

STRUCTURE-FUNCTION STUDIES OF THE HUMAN INTERFERON GAMMA

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the Requirements for the Degree of
Doctor of Philosophy

By
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ABSTRACT

The present study involves the investigation of the structure-function relationships of the human interferon gamma (HuIFN- γ). This study is divided into two parts. The first part deals with the exclusion of primary sequences from the carboxy-terminus (C-terminus) that are not involved in conferring the biological activities of the HuIFN- γ . The second part involves the investigation of the structural and/or functional importance of the individual conserved amino acid residues of the HuIFN- γ .

In the first part, deletion variants were either isolated as a proteolytic cleavage product or constructed by specific gene modifications. Two of the variant polypeptides which had, respectively, 15 and 21 amino acid residues deleted from the C-terminus were successfully expressed in Escherichia coli and were purified by immunoaffinity chromatography to homogeneity. These two variant polypeptides were shown to have specific activities comparable to the parental HuIFN- γ molecule. Thus, the functional site(s) of the HuIFN- γ must be located in the N-terminal 122 amino acid residues. The variant which had 21 residues deleted was treated with guanidine hydrochloride and was subsequently renatured in vitro. It was found that the antiviral specific activity of the renatured molecule was significantly reduced. Thus, although the C-terminus of HuIFN- γ is not involved in conferring its biological activities, this region may play a positive role in the proper folding of the IFN molecule in vitro. This may explain earlier reports which showed that recombinant deletion variants which had as few as 11 residues deleted from the C-terminus and which were extracted with chaotropic agent showed substantially diminished

specific activities. Deletion variant polypeptides which had, respectively, 26, 32, and 37 amino acid residues deleted from the C-terminus exhibited reduced intracellular accumulation and attempts to purify these polypeptides were unsuccessful. The biological activities of these latter variants were undetectable at the limit of sensitivity of the antiviral assay. It was estimated that the antiviral activity of these variants must be at least 1000-fold less than that of the parental full-length molecule to be undetectable. Thus, deletions of more than 21 residues from the C-terminus might have caused structural instability of the molecules which renders these molecules susceptible to proteolytic degradation inside the bacterial cell.

In the second part, single-site variants were constructed by site-directed mutagenesis. These variants were constructed based on analyses of primary sequences that are evolutionarily conserved, regions that are surface-exposed (hydrophilic), and areas that consist of conserved secondary structures. Four single-site variants were constructed. These variant polypeptides were purified by immunoaffinity chromatography. The first variant, which was constructed by the substitution of Asp⁴¹ for Ala, was found to have significantly reduced antiviral specific activity as well as diminished intracellular accumulation. Secondary structure analysis showed that a conserved β -turn was disrupted as a result of this substitution. Since this β -turn is highly exposed and is conserved among IFNs- γ from various vertebrate species, it is likely to play an important role in intermolecular interactions. A second variant was constructed by replacing Asp⁴¹ with Asn. This variant retained the conserved β -turn structure; however, the local hydrophilicity was reduced and the negative charge on the carboxyl group of Asp⁴¹ was abolished. This purified variant polypeptide was found to have antiviral specific activity comparable to the unmodified

molecule. Thus, it is likely that the whole conserved β -turn rather than individual residues that is essential for conferring the full biological activities of HuIFN- γ . Two variants were also constructed to study the possible influence of the adjacent secondary structures on the conserved β -turn. Two residues, Gln⁴⁶ and Gln⁴⁸, were individually replaced by Leu. Hydrophilicity and secondary structure analyses showed that these two substitutions resulted in the reduction of the local hydrophilicity without any alteration in the local secondary structures. Unexpectedly, these purified variant polypeptides were found to have approximately 30-fold increase in antiviral specific activity. These two amino acid substitutions might have enhanced the hydrophobic interactions between the IFN molecule and the effector molecule(s) and/or the cell-surface receptor.

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CHAPTER 1 INTRODUCTION

1.1 Classification

The interferons (IFN) are a family of glycoproteins first discovered by their ability to induce resistance to virus infection (an antiviral state) in susceptible cells (Isaacs and Lindenmann, 1957). At least 3 distinct classes of interferons have been identified. They are the α -, β -, and γ -interferons. Since IFNs- α and IFNs- β are induced by the same type of inducers, namely, viruses and double-stranded RNA (dsRNA), they have also been referred to as type I IFN, whereas IFN- γ has been classified as type II IFN which is predominantly induced by various mitogens. At least 15 IFN- α molecules, which have 70-90 % amino acid sequence homology over 166 amino acids, have been identified based on their immunological relatedness. Two IFNs- β have also been identified: IFN- β_1 and IFN- β_2 . While IFN- β_1 shares 30-40 % homology with the IFNs- α , IFN- β_2 does not appear to belong to the same type of IFN based on sequence homology. Only one IFN- γ has been identified and it appears to be encoded by a single gene. The IFNs- α are predominantly produced by leukocytes, IFNs- β are produced by fibroblasts, and IFN- γ is produced mainly by activated T-lymphocytes.

1.2 Mechanism of antiviral action

The antiviral state induced by IFN involves multiple pathways. One of these pathways involves inhibition of viral protein synthesis. Two enzyme systems are believed to play an important role in this mode of antiviral actions of IFN: (1) the (2'-5')-oligoadenylate [2,5-A_n] synthetase - nuclease system and (2) the P1/eIF-2a protein kinase - phosphoprotein phosphatase system. While both the (2-5)(A)_n synthetase and the p1/eIF-2a protein kinase are induced by interferon, they have to be activated with either natural or synthetic double-stranded RNA (dsRNA). Interferon treatment alone does not cause a significant increase of intracellular (2',5')-oligoadenylates or the extent of eIF-2a phosphorylation, whereas interferon treated cells that were either activated by dsRNA or were infected by various viruses resulted in activation of these two enzyme systems (Pestka et al., 1987). In addition to the two enzyme systems, the MHC class I and II molecules are also induced by IFN and have been shown to play important roles in enhancing cell-mediated immunity during viral infection. Other IFN-inducible proteins such as the Mx protein likely represent novel antiviral mechanisms that are yet to be elucidated.

1.2.1 The (2'-5')-Oligoadenylate Synthetase - Nuclease System

First discovered in the studies of rates of cleavage of reovirus mRNAs in extracts from IFN-treated EAT (Ehrlich Ascites Tumor) cells (Brown et al., 1976; Ratner et al., 1978; Lengyel 1981), the (2'-5')-oligoadenylate synthetase - nuclease system comprises three enzymes: the 2,5-

A_n synthetase that catalyzes the formation of oligonucleotides possessing 2',5'- phosphodiester bonds; the 2,5- A_n -dependent endoribonuclease (RNase L) that is activated by certain 2,5- A_n structures; and the (2',5') phosphodiesterase that catalyzes the hydrolysis of oligonucleotides possessing 2',5'-phosphodiester bonds (Pestka et al., 1987).

All three classes of IFN's (α , β , and γ) are able to induce 2,5- A_n synthetase in a variety of cells. The level of induction depends on the types of IFN, cell types, and growth state of cell. The enzyme is activated by dsRNA to catalyze the synthesis of a family of oligonucleotides of the general structure ppp(A2'p) $_n$ A with $n \geq 2$, abbreviated 2,5- A_n . The only well established biochemical function of 2,5- A_n is its ability to activate another enzyme, RNaseL. RNaseL is an endoribonuclease and is present as a latent protein in both untreated and IFN-treated cells. When activated, RNase L is able to cleave various single-stranded viral RNA as well as cellular mRNA. Double-stranded RNAs are, however, not cleaved (Ratner et al., 1977). The activated enzyme catalyzes the cleavage of single-stranded RNAs on the 3' side of -UpXp- sequences (predominantly UA, UG, and UU) to yield products with -UpXp 3'-termini. It was shown in the EMC virus - HeLa cell system that virus infection without IFN treatment functionally inactivates the 2,5- A_n -dependent RNase (Silverman et al., 1982). The appearance of 2,5- A_n oligonucleotides induced by interferon is only transient. This is due to the presence of another enzyme, the 2',5'-phosphodiesterase, that specifically degrades 2,5- A_n to yield AMP and ATP, thereby returning the activated 2,5- A_n -dependent RNase to an inactive state.

Although the activation of the 2,5- A_n synthetase - nuclease system has been implicated as one of the mechanisms that establishes the antiviral state in IFN-treated cells, the extent to which

this system may be involved in the antiviral actions is a question yet to be resolved. There is evidence to suggest that a functional 2,5- A_n system is neither sufficient nor required for the antiviral action of IFN against a variety of different viruses that are sensitive to IFN (Hersh et al., 1984; Kingsman and Samuel, 1980; Masters and Samuel, 1982; Ulker and Samuel, 1985; Whitaker-Dowling and Youngner, 1986; Rice, et al., 1984). Others have suggested that the 2,5- A_n pathway may play a more important role in events associated with cell-cycle progression (Wells and Mallucci, 1985) and cell differentiation (Krause et al., 1985; Michel and Chebath, 1986) rather than antiviral responses. The concentration of 2,5- A_n synthetase was found to increase during maturation of T-lymphocytes, erythroid cells, and monocytes (Michel and Chebath, 1986) and that the rise in 2,5- A_n synthetase was due to the production of endogenous IFN (Yarden et al., 1984).

1.2.2 The P1/eIF2a Protein Kinase - Phosphoprotein Phosphatase System

The process of phosphorylation-dephosphorylation has long been known to be a major mechanism for the regulation of intracellular events in eukaryotic cells (Alberts et al., 1989). Studies of the antiviral actions of IFN revealed the presence of a protein kinase - phosphatase system that was induced by IFN (Lengyel 1982). This system consists of at least two major protein substrates and two enzymes: (1) a ribosome associated protein, P1, (Lebleu et al., 1976; Zilberstein et al., 1978; Roberts et al., 1976), (2) the smallest subunit of the protein synthesis initiation factor eIF-2, eIF-2a (Farrell et al., 1978; Samuel, 1979), and (3) a protein kinase (Sen

et al., 1978; Kimchi et al., 1979) and a phosphoprotein phosphatase (Kimchi et al., 1979; Crouch and Safer, 1980) that regulate the phosphorylation state of P1 and eIF-2a.

The P1/eIF2a protein kinase - phosphoprotein phosphatase system was first discovered during the course of studying reovirus infection of IFN-treated cells (Lebleu et al., 1976). It was found that addition of either dsRNA from reovirus or poly(I).poly(C) to extracts from IFN-treated EAT cells or L cells resulted in phosphorylation of P1 and eIF-2a. The dsRNA-dependent protein kinase responsible for the phosphorylation can be induced by both type I and type II IFNs. The IFN-induced protein kinase is dependent upon dsRNA for activation (Berry et al., 1985). The mechanism by which the dsRNA activated protein kinase inhibits viral protein translation involves the phosphorylation of eIF2a which is rendered inactive due to the phosphorylation. Studies with cell-free protein synthesizing systems have shown that the P1/eIF2a protein kinase can efficiently inhibit the synthesis of protein in vitro. There is evidence to suggest that P1, which is induced by IFN, itself possesses the dsRNA-dependent protein kinase activity (Samuel, 1985). The phosphorylation of P1/eIF2a is reversible. Phosphoprotein phosphatase(s) was shown to dephosphorylate both P1-P and eIF-2a-P in untreated and IFN-treated cells (Kimchi et al., 1979; Crouch and Safer, 1980; Samuel and Knutson, 1982), thereby returning the cell to its uninduced state.

1.2.3 Major Histocompatibility Complex (MHC) Antigens

Antigen recognition by T lymphocytes is accomplished by binding of the antigen to antigen receptors in association with the MHC molecules on the cell surface (Kindred and Schreffler, 1972; Zinkernagel and Doherty, 1975; Katz and Benacerraf, 1975). Expression of these cell surface molecules is essential for cell-mediated immunity. The classical work of Zinkernagel and Doherty (1974) provided the first evidence that T-cell mediated cytotoxicity of viral infected cells is MHC restricted. It was observed that lymphocytic choriomeningitis virus (LCMV) infected target cells could only be killed by cytotoxic T cells (Tc) of the same haplotype (MHC class I H2-K or -D compatible). The restriction of Tc by the MHC class I molecules has since been confirmed in both mouse and human viral infections (Sissons and Oldstone, 1985). Cell mediated cytotoxicity of influenza virus infected cells has been shown to be both MHC class I and class II restricted (Braciale et al., 1987). Enhanced MHC antigen expression induced by IFN may also contribute to antiviral actions of IFN *in vivo* (Tominaga et al., 1985) through the enhancement of the antigen specific lytic effect of cytotoxic T cells. In addition to its role in cell-mediated cytotoxicity, IFN may be involved in the induction of MHC class II antigens on antigen presenting cells (APC). APC such as macrophages can only effectively present antigen to T helper cells when both express the same MHC class II antigens. Most macrophages do not express these antigens constitutively (Unanue, 1981) and have to be stimulated into expression of the class II antigens before they can act as accessory cells in presenting antigens to activated T lymphocytes.

Both type I and type II IFNs are able to induce the class I and class II major histocompatibility complex (MHC) antigens on a variety of different cell types (Heron et al., 1978; Basham et al., 1982; Fellous et al., 1982; Wallach et al., 1982; Yoshie et al., 1982; Satz and Singer, 1984; Burrone and Milstein, 1982; Collins et al., 1984; Dani et al., 1985). Cells of the immune systems (Wong et al., 1982; King and Jones, 1983; Koeffler et al., 1984; Capobianchi, et al., 1985) as well as nonimmune cells are able to express class II antigens upon induction by IFN- γ (Poher et al., 1983; Collins et al., 1984; Goldring et al., 1986; Wong et al., 1984; Capobianchi et al., 1985). Although some studies have shown that IFN- γ was more effective than IFN- α and IFN- β in the induction of class II antigens (Basham and Merigan, 1983; Zlotnik et al., 1983; Kelley et al., 1984; Virelizier et al., 1984), other studies showed that IFN- α and IFN- β were equally effective in stimulating these antigens (Kim et al., 1983; Dolei et al., 1983). Therefore, the effect of both classes of IFNs on class II expression probably depends on the types and stages of differentiation of the cells. This question is further complicated by the data of Rosa et al. (1983) who found that there was little correlation between the increase of class II antigen mRNA and the corresponding antigens expressed at the surface of cells treated with types I and II IFNs, suggesting that other regulatory mechanisms were involved.

1.2.4 Mx proteins

It is known that the antiviral state induced by IFN against influenza virus infection in the mouse is genetically determined. It was originally discovered that an inbred mouse strain A2G

had an inborn resistance to infection by influenza virus (Lindenmann et al., 1963). At least two genes in the mouse, designated Mx1 and Mx2, are believed to be responsible for the resistance. These genes have been cloned (Staeheli et al., 1986; Staeheli and Sutcliffe, 1988) and the cDNAs encode a 72 kD (Mx1) and a 74 kD (Mx2) polypeptide that are found to accumulate in the nucleus (Dreiding et al., 1985; Meier et al., 1988). The Mx1 gene has been mapped to the distal part of mouse chromosome 16 (Staeheli et al., 1986; Reeves et al., 1988). Chromosomal analysis has shown that the wild-type Mx1 gene consists of 14 exons distributed over at least 55 kb (Hug et al., 1988). Influenza virus-susceptible mouse strains were found to have mutations in the Mx1 gene which result in the failure of synthesis of the Mx1 gene product. The Mx phenotype of most inbred strains, including BALB/c, is due to deletion of Mx1 exons 9 through 11, whereas that of strain CBA arises from a nonsense mutation to exon 10 (Staeheli et al., 1988). The Mx2 gene was found to be expressed only in influenza virus-susceptible mice and could not be detected in influenza virus-resistant mice that contain the functional, nonmutated Mx1 alleles (Staeheli et al., 1988). However, in both cases, the Mx2 appeared to be nonfunctional (Staeheli and Sutcliffe, 1988). The latter results suggested that Mx2 could be a pseudogene and may not have physiological importance in the mouse. The Mx proteins are induced by IFN- α and - β but not by IFN- γ .

Recently, the human counterparts of the mouse Mx genes have been cloned (Aebi et al., 1989). Using the mouse Mx1 cDNA as a hybridization probe, two human Mx genes, designated MxA and MxB, were isolated from human fetal lung cells and the human glioblastoma cell line T98G. The MxA and MxB cDNAs encode two polypeptides with molecular masses of 76 and

73 kD, respectively. The expression of these genes was stimulated by IFN- α , IFN- β , Newcastle disease virus, and, to a lesser extent, IFN- γ . The Mx proteins of humans and mice have overall similarities of between 56 and 77% in amino acid contents. The human MxA and the mouse Mx2 are most closely related and are probably encoded by homologous genes, whereas the MxB and the Mx1 have relatively low degree of homology. In contrast to the mouse Mx proteins, which are nuclear proteins (Dreiding et al., 1985), the human Mx gene products could only be detected in the cytoplasm (Aebi et al., 1989). The significance of this difference is not yet known. It is interesting to note that of the three Mx proteins identified (encoded by three Mx-related genes) in the rat, two have also been found to localize in the cytoplasm (Meier et al., 1988). The molecular basis for the antiviral action of Mx is not known.

1.3 Mechanism of antiproliferative actions

The antiproliferative activity of IFN for cells was first recognized when proliferation of different cell types in culture were inhibited upon exposure to crude preparations of IFN (Paucker et al., 1962). Both type I and type II IFNs are able to inhibit growth of a variety of cell types with different sensitivities. IFN- γ has been shown to have more potent antiproliferative effect than that of IFN- α or IFN- β (Crane et al., 1978; Rubin and Gupta, 1980; Fleishmann, 1982). In addition, synergistic effects of antiproliferation between MuIFN- γ and either MuIFN- α or - β was observed suggesting that the two types of IFNs may have different mechanisms of action (Pestka et al., 1987).

A number of studies have shown that the antiproliferative action of IFN could affect all phases of the cell cycle (Clemens and McNurlan, 1985). It is generally assumed that IFN inhibits cell proliferation by impairing DNA synthesis. In fact, one of the most common assays for antiproliferation involves measurement of [³H]-thymidine uptake. However, these data could be complicated by the fact that membrane transport of thymidine and/or its subsequent intracellular phosphorylation by thymidine kinase are inhibited by IFN in some cell types (Clemens and McNurlan, 1985). In addition, cells that showed impaired uptake of thymidine could have normal incorporation of deoxyadenosine (Gewert et al., 1983). Human fibroblasts, for instance, have been shown to continue incorporating thymidine in the presence of IFN (Pfeffer et al., 1977). There were also reports of IFN-treated cells showing lower DNA polymerase activity (Lundblad and Lundgren, 1981; Lundblad and Lundgren, 1982) and delay in DNA ligation of Okazaki fragments (Moore et al., 1984), suggesting that the antigrowth effects of IFN were due to subtle disruptions of a series of steps in the DNA replication process rather than inhibition of entry into the S phase. However, these data should be interpreted with caution since it cannot be established whether these phenomena were the cause, or the effect of, the antiproliferative function of IFN.

There are relatively few reports regarding the antiproliferative activity of IFN as a result of inhibition of protein synthesis. While some studies showed a reduction of incorporation of radioactive amino acids in cells treated with IFN, other sensitive cell types showed very little inhibition of protein synthesis (Clemens and McNurlans, 1985). Although the role of 2,5-A_n has been implicated for the inhibition of protein synthesis in virus infected cells, its role in the antiproliferative action of IFN remains to be established. Results that showed an inverse

correlation between cell proliferation and levels of 2,5-A_n synthetase (Kirshnan and Baglioni, 1980, 1981; Etienne-Smekens et al., 1983; Kimchi et al., 1981; Jacobson et al., 1983; Creasey et al., 1983) contrast to reports that showed no correlation between these two parameters (Verhaegen et al., 1980; Hovanessian et al., 1980; Vandenbussche et al., 1981; Verhaegen-Lewalle et al., 1982; Silverman et al., 1982; Tomita et al., 1982; Tovey et al., 1983; Chapekar and Glazer, 1983; Chapekar and Glazer, 1984). It cannot be excluded, however, that localized effects of 2,5-A_n synthetase may play a role in antiproliferation. For instance, it has been shown that both 2,5-A_n synthetase and the protein kinase were activated in a compartmentalized manner in the vicinity of dsRNA (Nilsen and Baglioni; 1979; De Benedetti and Baglioni, 1984).

Recent reports by Ozaki et al. (1988) showed that induction of the enzyme indolamine 2,3-dioxygenase (IDO) may play a role in the antiproliferative effects of IFN. IDO, utilizing reduced flavin and the superoxide anion, catalyzes the oxygenative decyclization of L-tryptophan to form N-formyl-L-kynurenine (references 30-32 in Ozaki et al., 1988). This enzyme has been found in a variety of mammalian tissues (references 22-26 in Ozaki et al., 1988) and is induced by viral infection or treatment with IFN's in normal and malignant tissues (references 27-29 in Ozaki et al., 1988). The antiproliferative effects of IFN- γ were found to be cell dependent: causing a greater antiproliferative effect against cell lines that exhibited induction of IDO, such as KB oral carcinoma or WiDr colon carcinoma, than against those that lacked the enzyme activity, such as Sw489 colon adenocarcinoma or NCI-H128 small-cell lung carcinoma. Correlation of IDO with increased metabolism of L-tryptophan was shown by the depletion of this amino acid in the culture medium. While 70-80% of L-tryptophan remained in the medium

of IFN- α - or mock-treated cells, virtually all of this amino acid was depleted in the medium of the IFN- γ -treated group following 2-3 days of culture. Supplementing the growth medium with additional L-tryptophan reversed the antiproliferative effect of IFN- γ against KB cells in a dose- and time-dependent manner. However, tryptophan deprivation only offers an explanation for the short term antiproliferative effects of IFN- γ since cells after prolonged treatment with IFN- γ did not respond to supplementation of tryptophan. Therefore, long-term inhibition may be due to irreversible changes in cellular metabolism. The induction of IDO in monocytes may be responsible for the substantial reduction in serum L-tryptophan concentration observed in humans after the administration of IFN- γ (Datta et al., 1987). Recently, IFN- γ resistant human cervical carcinoma cell lines (ME180) have been isolated that were defective in the induction of IDO (Feng and Taylor, 1989). Studies of these mutant cell lines should provide further insight into the antiproliferative mechanism of IFN- γ .

The antiproliferative activities of IFN- γ have made this cytokine one of the major candidates in the search for potential antitumor therapeutic agents. The antitumor effects of IFN- γ on virally induced tumors could, at least partially, be explained by its ability to induce the expression of MHC class I antigens. It was shown that adenovirus type 12 induced tumor could be suppressed when treated with IFN and that the tumor suppression effect was likely due to augmentation of immune surveillance mediated through the upregulation of MHC class I antigens by IFN (Hayashi et al., 1985; Tanaka et al., 1987). Results from Taniguchi et al. (1987), on the other hand, have shown that enhancement of MHC-class I antigen by IFN- γ treatment resulted in the increase of metastatic ability of melanoma cells. It has also been shown that in human

melanoma, metastasis and poor prognosis are associated with high MHC class II expression in the primary tumor (Van Duinen et al., 1984). Therefore, the parameters responsible for the contrasting effects of IFN- γ on different tumors need to be defined in order for the successful use of IFN- γ for immunotherapy which would likely depend on the forms of malignancy. The efficacy of IFN as an antitumor agent would require not only better understanding of its immunoregulatory functions but also the interactions of effector cells responsible for tumor rejection.

1.4 Cell surface receptor for interferon gamma and mechanisms of signal transduction

1.4.1 Receptor for Interferon Gamma

The cell surface receptor for interferon gamma seems to serve at least three major purposes. First, the binding of IFN to the receptor transduces intracellular signals that are responsible for the various biological responses induced by this lymphokine. Second, it provides a means by which the interferon molecule is internalized. Internalization is essential for the establishment of activation of macrophages (Fidler et al., 1981; Fidler et al., 1985). It was shown that only liposome-encapsulated HuIFN- γ , but not free HuIFN- γ , could activate the tumoricidal activities of mouse peritoneal exudate macrophages. Third, the receptor provides a barrier that prevents cross-species binding. It has been shown that species specificity could be abrogated if the interferon molecule was encapsulated in liposomes (Fidler et al., 1985).

Different cell types may possess different forms of receptors for interferon gamma. It has been shown that IFN- γ receptors in HeLa cells and monocytes not only differed in size and molecular form but also differed in the internalization pathways (Rubinstein et al., 1987). In the case of HeLa cells, the IFN-receptor complex is dissociated in the endosome and the receptor is then recycled to the surface of the cell. The recycling is not affected by protein synthesis. Therefore, the IFN- γ receptor in HeLa cells serves as a "shuttle" which internalizes the ligand continuously. Similar results were observed in fibroblasts (Anderson et al., 1982; Rubinstein et al., 1987). On the other hand, IFN- γ receptors on monocytes are degraded rapidly once they are internalized. Therefore, these receptors are "down-regulated" as they are internalized. Nevertheless, the IFN- γ internalized via the two different pathways appears to be degraded in all of the aforementioned cell types. Therefore, the degradation pathway for IFN- γ itself seems to be similar in these different cell types.

The differences in the receptor cycling pathways may reflect different responses in various cell types to IFN- γ treatment. It has been shown that monocytes do not establish an antiviral state on exposure to IFN- γ (Orchansky et al., 1986). Since the induction of an antiviral state requires a prolonged (at least 9 hours) exposure to IFN- γ , the lack of response in monocytes may be due to the down-regulation of receptors during this period. It was therefore hypothesized that this may serve as one of the mechanisms that prevent immune cells from over-reacting in an inflammatory site (Rubinstein et al., 1987).

Recently, the cDNA for the HuIFN- γ receptor has been cloned (Aguet et al., 1988). The cDNA has the potential of encoding a protein of 489 amino acids. In addition, a 17 amino acid-

long putative signal peptide and a hydrophobic transmembrane region was also predicted. The discrepancy between the M_r of 54,000 as predicted from the cDNA deduced amino acid sequence and the apparent M_r of 90,000 for the purified natural receptor protein is likely due to the latter being glycosylated. From the deduced amino acid sequence, five potential N-linked glycosylation sites could be identified. Since 12% of the all the amino acids are serines, there is a potential for O-linked glycosylation. The receptor protein appears to have an extracellular N-terminus which likely carries the ligand binding site, while the 212 amino acid-long intracellular portion is presumably involved in signal transduction. In agreement with an earlier report (Rashidbaigi et al., 1986), the HuIFN- γ receptor gene was mapped to the long arm of chromosome 6. When the cloned human receptor gene was transfected into mouse cells, the human receptors were found to correctly express on the cell surface. The dissociation constants of these receptors were also found to be comparable with those on other human cells. However, none of these transfectants was able to enhance expression of MHC class I antigens nor the induction of 2-5A_n synthetase upon treatment with HuIFN- γ . Therefore, the expression of the HuIFN- γ receptor gene in heterologous cells is apparently not sufficient to elicit the biological responses to HuIFN- γ . This seems to confirm an earlier report by Jung et al. (1987) who showed that, in addition to human chromosome 6, chromosome 21 in human cells was also required for response to HuIFN- γ and that the factors encoded by these two chromosomes were species specific. It is interesting to note that an earlier unconfirmed report which demonstrated that the HuIFN- γ gene expressed intracellularly in mouse cells could establish an antiviral state (Sanceau et al., 1987), suggesting that an intracellular receptor or binding factor for HuIFN- γ is involved in signal transduction.

1.4.2 Mechanisms of Signal Transduction

The binding of a specific ligand to its cell surface receptor initiates a variety of intracellular signals that lead to rapid execution of functions and regulations of gene expression. These events could be rapid (within seconds or minutes), or intermediate in time (within minutes to hours), or slow (within hours to days) (Adams and Hamilton, 1987).

A number of rapidly induced biochemical products have been described that have the potential of acting as "second messengers". These include production of the cyclic nucleotides cAMP and cGMP (Fesenko et al., 1985; Yau and Nakatani, 1985; Matthews et al., 1985; Cobb and Pugh, 1985; Neer and Clapham, 1988); hydrolysis of polyphosphoinositides which leads to release of inositol 1,4,5 triphosphate (Berridge, 1985), increase of intracellular Ca^{++} , and the generation of diacylglycerol which in turn stimulates protein kinase C (Nishizuka, 1984, 1986, 1988); opening of ion channels in the membrane, leading to changes in intracellular concentrations of Na^+ , K^+ or Ca^{++} or alteration of intracellular pH (Alberts et al., 1989); and stimulation of autophosphorylation of tyrosine of the receptor (reviewed by Jove and Hanafusa, 1987). These rapid events may be followed by intermediate events that include activation of various enzymes and proteins due to changes in pH or level of ions; and activation of enzymes and proteins by covalent modifications such as phosphorylation; and expression of specific genes whose products may function to regulate other genes. The slow events usually involve manifestation of various cellular functions resulting from the activation of the intermediate specific gene products.

Signal transduction events induced by IFN- γ are not well understood. Among the limited number of studies, most have focused on the rapid and intermediate events. There have been reports of elevation of intracellular diacylglycerol and inositol triphosphate in fibroblasts and human B-cell-like lymphoblastoid cells (Yap et al., 1986) within 30 s after treatment with IFN- γ . However, the requirement of 20,000 units/ml of IFN raises questions concerning the physiological significance of such observation. Recently, observation of influx of Na⁺ in murine macrophages treated with IFN- γ has been reported (Prpic et al., 1989). It was shown that IFN- γ treatment was able to raise the cytosolic pH by 0.1 unit within 1 min. The findings that alkalination was inhibited by amiloride suggested the involvement of the Na⁺/H⁺ antiporter. The influx of Na⁺ was also inhibited by amiloride. Both the increase of cytosolic pH and Na⁺ influx could be induced by 1 unit/ml and 0.1 unit/ml of IFN- γ , respectively. The two effects were correlated with expression of two IFN-inducible cellular genes, JE and I-A _{β} . The gene induction was apparently due to cytosolic alkalination, since monesin was ineffective in inducing JE, whereas significant accumulation was observed on a pharmacologic increase in cytosolic pH. Observation of IFN- γ stimulated cytosolic alkalization was also reported in a pre-B lymphocyte cell line (Smith et al., 1988).

In addition to the early signals described above, studies on the IFN- γ induced transcription in macrophages suggested that the activation process may be mediated by protein kinase C (PKC) (reviewed by Adams and Hamilton 1987; Hamilton et al., 1985; Fan et al., 1988). The macrophage-specific gene, γ .1, could be detected within 20 min upon addition of IFN- γ and accumulated thereafter to maximum level in 3 h. The transcriptional activation of γ .1 was found

to correspond remarkably well with the kinetics and dose dependence of PKC translocation in IFN- γ treated macrophages. Activation of PKC by phorbol esters, such as PMA, is due to the induction of translocation of the enzyme from cytosol to membrane (Nishizuka, 1986). Two PKC inhibitors, H7 and sphingosine, were also shown to inhibit the transcriptional activation of the γ .1 gene induced by IFN- γ and by PMA in a dose-dependent manner. Activators of cAMP- or cGMP-dependent kinases either inhibited the induction of γ .1 mRNA or had no effect. However, not all genes that have been reported to be transcriptionally activated by IFN- γ were inhibited by PKC inhibitors. For instance, while HLA-DR or IP-30 (Luster et al., 1988) were inhibited by H7, IP-10 (Luster and Ravetch, 1985) was not affected by sphingosine (Fan et al., 1988). Therefore, PKC is likely to be involved in the signal transduction pathway leading to transcriptional activation of some, but not all, IFN- γ -induced genes.

1.5 Interactions of interferon gamma with other cytokines

1.5.1 Interactions with interleukin 1 (IL-1)

Interleukin-1 is one of the many soluble immunoregulatory molecules produced by LPS-activated monocytes which, when stimulated by LPS and IFN- γ , are capable of presentation of antigens to T lymphocytes. IL-1 has been shown to have a broad spectrum of activities including induction of lymphokine release from activated T cells (Gillis and Mizel, 1981; Oppenheim et al., 1986), increase in natural killer cell activity (Oppenheim et al., 1986) and in the presence of

B-cell growth factor, proliferation of B-cells (Falcoff et al., 1983; Pike and Nossal, 1985). It has also been known to be one of the endogenous pyrogens (Oppenheim et al., 1986).

The production of IL-1 is regulated by other lymphokines such as IFN- γ . Fresh monocytes gradually lose their ability to produce IL-1. These cells can be re-stimulated to secrete IL-1 by IFN- γ in combination with LPS (Arenzana-Seisdedos et al., 1985). Although it was found that IL-1 β mRNA was increased in the presence of IFN- γ /LPS/CHX, there was no correlation of mRNA level and IL-1 production (Arend et al., 1988).

Opposing effects of IFN- γ and IL-1 have been described. IFN- γ -induced Fc receptor expression on human monocytes was shown to be inhibited by IL-1 and tumor necrosis factor (TNF) produced by the same cells (Arend et al., 1987). In other cell systems, it was shown that secretion of type I and III collagen and of fibronectin by rheumatoid synovial fibroblasts was stimulated by IL-1 (Krane et al., 1985) and inhibited by IFN- γ (Amanto et al., 1985). On the other hand, IL-1 and IFN- γ share many biologic activities including induction of IL-2 receptors on NK cells (Shirakawa et al., 1986), stimulation of expression of MHC class I molecules on tumor cells (Lovett et al., 1986), and enhancement of production of intercellular adhesion molecules (Dustin et al., 1986). However, the molecular mechanisms of the interactions between these two cytokines are poorly understood.

1.5.2 Interactions with interleukin 2 (IL-2)

The immunoregulatory functions of interferon gamma are exemplified by its regulation of helper T cells. It was shown that human peripheral T cells, but not non-T cells, expressed receptors for IL-2 when treated with purified HuIFN- γ (Johnson and Farrar, 1983). The IFN- γ -induced expression of IL-2 receptors is blocked by treatment of IFN- γ with anti-IFN- γ antibody or by destruction of IFN- γ at acid pH. The observations that IL-2 could directly induce untreated mouse and human lymphocytes to produce small amounts of IFN- γ early in culture without additional stimulation provided by mitogen or antigen (Handra et al., 1983; Kawase et al., 1983; Weigent et al., 1983; Yamamoto et al., 1982) suggested the action of a positive feedback amplification loop. Interleukin 2, acting on a small populations of cells, would induce the production of IFN- γ , which in turn would enhance the expression of IL-2 receptors on cells. The expression of these receptors could thus augment the helper effects of IL-2 in further IFN- γ production and at the same time could commit lymphocytes to cell-cycle progression when stimulated by IL-2.

Both IL-2 and IFN- γ were also shown to play an important role in B cell proliferation and differentiation. It has previously been demonstrated that rIFN- γ did not have any direct effect on either proliferation and differentiation of B cells (Nakagawa et al., 1985). However, in combination with IL-2, antibody secretion was promoted in Staphylococcus aureus (SA)-induced tonsillar B cells. Therefore, IFN- γ appeared to play an amplifying role in B cell activation, but the mechanisms of its action are not known. Studies carried out by Jelinek et al. (1986) showed

Table 1.1 Production of lymphokines in mouse T cell clones

	T _H 1	T _H 2
IL-2	+	-
IFN- γ	+	-
IL-3	+	+
GM-CSF	+	+
IL-4	-	+
IL-5	-	+

Adapted from Miyajima et al., 1988.

that IFN- γ could play at least two distinct roles in B cell activation. First, it could deliver a signal during initial activation with killed SA that augmented subsequent proliferation and generation of antibody secreting cells supported by IL-2. Second, IFN- γ could amplify the capacity of IL-2 to promote differentiation of antibody secreting cells but not proliferation of B cells activated initially by SA in the absence of T cell influences.

1.5.3 Interactions with interleukin 4 (IL-4)

Interleukin 4 was first discovered by its ability to promote B cell growth (Howard et al., 1982) and later found to have additional effects on small resting B cells, such as the induction of MHC class II antigens (Noelle et al., 1984; Roehm et al., 1984), induction of a low-affinity receptor for IgE (also known as CD23) (Kikutani et al., 1986; DeFrance et al., 1987; Hudak et al., 1987), increase in the expression of its own receptor on resting B cells (Paul and Ohara, 1987), and enhancement of IgG1 production (Snapper and Paul, 1987). In long term lines of murine T lymphocytes, IL-4 is produced by the T_H2 cells but not T_H1 cells (Table 1.1) (Mossmann et al., 1986). However, such simplistic clonal distinction may be valid only for long-term T-cell lines, since studies with allogeneic mixed leukocytes showed that lymphokines listed in Table 1.1 could be produced at random in both $CD4^+$ as well as $CD8^+$ cells and that production of these lymphokines was not mutually exclusive (Kelso and Gough, 1988). In the presence of anti-Ig, IL-4 acts as a costimulant of B cell activation. Like most cytokines, IL-4 is capable of acting on more than one cell type. For instance, it can act on resting T-cells to

maintain their viability and, in the presence of phorbol ester or antireceptor antibodies, stimulate the division of resting T cells (Hu-Li et al., 1987). In collaboration with other lymphokines, IL-4 was found to act on multiple hematopoietic lineages (Paul, 1986). The cDNA for IL-4 has been cloned (Noma et al., 1986; Lee et al., 1986) and studies using the purified recombinant IL-4 have provided important insights not only into its diverse functions on different cell types but also its regulation of and interactions with other lymphokines.

Interactions between IFN- γ and IL-4 were first recognized when it was observed that these two lymphokines reciprocally regulate Ig isotype production (Coffman and Carty, 1986; Snapper and Paul, 1987). For instance, IL-4-mediated expression of IgG1 and IgE by LPS-stimulated murine B cells was shown to be suppressed by IFN- γ . At the molecular level, there is data to suggest that IL-4 may direct specific switching events by transcriptional activation of the heavy chain switch regions (Berton et al., 1989) and that addition of IFN- γ markedly inhibited the expression of germ-line γ 1 transcripts without affecting cell viability or proliferation. This inhibition could be partially reversed by increasing the dose of IL-4. However, whether this inhibition was due to direct effects of IFN- γ or the expression of germ-line γ 1 transcripts or due to interference with the delivery of the IL-4-mediated signal is not known. Interestingly, although IL-4 and IFN- γ do not bind the same receptor (Mond et al., 1986), significant amino acid sequence homology exists between these two lymphokines (Noma et al., 1986).

There is evidence to suggest that interactions between IL-4 and IFN- γ differ in the murine and human systems (O'Garra et al., 1988). Although IL-4-induced B-cell activation (Hudak et al., 1987; Mond et al., 1986; Rabin et al., 1986) and Fc $_{\epsilon}$ receptor expression (DeFrance et al.,

1987; Rousset et al., 1988) were inhibited by IFN- γ in both systems, only human cells proliferated when co-stimulated with anti-Ig and IFN- γ (DeFrance et al., 1987; Romagnani et al., 1986; DeFrance et al., 1986). Therefore, IFN- γ is able to potentiate the IL-4-induced proliferation of preactivated human B cells, in contrast to its antiproliferative effects on other cell types.

Both IL-4 and IFN- γ can influence the antigen presenting ability in thymic epithelial cells. It has been shown that these T-cell derived lymphokines could enhance antigen presentation by thymic macrophages and could therefore influence the interaction between thymic stromal cells and immature thymocytes (Ransom et al., 1987). It was found that both fetal immature thymocytes and adult L3T4/Lyt-2 thymocytes could be activated to produce IL-4 and IFN- γ and that proliferation of these thymocytes in response to phorbol ester and IL-4 was inhibited by IFN- γ . However, the proliferative response of a thymocyte population enriched in mature cells was not affected by IFN- γ . These findings suggested that both IL-4 and IFN- γ may be directly involved in immature thymocyte differentiation and that IFN- γ may function as a differentiation factor for thymocytes.

1.5.4 Synergism with tumor necrosis factors

The tumor necrosis factor (TNF) was first described as a cytotoxin released by macrophages which had been treated with bacillus Calmette-Guerin (BCG) and LPS. This cytotoxin was shown to induce haemorrhagic necrosis of methylcholanthrene-induced (Meth A)

sarcomas in BALB/c mice while leaving surrounding normal cells unaffected (Ruddle and Waksman, 1967; Granger and Williams, 1968; Carswell et al., 1975; Helson et al., 1975; Matthews and Watkins, 1978; Aggarwal et al., 1985a; Pennica, et al., 1984; Pennica, et al., 1985). Two types of TNFs have been identified: (a) TNF- α , produced by activated macrophages and (b) TNF- β (also known as lymphotoxin), produced by lymphocytes (Shalaby et al., 1985). In addition to their tumoricidal activity, TNFs have been demonstrated to have mitogenic effects (Vilcek et al. 1985; Sugarman et al., 1985), to induce IFN- β_2 in fibroblasts (Kohase et al., 1986), to stimulate IL-1 synthesis in monocytes and endothelial cells (Dinarello et al., 1986; Nawroth et al., 1986), to stimulate the production of collagenase and prostaglandin E₂ (Dinarello et al., 1986; Dayer et al., 1985), to suppress adipocyte gene expression (Torti et al., 1985), to activate granulocyte functions (Shalaby et al., 1985; Klebanoff et al., 1986), to stimulate synthesis of granulocyte-macrophage colony stimulating factor (GM-CSF) (Munker et al., 1986), and to regulate hemostatic properties in endothelial cells (Nawroth et al., 1986). TNF was also shown to be involved in the acute phase of infections. It was found that parasite infected animals produced a blood-borne factor (cachetin) that showed significant homology to HuTNF- α , suggesting that TNF was involved in cachexia which was found in a variety of disease, including cancer and infection (Beutler et al., 1985; Beutler and Cerami, 1986). Because of its potential as an antitumor agent as well as an important immunoregulatory molecule, TNFs have been studied intensively both *in vitro* and *in vivo* in order to understand its role in the immune system.

TNFs and IFN- γ have been shown to have reciprocal synergism. For instance, IFN- γ was shown to enhance the cytotoxic effects of TNFs (Williams and Bellanti, 1983; Lee et al., 1984;

Stone-Wolff et al., 1984; Feinman et al., 1987). Both TNF- α and - β are also able to synergize with IFN- γ to inhibit virus replication (Wong and Goeddel, 1986; Mestan et al., 1986). Although TNFs were shown to possess antiviral activities, TNFs and IFN- γ likely exert their antiviral effects through independent mechanisms. It was shown that both types of TNFs induced 2-5-A_n synthetase and IFN- β (Kohase et al., 1986). However, the TNF antiviral effect was only partially inhibited by anti-IFN- β antibodies and there was no evidence for synthesis of any other class of IFN or IFN mRNA (Mestan et al., 1986). In addition to their synergism in the induction of antiviral activity, TNF- α and IFN- γ synergistically enhance MHC class I and class II genes (Pujol-Borrell et al., 1987), MHC-associated β 2-microglobulin (Wong and Goeddel, 1988), and 2,5-A_n synthetase (Wong and Goeddel, 1986). The synergism between TNFs and IFNs could partially be explained by the upregulation of TNF receptors by IFNs (Aggarwal et al., 1985; Tsujimoto et al., 1986; Scheurich et al., 1986). In vivo studies, however, showed that IFN- γ and TNF- α exhibited rather different properties (Jacob et al., 1988). These authors showed that while IFN- γ treatment aggravated a systemic lupus-like autoimmune disease in the mouse, anti-IFN- γ treatment or administration of TNF- α significantly improved the disease state and increased the survival rate of the animals. Therefore, the exact nature of synergism between IFN- γ and TNF remains to be established.

Studies with acute phase of infections suggested that during the challenge, TNF/cachetin would induce a net flux of lipid into the circulation (Beutler et al., 1985; Beutler and Cerami, 1986). The lipid being released would be used as a source of energy. However, with chronic infection, the continual release of lipid would result in wasting and eventually death. This

hypothesis prompted the studies of the effects of other cytokines on lipid metabolism, since a number of cytokines are synthesized and released during infection. There is evidence to show that IFN- γ , which is known to synergize with TNF, is able to induce hypertriglyceridemia and inhibits post-heparin lipase activity in cancer patients (Kuzrock et al., 1986). *In vitro* studies (Patton et al., 1986) also showed that both IFN- γ and TNFs were able to suppress lipid anabolism and enhance secretion of free fatty acids, supporting the hypothesis of their roles in acute phase infection and cachexia. Therefore, synergism between IFN- γ and TNF is not limited to antitumor activities but also acts on general cell metabolism during the acute phase infections.

1.6 Interferon gamma – gene and protein

1.6.1 Organization of the human interferon gamma gene

The genomic sequence of HuIFN- γ has been described (Gray and Goeddel, 1982). The IFN- γ gene comprises four exons and three introns spanning 33.5 kb that was later mapped to chromosome 12 (Gray et al., 1982; Devos et al., 1982; Gray and Goeddel, 1982; Naylor et al., 1983). The 1.2 kb mature message was generated by splicing of the four exons. The mature message encodes a polypeptide of 143 amino acid residues plus a 23 amino acid-long signal peptide (Gray and Goddel, 1982).

Sequences important for gene regulation have been identified in the 5' untranslated region of the IFN- γ gene. For instance, a consensus sequence TATAAATA was located 28 bp upstream

from the 5' end of the RNA initiation site of the IFN- γ gene. This sequence is similar to the Goldberg-Hogness box (5' TATA(A/T)A(A/T) 3') (Gannon et al., 1979) which has been thought to play an important role in determining the specificity of the initiation of RNA synthesis by RNA polymerase II (Grosschedl and Birnstiel, 1980; Wasyluk et al., 1980; Grosveld et al., 1981). Another 5' sequence GTCACCATCT was found 89 bp upstream from the 5' end of IFN- γ mRNA and was similar to the consensus sequence GG(T/C)CAATCT (Benoist et al., 1980) thought to be responsible for modulating transcription by RNA polymerase II (Grosschedl and Birnstiel, 1980). More detailed analysis (Hardy et al., 1985) has revealed additional potential regulatory sequences. A strong DNase I hypersensitive site (HS) was identified in the first intron (Hardy et al., 1985). DNase I HS has been used to localize genomic regions functionally linked to gene regulation. The HS of the IFN- γ gene was only found in cells capable of expressing IFN- γ . However, purified genomic DNA from Jurkat cells did not show hypersensitivity in this region, suggesting that DNA-protein interactions, and not DNA sequence alone, were responsible for DNase I hypersensitivity.

Other evidence concerning the expression of the HuIFN- γ gene suggested that post-transcriptional regulation may play an important role (Lebendiker et al., 1987). It was shown that the HuIFN- γ gene could be superinduced by inhibitors of translation or low doses of gamma-irradiation. The latter treatment was known to prevent activation of suppressor T cells. It was hypothesized that the HuIFN- γ gene was normally regulated by a labile protein that prevented the accumulation of mature IFN- γ mRNA (Lebendiker et al., 1987).

1.6.2 Production of the human interferon gamma protein

IFN- γ is predominantly produced by T cells bearing the IL-2 receptors and its release is at least partially triggered by helper T-cell-derived IL-2 (Sandvig et al., 1987). However, other cells such as natural killer (NK) cells, mononuclear blood cells (MNC), and CD11b⁺ cells (IFN- γ producing cells bearing this complement receptor are likely to be T-suppressor cells which also express the CD8 marker) are also known to produce IFN- γ (Handa et al., 1983; Sandvig et al., 1987; Young and Ortaldo, 1987). In addition, mice with severe combined immunodeficiency, which do not express detectable T- and B-cell function, and athymic nude mice can also produce IFN- γ (Bancroft et al., 1987; Wentworth and Toy, 1987).

The production of IFN- γ by T cells upon contact with antigen presenting cells is antigen specific (McKimm-Breschkin et al., 1982) and MHC restricted (Morris et al., 1982). In addition, a number of stimuli are also capable of inducing IFN- γ production which include phytohemagglutinin (PHA), concanavalin A (Con A), Epstein-Barr virus, and OKT3 antibody.

The natural HuIFN- γ exists as a dimer (Yip et al., 1981; Rinderknecht et al., 1984) or tetramer (Pestka et al., 1983). Natural HuIFN- γ monomers are heterogeneous in size with the major species having 20 kD and a 25 kD in mass. The difference in the molecular mass of the monomers is a result of different extent of glycosylation of a single polypeptide species (Rinderknecht et al., 1984). Smaller polypeptides also exist as a result of the loss of up to 15 amino acid residues from the C-terminus due to proteolytic cleavage either during or after the secretion of IFN- γ (Rinderknecht et al., 1984).

1.7 Genes induced by interferon gamma

The binding of IFN to the cell surface receptor results in the induction of a number of genes (Revel and Chebath, 1986), as shown by RNA synthesis *in vitro* in nuclei isolated from cells exposed to IFN and hybridization of the labelled RNAs to their corresponding cDNAs. Many of these IFN-inducible genes and their gene products have been implicated in the antiviral, antiproliferative, and immunoregulatory functions of interferon. Based on functional studies, IFN-inducible genes can be divided into three groups. The first group of genes are induced by both types of IFNs: the gene that encodes the 2,5 (A)_n synthetase (Benech et al., 1985a; Benech et al., 1985b) and the 1-8 gene family (Friedman et al., 1984). The second group of genes are predominantly induced by Type I IFN. They include the ISG 15 and ISG 54 genes (Larner et al., 1984; Levy et al., 1988); Mx (Staeheli et al., 1984; Staeheli et al., 1986); and the 6-16 genes (Kelly et al., 1985; Porter et al., 1988). The third group of genes are preferentially induced by type II IFN. They include genes of the MHC class-I antigens (Fellous et al., 1982; Wallach et al., 1982), class II (Basham and Merigan, 1983), and class III (Strunk et al., 1985); a gene encoding the Fc receptor for IgG (Guyner et al., 1983); IP-10 and IP-30 genes (Luster et al., 1985; Luster et al., 1988); a gene encoding the heavy chain of the chronic granulomatous disease protein (Neuberger et al., 1988); and a macrophage specific gene, γ .1 (Fan et al., 1989).

Both transcriptional and post-transcriptional regulations have been shown to result in the induction and accumulation of the IFN-inducible RNA. The transcriptional activation of these genes are, at least in part, mediated through cis-acting sequence at their 5' untranslated regions.

An interferon consensus sequence (ICS) was originally described by Friedman and Stark (1985) as a homologous region among various genes induced by IFN- α . A similar interferon response sequence (IRS) has also been found in genes induced by IFN- β and IFN- γ . Genes that are induced by both types of IFNs appear to contain similar IRS. Indeed, a single IRS that can confer inducibility by both IFN- α and IFN- γ has been identified (Reid et al., 1989). Cis-acting elements other than IRS are also involved in transcriptional activations of IFN-inducible genes. In the mouse MHC H-2K^b and H-2L^d, an enhancer sequence upstream from and overlapped with an IRS was found to be required for induction by both type I and type II IFNs (Kimura et al., 1986). Furthermore, an additional enhancer has been found to locate in the first intron of the H-2K^b gene that potentiates the response of H-2K^b to both types of IFNs (Israel, et al., 1986). The location of IFN-responsive enhancer sequences are likely to be different in different cell types. Tsang et al. (1988) have described the presence of a transcriptional enhancer in the first intron of the HLA-DRA that may be responsible for its B-cell-specific expression and may involve cooperative interactions between the promoter and the enhancer. IRS different from the Friedman-Stark consensus sequence has also been described in the MHC class II genes. Basta et al (1988) have shown by deletion analysis that a 5' upstream region between -141 and -109 of the HLA-DR α gene contained a critical IFN- γ -responsive element that was different from the Friedman-Stark sequence. Therefore, the regulations of IFN-inducible genes likely involve multiple cis-acting elements whose arrangements may differ in different cell types.

