endogenous γ-globin gene is heavily methylated and unexpressed, yet unmethylated copies of this gene are transcriptionally active when introduced into the same cell. Genes methylated in vitro and introduced in the same transfection assay are inactive, illustrating the role that methylation plays in the regulation of some genes.

The availability of cell lines that undergo differentiation in tissue culture allowed the direct examination of whether methylation of the genome was involved in the developmental regulation of gene expression following differentiation. Mouse erythroleukemia cells undergo erythroid differention after exposure to chemical inducers, which results in an induction of globin mRNA (Gambari et al., 1979) and correlates with a decrease in the level of DNA methylation (Christman et al., 1977, 1980).

A more direct correlation between methylation, gene expression and differention was demonstrated by Yisraeli et al. (1986) who showed that a fully methylated skeletal muscle α-actin gene transfected into fibroblasts is inactive, whereas when introduced into myogenic cells it is demethylated and expressed. Tissue-specific repression of gene function as a consequence of de novo methylation has also been observed. Gautsch and Wilson (1983) showed that in undifferentiated EC cells replication of Moloney murine leukemia virus (M-MuLV) is blocked as a result of de novo methylation of the proviral DNA at sites not methylated in virus replicating in differentiated cells. Furthermore, Speers et al., (1980) showed that M-MuLV-infected EC cells could be induced to produce virus following differentiation.

Clearly the pattern of methylation of a particular gene can influence its expression, and differentiation can influence the degree to which a particular gene is methylated. Consequently, methylation could conceivably be involved in the differential control of the IFN genes. In different cell types there may be tissue-specific regulators of methylation with varying affinities for CpGs depending on their flanking sequences and hence may display specificity for a particular set of interferon genes and in so doing influence the pattern of expression of these genes.

b)   Changes in chromatin nuclease hypersensitivity.

Sites of nuclease hypersensitivity present in chromatin are thought to correspond to nucleosome free regions that represent an opening up of specific tracts of the chromatin to allow access of trans-activating factors to important cis-acting DNA sequences (reviewed in Gross and Garrard, 1988). Nuclease hypersensitive sites are usually located in the promoter regions of genes committed to transcriptional activation (Wu, 1980; Lowenhaupt et al., 1983; Vitek and Berger, 1984; Azizkhan et al., 1986; Bentley and Groudine, 1986; Cartwright and Elgin, 1986; Shimada et al., 1986). Changes in the chromatin structure following differentiation have been studied by investigating the susceptibility of sequences surrounding tissue-specific genes to digestion with nuclease and correlating this to gene expression. Such an approach revealed that hypersensitive sites were associated with tissue-specific expression, appearing in a tissue-specific, transcription-dependent manner (Shermoen and Beckendorf, 1982; Crowder and Merlie, 1986; Renaud and Ruiz-Carrillo, 1986; Ayres et al., 1987).

Coverney et al. (1984) studied the changes in the DNaseI hypersensitivity of the IFN-β gene following differentiation and/or induction in EC cells. The appearance of DNaseI hypersensitive sites in the IFN-β gene correlated with its induction in differentiated cells only; in non-differentiated EC no changes were detected following induction. Differentiation of the EC cell failed to elicit any alterations in chromatin structure of the IFN-β gene as judged by DNaseI hypersensitivity. A similar correlation of increased DNaseI sensitivity of this gene following induction was reported by Higashi (1985).

These results appear to correlate the relaxing of the chromatin structure of IFN-β locus with induction (ie. to the activation of transcription) rather than with changes associated with differentiation. It therefore seems rather unlikely that changes in chromatin structure determine which of the IFN genes are in a conformation amenable to viral induction.

An alternative mechanism by which differentiation may influence the expression of the IFN genes could be based on the expression of trans-activating factors that interact directly with the IFN regulatory elements either to activate or repress their expression. Evidence in support of such a mechanism was provided by Haggarty et al. (1988), who found that the pattern of DNA-binding proteins with specificity for the IFN-β promoter different between differentiated and non-differentiated EC cells. In gel retardation experiments using nuclear extracts prepared from differentiated and non-differentiated EC cells a common band was observed interacting with the promoter region of the IFN-β gene, while each extract had at least one additional distinct band. These extract-specific DNA/protein interactions may reflect the binding of tissue-specific factors to the IFN-β promoter. Furthermore, it was found that cell fusions between EC and differentiated cells (which were normally competent for the IFN gene expression) produced hybrids that were unable to produce IFN (like the parental EC cells). The ability of these cells to produce IFN was recovered following differentiation, indicating that the EC cells contain what appears to be a tissue-specific repressor of IFN-β transcription. If an alteration of chromatin following differentiation were to influence the expression of such tissue-specific regulators of IFN expression in different cell types, then this may provide an explanation as to how the differential expression of the IFN genes is achieved.

3.1.4 A hybrid IFN-α1/β-globin gene but not the endogenous IFN-α1 gene is virus inducible in HeLa cells: The implications for the mechanisms of differential expression of the IFN genes.

The observation that in HeLa cells a hybrid reporter gene under the control of two copies of the VREα1, but not the endogenous IFN-α1 gene is virus inducible (see Section 2.1) has important implications as to which of

the mechanisms discussed above may be involved in the regulation of differential expression of this gene. This finding indicates that VREα1 contains all the sequences necessary to confer virus inducibility upon a truncated rabbit β-globin gene in HeLa cells, although when in the context of the endogenous gene these sequences are no longer sufficient for induction. This is consistent with the endogenous gene being under some form of negative control that the plasmid-borne sequences are able to evade. Such a situation may occur if either sequences up or downstream of VREα1 are important in the repression of the expression of this gene or if the chromatin structure plays a role in determining inducibility. Alternatively virus induced-expression of the hybrid reporter may arise if messenger stability plays an important role in the level of expression.

The failure of the endogenous IFN-α1 gene to be induced by virus in HeLa cells could simply reflect the interaction of negative regulating cis-acting DNA sequences with repressor molecules. It is conceivable that such elements are present within the IFN-α1 promoter, but outside the VREα1 element. Although no such elements were identified during the deletion analysis of the IFN-α1 promoter in L929 cells (Ragg and Weissmann, 1983; Ryals et al., 1985) this does not rule out the possibility that a tissue-specific negative regulatory element may be active in HeLa cells.

The role of messenger stability in regulating the level of IFN-α1 production in HeLa cells cannot be addressed by the experiment performed in Section 2.1. As the expression from the two promoters was assayed by measuring the levels of induction of two different transcripts, the effects of messenger stability and promoter activity cannot be distinguished; therefore such a mechanism is not excluded.

The results of Section 2.1 are also compatible with the repression of the IFN-α1 gene being a result of its epigenetic state, since the transiently introduced IFN-α1/β-globin gene would be unaffected by changes in chromatin structure and/or methylation of the DNA. Consequently the introduced gene would be able to avoid any negative regulation that arises as a result of epigenetic changes that are imposed upon the endogenous gene.

To determine whether any of these mechanisms are involved in the control of expression of the IFN-α1 gene in HeLa cells, a number of

additional experiments would need to be done. By using longer IFN-α1 promoter fragments connected to the β-globin gene the question of whether tissue-specific regulatory elements are present further up or downstream of the VREα1 may be answered. The role of chromatin structure and methylation in the tissue-specific regulation of the IFN genes could be studied using homologous recombination to substitute the IFN-α1 gene with that of the hybrid IFN-α1/β-globin or a 'tagged' IFN-α1 gene to determine whether these sequences when in the same context as the endogenous gene are repressed or not. The function that methylation plays could be addressed by treating HeLa cells with 5-azacytidine, a potent demethylating agent, and determining whether the pattern of expression of the IFN-α1 gene alters. Alternatively, hybrid genes could be methylated in vitro (Busslinger et al., 1983) prior to introducing them into cells to see if they are repressed in cell lines that do not normally express the IFN-α1 gene and conversely, whether they are demethylated and consequently expressed in cell lines that do express IFN-α1.

The human β-like-globin genes are a cluster of five active genes comprising of approximately 60 kb of DNA that are expressed in a developmental and tissue-specific manner. Although all the sequences necessary for the induction of the β-globin gene during erythroid differentiation are located immediately 5' inside and 3' of the β-globin gene (Charnay et al., 1984; Wright et al., 1984) when introduced into transgenic mice this gene is weakly expressed in a position dependent manner (Chada et al., 1985; Magram et al., 1985; Kollias et al., 1986). Using regions corresponding to erythroid-specific DNaseI hypersensitive sites (Groudine et al., 1983; Tuan et al., 1985; Forrester et al., 1986) as a guide, Grosveld et al. (1987) constructed a 'minilocus' consisting of the β-globin gene and sequences originally located approximately 50 kb 5' and 20 kb 3' of the β-globin gene. This 38 kb minilocus was expressed in a tissue-specific, position independent manner in transgenic mice at a level comparable to that of the endogenous mouse β-globin gene and proportional to the copy number. Thus it appears that at least for the β-globin gene sequences flanking the gene considerable distances away can influence its expression.

A similar situation may exist for the IFN-α gene locus. If tissue-specific activator or repressor sequences were scattered through out the IFN-α gene locus then such sequences might regulate in a tissue and

developmental manner the expression of the IFN genes. It has been illustrated that such sequences can operate over distances of up to 50 kb, (Grosveld et al., 1987) conceivably when even further away from the transcription start their effect may be less pronounced and the distance a gene is from such an active activator and/or repressor sequences may determine to what extent it is expressed in a particular cell type.

