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# How Many Human Interferons Are There?

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## I WHAT ARE INTERFERONS?

Investigations carried out in our laboratory in recent years have suggested that the inventory of human interferons is substantially larger than has been generally appreciated. This presentation attempts to retrace our steps towards this inference and to highlight the major themes involved in our current research.

In order to answer the question posed in the title of this article it is necessary to have a clear definition of what constitutes an interferon. The official definition states "to qualify as an interferon a factor must be a protein which exerts virus non-specific antiviral activity at least in homologous cells through cellular metabolic processes involving synthesis of both RNA and protein" (Interferon Nomenclature, 1980). The definition of an interferon does not involve any obligatory notion about sequence relatedness either at the nucleotide or the amino acid levels. Since the definition of an interferon is essentially biological, it allows of the possibility that a wide variety of proteins could exhibit the common biological property of exerting a broad spectrum antiviral effect via various biochemical mechanisms and yet be

grossly different chemically. Similarly, although all interferons that have been studied to date appear to be proteins secreted by cells into the extracellular compartment which then can affect other cells, the notion that interferons are secretory proteins is not intrinsic to the basic definition. There is a clear experimental bias in conventional procedures used in interferon research since these almost exclusively involve the detection and characterization of interferons secreted into the cell culture medium or into extracellular compartments *in vivo*. In theory, a protein which remains embedded in the cell membrane and exerts an antiviral effect on other cells following cell-to-cell contact could be called an interferon. The secretory and membrane-bound immunoglobulins come to mind as an analogy (reviewed in Cheng *et al.*, 1982). Lin *et al.* (1982) suggest that such membrane-bound interferons may indeed exist.

In our experiments we have obtained polyadenylated RNA from appropriately induced human cells, translated it by microinjection into oocytes of *Xenopus laevis* (Reynolds *et al.*, 1975; Sehgal *et al.*, 1977) and have usually assayed the protein products exported into the oocyte culture medium (Colman and Morser, 1979) for antiviral activity in appropriate human or other indicator cell strains using vesicular stomatitis virus as the challenge virus in conventional cytopathic effect inhibition assays for interferon (Armstrong, 1971; Havell and Vilček, 1972). In every instance the antiviral activity observed was blocked by an excess of appropriate anti-human interferon antisera. We therefore consider these proteins to be human interferons. We have then fractioned mRNA preparations by electrophoresis through agarosemethylmercury gels under stringent denaturing conditions and have used the *Xenopus* oocyte translation assay to detect human interferon mRNA species (Sehgal and Sagar, 1980). Our experiments have revealed unexpected heterogeneity in human interferon mRNA species. Available evidence indicates that at least some of this heterogeneity may be attributed to the existence of novel human interferon genes which had largely escaped detection using conventional procedures.

The major advantage of the procedures used by us resides in the fact that we have made no assumptions about nucleic acid or amino acid sequence relatedness or about the physical properties of the interferons and their mRNA species. The procedures used by us closely follow the basic biological definition of interferons which is based upon their antiviral effects. Although neutralization tests with appropriate anti-interferon antisera have clearly provided us with valuable information about our antiviral products, this procedure, with its underlying assumption of amino acid sequence relatedness between some interferons, is not an obligatory step in our analysis.

The most gratifying aspect of our work is that it has provided a basis for the resolution of the chromosomal localization problem that has existed in the

IFN-$\beta$ field for the last few years (Tan *et al.*, 1974; Morgan and Faik, 1977; Slate and Ruddle, 1979, 1980; Meager *et al.*, 1979a, b). Our data indicate that all of the observations reported by previous investigators about human IFN-$\beta$ production in human–rodent somatic cell hydrid experiments were essentially correct, but that the earlier apparently contradictory interpretations of the then available data can be reconciled based upon (1) the existence of distinct IFN-$\beta$ genes located on several different human chromosomes (at least three), and (2) the grossly non-coordinate expression of these genes (Sehgal *et al.*, 1981a, Sagar *et al.*, 1982). Although a great many details still remain to be worked out, we believe that the essence of this story is in hand.