The kinetics of the two types of IFNs on gene expression also differ. The effect of IFN- γ on gene expression is slow compared to that of IFN- α and IFN- β (Michel and Chebath, 1986).

For instance, studies with HLA and $\beta 2m$ gene transcription in melanoma cells showed that transcription did not increase until 90 min after treatment with IFN- γ and reached its maximum level after 16 h versus 4 h for IFN- α (Rosa and Fellous, 1984). Post-transcriptional regulation of IFN-inducible genes is not well understood. In the case of IFN- γ , cyclohexamide inhibits accumulation of 2,5-A_n synthetase and C56 mRNAs, suggesting protein synthesis or some kind of protein factors are needed for some actions of IFN (Faltynek, et al., 1985).

1.8 Proteins induced by interferon gamma

Interferon gamma is known to induce the synthesis of a number of cell surface proteins including the MHC class I and class II antigens in different cell types. Expression of the MHC class I molecules has been shown to facilitate immune recognition of tumor cells (Tanaka et al., 1987) and may, at least in part, explain some of the antitumor effects of IFN. Surface expression of the MHC class II molecules is also enhanced by IFN- γ . These molecules are essential for macrophages to present antigens to T-lymphocytes. Interestingly, lipopolysaccharide (LPS), which is known to enhance the tumoricidal activity of macrophages induced by IFN- γ , has been shown to suppress IFN- γ -mediated expression of the MHC class II molecules (Steeg et al., 1982).

Recently, IFN-inducible protein factors have been identified to bind specific DNA sequences and may be involved in transcriptional activation of specific genes. These proteins have been shown to bind some of the ICS and/or IRS previously identified. A DNA-binding factor (IBP-1) was identified and was shown to induce the MHC class I antigen H-2K^b by binding to its

interferon response sequence (IRS) at the 5' untranslated region (Blanar et al., 1989). IBP-1 activity was induced in HeLa cells by treatment with IFN- γ and maximal levels of binding activity were observed within 45 min following interferon treatment. The sequence recognized by IBP-1 was found to be highly homologous to IRS of interferon- β_1 promoter, the heat shock protein HSP70, and the immunoglobulin kappa (κ) enhancer (IG κ) (Figure 1.1) (Blanar et al., 1989). However, IBP-1 was found to bind IRS of interferon- β_1 promoter but not that of the latter two genes. The sequence specificity was also shown to be important for IBP binding. When two guanine residues, G₁₄₄ and G₁₄₆, of the non-coding strand were methylated, binding of IBP-1 to the sequence, 3' AGGTGCAGAAAGTGAAACTGTGGA 5', was blocked, while methylation at any of the guanine residues on the coding strand did not interfere with IBP-1 binding. Substitution of these two guanine residues for thymines also abolished IBP-1 binding. This DNA-binding factor was characterized by means of photoactivated DNA-protein crosslinking analysis which showed that the estimated molecular weight of IBP-1 to be 59,000. IBP-1 binding alone, however, may not be sufficient for induction since oligonucleotide containing the H-2K^b sequence from -156 to -135 was unable to confer IFN- γ responsiveness to a thymidine kinase promoter-CAT construction. Induction of IBP-1 binding activity was also blocked by treatment with cyclohexamide (CHX), suggesting a requirement for protein synthesis. Similar or identical inducible DNA-binding factors have also been observed by others (Keller and Maniatis, 1988; Shirayoshi et al., 1988; Levy et al., 1988).

Recently, Miyamoto et al. (1988) have isolated a gene that encodes a nuclear factor which is inducible by either viruses or IFN. This nuclear factor, termed interferon regulatory factor

(IRF-1), was shown to bind specifically to IFN gene regulatory elements of the MHC class I genes. Specifically, it was shown to bind efficiently to repeated hexamer motifs (such as AAGTGA) present in IFN genes, which function as virus-inducible enhancer elements (Fujita et al., 1989). Interestingly, the AAGTGA core is identical to the core sequence of IBP binding sites in H-2K^b and IFN- β . However, IRF-1 was found to be induced only by type I IFN while IBP was induced by type II IFN. Therefore, other cis-acting elements or protein factors may be involved in conferring the binding specificity. High level expression of IRF-1 cDNA in transfected COS cells showed that IRF-1 functioned as a positive regulatory factor that induce endogenous IFN- α and IFN- β genes (Fujita et al., 1989). A second regulatory factor, IRF-2, has recently been identified and was also found to be induced by virus or IFN (Harada et al., 1989). Unlike IRF-1, IRF-2 was found to show repressor-like activity towards the repeated AAGTGA hexamers. The two factors exhibited different affinities for the hexamer repeats; IRF-2 exhibited approximately 5-fold higher binding activity. Therefore, the regulation of binding is likely mediated through competitive binding of the two factors for the same interferon responsive sequence. It was found that upon virus induction of the cells, the silencing effect of IRF-2 was fully reversed by the binding of IRF-1. Therefore, it was hypothesized that IRF-2 normally bound to the IFN genes in uninduced cells to the same sequence elements as IRF-1. Virus or IFN induction resulted in the replacement of IRF-2 with IRF-1, which would cooperate with other factors to promote transcription. In the case of the IFN- β gene, the H2TF-1/NF- κ B-like factor (Sen and Baltimore, 1986; Baldwin and Sharp, 1988; reviewed by Lenardo and Baltimore, 1989) that bound to a decamer sequence at -64 to -55 (GGGAAATTCC) (Keller and Maniatis, 1988)

appears to play a role in inducing transcription in cooperation with IRF-1. Moreover, IRF-1 may undergo qualitative modification such as phosphorylation (Zinn et al., 1988) which renders it more efficient in binding the hexamer motifs.

Since both IRF-1 and IRF-2 genes are virus- and IFN-inducible and since both bind to the same regulatory elements within virus-inducible IFN genes as well as IFN-inducible MHC class I genes, the IRF-1/IRF-2 system suggests the presence of a positive feedback mechanism in IFN induction. Further studies of this system should shed some light on the fine regulation of the antiviral mechanism as well as the multiple effects of IFN on cellular genes and cell growth.

2.1 Extraction and Purification of rHuIFN- γ and its deletion variants

The respective recombinant proteins were isolated from the transfected *E. coli* host LE392. Individual clones were grown overnight on a rotary shaker at 37°C in 500 ml T broth (1.2 % tryptone/2.4 % yeast extract/0.4 % glycerol/0.17 M KH_2PO_4 /0.72 M K_2HPO_4) (Tartof and Hobbs, 1987) containing 20 $\mu\text{g}/\text{ml}$ of tetracycline (Sigma). Cells were harvested by centrifugation for 10 min at 4°C in a Sorvall GSA rotor at 5000 rpm and were washed twice with an equal volume of TN buffer (50 mM Tris.HCl, pH 8.0/30 mM NaCl/1 mM ZnCl_2) at 4°C. The final cell pellet was re-suspended in 10 ml of TN buffer, frozen at -20°C, and disrupted by passing the cells through a Carver press (Fred S. Carver Inc., New Jersey) twice at -20°C. The cell debris was collected by centrifugation in a Sorvall HB4 rotor at 10,000 rpm for 60 min at 4°C and the supernatant was collected for immunoaffinity purification.

The HuIFN- γ and its variant polypeptides were purified by immunoaffinity column chromatography. The affinity column was prepared by coupling purified anti-HuIFN- γ monoclonal antibody, MIF3052 (Alfa and Jay, 1988), to CNBr-activated Sepharose GMB (Pharmacia) (Section 2.2). Supernatants of *E. coli* cell lysates were adjusted to 50 mM Tris.HCl pH 8.0/500 mM NaCl (column buffer) and 1% NP40 before applying onto the affinity column. The column was washed with the same buffer at pH 9.0 followed by 50 mM diethylamine hydrochloride (Sigma), pH 10. The adsorbed rHuIFN- γ was then eluted with 50 mM

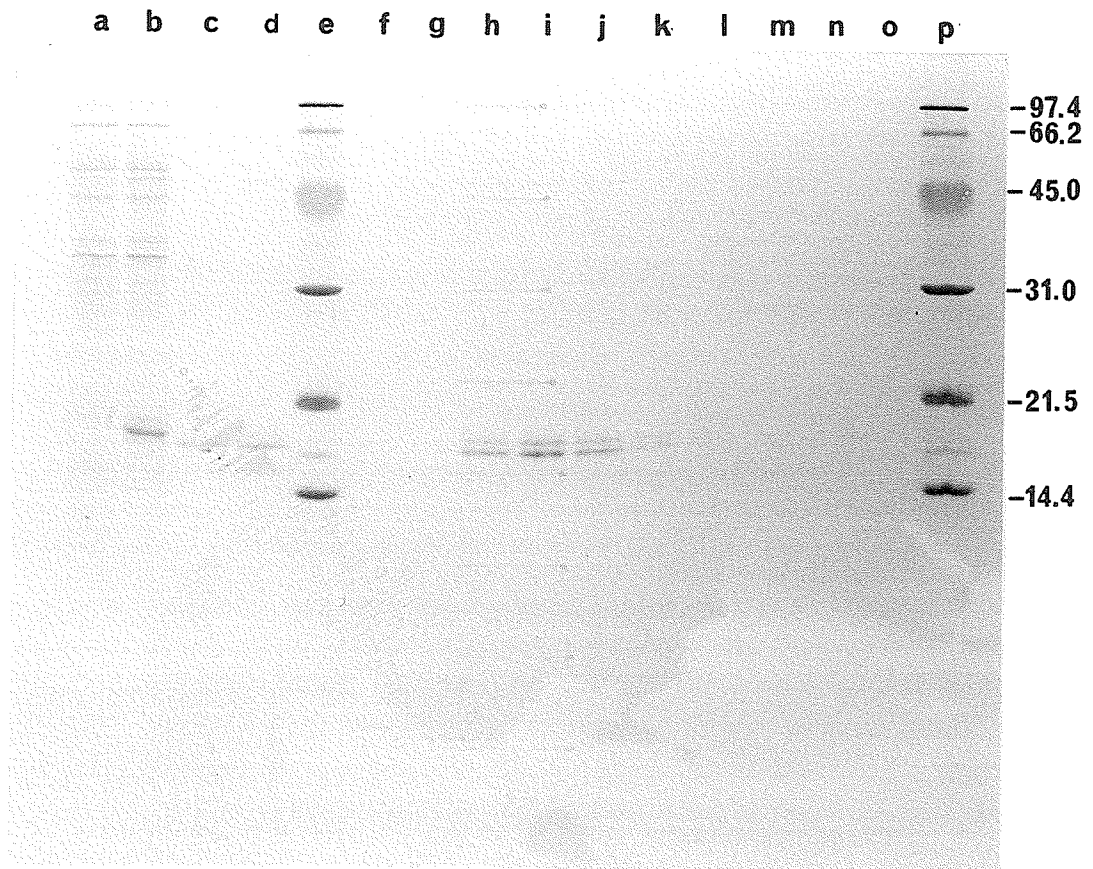


Figure 2.1 SDS-PAGE analysis of rHuIFN- γ purified on the monoclonal antibody MIF3052-coupled affinity column. Lanes: a, cell lysates of *E. coli* containing the expression vector without the IFN- γ insert; b, P₁₄R₃IFN cell lysates; c, pooled fraction (fractions 3 to 6) of affinity-purified Q46L; d, pooled fraction (fractions 3 to 6) of affinity-purified P₁₄R₃IFN; e and p, molecular mass standards (kilodalton); f to o, eluted fractions 1 to 10, respectively.

diethylamine hydrochloride, pH 11.2. Fractions (1.0 ml per fraction) were immediately neutralized by collecting into tubes containing 0.10 ml of 2 M Tris.HCl (pH 7.5). Purified fractions were stable at 4°C for up to two months. Protein concentration was estimated using an Amido Black (Sigma) adsorption micro-assay method according to Schaffner and Weissmann (1973) (Section 2.7).

Purified fractions were also characterized by SDS-PAGE. Typically, 100 ul of individual fractions or pooled fractions were precipitated with 11 ul of 100% trichloroacetic acid, incubated at 4°C for 10 min, and centrifuged for 15 min at 12,000 x g. The pellet was washed 2 times with 5 volumes of ice-cold 80% acetone. The final pellet was air-dried and resuspended in 10 ul of SDS sample buffer. The purified proteins were electrophoresed on a 15% SDS polyacrylamide gel and were stained with Coomassie Blue Stain (0.04% Coomassie Brilliant Blue/20% methanol/10% TCA/7.5% acetic acid). A typical profile of the purified fractions as analysed by SDS-PAGE is shown in Figure 2.1.

To assess the effect of denaturation and renaturation on the biological activities, 2 ml of the affinity-purified rHuIFN- γ polypeptides were added to 12 ml of 7 M guanidine hydrochloride (Sigma), pH 7.0 and incubated at 4°C for 2 h. The samples were renatured by diluting with 5-volumes of RPMI 1640 medium (Gibco) and then dialyzed in the same medium for 8 h with 3 changes of medium at 4°C. Dialyzed samples were assayed for their biological activities (Section 2.8) and the amount of proteins were estimated as described in Section 2.7.

2.2 Covalent Coupling of Monoclonal Antibody to CNBr-activated Sepharose 6MB

Protein-A purified monoclonal antibodies (10 mg) were dialyzed in 0.2 M NaHCO₃/0.5 M NaCl (pH 8.5) for 16 hours with 3 changes of buffer. One gram of CNBr-activated Sepharose 6MB (Pharmacia) was swelled in 200 ml of 1.0 mM HCl for 20 min and then washed with 1.0 mM HCl for 15 min on a sintered glass filter. Antibodies were mixed with Sepharose gel (5 to 10 mg antibodies per ml gel) in 5.0 ml coupling buffer (0.2 M NaHCO₃/0.5 M NaCl (pH 8.5)) in a polypropylene tube and rotated end-over-end for 16 h at 4°C. Unbound materials were washed 3 times with coupling buffer. The remaining active groups on the Sepharose were blocked with 1.0 M ethanolamine (pH 8.0) for 16 h at 4°C or 2 h at room temperature. Non-covalently adsorbed proteins were washed away with 0.1 M NaOAc/0.5 M NaCl (pH 4.0) and then with 0.1 M sodium borate/0.5 M NaCl (pH 8.0). The washing steps were repeated three times. The coupled antibody-coupled sepharose gel was equilibrated with eluting buffer and then with column buffer before use.

2.3 Plasmid Isolation (Miniprep)

A single bacterial colony was inoculated into 2 ml T broth (Tartof and Hobbs, 1987) containing 20 ug/ml tetracycline and incubated at 37°C overnight with vigorous shaking. The overnight culture (1.5 ml) was then transferred to a microcentrifuge tube and the bacterial cells were collected by centrifugation at 12,000 x g for 2 min. The medium was removed by aspiration

and the cell pellet was resuspended in 100 ul of freshly prepared 25 mM Tris.HCl (pH 8.0)/10 mM EDTA/15 % sucrose/2 mg/ml lysozyme and incubated for 5 min at room temperature. The cells were lysed by adding 200 ul of a solution containing 0.2 N NaOH/1 % SDS. The suspension was mixed by inverting the tube 3 to 4 times and the incubated at 4°C for 5 min. The chromosomal DNA was precipitated by adding 150 ul of 5 M potassium acetate (pH 5), mixed by inversion, and incubated at 4°C for 5 min. Chromosomal DNA was then pelleted by centrifugation at 12,000 x g for 15 min and the supernatant which contained the plasmids was collected. DNase-free RNase A (Boehringer Mannheim) was added to the supernatant in a final concentration of 20 ug/ml and incubated at 37°C for 20 min. The supernatant was then extracted with phenol/chloroform/isoamyl alcohol (48:48:4) three times and chloroform/isoamyl alcohol (48:2) three times. Plasmid DNA was precipitated by adding 5 M sodium acetate (pH 7) to a final concentration of 0.3 M and two volumes of absolute ethanol and then incubated in a dry ice/ethanol bath for 20 min. The plasmid DNA was pelleted by centrifugation at 12,000 x g at 4°C for 15 min, washed with an equal volume of 70 % ethanol, and once again collected by centrifugation. The pellet was then air-dried and resuspended in 30 ul of 10 mM Tris.HCl/1 mM EDTA (pH 8.0).

2.4 Western Immunoblot (Burnette, 1981)

Proteins separated by SDS-PAGE was transferred electrophoretically onto nitrocellulose membrane (Schleicher and Schuell, BA83, 0.2 um pore size) at 300 mA for 2 hours. Nonspecific

binding sites on the nitrocellulose membrane was blocked with 4 % bovine serum albumin (BSA) (Sigma) in 20 mM Tris.HCl/500 mM NaCl/0.05 % Tween 20 (TTBS) for 2 h at 37°C. Primary antibody was then added (1:2000 for polyclonal antibody or 1:200 for monoclonal ascites fluid) in 1 % BSA/TTBS and incubated overnight at 4°C. The nitrocellulose membrane was washed with TTBS three times (10 min each time) at room temperature. Peroxidase-conjugated secondary antibody was added (1:1000) in 1 % BSA/TTBS and incubated for 1 h at room temperature. The membrane was once again washed three times with TTBS. The blot was then developed in 100 ml TBS, 60 mg of 4-chloro-1-naphthol (BioRad) that was dissolved in 20 ml of ice-cold methanol, and 60 ul of hydrogen peroxide.

2.5 Transformation of competent E. coli host LE392

A single colony of LE392 from YT¹ agar plate was inoculated into 2 ml of 2x YT medium² and allowed to grow overnight at 37°C on a rotary-shaker. The overnight culture was then diluted (1:100) with 2x YT medium and was allowed to grow with vigorous aeration until logarithmic phase ($OD_{600} = 0.3$ to 0.4). Cells were chilled on ice and were collected by centrifugation at 4000 rpm using a Sorvall SS34 rotor for 10 min at 4°C. The cell pellet was resuspended in equal volume of ice-cold ST buffer (ST buffer = 25 mM Tris.HCl pH 7.5/10 mM NaCl) and collected by centrifugation as before. The cell pellet was then resuspended by swirling in 1/2 volume of ice-cold CaST buffer (CaST buffer = 50 mM CaCl₂ in ST buffer) and incubated on ice for 20 min. Cells were once again collected by centrifugation and the pellet

was resuspended in 1/10 volume of ice-cold CaST buffer. The cells so treated are competent to take up exogenous DNA. One to five microliters of plasmid DNA (1-5 ng) was added to 100 ul of CaST and 0.3 ml competent cells. The cells were incubated on ice for 40 min and then heat-shocked at 42°C for 2 min. Four hundred microliters of S.O.C. medium³ was added to the cells and incubated at 37°C in a shaker-bath at 150-200 rpm for 1 h. The cells were diluted 5-fold with 2xYT medium and 250 ul was spreaded onto 2x YT agar plates containing 20 ug/ml of tetracycline. The plates were incubated overnight at 37°C.

- 1 YT agar: 7.5 g of Bacto-agar per 500 ml of YT medium
 2 YT medium (per liter): 8.0 g of Bacto-tryptone, 5.0 g of Bacto-yeast extract, and 5.0 g of NaCl dissolved in double-distilled water.
 3 S.O.C. medium (Hanahan, 1983):

REAGENT	CONCENTRATION	AMOUNT per 100 ml
Bacto-tryptone	2 %	2.0 g
Yeast Extract	0.5 %	0.5 g
NaCl	10 mM	1.0 ml of 1.0 M NaCl
KCl	2.5 mM	0.25 ml of 1.0 M KCl
MgCl ₂ , MgSO ₄	20 mM (10 mM each)	1.0 ml of 2.0 M Mg Stock
Glucose	20 mM	1.0 ml of 2.0 M Glucose
Distilled water	up to 100 ml	

2.6 Cloning of DNA into M13mp8 vector for site-directed mutagenesis and/or dideoxy DNA sequencing - general characteristics of the vector

A range of cloning vehicles were constructed using the single-stranded DNA bacteriophage M13 (Messing et al., 1977; Gronenborn and Messing, 1978; Messing et al., 1981; Messing and Vieira, 1982; Norrander et al., 1983). These vectors have several special features that make them particularly suitable for sequencing DNA of varying sizes: (i) they contain multiple cloning site (MCS) polylinkers of some of the most common restriction enzymes thereby allowing rapid cloning of DNA that have the same enzyme sites; (ii) part of the *E. coli* β -galactosidase (β -gal) gene has been genetically engineered into the MCS. The lac region therefore serves as a marker system for distinguishing wild type phage (blue plaque formers) from recombinant phage (white plaque formers) when grown on lac⁻ *E. coli* in the presence of the β -gal inducer IPTG (isopropylthio- β -D-galactoside) and the chromogen X-gal (5-Bromo-4-chloro-3-indolyl- β -galactopyranoside); (iii) pairs of vectors of opposite orientations are available, allowing cloning and sequencing from either orientation of the DNA of interest; (iv) since M13 is a ssDNA bacteriophage, the ssDNA phage genome containing the gene of interest can be rapidly isolated and sequenced by the dideoxy chain termination method (Sanger et al., 1977); (v) a number of sequencing primers flanking the MCS are commercially available.

2.7 Dideoxy DNA Sequencing

The following procedures for sequencing ssDNA are essentially the same as that described by Sanger et al. (1977) and Biggin et al. (1983) with modifications according to the protocol recommended by BioRad.

2.7.1 Preparation of single-stranded phage DNA

A single plaque was inoculated into 2 ml culture of logarithmic growing *E. coli* strain JM103 and was allowed to grow for 6.5 h at 37°C on a rotary-shaker. Cells were pelleted in a microcentrifuge for 10 min and the supernatant was collected. The phages were precipitated by the addition of 200 ul of 20% PEG/2.5 M NaCl to the supernatant and were incubated for 15 min at room temperature. The phages were then collected by centrifugation in a microcentrifuge for 5 min and the pellets were resuspended in 150 ul of 20mM Tris.HCl(pH 7.5)/0.1 mM EDTA/10mM NaCl (TES buffer). The suspension was extracted with phenol/chloroform/isoamyl alcohol (50:50:2) 3 times and chloroform/isoamyl alcohol (50:2) 3 times. The final liquid phase was adjusted with 5 M NaOAc to 0.3 M and the phage DNA was precipitated with 2 volumes of absolute alcohol for 30 min in an ethanol/dry ice bath. The precipitate was washed 2 times with ice-cold 80% ethanol. The final pellet, which contained the purified single-stranded phage DNA, air-dried, and resuspended in 20 ul of TES buffer. The amount of ssDNA was measured using a Shimadzu UV-160 spectrophotometer.

2.7.2 Annealing and Primer Extension

Single-stranded M13mp8 phage DNA containing the insert of interest was mixed with the sequencing primer. Typically, 1 ug of ssDNA template was annealed with 1 ng of primer. The primer is usually between 15 to 20 bases long and at least 30 bases downstream of the site to be sequenced. The annealing reaction was adjusted with 1.5 ul of 10x Klenow reaction buffer (100 mM Tris.HCl, pH 8.0/ 100 mM MgCl₂,/ 300 mM NaCl) and distilled water to a final volume of 10.0 ul. The reaction mix was then incubated at 68°C for 5 min, briefly centrifuged, and cooled at room temperature for 30 min. Five microliters of [α -³⁵S]dATP (500 Ci/mmol) (NEN) and 5 units of Klenow enzyme was added to and mixed with the template/primer mix before aliquoting 3 ul each into 4 tubes labelled G, A, T, and C. Each of the four tubes contained a predetermined ratios of deoxynucleotides and dideoxynucleotides (see below). The tubes were incubated at 37°C for 20 min and then 1 ul of chase solution (0.5 M dATP) was added into each tube. The reactions were further incubated at 37°C for 15 min. The reactions were stopped by adding 5 ul of stop buffer (0.1 % xylene cyanol, 0.1 % bromophenol blue, 10 mM EDTA, 95 % deionized formamide).

The components of the deoxynucleotides and dideoxynucleotide mixes were the same as those recommended by BioRad:

Prepare 0.5 mM dilutions of each 10 mM dNTP stock except dATP, then mix (in μ l):

Component	G	A	T	C
0.5 mM dGTP	1	20	20	20
0.5 mM dTTP	20	20	1	20
0.5 mM dCTP	20	20	20	1
1x TE Buffer	20	20	20	20

Dilute the 2.5 mM ddNTP stocks to the following working solutions (in μ l):

Component	ddG	ddA	ddT	ddC
2.5 mM ddGTP	6			
2.5 mM ddATP		2		
2.5 mM ddTTP			12	
2.5 mM ddCTP				4
Distilled water	44	48	38	46

2.7.3 Electrophoresis of Sequencing Gel

Typically, sequencing reactions were resolved on a 6 % sequencing gel (see Appendix 1 for detail components) using a BioRad Sequencing cell (Sequi-Gen™, 21 x 40 IPC Assembly) in Tris-borate buffer (Appendix 1). In addition, wedged-shaped spacers (0.2-0.4 m thick) were used to generate a gel gradient. Sharkstooth comb (24 wells) was used which allowed better alignment of the sequence ladders. The gel was pre-run for 15 to 20 min at a constant power of 60 watts until the gel temperature reached between 50° and 55°C. The sequencing reaction was then heated at 95°C for 3 min before 1 to 2 ul of each sample were loaded into individual wells. The samples were electrophoresed for 1.5 to 2 h at a constant power of 60 watts. The gel was fixed with 10 % methanol/10 % acetic acid for 15 min, dried under vacuum, and exposed to Kodak XAR film for at least 16 h.

2.8 Protein Estimation (Schaffner and Weissmann, 1973)

A standard curve for calibrating protein concentration of an unknown sample was prepared by mixing the following:

Water	Bovine Serum Albumin (BSA)
(ul)	0.1 mg/ml (ul)
270	-
265	5
260	10
255	15
250	20
245	25
240	30
220	50

The unknown sample was added into a separate tube and adjusted to 270 ul with double-distilled water. Thirty microliters of 1.0 M Tris.HCl/1% SDS (pH 7.5) and 60 ul of 60% trichloroacetic acid (TCA) were added into each tube and were mixed by vortexing. Two milliliter of 10% TCA was added into each tube and mixed before being poured onto moistened GSWP filter (Millipore). The precipitated proteins were filtered onto the membranes by vacuum suction. Each of the tubes was rinsed twice with 1.0 ml of 6 % TCA and poured onto filters. The filters were stained in 0.1 % AmidoBlack (Sigma) in methanol/acetic acid/water (45:10:45, v/v) for 3 min and rinsed in distilled water for 1 min. Filters were then destained with methanol/acetic acid/water (90:2:8, v/v) three times or until background on the blank filters were clear. The filters were rinsed in distilled water, and then air-dried. The adsorbed dye was eluted from the filters with 1.5 ml of 25 mM NaOH/0.05 mM EDTA/50% ethanol and incubated at room temperature for 20 min. Optical absorbance of eluant was measured at 630 nm using a Shimadzu UV-160 dual beam spectrophotometer.

2.9 Biological Assays

2.9.1 In Vitro Antiviral Assay

The human lung carcinoma cell line A549 was used to assay for the antiviral activities of the various IFN preparations. Cells were seeded in individual wells of 96-well tissue culture plates (Corning, New York) at a concentration of 4×10^4 cells/well and were allowed to attach at 37°C for 4 h in 5% CO₂. The cells were then pretreated for 16 h with diluted IFN preparations (range from 1:1 to 1:10⁵, depending on the activities of preparations) before being serially diluted 1:1 in each row (12 wells) of wells. One hundred microliters of encephalomyocarditis virus (EMCV) was inoculated into each well at a multiplicity of infection (MOI) of 10 and the cells were further incubated for 24 h. The supernatants were removed and the cells were fixed and stained with 0.25% crystal violet/20 mM Tris.HCl pH 7.5/0.9 % NaCl/20% methanol. The extent of EMCV infection was determined by spectrophotometric estimation of viral cytopathic effect (CPE): the absorbance of the amount of dye uptake is proportional to the number of viable cells and inversely proportional to the amount of CPE. The absorbance at 590 nm was determined using an EIA Reader EL308 (Biotek Instrument). The average absorbance of untreated cells that were infected with EMCV was used as the calibration for 100% CPE. The end-point was defined as the reciprocal of the highest serial dilution that produced 50% CPE relative to 100% CPE control. A laboratory IFN- γ standard calibrated against the NIH standard (Gg23-901-530) was included in each assay for the conversion into international units.

2.9.2 In Vitro Antiproliferative Assay

The human glioblastoma cell line T98G was used to assay for the antiproliferative activities of the various IFN preparations. Cells were seeded in individual wells of 96-well tissue culture plates (Corning, New York) at a concentration of 4×10^3 cells/well and were allowed to attach at 37°C for 4 h in 5% CO₂. Diluted IFN preparations were serially diluted into each well and the cells were allowed to grow for 72 h. The antiproliferative activity of the IFN preparation was determined as a percent of normal growth. The absorbance at 590 nm of cells not treated with IFN and stained with crystal violet (same concentration as the antiviral assay) served as a standard for normal growth. For cells treated with IFN, the absorbance was indirectly proportional to the percent of growth inhibition relative to that of normal growth. The end point was defined as the reciprocal of the highest serial dilution that inhibited 50% cell growth.

		105		110		115											
	D	L	N	V	Q	R	K	A	I	H	E	L	I	Q	V	M	Oc
(1)	AGATCTGAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGATGTAAGC																
(2)	ACTTACAGGTTGCGTTTCGTTATGTA																
		▲															†
		105		110													
	D	L	N	V	Q	R	K	A	I	H	Oc						
(3)	AGATCTGAATGTCCAACGCAAAGCAATACATTAAGC																
(4)	ACTTACAGGTTGCGTTTCGTTATGTAATT																
		▲															†
		105															
	D	L	N	V	Q	Oc											
(5)	AGATCTGAATGTCCAATAAGC																
(6)	ACTTACAGGTTATT																
		▲															†

Figure 2.2 Sequences of synthetic oligonucleotides for replacing the BglII/SstII fragment of the rHuIFN- γ . Fragments 1 and 2, 3 and 4, and 5 and 6 are complementary strands. The single amino acid code is used. The restriction enzyme sites are denoted by ▲ (BglII) and † (SstII).

2.10 Purification of Synthetic Oligonucleotides

A total of 6 synthetic deoxyoligonucleotides ranging from 53 to 14 nucleotides were synthesized (Figure 2.2). To separate the full-length products from the truncated by-products, synthetic oligonucleotides of 53, 47, and 35 nucleotides in length were separated on a 12 % polyacrylamide-urea sequencing gel (Section 2.7.3) while those of 29, 20, and 14 nucleotides in length were separated on an 18 % polyacrylamide-urea sequencing gel. Typically, 5.0 OD₂₆₀ units (1 OD₂₆₀ unit is equivalent to approximately 40 ug/ml of single-stranded DNA) were loaded onto a 1.5 mm thick gel and electrophoresed at 22 mA for 2 hours. Oligonucleotides were visualized under ultraviolet light shadowing on a fluorescent background of a thin layer chromatography (TLC) plate. The uppermost bands from each track, which represented the full-length oligonucleotide, were excised from the gel. The excised gel pieces briefly washed once with 1.0 ml of sterile double-distilled water to rinse out the excess urea, separately pulverized in polypropylene tubes, and resuspended in 1.0 ml of autoclaved 10 mM Tris.HCl/1 mM EDTA (pH 8.0). The gel suspensions were incubated at 37°C with shaking for 16 h. The supernatants, which contained the oligonucleotides that had diffused from the polyacrylamide gel, were collected.

Supernatants containing the respective oligonucleotides were loaded onto 1.5 ml DEAE-Sephacel (Pharmacia) columns had been washed with 3 bed volumes of 10 mM Tris.HCl/1 mM EDTA/1 M NaCl (pH 8.0) and equilibrated with 3 column volumes of 10 mM Tris.HCl/1 mM EDTA (pH 8.0). The columns were washed with 1.5 bed volumes of 10 mM Tris.HCl/1 mM

EDTA/50 mM NaCl (pH 8.0). The bound oligonucleotides were then eluted with 0.5 ml 10mM Tris.HCl/1 mM EDTA/1 M NaCl. Five microliters of each sample was used for optical density (OD_{260}) estimation.

2.11 Cloning of synthetic oligonucleotide fragments

2.11.1 Phosphorylation and Annealing of synthetic oligonucleotides

Synthetic oligonucleotides (48 pmol) were individually phosphorylated by adding 5 ul of 10x kinase buffer, 5 units of T4 DNA kinase (10 units/ul) (5-molar excess over 5' end of the oligonucleotides), 1 ul of 150 mM of ATP, and adjusted to 50 ul with double-distilled water. The reaction was incubated at 37°C for 30 min. Phosphorylated oligonucleotides were extracted with phenol/chloroform/isoamyl alcohol (48:48:4, v/v) 3 times and then extracted with chloroform/isoamyl alcohol (48:2, v/v) 3 times. The liquid phase of each sample was collected, adjusted to a final concentration of 0.3 M NaCl, and precipitated with 2 volumes of absolute ethanol. The samples were then frozen in an acetone/dry ice bath for 15 min to facilitate precipitation. The samples were centrifuged at 10,000 rpm for 30 min, air-dried, and resuspended in 10 mM Tris.HCl/1 mM EDTA (pH 8.0). The samples were further precipitated twice to remove the excess ATP.

Equimolar (0.0065 pmol) of the ssDNA fragments were mixed as follow: fragment 1 and fragment 2, fragment 3 and fragment 4, and fragment 5 and fragment 6. Each set of fragments

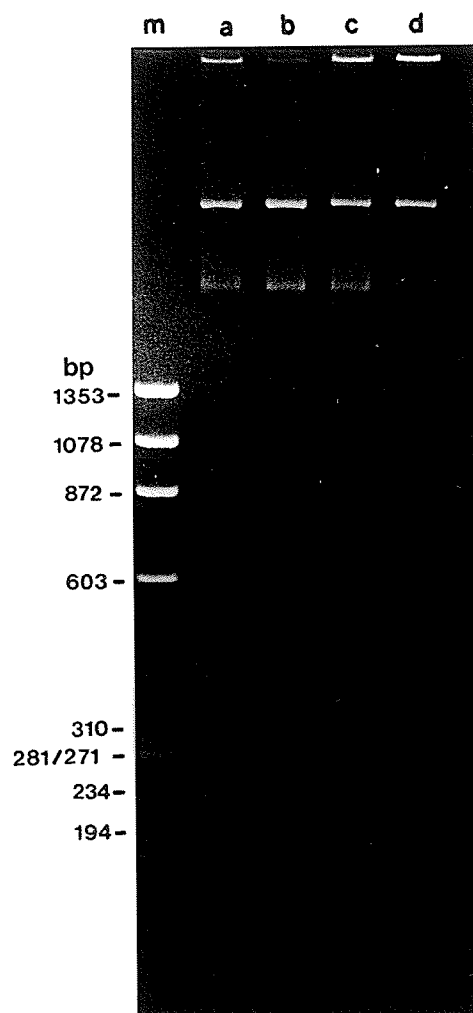


Figure 2.3 Restriction endonuclease analysis of deletion variants. Plasmids containing the inserts of various length were digested with HindIII and separated in a 1.5 % agarose gel containing 1 ug/ml of ethidium bromide. Lanes: m, molecular size markers (ϕ X174 DNA digested with HaeIII); a, pP₁₄R₃IFN; b, pDel-117; c, pDel-111; d, pDel-106. The inserts of plasmids were, respectively, 440, 428, 410, and 395 base pairs.

were adjusted to 13.0 ul with 1.5 ul of 10x ligase buffer, and 4 ng of BglII/SstII linearized, dephosphorylated vector DNA. The samples were heated to 68°C for 1 h and then slow-cooled to 4°C. One microliter of 15mM ATP and 2.4 units of T4 DNA ligase were added to a final volume of 15 ul. Ligation was carried out at 11°C for 16 h. Ligated DNA were directly used to transform competent E. coli host LE392.

Transformation of E. coli was carried out as described in the Section 2.5. Five independent transformants of each variant were inoculated into 2.0 ml LB broth and were grown at 37°C for 16 h on a rotary-shaker. Plasmid DNA were isolated from these transformants as described in Section 2.3 and were characterized by restriction digestion with HindIII and electrophoresis on 1.8% agarose gels (Figure 2.3).

2.12 Subcloning of deletion variants Del-117, Del-111, and Del-106 into M13mp8 vectors for sequence analyses

In order to confirm the nucleotide sequences of the deletion variants, the coding sequences of these variants were subcloned into the M13mp8 vector (see Section 2.6 for general characteristics of the M13 cloning vectors). Purified plasmid DNA (0.5 ug) of Del-117, Del-111, and Del-106 were digested by adjusting to 20 ul with 2.0 ul of the BRL REact2™ buffer (50 mM Tris.HCl pH 8.0/10 mM MgCl₂/50 mM NaCl), double-distilled water, and 10 units (10 units/ul) of HindIII and PstI. The reactions were incubated at 37°C for 2 h. The digested DNA were extracted with phenol/chloroform/isoamyl alcohol (48:48:4, v/v) 3 times,

chloroform/isoamyl alcohol (48:2, v/v) 3 times, and then precipitated in a final concentration of 0.3 M sodium acetate (pH 7.0) and 2 volumes of absolute ethanol at -20°C for 30 min. The DNAs were pelleted in a microcentrifuge for 15 min at 4°C, washed once with 70% ethanol, and the pellets were dried in vacuo.

The M13mp8 vector (2 ug) was digested by adjusting to a final volume of 20 ul with REact2™ buffer, water, and 10 units of HindIII (10 units/ul). The reaction was incubated at 37°C for 2 h. The reaction was then adjusted to 300 ul by adding 27 ul of double-distilled water, 3 ul of 5 M sodium acetate (pH 7.0), and 250 ul of absolute ethanol. The digested vector DNA was precipitated at -20°C for 30 min and pelleted in a microcentrifuge for 15 min at 4°C. To dephosphorylate the 5'-phosphate group of the vector, the dried DNA pellet was resuspended in 44 ul of water, 5 ul of 10x CIP buffer (0.5 M Tris.HCl pH 9.0/10 mM MgCl₂/1mM ZnCl₂/10 mM spermidine), and 2.5 units of calf intestine phosphatase (CIP)(2.5 units/ul). The reaction was incubated at 37°C for 30 min. Another 2.5 units of CIP was added and was further incubated for 30 min. The reaction was adjusted with 40 ul of water, 10 ul of 10x STE (100 mM Tris.HCl pH8.0/1M NaCl/10 mM EDTA), and 5 ul of 10% SDS. The reaction was inactivated by heating to 68°C for 10 min and then extracted by phenol/chloroform extraction, precipitated, and dried as before.

M13mp8 vector was ligated with deletion variants with a vector to insert ratio of 5:1. Deletion variants (0.02 pmol) were mixed with 0.1 pmol of dephosphorylated M13mp8 vector, 5x ligase buffer, 1 unit of T4 DNA ligase (1 unit/ul), and adjusted to 20 ul with water. Ligation was carried out at 12.5°C overnight. Each ligation reaction was diluted 1:5 before using for

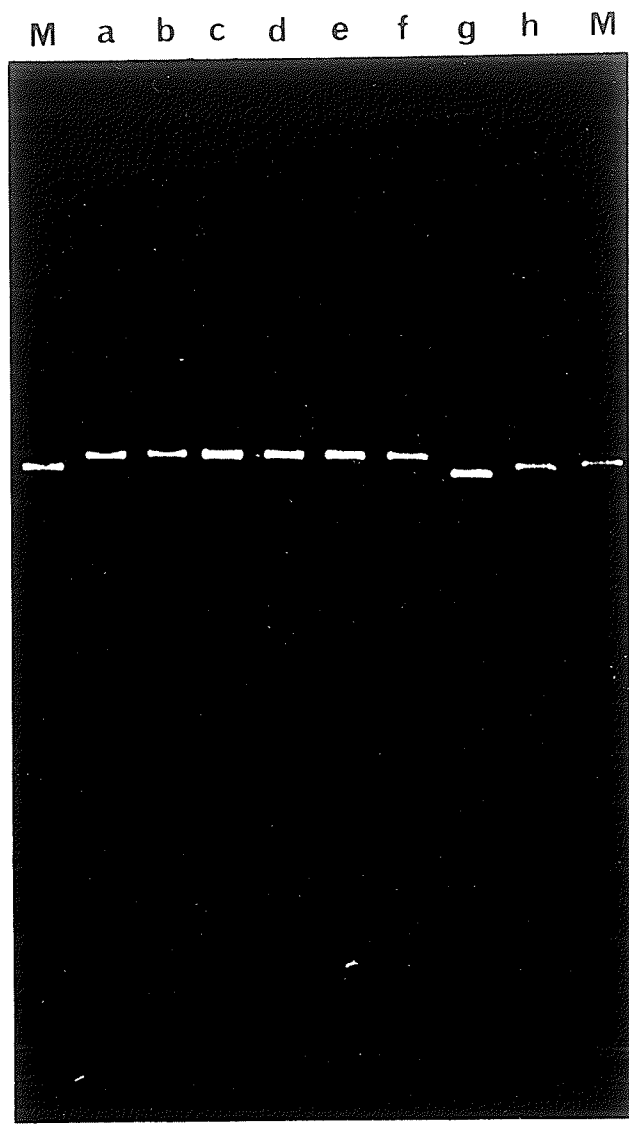


Figure 2.4 Analysis of deletion variants subcloned into M13mp8 vector. Single-stranded DNA from phages containing (M) M13mp8 vector, vector with insertion of (a and b) Del-117, (c,d, and e) Del-111, and (f,g, and h) Del-106, were separated on a 0.8% agarose gel by electrophoresis.

transforming competent E. coli JM103 (Section 2.18.4).

Independent colorless plaques generated from ligation between M13mp8 and the deletion variants were isolated: 2 plaques from Del-117 and 3 of each from Del-111 and Del-106. The ssDNA was prepared as described in Section 2.6.1 and electrophoresed on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light (Figure 2.4)¹.

2.13 Purification of deletion variant polypeptides Del-117, Del-111, and Del-106

Deletion variant polypeptides were extracted and purified essentially as described for that of parental full-length molecule (Section 2.1). Fractions collected were tested for biological activities as well as analysed by SDS-PAGE (Figure 2.5). However, due to their lack of accumulation inside the E. coli cells, attempts to purify these polypeptides were unsuccessful.

¹ Figure 2.4 is the same as Figure 3.9 (Chapter 3).

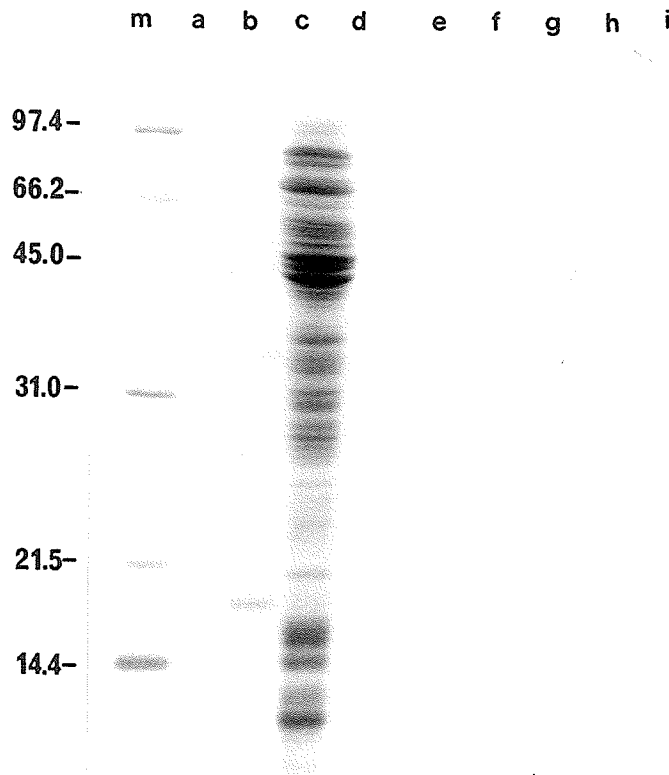


Figure 2.5 SDS-PAGE analysis of deletion variant Del-117 purified on MIF3052-coupled affinity column. Fractions collected were precipitated and electrophoresed on a 15% SDS polyacrylamide gel. Total proteins were stained with Coomassie blue. Lanes: m, molecular mass standards (kDa); a, lysates of cells harboring the expression vector without the IFN insert; b, lysates of cells harboring the plasmid pP₁₄R₃IFN; c, lysates of cells harboring the plasmid pDel-117; d to i, affinity-purified Del-117 fractions 1 to 6.

2.14 In vitro Transcription-Translation

In vitro transcription-translation experiments were performed to evaluate the efficiency of expression of deletion variants Del-117, Del-111, and Del-106. A bacterial cell-free coupled transcription-translation system kit was purchased from Amersham (Arlington Height, IL). This commercial kit was based on the system as described by De Vries and Zubay (1967) and modified by Zubay (1973) and Collins (1979).

Equal amount (0.6 ug) of plasmids containing the various deletion variant coding sequences under the control of a T₇ promoter and a ribosome binding site (Jay et al., 1984) were used in the in vitro transcription-translation system. A positive plasmid control which contained the full-length HuIFN- γ coding sequence and a negative control which consisted of the expression vector without the IFN- γ insert were included. The amount of DNA and the reaction reagents used in the entire experiment were 1/4 of those recommended by the manufacturer. The following reagents supplied in the kit were added to the DNA template: 1.9 ul Supplement Solution, 0.75 ul of Amino Acid Mix Minus Methionine, 0.5 ul of L-[³⁵S]-labelled methionine, and 1.25 ul of S-30 extract. The mixture was then adjusted to a final volume of 7.5 ul with Dilution Buffer and incubated at 37°C for 60 min. Methionine Chase Solution was added (1.25 ul) and then incubated at 37°C for 5 min. The reaction was terminated by cooling at 0°C. The sample was diluted 1:1 with SDS sample buffer and boiled for 2 min before analyzed by SDS-PAGE. These polypeptides were then resolved on a 15 % SDS polyacrylamide gel and visualized by fluorography. ¹⁴C-labelled molecular weight standards were purchased from Bethesda Research Laboratory.

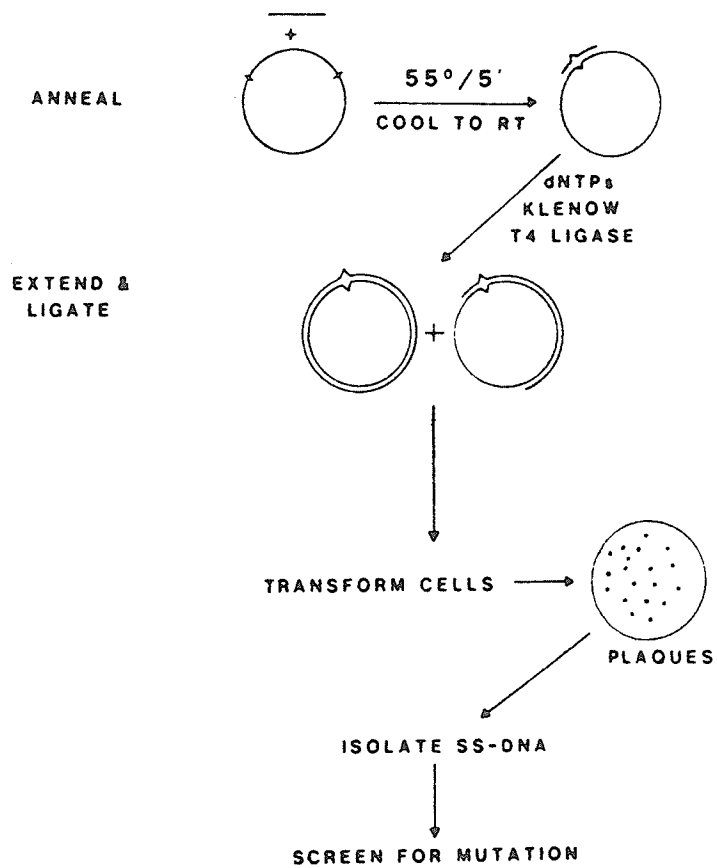


Figure 2.6 Principle of site-specific mutagenesis:

2.15 Site-directed mutagenesis (Zoller and Smith, 1983)

The principle of site-directed mutagenesis is schematically shown in Figure 2.6. Complementary synthetic oligonucleotide with one nucleotide mismatch is annealed with ssDNA template to the site of mutation and is extended by DNA polymerase. The circular heteroduplex is then ligated. Semiconservative replication of the closed circular heteroduplex will generate, theoretically, 50% of mutant and parental molecules. However, in practice, the low efficiency of primer extension will greatly reduce the percentage of mutant molecules generated. These molecules will be used to transform the *E. coli* strain JM103 made competent according to the procedure described in Section 2.18.4. The plaques are screened according the procedures described in Section 2.18.5 using ³²P-labelled synthetic oligonucleotide and the desired mutation could then be confirmed by DNA sequencing (Section 2.7).

2.16 Subcloning of rHuIFN- γ into M13mp8 replicative form (RF)

Site-directed mutagenesis was carried out in the M13 bacteriophage system as described by Zoller and Smith (1983). The entire 440-base pair rHuIFN- γ coding sequence was excised from the expression vector pP₁₄R₃IFN at the two flanking HindIII sites (Figure 2.7). The rHuIFN- γ coding sequence was cloned into the multiple cloning site (MCS) of the linearized M13mp8 vector (Messing, 1983). Successful insertions into the MCS were identified by appearance of colorless plaques due to interruption of the β -galactosidase gene within the MCS. Annealing of

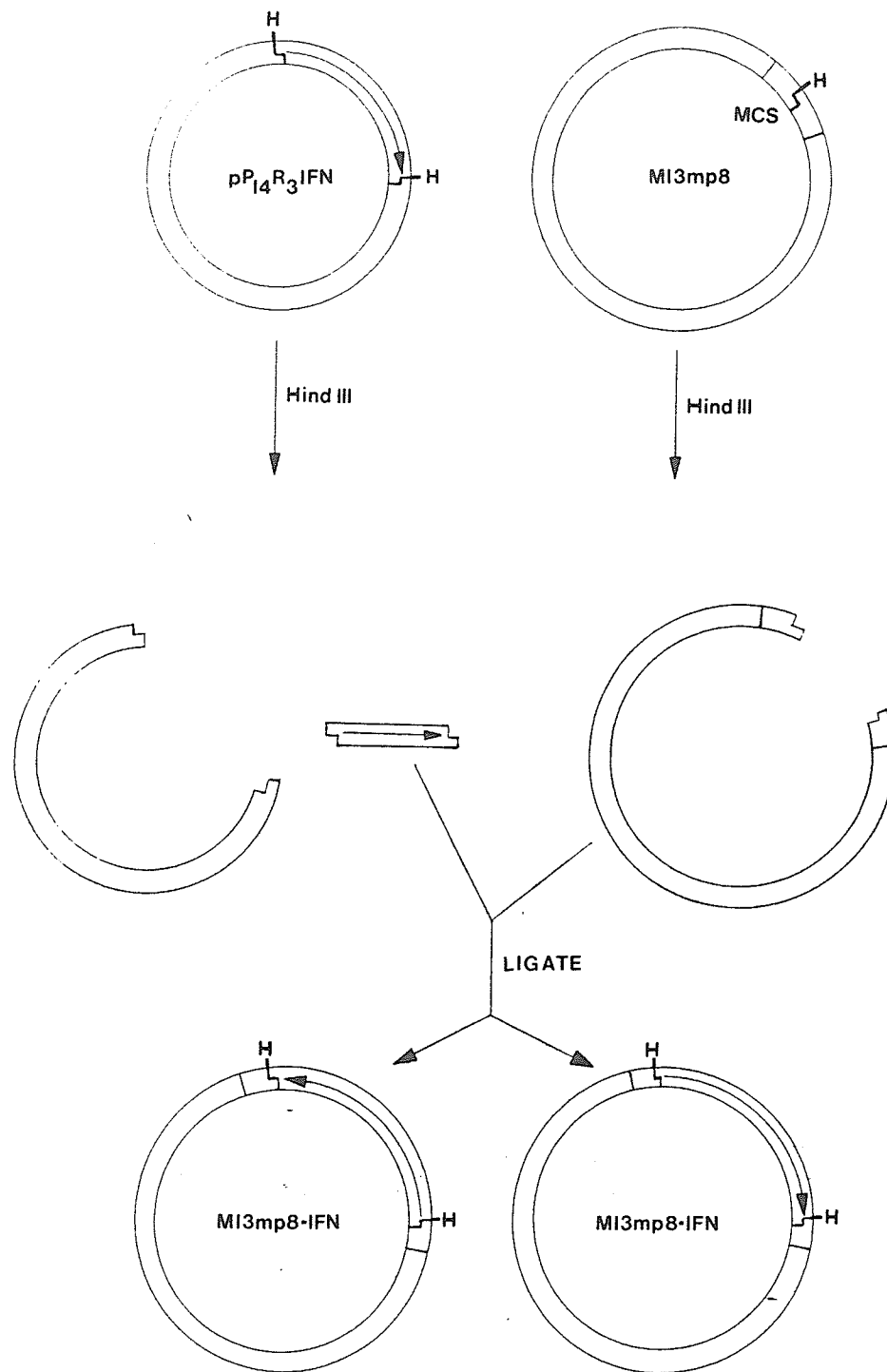


Figure 2.7 Subcloning of HuIFN- γ into M13mp8 vector. The M13mp8 cloning vector and the expression vector that contains the HuIFN- γ coding sequence ($pP_{14}R_3IFN$) are cleaved with HindIII. Due to the flanking HindIII sites of the HuIFN- γ coding sequence, the HindIII fragment subsequently cloned into the M13mp8 cloning vector could be in two opposing orientations (the direction of the transcription is denoted by an arrow). H: HindIII site. MCS: multiple cloning site.