Although a variety of mechanisms can be postulated to explain the repression of the IFN-α1 gene in HeLa cells the possibility that it is the result of the inactivation of the endogenous gene due to some deletion or rearrangement cannot be ruled out.

3.2 Tetrameric hexanucleotides mediating virus inducibility are of distinct types.

Oligonucleotides of the type $(GAAANN)_4$ mediating virus inducibility were classified into three types based on their in vivo properties. Furthermore, gel retardation analysis indicated that members of each type of oligonucleotide bound a similar set of proteins and that type I oligonucleotides and VREβ shared affinity for some of the same proteins, as did the type II oligonucleotide and VREα1.

3.2.1 Type I oligonucleotides.

Type I oligonucleotides (GT_4, GC_4, CT_4 and CC_4) showed no constitutive expression, were induced not only by virus and IFN-γ but also by cotransfection with an IRF-1 expression plasmid and displayed inducible enhancer coupling (Section 2.2). Furthermore, it was demonstrated that these oligonucleotides were also induced by IFN-α and when placed between the TATA box and a close-by SV40 enhancer, silenced the transcriptional activation attributed to the enhancer (MacDonald et al., 1990 [2]).

Using ^{32}P-labeled GT_4 as a probe in gel retardation competition analysis it was shown that type I oligonucleotides were equally efficient

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[2]   All references to MacDonald et al. (1990) refer to work done by the co-authors.

competitors of the retarded bands (Section 2.3.1). Subsequently, it was demonstrated that all type I oligonucleotides generated the same pattern of retarded bands when used as probes and that all bound recombinant IRF-1 (MacDonald et al., 1990).

These findings support the conclusion (Miyamoto et al., 1988; Fujita et al., 1989a; Harada et al., 1989) that IRF-1 is responsible, directly or indirectly, for the activation of transcription by type I oligonucleotides. Uninduced L929 cells contain low levels of IRF-1 (T. Taniguchi, personal communication) but exposure to virus (or IFN-α) causes a strong increase in the level of IRF-1 mRNA which precedes the appearance of IFN-β mRNA (Miyamoto et al., 1988; Harada et al., 1989). Miyamoto et al. (1988) proposed that the increase in level of IRF-1, and perhaps in addition a modification of the pre-existing (possibly inactive) IRF-1, activates transcription.

De novo synthesis of IRF-1 by itself is likely not the only physiological mechanism by which virus induces type I oligonucleotide promoters, because full virus inducibility is observed in the presence of cycloheximide, under conditions where protein synthesis is >97% inhibited (Hug, 1988; Ruffner, 1990). It must therefore be concluded that either pre-existing IRF-1 is released or activated by virus induction, that there is an alternative pathway for inducing type I oligonucleotide promoters in L929 cells, or less likely that residual IRF-1 in the presence of cycloheximide suffices for full induction.

Assuming that IRF-1 were the sole activator of type I oligonucleotide promoters then a latent form would have to exist that following induction could be activated without the requirement for protein synthesis. Activation of pre-existing IRF-1 may be achieved by dissociation from an inhibitory molecule that represses the activating activity of IRF-1. Alternatively some post-translational modification that either directly activates the IRF-1, increases its DNA binding affinity or alters its location could account for this activation in the absence of protein synthesis. Although there are no indication as to how such a modification may occur with IRF-1, precedents for each of these modes of activation have been described in other systems.

NF-κB, the B cell-specific DNA binding protein that recognizes cis-regulatory sequences within the enhancer of immunoglobulin genes

(Sen and Baltimore, 1986a) is thought to be important in regulating the tissue-specific developmental expression of the immunoglobulin genes. The induction of NF-κB does not require protein synthesis, although no NF-κB binding activity is detectable in either cytoplasmic or nuclear extracts prepared from pre-B cells (Baeuerle and Baltimore, 1988a). However, NF-κB can be activated in pre-B cells following induction with LPS or phorbol esters (Sen and Baltimore, 1986b) and in cytosolic extracts from uninduced cells treated in vitro with denaturing agents such as deoxycholate (Baeuerle and Baltimore, 1988a). In pre- and non-B cells NF-κB is associated with a cytoplasmic inhibitor (I-κB) and upon induction this complex dissociates to release active NF-κB which is translocated to the nucleus whereupon it interacts with its binding sites to stimulate transcription of the immunoglobulin genes. (Baeuerle and Baltimore, 1988a, 1988b, 1989). Recent evidence indicates that a phosphorylation event is involved in the activation and translocation of NF-κB from the cytoplasm of non-B cells (Shirakawa and Mizel, 1989).

The heat shock genes are induced following exposure to increasing temperature (reviewed in Lindquist, 1986). This response is mediated by the heat shock transcription factor (HSTF), which binds the cis-regulatory heat shock element (HSE) and occurs in the absence of fresh protein synthesis (Pelham, 1982; Parker and Topol, 1984; Wu, 1984; Kingston et al., 1987; Sorger et al., 1987; Wiederrecht et al., 1987; Wu et al., 1987). Regardless of their origin, eukaryotic HSE are nearly invariant and HSTFs do not distinguish between the HSE from various species (Sorger et al., 1987; Wiederrecht et al., 1987). Surprisingly though the HSTFs of yeast and higher eukaryotes are regulated differently.

In yeast, levels of HSTF/HSE binding are similar in heat shocked and control cells (Sorger et al., 1987). Following heat shock, the yeast HSTF has altered electrophoretic mobility, a transition that can be reversed by phosphatase treatment (Sorger et al., 1987; Sorger and Pelham, 1988). Consequently it appears that yeast HSTF can activate transcription only after heat shock-dependent phosphorylation, which may create an acidic activation domain analogous to those of GCN4, GAL4 and VP16 (Hope and Struhl, 1986; Ma and Ptashne, 1987; Triezenberg et al., 1988).

In contrast to yeast, HSTF binding activity of higher eukaryotes can only be demonstrated in cell extracts prepared from heat shocked cells

(Kingston et al., 1987; Sorger et al., 1987; Zimarino and Wu, 1987). Thus, unlike yeast, the DNA binding activity of HSTF in higher eukaryotes appears to be regulated by heat shock. Activation of DNA activity must involve some form of post-translational modification of pre-existing HSTF since it can be detected in extracts from cells heat shocked in the presence of cycloheximide (Kingston et al., 1987; Sorger et al., 1987; Zimarino and Wu, 1987). The DNA binding activity of HeLa HSTF can be activated in vitro by heat shocking extracts prepared from control cells (Larson et al., 1988). However, unlike the activation of yeast HSTF this does not appear to require protein phosphorylation, since it can occur in the absence of ATP (Larson et al., 1988). Although phosphorylation is not required in the activation of HeLa HSTF DNA binding activity evidence suggests that the activation of its transcriptional activity does require phosphorylation (Larson et al., 1988).

Activation of gene expression in response to hormones can also proceed in the absence of protein synthesis (reviewed in Yamamoto, 1985). The best understood hormone-mediated activation of gene expression is that of the glucocorticoid receptor. The glucocorticoid receptor is a cytosolic protein that interacts both with ligand and the genes it activates. Upon the binding of hormone the glucocorticoid receptor undergoes a conformational transition which results in the translocation of the receptor/ligand complex to the nucleus where it binds directly to cis-regulating DNA sequences to stimulate transcription from adjacent promoters (Picard et al., 1988). Although nuclear localization is not sufficient for transcriptional activation by the glucocorticoid receptor it is necessary and illustrates how nuclear localization may play an important role in the regulation of gene expression by latent activators (Picard et al., 1988).

The mechanism by which plasmid-directed IRF-1 expression activates type I oligonucleotide promoters is not clear. Perhaps, IRF-1 is bound by an inhibitor shortly after synthesis, that dissociates following induction. Such a mechanism requires a delicate balance of activator and inhibitor in order to maintain the balance. Thus, over-expression of IRF-1 from an expression plasmid may upset this balance, titrating out the inhibitor and thus leaving the excess IRF-1 free to stimulate transcription.

If activation of IRF-1 were the result of a post-translational modification, then stimulation of type I oligonucleotide promoters by cotransfection with IRF-1 may occur in a variety of ways. For example, if IRF-1 were synthesized in an active form then the excess of newly synthesized factor may be able to stimulate transcription before being converted to an inactive state. Alternatively, if IRF-1 were produced in an inactive form, then a low basal activating capacity may convert sufficient amounts of the over-expressed factor to the active form to enable activation of the oligonucleotide promoters.

If the activation of transcription by IRF-1 were to involve its transfer from a cellular compartment where it is unable to interact with the DNA to one where it can, then stimulation of transcription by cotransfection with the IRF-1 expression plasmid may occur if the excess of newly synthesized IRF-1 is able to stimulate transcription prior to it becoming compartmentalized. Alternatively, the over-expression of IRF-1 may simply overwhelm the storage facilities, resulting in the excess being free to migrate to the nucleus or a basal translocation activity may result in the transfer to the nucleus of sufficient amounts of the over-expressed IRF-1 to stimulate transcription.

In addition to being induced by virus, type I oligonucleotide promoters are also induced by both IFN-α and -γ (Section 2.2.5 and MacDonald et al., 1990). Type I IFN leads to a rapid increase in IRF-1 mRNA (and presumably protein; Harada et al., 1989), indicating that IFN induction of type I oligonucleotides probably occurs as a result of the synthesis of IRF-1.