## II THE CLASSICAL ANSWER

The classical answer to the question posed in the title of this paper derives from two major lines of work – the purification and characterization of interferon proteins and, more recently, the application of recombinant DNA procedures to clone and characterize various IFN cDNA species and their corresponding genes. It is important to emphasize that these studies were carried out in more or less conventional experimental systems and, generally speaking, only those species of IFNs or IFN mRNAs which were the dominant molecules were characterized.

The antigenic characterization of human interferons obtained from a variety of experimental systems has led to the clear recognition of three distinct serologic classes of interferons (Havell *et al.*, 1975; De Ley *et al.*, 1980, 1981). The $\alpha$ interferons represent the major antigenic species in preparations derived from Sendai virus-induced human leukocyte cultures, the $\beta$ interferons represent the major or the only antigenic species in preparations of poly(I).poly(C)-induced human fibroblast cultures and the $\gamma$ interferons represent the major antigenic species in preparations derived from immunologically stimulated lymphocytes. Antisera have been raised against these three distinct serologic types which exclusively neutralize only the homologous interferons. Thus these antisera have served as valuable tools to distinguish readily between the three human interferon serotypes. That the $\alpha$ and $\beta$ human interferons represent expression products of distinct genes and not merely differences at some post-translational antigenic determinants was strongly supported by the demonstration that there existed distinct mRNA species coding for human $\alpha$ and $\beta$ IFNs (Cavalieri *et al.*, 1977). That there exist distinct mRNA species coding for human $\gamma$ interferons was reported last year (Wallace *et al.*, 1981; Taniguchi *et al.*, 1981).

During the late 1970s a number of laboratories successfully purified human interferons to homogeneity (Knight, 1976; Rubinstein *et al.*, 1978;

Tan *et al.*, 1979). Polyacrylamide gel electrophoresis of human $\alpha$ IFNs had already revealed some heterogeneity in these proteins (Stewart and Desmyter, 1975). Purification of these proteins to homogeneity revealed that there existed 8–10 distinct IFN polypeptides which differed in primary structure in preparations of IFN-$\alpha$ (Allen and Fantes, 1980; Rubinstein *et al.*, 1981). Polyacrylamide gel electrophoresis of human $\beta$ IFN preparations appeared to suggest that there existed a single species of IFN-$\beta$ (Reynolds and Pitha, 1975; Knight, 1976). Purification of the major IFN-$\beta$ species produced by poly(I).poly(C)-induced human fibroblasts led to the recognition of a single IFN-$\beta$ protein with a unique *N*-terminal amino acid sequence which was distinct but vaguely related to those deduced for human IFN-$\alpha$ (Knight *et al.*, 1980; Zoon *et al.*, 1980). This *tour de force* had such an overwhelming effect on the field that it created the strong impression that there existed a single human $\beta$ interferon.

The availability of a simple and highly sensitive *Xenopus* oocyte assay for interferon mRNA (Reynolds *et al.*, 1975) allowed a number of investigators to begin to characterize these molecules as expressed in the induction systems of the day. Thus human IFN-$\alpha$ and $\beta$ mRNA species were found to be polyadenylated and to sediment in sucrose gradients mainly around 12 *s* (Sehgal *et al.*, 1978a; Morser *et al.*, 1979; Berger *et al.*, 1980). Given this kind of information a number of investigators well versed in recombinant DNA techniques began to clone cDNA copies of IFN mRNAs derived from the 12 *s* region of sucrose gradients. On the $\alpha$ side, Nagata and his colleagues (Nagata *et al.*, 1980a) used a positive hybridization-translation screen (this screen has the advantage that it does not commit the investigator to a predetermined sequence nor is it necessary to have any such information) to isolate a single cDNA clone that hybridized translationally active human IFN-$\alpha$ mRNAs. This was then used to pick up a further crop of strongly-hybridizing and weakly-hybridizing clones from the 12 *s* cDNA library (Nagata *et al.*, 1980a; Streuli *et al.*, 1980). These two sets turned out to represent IFN-$\alpha_1$ and $\alpha_2$ genes respectively. These investigators have since used the IFN-$\alpha_1$ cDNA clone to search extensively for related cross-hybridizing genes in a human DNA gene bank and have successfully detected and characterized about eleven "closely-related" distinct genes and pseudogenes and about seven "distantly-related" genes and pseudogenes (Nagata *et al.*, 1980b; Brack *et al.*, 1981; reviewed in Weissmann, 1981). All of these IFN-$\alpha$ genes lack introns and many of these have been expressed in *E. coli* to yield biologically active human IFN-$\alpha$. Similarly, Goeddel and his colleagues have used a single IFN-$\alpha$ cDNA clone to screen a 12 *s* IFN-cDNA library and have isolated numerous IFN-$\alpha$ cDNA clones which correspond to eight distinct but cross-hybridizing genes (Goeddel *et al.*, 1980a, 1981). These and other investigators have also screened human DNA gene banks using these cDNA clones or using oligonucleotide probes corresponding to appropriate