HindIII-linearized M13mp8 RF vector and HuIFN- γ fragment was carried out with a vector to insert molar ratio of 1:3, incubated at 56°C for 15 min, and then slow-cooled to 4°C. Annealed fragments were adjusted to 15 μ l with 1 μ l of 1mM ATP and 1.2 units of T4 DNA ligase. Ligation was carried out overnight at 10°C. The reaction mix was then adjusted with 5 M NaCl to 0.3 M, and was precipitated by the addition of 2.5 volumes absolute ethanol and incubated in an ethanol/dry ice bath for 30 min. Ligated DNA was pelleted in a microfuge for 15 min and the pellet was washed with 70% ethanol, centrifuged, lyophilized, and resuspended in TE buffer. Transformation was carried out as described in Section 2.18.5.

2.17 Screening for orientation of inserts

Successful subcloning was indicated by the appearance colorless plaques as a result of disruption of the β -galactosidase gene at the multiple cloning site. A total of 9 colorless plaques and 1 blue plaque were selected and separately inoculated into 2 ml cultures of logarithmically growing ($OD_{600}=0.3$ to 0.4) *E. coli* host JM103 and were allowed to grow for 6.5 h at 37°C on a rotary-shaker. Cultures (1.5 ml) were then dispensed into 1.5-ml Eppendorf tubes and pelleted in a microfuge for 10 min. The cell pellets were saved for the isolation of intracellular RF. The supernatant, which contained the packaged single-stranded M13 phage, was used for the isolation of single-stranded DNA (ssDNA) as well as for quick screening by direct gel electrophoresis (DIGE). The single-stranded phage DNA was isolated by adding 20 μ l of supernatant to an Eppendorf tube that contained 1.0 μ l of 2% SDS and 3.0 μ l of gel loading buffer. Ten microliters

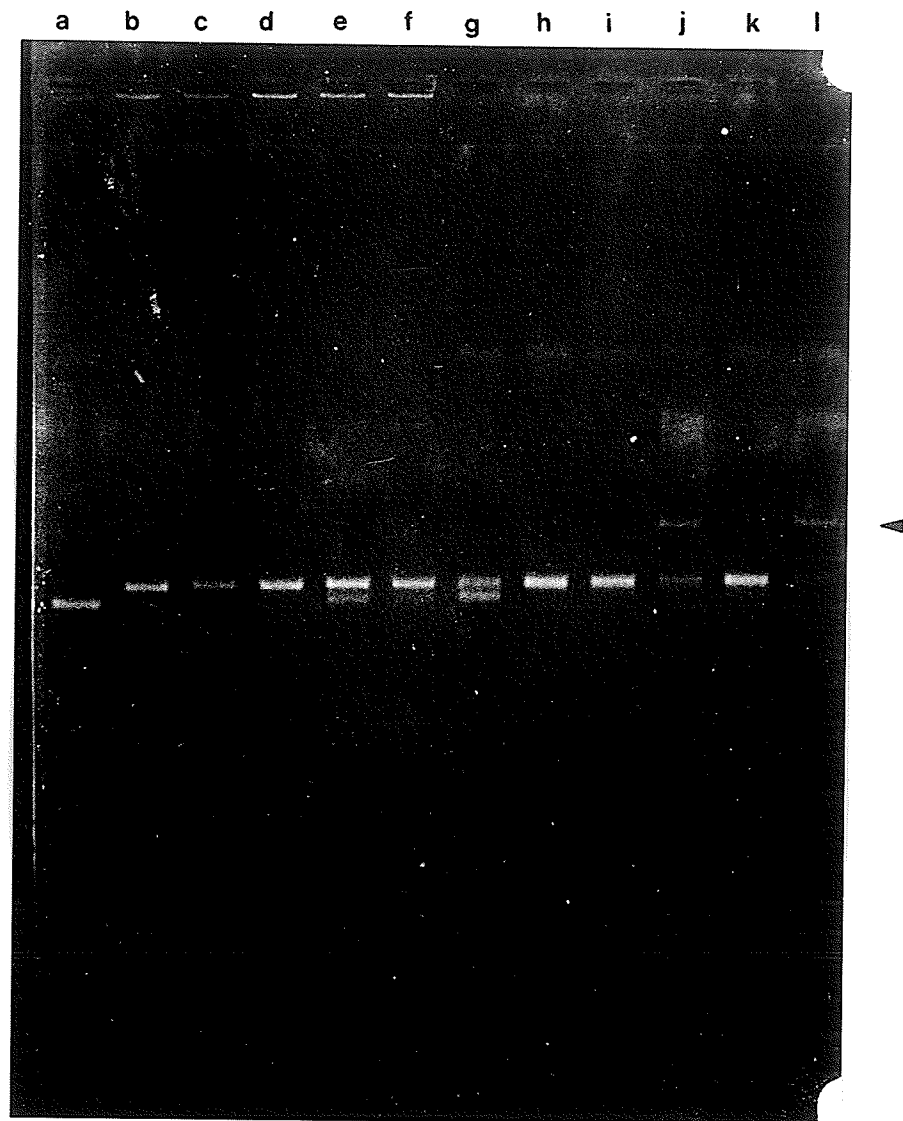


Figure 2.8 Screening of M13mp8/IFN subclones and 'Figure-8' Analysis. Samples were prepared as described in the text and electrophoresed on a 0.7 % agarose gel. Lanes: a, M13mp8 vector; b to f, respectively, clones 4, 6, 8, 11, and 13 which contained the HuIFN- γ inserts; g to l, respectively, M13mp8 vector, clones 4, 6, 8, 11, and 13 hybridized with clone 4. 'Figure-8' structures exhibited as slower migrating bands (arrow head) in lanes j and l, representing hybridization of clone 4 with clones 8 and 13.

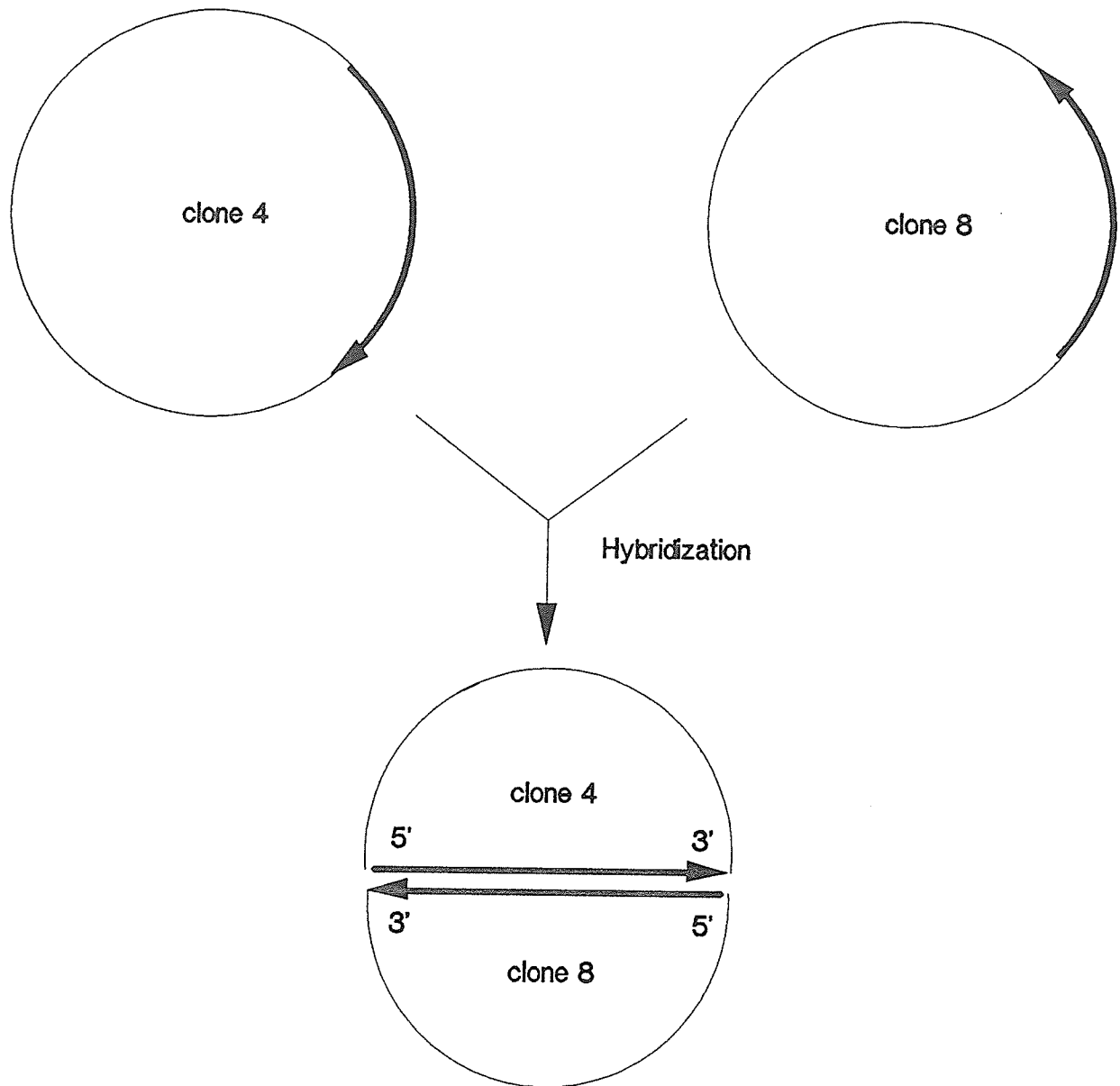


Figure 2.9 Schematic of the 'Figure-8' structure. Clones 4 and 8 represent two independent M13mp8 subclones that contain the HuIFN- γ inserts that are of opposing orientations. As shown in Figure 2.7, since the inserts are cloned into the unique HindIII site of the M13mp8 vector, the inserts could be in either orientations. Since the M13 phage only package the plus-strand of the replicative form DNA, clones 4 and 8 thus contain ssDNA that are complementary to each other. Single-stranded DNA extracted from these phages would hybridize under the conditions described in the text. Thick arrows represent the direction of the ssDNA of the IFN coding sequence.

of the samples were separated by electrophoresis on a 0.7% agarose gel containing 1.0 ug/ml ethidium bromide and the ssDNA were visualized by short UV light. As shown in Figure 2.8, clones that contained the IFN inserts (lanes b to f) exhibited slower migration on the agarose gel due to their increase in size compared to the M13 vector alone (lane a). The presence of mixed populations were also evident in clone 11 (lane e).

Since the HuIFN- γ fragment was flanked by two HindIII sites, it could be inserted in either orientation into the HindIII-linearized M13mp8 vector, the 'figure-8' analysis (Messing, 1983) was performed in order to identify the orientations of the 5 clones that were selected. The analysis was based on the fact that M13 phage only package its positive ssDNA. An arbitrarily chosen reference phage was then used to screen phages that contained single-stranded inserts of the opposite orientation. Phages whose ssDNA of the same inserts but in opposite orientation relative to that of the M13 sequence would hybridize via the inserted sequence with the reference phage insert that carries the complementary strand of the insert. The heteroduplex thus formed resembles the figure '8' as a result of the hybridization between the inserts whereas the rest of the circular M13 DNA remains single-stranded (Figure 2.9). On the other hand, if the ssDNA insert of the reference phage were of the same orientation as that of the tested phage, no annealing would occur.

The formation of the 'figure-8' structure was then analyzed by gel electrophoresis (Figure 2.8). Due to the increase in size of the 'figure-8' structure compared to the non-hybridizing single-stranded circular DNA, a slower migrating band would be expected. Twenty microliters of the culture supernatant of a reference phage (clone 4) was mixed with 20 ul of supernatants

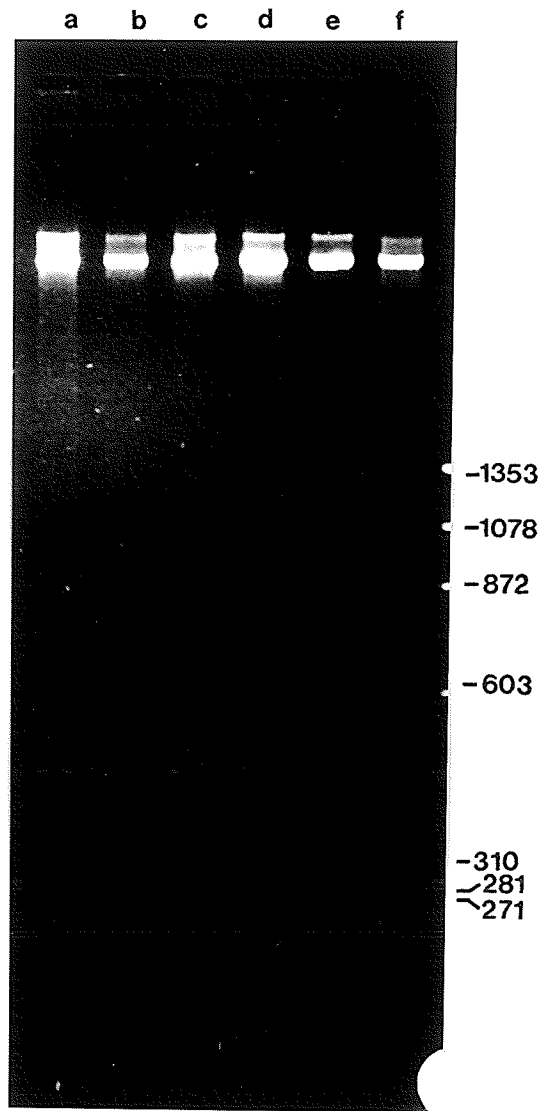


Figure 2.10 Restriction endonuclease analysis of M13mp8/IFN subclones. Intracellular RF's were isolated from the positive clones as described in the text and digested with HindIII. The DNA were electrophoresed on a 1.5% agarose gel and stained with 1 ug/ml ethidium bromide. Lanes: a, clone 4; b, clone 6; c, clone 8; d, clone 11; e, clone 13; f, M13mp8 vector without the IFN insert. Molecular size standards in base pairs were ϕ X174 DNA digested with HaeIII.

of the tested phages. Two microliters of 2% SDS and 6 ul of gel loading buffer were added into each reaction. Hybridization was carried out at 67°C for 1 h and the reactions were analyzed in a 0.7% agarose gel. As shown in Figure 2.8, the presence of a slower migrating band in addition to the parental band is indicative that clones 8 and 13 (lanes h and i, respectively) are of opposite orientation relative to the reference phage (clone 4). The presence of the parental band was likely due to incomplete hybridization. On the other hand, no hybridization was observed between clone 4 and the control phage that had no insert (lane g).

In addition to the 'figure-8' analysis, which was used to screen and to group phages that contained inserts of opposite orientations, restriction enzyme analysis of the intracellular RF was also performed in order to identify the exact orientation of the inserts. Intracellular RF were isolated essentially the same as described in Section 2.3. Supercoiled RF's were digested with HindIII (Figure 2.10) or EcoRI and KpnI (Figures 2.11 and 2.12). Restriction digestion of the RF with HindIII re-generated the full-length rHuIFN- γ insert (440 bp) (Figure 2.10, lanes a to e), while double digestion of clone 4 and clone 8 with EcoRI/KpnI yielded the diagnostic fragments (Figure 2.12) that were either 124 bp or 383 bp in length (Figure 2.12, lanes a and b, respectively). Clone 4, whose 5'-end was adjacent to the EcoRI site of the multiple cloning site (Figure 2.11), was used for site-directed mutagenesis throughout this study.

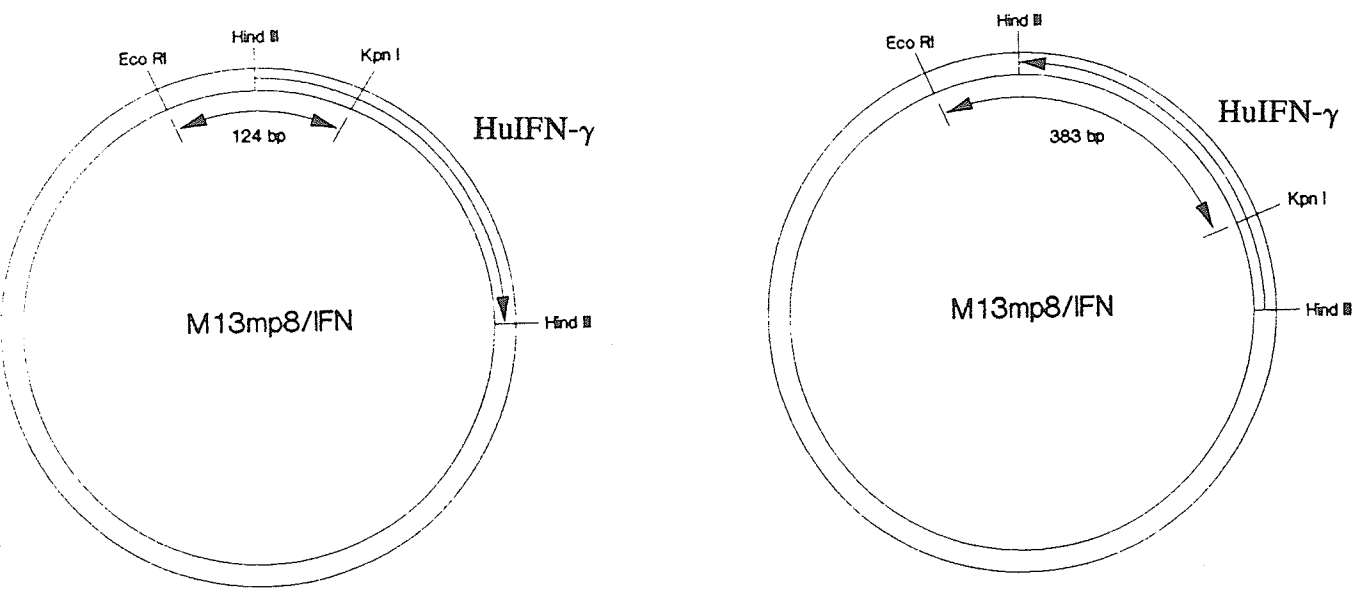


Figure 2.11 Schematic of restriction endonuclease digestions for distinguishing the orientations of M13mp8/IFN clones. The two orientations could be distinguished by the diagnostic fragments of either 124 bp or 383 bp generated by double-digestions of the intracellular RF's with EcoRI and KpnI.

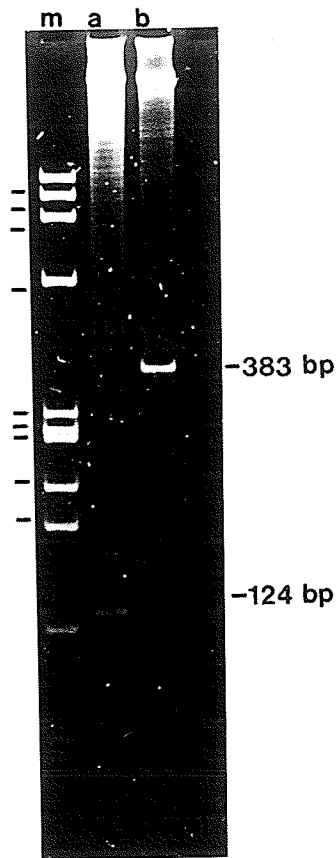


Figure 2.12 Polyacrylamide gel electrophoresis of EcoRI/KpnI digestion of M13mp8/IFN clones. Clone 4 (lane a) and clone 8 (lane b) were digested with EcoRI and KpnI, electrophoresed in a 5 % polyacrylamide gel (1.5 mm thick), and stained in 1 ug/ml ethidium bromide. The positions of the 124-bp and the 383-bp fragments are indicated on the right. Molecular size standards (lane m) were ϕ X174 DNA digested with HaeIII (from top to bottom): 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, 271 bp, 234 bp, 194 bp, 118 bp, and 72 bp.

2.18.1 Design of Oligonucleotide Probes

Synthetic oligonucleotides were designed to enable efficient site-directed mutagenesis and to allow highly specific screening of single-site mutants. The effective use of synthetic oligonucleotide for these purposes depend on a number of factors (Wallace and Miyada, 1987). Three of the major factors will be considered here: (i) the G+C content, (ii) length of the oligonucleotides, and (iii) the position of the mismatch.

DNA-DNA hybridization depends on the pairing of individual nucleotide bases on two complementary strands through hydrogen bonding. Oligonucleotides hybridize to complementary sequences with a high degree of specificity (Wallace et al., 1979; Wallace et al., 1981). It has been shown that the G+C content of an oligonucleotide is important in duplex stability (Suggs et al., 1981). When the dissociation of an oligonucleotide-DNA duplex is determined as a function of temperature with the G+C content taken into account, an empirical relationship (Wallace Rule) can be derived (Wallace et al., 1979):

$$T_m = 2^{\circ}\text{C}(\text{number of A} + \text{T residues}) + 4^{\circ}\text{C}(\text{number of G} + \text{C residues})$$

where T_m is the melting temperature at which 50 % of the duplex is dissociated. This relationship is only valid for duplexes that are 11-23 bases long in 1.0 M Na^+ (6x SSC) (Wallace et al., 1979). The T_m of short oligonucleotides with one mismatch is 7-9°C lower than that of the perfect-matched duplex. Therefore, oligonucleotides of such lengths could be used to distinguish

a single mismatch from the perfect-matched duplex by adjusting the temperature of hybridization and the temperature of washing.

The hybridization kinetics is governed by the length of the oligonucleotide probe: the longer the sequence, the lower the temperature is required for hybridization. However, hybridization at low temperature would increase the chances of non-specific hybridization. The length of the probe also determines the temperature required for screening mutants that have one nucleotide mismatch. Long probes may allow more efficient hybridization but may not be able to distinguish single-nucleotide mismatches. Short oligonucleotide probes that are of 15 to 20 nucleotides in length are usually suitable for such purposes.

The position of the mismatch is also important for efficient mutagenesis. The single-nucleotide mismatch should be at the middle of the oligonucleotide for two main reasons. First, the 5' end of the oligonucleotide forms a perfect duplex with the template so that repairing does not cause the displacement of the oligonucleotide by the Klenow fragment of *E. coli* DNA polymerase I. Second, the 3' end of the oligonucleotide forms a sufficiently stable duplex with the template so as to prevent exonucleolytic degradation by the Klenow fragment.

2.18.2 Oligonucleotides used to direct mutagenesis of Asp⁴¹, Gln⁴⁶, and Gln⁴⁸.

Based on the criteria described in Section 2.18.1, oligonucleotides were designed to direct mutagenesis at residues 41, 46, and 48. Four oligonucleotides were designed to specifically substitute Asp⁴¹ for Ala, Asp⁴¹ for Asn, Gln⁴⁶ for Leu, and Gln⁴⁸ for Leu:

Clone Names	Synthetic Oligonucleotides	T _m (°C)	T _m with one mismatch(°C)
D41A	5' TTTACGGG <u>C</u> ACTCTCC 3'	50	43
D41N	5' ATTTTACGGT <u>T</u> ACTCTCCTCT 3'	58	
Q46L	5' TTGGCTC <u>A</u> GCATTAT 3'	42	35
Q48L	5' GACAATT <u>A</u> GGCTCTG 3'	44	37

(The single-nucleotide mismatch is underlined)

To increase the efficiency as well as the stability of annealing of the oligonucleotide with the single-stranded template, the oligonucleotides were designed so that the single-nucleotide mismatch was in the middle of the strand.

The T_m of these oligonucleotides with one mismatch shown above indicated that in order to facilitate efficient annealing, the hybridization temperature should be maintained below 18°C (low stringency hybridization). Thus, hybridization of the ssDNA template with all of the oligonucleotides, except that of D41N which is described in Appendix 2, was carried out at 12.5°C.

To screen for the desired mutants, the same oligonucleotides were used. Since the T_m of a short heteroduplex with one mismatch is between 7-9°C lower than that of the perfect-matched duplex, the hybridization temperatures were adjusted to the T_m's that would allow 50%

hybridization between the oligonucleotide and the mutant (high stringency hybridization). As a result, the efficiency of hybridization between the oligonucleotide and the mismatched-parental molecule would be substantially reduced at these temperatures. This method thus allows the differentiation between the mutants and the parental molecules. Any nonspecific hybridization could either be washed away at the hybridization temperatures or by increasing the temperatures of the washing steps 1-2°C above the hybridization temperatures. Thus, the screening of D41A was performed at 42°C and washed at 45°C whereas both Q46L and Q48L were hybridized and washed at 37°C.

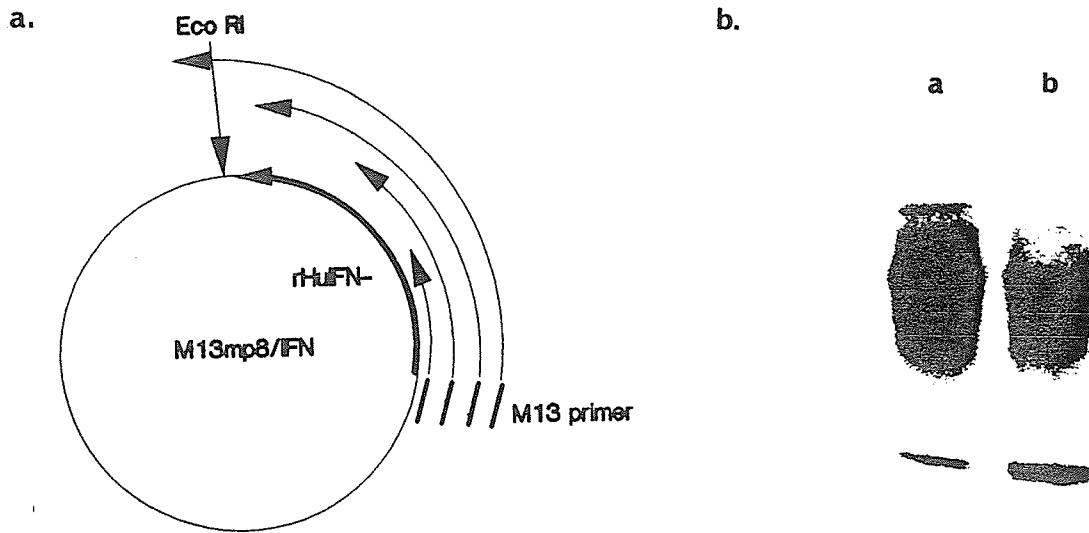


Figure 2.13 a, Schematic of M13 ssDNA template titration. The rHuIFN- γ coding sequence (thick arrow) was subcloned into the multiple cloning site of the M13mp8 vector. The M13 15-base universal primer downstream of the IFN insert was used for enzymatic extension. The direction of the extension is indicated (thin arrows). The distance between the primer and the EcoRI site is approximately 500 bases. b, Electrophoresis analysis of template titration. The reaction samples were resolved on a 4% sequencing gel. The amount of ssDNA template used for titration were: lane a, 0.18 μ g and lane b, 0.6 μ g. The lower band of each lane represents the EcoRI-linearized dsDNA resulted from primer-extension.

2.18.3 M13mp8/IFN template DNA titration

The ratio of M13 ssDNA template to synthetic primers is one of the critical factors in ensuring efficient primer-extensions. Template titration was therefore performed in order to determine the optimal template/primer ratio. Two different concentrations of single-stranded M13mp8/IFN template DNA, 0.6 ug and 0.18 ug, were used while the amount of primer was kept constant. The DNA templates were adjusted to 12.5 ul with 1.0 ul of 10x HIN buffer (0.5 M Tris.HCl, pH 7.5/0.1 M MgCl₂/0.05 M DTT) and 20 pmol of the 15-base universal primer (5' CCCAGTCACGACGTT 3') (Pharmacia) located at 17 bp downstream of the 3'-HindIII site of the multiple cloning site (Figure 2.13a). Hybridization was carried out sequentially at 68°C for 10 min, 42°C for 10 min, and 25°C for 60 min. The hybridized primers were enzymatically extended by adding into the hybridization mix 1.0 ul each of 0.1 M DTT, 1.0 mM TTP, 1.0 mM dATP, 1.0 mM dGTP, and 2.0 ul of [α -³²P]dCTP (4000 Ci/mmol, 10 mCi/ml), 1.0 unit DNA polymerase (Klenow fragment) (1.67 units/ul), and incubated at 25°C for 30 min. The reactions were then supplemented with 7.0 ul of chase mix (2.0 mM of each of the four dNTP) and further incubated for 20 min at 25°C. Reactions were inactivated by heating to 68°C for 10 min. In order to determine the length of the extension, both of the extended templates were digested with 40 units of EcoRI at 37°C for 2 h and separated on a 4% urea-PAGE. The gel was covered with food wrap and exposed to XAR-2 (Kodak) film. Successful extension of the template was indicated by the presence of the EcoRI-digested fragment. As shown in Figure 2.13, increasing amount of the EcoRI fragment was seen in the reaction where 0.6 ug of template was used. As

evident from the autoradiograph, the amounts of template that were used had not reached saturation, indicating that the amount of template could be increased further. Therefore, in the primer-extension experiments described below, between 0.8 to 1.0 ug of template was used. Accordingly, the primer to template ratio of 1:33 was used in the primer extension experiment as described in Section 2.18.4.

2.18.4 Primer-extension

The principle of site-directed mutagenesis is illustrated schematically in Figure 2.6. Four synthetic primers were designed to direct mutagenesis at Asp⁴¹, Gln⁴⁶, and Gln⁴⁸:

Amino Acid Changes	Clone Names	Synthetic Oligonucleotides	Nucleotide Changes
Asp41 to Ala41	D41A	5' TTTACGGG <u>C</u> ACTCTCC 3'	T to G
Asp41 to Asn41	D41N	5' ATTTTACGGT <u>T</u> ACTCTCCTCT 3'	C to T
Gln46 to Leu46	Q46L	5' TTGGCTC <u>A</u> GCATTAT 3'	T to A
Gln48 to Leu48	Q48L	5' GACAATT <u>A</u> GGCTCTG 3'	T to A

The primers were radiolabelled at the 5' end with [γ -³²P]ATP using polynucleotide kinase as described in Section 2.11.1. Twenty picomoles of each primer were mixed with 3.0 ul of 10x HIN buffer, 0.84 ug of DNA template (0.36 pmol), and adjusted to 20 ul with sterile double-

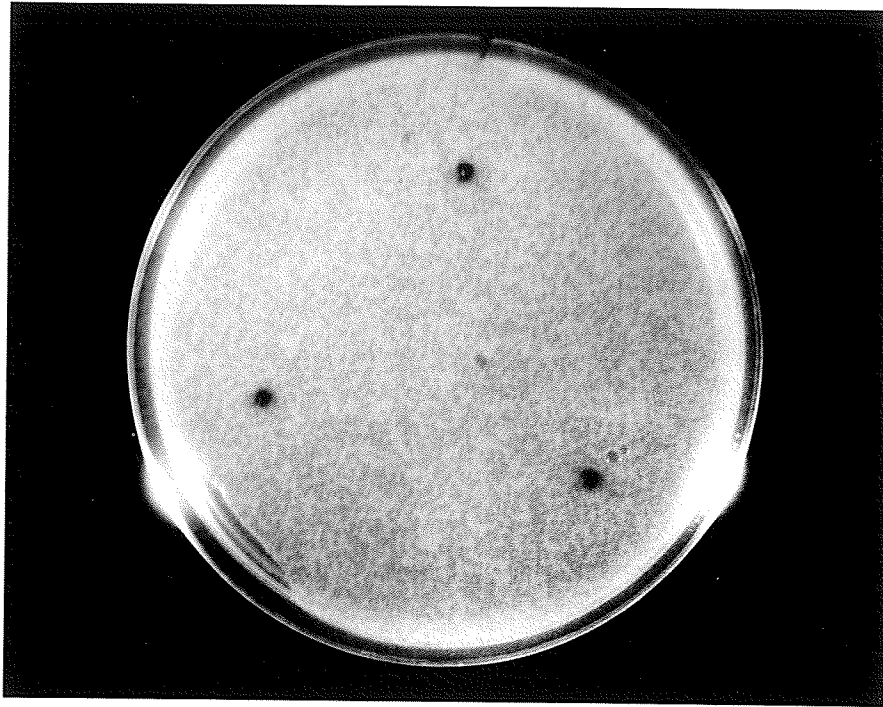
distilled water. To serve as a control, a parallel reaction was carried out except that the primer was omitted. The M13mp8/IFN single-stranded DNA template was allowed to hybridize with the respective primers sequentially at 68°C for 10 min, 42°C for 10 min, and 20°C for 60 min. Enzymic extension was carried out by adding 1.5 ul of each of the 10 mM dNTP (final concentration = 0.5 mM), 3.0 ul of 10 mM ATP (final concentration = 1.0 mM), and 5 units of DNA polymerase (Klenow fragment). The reactions were incubated at 20°C for 5 h before an additional 5 units of Klenow fragment was added. One unit of T4 DNA ligase was then added and the reactions were incubated at 12.5°C overnight. An additional one unit of T4 DNA ligase was added the next day and incubated for 2 to 3 h. The primer-extended DNAs were used directly for transformation as described in Section 2.18.5.

Variant D41N was constructed using an Amersham mutagenesis kit based on the method of Eckstein and coworkers (Taylor et al., 1985a; Taylor et al., 1985b; Nakamaye and Eckstein, 1986). The detailed methodology has been described in Appendix 2.

2.18.5 Transformation of primer-extended DNA into bacterial (JM103) host

A single colony of JM103 from minimal agar plate was inoculated into 2 ml 2x YT medium and allowed to grow overnight at 37°C on a rotary-shaker. The overnight culture was then diluted 1 in 100 with 2x YT medium and was allowed to grow with vigorous aeration until logarithmic phase ($OD_{600} = 0.3$ to 0.4). Cells were chilled on ice and were collected by centrifugation at 4000 rpm in a SS34 rotor for 10 min at 4°C. The cell pellet was resuspended

A



B

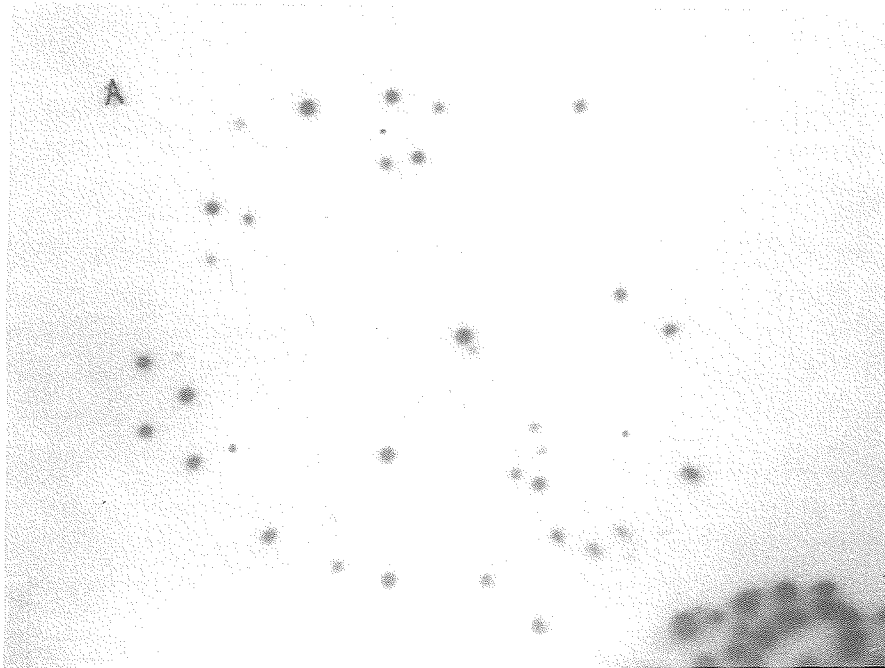


Figure 2.14 Screening of single-site variants by colony hybridization. a, plaques of M13mp8/IFN- γ variants (> 1000) were generated. The three dominant black dots are India ink markers used to identify the orientation of the nitrocellulose filters. b, plaques in (a) were transferred onto nitrocellulose, hybridized with the appropriate ^{32}P -labelled oligonucleotide probe, and exposed to X-ray film. The labelled oligonucleotide probe would only hybridize with the mutants but not with the parental molecules which had one mismatch relative to the probe.

in equal volume of ice-cold ST buffer (ST buffer = 25 mM Tris.HCl pH 7.5/10 mM NaCl) and once again collected by centrifugation as before. The cell pellet was then resuspended by swirling in 1/2 volume of ice-cold CaST buffer (CaST buffer = 50 mM CaCl₂ in ST buffer) and incubated on ice for 20 min. Cells were once again collected by centrifugation and the pellet resuspended in 1/10 volume of ice-cold CaST buffer. The cells thus treated had become competent to take up exogenous DNA. Five to ten microliters of primer-extended DNA (0.1-0.2 ug) was added to 100 ul of CaST and 0.3 ml competent cells. The cells were incubated on ice for 40 min and then heat-shocked at 42°C for 2 min. The cells were diluted 10-fold and were added 10 ul 100 mM IPTG, 50 ul 2% Xgal, 0.2 ml log phase JM103, and 3 ml soft agar (45°C) before pouring onto pre-warmed 2x YT agar plates. The soft agar was allowed to air-dry at room temperature and the plates were incubated overnight at 37°C. Plaques appeared the next day (Figure 2.14a) were screened by colony hybridization.

2.18.6 Screening for single-site variants

Variants D41A, Q46L, and Q48L were initially screened by colony hybridization. Plaques were transferred onto 0.22 um nitrocellulose membrane by plaque-lifting and were allowed to air-dried. Cells that were transferred were lysed with 0.5 M NaOH/1.5 M NaCl for 5 min and then neutralized with 0.5 M Tris.HCl (pH 7.5)/1.5 M NaCl for 5 min. The membranes were further treated with 3x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate (pH 7.0)) for 5 min before oven-baked for 3 h at 80°C. The membranes were then prehybridized in 6x SSC/10x

Denhardt solution (1X Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin dissolved in water) for 2 h at 68°C. The pre-hybridization solution was completely drained and the membranes were hybridized in 6x SSC/10x Denhardt solution in the presence of the [γ - 32 P]ATP-labelled probes (1×10^6 cpm in 5 ml). Oligonucleotides used for mutagenesis (15- and 16-mer) were used as probes for hybridization. Hybridization was carried out overnight at 42°C for variant D41A and at 37°C for variants Q46L and Q48L. The nitrocellulose membranes were then washed five times with 6x SSC at hybridization temperatures and were air-dried. Positive clones were identified by exposing the dried filter to XAR (Kodak) film with an enhancing screen (DuPont) at -70°C (Figure 2.14b).

Two individual positive clones from each variant were subjected to another round of re-plaquing and re-hybridization under the conditions described above. Two individual positive clones from the second round of hybridization were again isolated. Single-stranded phage DNA from these clones were prepared and the single nucleotide mutations were confirmed by DNA sequencing (see Results of Chapters 4 and 5).

Variant D41N clones were screened directly by dideoxy chain termination DNA sequencing. Of the four individual plaques being screened, two were found to have the desired single-nucleotide mutation (see Results of Chapter 4).

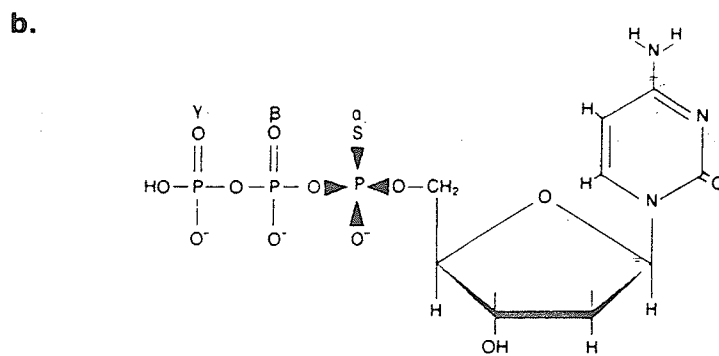
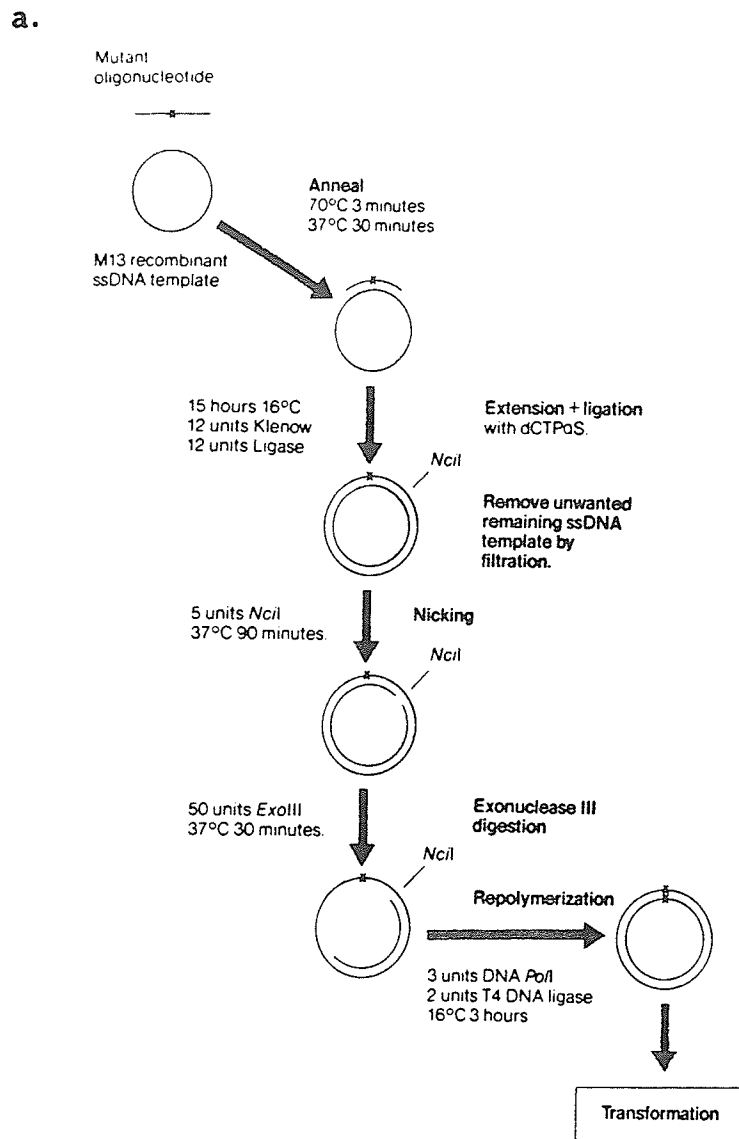


Figure 2.15 a, Schematic of the Amersham site-directed in vitro mutagenesis system. b, The structure of dCTP α S analogue.

2.19 Construction of single-site variant using the 'Eckstein' method

The low efficiency of generating single-site mutants by the conventional site-directed mutagenesis described in Section 2.15 is due to several reasons intrinsic to the mutagenesis system. Firstly, the heteroduplex dsDNA closed circular DNA produced by primer-extension contain a mixed population of single-stranded non-mutant template DNA that has remained uncopied and partially replicated dsDNA. Secondly, following transformation and DNA synthesis *in vivo*, segregation of the two strands of the heteroduplex molecules occurs resulting in a mixed population of mutant and non-mutant phages. Although there should, in theory, be equal number of mutants and wild-types plaques, the percentage of mutants is always substantially lower. This is due to the fact that the methyl-directed mismatch repair system in *E. coli* favors the repair of non-methylated DNA. Newly synthesized DNA strands which have not yet been methylated are preferentially repaired at the position of a mismatch, thus preventing a mutation. Similarly, the mutant strands generated *in vitro* are not methylated and are repaired by the cell. Thirdly, since the mutant oligonucleotide is susceptible to digestion by 5'-3' exonucleases present in some preparations of DNA polymerase I 'Klenow' fragment, digestion beyond the position of the single base mismatch before the oligonucleotide is enzymatically extended and ligated would give rise to wild-type molecule.

The 'Eckstein' method of site-directed mutagenesis circumvents some of the problems described above by enriching the primer-extended dsDNA by simple filtration and enzymatic removal of the wild-type strand. A schematic of this method is shown in Figure 2.15a. Mutant

oligonucleotide was annealed with the template and then extended with dNTP except that dCTP was replaced by the analogue dCTP α S which contained a sulfur atom instead of an oxygen on the α -phosphate of dCTP (phosphorothioate) (Figure 2.15b). The completely extended DNA was subsequently ligated while any remaining ssDNA template was removed by filtration. This step minimizes the percentage false positives resulting from unmutated ssDNA template. The repaired circular dsDNA was digested with the restriction endonuclease NciI which recognizes the sequence: CC(C/G)GG. Since the mutant strand was synthesized using the dCTP α S analogue all the NciI sites on this strand would be 'protected' (Taylor et al., 1985; Nakamaye and Eckstein, 1986), whereas the non-mutant (non-phosphorothioate) strand would be nicked by NciI. The nicked DNA was then digested with the enzyme exonuclease III which recognizes free 3'-ends and degrades dsDNA in a 3' to 5' direction. Thus, only the nicked non-mutant strand of the heterduplex is attacked by this enzyme. The DNA was then re-polymerized to generate double-stranded closed circular DNA in the form of homoduplex mutant molecules.

The experimental procedures for the Eckstein method has been outlined in detail in the handbook supplied with the mutagenesis kit (Appendix 2). The procedures for the construction of single-site variant D41N were carried out exactly as described in the handbook.

CHAPTER 3 STRUCTURE-FUNCTION STUDIES OF THE HuIFN- γ BY DELETION ANALYSIS

3.1 Introduction

One of the most intriguing questions concerning the properties of cytokines is their pleiotropic activities on a variety of cells. Although IFN- γ was first identified by its antiviral effect *in vitro* (Wheelock, 1965; Wheelock and Toy, 1973), a number of immunoregulatory functions have been attributed to the activities of IFN- γ (Pestka et al., 1987). Since IFN- γ was initially purified from mitogen-stimulated cultured lymphocytes, some of these activities have been due to contamination by other cytokines including the tumor necrosis factors (TNF) (Old, 1985; Beutler and Cerami, 1986; Aggarwal et al., 1985; Trinchieri et al, 1986). However, with the advent of recombinant DNA technology, a homogeneous source of cloned IFN- γ expressed in *E. coli* is now available for structural and functional studies. The rHuIFN- γ has been shown to exhibit many of the biological activities that were previously identified (Pestka et al., 1987). Despite these major advances, the structure/function relationships of HuIFN- γ remain poorly understood. It is still not known if binding of HuIFN- γ to the cell surface receptor is sufficient to initiate signal transduction or if an additional effector site on the HuIFN- γ molecule is required. Furthermore, neither the receptor binding site nor the effector site has been identified. Therefore, in order to dissect the various modes of action of the HuIFN- γ , it is imperative to identify its functional site(s). The strategies utilized in the present study involve the design and

construction of rHuIFN- γ variant molecules that either have various deletions or variant molecules that contain specific amino acid modifications. These approaches should facilitate the delineation of the functional and/or receptor binding domains of HuIFN- γ .

3.2 Studies of deletion variants

The study of genetically mutated molecules has been one of the most powerful methods for understanding the molecular biology and normal functions of a macromolecule. However, naturally mutated molecules are difficult to isolate. *In vivo* mutagenesis, either using chemical carcinogen or ultraviolet light, has provided alternative ways for generating genetic mutants in spite of the nonspecific nature of these methods. With the advent of molecular cloning, it is now possible to engineer specific mutations into any coding sequence and to produce the mutated gene products in sufficient quantities for functional analyses.

Since the bioactivity of a particular protein requires the proper folding of its tertiary structure which, in turn, is dictated by the information contained within its primary sequence (Anfinsen, 1973), it is therefore possible to study the functions of a macromolecule by altering specific regions or amino acid residues in the primary sequence. However, aside from the technical difficulties in the production of recombinant proteins, there is the formidable problem of choosing, among the astronomical number of possible combinations of mutations, target amino acid residues that may be informative. Therefore, in order to study the structure-function relationships of a particular protein, it is imperative to devise practical strategies that would limit

the search for its functional site(s). One of the approaches is to conduct deletion analyses which would exclude sequences that are not involved in the functions of the protein.

Although the cDNA of HuIFN- γ has been cloned (Gray et al., 1982; Devos et al., 1982) and the recombinant HuIFN- γ polypeptide has been successfully expressed *in vitro* (Jay et al., 1984b), information is scarce regarding the relationships between its primary sequence and its functions. The objective of the present study was to determine the maximum amount of sequence that may be deleted from the C-terminus without losing the functional activity. Thus the functional sites may be localized to the retained sequences and the deleted amino acid sequences may be excluded from further search for the location of the active sites. A series of carboxy-terminus deletion variant molecules were constructed and the resulting truncated polypeptides were tested for their biological activities. Three different approaches were employed to generate these variants. They were (i) isolation of proteolytic product, (ii) frameshift mutation, and (iii) restriction fragment replacement.

3.3 Materials and Methods

Experimentation undertaken in the entire study made use of the rHuIFN- γ coding sequence that had been chemically synthesized by Jay et al (1984a). Deletion variant Del-128 was extracted and purified essentially as described for rHuIFN- γ in Section 2.1 except that 1 mM of ZnCl₂ was omitted in the extraction procedures. Western immunoblot analysis was carried out as described in Section 2.2.