The SV40 enhancer, when placed closely upstream of a minimal promoter, displays a strong distance-dependent stimulation of transcription (Pelham, 1982; Hen et al., 1982; Treisman and Maniatis 1985; Kuhl et al., 1987); this can be overcome when certain upstream elements are placed between the TATA box and the enhancer. The ability of upstream sequences to mediate the enhancer effect upon a minimal promoter was termed enhancer coupling (see Section 2.2.4). Enhancer coupling probably reflects the extent to which the SV40 enhancer or associated proteins can stabilize the binding of factor(s) to the tetrameric hexanucleotides present in the promoters of the test genes. The amount of enhancer coupling displayed by a particular element probably reflects its affinity for the cognate binding factors, that is, the stronger the affinity of a

factor for a particular element, the less 'stabilization' is required for maximal cooperation between itself and the transcription initiation complex, which results in a low coupling index. Conversely, CC_4 (induced coupling index 2.4) following this hypothesis should have stronger affinity for its factor (IRF-1 ?) than does GT_4 (induced coupling index 16), whether this is the case or not has never been determined.

Silencing (Kuhl et al., 1987) which was mediated by all type I oligonucleotides, was abolished by viral induction (MacDonald et al., 1990). Presumably, factors binding to type I oligonucleotides in the uninduced state prevent the productive interaction between the enhancer and the TATA box, either by steric hindrance or by entering into an unproductive association with the transcription initiation complex.

IRF-2, which is related to IRF-1 and has the same binding specificity but no activating capacity, may be responsible for this effect. R66 cells which lack IRF-like activity neither respond to viral induction nor silence enhancer activity (Fujita et al., 1988). Harada et al. (1989) were able to reconstitute silencing in R66 cells by cotransfecting a reporter gene under the control of $(AAGTGA)_4$ (a permutation of GT_4) with an IRF-2 expression plasmid, illustrating the potential role that IRF-2 may play in silencing.

IRF-1 and -2 display striking homology in their N-terminal regions, the DNA binding domain, while the C-terminal region, which in IRF-1 is responsible for transcriptional activation, displays little if any homology (Fujita et al., 1989a; Harada et al., 1989). If the region involved in cooperation between IRF-1 and the TATA box factors resides in the N-terminal portion then it is conceivable that the corresponding domain of IRF-2 interacts with the initiation complex forming an unproductive association that blocks the effects of the enhancer. A. Keller and T. Maniatis (personal communication) characterized an 88 kD polypeptide containing 5 zinc fingers, PRDI-BF1, which is clearly different from IRF-1 and -2 and blocks expression mediated by PRDI; it is therefore another candidate for a silencing effector.

The hexanucleotides which were examined as tetramers do not on their own constitute recognition sequence subunits, as shown by the finding that they do not confer inducibility on a minimal promoter even when assayed in conjunction with an enhancer (D. Näf and S. Hardin; unpublished results). They are therefore not analogous to the enhansons of the SV40 enhancer

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(Ondek et al., 1988; Kanno et al., 1989), but rather the result of the fortuitous generation of sequences resembling natural IRF binding sites, when tandemly repeated. Harada et al. (1989) have suggested that the sequence recognized within $(AAGTGA)_4$ is AA(G/A)TG(A/G)AA while Keller and Maniatis (1988) proposed AAGTGAAAGT. D. Näf and S. Hardin (unpublished results) have found that a dimer of GAAAGT, but not a dimer of AAGTGA, mediates virus inducibility, and deduced from this and other findings that the active sequence in GT_4 is (T)GAAAGTGAAAG(T).

3.2.2   Type II oligonucleotides.

The only representative, TG_4, shows no constitutive activity, mediates induction by virus infection, but is stimulated neither by IRF-1 nor IFN and does not show silencing (Section 2.2; MacDonald et al., 1990). In contrast to type I and type III oligonucleotides, TG_4 mediates significant virus inducibility only when it is associated with an enhancer which is reflected in the inordinately high enhancer coupling index of >70 (Section 2.2). However, the 8 fold repeat of GAAATG, TG_8, is strongly virus inducible even in the absence of an enhancer (Ruffner, 1990). Although TG_4 is inducible by virus, cotransfection with the IRF-1 expression plasmid fails to activate it, indicating that a pathway involving a factor(s) other than IRF-1 is regulating the virus induced expression of this sequence.

TG_4 exhibits a distinctive gel retardation band pattern of 3 bands (MacDonald et al., 1990). One of the TG_4-retarded bands (with intermediate mobility) was competed by all NN_4 oligonucleotides and was thus considered to be unspecific (MacDonald et al., 1990). The remaining two bands were efficiently competed by both TG_4 and VREα1 but none of the other oligonucleotides, and were thus considered potential candidates for the factor mediating the virus induction of the type II oligonucleotide and VREa1 (MacDonald et al., 1990).

A striking similarity exists between the octamer sequence ATGCAAAT (Falkner and Zachau, 1984; Parslow et al., 1984; Schreiber et al. (in press)), TG_4 and to a lesser degree with a sequence in VREα1 designated 'TG sequence':

(GAAATG)$_4$	GAA<u>ATG</u>G<u>AAATG</u>G<u>AAATG</u>G<u>AAATG</u>
octa sequence	ATGCAAAT ATGCAAAT
'TG sequence'	GA<u>AATGG</u>A<u>AA</u>

A comparison of the gel retardation patterns given with uninduced Namalwa extract by TG$_4$ and the 'hepta-octamer' sequence GTG<u>CTCATGA</u>AT<u>ATGCAAAT</u>CAATTGG (from the immunoglobulin heavy chain promoter; Ballard and Bothwell,1986) was made to determine whether the sequence similarity between these two elements had any significance. Both oligonucleotides gave the upper of the TG$_4$-retarded bands, while only TG$_4$ showed the two lower bands (MacDonald et al., 1990). Competition of labeled TG$_4$ with the hepta-octa sequence abolished the upper but not the lower bands, indicating that the upper band results from the interaction of an octamer factor with the octamer-like sequence (MacDonald et al., 1990). The hepta-octa sequence interacts with two molecules of the ubiquitous OTF-1 or the lymphoid-specific OTF-2 (as homo- or heterodimers; Poellinger and Roeder, 1989). As TG$_4$ gave an octa-factor-like band shift with L929 cell extract (a fibroblastic cell line) it suggests that it is interacting with the ubiquitous octamer factor, OTF-1 rather than lymphoid-specific, OTF-2.

The observation that OTF-1 bound TG$_4$ raised the question as to whether this interaction mediates the virus inducibility of this oligonucleotide. To address this question, a dimer of hepta-octa in conjunction with the SV40 enhancer was tested for virus inducibility. Though this sequence did mediate low constitutive activity, induction by virus increased the expression only about twofold (Ruffner, 1990) whereas under similar conditions TG$_4$ was induced >70 fold. Thus, it seems unlikely that OTF-1 is mediating the virus inducibility of this oligonucleotide through its interaction with TG$_4$.

The third of the TG retarded bands (the fastest migrating) was specifically competed by TG$_4$ and VREα1, but by none of the other oligonucleotides tested (MacDonald et al., 1990). The protein giving rise to this band was designated 'TG protein' and was subject to further analysis. Methylation interference experiments indicated that TG protein forms similar contacts with a 10-nucleotide sequence common to both TG$_4$ and VREα1,

GAAATGGAAA ('TG sequence'; see above). Consequently, TG protein is considered to be a potential candidate for a factor mediating the virus inducibility of both TG_4 and $VRE\alpha1$ by a pathway other than that by IRF-1.

3.2.3   Type III oligonucleotides.

The type III oligonucleotide CG_4 mediates induction by virus but responds neither to IRF-1 nor to IFN and does not silence. The characteristic features of CG_4 are its strong constitutive activity and distinctive gel retardation pattern, both of which were attributed to its capacity to interact with the ubiquitous transcription factor IEF_{ga} (LaMarco and McKnight, 1989; MacDonald et al., 1990). IEF_{ga} binds to the 'GA' sequence in the immediate early ICP4 promoter of Herpes Simplex virus I and is believed to mediate the trans-activating effect of VP16 (Post et al., 1981; Campbell et al., 1984; Triezenberg et al., 1988). LaMarco and McKnight (1989) pointed out that CG_4 contains a sequence which resembles 'GA' sequence at 15 out of 17 positions:

'GA' sequence   GC**G**G**AAC**G**G**AA**G**C**G**G**AAAC**

$(GAAACG)_4$ GA**AAC**G**G**AAA**C**G**G**AAA**C**G**G**AAA**C**G

LaMarco and McKnight (1989) found that IEF_{ga} gives a strong footprint on CG_4 and it was determined that IEF_{ga} gave the same strong band retardation pattern with both CG_4 and the 'GA' oligonucleotide, similar to that found with Namalwa nuclear extract (MacDonald et al., 1990). Type I oligonucleotides did not bind IEF_{ga}, while GG_4 showed weak and TG_4 barely detectable binding (MacDonald et al., 1990).

This suggested that IEF_{ga} was responsible for constitutive expression mediated by CG_4 and raised the question as to whether it could also mediate virus induction, perhaps as a consequence of being trans-activated by VP16. Both the 'GA' sequence and CG_4 promoted the same constitutive transcript levels, but whereas CG_4 was 7 fold induced by virus, there was no significant stimulation in the case of the 'GA' sequence (data

not shown). Furthermore, cotransfection with a VP16 expression plasmid (pMSVP16; gift of K. LaMarco) failed to activate transcription of either the 'GA' sequence or CG_4-containing promoters (whether VP16 was expressed was not determined) data not shown)). Thus it was concluded that whereas constitutive activity of CG_4 may well be mediated by IEF_{ga}, virus inducibility depends on some other or on additional proteins. Furthermore, the inducibility of CG_4 appears not to be the result of VP16 trans-activation of CG_4 via protein/protein interactions with IEF_{ga}.

With the the exception of its constitutive activity, CG_4 shares properties with that of the Type II oligonucleotide, TG_4. Thus, while the constitutive activity of CG_4 may be mediated by IEF_{ga}, its virus inducibility may be mediated by TG protein. The sequence similarity between CG_4 and TG_4 oligonucleotides is such that if TG protein were to bind to CG_4 it could do so by making contacts similar to those shown to be important for TG protein binding to TG_4 (see Figure 15).

The oligonucleotide GG_4 gives the same gel retardation band pattern as CG_4, however with a much lower intensity (MacDonald et al., 1990). It shows moderate constitutive activity in some experiments and is stimulated by cotransfection with IRF-1, for which it also shows weak binding activity. It thus has properties of both type I and type III oligonucleotides.

3.3 The relationship of the NN_4 oligonucleotides to the IFN-α1 and IFN-β promoters.

3.3.1 The IFN-β promoter.

Maniatis and coworkers identified a 40 bp sequence (VREβ; -79 to -39) of the IFN-β promoter as being essential for the maximal induction of this gene in C127 cells (Goodbourn et al., 1985). Subsequently, by examining the effect of a large number of single point mutations, VREβ was found to consist of two functionally distinct positive regulatory domains (PRDI and PRDII) and one negative regulating element ((NRDI) Goodbourn and Maniatis, 1988).

PRDI (-79 to -66), contains the sequence <u>AG</u>AAGTGAAAG which differs by two (underlined) nucleotides from GAAAGTGAAAG, the sequence identified by D. Näf and S. Hardin (unpublished results) as being the

minimal sequence essential for the virus induction of multimers of GAAAGT and AAGTGA. In L929 cells a second PRDI-like sequence (-91 to -80), <u>A</u>AAA<u>C</u>TGAAA**G**, is required for optimal inducibility (Fujita et al., 1985, 1987; Dinter and Hauser, 1987). This sequence differs by two nucleotides (underlined) from the minimal virus inducible sequence (see above) and by two nucleotides (bold) from the sequence GAAACTGAAA repeated three times in the type I oligonucleotide CT_4.

Keller and Maniatis (1988) identified two factors that bind PRDI, one in uninduced (PRDI-BFc) the other in induced cells (PRDI-BFi), which display the same binding specificity, but result in the formation of complexes with different electrophoretic mobility. Although the relationship between the PRDI binding factors of Keller and Maniatis and IRF-1 and -2 is not clear, it seems reasonable to assume that the positive effects of PRDI are mediated at least in part by IRF-1. PRDI is itself both virus and IRF-1 inducible (D. Näf, unpublished results) and has been shown to bind IRF-1 in vitro (Harada et al 1989). Consequently it would appear that the virus and IFN inducibility of the type I oligonucleotides is due to the fortuitous generation of PRDI-like sequences created as a result of the multimerization of the hexamers, which like PRDI are probability activated by IRF-1.

Keller and Maniatis (1988) identified a factor, PRDII-BF, that binds to the PRDII sequence (-68 to -57). Clark and Hay (1989) and Hiscott et al. (1989) pointed out the resemblances between PRDII (GGGAAATTCC), the EBP1 binding site in the SV40 enhancer (<u>GG</u>A<u>AA</u>G<u>TC</u>CC), the NF-κB binding site in the κ light chain enhancer (C<u>GGAAAG</u>T<u>CC</u>) and the H2TF1 binding site in the mouse class I histocompatibility gene enhancer (<u>GGG</u>G<u>A</u>T<u>TC</u>CC). Lenardo et al. (1989) and Visvanathan and Goodbourn (1989) argue convincingly that PRDII-BF is indistinguishable from NF-κB. This factor is constitutively active in B cells and is released from an inactive complex (Baeuerle and Baltimore, 1988b) by LPS or TPA in pre-B cells (Sen and Baltimore, 1986a) or with TPA in non-B cells (Sen and Baltimore, 1986a, 1986b). Moreover, NF-κB can also be activated by double-stranded RNA (Lenardo et al., 1989; Visvanathan and Goodbourn, 1989), virus infection (Fujita et al., 1989b) TNF-α and IL-1 (Osborn et al., 1989). In line with these findings, dimeric PRDII mediates constitutive activity in B cells (Lenardo et al., 1989) and virus inducibility in non-B cells such as L929 cells (Fan and Maniatis, 1989). However, some L929 sublines contain

constitutively active NF-κB and in such cell, when tandemly repeated, PRDII is constitutively active (Fujita et al., 1989b).

Thus, the induction of the IFN-β promoter is envisaged as coming about by (a) the activation (and/or de novo synthesis) of IRF-1 or an IRF-1-like factor (for potential mechanisms see Section 3.2.1) and (b) release of NF-κB as a consequence of exposure to virus or double-stranded RNA. This results in the simultaneous binding of one or two 'activated' factor molecule(s) to the IRF binding sites (one of which is PRDI, the other 'PRDI-like' site is located between -91 to -80) and of an NF-κB molecule to PRDII; the requirement for cooperative binding has been proposed by Fan and Maniatis (1989), and Fujita et al. (1989b). The binding of only one of these factors is insufficient to activate transcription as judged by the fact that IRF-1 expression alone gives only 2.7 fold stimulation of VREβ, and that the IFN-β promoter is not active in NF-κB-containing B cells. In support of the hypothesis that expression of the IFN-β gene requires the simultaneous binding of two of more molecules of NF-κB and/or IRF-1 is the observation that single copies of PRDI or PRDII alone are insufficient to mediate virus induction while multimers of either PRDI or PRDII can. Presumably once multimerized these elements bind at least two molecules of either NF-κB or IRF-1 and in so doing can function as virus-inducible regulatory elements (Fujita et al., 1989b; Fan and Maniatis, 1989).

When used in conjunction with an SV40 enhancer at a distance, a single copy of PRDI can confer both virus and IRF-1 inducibility upon a minimal promoter (D. Näf, unpublished result). This finding indicates that the binding of a single IRF-1 molecule is sufficient to activate transcription when supported by an enhancer. Whether this effect is the consequence of enhancer coupling 'stabilizing' the IRF-1/PRDI interaction or of the enhancer supplementing some function normally supplied by the binding of a second molecule is not known. Even when supported by an enhancer, VREβ, which contains a single PRDI element, is only stimulated 2.7 fold comparing to the 31 fold inducibility of PRDI. This indicates that activation of VREβ, in addition to requiring the simultaneous binding of two molecules may also require the disengagement of putative repressors from PRDI and/or NRDI, an event also elicited by induction.

In addition to being inducible to virus, multimers of a PRDI-like sequence ((AAGTGA)$_2$), (but not PRDII) are inducible by IFN (Fan and Maniatis,

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1989). Although VREβ contains a single PRDI domain, it is not inducible by IFN (M. Chaturvedi, unpublished result). The inability of IFN to activate VREβ although it can induce the expression of IRF-1 (Harada et al., 1989), may be due to its inability to elicit the release of active NF-κB and/or relieve VREβ of its negative repression. Alternatively, it may be that simultaneous binding of two molecules of IRF-1 is required for activation by IRF-1 and thus a second PRDI site required, such as the PRDI-like sequence present in VREβ' but absent from VREβ (Figure 17).

The role of IRF-2 in the induction of the IFN-β gene is not clear. It has been suggested that it may serve as repressor (T. Taniguchi personal communication to C. Weissmann) or placeholder for IRF-1 in chromatin (Harada et al., 1989). Presumably, IRF-2 would keep the chromatin in an open configuration that would be amenable to the rapid binding of IRF-1 following induction, leading to expression of this gene

The contribution of repression and cooperative binding of IRF-1 and NF-κB to the regulation of VREβ could be assessed by comparing the transcriptional properties of VREβ and PRDI-PRDII in B and non-B cells under different induction conditions. If the binding of a single molecule of either IRF-1 or NF-κB were sufficient to activate transcription and no repression were operative, then both constructs would be constitutively expressed in B cells (which contain active NF-κB) but would require either cotransfection with an IRF-1 expression or TPA induction (to release active NF-κB) to be active in non-B cells. If on the other hand, a repressor were operative, then the PRDI-PRDII construct would be active as described above, while the VREβ promoter (which contains the NRDI element; Goodbourn and Maniatis, 1988) would remain repressed under all the above conditions.

If simultaneous binding of IRF-1 and NF-κB were required for activation then both constructs would be constitutive when cotransfected with an IRF-1 expression plasmid in B cells and TPA inducible in non-B cells. If repression were involved, the VREβ construct but not the PRDI-PRDII element would be inactive under these conditions.