3.3.1 Extraction and purification of Del-128

The procedures for extraction and purification of Del-128 were essentially the same as those used for isolation of the full-length rHuIFN- γ except that 1 mM ZnCl₂ was omitted. The purified fractions were pooled and 100 μ l of affinity-purified sample was precipitated in a final concentration of 10 % (v/v) trichloroacetic acid (TCA). The precipitated protein was pelleted by centrifugation at 10,000 rpm, washed with 10 volumes of ice-cold 100% acetone, air-dried, and resuspended in 9 μ l of water and 3 μ l of 4x SDS sample buffer. The sample was boiled for two minutes before loading onto a 15% SDS polyacrylamide gel and separated by electrophoresis. The proteins were detected by staining the gel with Coomassie Blue (Section 2.1) (Figure 3.1).

3.3.2 Susceptibility of the rHuIFN- γ to OmpT

A 300 ml-overnight culture of P₁₄R₃IFN was divided into two 150 ml portions and the cells were collected by centrifugation at 5000 rpm in a Sorvall GSA rotor at 4°C. The pellets were washed with an equal volume of 50 mM Tris.HCl (pH 8.0)/30 mM NaCl and once again collected by centrifugation. The pellets were resuspended separately in 6 ml of (i) 50 mM Tris.HCl (pH 8.0)/30 mM NaCl and (ii) 50 mM Tris.HCl (pH 8.0)/30 mM NaCl/1 mM ZnCl₂. Thirty microliters were taken from each of the cell suspension, mixed with 10 μ l of 4x SDS sample buffer, boiled for 2 min, and were used as controls for cells directly lysed with SDS sample buffer. The resuspended pellets were sonicated 4 times for 30 sec at 0°C. The cell debris

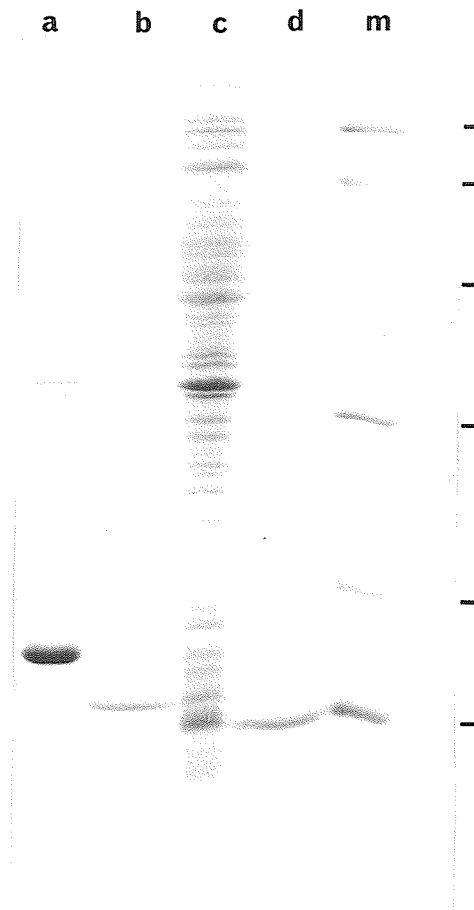


Figure 3.1 SDS-PAGE analysis of immunoaffinity purified Del-128 and Del-122. Lanes: a, P₁₄R₃IFN cell lysate; b, purified Del-128; c, Del-122 cell lysate; d, purified Del-122; m, molecular weight markers (from top to bottom) were phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

were removed by centrifugation at 10,000 rpm at 4°C for 30 min in a Sorvall SS34 rotor and the supernatants were collected.

The time course experiment was carried out by incubating 12 ul of each of the two supernatants for 0, 5, 10, 20, and 50 min at 37°C. At each time point, the incubation was stopped by the addition of 4 ul of 4x SDS sample buffer and the samples were boiled for 2 min. Each sample was diluted 1:10 before loading 5 ul onto a 15% SDS polyacrylamide gel. A control consisting of total cell protein directly lysed with SDS sample buffer was diluted 1:50 before loading 4 ul onto the gel. A negative control was similarly prepared from cells which carried the expression vector without the rHuIFN- γ insert. The separated proteins were then blotted onto nitrocellulose electrophoretically as described in Section 2.2. The proteins were detected using a rabbit polyclonal serum against denatured rHuIFN- γ that was diluted 1:1000 and the secondary antibody was a peroxidase-conjugated goat anti-rabbit antibody (Nordic Immunology) that was diluted 1:2000. Molecular weight markers were biotinylated standards diluted 1:4 as recommended by the manufacturer (BioRad).

3.3.3 Construction of deletion variant Del-122 by frameshift mutation*

Since the deletion of the C-terminal 15 amino acid residues of rHuIFN- γ did not affect the biological activities, variants which had more extensive deletions upstream from residue 128 were constructed to further exclude sequences that are not involved in conferring the biological activities of HuIFN- γ .

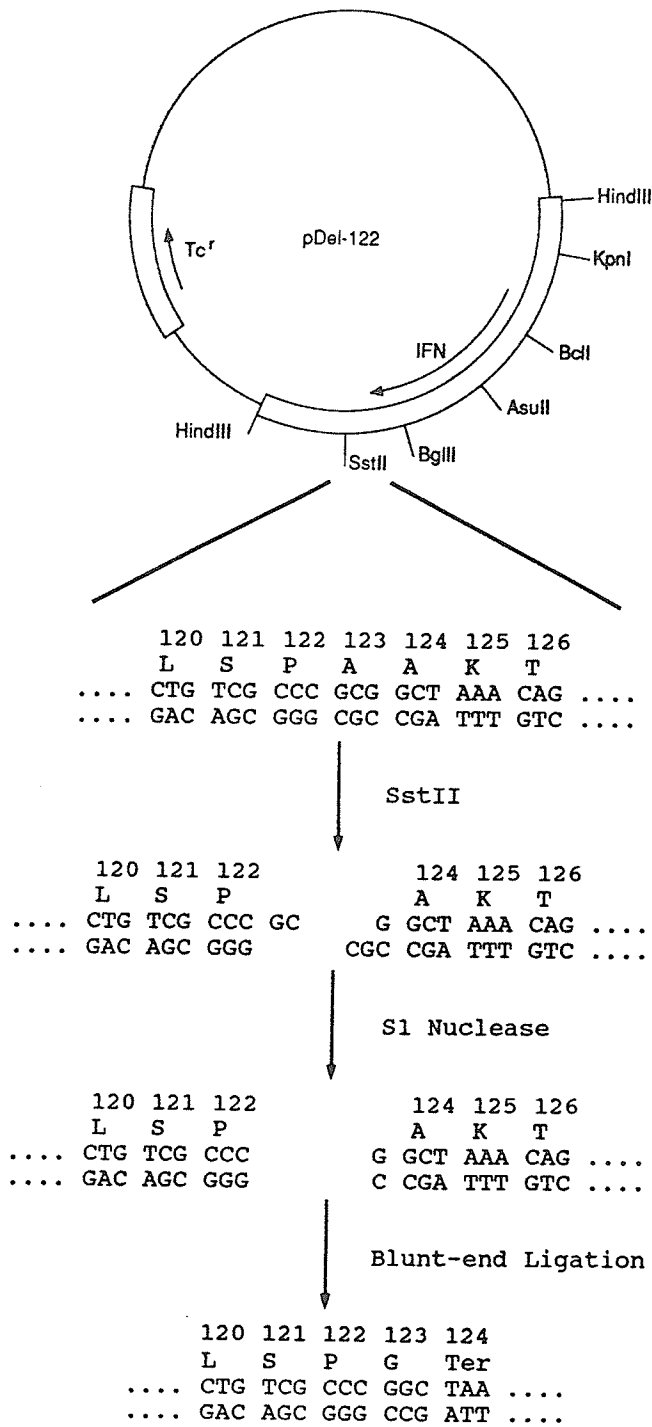


Figure 3.2 Construction of pDel-122 by frameshift mutation. pJP₁₄R₃-IFN is linearized with SstII and the two-nucleotide overhang is removed by S1 nuclease. Subsequent re-circularization by blunt-end ligation results in a reading frameshift which brings in an in-frame translational termination signal (TAA) at position 124. The single-letter code for amino acids is used. Tc^r, tetracycline resistance gene.

Construction of the deletion variant Del-122 which had deletion further upstream from amino acid residue 128 was achieved by specific gene modifications. Variant Del-122 was derived from frameshift mutation at the unique SstII restriction enzyme site which was incorporated into the coding sequence of rHuIFN- γ during chemical synthesis (Jay et al., 1984b). The construction of deletion variant Del-122 is shown schematically in Figure 3.2. The parental plasmid encoding the rHuIFN- γ coding sequence was linearized at the unique SstII site between amino acid residues 122 and 124. The two-nucleotide 5' overhang generated was removed by S1 nuclease and the blunt ends were subsequently ligated to generate a molecule which had two base pairs deleted from the Sst II site. The deletion of these nucleotides caused a frameshift of the coding sequence starting at amino acid residue 123, resulted in the replacement of alanine by glycine which was immediately followed by an in-frame termination codon (TAA). This modified plasmid was used to transform the *E. coli* strain LE392 made competent according to the procedures described in Section 2.5. This deletion variant, referred to as Del-122, produced a truncated polypeptide which lacked the C-terminal 21 amino acid residues. This deletion represented 14.7% of the rHuIFN- γ molecule. The mutation was confirmed by DNA sequencing as described in Section 2.7. The insert was sequenced in its entirety to ensure that no secondary mutation was introduced.

* Deletion variant Del-122 was designed and constructed by Dr. Ernest Jay.

3.3.4 Denaturation and Renaturation of rHuIFN- γ and Del-122 with 6 M Guanidine hydrochloride (GuHCl)

In order to determine the effects of chaotrophic agent on the biological activities of rHuIFN- γ , affinity-purified rHuIFN- γ and Del-122 were treated with 6 M GuHCl. All procedures were carried out at 4°C. One milliliter of the purified samples were added into 6.0 ml of 7 M GuHCl and incubated for 4 h. Denatured samples were renatured by diluting with 5 volumes of RPMI 1640 medium to 1.0 M with respect to GuHCl, dialyzed extensively in RPMI 1640 medium (Gibco), and aliquots were assayed for biological activities. To determine the activities of the renatured samples upon re-purification by affinity chromatography, the renatured samples were dialyzed in 50 mM Tris.HCl (pH 8.0)/30 mM NaCl before purifying by affinity chromatography as described in Section 2.1.

3.3.5 Construction of deletion variants Del-117, Del-111, and Del-106 by restriction fragment replacement

In order to exclude primary sequences upstream from residues 122 that are not required for conferring the biological activities of the HuIFN- γ , variants were constructed which had 26, 32, and 37 amino acid residues deleted from the C-terminus. Synthetic oligonucleotides were designed to replace the 60-bp BglII/SstII fragment that spanned residues 102 and 123 (Figure 3.3). These modified fragments were similar to the parental BglII/SstII fragment except that they

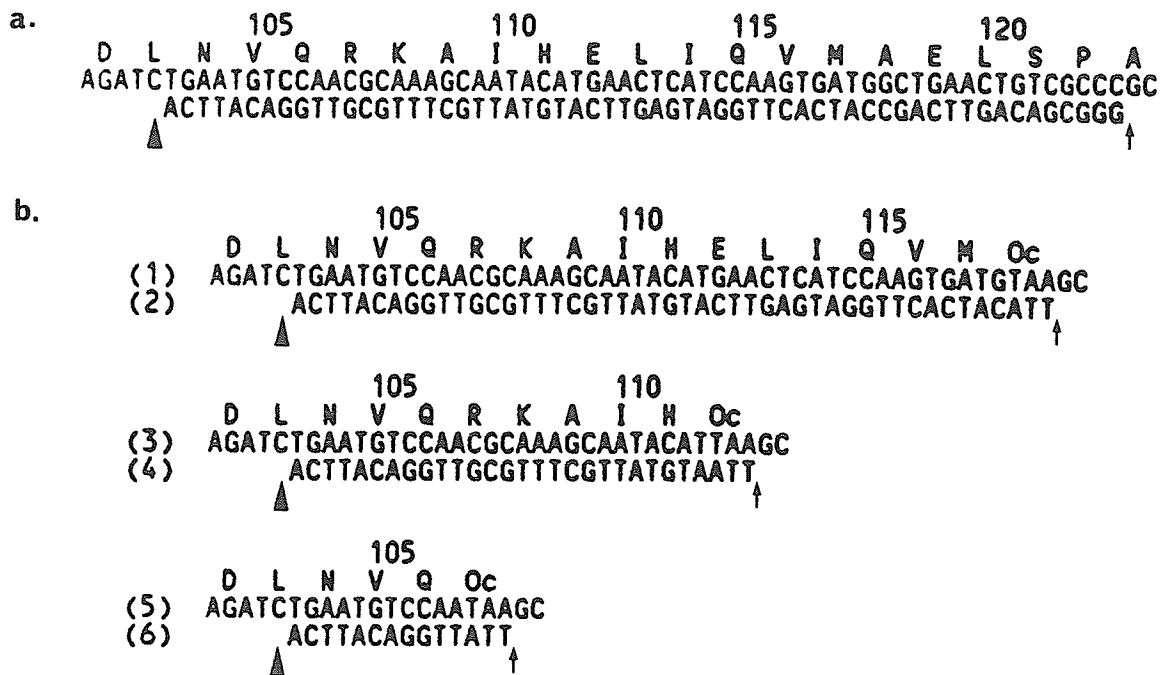


Figure 3.3 Nucleotide sequences of synthetic oligonucleotides used for fragment replacement. Cohesive ends are generated following annealing of the single-stranded oligonucleotides. The three sets of oligonucleotides are flanked by Bgl II (5' end) and Sst II (3' end). Fragments used to replace the unmodified Bgl II/Sst II fragment (a) are shown in (b): fragments 1 and 2 generate pDel-117, fragments 3 and 4 generate pDel-111, and fragments 5 and 6 generate pDel-106. Single-letter code for amino acids is used. The restriction enzyme sites are denoted ▲ (BglII) and ↑ (SstII).

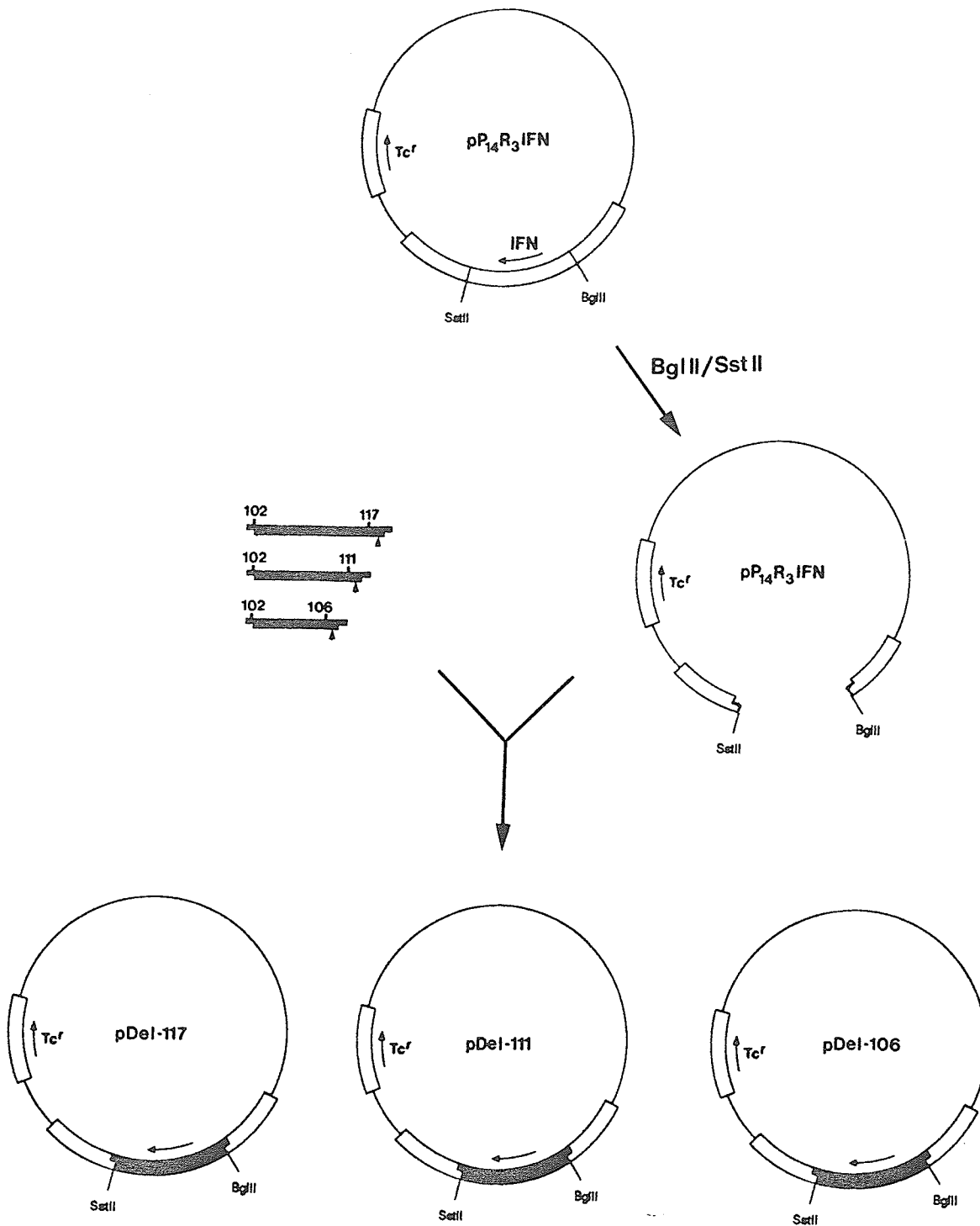


Figure 3.4 Construction of pDel-117, pDel-111, and pDel-106 by restriction fragment replacement. The BglII/SstII fragment of the rHuIFN- γ coding sequence is replaced with homologous double-stranded synthetic DNA fragments that have various deletions at the 3' ends and immediately followed by an in-frame translation termination signal (Δ). The BglII and SstII sites are conserved in these fragments.

were truncated at the C-terminus. In addition, an in-frame termination codon (TAA) was installed at the 3' end of the sense strand of each set of fragment. The strategy for the construction of these variants is schematically shown in Figure 3.4. When these variants were subsequently expressed in *E. coli*, truncated polypeptides were produced which lacked the C-terminal 26, 32, and 37 amino acid residues.

3.4 Results

3.4.1 Isolation and Characterization of Del-128

It was observed that truncated polypeptides were generated during the course of extraction and purification of the full-length rHuIFN- γ molecule ($M_r = 16,900$). Kung et al. (1987) have isolated a 15 kD polypeptide which was a proteolytic product from the N-terminus of the full-length rHuIFN- γ . The proteolytic cleavage site was shown to be located between Lys¹²⁸ and Arg¹²⁹ at the C-terminus (Kung et al., 1987). Subsequent analyses showed that the proteolytic cleavage was associated with the outer membrane of *E. coli* (Sugimura and Higashi, 1988) and that the membrane-associated protease OmpT was responsible for the proteolytic activity (Sugimura and Nishihara, 1988; Grodberg and Dunn, 1988). This protease, however, could be inhibited by divalent cations such as Cu⁺⁺ or Zn⁺⁺ (Sugimura and Nishihara, 1988). In addition, three other proteolytic products, which had M_r 's of 16,500, 16,000, and 15,400, were also observed in cell extracts prepared in the absence of ZnCl₂. In the present study, a 15 kD truncated form of the

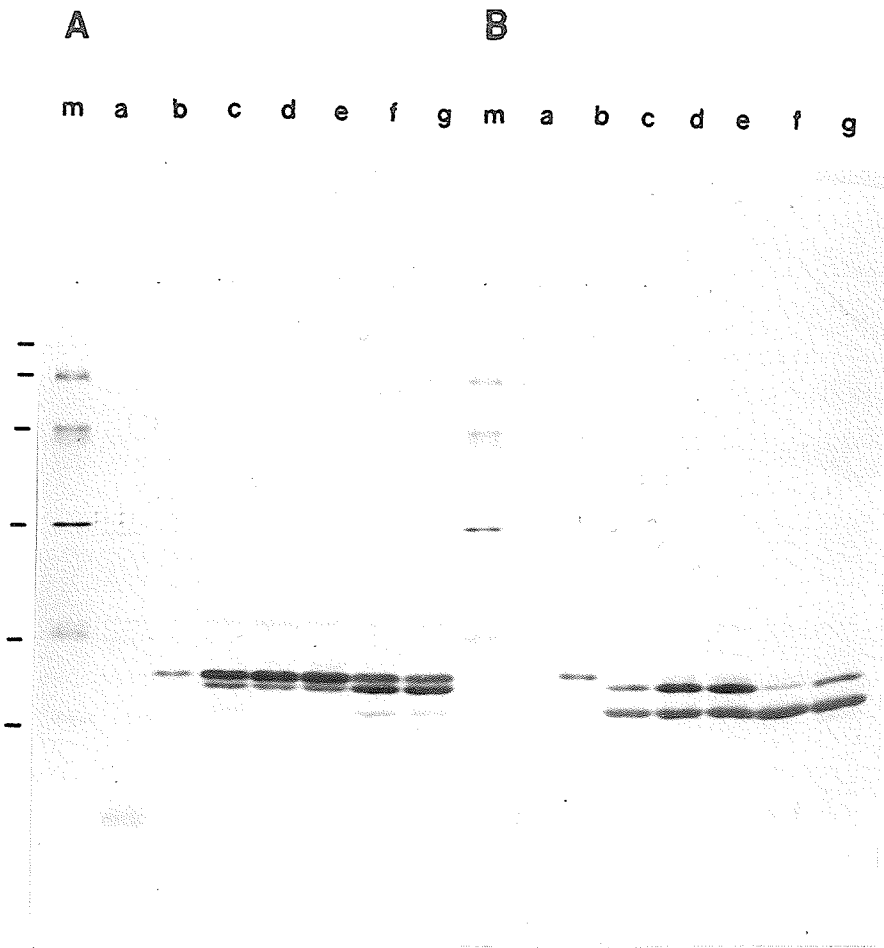


Figure 3.5 Western immunoblot analysis of proteolytic digestion of rHuIFN- γ . Soluble fraction of the sonicated cell extract of P₁₄R₃IFN was incubated in the presence (panel A) or absence (panel B) of 1 mM ZnCl₂ for different time intervals at 37°C. Lanes: m, biotinylated molecular weight markers (from top to bottom: rabbit muscle phosphorlyase b, 97,400; bovine serum albumin, 66,200; hen egg white ovalbumin, 42,699; bovine carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; hen egg white lysozyme, 14,400); a, lysate from cells containing the expression vector without IFN- γ insert; b, P₁₄R₃IFN lysate control; c to g, P₁₄R₃IFN X-pressed supernatant incubated at 37°C in the presence (Panel A) or absence (Panel B) of 1 mM ZnCl₂ for 0, 5, 10, 20, and 50 min, respectively.

rHuIFN- γ , designated Del-128, was also isolated. Since the purpose of the present study is to define the maximum amount of sequences that may be excluded, only the 15 kD polypeptide was purified and tested for biological activities.

Time course experiments were carried out to assess the susceptibility of the rHuIFN- γ to the membrane-associated protease OmpT. Supernatants from cells disrupted by sonication either in the presence or in the absence of 1 mM of ZnCl₂ were incubated at 37°C for 0, 5, 10, 20, and 50 min. The rHuIFN- γ related polypeptides were analysed by Western immunoblot. As shown in Figure 3.5, in the presence of ZnCl₂ at zero time, the rHuIFN- γ related polypeptides comprised approximately 70% of the full-length species and 30% of the 16.5 kD species in the presence of 1 mM of ZnCl₂ (Panel A, lane c), whereas almost all of the full-length molecules were converted into the 16.5 kD and 15 kD species in a ratio of 1:2 when ZnCl₂ was omitted (Panel B, lane c). As the incubation time progressed, the 16.5 kD became more prominent in the absence of ZnCl₂, approaching 55% of the total rHuIFN- γ protein (Panel B, lanes d and e) while the ratio of the full-length to the 16.5 kD species remained unchanged in the presence of ZnCl₂ (Panel A, lanes d and e). Furthermore, whereas more than 40% of the protein were converted to the 15 kD species in the absence of ZnCl₂ (Panel B, lanes f and g), only less than 5% of the rHuIFN- γ was cleaved in the presence of the cation inhibitor in 20 and 50 min at 37°C (Panel A, lanes f and g). The protease OmpT was only released after disruption of the cell since direct lysis of the cell pellets yielded essentially the full-length rHuIFN- γ (Figure 3.5, lane b of Panels A and B). Variant Del-128 was, therefore, not produced as a result of intracellular degradation.

The 16.5 kD polypeptide was equivalent to the deletion of 4 amino acid residues. Based

122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137
Pro Ala Ala Lys Thr Gly Lys Arg Lys Arg Ser Gln Met Leu Phe Arg
 ▲ ▲

138 139 140 141 142 143
Gly Arg Arg Ala Ser Gln
 ▲

Figure 3.6 Amino acid sequence of the C-terminus of the HuIFN- γ . Proteolytic cleavage sites of OmpT are indicated by arrowheads.

on the sensitivity to $ZnCl_2$, the cleavage site of this truncated product is likely to be between Arg¹³⁹ and Arg¹⁴⁰ (Figure 3.6), in accordance with the results reported by Sugimura and Higashi (1988). The presence of 1 mM $ZnCl_2$, however, did not completely inhibit the proteolytic cleavage at the Arg¹³⁹-Arg¹⁴⁰ site. The fact that the 16.5 kD polypeptide was generated at time zero was also in agreement with the results of Sugimura and Higashi (1988) that the peptide bond between Arg-Arg seemed to be more susceptible to OmpT than that of Lys-Arg.

Deletion variant Del-128 was purified as described in Section 2.1 and was found to have biological activities comparable to those of the full-length molecule (Table 3.1). Therefore, the C-terminal 15 amino acid residues are not involved in the biological activities of HuIFN- γ . The results on these data will be discussed in detail in Section 3.5.

3.4.2 Characterization of variant Del-122 polypeptide

Deletion variant Del-122 polypeptide was extracted and purified according to the procedures as described in Section 2.1 and analyzed by SDS-PAGE. As shown in Figure 3.1 (lane d), the 14.4 kD variant Del-122 polypeptide was purified to homogeneity as estimated by SDS-PAGE analysis. The immunological identity of this polypeptide was confirmed by its positive reaction in a Western immunoblot analysis using a polyclonal antiserum against denatured rHuIFN- γ as the detecting agent (Figure 3.7). The apparent molecular weight of the truncated polypeptide Del-122 (lane c) was estimated to be 14,400, in accordance with the calculated molecular weight of 14,300 for a truncated rHuIFN- γ molecule that had deletion of 20 amino

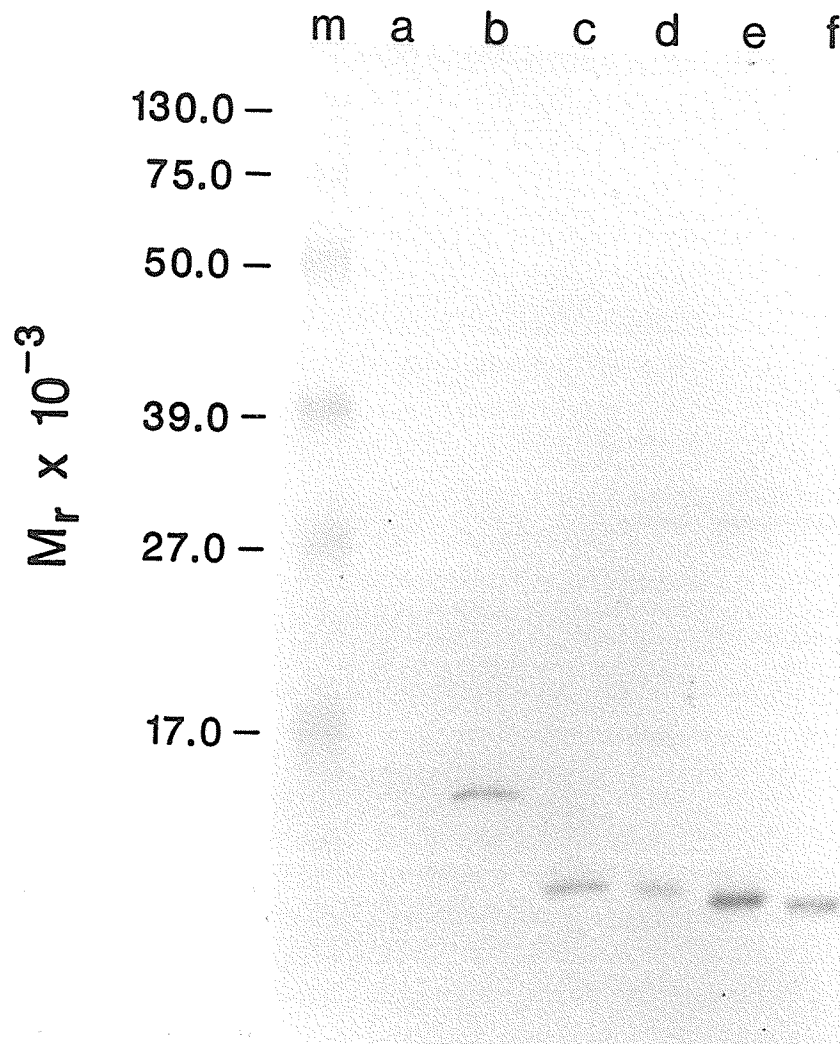


Figure 3.7 Western immunoblot analysis of the rHuIFN- γ and C-terminal deletion variant polypeptides. Cells harbouring the plasmids pJP₁₄R₃ (a), pJP₁₄R₃-IFN (b), pDel-122 (c), pDel-117 (d), pDel-111 (e), and pDel-106 (f) were lysed and were separately resolved on a 15% SDS polyacrylamide gel. Total proteins were blotted electrophoretically onto nitrocellulose and then probed with a rabbit polyclonal antiserum against purified rHuIFN- γ . The pre-stained molecular weight markers (m) were (from top to bottom): phosphorylase b (130,000), bovine serum albumin (75,000), ovalbumin (50,000), carbonic anhydrase (39,000), soybean trypsin inhibitor (27,000), lysozyme (17,000).

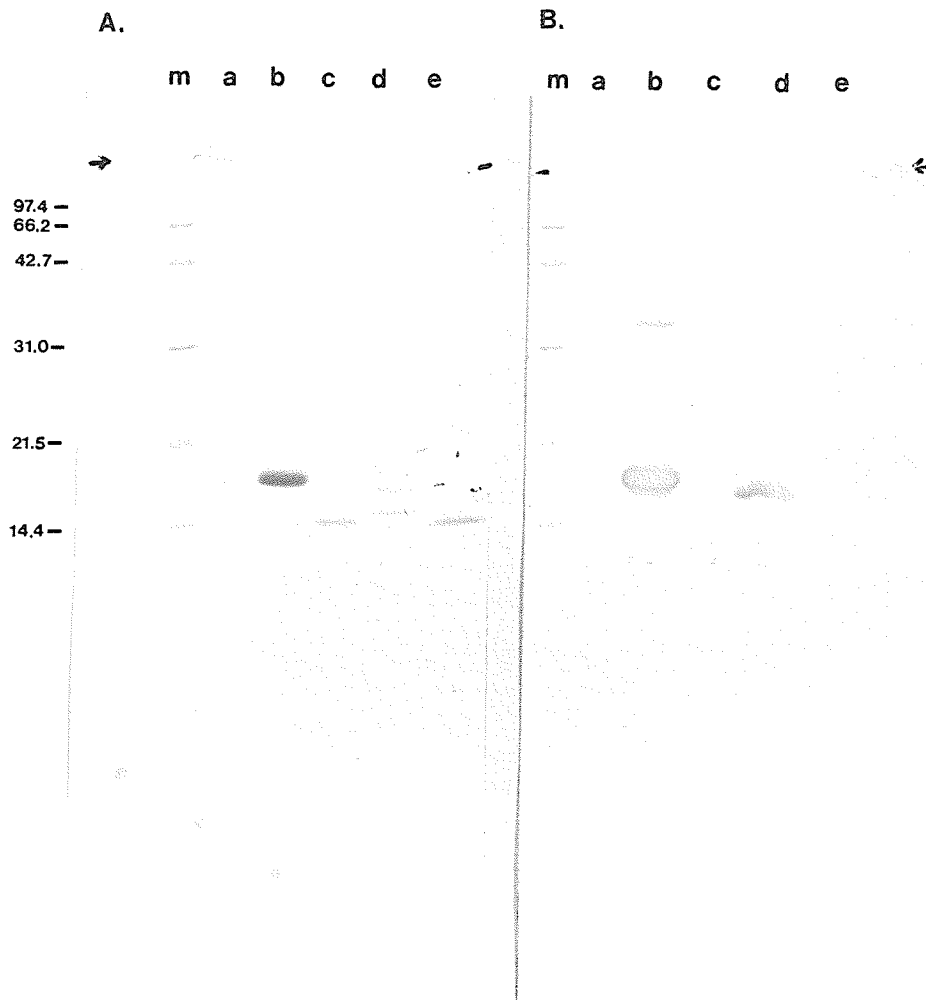


Figure 3.8 Western immunoblot analysis of Del-122. Deletion variants Del-128 and Del-122 were detected with MAbs MIF3061 (Panel A) and MIF3037 (Panel B). Lanes: a, lysates of cells harboring the expression vector without the IFN- γ insert; b, lysates of cells harboring the plasmid pP₁₄R₃IFN that contained the full-length IFN- γ gene; c, lysates of cells containing the plasmid pDel-122; d, supernatant of disrupted cells containing pP₁₄R₃IFN in the absence of 1 mM ZnCl₂; e, supernatant of disrupted cells containing pDel-122 in the absence of 1 mM ZnCl₂; m, biotinylated molecular weight standards (kDal).

acid residues from its C-terminus.

To determine whether the Del-122 polypeptide still contained one of the neutralizing epitopes as defined by Alfa and Jay (1988), western immunoblot analysis was carried out using two monoclonal antibodies (MAb) as primary detecting antibodies: a neutralizing MAb MIF3061 and a non-neutralizing MAb MIF3037 (Figure 3.8). Both of these MAbs were able to detect the full-length molecule (Panels A and B, lane b). While MIF3061 was able to recognize Del-128 (Panel A, lower band of lane d) and Del-122 (Panel A, lanes c and e), MIF3037 was not able to detect these two deletion variant polypeptides (Panel B, lane c to e). It is also of interest to note that both MAbs were able to recognize the truncated IFN- γ polypeptide that was generated by OmpT cleavage between Arg¹³⁹ and Arg¹⁴⁰ (Panels A, upper band of lane d; Panel B, lane d). In addition, the dimeric form of IFN- γ could also be detected by MIF3037 (Panel B, upper band of lane b).

3.4.3 Characterization of DNA inserts in deletion variants by restriction enzyme digestion and DNA sequencing

Restriction enzyme digestion was performed on representative plasmids encoding the deletion variants Del-117, Del-111, and Del-106 which corresponded, respectively, to deletions of 26, 32, and 37 amino acids from the C-terminus (Figure 3.9). Since all of the variants were flanked by HindIII sites, the inserts were excised with HindIII and separated by electrophoresis on a 1.5% agarose gel. The sizes of the DNA inserts of pDel-117, pDel-111, and pDel-106 were

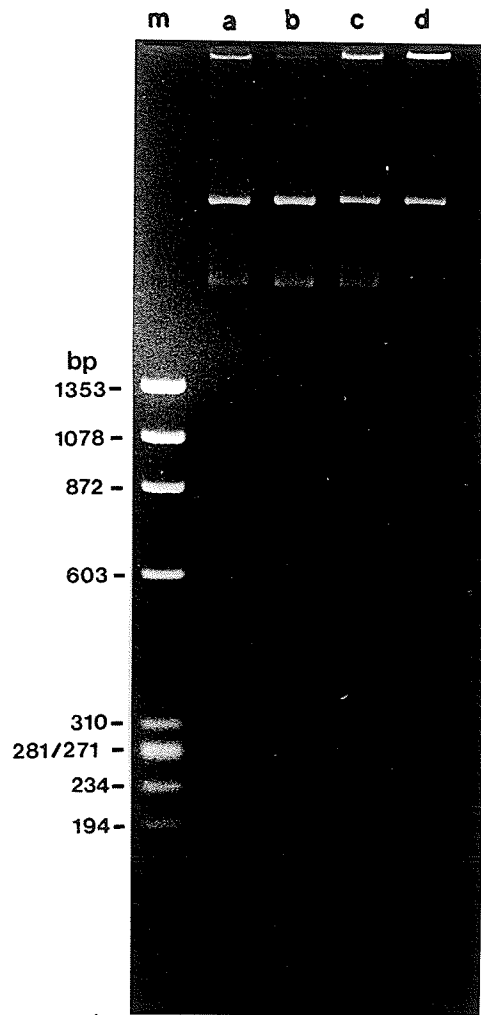


Figure 3.9 Restriction endonuclease analysis of deletion variants. Plasmids containing the inserts of various length were digested with Hind III and separated in a 1.5 % agarose gel containing 1 ug/ml of ethidium bromide. Lanes: m, molecular size markers (ϕ X174 digested with Hae III); a, pP₁₄R₁IFN; b, pDel-117; c, pDel-111; d, pDel-106. The inserts of plasmids were, respectively, 440, 428, 410, and 395 base pairs.

428, 410, and 395 base pairs (bp), respectively, as expected.

To confirm the nucleotide sequences of deletion variants Del-117, Del-111, and Del-106, coding sequences of the respective plasmids were subcloned into M13mp8 vector. A total of 8 independent plaques (two of Del-117 and three of each of Del-111 and Del-106) were screened for insertion into M13mp8 vector and their ssDNA were analysed on a 0.8% agarose gel (Figure 3.10). Single-stranded DNA isolated from each of the plaques (lanes a to h), except those of the two plaques from Del-106 (lanes g and h), had slower migration rate compared to the M13mp8 vector control (lanes M), an indication of increased sizes due to insertion of the respective HuIFN- γ DNA inserts.

The ssDNA were sequenced by the dideoxynucleotide chain termination method (Section 2.3). Since the sequencing primer used was located at the 3'-end of the insert, the sequence ladders shown in Figure 3.11 represent nucleotide sequences 5' to 3' from the top of the gel to the bottom. Only sequences at the two junctions (BglII and SstII) into which the modified synthetic oligonucleotides were inserted are shown in Figure 3.11.

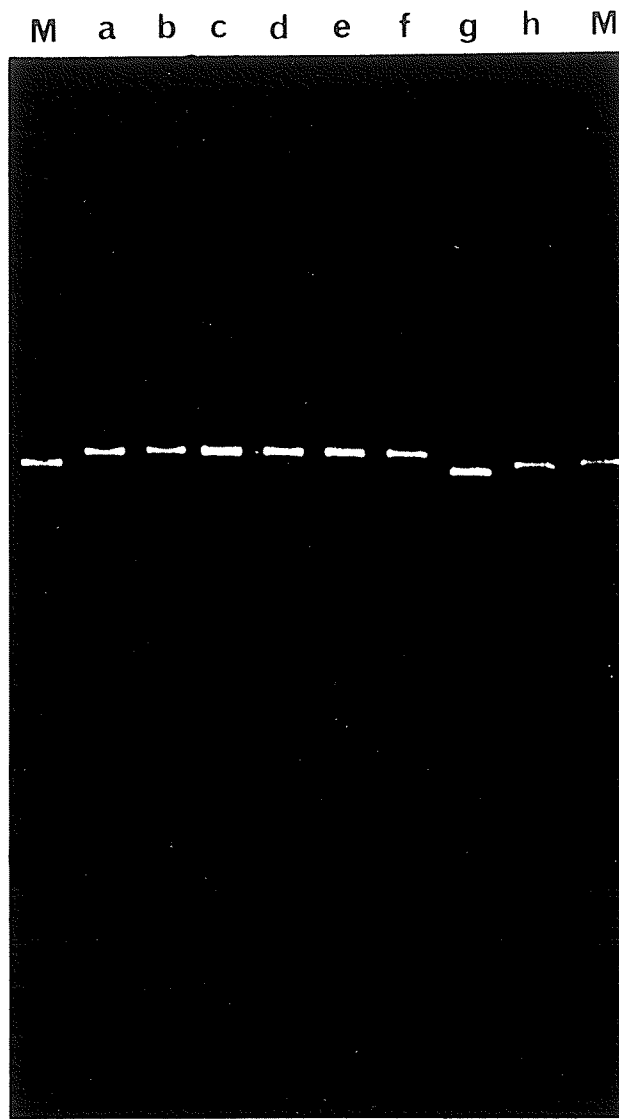


Figure 3.10 Analysis of deletion variants subcloned into M13mp8 vector. Single-stranded DNA from phages containing (M) M13mp8 vector, vector with insertion of (a and b) Del-117, (c,d, and e) Del-111, and (f,g, and h) Del-106, were separated on a 0.8% agarose gel by electrophoresis.

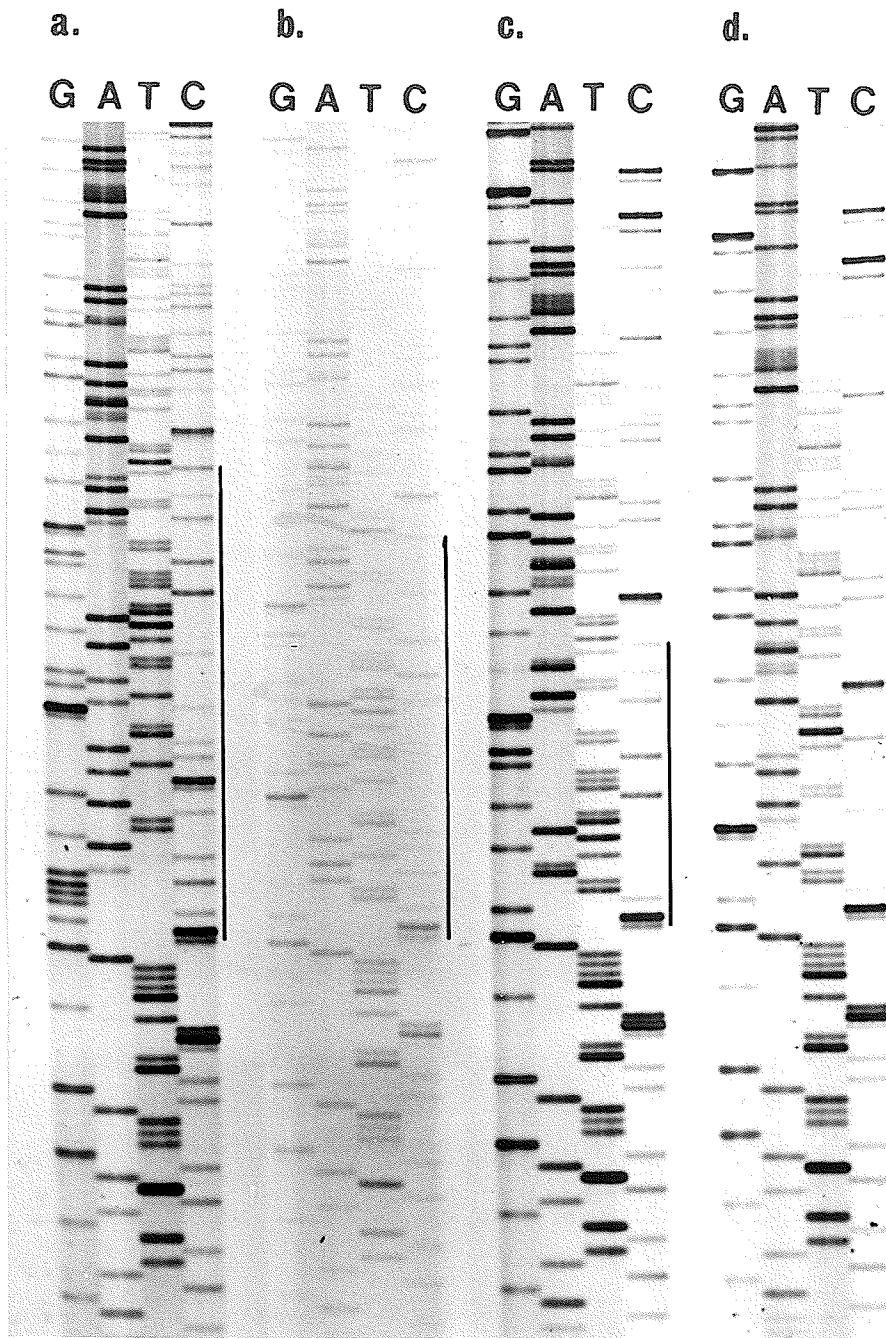


Figure 3.11 DNA sequence analysis of DNA inserts of (a) unmodified HuIFN- γ , deletion variants (b) Del-117, (c) Del-111, and (d) Del-106. Four M13 phage containing the respective inserts were sequenced by the dideoxynucleotide chain-termination method and separated on a 6% sequencing gel. Vertical bars in b, c, and d denote the extent of sequences of the modified oligonucleotides compared to that of the corresponding unmodified sequence (a).

3.4.4 Characterization of deletion variant polypeptides of Del-122, Del-117, Del-111, and Del-106

One hundred microliters of overnight cultures of the deletion variants Del-122, Del-117, Del-111, and Del-106 were pelleted by centrifugation, solubilized in 40 μ l of 1x SDS sample buffer, and resolved on a 15 % SDS polyacrylamide gel. Total proteins were blotted electrophoretically onto nitrocellulose membranes and were detected with a polyclonal rabbit antiserum against denatured rHuIFN- γ (Figure 3.7). The immunological identities of the variants were confirmed by their positive immunoblot reactions. The apparent molecular weights of the variant polypeptides were 14,300, 13,600, 12,900, and 12,200, which corresponded, respectively, to 20, 26, 32, and 37 amino acid residues deleted from the parental molecule. In order to obtain bands of similar intensity in the Western immunoblot, the amounts of variant polypeptides from clones Del-117, Del-111, and Del-106 were loaded onto the gel at 50-fold that of the full-length molecule. Based on the relative amounts of proteins loaded on the gel and the band intensity, the intensity of the variant polypeptide bands were about 2-fold less than that of P₁₄R₃IFN. The amount of these variant polypeptides accumulated in the *E. coli* cells were estimated to be approximately 100-fold less than that of the full-length molecule.

3.4.5 In vitro transcription-translation of deletion variants

In order to evaluate whether the lack of accumulation of the variant polypeptides was due to their inefficient expression or due to rapid degradation inside the E. coli cell, an in vitro transcription-translation experiment was carried out as described in Section 2.14.

As shown in Figure 3.12, equal amounts (0.6 ug) of plasmid DNA that contained the coding sequences of the deletion variants were used to direct the synthesis of the respective polypeptides. The ³⁵S-methionine labelled polypeptide products were resolved by SDS-PAGE and visualized by fluorography. The expression plasmid pP₁₄R₃ was shown to direct the synthesis of 3 polypeptide bands (lane a). Each of the plasmid carrying an IFN- γ insert directed the synthesis of an additional polypeptide with apparent molecular weights as expected of the specific variant molecules (lanes b to f). The amount of each of the variant polypeptide synthesized was comparable to that of the parental molecule from an equivalent amount of plasmid DNA (lane b). Thus, the low levels of in vivo accumulation of Del-117, Del-111, and Del-106 polypeptides were likely due to the instability of these molecules in the bacterial cell rather than the deficiency of expression.

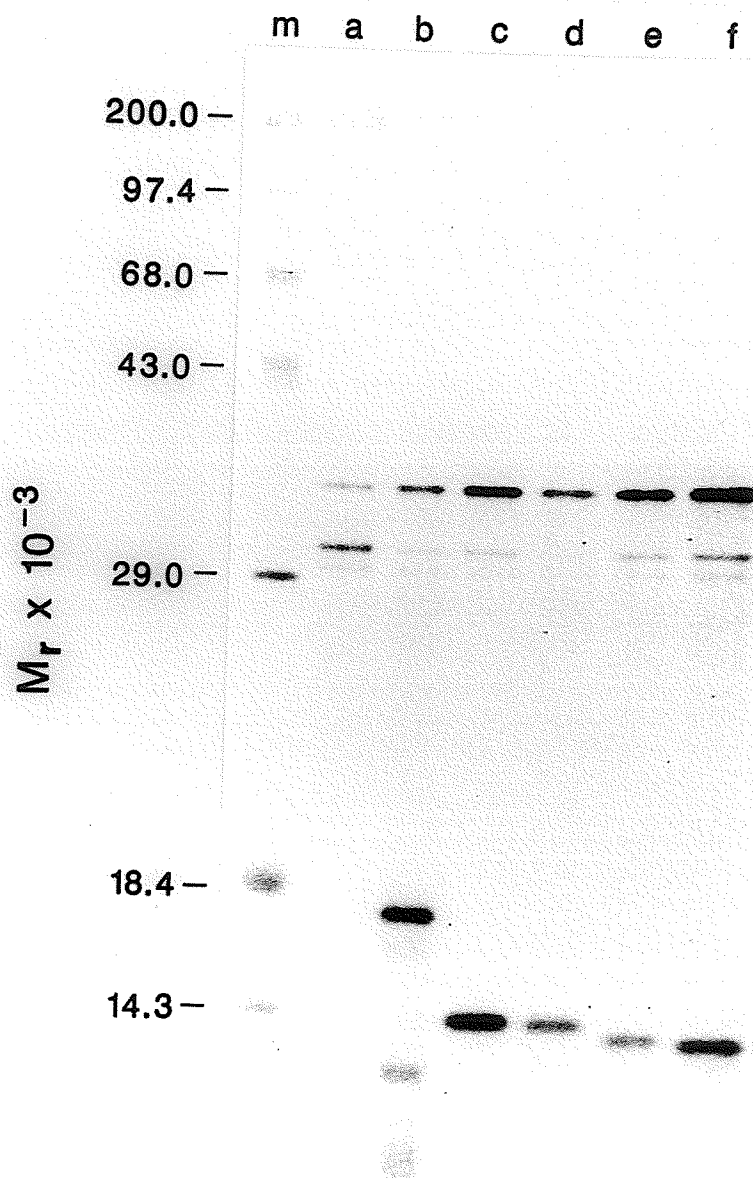


Figure 3.12 In vitro Transcription-Translation Analysis of Plasmid DNA. Equivalent amounts (0.6 ug) of pJP₁₄R₃ (a), pJP₁₄R₃-IFN (b), pDel-122 (c), pDel-117 (d), pDel-111 (e), and pDel-106 (f) were used to direct the synthesis of the respective rHuIFN- γ -related polypeptides in a prokaryotic DNA-directed translation assay (Amersham) as described in Section 2.14. The samples were resolved by SDS-polyacrylamide gel electrophoresis on a 15% gel and the ³⁵S-methionine-labelled polypeptides were detected by fluorography. The ¹⁴C-labelled protein molecular weight standards (m) were (from top to bottom): Myosin (H-chain) (200,000), phosphorylase b (97,400), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), β -lactoglobulin (18,400), lysozyme (14,300).