IFN-β

VREβ'

```
                    "TG"-like        PRDI-like
AACTACTAAAAATGTAAATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGGGGAAATTCCTCTGAATAGAGAGAGACCATCTCATATAAATAGG
      |          |gAAAATGgAAATG|  |gAAACTGAAAGG| |gAAAGTGAAAGII|                  |          |       |
    -117        -96            -79                -57              IRF            -39        -31     -22
                                                          PRDI   PRDII   NRDI
                                                          gGGGActTTCC
                                                                  NF-κB
```

VREβ

IFN-α1

VREα1

repA

```
                                            PRDI-like                    "TG"                         PRDI-like
TGTAAAGAGTGCATGAAGGAAAGTGCATGAAGGAAAGCAAAAACAGAAAATGGAAAGTGGCCCAGAAGCATTAAGAAAGTGGAAATCAGTATGTTCCCTATTTAAGGC
  |      |         |GAAAGCgAAAgG|    |GAAATGGAAAtg|                              |GAAAGTGaAAgII|       |
-115   -109                                                                    -64                    -32
```

Figure 17   Sequence of the IFN-α1 and -β promoters with established and putative factor binding sites.

Established and putative factor binding sites within the IFN-α1 and IFN-β promoters. Numbering is from the cap site of the human IFN-β (Fujita et al., 1985) and IFN-α1 gene (Ryals et al., 1985), respectively. PRDI, PRDII and NRDI are delineated according to Fan and Maniatis (1989). VREβ and VREβ' are the minimal inducible sequences determined by Goodbourn et al. (1985) and Fujita et al. (1985), respectively. VREα1 and repA were described by Kuhl et al. (1987). Solid frames, regions believed to be functionally important for induction; dotted frames, similarities to binding sites. IFN-β promoter: Binding to the sites designated "IRF" is supported by experiments with recombinant IRF-1 (Miyamoto et al., 1988; Harada et al., 1989). The NF-κB binding site was characterized by Fujita et al., (1989b), Lenardo et al., (1989) and Visvanathan and Goodbourn (1989). The short sequences within the "PRD1-like" boxes indicate the match to a (GAAANN)4 oligonucleotide known to respond to IRF-1. Italicized sequence in the "NF-κB" box represents a typical NF-κB element (Sen and Baltimore, 1986a; mismatches to the IFN sequences are indicated in small letters). IFN-α1 promoter: The binding of "TG" factor to the "TG" sequence is based on band shift competition and methylation interference data. There is no evidence that IRF-1 binds to either of the "PRDI-like" sequences or that the downstream "PRDI-like" sequence is functionally important.

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3.3.2   The IFN-α1 promoter.

A 46-bp region within the 5' flanking sequences of the human IFN-α1 gene (VREα1; -109 to -64) was identified as being sufficient to mediate full induction by virus in L929 cells (Ryals et al., 1985). VREα1, while mediating induction by virus, shows no constitutive activity and is neither stimulated by cotransfection with an IRF-1 expression plasmid nor by IFN, and does not silence.

The IFN-α1 promoter contains two sequences reminiscent of PRDI sites. One, GAAAGAAAAAC, is within VREα1 between positions -97 and -86, while the other GAAAGTGGAAATC (-56 to-45) lies between VREα1 and the TATA box ('PRDI-like sequences' in Figure 17). Despite the presence of an PRD1-like sequence, the binding of IRF-1 to VREα1 is much weaker than to VREβ, which is in turn weaker than to $(AAGTGA)_4$ or $(GAAAGT)_4$ (Harada et al., 1989; MacDonald et al., 1990). Furthermore, unlike PRDI, (believed to be the physiological target IRF) neither of the IFN-α1 PRDI-like binding sites mediate virus inducibility when assayed in conjunction with the SV40 enhancer at a distance (D. Näf, unpublished result). Taken together with the observation that an IRF-1 expression plasmid fails to activate VREα1, these findings imply that the virus inducibility of VREα1 and therefore presumably of the IFN-α1 gene occurs by a pathway other than that involving IRF-1.

Although the 5' portion of VREα1 does not appear to be involved in the IRF-1 activation, 5' truncation analysis (Ragg et al., 1983; Ryals et al., 1985) as well as the fact that an A->C conversion in GA<u>A</u>AGCAAAAAC of VREα1 greatly reduces its virus inducibility (MacDonald et al., 1990) show that this region contributes significantly to the virus response of VREα1. Kuhl et al. (1987) showed that a tetramer of a 20 bp subsequence of VREα1, tetra-repA (which contained a small deletion causing the two central units to overlap by one nucleotide), efficiently mediated virus inducibility. Consequently it would appear that either repA (-101 to -82; see Figure 17) or a sequence generated as a result of its multimerization may be contributing to the virus inducibility of VREα1. This element may be functionally equivalent to PRDI of VREβ, as in addition to being inducible to virus, tetra-repA was also shown to silence (Kuhl et al., 1987), a characteristic of PRDI (and PRDI-like elements). To date nothing is known about factors interacting with repA, though it appears that neither IRF-1 nor

-2 interact with this sequence as judged by methylation interference experiments (Harada et al., 1989).

VREα1, in contrast to VREβ, does not bind NF-κB (MacDonald et al., 1990), in agreement with the statement of Lenardo et al. (1989) that no NF-κB activation is operative in induction of the IFN-α1 promoter. Hiscott et al. (1989), in gel retardation experiments with nuclear extracts from U937 cells, have noted that the complexes formed with a fragment slightly shorter than VREα are not competed by VREβ and vice-versa. Thus, neither IRF-1 nor NF-κB, the two factors implicated as being responsible for the virus induction of the IFN-β gene appear to play a role in the virus induction of the IFN-α1 gene.

Gel retardation competition analysis revealed that none of the tetrameric hexanucleotides, with the exception of TG_4, competed the VREα1-retarded bands (Figures 13 and 14). Furthermore, VREα1, but not VREβ is a strong competitor of the proteins binding TG_4, namely TG protein and OTF-1 (MacDonald et al., 1990). A perfect match of 10 nucleotides (GAAATGGAAA) exists between a sequence in TG_4 and one in VREα1 (designated TG sequence; see Figure 17). This sequence has been postulated to be a virus inducible element, contributing to the virus inducibility of both the VREα1 and the type II oligonucleotide, TG_4.

In support of the functional importance of the TG sequence are the findings that a single A->C substitution (GAAATGGA<u>A</u>A) in the TG sequence of VREα1 reduces inducibility more than 7-fold (MacDonald et al, 1990) and that the VREα1-derived oligonucleotide A<u>GAAATGGAAA</u>GTG (which contains the TG sequence, underlined) mediates inducibility by virus (D.Näf, unpublished results). Surprisingly the A->C change in the TG sequence of VREα1, which reduces its inducibility, does not diminish the binding of the TG protein (D. Maguire,unpublished results). Such a finding might indicate that a protein other than the TG protein is conferring the virus inducibility to VREα1 or that the DNA sequence required for binding and activation by TG protein are not the same.

Both TG protein and OTF-1 can bind to VREα1 as with TG_4 and consequently they were candidates for mediating the virus inducibility of these oligonucleotides (MacDonald et al, 1990; D. Maguire, unpublished result). It seems unlikely that octa factors are involved in the viral induction of VREα1 because an authentic dimeric hepta-octa sequence is not virus

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inducible in our system. The TG protein and its binding is strongly impaired by methylation of the same GG doublet in the TG sequence of both VREα1 and TG$_4$ (ie. GAAAT<u>GG</u>AAA). In agreement with this result is that conversion of the TGG sequence (GAAA<u>TGG</u>AAA) to CCC in VREα1 virtually abolished the binding of TG protein (D. Maguire,unpublished results) Curiously, Harada et al. (1989) showed that methylation of the GG doublet within the TG sequence of VREα1 impaired the weak binding of IRF-1 to VREα1. Nevertheless, whether or not the TG protein plays a role in the virus induction still remains to be established though it interacts with the TG sequences of both TG$_4$ and VREα1.

The VRE of the murine IFN-α4 promoter which has similar boundaries to its human IFN–α1 counterpart, also contains a region resembling RepA (<u>T</u>AA<u>A</u>GAAAG<u>TG</u>AAAA<u>G</u>A<u>C</u>AA; differences underlined) which contains a PRDI-like element (bold), and a sequence very similar to the TG sequence (GAA<u>T</u>TGGAAA; Raj et al., 1989). Thus, like the human IFN-α1 gene, virus induction of the murine IFN-α4 gene may be mediated by a TG-like sequence and some as yet unidentified factor interacting with sequences contained within the RepA-like domain.

Surprisingly, the natural IFN-α1 promoter, extending either from -675 or from -141 to -6 is considerably less active than the VREα1 segment joined to a minimal β-globin promoter in both Namalwa (Figure 16) and L929 cells (Näf, 1989). Moreover the VREα1 dimer is more than 20 times more active than the natural IFN-α1 promoter segment. This suggests that even the largest IFN-α1 promoter segment tested may lack an enhancer, or contain inhibitory sequences.

3.4   Conclusions.

At least two different elements in the IFN-β promoter respond to virus induction, namely PRDI and PRDII, which interact with IRF-1 and NF-κB, respectively. A novel virus-responsive element in the IFN-α1 promoter, the TG sequence which is different from both PRDI and PRDII and interacts with a protein distinct from IRF-1 and NF-κB has been identified. Thus, there are at least three separate terminal pathways for virus-induced signal transmission and the promoters of the IFN-α1 and IFN-β genes, despite their apparent homology, contain different response elements. The

functional differences between the promoters could explain, at least in part, the tissue specific expression patterns of the two IFN subfamilies.

4. EXPERIMENTAL PROCEDURES.

4.1   Plasmid constructs.

Constructs were carried out by standard procedures (Maniatis et al., 1982). Plasmids containing tetrameric GAAANN, the 24 bp pBR spacer (pBR322, positions 968-991; Sutcliffe, 1978) and the VRE elements (Kuhl et al., 1987; MacDonald et al., 1990; and this work) were constructed as follows; all test sequences were synthesized chemically and were flanked by a 5'-ClaI-compatable overhang (CGAT) on the top and 5'-HindIII-compatable overhang (TTCGA) on the bottom strand (see below). The test sequences were inserted between the ClaI and HindIII sites of 42P (no enhancer; Kuhl et al., 1987). For constructs with the enhancer at a distance, the test sequences were inserted between the ClaI and HindIII sites of a plasmid derived from 39P by replacing the XhoI-ClaI fragment (see legend, Figure 7) with the 1291 bp XhoI-ScaI rabbit β-globin fragment (ClaI-linkered at the ScaI end) (see Table 1, fragment XXVI, in Kuhl et al., 1987). All constructs were verified by sequencing.

Tetrameric hexanucleotides;

```
5'-cgat GAAANN GAAANN GAAANN GAAANN a-3'
      ta CTTTNN CTTTNN CTTTNN CTTTNN ttcga
```

VREα1;

```
5'-cgatGAGTGCATGAAGGAAAGCAAAAACAGAAATGGAAAGTGGCCCAGAa-3'
     taCTCACGTACTTCCTTTCGTTTTTGTCTTTACCTTTCACCGGGTCTttcga
```

VREβ;

```
5'-cgatGAGAAGTGAAAGTGGGAAATTCCTCTGAATAGAGAGAGGACa-3'
     taCTCTTCACTTTCACCCTTTAAGGAGACTTATCTCTCTCCTGttcga
```

RβGα-141/-6 was constructed by inserting the 73 bp ClaI/BspI fragment of CF-2 (IFN-α1 -141 to -70; ClaI-linkered at position -141) and the 64 bp BstI/StuI fragment of JM12-13 (IFN-α1 -70 to -6) into ClaI/PvuII digested ID1. Plasmids CF-2 and JM12-13 were constructed by C. Filippini and H. Ragg respectively.

Plasmids ID1 and 12P (HP6Δ-109) are from Ryals et al. (1985), 13P and 61P from Kuhl et al. (1987), CF1/42P and CF2/42P from C. Filippini (unpublished result) and pIG from Weidle and Weissmann (1983). The internal reference plasmids pSTC407-556 and 30P are from Severne et al. (1988) and Kuhl et al. (1987) respectively, the IRF-1 expression plasmid, pIRF-L from Miyamoto et al. (1988) and the expression vector, CDM8 from Seed, (1987).

4.2   Purification of oligonucleotides.

Oligonucleotides, synthesized on an Applied Biosystems DNA synthesizer (Model 380A) were deblocked by incubation with an equal volume (vol) of conc. NH$_4$OH (25%) at

55 °C for 8 hr. After lyophlizing, the oligonucleotides were dissolved in 150 μl formamide loading dye (90% formamide (v/v), 1 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), heated 2 min at 90 °C, chilled on ice and loaded on to a pre-run (1-2 hr) 40x28x0.2 cm, 12% polyacrylamide (20:1 cross-link), 7 M urea, 89 mM TBE (89 mM Tris-base, 89 mM boric acid, 1 mM EDTA) gel. Gels were run 600-700 volts until the bromophenol blue had migrated ~30 cm (length of run depended on the size of the oligonucleotide being purified). The gel was sandwiched between two sheets of Saran Wrap placed on a thin layer chromatography 60 F_{254} plate (Merck), DNA visualized by illuminating the gel from above with a 254 nm UV lamp and the slowest migrating band excised. The excised gel fragments were placed in 10 ml Greiner tubes, crushed with a glass rod and the DNA eluted overnight while agitating with 4 ml of H_2O at room temperature. The supernatant was passed through a glass wool plug prior to being lyophilized, the resulting pellet was resuspended in 400 μl of TNE (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA) and fractionated over a 10 ml Sephadex G-25/H_2O column eluted with H_2O. The DNA was localized by measuring the absorbance at 260 nm (1 in 30 dilutions), DNA containing fractions pooled, lyophilized and the resulting pellets were dissolved in 100 μl of H_2O. The concentration of the purified oligonucleotides was determined by UV absorbance (260 nm).

4.3 Large scale plasmid DNA preparation.

250 ml of N-medium (10 mg/ml Bacto-tryptone, 1 mg/ml yeast extract, 1 mg/ml glucose, 8 mg/ml NaCl, 0.3 mg/ml $CaCl_2$, 10 mM $MgSO_4$) plus antibiotic (100 μg/ml ampicillin and/or 10 μg/ml tetracyclin) was inoculated with 0.75 ml of an overnight culture and incubated 37 °C, 250 rpm until the OD_{650} was between 0.9 and 1.2 (~6-9 hr). The cells were collected at 4500 rpm, room temperature for 15 min (Sorvall GSA rotor), the cell pellet resuspended in 250 ml of prewarmed (37 °C) N-medium (+ antibiotic) which contained 80 μg/ml of chloramphenicol (freshly prepared) and incubated overnight (~12-16 hr), 37 °C, 250 rpm. The cells were harvested at 6000 rpm, 4 °C, for 20 min (Sorvall GSA rotor) and resuspended in 12 ml of 50 mM Tris-HCl (pH 8). 3 ml lysozyme (10 mg/ml in 50 mM Tris-HCl (pH 8); freshly prepared)), 1.8 ml 0.5 M EDTA and 0.75 ml 2% Triton X-100 added with 10 min incubations on ice between the addition of each. The cells were then incubated on ice for 45-60 min prior to being centrifuged for 30 min at 15000 rpm, 4 °C (Sorvall SS34 rotor). The supernatant was adjusted to a pH of ~12.5 by the addition of 1.8 ml of 1N NaOH (freshly prepared) while stirring, and was stirred for an additional 10 min at room temperature. The pH was subsequently lowered to ~8.3-8.5 by the addition of 8 ml of 1 M Tris-HCl (pH 7.5) and stirred for 3 min before the addition of 3 ml of 5 M NaCl and 1 vol phenol (saturated with 0.5 M NaCl; unless otherwise stated all phenol, phenol/chloroform and chloroform extractions were done using one volume and with phenol equlibrated against TNE). The aqueous phase was extracted twice with chloroform then incubated for 30-60 min with 60 μl of

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RNaseA (10 mg/ml in TNE; preincubated at 85 °C for 10 min) at 37 °C. After precipitation in 0.65 M NaCl and 6.5% PEG 6000 for 90 min at -10 °C or overnight on ice the DNA was collected by centrifugation for 20 min, 9000 rpm, 4 °C (Sorvall HB4 rotor) and resuspended in 400 µl TNE. Incubation for 30 min at 37 °C with 20 µl pronase (20 mg/ml; preincubated for 2 hr at 37 °C) in 1% SDS followed, and then the solution was extracted 3 times with 0.75 vol phenol and 0.25 vol chloroform and precipitated with 2 vol ethanol for 30 min at -80°C. After centrifugation the DNA pellet was resuspended in 400 µl TE and loaded on to 12 or 36 ml sucrose gradients (0.1-0.5 mg and 0.5-1 mg of DNA per gradient respectively) with six different density layers (5%, 8.6%, 12.2%, 15.8%, 19.4% and 23% in 50 mM Tris-HCl (pH 8) and 1 mM EDTA) which were centrifuged for 15 hr at 20000 rpm, 15 °C in Beckman SW41 or SW27 rotors. The gradients were fractionated, DNA containing fractions pooled, adjusted to 1x TNE and precipitated with 2.5 vol EtOH -20 °C overnight. The DNA was subsequently dissolved in 200-400 µl TE and the yield determined by UV spectroscopy. DNA prepared in such a way was used for all purposes without further purification.

4.4   Isolation of plasmid DNA from mini-preps.

3 ml of N-medium plus antibiotic (100 µg/ml ampicillin and/or 10 µg/ml tetracyclin) was inoculated with a bacterial colony and shaken 20-24 hr, 250 rpm at 37 °C. The bacterial pellet from 1.5 ml of culture was redissolved in 100 µl of TELT buffer (50 mM Tris-HCl (pH 7.5), 62.5 mM EDTA, 0.4% Triton X-100, 2.5 M LiCl). After the addition of 10 µl lysozyme (10 mg/ml; freshly prepared) the suspension was incubated in a boiling water bath for 1 min and cooled on ice 5 min. The precipitate was removed by spinning in an Eppendorf centrifuge for 8 min at room temperature. The DNA in the supernatant was precipitated with 2.5 vol of ethanol (EtOH) and resuspended in 30-50 µl TE (10 mM Tris-HCl (pH 8), 0.5 mM EDTA). The DNA was used directly for restriction analysis but prior to DNA sequencing contaminating RNA was removed either by running over a low gelling temperature agarose gel or by PEG precipitation.

DNA was incubated in agarose gel loading buffer which contained 50 µg/ml RNaseA for 5 min before being applied to a 0.8% TAE (Maniatis et al., 1982), low gelling temperature agarose (FMC) gel and purified by electrophoresis. The band corresponding to the supercoiled plasmid was excised and incubated at 65 °C for 15 min during which time the agarose concentration was adjusted to 0.5% with 20 mM Tris-HCl (pH 7.5) and sodium acetate (pH 6) added to a final concentration of 0.3 M. The sample was incubated at 37 °C, extracted three times with phenol and once with chloroform. The aqueous phase was EtOH precipitated with 2.5 vol and the pellet dissolved in 10 µl TE. The yield was estimated by running 1 µl in parallel with known amounts of DNA standards on a 1% agarose gel.