3.4.6 Determination of the biological activities of deletion variants

Affinity-purified Del-128 and Del-122 were assayed for the antiviral as well as antiproliferative activities essentially as described in Section 2.9. As shown in Table 3.1, the antiviral specific activities of variant Del-128 and Del-122 were, respectively, 2.3×10^7 units/mg and 1.03×10^7 units/mg, comparable to that of the full-length rHuIFN- γ molecule which had an antiviral specific activity of 2.25×10^7 . Similarly, the specific antiproliferative activities of Del-128 and Del-122 were 2.3×10^6 units/mg and 3.00×10^5 units/mg, respectively. These values were within 2- to 3-fold of the specific activities of the full-length molecule which had an antiproliferative specific activity of 1×10^6 units/mg. These results therefore indicate that deletions of up to 21 amino acid residues from the C-terminus of the HuIFN- γ did not affect either the antiviral or antiproliferative activities.

Attempts to purify variants Del-117, Del-111, and Del-106 have not been successful due to the lack of adequate accumulation of these polypeptides inside the *E. coli* cell. Therefore, the relative activities of these variants were evaluated using clarified supernatant from the 50-fold concentrated culture. As shown in Table 3.1, no activity could be detected from any of these variants. Since the antiviral assay has a limit of detection of 50 IU/ml (or 5 units per 100 μ l, which is equivalent to 1 unit in the second or third well if the sample were serially diluted 1 in 2), these variants must be at least 1000-fold less active than the parental molecule to have undetectable activity.

Table 3.1 Antiviral and Antiproliferative Activities of HuIFN- γ and C-terminal Deletion Variants

Clone	Extent of Deletion		Specific Activity (U/mg) ^b	
	Residues	% Molecule	Antiviral	Antiproliferative
pJP ₁₄ R ₃ Vector ^a	--	--	< N.D.	< N.D.
HuIFN- γ	0	0	2.25 x 10 ⁷	1.00 x 10 ⁶
Del-128	15	10.5	2.30 x 10 ⁷	2.30 x 10 ⁶
Del-122	21	14.7	1.03 x 10 ⁷	3.00 x 10 ⁵
Del-117	26	18.2	< N.D.	< N.D.
Del-111	32	22.4	< N.D.	< N.D.
Del-106	37	25.9	< N.D.	< N.D.

a, Expression vector without HuIFN- γ insert; N.D., activity not detectable at an assay sensitivity of 50 unit/ml supernatant prepared from 50-fold concentrated X-press disrupted culture.

b, The variation of each of the values was within 2-fold.

3.4.7 Determination of biological activities of denatured/renatured rHuIFN- γ and variant Del-122

To evaluate the possible effect of the C-terminus of HuIFN- γ upon treatment with chaotropic agent and its ability to renature in vitro, purified HuIFN- γ and Del-122 were treated with 6 M guanidine hydrochloride (GuHCl). The proteins were renatured by dilution to 1 M with respect to GuHCl with RPMI 1640 medium, and then dialyzed against the same medium before being tested for their antiviral activities. As shown in Table 3.2, the denaturation and subsequent renaturation of HuIFN- γ resulted in 10-fold reduction in the antiviral specific activity while similar treatment of Del-122 resulted in more than 25-fold reduction in specific activity. These results indicate that the highly hydrophilic C-terminus may play a positive role in the in vitro renaturation of the rHuIFN- γ molecule and that the loss of the C-terminus may affect the efficiency of Del-122 to renature in vitro. However, upon re-purification by immunoaffinity chromatography using monoclonal antibody MIF3052, the specific activities of both renatured preparations were comparable to the original levels of the respective molecules, indicating that MIF3052 was able to discriminate between the functional molecules and molecules that were not properly folded in the in vitro renaturation process.

Table 3.2 Effect of the C-terminus on the refolding of denatured HuIFN- γ and Del-122 in vitro.

	Antiviral Specific Activity ^a (Units/mg)		
	Native ^b	Renatured ^c	Affinity Chromatography Re-Purified ^d
HuIFN- γ	$(3.1 \pm 1.1) \times 10^7$ ^e	$(2.1 \pm 2.3) \times 10^6$ ^f	$(1.8 \pm 0.9) \times 10^7$ ^g
Del-122	$(7.3 \pm 2.5) \times 10^6$ ^h	$(7.5 \pm 1.6) \times 10^4$ ⁱ	$(5.9 \pm 1.7) \times 10^6$ ^j

a, Results are the means \pm SD of 3 separate experiments (purifications) assayed in triplicate and statistical analysis was carried out using the Student's t-test.

b, Affinity-purified native rHuIFN- γ .

c, Affinity-purified rHuIFN- γ , obtained in **b**, was treated with 6.0 M guanidine hydrochloride and subsequently renatured by 6-fold dilution in RPMI1640 medium and then dialyzed in the same medium.

d, rHuIFN- γ , obtained in **c**, was re-purified by affinity chromatography using monoclonal antibody MIF3052.

e/h, e/f, Significant difference at $p < .025$.

h/i, Significant difference at $p < .01$.

f/i, Significant difference at $p < .001$.

e/g, h/j, No significant difference.

3.5 Discussion

3.5.1 Biological Activities of Deletion Variants

Several reports (Burton et al., 1985; Czarniecki et al., 1985; Arakawa et al., 1986; de la Maza et al., 1987; Garotta et al., 1987; Leinikki et al., 1987; Seelig et al., 1988; Arakawa et al., 1989) have shown that deletions of as few as 11 amino acid residues from the C-terminus of HuIFN- γ resulted in significant reduction in its biological activities, suggesting the involvement of these amino acid residues in either receptor binding (Arakawa et al., 1989) or in maintaining the tertiary structure of the molecule (de la Maza et al., 1987). In contrast, several studies (Honda et al., 1983; Rose et al., 1983; Kung et al., 1986; Kitano et al., 1988) have shown that rHuIFN- γ molecule that lacked the C-terminal 15 amino acids retained full biological activities. In addition, Rinderknecht et al. (1985) have previously shown that the natural HuIFN- γ was processed posttranslationally at the C-terminus and that molecules that lacked the C-terminal 15 amino acid residues were found to be fully active. Therefore, in order to establish the minimal structural requirement for the biological functions of the HuIFN- γ , a series of C-terminal deletion variants were either isolated as proteolytic product or constructed by means of specific gene modifications.

Deletion variant Del-128 was isolated as a 15 kD proteolytic product of the full-length rHuIFN- γ . This truncated polypeptide was generated during the extraction of the rHuIFN- γ from the *E. coli* cell. The proteolytic activity was subsequently shown to be associated with the *E. coli*

membrane-bound protease OmpT (Sugimura and Nishihara, 1988; Grodberg and Dunn, 1988). OmpT was shown to be an endopeptidase that had serine protease-like activity and had unique substrate specificity for paired basic residues (Sugimura and Nishihara, 1988). It was shown that truncated molecules of the rHuIFN- γ were generated during extraction from the *E. coli* cell and that three pairs of basic residues at the C-terminus were susceptible to the protease OmpT (Sugimura and Higashi, 1988). The peptide bond between Arg¹³⁹/Arg¹⁴⁰ was more susceptible to OmpT than those of Arg¹²⁹/Lys¹³⁰ and Lys¹²⁸/Arg¹²⁹ (Sugimura and Higashi, 1988). Results from the time course experiment shown in Figure 3.5 supported this conclusion. It was also confirmed that ZnCl₂ was an inhibitor of OmpT. However, the 16.5 kD polypeptide was detected, even in the presence of ZnCl₂, when the cells were disrupted by sonication and at time zero of incubation (Panel A, lanes c), indicating that Arg¹³⁹/Arg¹⁴⁰ was highly susceptible to OmpT cleavage. The 15 kD polypeptide, on the other hand, was only detectable after incubation at 37°C for 20 min or longer (lanes f and g). In the absence of ZnCl₂, however, the full-length molecule was rapidly cleaved to generate both the 16.5 kD and 15 kD polypeptides (Figure 3.5, lanes c to g). Therefore, based on the apparent molecular weight of the 15 kD polypeptide, 15 residues were deleted in this molecule. The inhibitory effects of ZnCl₂ on the proteolytic activity confirmed previous findings that the full-length rHuIFN- γ was cleaved by the membrane-associated protease OmpT. Since OmpT was shown to have a specificity for paired basic residues, it was concluded that Del-128 was generated by cleavage at Lys¹²⁸/Arg¹²⁹ resulting in the deletion of 15 residues from the C-terminus. This variant was purified to homogeneity (Figure 3.1) and the immunological identity of this variant was also confirmed by Western immunoblot analysis

(Figure 3.5). The purified Del-128 polypeptide was tested for its biological activities and the specific activities of Del-128 were found to be comparable to those of the full-length rHuIFN- γ (Table 3.1). Therefore, in contrast to results reported by several groups (Burton et al., 1985; Czarniecki et al., 1985; Arakawa et al., 1986; de la Maza et al., 1987; Garotta et al., 1987; Leinikki et al., 1987; Seelig et al., 1988; Arakawa et al., 1989), the deletion of 15 amino acid residues from the C-terminus of HuIFN- γ did not affect its biological activities, indicating that the functional site(s) of HuIFN- γ must be located within the N-terminal 128 amino acids.

In order to further define the primary sequences of the HuIFN- γ that confer its biological activities, variant molecules which had more extensive deletions at the C-terminus were constructed. By means of specific gene modifications, truncated molecules were constructed which lacked the C-terminal 21, 26, 32, and 37 residues, respectively. These variants were expressed in *E. coli* and their immunological identities were confirmed in Western immunoblot analysis using a rabbit polyclonal antiserum against rHuIFN- γ (Figure 3.7, lanes c to f). Based on their apparent molecular weights, the molecular sizes of the respective variant polypeptides were in accordance with the calculated molecular weight of 14.3 kD, 13.6 kD, 12.9 kD, and 12.2 kD, as expected of truncated rHuIFN- γ molecules which had, respectively, 20, 26, 32, and 37 residues deleted from the C-terminus. It was also found that the level of accumulation of variant polypeptides Del-117, Del-111, and Del-106 in the *E. coli* cells was substantially lower than the full-length molecule. While the accumulation of Del-122 was within 3- to 5-fold lower compared to that of the full-length molecule, the amount of Del-117, Del-111, and Del-106 found in the bacterial cell was only at least 50-fold lower than that of the full-length molecule. The respective

variant polypeptides were not discernable in the SDS-PAGE pattern of the whole cell protein and were only detectable in Western immunoblot (Figure 3.7).

The biological activities of these variants are shown in Table 3.1. Affinity-purified Del-122 were found to have specific activities were comparable to that of the parental full-length molecule. The antiviral specific activity of Del-122 was 1.03×10^7 units/mg, or approximately 2- to 3-fold lower than that of the full-length rHuIFN- γ which was 2.25×10^7 units/mg. Similarly, the antiproliferative activity of Del-122 was 3.0×10^5 units/mg, which was also 3-fold lower than that of the rHuIFN- γ which was 10.0×10^5 units/mg. Further deletions, however, resulted in the reduction of activities to undetectable level. Due to the lack of accumulation of Del-117, Del-111, and Del-106, purification of these latter polypeptides has been unsuccessful. Thus, the relative activities of these variants were determined by using clarified cell supernatants prepared from the 50-fold concentrated culture disrupted by sonication (Table 3.1). Accurate estimation of the specific activities of these variants, however, must await the purification of the variant polypeptides. It is possible that C-terminal deletions beyond residue 122 may have rendered these molecules unstable in the condition (pH 11.2) required for elution from the immunocomplexes. However, based on the sensitivity of the antiviral activity assay, which was 50 units/ml culture supernatant, and the relative concentrations of these variants in the cell lysates which was less than 1% of that of the full-length molecule as estimated by Western immunoblot analysis, the three variant polypeptides Del-117, Del-111, and Del-106 must have specific activities, if any, at least 1000-fold lower than those of the full-length molecule to be undetectable in the antiviral activity assay. Thus, the modified HuIFN- γ molecules appeared to have lost their

structural integrity and have become unstable or have lost their functional site(s) upon C-terminal deletions in excess of 21 amino acid residues. However, whether these latter deletions represented primary sequences that comprised the receptor binding site and/or the effector site could not be ascertained due to lack of the respective purified polypeptides. Nevertheless, the present study has definitively shown that the functional site(s) of HuIFN- γ must be located within the N-terminal 122 amino acid residues since variant Del-122 is fully active and therefore must contain the functional site(s). In corroboration with the present conclusion which showed that the C-terminal 21 residues are not involved in conferring the biological activities of HuIFN- γ , it was also found that neither Del-128 nor Del-122 were recognized by the monoclonal antibody MIF3037 (Figure 3.8). The fact that MIF3037 is non-neutralizing and immunoblot positive (Alfa and Jay, 1987) suggest that MIF3037 binds to a contiguous epitope at the C-terminus of the HuIFN- γ polypeptide that is not involved in conferring its functions and that the binding of this MAb to the C-terminus does not interfere with the functions of the HuIFN- γ molecule. In addition, since both Del-128 and Del-122 could be detected by the neutralizing MAb3061, these two deletion variants must still contain the functional site(s).

On the other hand, variants which had deletions of more than 21 residues and which lacked detectable biological activities may have resulted from deletions of sequences that constituted either the receptor binding site or the effector site. However, neither of these possibilities can be addressed due to the lack of purified materials. Nevertheless, the fact that both Del-128 and Del-122 retain almost full activities indicates that both the receptor binding site and the effector site for signal transduction must be within the N-terminal 122 amino acid residues.

3.5.2 Intracellular stabilities of deletion variants

Although the intracellular stability of variant polypeptide Del-122 was found to have decreased by about 2-fold, as reflected by its slight decrease in accumulation (Figure 3.1, lane c), its specific activities were not affected. However, further deletions of 26 to 37 amino acid residues from the C-terminus of HuIFN- γ in Del-117, Del-111, and Del-106, resulted not only in the loss of biological activities (Table 3.1) but also in reduction in the accumulation of these variant polypeptides.

In order to examine if the reduced accumulation of the variant polypeptides was due to inefficient expression of the modified coding sequences, the respective polypeptides were expressed in an *in vitro* transcription-translation system. The *in vitro* cell-free coupled transcription-translation system has several important features as a means to verify the gene products of interest. Firstly, since the cell free extracts contain all the essential components for transcription and translation, any coding sequence that contains a prokaryotic promoter and a ribosome binding site can be readily translated and characterized. Secondly, since radioactive amino acids (^{35}S -methionine) are incorporated into the synthesized polypeptides, only small amounts of plasmid DNA is needed (less than 0.5 μg) to direct the synthesis of sufficient polypeptides for easy detection and analysis. Furthermore, incorporation of radioactive label into protein is far more efficient than is possible using *in vivo* methods (Dougan and Sherratt, 1977; Sancar et al., 1981). Thirdly, due to the short incubation time (30 min), polypeptides synthesized *in vitro* would not be affected by degradation processes that commonly occur *in vivo* (Itakura et

al., 1977; Talmadge and Gilbert, 1982; Goff and Goldberg, 1985; Kitano et al., 1987). Finally, the in vitro system may be used to indirectly confirm the effectiveness of the vector designed for high level expression of cloned genes.

Using the same amount of plasmid DNA, the level of in vitro production of the variant polypeptides (Figure 3.12, lane c to f) were found to be comparable to the parental full-length molecule (Figure 3.12, lane b), suggesting that the efficiency of expression of each of the modified HuIFN- γ coding sequences was not different from that of the full-length parental sequence. Therefore, the lack of accumulation of these polypeptides in the bacterial cell are likely due to degradation in vivo. It is conceivable that the reduction in protein accumulation was due to deletions of the carboxy-terminal residues, thereby rendering the variant proteins more susceptible to proteolytic degradation inside the bacterial cell. Indeed, the problem of degradation of foreign proteins expressed in E. coli is well known (Itakura et al., 1977; Talmadge and Gilbert, 1982; Goff and Goldberg, 1985; Kitano et al., 1987). Genetically engineered mutant proteins of the bacteriophage P22 Arc repressor has been shown to display structural instability and failed to accumulate to high steady-state levels in the bacterial cell due to rapid proteolysis (Vershon, et al., 1986; Bowie and Sauer, 1989a).

It is possible that the deletions introduced in these variants had prevented the proper folding of these molecules. This question may be addressed by conducting kinetic studies or by directly measuring the protein folded state using physical chemical methods such as 2-dimensional nuclear magnetic resonance (2-D NMR) and circular dichroism (CD) spectral analysis. This, however, is not the objective of the present study. Nevertheless, the important conclusion is that

deletions further upstream from residue 122 resulted in the loss of structural stability inside the E. coli cell.

3.5.3 Role of carboxy-terminus of rHuIFN- γ in refolding in vitro

Proteins overexpressed in E. coli often result in the formation of insoluble aggregates as inclusion bodies due to extensive intra- and intermolecular interactions (reviewed by Mitraki and King, 1989). Many of these intermediates are products of off-pathway folding. This is because protein folding pathways involve multiple steps and misfolded intermediates may accumulate due to changes in the intracellular environment, resulting in the failure in continuing the proper folding pathway that leads to maturation of the native polypeptide. In order to maximize recovery of the overexpressed proteins, it is a common practice to solubilize proteins from the inclusion bodies by chaotropic agents and then renature the unfolded proteins in vitro. Chaotropic agents such as urea or guanidine hydrochloride are the most commonly used agents to solubilize polypeptides by denaturing the molecule to reduce intermolecular complexing. The denatured polypeptides are usually purified by a number of chromatography steps before being renatured which, in many cases, simply require rapid removal or dilution of the chaotropic agent in the sample. While such in vitro denaturation-renaturation procedure has been successful in extracting and purifying a number of proteins expressed in E. coli, a small percentage of these polypeptides could not be properly refolded by means of denaturation-renaturation in vitro. Furthermore, the efficiency of re-folding proteins to their functional forms not only depends on the peptide

sequences but also on environmental factors such as temperature (Mizukami et al., 1986; Schein and Noteborn, 1988; Takagi, et al., 1988) and pH (Creighton 1984) as well as protein factors such as the molecular chaperones (Randall et al., 1987; Collier et al., 1988; Hemmingsem et al., 1988; Rothman, 1989).

Recombinant deletion variants prepared by the denaturation-renaturation procedures were shown to have drastic reductions in their biological activities (Burton et al., 1985; Czarniecki et al., 1985; Arakawa et al., 1986; de la Maza et al., 1987; Garotta et al., 1987; Leinikki et al., 1987; Seelig et al., 1988). Whereas these studies showed that deletion of as few as 11 amino acid residues from the C-terminus of HuIFN- γ resulted in 100- to 1000-fold reduction in the biological activities, results from the present study demonstrated that up to 21 amino acid residues of HuIFN- γ may be deleted from the C-terminus without any significant effect on its biological functions.

The deletion variants analyzed in the present study, however, were extracted and purified in their native forms without any treatment with chaotropic agents. It is conceivable that deletions of as few as 11 amino acid residues from the C-terminus of HuIFN- γ could have prevented the correct re-folding of the polypeptide *in vitro*. To test this possibility, affinity-purified rHuIFN- γ and Del-122 were subjected to denaturation-renaturation as described in Section 3.4.7. As shown in Table 3.2, denaturation-renaturation resulted in 10- and 25-fold reduction in the antiviral specific activity for rHuIFN- γ and Del-122, respectively. When these renatured molecules were re-purified by affinity chromatography using a neutralizing monoclonal antibody, the antiviral specific activities were found to be comparable to those of the untreated

molecules. These results suggested that denaturation-renaturation may have generated inactive molecules due to improper re-folding or off-pathway folding and that the re-purification procedure must have allowed selection of the functional molecules. Thus, the specific activities of a biologically active protein could be greatly affected by extraction and purification procedures specifically designed for that particular protein. In the present study, both the HuIFN- γ and the variants were maintained in their native forms throughout the extraction and purification procedures. This would minimize the chance of inadvertently introducing inactive molecules in and lowering the specific activities of the preparations.

Therefore, although the C-terminal 15 residues of HuIFN- γ are not involved in conferring its biological activities, they may facilitate the proper re-folding of the HuIFN- γ molecule in vitro. It is not clear, however, if the effect of the C-terminus on the potential for re-folding in vitro explains the failure of several groups to detect biological active HuIFN- γ variant molecules upon removal of 11 to 18 residues from the C-terminus. This observation illustrates that caution must be exercised in the interpretation of negative results in this type of study. Thus, it would not be appropriate to infer a direct involvement of residues 106 to 122 (Del-106, Del-111, and Del-117) in the biological activity of HuIFN- γ based on the results presented.

CHAPTER 4 STRUCTURE-FUNCTION STUDIES OF A CONSERVED β -TURN IN THE HuIFN- γ BY SITE-DIRECTED MUTAGENESIS

4.1 Introduction

Deletion analyses described in Chapter 3 have defined sequences that could be excluded for further search of the functional site(s) of the HuIFN- γ . However, since functional site(s) of protein molecules often involve only a few critical amino acids, it is imperative that individual amino acid residues be systematically modified and analysed in order to identify these crucial residues. The technique of site-directed mutagenesis has allowed rapid and precise alteration of specific single amino acids and has proved to be a powerful tool in analyzing the importance of individual amino acids in the functional sites of proteins (Zoller and Smith, 1983). However, since the combinations of mutations that could possibly be introduced would be astronomical, empirical criteria must be developed as guidelines for selecting potentially important regions for study. In the present study of the structure/function relationships of the HuIFN- γ , the potential regions and residues for modifications were selected based on the empirical guidelines developed upon analyses of the extent of homologous amino acid residues, the local structural hydrophilicity, and conserved secondary structures.

4.2 Analysis of evolutionarily conserved regions

Comparison of amino acid sequences derived from cDNAs of IFNs- γ of different vertebrate species revealed regions of sequence homology (Figure 4.1) (Pestka, 1986). It is generally accepted that regions of homology in functionally related proteins represent evolutionarily conserved regions and may play important structural or functional roles. Although IFNs- γ are known to have strict species specificity, the spectrum of activities induced by this cytokine is very similar in the respective species (Pestka et al., 1987). It is possible that the species specificity of IFNs- γ is a result of evolutionary divergence in the receptor binding site while the effector site(s) is well conserved among the IFNs- γ from various vertebrate species. As shown in Figure 4.1, the sequences that spanned residues 39 and 60 contained the longest consecutive stretch of conserved residues in all of the IFNs- γ from the four animal species being compared: 10 out of 22 residues (45.5 %) are perfectly conserved and 6 out of 22 residues (27.3 %) are conserved substitutions that are of the same polarity. This gives a very high degree of overall homology of over 70 %. Interestingly, part of this conserved region also overlaps with one of the most hydrophilic peaks (residues 36 to 46) as predicted in the Section 4.3. The fact that such an exposed region has been so well conserved suggests that there are certain important structural or functional features within this region that are preserved during evolution.

	SI	S10	S20	S23																										
Hu-IFN- γ	M	K	Y	T	S	Y	I	L	A	F	Q	L	C	I	V	L	G	S	L	G	C	Y	C							
Bo-IFN- γ	F	.	.	.	L	L	.	.	G	L	.	.	F	S	.	S	.	G							
Mu-IFN- γ	.	N	A	.	H	C	.	.	.	L	.	.	.	F	L	M	A	V							
Ra-IFN- γ	.	S	A	.	R	R	V	.	V	L	.	.	.	L	M	A	L								
	♦♦																													
	1	10	20	30																										
Hu-IFN- γ	Q	D	P	Y	V	K	E	A	E	N	L	K	K	Y	F	N	A	G	H	S	D	V	A	D	N	G	T	L	F	L
Hu-IFN- γ (protein)	e
Bo-IFN- γ	.	G	Q	F	F	R	.	I	E	.	.	.	S	S	P	.	.	.	K	G	.	P	.	.	S	
Mu-IFN- γ	H	G	T	V	I	E	S	L	.	S	.	N	N	.	.	S	S	G	I	.	.	-	E	E	K	S	.	.	.	
Ra-IFN- γ	.	G	T	L	I	E	S	L	.	S	.	.	N	.	.	S	S	S	M	.	.	A	M	E	G	K	S	.	L	
	♦♦♦																													
	40	50	60																											
Hu-IFN- γ	G	I	L	K	N	W	K	E	E	S	D	R	K	I	M	Q	S	Q	I	V	S	F	Y	F	K	L	F	K	N	F
Hu-IFN- γ (protein)
Bo-IFN- γ	D	D	K	.	I	
Mu-IFN- γ	D	.	W	R	.	Q	K	D	G	.	.	M	.	L	I	.	.	.	L	R	.	E	V	L	.	
Ra-IFN- γ	D	.	W	R	.	Q	K	D	G	.	N	T	.	L	E	.	.	.	I	.	.	.	L	R	.	E	V	L	.	
	♦♦																													
	70	80	90																											
Hu-IFN- γ	K	D	D	Q	S	I	Q	K	S	V	E	T	I	K	E	D	M	N	V	K	F	F	N	S	N	K	K	R	D	
Hu-IFN- γ (protein)
Bo-IFN- γ	.	.	N	.	V	.	R	.	M	.	D	I	.	Q	.	F	Q	.	.	.	L	.	G	S	S	E	.	L	E	
Mu-IFN- γ	.	.	N	.	A	.	S	N	N	I	S	V	.	E	S	H	L	I	T	.	.	S	N	S	.	A	.	K	.	
Ra-IFN- γ	.	.	N	.	A	.	S	N	N	I	S	V	.	E	S	H	L	I	T	.	.	S	N	S	.	A	.	K	.	
	♦♦♦♦																													
	100	110	120																											
Hu-IFN- γ	D	F	E	K	L	T	N	Y	S	V	T	D	L	N	V	Q	R	K	A	I	H	E	L	I	Q	V	M	A	E	L
Hu-IFN- γ (protein)
Bo-IFN- γ	.	.	K	.	.	I	Q	I	P	.	D	.	Q	I	N	.	.	K	N	D	
Mu-IFN- γ	A	.	M	S	I	A	K	F	E	.	N	N	P	Q	.	.	Q	.	F	N	.	.	R	.	V	H	Q	.	.	
Ra-IFN- α 1	A	.	M	S	I	A	K	F	E	.	N	N	P	Q	I	.	H	.	V	N	.	.	R	.	I	H	Q	.	.	
	♦♦♦																													
	130	140	143																											
Hu-IFN- γ	S	P	A	A	K	T	G	K	R	K	R	S	Q	M	L	F	R	G	R	R	A	S	Q							
Hu-IFN- γ (protein)							
Bo-IFN- γ							
Mu-IFN- γ	L	.	E	S	S	L	R							
Ra-IFN- γ	.	.	E	S	S	L	R							
	♦♦♦																													

Figure 4.1 Comparison of sequence homology of IFNs- γ from different vertebrate species (Adapted from Peskta, 1986). The structure derived by direct amino acid sequencing of natural IFN- γ is denoted as "HuIFN- γ (protein)" (Rinderknecht et al., 1984). The first four rows of sequence represent the signal peptides of the respective IFN- γ . The single-letter code for amino acids is used. Conserved amino acids were represented as (.) below the HuIFN- γ sequence. Horizontal bars above residues represent hydrophilic regions. Relative degree of hydrophilicity is represented as (+).

4.3 Analysis of hydrophilicity

One of the important parameters used for studying the conformation of a protein is the van der Waals' interactions of the amino acid side chains of a protein with the surrounding water molecules (Levitt, 1976). However, the full mathematical treatment of protein-water interactions is extremely complex (Lee and Richards, 1971) due to the fact that different amino acid side chains have very different affinities for water and that water molecules carry a large dipole moment, are in continual thermal motion, and tend to favor specific orientations around the protein due to hydrogen bonds. Therefore, it is usually assumed that the interaction energy of a side chain with water is proportional to the amount of water in contact with the group (Levitt, 1971). Based on these considerations, each type of side chain has been assigned a solvent interaction energy estimated from solubilities of amino acids in water and ethanol (Nozaki and Tanford, 1971). By combining and modifying these solvent interaction energy values (hydrophilicity values) from a number of studies (Nozaki and Tanford, 1971; Chou and Fasman, 1974; Levitt, 1976), Hopp and Woods (1981) have successfully predicted the antigenic determinants of 17 different proteins based on these values. A computer program based on the methods and hydrophilicity values developed by Hopp and Woods (1981) were used in the present study to predict the overall hydrophilicity of the HuIFN- γ .

Since the hydrophilicity of a particular residue is influenced by its adjacent residues, the hydrophilicity values for amino acids are repetitively averaged over the length of the polypeptide chain, generating a set of local hydrophilicity values. An averaging group length of six amino

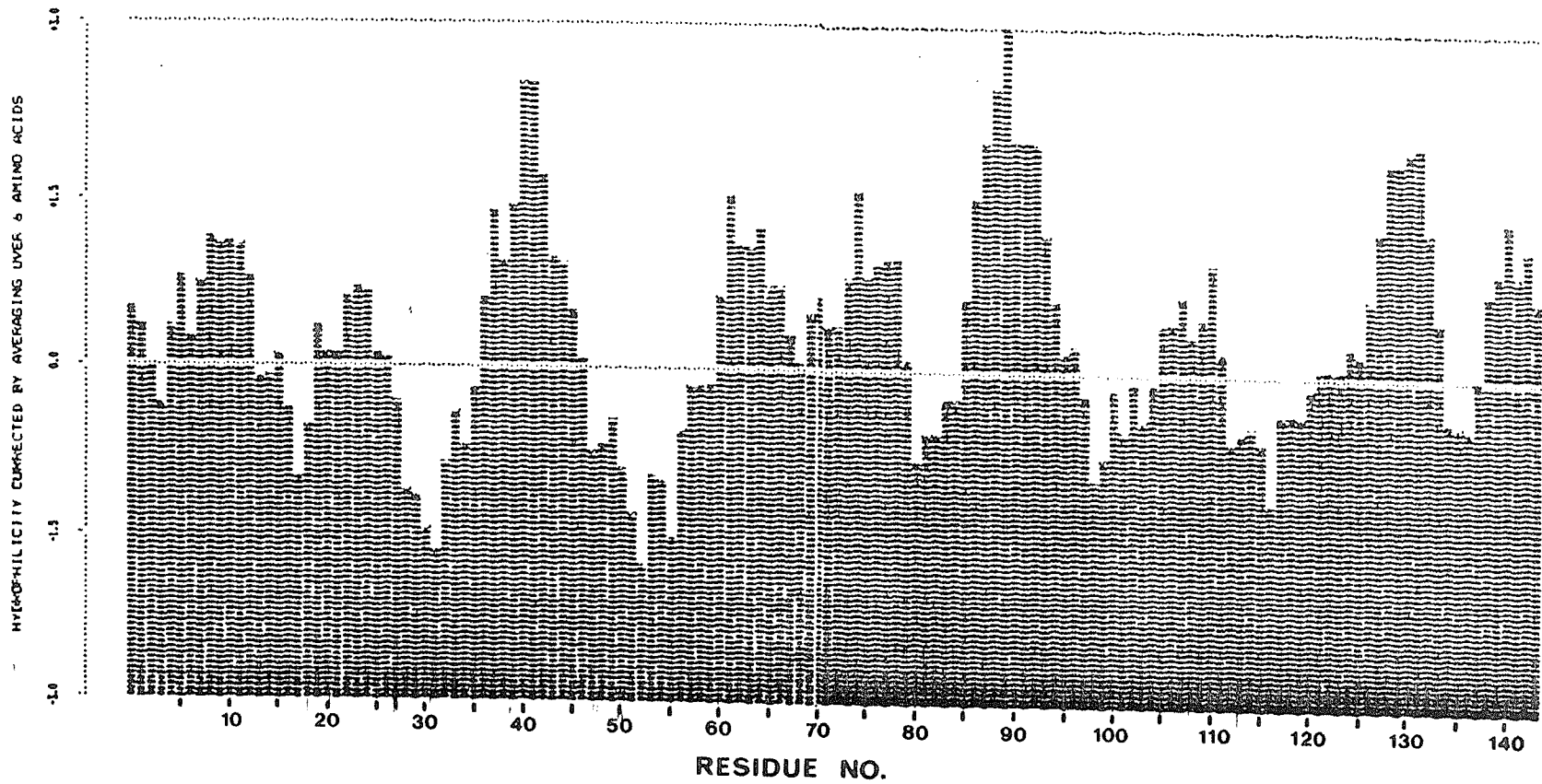


Figure 4.2 Hydrophilicity profile of rHuIFN- γ . Algorithm based on that of Hopp and Woods (1981) was used. The hydrophilicity values were averaged over 6 amino acids. Residues with positive values are hydrophilic while those with negative values are hydrophobic. Regions on the dotted line represent neutral area. The single-letter code for amino acid is used.

acids has been shown by Hopp and Woods (1981) to give the best prediction value compared to group lengths ranging from 2 to 10 residues. The hydrophilicity profile of HuIFN- γ shown in Figure 4.2 represented values that were averaged over six amino acids for each repetition. As shown in Figure 4.2, a number of hydrophilic regions on the HuIFN- γ were predicted. Among these hydrophilic peaks, three of the most hydrophilic regions were located between residues 36 to 46, 84 to 96, and 124 to 133. The hydrophilic peak between residues 36 and 46 was also found to reside in a conserved region as analysed in Section 4.1. The occurrence of such a conserved segment on the surface of the HuIFN- γ molecule suggests that these residues are more likely to be involved in intermolecular interactions. These residues were then further analyzed for the types of secondary structures they were likely to adopt.

4.4 Analysis of secondary structures

Although the information that dictates the specific folded conformation of a protein is contained in the amino acid sequence, it is yet impractical to predict tertiary conformations based on the primary sequence. The number of theoretically possible conformations of a 100 amino acid long polypeptide is estimated to be 3^{100} (Creighton, 1984), an astronomical number that could not be easily computed. Therefore, empirical rules have been developed to restrict the possible number of predicted conformations based on the database of crystal structures of a number of different proteins.

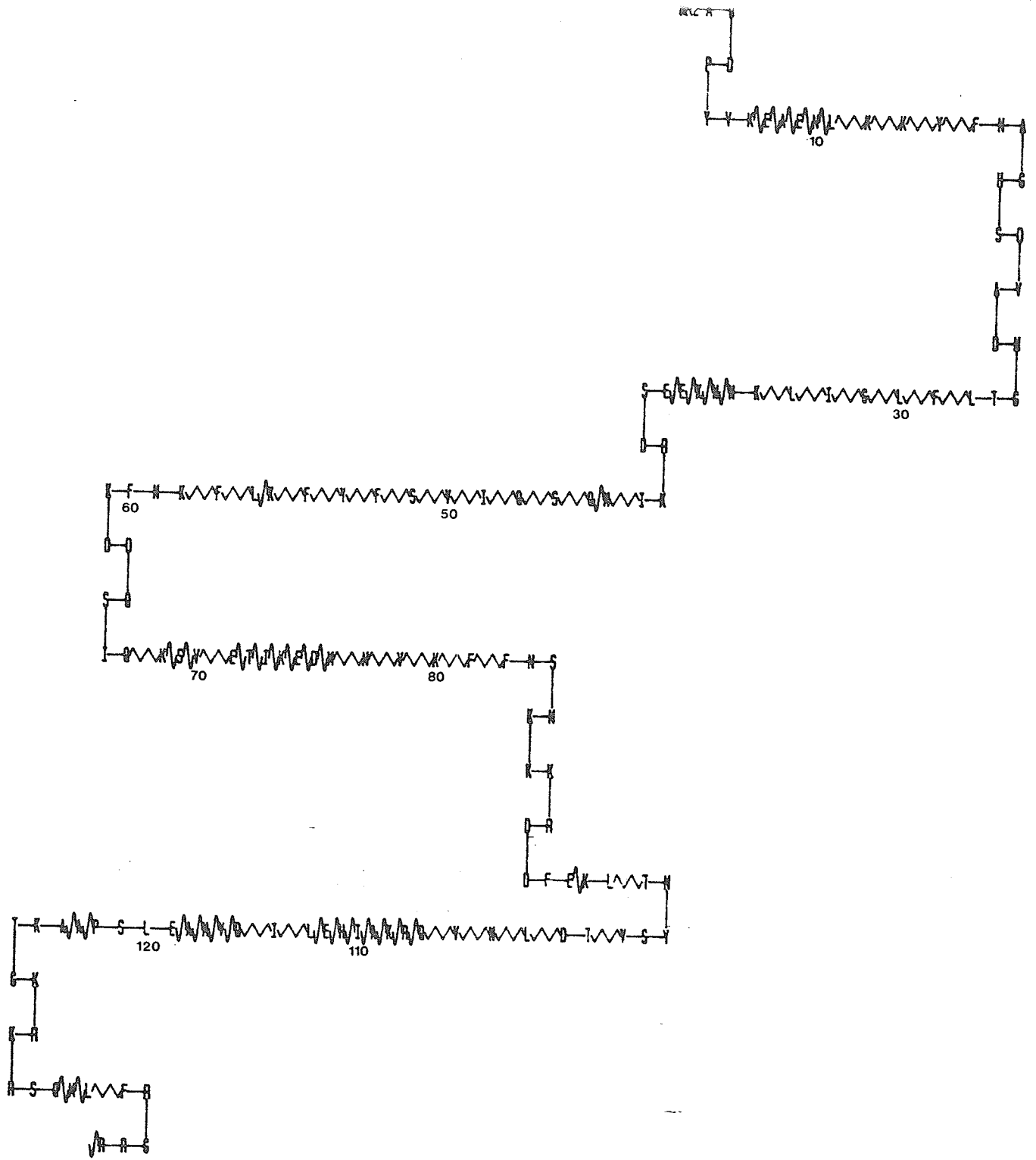


Figure 4.3

Secondary structure prediction of rHuIFN- γ . Algorithm based on that of Chou and Fasman (1978) was developed by Glorioso and Black (1984). The single-letter code for amino acids is used. Secondary structures are represented graphically as: W (α -helix), V (β -sheet), U (β -turn), and — (random coil). Secondary structures of residues 1 to 140 are shown. Residues 141 to 143 are not shown due to possible false structures.

One of the approaches used to study protein structures is the prediction of secondary structures. Since almost 90% of the residues in most proteins are involved in the three secondary structures: alpha-helix (α -helix) (30%), beta-sheet (β -sheet) (20%), or beta-turn (β -turns) (32%) (Creighton, 1984), it may be feasible to learn how to pack them together to generate the folded conformation if the secondary structure elements could be predicted accurately. A number of prediction schemes based on such empirical observations have been proposed (Lim, 1974; Chou and Fasman, 1978; Argos et al., 1978). One of these schemes is that of Chou and Fasman (1978), which classifies the amino acids as favoring, breaking, or being indifferent to each type of conformation. However, since α -helices, β -sheets, and β -turns are determined not by individual residues but by a number of adjacent residues in the sequence, consecutive stretches of residues were computed repetitively along the length of the polypeptide so that the probability of several sequential residues tend to prefer that particular structure can be calculated. Computer programs based on such prediction schemes have been developed over the years. A computer program developed by Black and Glorioso (1985) based on the algorithm of Chou and Fasman (1978) was employed in the present study for the prediction of secondary structures of the rHuIFN- γ .

The overall secondary structures of the HuIFN- γ in 2-dimensional forms are shown in Figure 4.3. Analysis of a number of secondary structures revealed a conserved and highly exposed region between residues 39 and 43. Further comparison of secondary structures of IFNs- γ from various vertebrate species between residues 30 to 50 revealed a β -turn structure (residues 39 to 43) that is conserved among the four animal species (Figure 4.4). Since β -turns and large

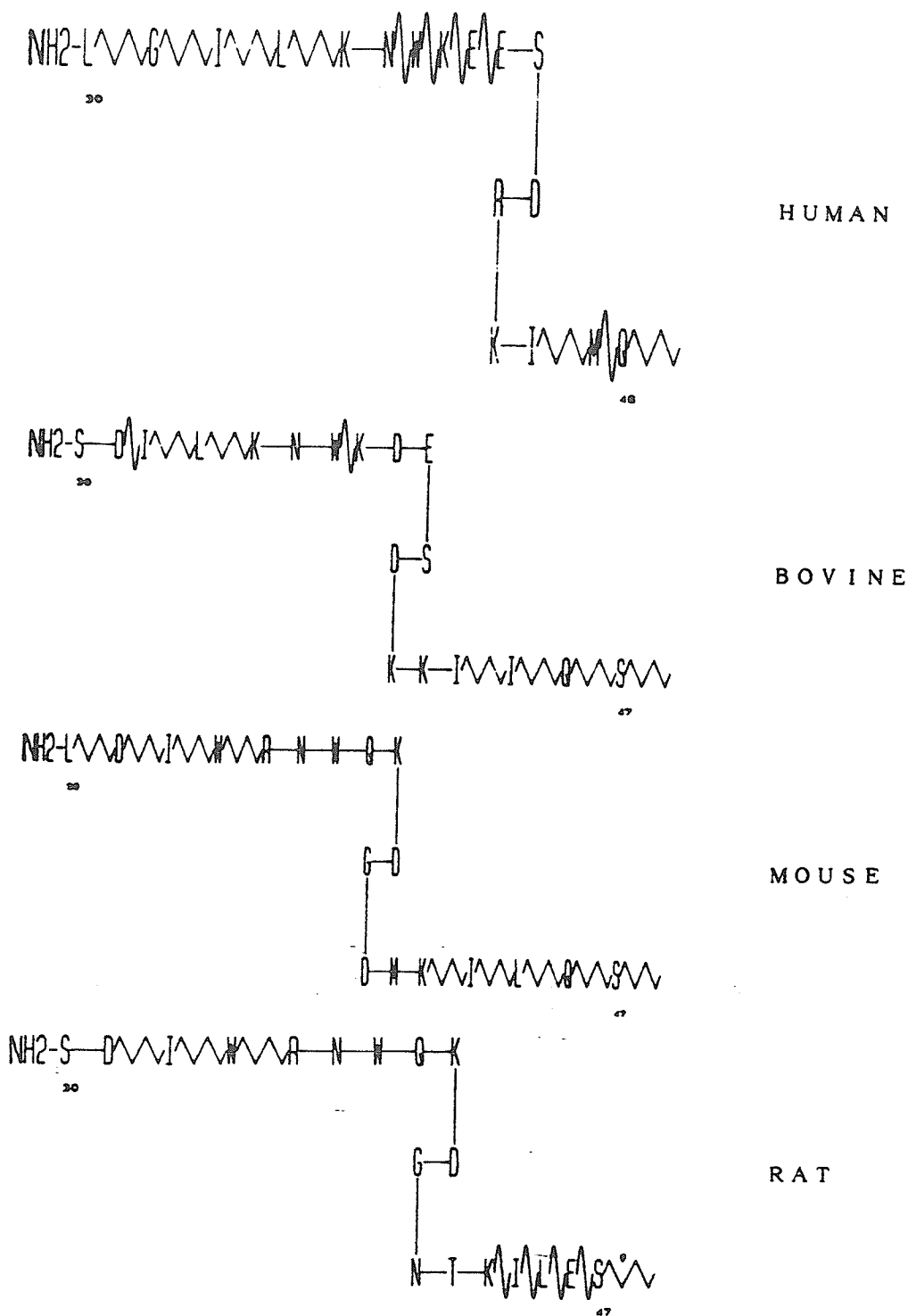


Figure 4.4 Comparison of a conserved β -turn (residues 30 to 46) of IFNs- γ from various vertebrate species. Algorithm based on that of Chou and Fasman (1978) was developed by Glorioso and Black (1984). The single-letter code for amino acids is used. Secondary structures are represented graphically as: β (alpha-helix), β (beta-sheet), β (beta-turn), and — (random coil).

loop structures have been shown to represent a significant fraction of globular protein surfaces, they are also known to play an important role in determining the conformation and specificity of enzyme active sites as well as antibody-combining sites (Rose et al., 1985; Kuntz, 1972). The conserved β -turn structure that spans residues 39 and 43 in the HuIFN- γ molecule may represent a region on the protein surface that has been conserved for its structural and/or functional roles. As a first step, mutagenesis experiments were designed to disrupt this conserved β -turn structure in order to determine its roles. Although it is possible to delete this region entirely, such an approach may affect the tertiary structure of the molecule. Therefore, single amino acid substitutions were designed to disrupt the local β -turn without altering the adjacent sequences and secondary structures.

4.5 Choice of amino acid residues for modifications

The conserved β -turn structure (residues 39 to 43) is made up of 5 polar amino acids, namely Glu³⁹, Ser⁴⁰, Asp⁴¹, Arg⁴², and Lys⁴³. One of the most hydrophilic (Figure 4.5a) and most conserved residues is Asp⁴¹. This residue is conserved in human, bovine, and mouse IFNs- γ . A conserved substitution of Asn at position 41 is present in the rat IFN- γ (Figure 4.4). Therefore, this residue would serve as a starting point for the present mutagenesis study. The choice of amino acids for replacement was selected based on the following rationale: (i) since the objective was to disrupt the local β -turn which was comprised of polar residues, Asp⁴¹ should, at the first instance, be replaced with a nonpolar residue; (ii) since bulky side chains such as those of Phe

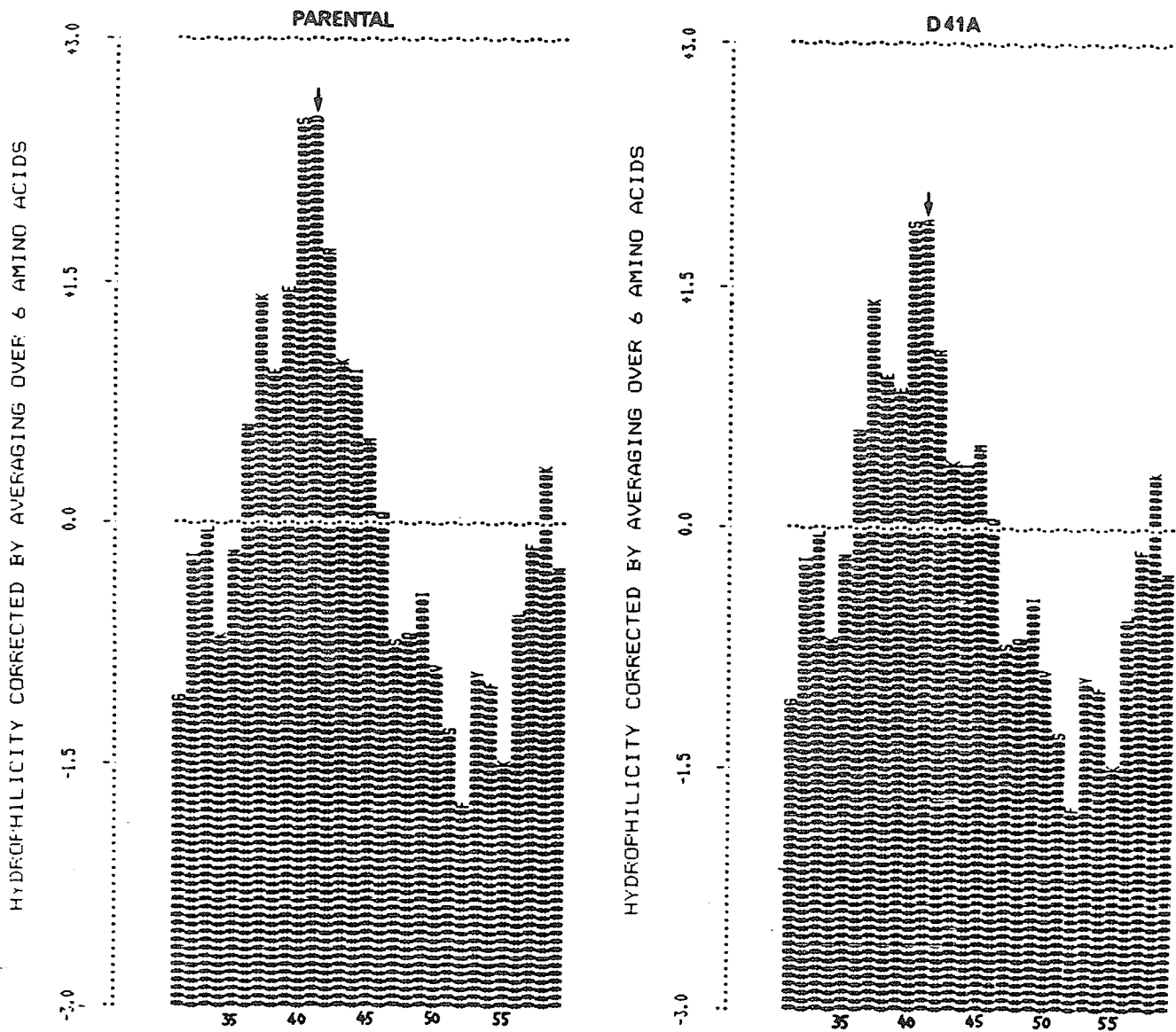
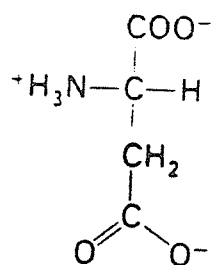
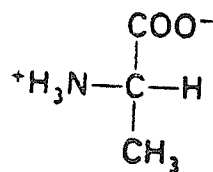


Figure 4.5 Comparison of hydrophilicity profiles of unmodified HuIFN- γ and single-site variant D41A. The algorithm of Hopp and Woods (1981) was used. Only regions between residues 30 and 60 are shown. Arrows denote Asp and Ala in the unmodified molecule and variant D41A, respectively. The hydrophilicity values were averaged over 6 amino acids. Residues with positive values are hydrophilic while those with negative values are hydrophobic. Regions on the dotted line with represents neutral area. The single-letter code for amino acid is used.

and Trp may significantly affect the local volume due to steric hindrance, nonpolar amino acids that have similar side chains to that of Asp should be selected in order to minimize the steric effect. Analyses of side chains from all the nonpolar amino acids showed that alanine (Ala) had the most similar side chain compared to that of Asp:



Aspartate
(Asp)



Alanine
(Ala)

The possible effects of this substitution on the local secondary structures were then simulated using the secondary structure prediction program described in Section 4.4. The replacement of Asp⁴¹ with Ala was shown to completely disrupt the β -turn that spanned residues 39 and 43 while extending the α -helix between residues 35 and 44 (Figure 4.6). The local hydrophilicity value (mean of values between Glu³⁹ and I⁴⁴) was also reduced by approximately 0.78 unit (Figure 4.5b). Therefore, the replacement of Asp⁴¹ with Ala should generate useful information regarding the significance of this β -turn.