PEG precipitations were performed by resuspending the pellet in 200 µl TE to which was added 1 µl RNaseA (10 mg/ml in TNE; preincubated at 85 °C for 10 min), incubating 30 min

37 °C, after which 40 µl 5 M NaCl and 67 µl 30% PEG 6000 were added, the mixture was vortexed and the DNA precipitated at 90 min -10 °C or overnight on ice. The resulting pellets were washed with 80% EtOH, air dried, resuspended in 10 µl TE and used directly for DNA sequencing.

4.5   Sequencing of plasmid DNA.

1 µg of DNA in 8 µl TE was denatured with 2 µl of 5x denaturing buffer (1M NaOH, 1 mM EDTA) for 5 min at room temperature. The mix was neutralized by the addition of 1 µl of 10 x neutralization buffer (2M NH_4OAC pH 4.5) and precipitated with 2.5 vol EtOH. The precipitate was collected by centrifugation, washed with 80% EtOH and air dried.

Hybridization and primer elongation reactions were performed simultaneously for 5 min at room temperature in 33 mM Tris-HCl (pH 7.5), 33 mM NaCl, 13 mM $MgCl_2$, 6.5 mM DTT, 0.1 mM EDTA, 0.2µM of each of the dNTPs, containing 1.75 pmol sequencing primer (see below), 5 pmol α-^{35}S-dATP (600 Ci/mmol; Amersham) and 3.2 units Sequenase enzyme (a modified form of T7 polymerase; United States Biochemical Corporation) in a final volume of 15.3 µl. 3.5 µl of the reaction mix were transferred to one of 4 tubes containing 2.2 µl of either ddATP-, ddTTP-, ddCTP- and ddGTP-tremination mix (80 µM each of the 4 NTPs, 8 µM of the corresponding ddNTP, 50 mM NaCl) and incubated at 37 °C for between 5 and 20 min. Reactions were stopped by the addition of 4 µl formamide loading dye.

After denaturing the samples at 80 °C for 2 min, the fragments were resolved on a 40x28x0.03 cm, 5.5% polyacrylamide (20:1 cross-link), 7.7 M urea, 89 mM TBE gel using a gradient in buffer concentration (89 mM TBE in the upper reservoir, 445 mM TBE in the lower reservoir). Gels were run until the bromophenol blue had reached the bottom, transferred to Whatman 3MM paper and dried for 30 min at 80 °C under vacuum.

The 'new globin sequencing primer' which annealed to positions +26 to +40, was used to sequence all rabbit β-globin expression vectors.

3'-ATGAACGTTAGGGGG-5'

4.6   Preparation of 5'-^{32}P-labeled S1 mapping probes.

In general, 15 µg of plasmid DNA was digested using the appropriate restriction enzyme as recommended by the suppliers after which 2 vol of 0.1 M Tris-HCl (pH 8.7) and 1 µl of calf intestinal alkaline phosphatase (Pharmacia; 5000-10000 units/ml) were added. Following incubation at 37 °C for 30 min, the enzyme was inactivated at 65 °C for 15 min. The supernatant was phenol/chloroform (1:1) extracted and the aqueous layer fractionated over a 1.5 ml Sephadex G-50/TNE column. Peak fractions were pooled, EtOH precipitated and the pellets obtained following centrifugation dissolved in 30-50 µl H_2O. Recovery was determined by UV spectroscopy.

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Kinase reactions were carried out at 37 °C for 30 min in a solution containing 50 mM Tris-HCl (pH 9.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 µg/ml dephosphorylated DNA, ~0.05 units/µl T4 polynuclease kinase (K. Murray, University of Edinburgh) and a 5-10 fold molar excess of γ-^{32}P-ATP (6 pmol/µl at 5000-7000 Ci/mmol). The DNA was phenol/chloroform (1:1) extracted and the aqueous phase loaded onto a 1.5 ml Sephadex G-50/TNE column. Peak fractions were pooled, EtOH precipitated and subjected to a second restriction digestion using the appropriate restriction enzyme. The resulting fragments were separated on 1-2% low gelling agarose gels with standards were run in parallel to allow estimation of recovery. The desired fragments were excised, recovered from agarose (see Section 4.4) dissolved in 80% FAHB (80% formamide, 400 mM NaCl, 40 mM Pipes (pH 6.4), 1 mM EDTA), such that the final concentrations were 0.01 pmol/10 µl 80% FAHB. Specific activities of probes ranged from 2-6x10^{6} Cerenkov cpm/pmol

probe	plasmid	primary restriction digest	secondary restriction digest	fragment isolated
rabbit β-globin	rβGA4	BamHI	PstI	452 bp
CMV-TK	pSTC407-556	BamHI	SacI	167 bp
3' end Hu IFN-α1	SP6α1 3'	EcoRI	RsaI	360 bp [1]

rβGA4	Weber et al.,(1981)	[1] copurified with a 386 bp
pSTC407-556	Severne et al. (1988)	non-hybridising fragment
SP6α1 3'	Streuli, (1986)	

4.7 Synthesis of γ-^{32}P-ATP.

Prepared the following solutions;

Mix A

50 µl	500 mM Tris-HCl (pH 9)
30 µl	100 mM DTT
25 µl	2.4 mM L-α-glycerol phosphate
25 µl	10 mM β-NAD
20 µl	300 mM $MgCl_2$
12.5 µl	2 mM Na-ADP
12.5 µl	40 mM Na-pyruvate

Mix B

50 µl	Cysteine Solution (see below)
10 µl	H_2O
7 µl	500 mM Tris-HCl (pH 9)
5 µl	Enzyme Solution (see below)

Cysteine Solution

Dissolved 47 mg cysteine in 5 ml H_2O into which was dissolve 70 mg of Trizma base.

Enzyme Mix

100 µl	Glycine-3-phosphate dehydrogenase (2 mg/ml; Boehringer)
1 µl	Triosephosphate isomerase (2 mg/ml; Boehringer)
20 µl	Glycerinaldehyde-3 phosphate dehydrogenase (10 mg/ml; Boehringer)
2 µl	3-phosphoglycerate kinase (10 mg/ml; Boehringer)
20 µl	Lactate dehydrogenase (5 mg/ml; Boehringer)

Resuspended the Enzyme Mix by inverting the tube a few times. Removed 15 µl, transferred to a fresh tube, spun 3 min, sucked off the supernatant and dissolved the pellet in 15 µl of 50 mM Tris-HCl (pH 9), to generate the Enzyme Solution.

Synthesis Reaction

To 60 µl ^{32}P-orthophosphate (15 mCi; Amersham) added 32 µl Mix A and 8 µl Mix B, vortexed gently and incubated at room temperature for 1 hr (incorporation usually >80%). Added 100 µl H_2O, phenol/chloroform extracted once (1:1), ether extracted twice, diluted the aqueous phase to ~40-50 µCi/µl in 5 mM DTT, prepared aliquotes and stored -20 °C.

4.8 Cell culture and DNA transfections.

4.8.1 HeLa cells.

HeLa cells were maintained in Iscov 's medium (Gibco) supplemented with 5% fetal calf serum (FCS) at 37°C in an atmosphere of 5% CO_2. For transfection 1.5-2x10^5 cells were seeded in a 60-cm dish. After 18-24 hr, 10 µg plasmid DNA (in 120 µl H_2O) and 120 µl 4x Ca-Hepes were mixed by tapping and then incubated 5' at room temperature. Following the addition of 240 µl 2x Hepes-phosphate the DNA solution was incubated for an additional 15 min at room temperature, pipetted onto the cells and incubated for 12 hr at 37 °C. The medium was removed, cells washed with PBS (0.8% NaCl, 0.1% KCl, 0.72% Na_2HPO_4, 0.1% KH_2PO_4), fresh medium added and the plates returned to the incubator for an additional 36 hr.

4x Ca-Hepes		2x Hepes-phosphate	
10 ml	0.5 M Hepes [1]	10 ml	0.5 M Hepes [1]
12.5 ml	2M $CaCl_2$	10 ml	2.8 M NaCl
27.5 ml	H_2O	80 ml	H_2O
		300 µl	0.5 M NaPi [2]

[1] = (10 ml 0.5M Hepes pH 6.5 + 2.5 ml 0.5M Hepes pH 7.3)
[2] = (50 ml 0.5 M NaH_2PO_4 + 50 ml 0.5 M Na_2HPO_4); added after autoclaving the other components.

4.8.2 L929 cells.

Murine L929 cells were grown in DMEM medium (Gibco) supplemented with 5% FCS in a 37 °C, 5% CO_2 incubator. For transfection (2.8x10^6) cells were seeded in a 10-cm dish. After 18-24 hr they were washed once with TBS (Fujita et al., 1986) and 7 µg of each test and reference plasmid, and 7 µg of either the IRF-1 expression plasmid, pIRF-L ('with IRF-1') or the corresponding 'empty' expression vector CDM8 ('no IRF') in 1.12 ml of 0.5 mg/ml DEAE Dextran (in TBS) were added. After 30 min at room temperature the cells were

shocked by adding 1 vol of 20% dimethylsulfoxid (DMSO) in TBS for 60 sec, rinsed twice with TBS and incubated at 37°C in DMEM/ 5% FCS for 48 hr.

4.8.3   Namalwa cells.

Namalwa (Human lymphoblastoid) cells were grown in suspension in RPMI-1640 medium (Gibco) supplemented with 10% FCS in an atmosphere of 5% CO_2 at 37°C. Transfections were preformed in 15 ml Falcon tubes using 8×10^6 cells per transfection. The cells were pelleted at 1500 rpm for 5 min and washed twice with 8 ml TBS, after which the cells were gently resuspended in 600 µl 0.5 mg/ml DEAE Dextran (in TBS) containing 2.5 µg test and 2.5µg reference plasmid (30P; Kuhl et al., 1987). After 30 min at room temperature the cells were shocked by adding 1 vol of 12.5% DMSO in TBS for 3 min, rinsed twice with TBS, returned to 10-cm dishes and incubated at 37°C in RPMI-1640/10% FCS for 48 hr.

4.9   Inductions.

48 hr after the completion of transfections cell were either NDV, IFN or mock induced. Induction was with 1000 units/ml of rat IFN-γ (originating from H. Schellekens) or with NDV in serum-free medium (1.2 ml or 3.4 ml per 6-cm and 10-cm plate respectively of a dose previously determined to give maximal IFN production in L929 cells) for 1 hr, whereupon 9 ml of medium supplemented with FCS were added. Total RNA was prepared 8 hr after beginning of induction.

4.10   Isolation of RNA.

Cells were washed with PBS prior to being lysed with 5 ml of lysis buffer (6 M urea, 3 M LiCl, 50 mM NaOAc, 200µg/ml heparin, 0.1% SDS). The lysate was homogenized twice for 1 min on ice at position 5 using a Sorvall omnimixer, transferred to 15 ml Corex tubes and precipitated at 4°C overnight. The precipitate was collected at 10000 rpm, 25 min, 4 °C (Sorvall HB4), washed with 5 ml washing buffer (8M urea, 4 M LiCl), air dried and dissolved in 600 µl 200 mM NaOAc (pH 5), 0.2% SDS, 1 mM EDTA. The RNA was extracted with phenol, phenol/chloroform (1:1) and then chloroform and precipitated overnight with 2 vol EtOH. The RNA pellet was wash once with 80% EtOH, air dried, resuspended in 100-300 µl H_2O and stored at -70 °C (for longer periods it was stored as an EtOH precipitate at -20 °C). RNA concentrations were determined spectrophotometrically.

4.11   S1 Mapping.

Lyophilized RNA (50 µg), was dissolved in 20µl 80% FAHB buffer containing 0.01 pmol of probe, over laid with a drop of paraffin oil, denatured at 65 °C for 15 min and hybridized at 49 °C overnight (usually 12-16 hr). Globin standard RNA was either reticulocyte poly(A)+ RNA titrated as described in Ryals et al. (1985) or rabbit globin RNA (BRL) of which 50% was

assumed to be β-globin (Morrison et al., 1974), in all cases 50 µg of yeast carrier was added and the standards were treated as the test RNA.

S1 digestion was done by adding 250 µl S1 Buffer (250 mM NaCl, 30 mM NaOAc (pH 4.5), 1 mM $ZnSO_4$, 20 µg/ml alkali-denatured carrier DNA) which contained 400 units/ml S1 (Pharmacia) to the hybridization mixture and incubating at 30 °C for 90 min. The reaction was stopped by transferring the reaction mix to tubes containing 300 µl phenol/chloroform (1:1), samples were vortexed and placed on ice until all tubes had been processed. After centrifugation to separate the phases the aqueous layer was EtOH precipitated in the presence of 20 µg yeast carrier RNA, the resulting pellets were air dried and resuspended in 8 µl formamide loading dye.

Samples were denatured in a boiling water bath for 1.5 min, transferred to ice and loaded directly onto a pre-run (2-4 hr) 40x28x0.1 cm, 6% acrylamide (20:1 cross-link), 7 M urea, 50 mM TBE (50 mM Tris-base, 50 mM boric acid, 1 mM EDTA) gel. Gels were run at 1000-1200 volts until the xylene cyanol had reached the bottom. ^{32}P-labeled fragments were visualized by autoradiography.

Radioactivity in test, reference, standard bands and blank areas was determined by Cerenkov counting. After background subtraction, uninduced and induced test signals were normalized to the cognate references signals and strands per cell were calculated using the following relationships:

1. Normalized test signal (cpm) $T^* = \dfrac{T \times (\Sigma R)}{R}$ where T, test signal (cpm); R, reference signal (cpm).

2. strands/cell $= \dfrac{T^* \times 3 \times 10^6}{C \times b}$ where C, number of cells; b, radioactivity (cpm) recovered after S1 mapping 1 pg standard β-globin RNA (equivalent to 3x 10^6 strands).

4.12 Nuclear extracts.

Namalwa cells were grown in 0.5-3 liter spinner flasks to a density of ~1x10^6 cells/ml in RPMI-1640 containing 10% FCS at 37 °C. Nuclear extracts were prepared as described in Dignam et al. (1983) with minor modifications. After resuspending the nuclei, the supernatant was ammonium sulphate precipitated (0.33 g/ml) and resuspended in and dialysed against 20 mM Hepes (pH 7.9), 20% glycerol, 20 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF.

4.13   Preparation of probes for gel retardation assays.

1-3 pmol of single stranded oligonucleotide were 5'-^{32}P-labeled in 10 μl containing 50 mM Tris-HCl (pH 7.5), 6.6 mM MgCl$_2$, 5 mM DTT, 1 mM spermidine, ~0.05 units/μl T4 polynuclease kinase (K. Murray, University of Edinburgh) and a 3-5 fold molar excess of γ-^{32}P-ATP (6 pmol/μl at 5000-7000 Ci/mmol) at 37 °C for 30 min. The reaction was stopped by the addition of 50 μl 10x TNE (which also improves the resolution of the column) and fractionated over a 1.5 ml Sephadex G25/H$_2$O column eluted with H$_2$O. Peak fractions were pooled and lyophilized.

The 5'-^{32}P-labeled oligonucleotides were annealed to a threefold molar excess of their opposite strand in 10 μl 50 mM Tris-HCl (pH 7.5), 6.6 mM MgCl$_2$,5 mM DTT, 1 mM spermidine. The mixture was heated to 85 °C for 10 min and then allowed to cool slowly to room temperature (≥ 3 hr). Probes prepared in such a way usually had a specificity activity of between 2 and 8x10^6 Cerenkov cpm/pmol.

Unlabeled oligonucleotides used in competition experiments were annealed in a similar manner except that equal amounts of both strands were used.

4.14   Gel retardation assays.

Crude nuclear extracts were used where not stated otherwise. Tenfold enriched TG protein (step I) was obtained by heating crude Namalwa nuclear extract at 85 °C for 5 min and removing the resulting precipitate by centrifugation, step II preparations were obtained by affinity chromatography (Kadonaga and Tjian, 1986) on immobilized VREα1 (A. Sailer and D. Maguire unpublished results). Binding reactions for the gel retardation assay (final volume, 10 μl) contained 5'-^{32}P-labeled oligonucleotide, non-specific and specific competitor DNA and nuclear extract as indicated in the figure legends. Incubations were done at room temperature for 30 min in 11 mM Hepes (pH 7.9), 45 mM KCl, 0.2 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 4% Ficoll 400, 2% glycerol. After adding 2 μl of loading dye (4% Ficoll 400, 10 mM Hepes (pH 7.9), 0.1% bromophenol blue) the samples were loaded onto a 20x20x0.1 cm, 4% polyacrylamide gel (30:1 cross-link), 25 mM TBE (25 mM Tris-base, 25 mM boric acid, 1.25 mM EDTA) gel, pre-run for 1 hr at 4 °C. Electrophoresis was for 2-2.5 hr at 4 °C, 250 volts until the bromophenol blue was ~5 cm from the bottom of the gel. Gels were transferred to Whatman 3MM paper, fixed for 10 min in 10% acetic acid/5% methanol before being dried under vacuum at 60 °C for 1.5 hr. For quantitation, bands were cut from the dried gel.

4.15   Methylation interference analyses.

5'-^{32}P-labeled oligonucleotides were partially methylated with dimethyl sulphate for between 4 and 8 min at room temperature (Siebenlist and Gilbert, 1980) and used as probes in scaled up gel retardation binding reaction (between 10 and 20 times). After

electrophoretic separation over a conventional band shift gel (see Section 4.14) the DNA in the protein /DNA complex (retarded band) and the free DNA were excised from the polyacrylamide gel. The excised gel fragments were placed in 10 ml Greiner tubes, crushed with a glass rod and the DNA eluted overnight while agitating with 8 ml of 150 mM NaCl/TE (150 mM NaCl, 10 mM Tris-HCl (pH 8), 0.5 mM EDTA) at room temperature. The DNA was purified by DEAE cellulose (DE 52, Whatman) chromatography and EtOH precipitated.

Alkali cleavage (G>A reaction) was performed by dissolving the pellet in 20 µl of 10 mM $NaPO_4$ (pH 7), 1 mM EDTA, incubating 90 °C for 10 min, chilling on ice, adding 100 µl 100 mM NaOH, 1 mM EDTA and continued to incubate at 90 °C for an additional 30 min. The pH was adjusted to 7 with 25 µl 1 M Tris-HCl (pH 7.5), 25 µl 2M NaOAc (pH 7), and 100 µl 200 mM NaOAc (pH 4.7) and the DNA EtOH precipitated in the presence of 10 µg yeast carrier RNA. Pellets were resuspended in formamide loading dye and the products separated on 40x28x0.03 cm, 12-18% polyacrylamide (30:1 cross-link), 7 M urea, 50 mM TBE gels.

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