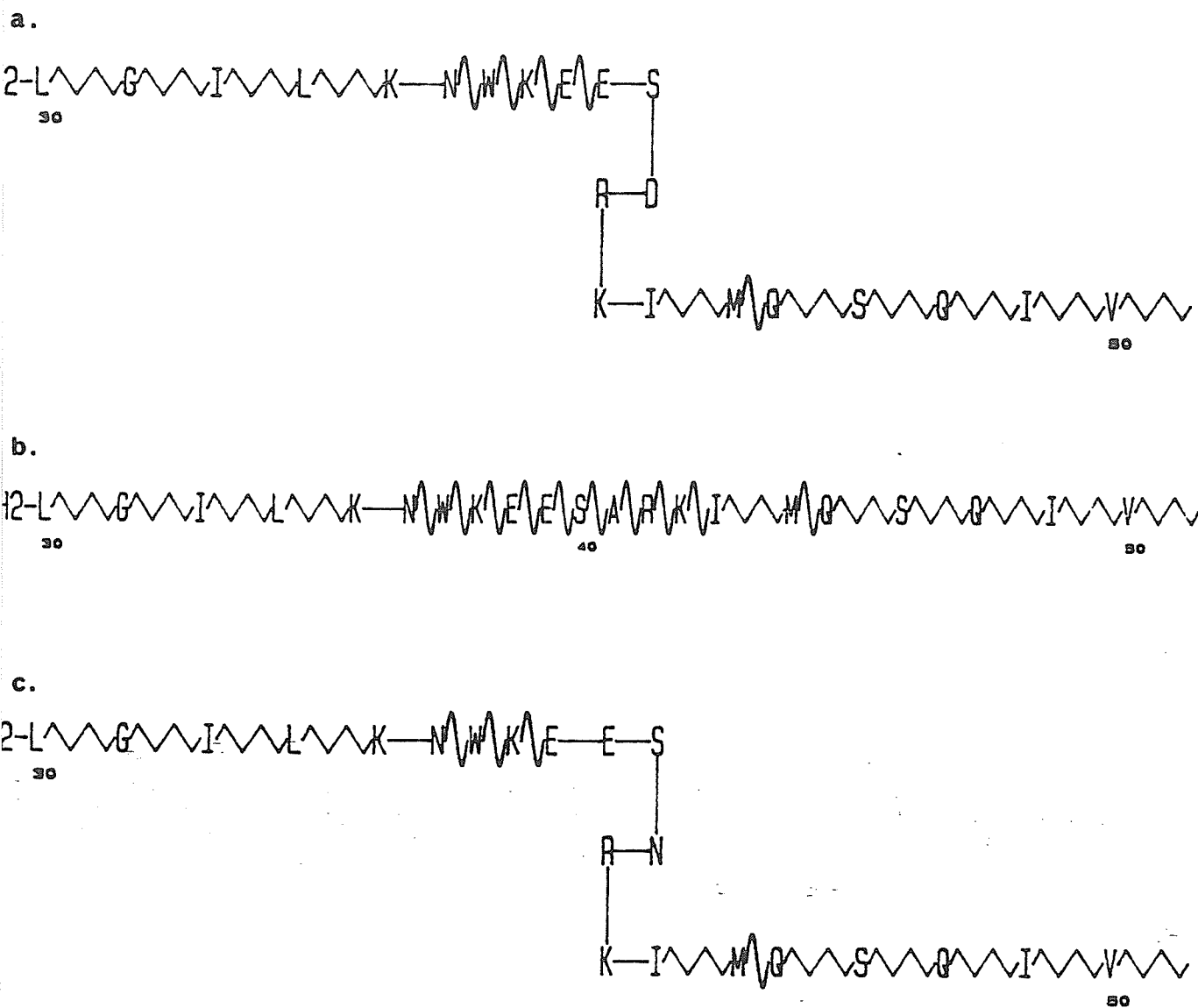


Figure 4.6 Comparison of secondary structures of unmodified HuIFN- γ and single-site variants D41A and D41N. Only structures between residues 30 and 60 are shown. a, unmodified HuIFN- γ ; b, variant D41A; c, variant D41N. Algorithm based on that of Chou and Fasman (1978) was developed by Glorioso and Black (1984). Secondary structures are represented graphically as: $\sim\sim\sim\sim$ (α -helix), $\wedge\wedge\wedge\wedge$ (β -sheet), \lrcorner (β -turn), and --- (random coil).

4.6 Materials and Methods

The substitution of Asp⁴¹ for Ala was achieved by site-directed mutagenesis described in Section 2.15. The detailed methodology for the construction of this variant, designated D41A, has been described in Section 2.18. The mutation of Asp⁴¹ was achieved by the substitution of an adenosine (A) for cytosine (C) which altered the codon GAC to GCC:

D41A

```

      38      39      40      41      42      43      44
      Glu    Glu    Ser    Asp    Arg    Lys    Ile
5' . . . . G A G G A G A G T G A C C G T A A A A T A . . . . 3'
      3' C C T C T C A C G G G C A T T T 5'
                    G C C
                    Ala
```

The primer-extended DNA were used to transform competent *E. coli* strain JM103 and the transformants were screened by colony hybridization as described in Section 2.18.6. Single-stranded DNA were isolated from the positive transformants (Section 2.7.1) and the nucleotide substitutions were confirmed by DNA sequencing (Section 4.7.1) using the dideoxynucleotide chain termination method (Section 2.7). A 15-base primer that spanned residues 62 and 66 (3' CTACTAGTCTCGTAG 5') was used for sequencing. Intracellular RF's were prepared (Section 2.3) from the transformants that had been confirmed by DNA sequencing. The mutated regions were then retrieved by excising the RF's with the restriction enzymes KpnI and AsuII and were subcloned into the expression vector P₁₄R₃ similarly linearized by the same enzymes. A schematic

of the subcloning procedure is shown in Figure 4.7. Both the expression vector and the restriction enzyme fragments were separated on a 1% agarose gel (Figure 5.5). The corresponding bands were excised from the gel and the DNA were eluted by electrophoresis into dialysis tubing. Eluted DNA were extracted with phenol/chloroform/isoamyl alcohol (49:49:2) 3 times, chloroform/isoamyl alcohol (48:2) 3 times, and precipitated with a final concentration of 0.3 M sodium acetate (pH 7.0) and two volumes of absolute ethanol at -20°C. The purified KpnI/AsuII fragment were ligated with the linearized vector in a ratio of 1:5 (fragment:vector). Ligation condition were as described in Section 2.12. The ligation reaction was directly used to transform the E. coli strain LE392 that had been made competent according to the procedures described in Section 2.5. Transformants were randomly selected, separately grown in 2 ml T broth, lysed in SDS sample buffer, and characterized by SDS-PAGE (Section 4.7.2).

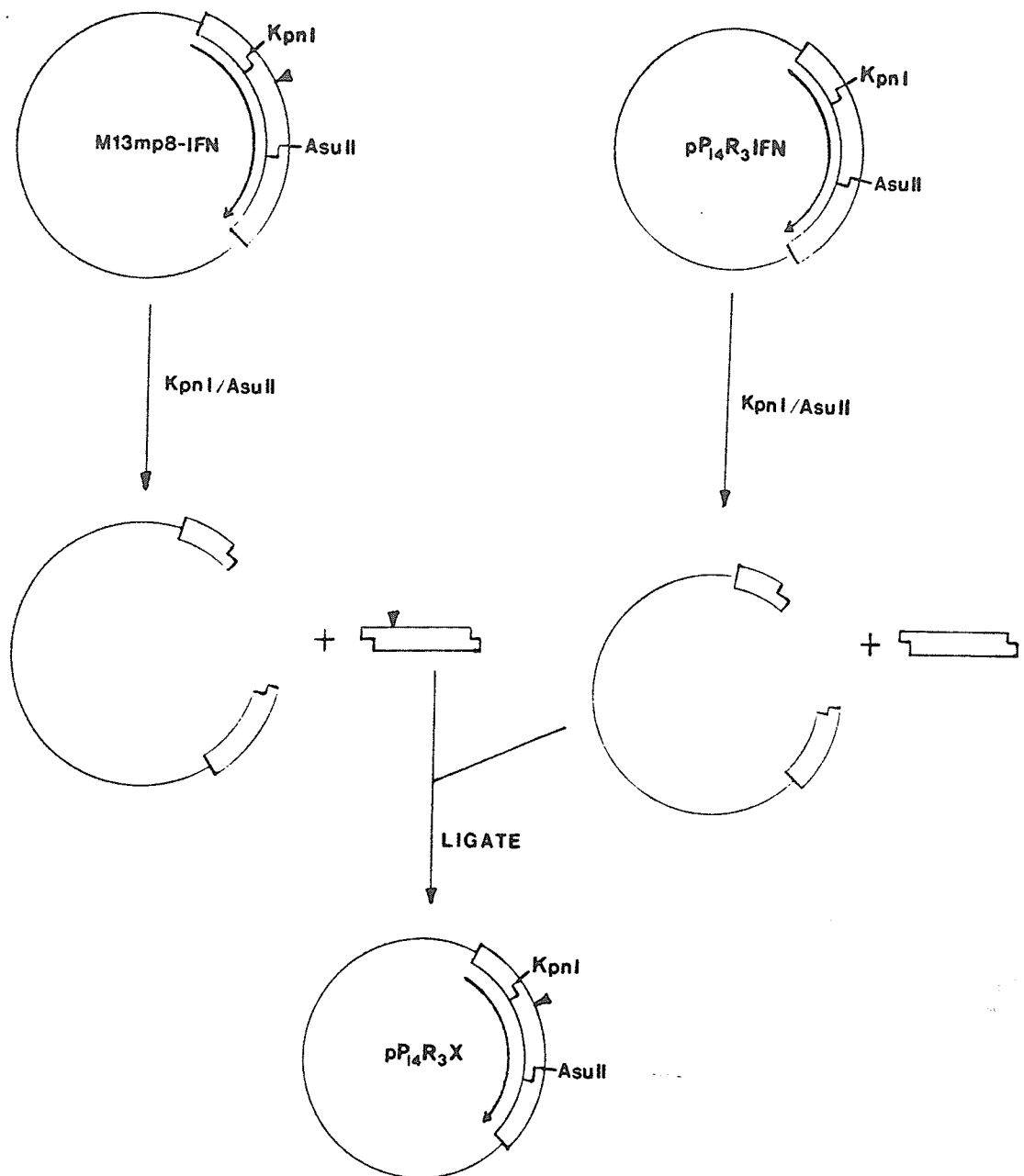


Figure 4.7 Subcloning of single-site variants into the expression vector P₁₄R₃. rHuIFN- γ coding sequence in the M13mp8 vector and the expression vector P₁₄R₃ were linearized by the restriction endonucleases KpnI and AsuII. The fragments were separated on a 1% agarose gel, electroeluted, and purified. The KpnI/AsuII fragment containing the mutation (∇) was subcloned into the purified expression vector linearized by the same enzymes. The resulting plasmid, pP₁₄R₃X (X: D41A, D41N, Q46L, or Q48L) contained the modified IFN- γ sequence that had the specific single-base substitution.

4.7 Results

4.7.1 Identification of single-site variant D41A

The method of colony hybridization was utilized to screen for variant D41A. As described in Section 2.18.6, this method allowed rapid screening of a large number of clones and reduced the number of clones that had to be analysed by DNA sequencing. After two rounds of colony hybridization, two independent positive clones were picked and sequenced to confirm the desired nucleotide change.

To determine the nucleotide sequence of the variant clones, ssDNA were prepared and sequenced using the dideoxynucleotide chain termination method as described in Section 2.7. A 15-base primer that spanned residues 62 and 66 (3' CTACTAGTCTCGTAG 5') was used to sequence the two mutants. Since the antisense strand was sequenced, the nucleotide change of T to G shown in Figure 4.8 represents the desired alteration of A to C on the complementary sense strand:

Clone	Nucleotide Change	Codon change
D41A	T → G	GAC → GCC sense strand CTG → <u>CGG</u> antisense strand

Only one clone was sequenced in its entirety to ensure no unwanted mutation was introduced. The mutated region of this clone was then subcloned into the expression vector P₁₄R₃, and expressed

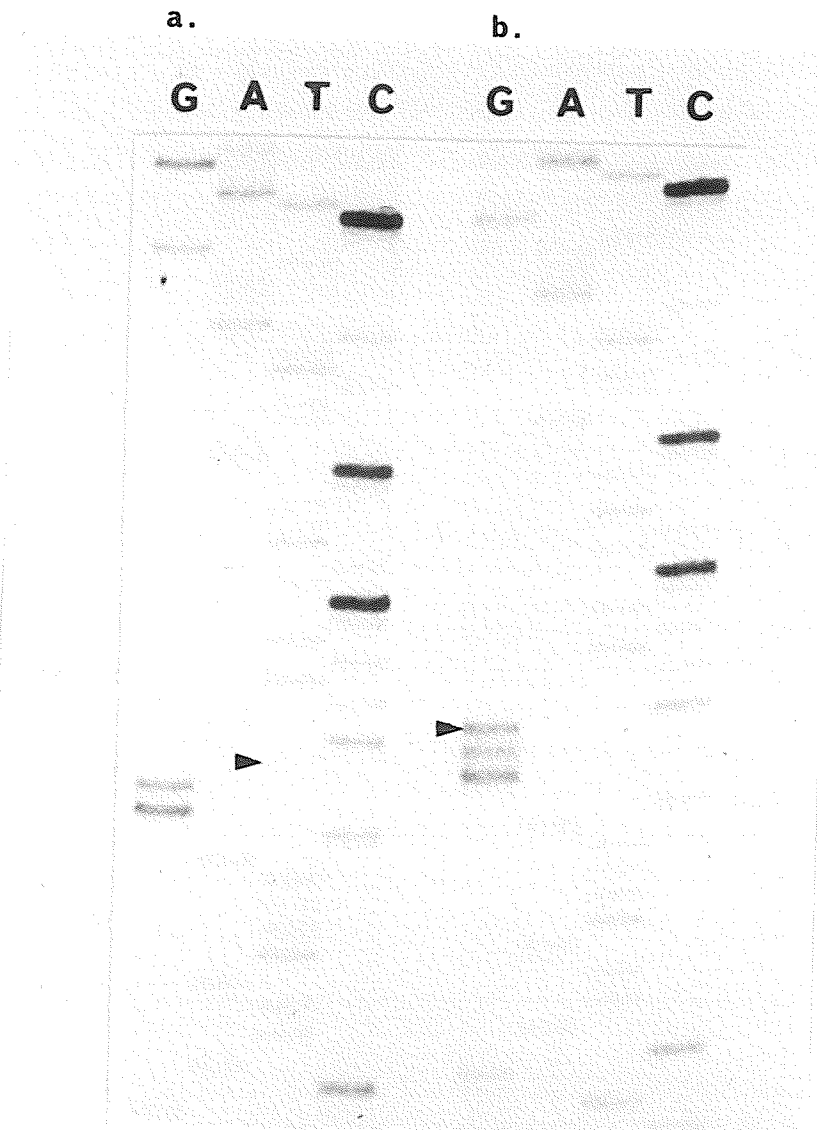


Figure 4.8 DNA sequence analysis of single-site variant D41A. The nucleotide sequences of (a) the unmodified IFN- γ and (b) variant D41A were analysed by dideoxynucleotide chain termination sequencing. The parental nucleotide (T) and the substitution in variant D41A (T \rightarrow G) are denoted by an arrowhead.

in the E. coli strain LE392 as described in Section 4.6.

4.7.2 Characterization of single-site variant D41A polypeptide

Single-site variant polypeptide D41A was expressed in the E. coli strain LE392. Cell lysates from 10 independent clones were prepared and electrophoresed on a 15 % SDS polyacrylamide gel. Total proteins were detected by silver stain. As shown in Figure 4.9, a 17 kD polypeptide was detected in the parental clone (lane b) whereas none of the D41A clones produced a polypeptide of similar size (lanes h to q), either due to their failure of expression or due to their lack of accumulation. Therefore, Western immunoblot analysis was carried out in order to ascertain the expression of the variant polypeptide (Figure 4.10, lanes h to q). These immunoblot results showed that low level of D41A were in fact expressed and could be detected by the rabbit polyclonal serum against purified rHuIFN- γ . Based on the intensity of the parental 17 kD band and that of the D41A, it was estimated that the amount of this variant polypeptide was at least 50-fold less that of the parental molecule.

To determine whether D41A could be recognized by two of the immunoblot positive Mab's, the polypeptide was detected in a Western immunoblot analysis using the neutralizing Mab MIF3061 and a non-neutralizing Mab MIF3037 (Figure 4.11, Panels A and B, respectively). A parallel analysis using the rabbit polyclonal antiserum was served as a positive control (Panel C). Results presented in Figure 4.11 showed that whereas both the polyclonal serum and MIF3037 could recognize the parental (Panels A and C, lanes a) as well as the D41A polypeptide

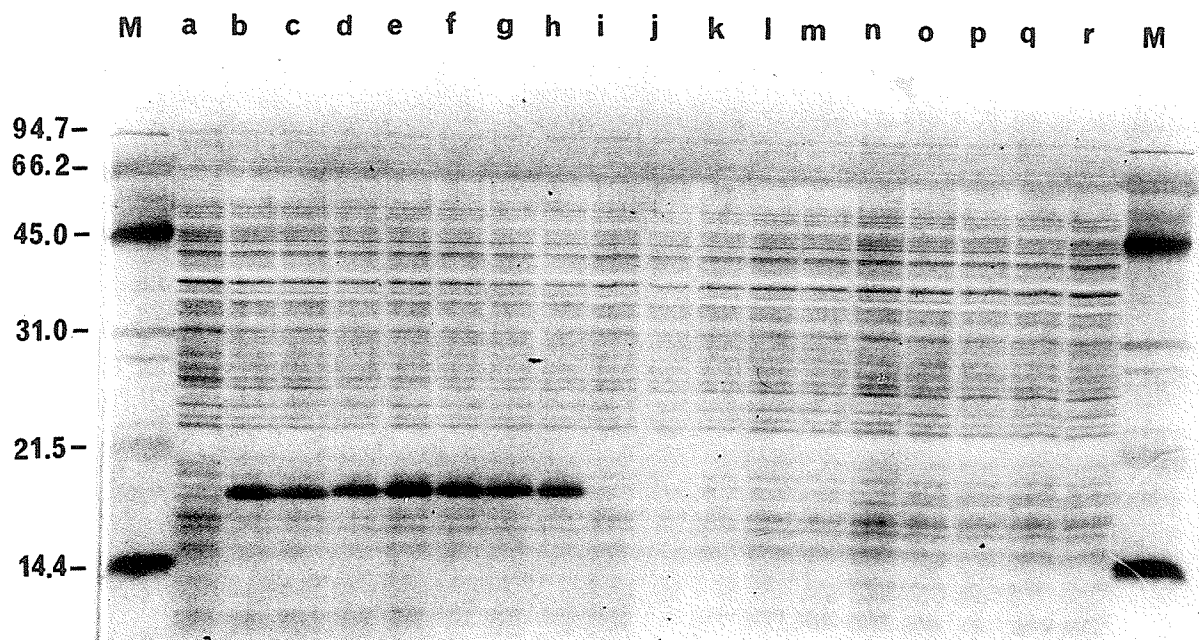


Figure 4.9 SDS-PAGE analysis of single-site variants Q46L, Q48L, and D41A. Independent clones of bacterial cells harboring the expression vector without the IFN insert (lane a), with the unmodified IFN insert (lane b), with the Q46L substitution (lanes c to e), with the Q48L substitution (lanes f to h), and with the D41A substitution (lanes i to r) were lysed and electrophoresed on a 15% SDS polyacrylamide gel. The total proteins were stained with silver. The molecular mass standards (lanes M) were in kilodaltons.

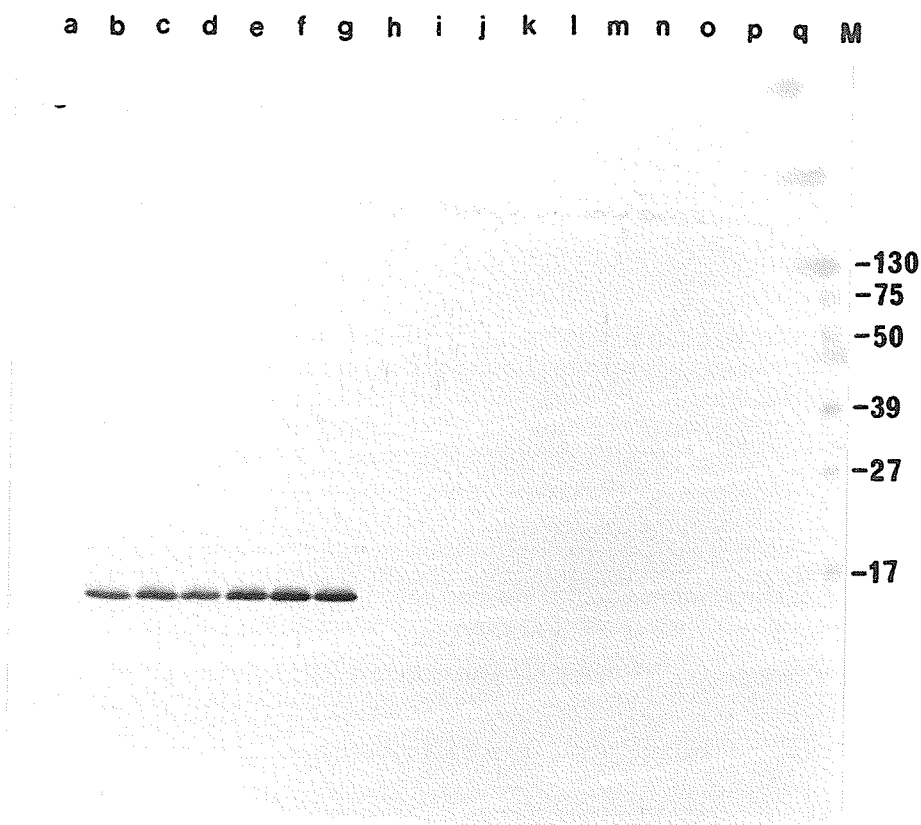


Figure 4.10 Western immunoblot analysis of single-site variants Q46L, Q48L, and D41A. Cell lysates were prepared and electrophoresed as described in Figure 4.9 except that 1/10 of the samples were loaded. Total proteins were blotted electrophoretically on to nitrocellulose membrane and probed with a rabbit polyclonal serum against purified IFN- γ . Lysates of cells containing the expression vector without the IFN insert (lane a), with the unmodified IFN insert (lane b), with the Q46L substitution (lanes c to e), with the Q48L substitution (lanes f and g), and with the D41A substitution (lanes h to q) are shown. The molecular mass standards (lane M) were pre-stained markers (in kilodalton).

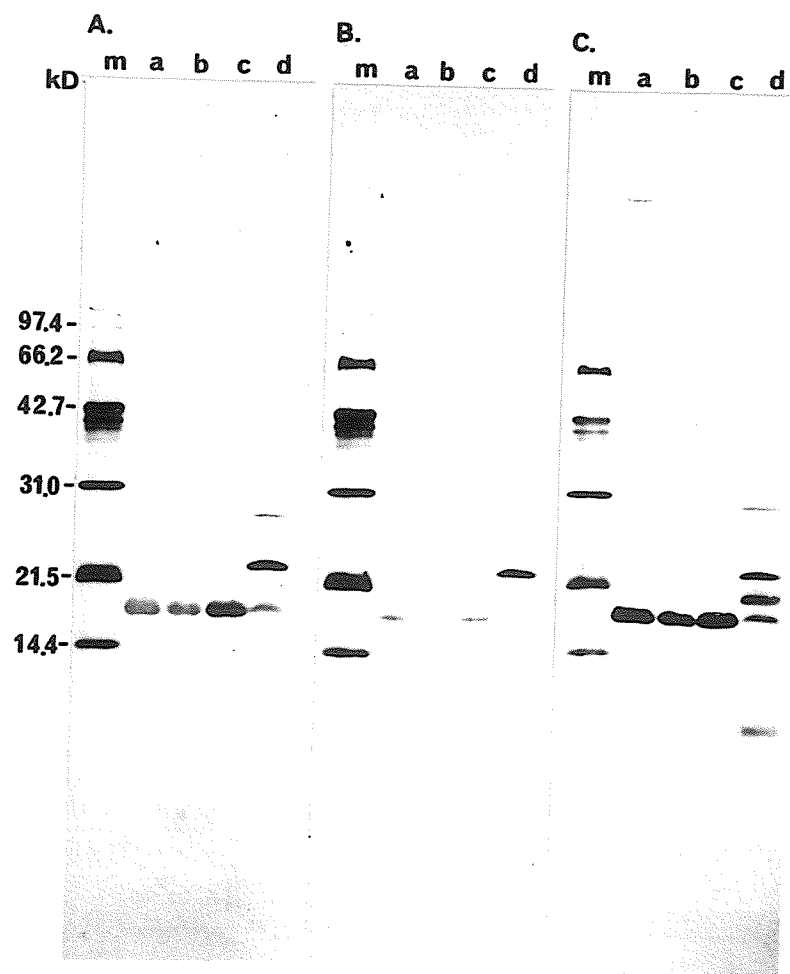


Figure 4.11 Western immunoblot analysis of single-site variants D41A, Q46L, and Q48L. Lysates of cells harboring the plasmids that expressed the unmodified IFN- γ (lanes a), the Q46L variant polypeptide (lanes b), the Q48L variant polypeptide (lanes c), the D41A variant polypeptide (lanes d) were separated on a 15% SDS polyacrylamide gel and blotted electrophoretically onto nitrocellulose membrane. These polypeptides were probed with a non-neutralizing monoclonal antibody MIF3037 (Panel A), a neutralizing monoclonal antibody MIF3061 (Panel B), and a polyclonal antiserum against the purified rHuIFN- γ (Panel C). The molecular mass standards (lane m) were biotinylated markers (in kilodalton).

(Panels A and C, lanes d), MIF3061 could no longer recognize D41A (Panel B, lane d), suggesting that an epitope normally recognized by this neutralizing Mab was abolished by the substitution of Asp⁴¹ for Ala. On the other hand, all of these three antibody preparations could recognize the parental molecule (lanes a of Panels A, B, and C) as well as variant Q46L (lanes b of Panels A, B, and C) and variant Q48L (lanes c of Panels A, B, and C). (The latter two variants will be discussed in detail in Chapter 5). The high molecular weight bands observed in lanes containing D41A were due to nonspecific reaction due to the amount of D41A cell lysates loaded, which was 50-fold more than that of the other clones.

4.7.3 Determination of antiviral activity of variant D41A

Variant D41A was extracted and purified according to the procedures essentially as those described for the parental molecule (Section 2.1). The purified fractions were pooled and assayed for antiviral activity as described in Section 2.9. The protein concentration of the pooled fraction was estimated according to the method of Schaffner and Weissmann (Section 2.8). It was noticed that the amount of purified D41A polypeptide was substantially less than that of the parental molecule. The specific activity was determined based on the amount of purified protein used in the assay. As shown in Table 4.1, the specific antiviral activity of D41A was 2.55×10^6 units/mg or approximately 10-fold less than that of the parental molecule. Taking into account the variations of the antiviral assay which is within 2-fold, the 10-fold difference in the antiviral specific activity between the parental molecule and D41A represents a significant reduction in

specific activity of the latter molecule.

4.8 Construction of single-site variant D41N

The mutagenesis study described above demonstrated that the replacement of Asp⁴¹ with Ala resulted in 10-fold reduction in specific antiviral activity. Although the objective of the substitution of Asp⁴¹ for Ala was to disrupt the local β -turn, it remained possible that the reduced activity in variant D41A was a result of the absence of the intrinsic properties of the amino acid Asp, either due to the contribution of its negative charge to the local ionic environment or its hydrophilic nature which might affect the surrounding surfaces. In order to investigate these possibilities, mutagenesis experiments were designed to replace Asp⁴¹ without disturbing the local β -turn.

The amino acid asparagine (Asn) was used to replace Asp⁴¹ for the following reasons: (i) Asn has a similar side-chain volume as Asp (117.7 Å and 111.1 Å, respectively) (Creighton, 1984) and is not likely to affect the interactions with its neighboring residues due to steric hindrance; (ii) based on secondary structure prediction shown in Figure 4.6, the substitution of Asp⁴¹ for Asn would maintain the conserved β -turn; (iii) although both are polar residues, Asp is substantially more hydrophilic than Asn (hydrophilicity values of 3.0 and 0.2, respectively) (Hopp and Woods, 1981). The local hydrophilicity profile of this substitution is shown in Figure 4.12. Therefore, any alteration induced by this substitution would be limited to the hydrophilicity effect and/or the abrogation of the negative charge on Asp⁴¹.

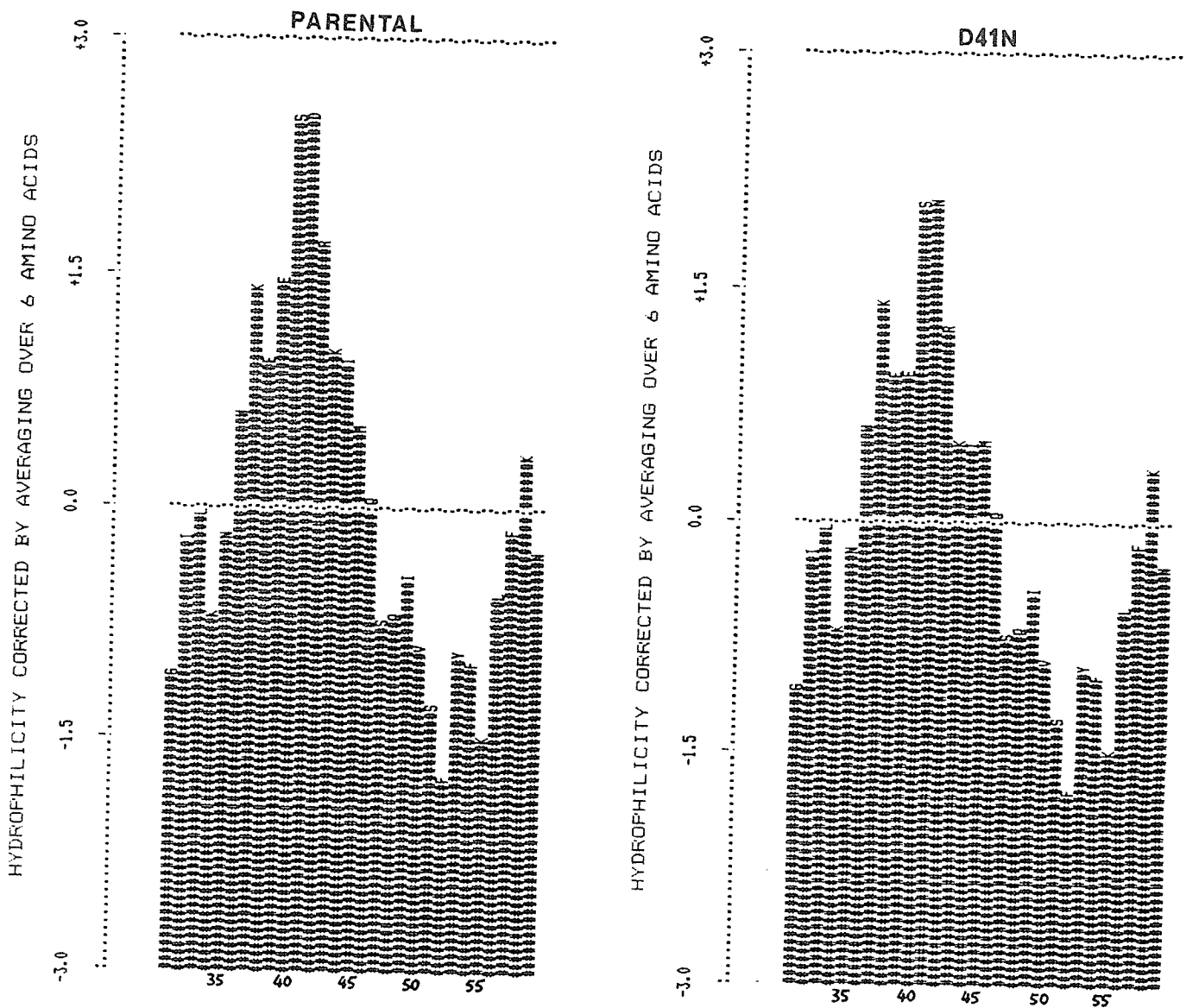


Figure 4.12 Comparison of hydrophilicity profiles of unmodified HuIFN- γ and single-site variant D41N. Algorithm based on that of Hopp and Woods (1981) was used. Only regions between residues 30 and 60 are shown. Arrows denote Asp and Asn in the unmodified molecule and variant D41N, respectively. The hydrophilicity values were averaged over 6 amino acids. Residues with positive values are hydrophilic while those with negative values are hydrophobic. Regions on the dotted line with represents neutral area. The single-letter code for amino acid is used.

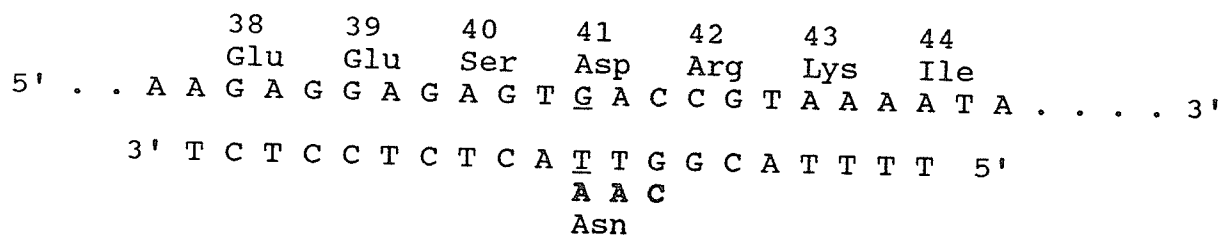
4.9 Materials and Methods

Variant D41N was constructed by the method of Eckstein as described in Section 2.19.

Synthetic oligonucleotide primer with one mismatch was synthesized and the sequence is shown

below:

D41N



Due to the high efficiency of this mutagenesis method, the transformants were directly screened by DNA sequencing using the dideoxynucleotide chain termination method (Section 2.7). Of the four transformants that were sequenced, two were found to contain the desired single-base substitution (Section 4.10.1). Subcloning and expression of this variant was essentially the same as described in Section 4.6.

4.10 Results

4.10.1 Determination of single nucleotide substitutions

Single-stranded DNA from variant clones were prepared according to the method described in Section 2.7.1. A synthetic primer located downstream from the mutation site was synthesized (Section 4.7.1) for sequencing regions where single-nucleotide substitution was introduced. Sequencing reactions were carried out as described in Section 2.7. The single-nucleotide substitutions are shown in Figure 4.13. Since the sequencing primer complementary to the sense strand were used, the nucleotide change shown in Figure 4.13 represents that on the antisense strand. The desired change of variant D41N was confirmed as follows:

Clone	Nucleotide Change	Codon change
D41N	C → T	GAC → AAC sense strand CTG → <u>TTG</u> antisense strand

4.10.2 Characterization of the variant D41N polypeptide

Single-site variant D41N was subcloned into the expression vector P₁₄R₃ and expressed in *E. coli* as described in Section 4.6. A total of 4 clones were lysed in SDS sample buffer and analysed by SDS-PAGE. As shown in Figure 4.14, all of the clones (lanes a to d) expressed the variant polypeptide in concentration that was comparable to that of the parental molecule (lane

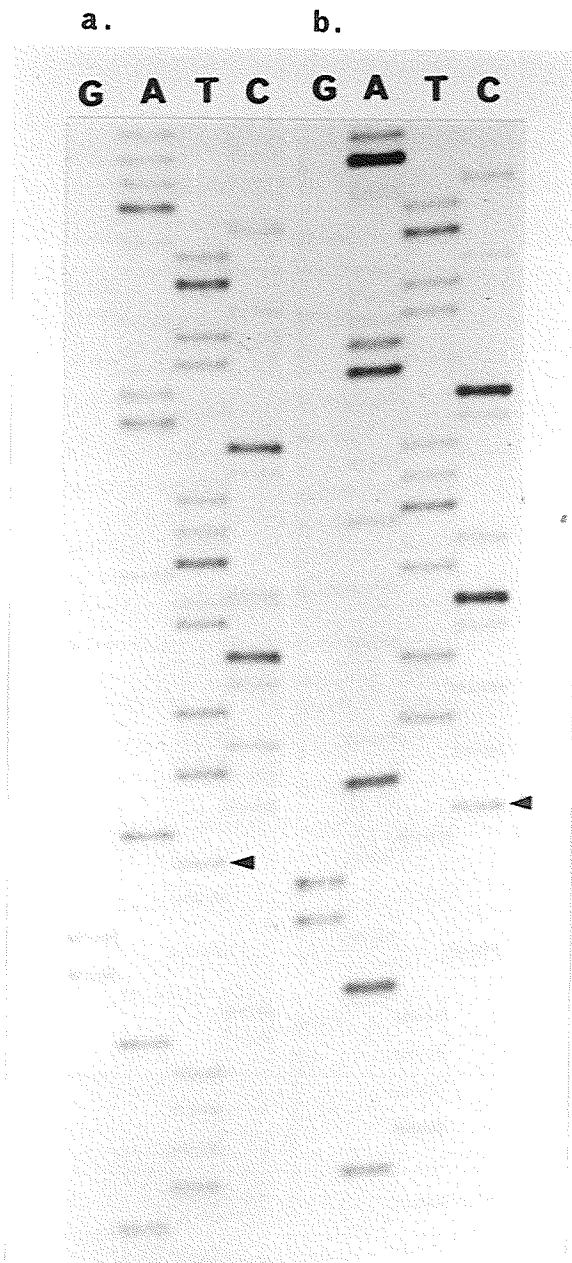


Figure 4.13 DNA sequence analysis of single-site variant D41N. The nucleotide sequences of (a) variant D41N and (b) the unmodified IFN- γ were analysed by dideoxynucleotide chain termination sequencing. The parental nucleotide (C) and the substitution in variant D41A (C \rightarrow T) are denoted by an arrowhead.

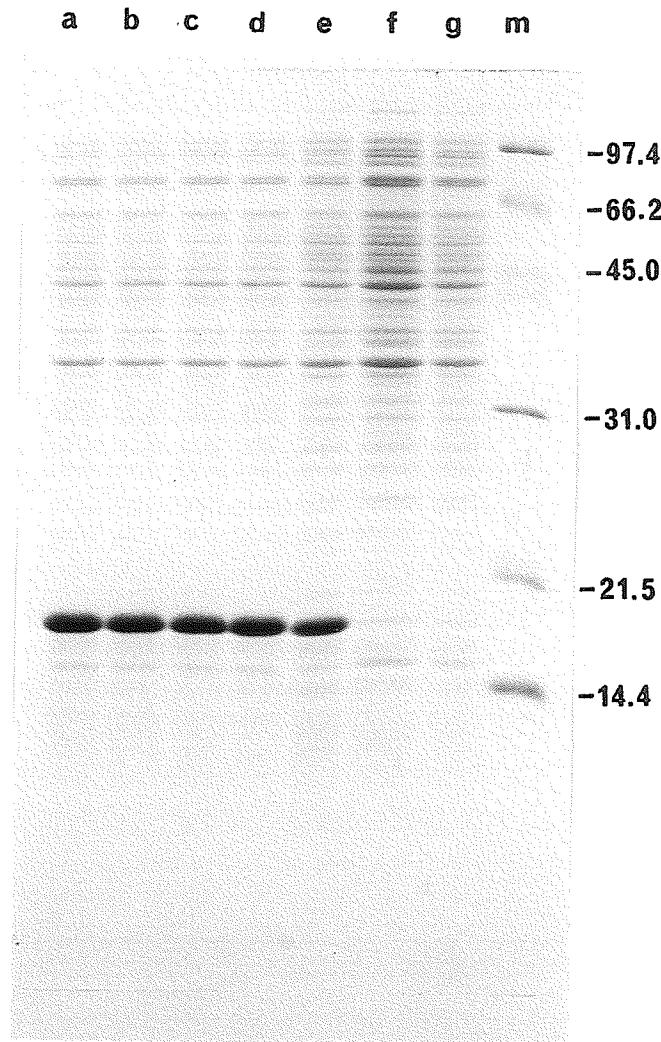


Figure 4.14 SDS-PAGE analysis of single-site variant D41N. Independent clones of bacterial cells harboring the expression vector without the IFN- γ insert (lanes f and g), with the unmodified IFN- γ insert (lane e), and with the D41N substitution (lanes a to d) were lysed and electrophoresed on a 15% SDS polyacrylamide gel. The total proteins were stained with Coomassie Blue. The molecular mass standards (lane m) were in kilodaltons.

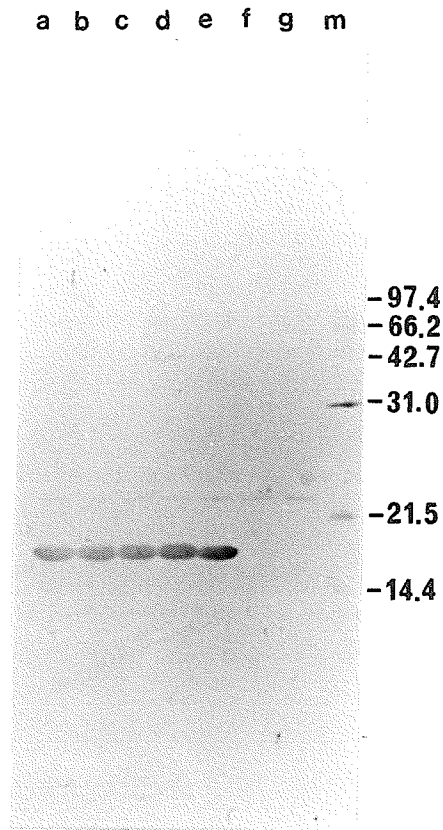


Figure 4.15 Western immunoblot analysis of single-site variants D41N. Cell lysates were prepared and electrophoresed as described in Figure 4.14 except that 1/10 of the samples were loaded. Total proteins were blotted electrophoretically on to nitrocellulose membrane and probed with a rabbit polyclonal serum against purified IFN- γ . Lysates of cells containing the expression vector without the IFN insert (lanes f and g), with the unmodified IFN insert (lane e), and with the D41N substitution (lanes a to d) are shown. The molecular mass standards (lane m) were biotinylated markers (in kilodalton).

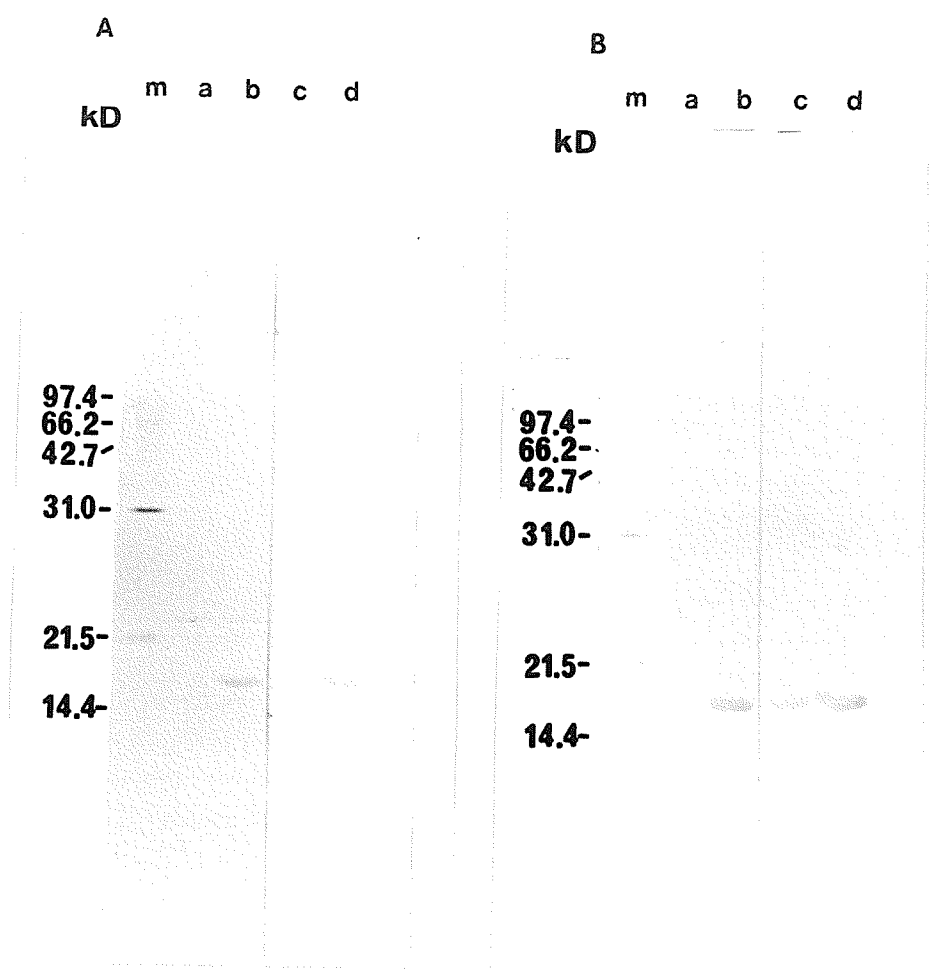


Figure 4.16 Western immunoblot analysis of single-site variants D41A and D41N. Cell lysates were prepared and electrophoresed as described in Figure 4.15. The polypeptides were probed with a neutralizing monoclonal antibody MIF3061 (Panel A) and a non-neutralizing monoclonal antibody MIF3037 (Panel B). Lanes: a, lysates from cells harboring the expression vectors without the IFN- γ insert; b, the unmodified IFN- γ polypeptide; c, variant D41A polypeptide; d, variant D41N polypeptide; m, biotinylated molecular weight markers.

e). The apparent molecular weight of 17,000 was also in agreement with the calculated molecular weight of 16,900. The negative controls were clones which contained the expression vector without the IFN- γ insert (lanes f and g). In a parallel experiment, these clones were analysed by Western immunoblot (Figure 4.15). All of the D41N polypeptides could be detected by the rabbit polyclonal antiserum against the purified rHuIFN- γ (lanes a to d) and the intensity of the protein bands were comparable to that of the parental molecule (lane e). Therefore, the variant D41N polypeptide appeared to have similar stability as the parental molecule. These variant polypeptides were further characterized with two immunoblot positive Mab, MIF3037 and MIF3061 (Figure 4.16). Both MIF3037 (Panel A) and MIF3061 (Panel B) were able to recognize the D41N polypeptide (lanes d).

4.10.3 Determination of antiviral activity of variant D41N

Variant D41N was extracted and purified as described in Section 2.1 and purified protein was assayed for its antiviral activity according to the procedure described in Section 2.9. The specific antiviral activity of the D41N was 1.02×10^7 units/mg, comparable to the activity of the parental molecule which was 2.30×10^7 units/mg (Table 4.1). Since the variation of the antiviral assay is within 2-fold, the specific activity of the parental molecule and variant D41N is approximately the same.

Table 4.1 Antiviral Specific Activities of Single-Site Variants D41A and D41N

Clones	Antiviral Specific Activity (units/mg) ^a
P ₁₄ R ₃ IFN	2.30 x 10 ⁷
D41A	2.55 x 10 ⁶
D41N	1.02 x 10 ⁷

a, The variation of each of the values was within 2-fold.

4.11 Discussion

4.11.1 Role of a conserved β -turn in the biological activity of HuIFN- γ

The purpose of the present study was to define the importance of individual amino acid residues that may be involved in the structure and/or functions of HuIFN- γ . Comparison of primary sequences of IFNs- γ from four vertebrate species revealed several highly conserved regions (Figure 4.1). Based on hydrophilicity analysis, the conserved residues between positions 36 to 46 were located in a highly hydrophilic region (Figure 4.2). Secondary structure analyses based on the algorithm of Chou and Fasman (Black and Glorioso, 1985) predicted that residues that spanned 39 and 43 constituted a β -turn structure. More importantly, this β -turn was shown to be highly conserved in all of the four vertebrate species being compared, despite their differences in primary sequences (Figure 4.4). The fact that a surface-exposed structure was so well conserved suggested that it may play a key role in the structure and/or functions of IFN- γ .

In order to study the significance of this conserved β -turn, single-site variants were constructed to either disrupt this structure or maintain the β -turn while altering the local charge. The construction of these variants was achieved by site-directed mutagenesis. Two single-site variants were constructed by replacing the most hydrophilic amino acid in this region, the negatively-charged Asp⁴¹, with (i) a hydrophobic amino acid (Ala) which resulted in the disruption of the local β -turn structure and (ii) an uncharged amino acid (Asn) which abolished the negative charge without disrupting the local secondary conformation.

The rationale for choosing the hydrophilic region between residues 39 to 43 was based on the assumption that the conserved hydrophilic domain would likely be located on the protein surface and be involved in intermolecular interactions, either with the receptor or with the effector molecule(s). By substituting Asp⁴¹ for a hydrophobic amino acid alanine (variant D41A), the local hydrophilicity of this region was predicted to decrease by 0.78 hydrophilic unit (Figure 4.5). On the other hand, the effect of this substitution on the local secondary structure was more drastic: the highly conserved β -turn between residues 39 to 43 was disrupted (Figure 4.6b).

The specific antiviral activity of variant D41A was found to be at least 10-fold less than that of the parental molecule (Table 4.1), indicating that the conserved β -turn is important for conferring the antiviral activity of the HuIFN- γ molecule. However, the fact that the activity was not completely abolished suggested that other functional domains must exist to confer the full biological activities. Since it has previously been demonstrated that the HuIFN- γ molecule had two distinct functional epitopes, E₁ and E₂, (Alfa and Jay, 1988), it is possible that the disruption of the β -turn had only affected one of the epitopes. Preliminary experiment supported this notion. While the D41A polypeptide could be recognized by both the polyclonal antiserum against purified HuIFN- γ (Figure 4.11, Panel C, lane d) and a Mab MIF3037 that recognized the C-terminus of the rHuIFN- γ (Figure 4.11, Panel A, lane d), it could only react very weakly with a neutralizing antibody MIF3061 (Figure 4.11, Panel B, lane d). Since MIF3061 belongs to the group of Mabs that neutralize the E₁ epitope (Alfa and Jay, 1988), the conserved β -turn may constitute part of the E₁ epitope. Immunoblot analysis of other Mabs that neutralize E₁ would confirm the finding that D41A had lost this functional epitope. It is not clear, however, whether

this epitope represents the receptor binding site or the effector site. Nevertheless, the fact that this region was conserved among the four vertebrate species suggested that it was not likely to be involved in receptor binding since IFNs- γ are known to be species specific (Pestka et al., 1987). Therefore, it is tempting to speculate that the conserved β -turn constitutes part of the effector site(s). It is possible that effector binding requires cooperative binding of both epitopes E_1 and E_2 and that the disruption of E_1 would only partially affect the binding capability.

In order to determine whether the loss of activity of D41A was due to the disruption of the β -turn or the direct involvement of the negatively charged-Asp⁴¹, this residue was replaced with Asn. Since both Asp and Asn have similar side-chain volume (Creighton, 1984), the substitution of Asp⁴¹ for Asn would not be expected to induce any significant changes in local secondary structures. Indeed, secondary structure prediction showed that the local β -turn was preserved (Figure 4.6c). Hydrophilicity analysis indicated that the local hydrophilicity would decrease by 0.55 hydrophilicity units as a result of this substitution (Figure 4.12).

The D41N polypeptide was expressed in *E. coli*, purified, and assayed for antiviral activity. As shown in Table 4.1, the specific antiviral activity of variant D41N was found to be comparable to that of the parental molecule. Therefore, it is likely that the whole β -turn structure, rather than individual residues or the local hydrophilic environment of this region, that contributes to the full activity of the HuIFN- γ molecule.

Other studies (Fish et al., 1988) have shown that disruption of a β -turn between residues 24 to 26 by replacing Asn²⁵ with Gln also resulted in a 10-fold reduction in antiviral activity and a 5-fold reduction in antiproliferative activity (Table 4.2). Receptor binding experiments showed

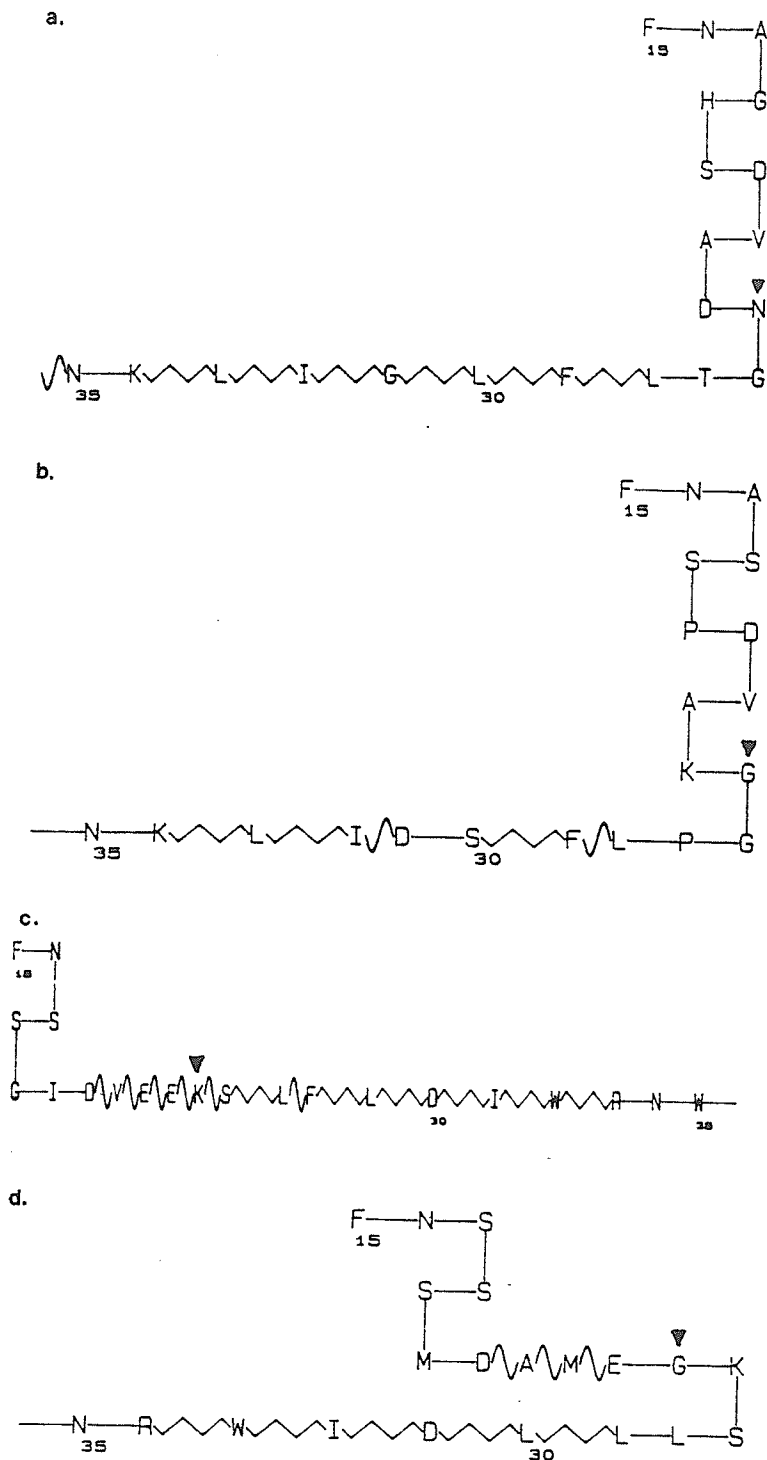


Figure 4.17 Comparison of local secondary structures between residues 15 and 35 from four vertebrate species. a, Human IFN- γ ; b, Bovine IFN- γ ; c, Murine IFN- γ ; d, Rat IFN- γ . Residue 25 of each of the IFN- γ is indicated with an arrowhead.

that the affinity of this variant was not affected. These results were interpreted as showing the involvement of the β -turn in effector functions (Fish et al., 1988). It is interesting to note, however, that this β -turn is only conserved in human and bovine IFN- γ , and only partially conserved in rat IFN- γ (Figure 4.17). Residue 25 in the murine IFN- γ is in fact located in the middle of a β -sheet (Figure 4.17d). Therefore, it appears that this β -turn is unique only in human and bovine IFN- γ . Furthermore, no conserved substitution was constructed to support the argument that the reduction of activities was due to the disruption of the β -turn. For instance, the replacement Asn²⁵ with Asp which would preserve the β -turn without altering neighboring structure. Instead, a double mutant was constructed (Fish et al., 1988). This second variant had a substitution of Asn⁷⁸ for Lys in addition to Gln²⁵. Although this mutation showed a further 20-fold and 10-fold reduction in antiviral and antiproliferative activities, respectively, the results from studies of this latter variant were not informative since the activities of Lys⁷⁸ alone was not shown. It is not clear whether this second mutation had an additive effect on the Asn²⁵ variant. In addition, all of these mutants were extracted by the denaturation/renaturation procedures described by Arakawa et al (1985). As already discussed in Chapter 3, the process of denaturation and renaturation could introduce inactive molecules into the preparation due to the formation of off-pathway intermediates (Mitraki and King, 1989) and may result in the underestimation of the specific activities of a biological active molecule. It is not clear whether the reduction in activities in the two mutants reported by Fish et al. (1988) resulted from the denaturation/renaturation procedures.

Table 4.2 Antiviral Specific Activities of recombinant HuIFN- γ Analogues (Fish et al., 1988).

Clones	Antiviral Specific Activity (units/mg)
IFN- γ	$1.5-2.2 \times 10^7$
N25Q	3.0×10^6
N25Q/N75K	7.0×10^5

4.11.2 Role of the conserved β -turn in the stability of the HuIFN- γ

It is interesting to note that production of the D41A variant polypeptide was also reduced by at least 50-fold, suggesting that the β -turn conformation may be important for the structural stability of the molecule. Since amino acid substitutions that affect stability do not generally occur at residues that are solvent-exposed or mobile in the folded protein (Alber et al., 1987; Alber et al., 1988), the fact that variant D41A significantly lost both its stability and activity further suggested that the conserved β -turn plays a key role in the structure/function of HuIFN- γ molecule. This is corroborated by the evidence that variant D41N had a similar stability as the parental molecule. Loss of stability due to disruption of a β -turn structure has also been demonstrated in other proteins such as the hemoglobins (Fermi and Perutz, 1981). β -turns are usually found on protein surfaces and have been considered to play an important role in protein folding pathways (Lewis et al., 1971; Zimmerman and Scheraga, 1977). The formation of β -turns is believed to significantly restrict the conformational space available to the folding polypeptide chain. By facilitating the positioning of distant parts of the polypeptide into close proximity, β -turns are important in promoting medium- and long-range intramolecular interactions which result in further stabilization of structure (Wright et al., 1988). Other evidence showing the importance of β -turns in protein folding came from the study of temperature-sensitive mutants of the bacteriophage P22 tailspike protein (Yu and King, 1988). It was found that single-site mutations that affect protein folding and assembly at restrictive temperature constituted regions which had a high probability of forming β -turns or areas of high solvent accessibility. Therefore,

different stages in the protein folding pathways may be guided by a few specific amino acid residues.

Therefore, the significance of the two mutations described in the present study was of several fold. First, the variant polypeptide D41A failed to accumulate to the same level as the parental molecule in the *E. coli* cell. Therefore, the substitution of Asp⁴¹ for Ala was deleterious to the structural stability of the HuIFN- γ , demonstrating the importance of the conserved β -turn in maintaining the overall structural stability. Second, the substitution of Asp⁴¹ for Asn resulted in the elimination of a local negative charge as well as the reduction in the local hydrophilicity without altering the secondary structure. Since this variant was found to retain its activity, the negative charge of Asp⁴¹ was not directly involved in the formation of the functional site(s). The full activity of the HuIFN- γ molecule thus requires an intact β -turn. Third, the specific antiviral activity of variant D41A was reduced by at least 10-fold which could not be accounted for by the lack of protein accumulation alone. Therefore, the conserved β -turn structure may represent part of a functional epitope. Fourth, a neutralizing monoclonal antibody MIF3061, which was immunoblot positive against the parental molecule, failed to detect or bound very weakly to the variant polypeptide D41A, whereas both polypeptides could be detected by a polyclonal antiserum against rHuIFN- γ , suggesting that an epitope that was normally recognized by this neutralizing monoclonal antibody was no longer present in the D41A variant. While increasing the amount of the other variant proteins loaded onto the gel resulted in an increase in sensitivity, corresponding increase was not observed with the D41A polypeptide. Thus, the weak reactivity is unlikely due to the low affinity of MIF3061. Furthermore, increasing the amount of proteins

also reduced the signal-to-noise ratio in all cases.

CHAPTER 5 SINGLE-SITE VARIANTS WITH ENHANCED ACTIVITIES

5.1 Introduction

Interferon gamma molecules from different animal species are known to exhibit strict species specificity (Pestka et al., 1987). However, these molecules are able to induce the same spectrum of biological responses in the respective animal species (Pestka et al., 1987), suggesting that they elicit the various biological responses through similar signal transduction pathways via their respective receptors. Therefore, it is possible that the receptor binding domain has diverged during evolution while certain essential functional domains on these IFN- γ molecules have been conserved in various vertebrate species. Criteria used to locate these domains have been described in Sections 4.2, 4.3, and 4.4. Based on these criteria, a conserved β -turn between residues 39 to 43 was identified and two single-site variants were constructed in order to specifically study this conserved secondary structure. The construction and the biological activities of these two variants have been described in Chapter 4. Since the disruption of this β -turn did not completely abolish the activities, other functional domains must exist in order to confer the full biological activity of the HuIFN- γ . These domains are likely to be located not only in regions of the molecule with high degree of sequence homology but also regions that have homologous secondary structures. Comparison of the primary sequences of IFN- γ from four vertebrate species revealed a highly conserved region adjacent to the β -turn between residues 43 to 57. Out of these 15 residues, 10 were 100% conserved while the other 5 were conserved substitutions (Figure

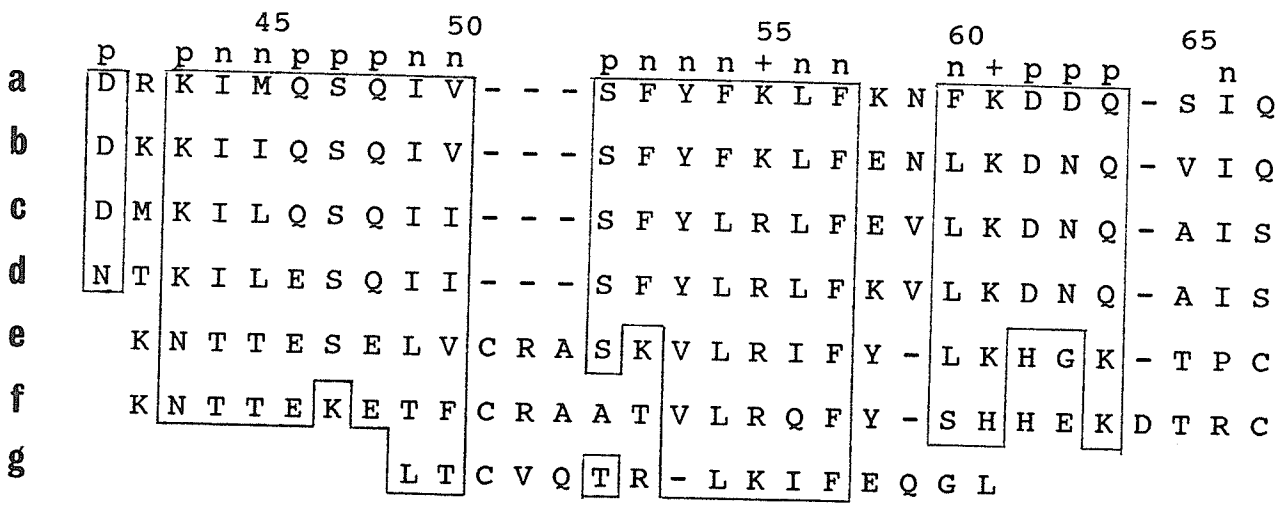


Figure 5.1 Comparison of primary sequence of IFNs- γ , ILs-4, and GM-CSF. a, human IFN- γ ; b, bovine IFN- γ ; c, murine IFN- γ ; d, rat IFN- γ ; e, murine IL-4; f, human IL-4; g, murine GM-CSF. The single amino acid code is used. Conserved residues are boxed. Gaps are introduced in order to allow alignment of sequences. The number of residues are according to the sequence of the IFNs- γ . Trends of a particular type of residue occurring in the same position are denoted as: p, polar residues; n, non-polar residues; +, basic residues.

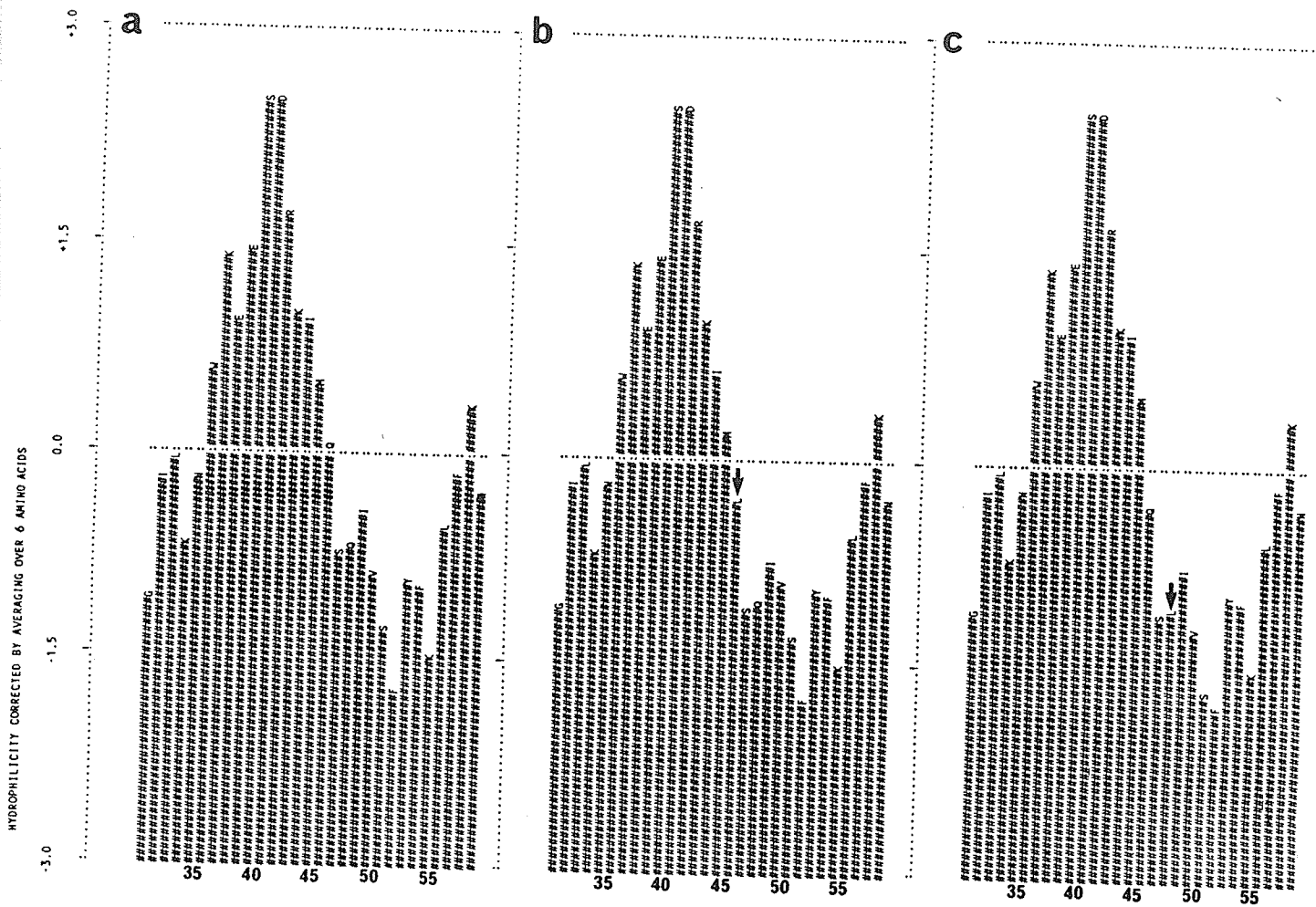


Figure 5.2 Comparison of hydrophilicity profiles of unmodified rHuIFN- γ and single-site variants. The algorithm of Hopp and Woods (1981) is used: (a) unmodified HuIFN- γ , (b) Q46L, and (c) Q48L. Arrows indicate the amino acid substitutions at positions 46 and 48. Hydrophilicity values are calculated by averaging over 6 amino acids. Only values of residues 31 to 59 are shown.

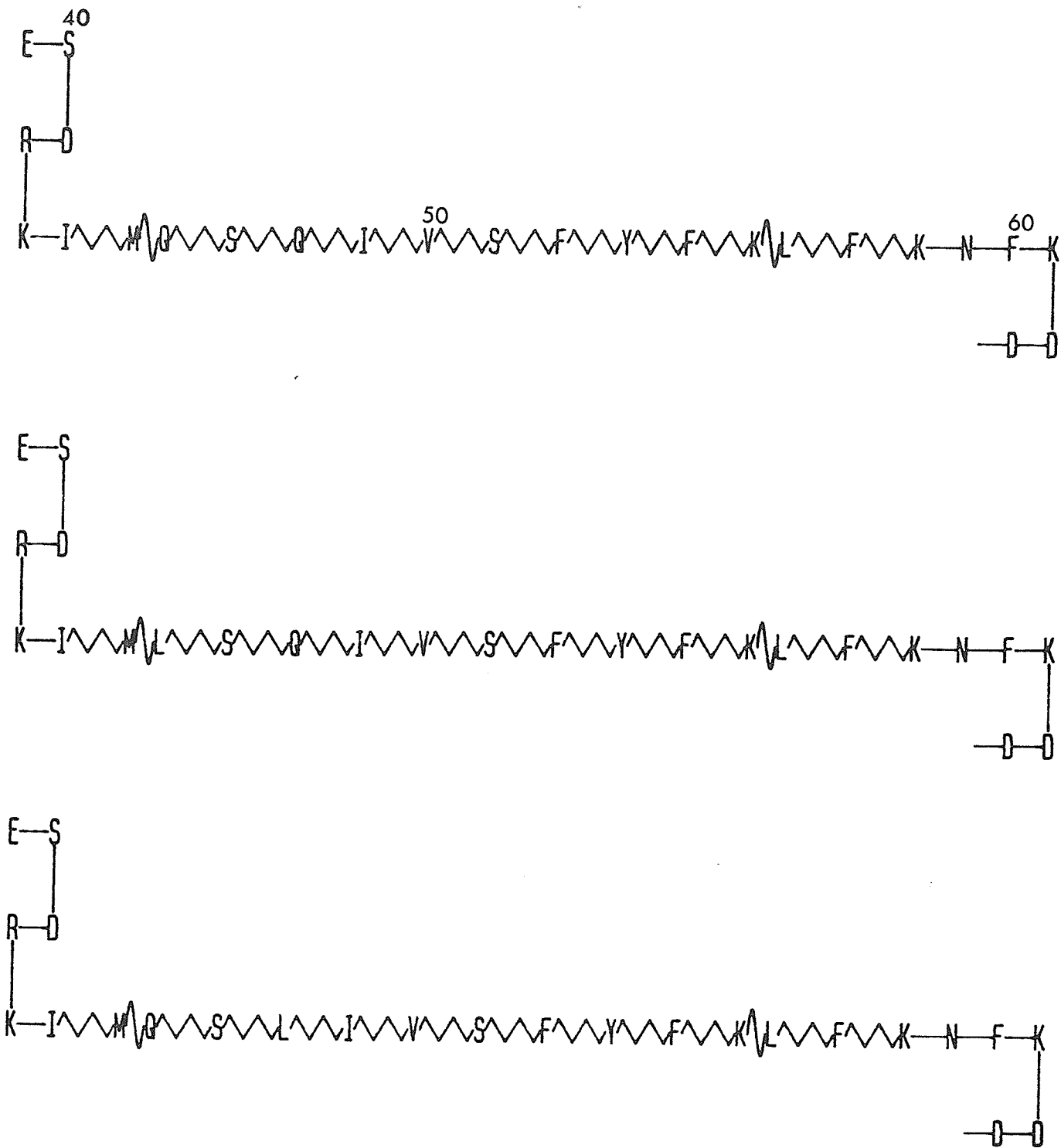


Figure 5.3 Comparison of secondary structures of (a) unmodified HuIFN- γ , single-site variants (b) Q46L and (c) Q48L. The algorithm based on that of Chou and Fasman (1978) was developed by Glorioso and Black (1984). Secondary structures are represented graphically as: $\wedge\wedge\wedge$ (α -helix), $\wedge\wedge\wedge$ (β -sheet), \lrcorner (β -turn), and --- (random coil).

5.1). In addition to the sequence conservation, these residues were also predicted to form a β -sheet (Figure 5.3). Analysis of the hydrophilicity of these residues indicated that they were located in a hydrophobic region (Figure 5.2). Although these residues are not likely to be surface-exposed, they could potentially have stabilization effects on the neighboring structures such as the conserved β -turn. It has been shown that the internal core of a protein is consisted primarily of hydrophobic side chains and that the shielding of these residues from solvent (hydrophobic effect) is thought to be one of the main sources of stabilization energy (Kauzmann, 1959; Tanford, 1980; Alber, 1989). Alternatively, the hydrophobic region may be located on the exterior of the protein as part of a β -barrel which, in conjunction with the conserved β -turn, may provide the molecular surface for protein-protein interactions. Although this hydrophobic region may or may not be part of the internal core, it nonetheless provides a starting point in an attempt to locate the structural and/or functional domains of the HuIFN- γ molecule.

5.2 Construction of Single-site Variants Q46L and Q48L*

5.2.1 Choice of amino acid for substitutions

Out of the 15 conserved residues between positions 43 to 57, two residues, namely Gln⁴⁶ and Gln⁴⁸, were chosen as the targets for amino acid substitution. These two residues were chosen based on the fact that since they were adjacent to the conserved β -turn and were highly polar, they may have significant influence on the exposed β -turn with respect to its role in

* The design and construction of variants Q46L and Q48L were done in collaboration with Dr. Gilbert Jay.

intermolecular interactions. It was rationalized that by replacing Gln⁴⁶ and Gln⁴⁸ with a hydrophobic residue, it may be possible to reduce the exposed surface for intermolecular interactions. The hydrophobic residue leucine (Leu) seemed to be the best choice for the replacement of Gln⁴⁶ and Gln⁴⁸ because of their similar side-chain volumes (Gln = 128.14 Å, Leu = 113.17 Å) compared to other hydrophobic residues (Creighton, 1984). Secondary structure prediction based on the algorithm of Chou and Fasman (1978) also showed that the substitutions of these two residues by Leu did not cause any structural changes (Figure 5.3). These considerations were aimed at minimizing steric hindrance effects that might have occurred if an aromatic, bulky residue such as tryptophan were used to replace the two glutamines.

5.2.2 Materials and Methods

The two single-site variants were constructed by site-directed mutagenesis utilizing the M13 system as described in Section 2.15. The subcloning of the rHuIFN- γ coding sequence into the M13mp8 vector has been described in Section 2.16. Two synthetic oligonucleotides were separately used as primers to generate the two independent mutations by replacing Gln⁴⁶ and Gln⁴⁸ with Leu:

Q46L

```
          43      44      45      46      47      48      49
          Lys   Ile   Met   Gln   Ser   Gln   Ile
5' . . . . A A A A T A A T G C A G A G C C A A A T T . . . . 3'
          3' T A T T A C G A C T C G G T T T 5'
                   C T G
                   Leu
```

Q48L

```
          45      46      47      48      49      50      51
          Met   Gln   Ser   Gln   Ile   Val   Ser
5' . . . . A T G C A G A G C C A A A T T G T C T C C . . . . 3'
          3' G T C T C G G A T T A A C A G 5'
                   C T A
                   Leu
```

The primer-extended DNA were used to transform *E. coli* strain JM103 which had been made competent according to the procedures described in Section 2.18.5. The transformants were screened by colony hybridization as described in Section 2.18.6. Single-stranded DNA were isolated from the positive transformants (Section 2.7.1) and the nucleotide substitutions were confirmed by DNA sequencing using the dideoxynucleotide chain termination method (Section 2.7). A 15-base primer that spanned residues 62 and 66 (3' CTACTAGTCTCGTAG 5') was used for sequencing.

Intracellular RF's were prepared (Section 2.3) from the transformants that had been confirmed by DNA sequencing. The mutated regions were then retrieved by cutting these RF's with the restriction enzymes KpnI and AsuII and were subcloned into the expression vector P₁₄R₃ similarly linearized by the same enzymes. A schematic of the subcloning procedure is shown in

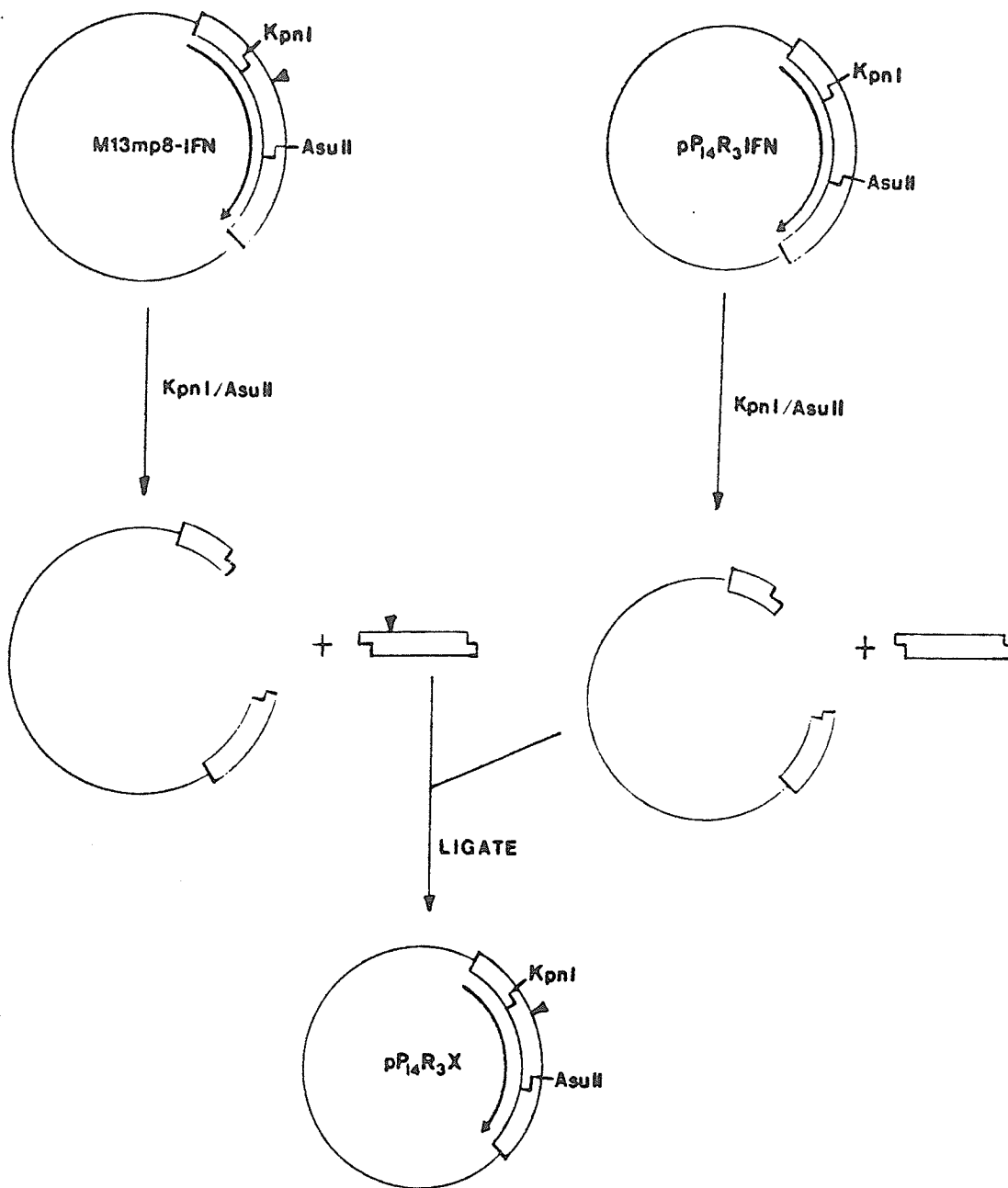


Figure 5.4 Subcloning of single-site variant into expression vector. The M13mp8 vector that carried the IFN- γ was excised with the restriction endonucleases KpnI and AsuII. This 200-bp fragment which contained the single-nucleotide substitution (denoted as ▼) was used to replace the unmodified IFN- γ coding sequence carried by the expression vector. The resulting plasmid, pP₁₄R₃X (X: D41A, D41N, Q46L, or Q48L) contained an modified IFN- γ sequence that has a single-nucleotide substitution.

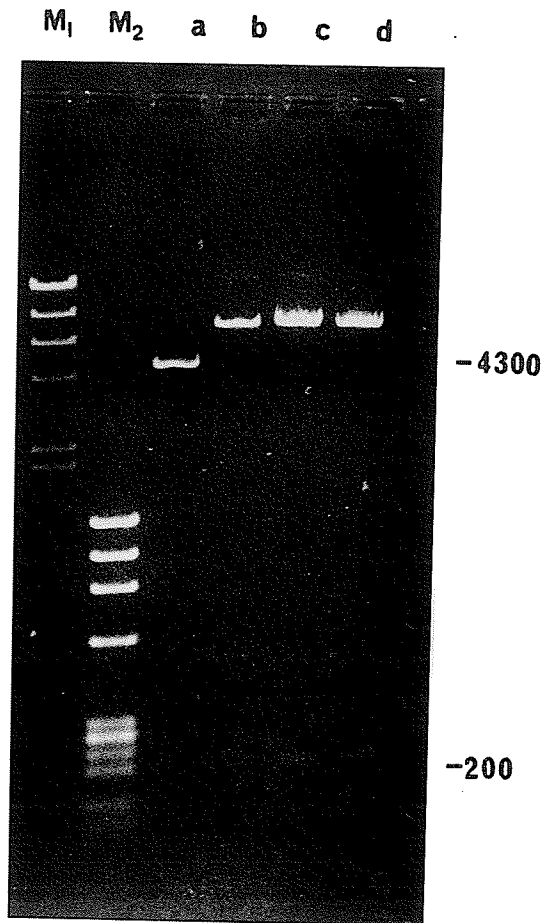


Figure 5.5 Restriction endonuclease digestion of the unmodified IFN- γ sequence and the modified sequence. Plasmids which contained (a) the unmodified IFN- γ sequence, P₁₄R₃IFN, the modified sequences (b) Q46L, (c) Q48L, and (d) D41A were digested with KpnI and AsuII, and electrophoresed on a 1% agarose gel. The 4300-bp band from (a) and the 200-bp band from (b),(c), and (d) were separately purified. Molecular size standards were (M₁) λ DNA digested with HindIII and (M₂) ϕ X174 DNA digested with HaeIII.

Figure 5.4. Both the expression vector and the restriction enzyme fragments were separated on a 1% agarose gel (Figure 5.5). The corresponding bands were excised from the gel and the DNA were eluted by electrophoresis into dialysis tubing. Eluted DNA were extracted with phenol/chloroform/isoamyl alcohol (49:49:2) 3 times, chloroform/isoamyl alcohol (48:2) 3 times, and precipitated with a final concentration of 0.3 M sodium acetate (pH 7.0) and two volumes of absolute ethanol at -20°C. The purified KpnI/AsuII fragment were separately ligated with the linearized vector in a ratio of 1:5 (fragment:vector). Ligation conditions were as described in Section 2.16. Each ligation reaction was separately used to transform the competent *E. coli* strain LE392. The transformants from each ligation were randomly selected and grown in 2 ml T broth. The bacterial cells were collected by centrifugation in a microcentrifuge, lysed in SDS sample buffer, and separated on a SDS polyacrylamide gel (Section 5.3.2).

5.3 Results

5.3.1 Identification of single-site variants Q46L and Q48L

The mutants generated were screened by two rounds of colony hybridization using the two respective mutant oligonucleotides. The conditions for colony hybridization has been described in Section 2.18.6. Two final positive clones were selected and ssDNA were prepared for sequencing. The desired nucleotide changes were confirmed as shown in Figure 5.6. Since the sequencing primer was complementary to the coding strand, the two nucleotide changes shown

were those on the antisense strand:

Clone	Nucleotide Change	Codon Change
Q46L	T → A	CAG → CTG sense strand GTC → GAC antisense strand
Q48L	T → A	CAA → CTA sense strand GTT → GAT antisense strand

5.3.2 Characterization of Q46L and Q48L polypeptides

Single-site variant polypeptides Q46L and Q48L were expressed in the *E. coli* strain LE392. Cell lysates from 8 independent clones of each variant were prepared and separated by SDS-PAGE. Total proteins were detected by silver stain. As shown in Figure 5.7, all of these clones produced a polypeptide which had an M_r of 17 kD (Q46L, lanes c to j; Q48L, lanes k to r) comparable to the parental molecule (lane b). This polypeptide band was absent in cell lysates from cells which contained only the expression vector without the IFN- γ insert (lane a). The immunological identities of these polypeptides were confirmed by Western immunoblot analysis (Figure 5.8). The 17 kD band of all the clones (Q46L, lanes c to j; Q48L, lanes k to r) could be detected by the rabbit polyclonal antiserum against purified rHuIFN- γ and their molecular sizes were indistinguishable from that of the parental molecule (lane b). This protein band was absent in cell lysates prepared from cells which contained the expression vector without the IFN- γ insert (lane a).

To determine whether the two variant polypeptides could be detected by a neutralizing monoclonal antibody MIF3061 and a non-neutralizing monoclonal antibody MIF3037, a Western

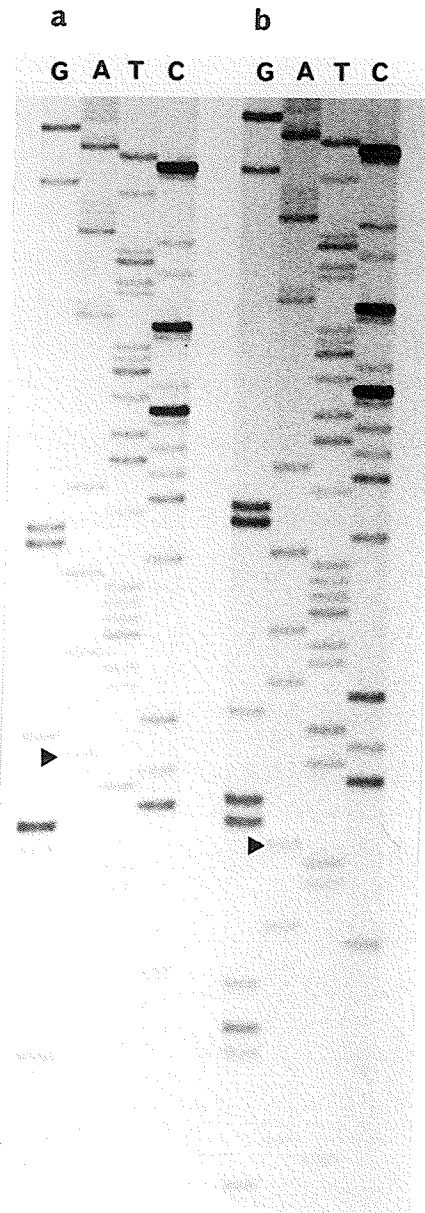


Figure 5.6 DNA sequence analysis of single-site variants Q46L and Q48L. The single-nucleotide substitutions of variants (a) Q46L and (b) Q48L were analysed by dideoxynucleotide chain termination sequencing. The substitutions in each set of sequence ladders (T → A) are denoted by an arrowhead.

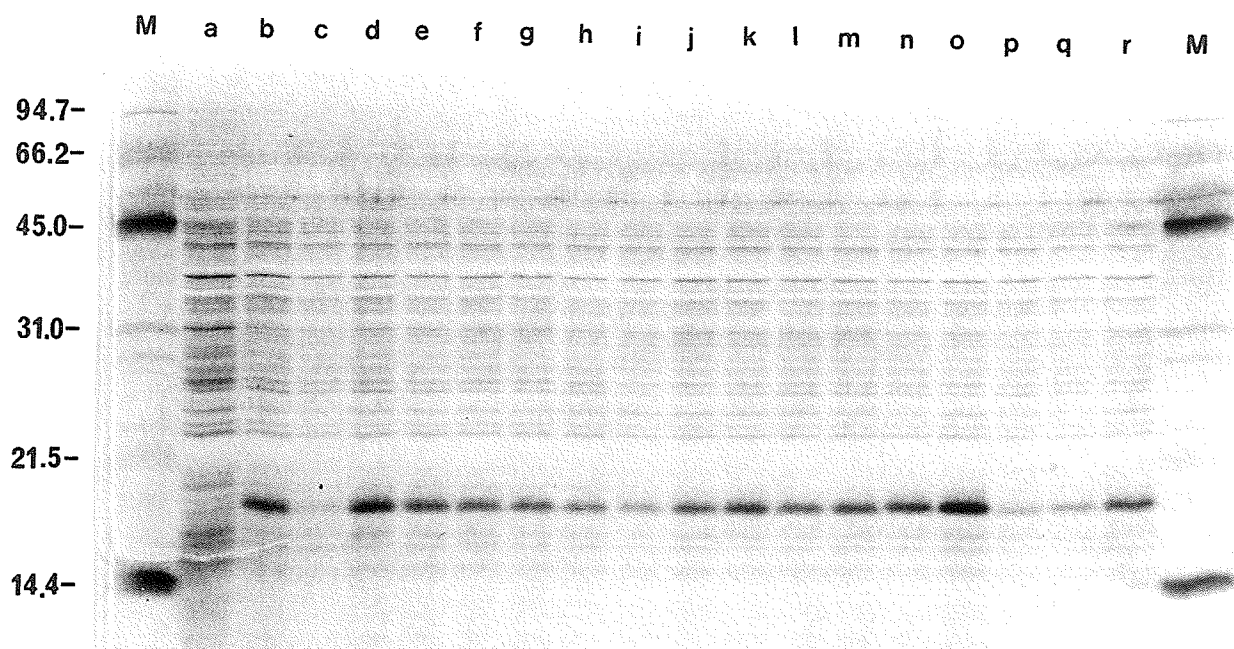


Figure 5.7 SDS-PAGE analysis of single-site variants Q46L and Q48L. Independent clones of bacterial cells harboring the expression vector without the IFN insert (lane a), with the unmodified IFN- γ insert (lane b), with the Q46L substitution (lanes c to j), with the Q48L substitution (lanes k to r) were lysed and electrophoresed on a 15% SDS polyacrylamide gel. Total proteins were stained with silver. The molecular mass standards (lanes M) were in kilodaltons.

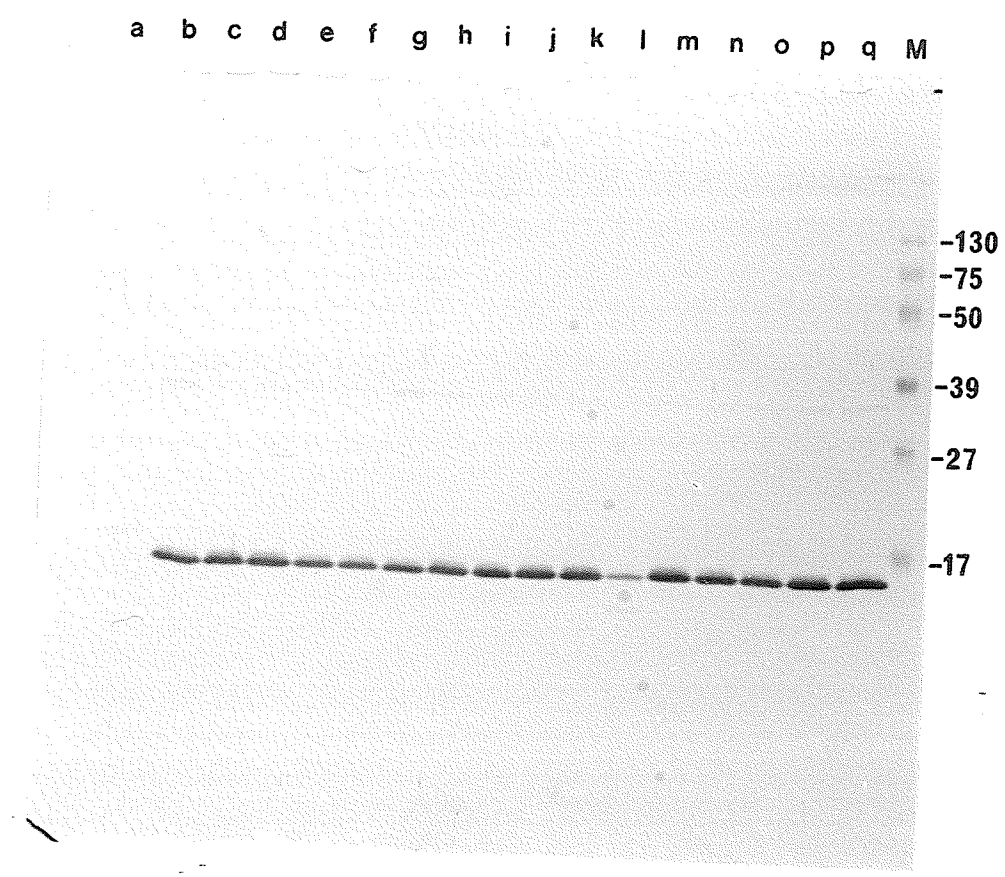


Figure 5.8 Western immunoblot analysis of single-site variants Q46L and Q48L. Cell lysates were prepared and electrophoresed as described in Figure 5.7 except that 1/10 of the samples were loaded. Total proteins were blotted electrophoretically on to nitrocellulose membrane and probed with a rabbit polyclonal serum against purified IFN- γ . Lysates of cells containing the expression vector without the IFN insert (lane a), with the unmodified IFN- γ insert (lane b), with the Q46L substitution (lanes c to j), with the Q48L substitution (lanes k to q) are shown. The molecular mass standards (lane M) were pre-stained markers in kilodalton.

blot analysis was performed by transferring the total proteins of the two clones onto nitrocellulose and then detected with the two monoclonal antibodies. As shown in Figure 4.11, both variant polypeptides (Panels A and B, lanes b and c) as well as the parental molecule (Panel A and B, lane a) could be recognized by these antibodies. As a control, all of these polypeptides could be detected by the rabbit polyclonal serum against purified rHuIFN- γ . Therefore, the neutralizing epitope(s) recognized by MIF3061 was retained in both variants Q46L and Q48L.

5.3.3 Determination of the biological activities of single-site variants Q46L and Q48L

Variants Q46L and Q48L were extracted and purified essentially the same as that for the parental molecule (Section 2.1). The purified fractions were pooled and were assayed for their antiviral activities (Section 2.9). The specific antiviral activity of both Q46L and Q48L were 5.8×10^8 units/mg or approximately 30-fold higher than that of the parental molecule (Table 5.1). Taking into account the variation of the antiviral assay which is within 2-fold, the 30-fold difference in specific activity between the parental molecule and the single-site variants is statistically significant.

Table 5.1 Antiviral Specific Activities of Single-Site Variants Q46L and Q48L

Clones	Antiviral Specific Activity (units/mg) ^a
P ₁₄ R ₃ IFN	2.30 x 10 ⁷
Q46L	5.80 x 10 ⁸
Q48L	5.80 x 10 ⁸

a, The variation of each of the values was within 2-fold.

5.4 Discussion

5.4.1 Enhanced activities of single-site variants

Two variants, Q46L and Q48L, were constructed to study two highly conserved Gln residues and their influence on the conserved β -turn between residues 39 to 43. The two hydrophilic residues selected, Gln⁴⁶ and Gln⁴⁸, were predicted to form part of a β -sheet adjacent to the conserved β -turn that appeared to play a key role in the biological activities of HuIFN- γ . These two amino acids were individually substituted with Leu. Analysis of the predicted secondary structures indicated that these two substitutions induced minimal structural change (Figures 5.3b and 5.3c).

Independent clones that produced the two variant polypeptides were successfully expressed in *E. coli* and the whole cell lysates were analysed by SDS-PAGE. It was observed that both of these polypeptides had comparable stability and yield as the parental molecule (Figure 5.7). Western blot analysis also showed that their immunological identities were indistinguishable from the parental molecule (Figure 5.8). When the biological activities of these variants were tested, both were found to have 30-fold increase in antiviral specific activity (Table 5.1). Such a significant increase in the antiviral specific activities was unexpected since it was originally rationalized that the replacement of Gln⁴⁶ and Gln⁴⁸ with the hydrophobic Leu would reduce the exposed surface and would therefore reduce the efficiency of intermolecular interactions. It is possible that the two substitutions have instead strengthened the interactions between the HuIFN-

γ molecule and the effector molecule. Alternatively, the substitutions may have improved the receptor binding affinity of the two variants. However, the latter possibility was deemed unlikely because Gln⁴⁶ and Gln⁴⁸ were located in a highly conserved region, whereas IFN- γ 's are known to be species-specific. It is conceivable that IFN- γ 's from different animal species have become evolutionarily diverged such that their receptor binding domains have become species specific. The evidence of such divergence may then be reflected in the divergence of their primary sequences. On the other hand, since IFNs- γ induce the same spectrum of biological activities in the respective animal species, the effector binding domains may have been conserved. Therefore, the results of these two substitution have reinforced the notion that these evolutionarily conserved residues play an important structural/functional role.

5.4.2 Sequence and Structural Characteristics of the Region between Residues 44 to 57

Comparison of the homologous amino acids revealed a highly conserved region that spanned residues 43 and 57, adjacent to the conserved β -turn. Out of these 15 residues, 10 were 100% conserved while the other 5 were conserved substitutions (Figure 5.1). Analysis of the hydrophilicity of these residues showed that they were located in a hydrophobic region (Figure 5.2). Such a conserved hydrophobic segment is reminiscent of the internal core of proteins first recognized in the hemoglobins (Perutz et al., 1965). In addition, based on the algorithm of Chou and Fasman (1978), this region was predicted to form a β -sheet (Figure 5.3). Since β -sheets are generally found in the interior of proteins (Creighton, 1984), this further supports the notion that

these residues are buried. There is evidence to suggest that the internal core of a protein play a key role in determining the protein's structure. First, the core is composed primarily of hydrophobic side chains and the shielding of these residues from solvent (hydrophobic effect) is thought to be one of the main sources of stabilization energy (Kauzmann, 1959; Tanford, 1980; Alber, 1989). Second, crystallographic studies have shown that core side-chains are very closely packed, occupying almost all available interior space with no steric overlaps (Chothia, 1975; Richards, 1977). A particular protein structure might therefore require a specific set of core residues which can maintain these properties (Lim and Sauer, 1989). Indeed, it has been suggested by Ponder and Richards (1987) and experimentally demonstrated by Lim and Sauer (1989) that by focusing on core residues, it may be possible to predict the structure that a protein sequence will adopt.

It is interesting to note that the hydrophobic region was conserved not only among IFN- γ from different vertebrate species, but also conserved with murine interleukin-4 (MuIL-4) and, to a lesser extent, granulocyte-macrophage colony-stimulating factor (GM-CSF) (Noma et al., 1986; Lee et al., 1986). A more detailed comparison of these residues revealed striking homology between a stretch of homologous amino acids spanning positions 49 and 57 (Figure 5.1). With the exception of a highly conserved basic residue (Lys⁵⁵ or Arg⁵⁵), these residues were almost exclusively hydrophobic. In addition to the sequence homology, these residues were predicted to have structural homology such that they all form β -sheets (Figure 5.9, a to d). Interestingly, the substitutions introduced in variants Q46L and Q48L have not only extended the hydrophobic region but also added two hydrophobic residues, Leu⁴⁶ and Leu⁴⁸, onto the same side of the β -

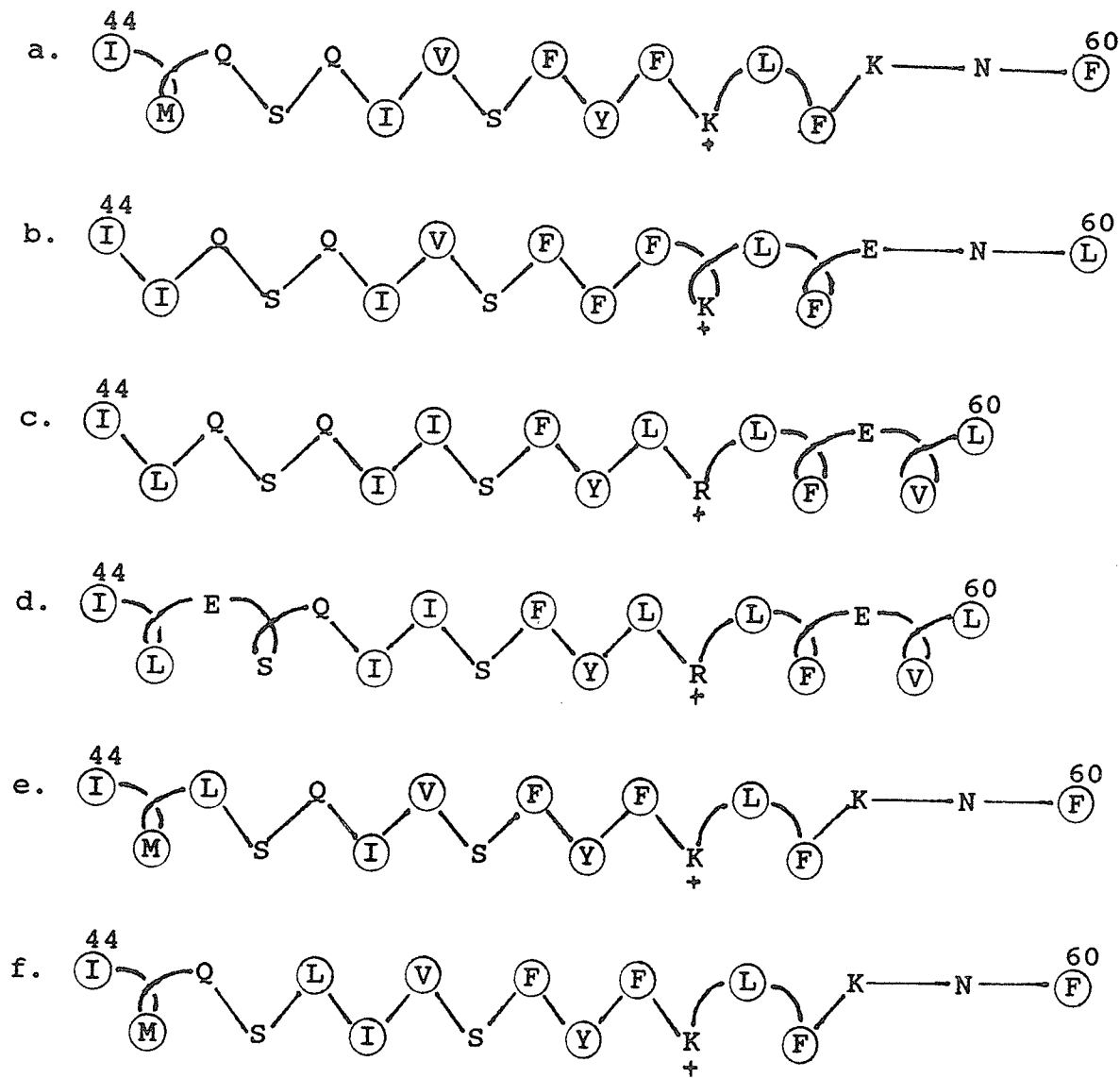


Figure 5.9 Comparison of secondary structures of IFNs- γ from various vertebrate species. The algorithm of Chou and Fasman (1978) was used to calculate secondary structures between residues 44 and 60 of (a) human IFN- γ , (b) bovine IFN- γ , (c) murine IFN- γ , (d) rat IFN- γ , (e) variant Q46L, and (f) variant Q48L. Single amino acid codes are used. Secondary structures are represented as: $\wedge\wedge\wedge$ (β -sheet); $\wedge\wedge\wedge$ (α -helix); — (random coil). The hydrophobic residues are circled. The conserved basic residues at position 55 is denoted +.

sheet (Figure 5.9, e and f). Therefore, the increase in specific activities observed in variants Q46L and Q48L may have been strengthened the hydrophobic interactions involved in protein-protein interactions. This is in contrast to the original assumption that the two substitutions may reduce the hydrophilic surface for intermolecular interactions.

The importance of the hydrophobic region between residues 43 to 57 was also demonstrated by Fish et al. (1988) who showed that the substitution of Met⁴⁵ by Thr reduced both the specific antiviral and antiproliferative activities by approximately 3 log. It was concluded that the reduced activities of this variant may have been due to its inability to form appropriately folded structures and that the hydrophobic core was essential for biological activity. Analysis of the local secondary structure of this variant supported this notion. The substitution of Met⁴⁵ by Thr disrupted the β -sheet that normally spanned residues 44 and 49 (Figure 5.10, a and b). Instead, a β -turn was formed between residues 45 to 48 such that Thr⁴⁵, Gln⁴⁶, Ser⁴⁷, and Gln⁴⁸ have become surface exposed. This further supports the notion that these conserved residues are involved in hydrophobic interactions. However, since this variant was extracted using the denaturation/renaturation procedures (Arakawa et al., 1985), the possibility that the variant was not re-folded properly cannot be excluded. Since neither variant Q46L and Q48L showed significant increases in stability, as judged by their normal accumulation in the *E. coli* cell compared to the parental molecule, the increase in activities observed in these two variants are likely to be due to better interactions with the effector molecule(s). However, the possibility of increased stability cannot be ruled out without thermodynamic data.

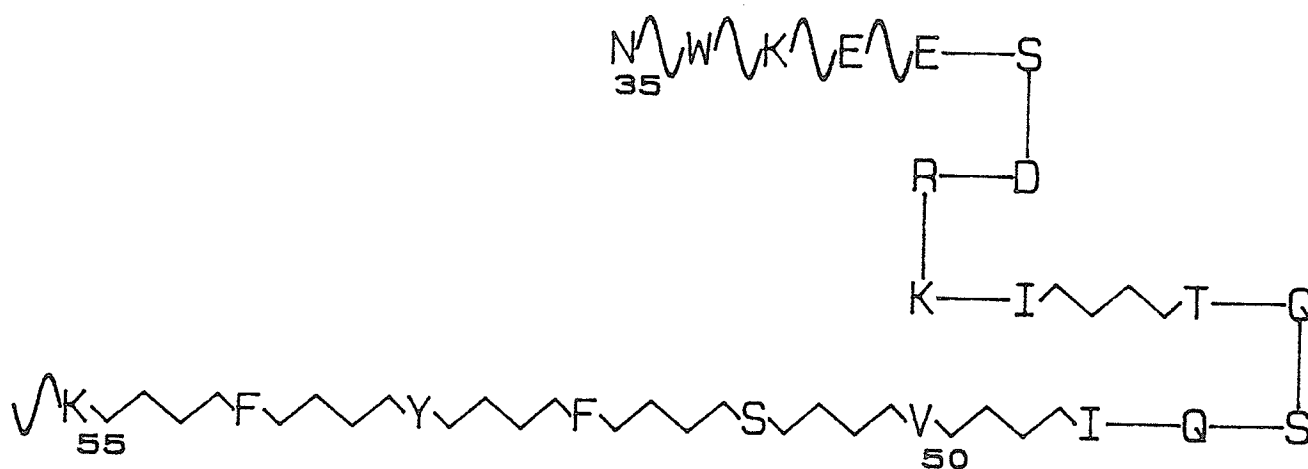
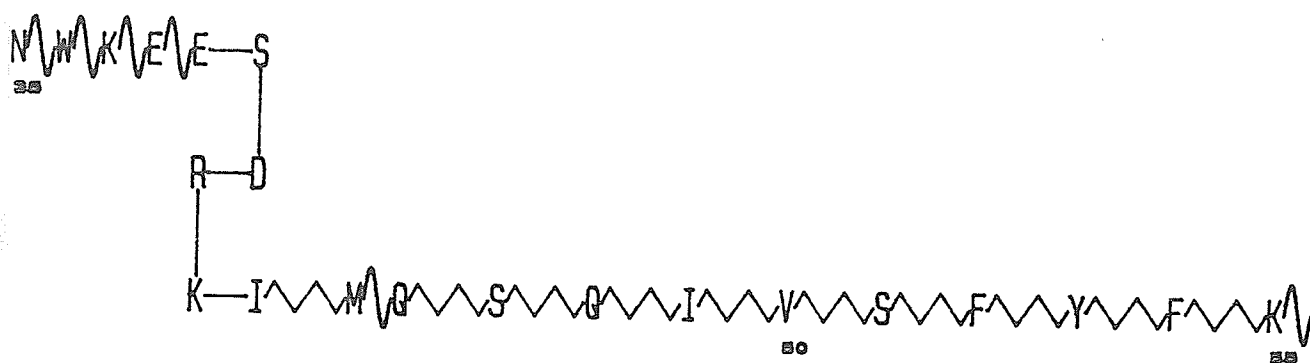


Figure 5.10 Comparison of secondary structures of HuIFN- γ and variant M45T. Secondary structures were predicted based on the algorithm of Chou and Fasman (1978). Only structures between residues 35 and 55 of (a) HuIFN- γ and (b) a variant with the replacement of Met⁴⁵ with Thr as described by Fish et al., (1988) are shown. The substitution of Met for Thr results in the partial disruption of the β -sheet structure between Thr⁴⁵ and Ile⁴⁹ which is converted instead to a β -turn. The single amino acid code is used. Secondary structures are represented graphically as: $\wedge\wedge\wedge$ (α -helix), $\wedge\wedge\wedge$ (β -sheet), \lrcorner (β -turn), and --- (random coil).

It is tempting to speculate that IFN- γ binds to the effector molecule(s) by hydrophobic interactions through the conserved β -sheet. The IFN- γ -effector heterodimer would either activate other intracellular signalling molecules or directly activate IFN-responsive genes. Such a mechanism may explain the multiple, yet specific, actions of IFNs- γ in different cell types. The permutations of the types of heterodimer generated would vastly increase the repertoire of regulatory molecules with distinct binding and functional specificities. These molecules would in turn differentially regulate IFN- γ responsive genes.

5.4.3 Heterodimer Formation between HuIFN- γ and Effector molecules – A Hypothesis

The concept of the formation of a heterodimer between two heterologous proteins which may be directly involved in gene regulation was first described in the 'leucine-zipper' model (Landschulz et al., 1988). It was observed that a stretch of 35 amino acids containing 4 to 5 leucine residues separated by 6 amino acids occurred in various DNA-binding proteins. Landshultz et al. (1988) thus proposed that the leucines were arranged on one side of an amphipathic α -helix and that the leucines on two such helices would interdigitate leading to dimerization. Such an arrangement of leucine residues thus resembled the "teeth of a zipper", thus the term 'leucine-zipper'. Furthermore, since the 'leucine-zipper' was immediately preceded by a region rich in positively charged amino acids (Figure 5.11), these authors further suggested that dimerization would arrange the basic regions of the two subunits in a configuration that allowed specific interactions with a DNA-recognition sequence. Although the 'leucine-zipper' was

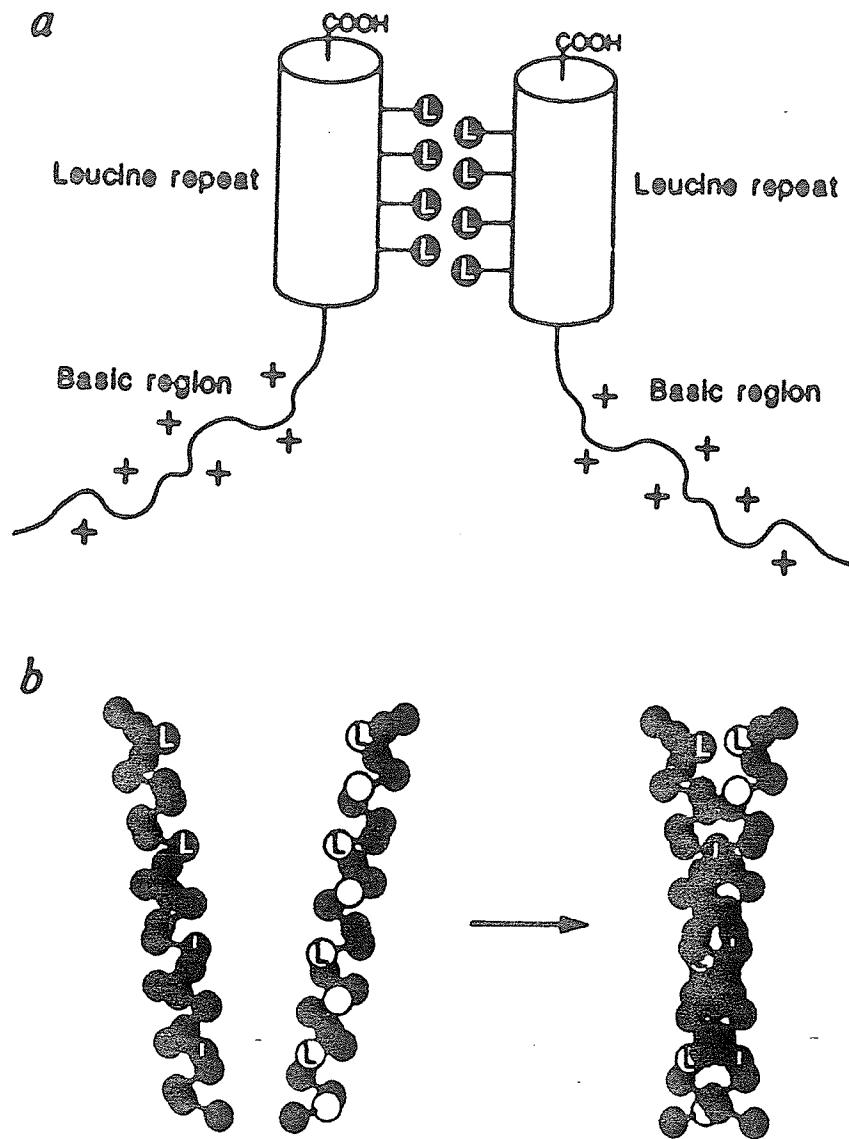


Figure 5.11 The leucine zipper model. a, The leucine repeat region, which could form an eight-turn α -helix (depicted as a cylinder), serves as a dimerization interface. The basic regions serve as the bifurcating arms that fit into the major groove of the DNA helix. b, The coiled-coil model for the structure of leucine zipper. The α -helical regions of two monomers are illustrated on the left; each residue is represented by a sphere centred on the C^α atom. In the dimer (right), these helices form a coiled coil, a structure in which two parallel helices are intertwined. The leucine residues are indicated by black or white balls marked with an L, and the alternating hydrophobic repeat (present in many, but not all, leucine zippers) by plain black or white balls. The two interspersed heptad repeats, known as 4-3 repeat, form the interaction surface between the two helices (Adapted from Abel and Maniatis, 1989).

first hypothesized to be directly involved in DNA-binding (Landschulz et al., 1988), studies with model peptides showed that the periodic leucine repeats were able to form stable dimer of α -helices with a parallel orientation forming a coiled-coil (O'Shea et al., 1989) (Figure 5.11). This latter study also suggested that the leucine sidechains might not interdigitate, but rather interact with the alternate, non-leucine, hydrophobic residues in the opposing helix. More recently, it was shown that the leucine repeats of two nuclear oncogene products could associate both in vivo and in vitro to form heterodimers (Kouzarides and Ziff, 1988; Sassone-Corsi et al., 1988; Turner and Tijian, 1989). The significance of the heterodimer formation was shown by Turner and Tijian (1989) in that the two oncogene products c-fos and c-jun were able to dimerize in vitro to bind a specific DNA sequence, whereas either protein alone had very little affinity for the same DNA sequence. It has since been hypothesized that the activation of certain target genes by these nuclear oncogene products required the direct binding of the heterodimer (Turner and Tijian, 1989). A more elaborate model was recently proposed which consisted of a dimerization interface involving the 'leucine-zipper' and a DNA contact surface termed the 'basic region' immediately NH₂-terminal to the leucine-zipper (Vinson et al., 1989). This bipartite structural motif was hypothesized to form a 'scissor-grip' with the 'basic-regions' from each polypeptide chain of the dimer function as two bifurcating arms clamping onto the major groove of the DNA, while the C-terminal 'leucine-zipper' interacted to maintain the dimer conformation (Vinson et al., 1989) (Figure 5.11).

It is important, however, to emphasize that the hydrophobic interactions hypothesized in the IFN--effector model differ from the 'leucine-zipper' model, which requires canonical leucine

repeats in an α -helix, in that hydrophobic interactions between two β -sheets are required to form the proposed IFN- γ /effector heterodimer. The latter configuration would allow not only hydrogen bond formation between internal polar residues on the two strands of β -sheets, as has been shown in bovine pancreatic trypsin inhibitor (BPTI) (Creighton, 1984 p.230-232), but would also accommodate the hydrophobic interactions of the nonpolar residues between the two strands. Such interactions would also provide stability to the folded conformation (Creighton, 1984). Alternatively, the hydrophobic β -sheets may be involved in homodimer formation of the IFN- γ molecules. It has been shown that upon gel filtration natural HuIFN- γ yields an apparent weight of 40-60 kD, suggesting that under physiological conditions IFN- γ may exist as a dimer (Yip et al., 1981; Rinderknecht et al., 1984). However, target analysis of both natural and recombinant HuIFN- γ suggested that the functional unit was a tetramer (Pestka et al., 1983). Nevertheless, no structural studies has been carried out to identify the dimerization mechanism. It is not known if the dimer or the tetramer persists intracellularly.

Despite the differences in the sequence requirement, certain similarities in the structural features of the two models are apparent. In the case of the 'leucine-zipper' model, two α -helices would interact through the leucine repeats. This is in accordance with our current understanding of the structural features of helices in that due to the amphipathic nature of α -helices, they tend to have polar side chains on one side and nonpolar side chains on the other along the axis of the helix. As a result, there is often a repeating pattern in the segment of the primary sequence. The repeats of leucines in the 'leucine-zipper' model occur every 3 to 4 residues which represent approximately one half of a helical turn, with the repeating leucines buried into the helix groove

of the apposing helix (Figure 5.11). However, this type of structural feature and interactions among proteins are not unique. Indeed, β -sheets near the protein surface tend to have polar side chains on the exterior side and the nonpolar side chains on the interior side. The result of which is also an alternating pattern of polar and nonpolar residues in the sequence (Creighton, 1984 p. 243). On the other hand, when helices or β -sheets are buried, all the side chains tend to be nonpolar; the central strands of a sheet tend to have the most hydrophobic residues. In the β -sheet conformation, the polypeptide chain is nearly fully extended and individual strands aggregate side by side forming hydrogen bonds between the carbonyl and NH groups of the backbone (Figure 5.12). In addition to the hydrogen bonds, the dipoles of the peptide bonds alternate along the chain, providing favorable conditions for interstrand interaction. Although extended strands of planar β -sheets do not appear to be helical, they may be considered a rather special helix with 2.0 residues per turn (Creighton, 1984 p.173), because most β -sheets are not planar but have a twisted conformation. Therefore, the hypothesis of IFN- γ /effector heterodimer formation resembles the leucine-zipper model in terms of (i) the utilization of hydrophobic interactions between two protein molecules to form heterodimer, (ii) the formation of heterodimer increase the permutation of regulatory potentials based on a limited repertoire of ligand/effector molecules, and (iii) the heterodimer formation can be mediated through interactions between either two helices or β -sheets.

The above model is testable. First, double mutation of Q46L and Q48L could be constructed. If the above hypothesis is correct, further increase in activities would be expected due to more efficient dimer formation. Second, the substitution of the Gln⁴⁶ and Gln⁴⁸ with Glu

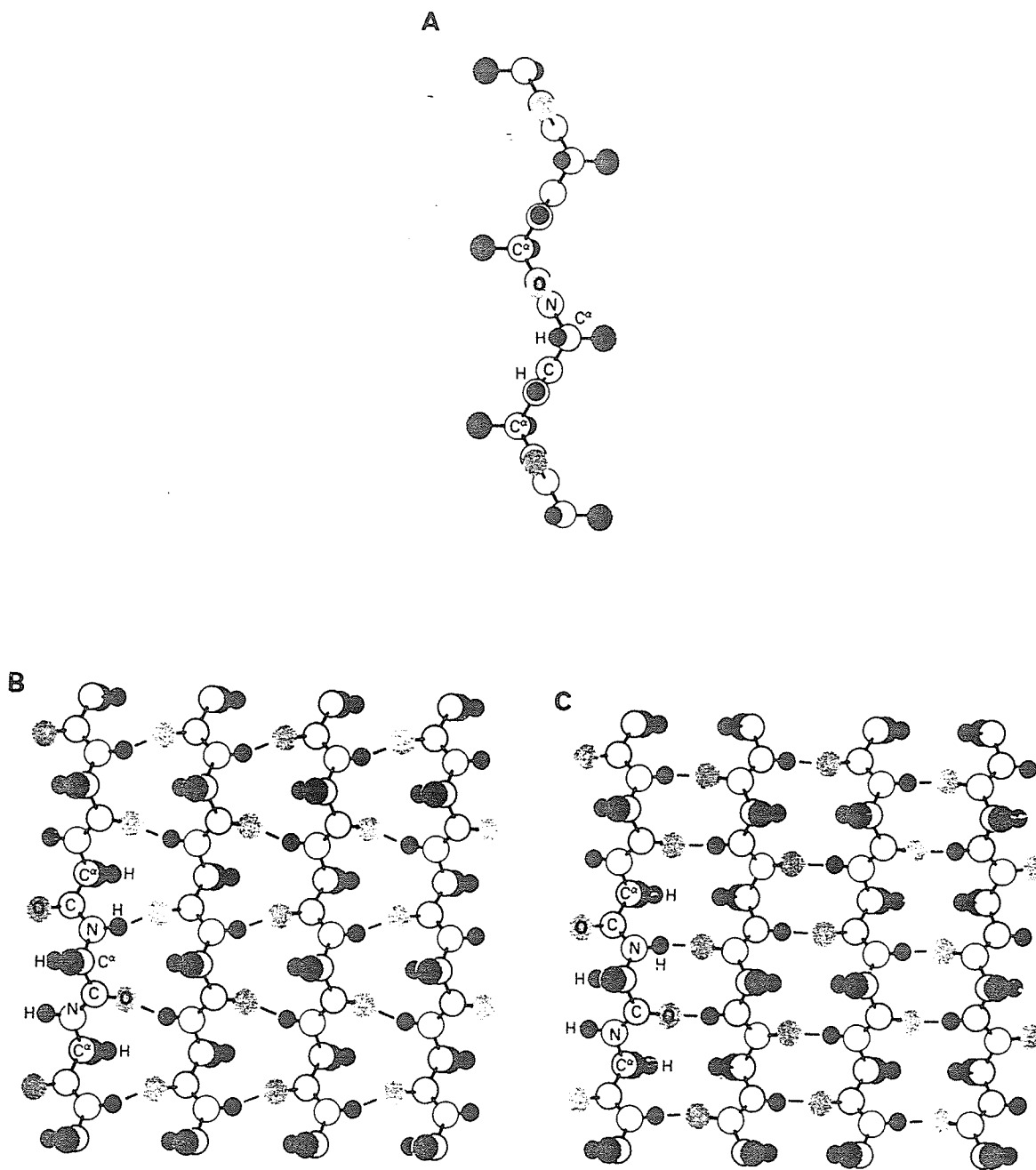


Figure 5.12 Drawings of a single, straight β -strand (A) and its incorporation into flat parallel (B) and antiparallel (C) pleated sheets. (Adapted from Creighton, 1984).

would not be expected to result in any increase in activities since Glu would not enhance hydrophobic interactions. Third, residue Phe⁵⁴ could be substituted by leucine. Since this leucine is conserved in murine and rat IFN- γ (Pestka et al., 1986) as well as IL-4 and GM-CSF (Noma et al., 1986), the substitution of Phe by Leu may increase the efficiency of binding of HuIFN- γ to the effector molecule(s) and may result in increase of activities. Fourth, detection of dimer formation could be achieved by immunoprecipitation of radiolabelled-IFN- γ treated cell extracts with polyclonal antiserum against HuIFN- γ and then resolved on native polyacrylamide gel. Fifth, substitutions of the conserved hydrophobic residues with hydrophilic residues would be expected to disrupt the proposed hydrophobic interactions. The replacement of Phe⁵², Tyr⁵³, Phe⁵⁴ are likely to yield useful information since these three bulky hydrophobic residues must have formed a very rigid hydrophobic pocket. Another candidate is Lys⁵⁵. This residue is the only hydrophilic and basic residue in a stretch of 9 hydrophobic residues. This residue may be involved in hydrogen bonding with the ligand-effector interactions since there is no hydrogen acceptor in the adjacent residues.

5.4.4 Interactions with Interleukin-4

The availability of cloned IL-4 has revealed some very interesting observations on interactions between this lymphokine and IFN- γ . It is known that certain actions of IL-4 can be inhibited by IFN- γ or vice versa in a dose-dependent manner (Coffman and Carty, 1986; Snapper and Paul, 1987). IL-4 has been shown to induce the IgE receptors and the induction could be

inhibited by exogenous IFN- γ . Evidence for the action of IL-4 at the transcriptional level has come from studies done by Berton et al. (1989) who showed that transcription of IgG₁ could be activated by IL-4 and that the activation could be inhibited by IFN- γ in a dose-dependent manner at the transcriptional level. A possible explanation for the antagonistic effects would be that both IL-4 and IFN- γ bind the same effector molecule(s) through their respective hydrophobic residues. The formation of an IL-4-effector heterodimer might enable its binding to the regulatory region of IgG₁ and its subsequent activation. The introduction of IFN- γ would therefore create a situation whereby the two cytokines would be competing for the limiting effector. This idea is testable.

In addition to its homology with IL-4, IFN- γ 's were also found to have a region of homology with mouse GM-CSF (Noma et al., 1986) (Figure 5.1). Interestingly, recent studies have shown that the mouse GM-CSF contained four critical regions (Shanafelt and Kastelein, 1989), one of which was the region of homology between MuIL-4 and IFNs- γ . This region also contained four conserved hydrophobic residues and one basic residue present in IFN- γ and IL-4 (LTCVQTRLKIFEQGL). Therefore, it is possible that IFNs- γ , ILs-4, and GM-CSF utilize a common set of effector molecule(s). The conserved hydrophobic β -sheets observed in cytokine molecules such as that in IFN- γ may represent part of a common site for cytokine-effector interactions. The differential actions of these cytokines could therefore be mediated through differential regulations of cellular genes resulting from the various permutations of ligand-effector heterodimers. If this hypothesis turns out to be correct, this will once again demonstrate one of the frugal strategies utilized by nature to achieve differential regulations based on a limited repertoire of genes or regulatory molecules. In eukaryotes, such strategies are the basis of

generating the diversity of antibodies (reviewed by Alt et al., 1987) and T-cell receptors (Marrack, P. and Kappler, 1987; recently reviewed by Lewis and Gellert, 1989). More recently, a number of other proteins have also been shown to form heterodimers such as the retinoic acid receptor - thyroid hormone receptor (Glass et al., 1989), the oncogene myc family, the transcriptional activator MyoD that converts fibroblast to myoblasts, and the *Drosophila* daughterless(da), achaet-scute, and the twist gene family (Murre et al., 1989). Therefore, the formation of dimers or multimers through the interactions between either hydrophobic residues or leucine repeats may well represent a highly conserved mechanism for cellular regulation.

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Appendix 1

Appendix 1 - Reagents for Sequencing Gels

Solution	Composition/Preparation				
40% acrylamide stock	380 g/l acrylamide and 20 g/l bisacrylamide Dissolve in H ₂ O with stirring. Bring to final volume of 1 l. Filter through Whatmann 3MM paper. Store at 4°C in dark. Stable indefinitely.				
10% (w/v) ammonium persulfate (APS)	1 g APS in 9 ml H ₂ O Store at 4°C. Stable 1-2 weeks.				
10X Tris-borate/EDTA buffer (TBE)	121.1 g Tris-base, 55 g boric acid, 7.4 g Na ₂ EDTA Dissolve in H ₂ O. Adjust to final volume of 1 l. pH should be 8.3. Store at room temperature.				
Instagel (500 ml)	<u>% Poly- acrylamide</u>	<u>40% Acrylamide Stock (19:1; ml)</u>	<u>Urea (g)</u>	<u>10X TBE (ml)</u>	<u>H₂O (ml)</u>
	6%	75	250	50	~75
	8%	100	250	50	~150
	12%	150	250	50	~100
	20%	250	250	50	0

Note: The approximate volume of water is given to aid in dissolving the urea. Mix vigorously with a magnetic stirrer; avoid heating. Check final volume for accuracy. De-gas the solution. Store at room temperature in a dark bottle. Stable approximately one month.

Appendix 2

Protocols for the Amerham Oligonucleotide-Directed Mutagenesis System

We recommend that you read the trouble-shooting guide (page 43) before beginning any experimental work

Note: It is advisable to practise the filtration step before using it in a mutagenesis reaction (see page 31).

Spare filters are provided.

The following protocols and recipes are described below:

- A. Growth and maintenance of M13 host cells
- B. Preparation of single-stranded template DNA
- C. 5'-phosphorylation of the oligonucleotide for mutagenesis
- D. Oligonucleotide-directed mutagenesis
- E. Transformation
- F. Media, buffers and reagents

The region of DNA to be mutagenized is first cloned into an M13 vector. Amersham supply M13 cloning kits and M13 vector kits for this purpose (page 48). Alternatively, a plasmid based system with a single-stranded phage origin of replication⁽¹²⁾ can be used, as long as there is a suitable *Nci*I site in the vector. The identity of the recombinant, and the orientation of its insert, should be determined by sequencing or by restriction analysis. The recombinant phage is then plaque-purified ready for the preparation of single-stranded template. The protocol for making single-stranded template has been designed by Eckstein and his co-workers⁽¹⁰⁾ to avoid contamination by cellular DNA and RNA fragments, which could give random priming and consequently generate unwanted mutations. This protocol has been tested and found to work well, without the need for RNase treatment or further purification steps. Following the design (page 39), synthesis and phosphorylation (page 27) of the mutant oligonucleotide, the Amersham *in vitro* mutagenesis system can be used to generate the desired base change in the target DNA sequence. Finally, the resultant homoduplex mutant RF DNA molecules are transformed into a suitable host cell to give mutant progeny. Due to the high level of efficiency of the Amersham system, no major screening program is usually required (see page 40). Simply sequencing a small number of putative mutants is all that is required to isolate the desired mutant with the sequence verified.

A. Growth and maintenance of host cells

A suitable *E. coli* host cell is required for the preparation of the single-stranded M13 DNA template, and also for transformation of the mutant progeny RF DNA to give mutant plaques. Any suitable M13 host cell can be used with these protocols, but the *E. coli* host strain TG1 (page 38) has been found to be particularly useful. It is a good all-purpose M13 host, and also gives a higher transformation efficiency with phosphorothioate DNA than many other similar strains. Its use in the transformation step, with mutant RF molecules generated *in vitro* by the mutagenesis protocol, is strongly recommended.

M13 is a male specific bacteriophage. It infects its host via the F-pilus or sex pilus, to which it adsorbs before penetrating the cell. Therefore, any host cell for M13 growth must carry the F-pilus, which is encoded by the F-plasmid. To ensure that the host cells do not lose the F-plasmid, most M13 hosts (for example, TG1, JM101, JM103) carry a deletion which makes them deficient in proline synthesis. The F-plasmid in these hosts carries the missing gene, and it is possible to select cells carrying the F-plasmid by growth on glucose/minimal medium, which requires the cell to be capable of proline synthesis.

The *E. coli* host strain TG1, which is supplied in 15% glycerol, should be stored at -20°C .

To retrieve cells:

1. Remove tube from freezer. Immediately scrape a clump of frozen cells off the top of the frozen mass using a sterile inoculating loop.
2. Return tube to freezer (it is important to minimize warming and thawing).
3. Touch the frozen clump of cells onto a glucose/minimal medium plate, where it will immediately melt.
4. Spread drop to isolate single cells.
5. Invert the plate and incubate overnight at 37°C to produce single colonies.

These plates can be used for up to one month to provide inocula for cultures. Long term stocks can be kept in 15% glycerol at -70°C, or as 2xTY agar stabs.

B. Preparation of single-stranded template DNA

The region of DNA to be mutagenized should be cloned into an M13 vector. The recombinant plaques should then be picked and recombinant phage stocks obtained.

Plaque purification of phage stocks

Note: It is best to store M13 phage as phage stocks (supernatants from a 1ml phage preparation grown from a single plaque). Store at 4°C, or long term at -20°C.

Do not store phage as plaques on agar plates.

1. To prepare a host cell lawn, pick a single TG1 colony from a glucose/minimal medium plate. Grow overnight in 10ml 2xTY medium, shaken at 37°C. Add one drop to 20ml of fresh medium, and shake at 37°C for 3-4 hours.
2. Dilute phage stock (see above) 1 in 10⁸ in 2xTY medium.
Alternatively, pick a single plaque into 1ml of 2xTY medium and dilute 1 in 10⁴ in 2xTY medium.
3. Prepare the following mix for each plate:
40µl IPTG 100mM (page 37)
40µl X-gal, 2% in dimethylformamide
200µl log. phase *E.coli* host cells from step 1.
4. To each IPTG/X-gal/cells mix, add 10µl of diluted phage.
5. To each tube add 4ml molten H top agar, kept at 45°C. Mix by rolling, and pour immediately onto a prewarmed (37°C) H plate. Leave at ambient temperature to set. Invert the plates. Incubate at 37°C overnight.

Single-stranded template DNA preparation

We recommend that you follow this protocol exactly (see page 25).

1. Pick a single TG1 colony from a glucose/minimal medium plate.
Grow overnight in 10ml 2xTY medium, shaken at 37°C.
Add one drop to 20ml of fresh medium, and shake at 37°C for 3 hours.
2. Inoculate 1ml 2xTY medium in a 10ml sterile culture tube with 100µl of 3 hour culture from step 1.
3. Inoculate the 1ml culture with a recombinant plaque, using a sterile wooden cocktail stick. (Toothpicks should not be used, as these are sometimes treated with anti-microbial agents).
4. Incubate for 4 hours with shaking, at 37°C. Transfer to a microcentrifuge tube.
5. Centrifuge for 5 minutes at ambient temperature. Pour supernatant into a fresh tube.
Store overnight at 4°C. Set up an overnight culture of TG1.
6. Inoculate 100ml 2xTY medium with 1ml of overnight TG1 culture, and shake at 37°C to an OD₅₅₀ of 0.3.
7. Add the phage supernatant from step 5 to the culture from step 6.
8. Incubate for 4 hours with shaking, at 37°C. Transfer to centrifuge tubes.
9. Centrifuge at 5000xg for 30 minutes at 4°C.
10. Transfer supernatant to a clean centrifuge tube. Take care not to carry over any

- cells — leave some of the supernatant behind if necessary. Discard pellet.
11. Add 0.2 volumes of 20% PEG in 2.5M NaCl (page 37) to the supernatant. Mix well and then leave to stand for 1 hour at 4°C.
 12. Centrifuge at 5000 xg for 20 minutes. Discard the supernatant.
 13. Centrifuge at 5000 xg for 5 minutes, and remove all remaining PEG/NaCl with a drawn-out Pasteur pipette.
 14. Resuspend the viral pellet in 500µl TE buffer (page 36) and transfer to a microcentrifuge tube (1.5ml).
 15. Centrifuge for 5 minutes in a microcentrifuge to remove any remaining cells. Transfer the supernatant to a fresh microcentrifuge tube.
 16. Add 200µl PEG/NaCl to the microcentrifuge tube. Mix well, then leave to stand for 15 minutes at ambient temperature. Alternatively, leave overnight at 4°C.
 17. Centrifuge for 5 minutes. Discard supernatant.
 18. Centrifuge for 2 minutes. Carefully remove all remaining traces of PEG with a drawn out Pasteur pipette. Wipe off any traces of PEG on the mouth of the tube with a tissue.
 19. Resuspend the viral pellet in 500µl TE buffer.
 20. Add 200µl of phenol saturated with TE buffer (page 37). Vortex 15-20 seconds.
 21. Stand tube for 15 minutes at ambient temperature. Vortex 15 seconds.
 22. Centrifuge for 3 minutes.
 23. Transfer upper (aqueous) layer to a fresh microcentrifuge tube.
 24. Repeat steps 20—23.
 25. Add 500µl diethyl ether. Mix well. Discard top layer.
 26. Repeat step 25 three times.
 27. Extract the aqueous phase with 500µl of chloroform (twice). Discard the lower organic layer.
 28. Divide the sample into two microcentrifuge tubes.
 29. Add 625µl ethanol and 25µl sodium acetate (3M) to each. Mix.
 30. Place each tube in a dry ice and ethanol bath for 20 minutes.
 31. Spin the tubes in a microcentrifuge for 15 minutes.
 32. Wash each pellet with 1ml cold (–20°C) ethanol. Pour off ethanol and leave to drain until dry.
 33. Redissolve each pellet in 50µl TE buffer.
 34. Measure the OD at 260nm by adding 1µl to 1ml of TE buffer in a 1ml quartz cuvette. 1 OD/ml corresponds to ~ 40µg/ml. Dilute DNA preparation to 1µg/µl. This procedure should yield 100-200µg DNA.
 35. Store at 4°C.

C. 5'-Phosphorylation of oligonucleotides

Note: The control mutant oligonucleotide supplied with the Amersham oligonucleotide-directed mutagenesis kit is already 5'-phosphorylated.

The following protocol is recommended for 5'-phosphorylation of the oligonucleotide for mutagenesis.

1. To a microcentrifuge tube add the following:

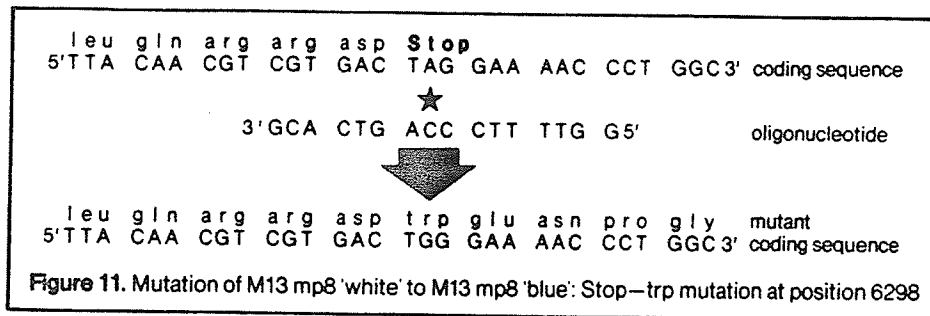
Oligonucleotide stock solution (5 OD units/ml; ~ 20nmol/ml for a 16 mer)	2.5µl
10x Kinase buffer (see page 36)	3µl
Water	25µl

- Add 2 units T4 polynucleotide kinase
Mix by gently pipetting up and down.
2. Incubate at 37°C for 15 minutes.
 3. Heat at 70°C for 10 minutes.
 4. Use immediately or store at –20°C.

D. Oligonucleotide-directed mutagenesis reaction

A mutagenesis control reaction has been included so that the Amersham oligonucleotide-directed *in vitro* mutagenesis system can be easily tested. It consists of a single-stranded DNA control template and a mutant oligonucleotide. The control template has been constructed from M13 mp8 by *in vitro* mutagenesis. A point mutation, which introduces a stop codon within the coding region of the β -galactosidase peptide, has been introduced at position 6298. This results in a colourless plaque phenotype (see figure 1 on page 6).

The control oligonucleotide changes the base at position 6298 back to the wild-type genotype (an A—G change), which restores β -galactosidase activity. Thus the reversion mutation can be observed by a white to blue phenotypic change (figure 11).



Using this oligonucleotide and control template with the Amersham *in vitro* mutagenesis system, we have observed mutation efficiencies of >95%.

The 'white' control template (1 μ g/ μ l) and the 'blue' control oligonucleotide (phosphorylated and at 0.5 OD units/ml) are supplied ready for direct use in the protocols.

All reactions should be carried out in 1.5ml microcentrifuge tubes. Suitable disposable gloves should be worn.

This protocol includes instructions for samples to be taken at critical stages, to analyse the progress of the mutagenesis reaction (page 44). The samples can be stored at -20°C and analysed, together with suitable controls, after completion of the reaction.

1. Annealing mutant oligonucleotide to single-stranded DNA template

Single-stranded DNA template (1 μ g/ μ l)	10 μ l
Phosphorylated mutant oligonucleotide (~ 1.6pmol/ μ l)	5 μ l
Buffer 1	7 μ l
Water	12 μ l
Total	34 μ l

Place capped tube in a 70°C water bath for 3 minutes, then place in a 37°C water bath for 30 minutes. Place on ice.

2. Synthesis and ligation of mutant DNA strand

To the annealing reaction add:

MgCl ₂ solution	10µl
Nucleotide mix 1	38µl
Water	12µl
Klenow fragment	12 units
T4 DNA ligase	12 units

Mix by pipetting up and down. Place tube in a 16°C water bath and leave overnight (~ 15 hours).

Remove 1µl of the reaction mix for analysis on an agarose gel (store at -20°C).

Sample 1 (see page 45).

3. Removal of single-stranded (non-mutant) DNA using nitrocellulose filters

Note: It is advisable to practise the filtration step before using it in a mutagenesis reaction (see page 31). Spare filters are provided.

To the reaction from step 2 add:

Water	170µl
5M NaCl	30µl

Mix well by repeated pipetting.

Pass the sample through the nitrocellulose filters, following carefully the protocol described on page 31. **Do not autoclave these filters. Autoclaved filters do not work in this application.**

To the filtrate add:

3M sodium acetate	28µl
Cold ethanol (-20°C)	700µl

Place in a dry ice and ethanol bath for 20 minutes. Centrifuge for 15 minutes. Wash the pellet with 1ml cold (-20°C) ethanol. Carefully remove the ethanol, taking care not to lose the pellet. Leave to drain until the pellet is dry. Resuspend the pellet in 50µl of buffer 2.

4. Nicking of the non-mutant strand of DNA using *NciI*

Remove 10µl of the filtered sample and place in a microcentrifuge tube. (The remaining 40µl can be stored at -20°C).

Add 65µl of buffer 3 and 5 units of *NciI* to the 10µl sample.

Place in a 37°C water bath and incubate for 90 minutes.

Remove 10µl of the reaction mix for analysis on an agarose gel (store at -20°C).

Sample 2 (see page 45).

5. Digestion of non-mutant strand using exonuclease III.

Note: To avoid stability problems, exonuclease III is supplied in concentrated form. Therefore, immediately before use take 1µl and dilute with a 1 in 10 dilution of buffer 4 to give a working stock of 25 units/µl. Discard excess working stock after use.

To the reaction mix from step 4 add:

500mM NaCl	12µl
Buffer 4	10µl
Exonuclease III working stock (50 units)	2µl

Place in a 37°C water bath and incubate for 30 minutes. (At 37°C, 50 units of exonuclease III will digest ~ 3,000 bases in 30 minutes. In mp8 the closest *NciI* site is 556bp away from the multiple cloning site).

Place in a 70°C water bath for 15 minutes to inactivate the enzymes. Spin briefly to collect condensate.

Remove 15 μ l of the reaction mix for analysis on an agarose gel (store at -20°C).
Sample 3 (see page 45).

6. Repolymerization and ligation of the gapped DNA

To the reaction mix from step 5 add:

Nucleotide mix 2	13 μ l
MgCl ₂ solution	5 μ l
DNA polymerase I	3 units
T4 DNA ligase	2 units

Place in a 16°C water bath and incubate for 3 hours.

Remove 15 μ l of the reaction mix for analysis on an agarose gel (store at -20°C).

Sample 4 (see page 45).

7. Transformation of competent host cells with the DNA

For transformation control see page 34.

Transform 300 μ l of competent TG1 cells (page 33) with 20 μ l of the reaction mix from step 6 (in duplicate) as described on page 34.

Store the rest of the reaction mix from step 6 at -20°C .

Invert the plates once set, and incubate overnight at 37°C .

8. Analysis of mutant progeny—see page 40.

It is advisable to check three or four clones by dideoxy sequencing, and to proceed to hybridization screening (page 41) if no mutant is found.

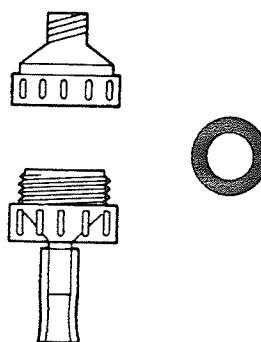
In all mutations the full sequence of the insert should be checked to ensure that no unwanted changes have occurred.

Removal of single-stranded DNA using nitrocellulose filters

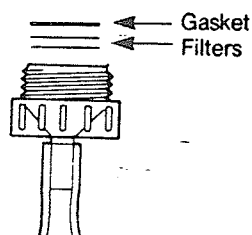
See troubleshooting guide (page 43) for further information on filtration step.

Note: This step should be practised with water to ensure success in the mutagenesis experiment. Spare filters are provided for this purpose. Also, sufficient single-stranded and double-stranded DNA is provided to permit a number of trials of the filtration step coupled with gel analysis, if desired. Add 1 µg of the RF DNA and 1 µg of the ssDNA control template to 170 µl of water, and add 30 µl of 5M NaCl. After filtration, add gel loading dye mix, and analyze by gel electrophoresis, with RF DNA and ssDNA size markers (see figure 13, page 45).

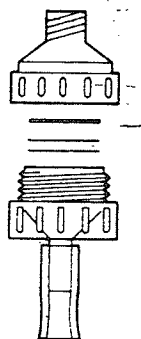
1. Unscrew the filter unit and remove the rubber gasket.



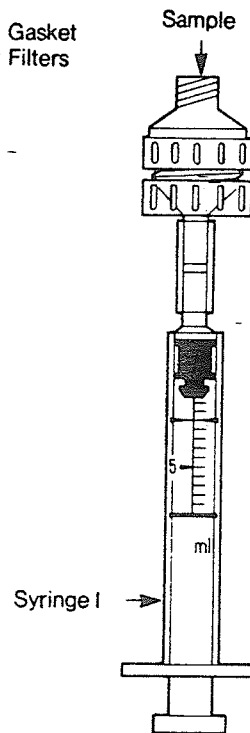
2. Using forceps, carefully place two nitrocellulose discs in the centre of the bottom half of the unit. Do not autoclave filters.



3. Carefully place the rubber gasket on top of the filters.



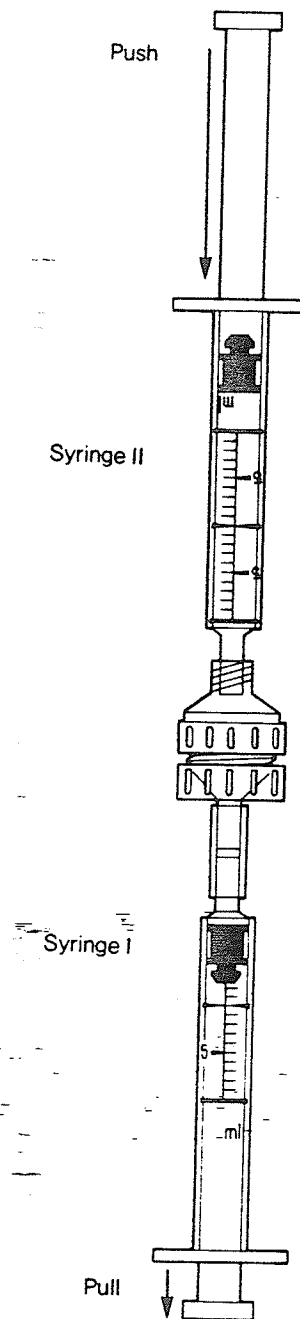
4. Place the top half of the filter unit on top of the gasket and carefully assemble the unit. It is important for the gasket to form a good seal. Any leakage will reduce the yield and efficiency of the procedure.



5. Insert a 2ml syringe (l) into the tubing connected to the bottom of the unit. Pipette half the sample (150 µl) into the top of the filter unit.

6. Insert a second 2ml syringe (II), with the plunger pulled up, into the luer fitting at the top of the filter unit.

7. **Slowly** pass the sample through the nitrocellulose filters by pushing down the plunger of syringe II and pulling the plunger of syringe I. The sample is collected in syringe I.



8. Transfer the filtered sample from syringe I to a microcentrifuge tube. Transfer any droplets remaining in the syringe by using a pipette. The sample does not always pass through the filters during the first filtration stage. The filtration of the second half of the sample (step 9) and the final wash stage (step 10) will ensure that all of the material will be collected.

9. Repeat steps 5–8 with the second half of the sample.

10. Pipette 100 μ l of 500mM NaCl into the top of the filter unit and wash through the filters as in steps 5–7. This material should also be transferred to the microcentrifuge tube with the filtered sample.

E. Transformation

E. coli host cells can be transformed by recombinant M13 RF DNA molecules only if they have been made 'competent'. There are several ways of achieving this. Two such methods are given here. Method A is a simple CaCl_2 treatment. This is a quick and easy method, but the competent cells produced in this way have only a limited storage life. They can be kept on ice, but the efficiency of transformation drops after 24 hours. Method B is based on the protocols of Hanahan⁽¹³⁾. It is more involved than method A, but it gives cells with a higher efficiency of transformation, which can be maintained upon storage, even for several months.

The use of the *E. coli* host strain TG1 (pages 25 and 38) is strongly recommended for use in these protocols.

Preparation of competent cells

Method A

For growth and maintenance of host cells see page 25.

1. Pick a single colony from a glucose/minimal medium plate. Grow overnight in 10ml 2xTY medium shaken at 37°C.
2. Inoculate 40ml 2xTY medium in a 250ml flask with 2ml of overnight culture, and shake at 37°C for 2 hours to $\text{OD}_{550} \sim 0.3$. At this time also inoculate 20ml of 2xTY medium with 1 drop of overnight culture to provide log. phase cells, which will be ready for use at the plating out stage (page 34). Shake at 37°C.
3. Spin down the cells from the 40ml culture (3000 x g for 5 minutes).
4. Resuspend cells in 1/2 volume (20ml) of sterilized 50mM CaCl_2 pre-chilled on ice. Leave on ice for 20 minutes.
5. Spin down cells (3000 x g for 2 minutes).
6. Resuspend in 1/10 volume (4ml) cold 50mM CaCl_2 .

These competent cells (4ml, enough for ~ 12 plates) can now be used directly for transformation. However, the efficiency of transformation obtained will be increased if the cells are left on ice for several hours before use. After 24 hours the efficiency of transformation begins to decrease.

Method B

For growth and maintenance of host cells see page 25.

1. Pick a colony from a glucose/minimal medium plate, and inoculate 30ml of SOB in a sterile 50ml polypropylene centrifuge tube.
2. Incubate overnight at 37°C, with moderate agitation.
3. Add 8ml of the overnight culture to a 2L flask containing 200ml SOB. Incubate at 37°C with moderate agitation to $\text{OD}_{550} \sim 0.3$.
4. Collect the culture in four 50ml sterile polypropylene centrifuge tubes, and chill rapidly in an ice-salt-water bath. Leave on ice for 15 minutes.
5. Pellet the cells by centrifugation at 3000 x g for 5 minutes at 4°C. Drain pellet thoroughly.
6. Resuspend the pellets gently by mild vortexing of each in 16ml transformation buffer 1 (page 36) (16ml per 50ml of initial culture). Incubate on ice for 15 minutes.
7. Pellet cells as before (3000 x g for 5 minutes, at 4°C).
8. Resuspend the pellet in a total of 16ml of transformation buffer 2 (page 36) (4ml per 50ml initial culture). Store at 4°C for no more than a few hours before use.
9. Alternatively, aliquot the cell suspension into microcentrifuge tubes. Flash freeze by dropping into liquid nitrogen until frozen, and place in -70°C freezer. This will give 16ml of competent cells — enough for ~ 50 plates.

In addition to preparing competent cells for the transformation stage, it is also

necessary to prepare fresh *E. coli* host cells for the plating out step. For method A, it is convenient to prepare the fresh cells for the lawn during the preparation of the competent cells. For method B, prepare the fresh cells as follows:

1. Pick a single colony from a glucose/minimal medium plate. Grow overnight in 10ml 2xTY medium shaken at 37°C.
2. Inoculate 20ml of 2xTY medium with a single drop of overnight culture approximately 4 hours before the fresh cells are required for the plating out stage. Incubate at 37°C with moderate agitation.

Transformation reaction (for methods A and B)

Note: Polypropylene culture tubes, such as those supplied by Elkay (sterile 15ml polypropylene, catalogue number 000-2098-STR), or by other manufacturers such as Falcon, have been found to give higher numbers of transformants than Eppendorf tubes or tubes made from polystyrene.

Control DNA is provided at 1mg/ml (see also page 43). Dilute this stock to 50ng/ml (1ng/20µl) in water, for use in transformation reactions.

1. If using frozen, stored competent cells, thaw on ice.
2. Prechill polypropylene tubes (see note above).
3. Aliquot 300µl of competent cells into chilled tubes.
4. Add ~ 20µl DNA by gently stirring the cells whilst pipetting. Roll tubes gently for a few minutes (on ice).
5. Incubate cells on ice for 40 minutes.
6. Heat shock cells at 42°C for 45 seconds. **Do not shake.**
7. Place on ice for 5 minutes before plating out.

Plating out

While the cells are on ice, prepare the following mixture for each tube (only transformation and mutagenesis controls should require this mixture):

IPTG 100mM (page 37)	40µl
X-gal 2% in dimethylformamide (page 37)	40µl
Log phase <i>E. coli</i> cells	200µl

Note: dimethylformamide is toxic, and will dissolve some plastics.

This solution may be prepared in bulk. The X-gal and IPTG should be freshly prepared and kept on ice. Cells should be from a fresh exponential culture of *E. coli*.

Add 200µl of log phase *E. coli* cells to each tube, and to the control tubes add 270µl of fresh cells/X-gal/IPTG mix.

Use a fresh pipette tip each time to prevent cross-contamination. To each tube add 3ml molten H top agar (page 35), kept at 45°C. Mix by rolling, and pour immediately onto a prewarmed (37°C) H plate (page 35). Leave at ambient temperature to set. Invert the plates and incubate at 37°C overnight.

Transformation control

Suitable controls should be included in the transformation reactions. Carry out each transformation on duplicate plates, and use the double-stranded RF DNA included in the kit as a transformation control. Transforming with 1ng of RF DNA per tube should give 200-300 blue plaques per plate.