IFN-$\alpha$ DNA sequences and have also isolated a collection of 10–12 distinct human IFN-$\alpha$ genes and pseudogenes which lack introns (Lawn *et al.*, 1981a, b; Mory *et al.*, 1981).

In a like manner several groups of investigators have used a sequence independent positive hybridization-translation procedure to screen for IFN-$\beta$ cDNA clones in 12 *s* cDNA libraries derived from poly(I).poly(C)-induced human fibroblasts and have isolated IFN-$\beta$ clones with a DNA sequence which corresponds to the Knight amino acid sequence for IFN-$\beta$ (Taniguchi *et al.*, 1979, 1980a; Derynck *et al.*, 1980a). This DNA has then been used to screen cDNA libraries and human DNA gene banks leading to the isolation of a single gene which cross-hybridizes the cDNA probe, which also lacks introns and which can be expressed in *E. coli* to yield biologically active IFN-$\beta$ (Taniguchi *et al.*, 1980b; Derynck *et al.*, 1980b; Goeddel *et al.*, 1980b; Houghton *et al.*, 1981; Tavernier *et al.*, 1981; Lawn *et al.*, 1981c; Ohno and Taniguchi, 1981). This gene is now designated IFN-$\beta_1$ (Sehgal and Sagar, 1980). Similarly several human IFN-$\gamma$ cDNA clones corresponding to a single gene have been isolated recently (Gray *et al.*, 1982). Screening of a human DNA gene bank with this cDNA clone has led to the isolation of a single human IFN-$\gamma$ gene which, like most mammalian genes, contains three large introns and four exons (Gray and Goeddel, 1982).

Thus the classical answer to the question posed in the title of this article is that there exists a family of perhaps 16–20 more or less related and cross-hybridizing human IFN-$\alpha$ genes which all lack introns, a single human IFN-$\beta$ gene which also lacks introns and a single human IFN-$\gamma$ gene which contains introns. Characterization of genomic clones and chromosome mapping studies using cDNA probes have indicated that these IFN-$\alpha$ genes are located more or less in tandem on human chromosome 9 (Nagata *et al.*, 1981; Brack *et al.*, 1981; Lawn *et al.*, 1981a; Owerbach *et al.*, 1981; Slate *et al.*, 1982), that the single IFN-$\beta$ gene is also located on human chromosome 9 (Owerbach *et al.*, 1981; Pitha *et al.*, 1982) and that the IFN-$\gamma$ gene is located on human chromosome 12 (Gray and Geoddel, 1982).

It is in the determination that there exists a single human IFN-$\beta$ gene which is located on human chromosome 9 that the body of information generated by using recombinant DNA techniques runs afoul of some rather carefully done somatic cell genetics experiments which had indicated that there should exist human IFN-$\beta$ genes on at least three distinct human chromosomes (Tan *et al.*, 1974; Slate and Ruddle, 1979, 1980). This situation has engendered two basic reactions. On the one hand one could dismiss the somatic cell genetics experiments as erroneous. After all, the interpretation of these experiments has been controversial (reviewed in Burke, 1980). On the other hand one begins to wonder whether the first wave of gene cloners may have overlooked substantial segments of the human interferon system. Could it be that there exist IFN-$\beta$ genes which do not cross-hybridize IFN-$\beta_1$

at least in conventional paper-bound hybridization format, but which nevertheless correspond to interferons which are serologically of the $\beta$ type? Could this also occur in the human IFN-$\alpha$ and IFN-$\gamma$ systems? The situation that proteins may be serologically related and yet their corresponding nucleic acids do not cross-hybridize is not without precedent. Our experiments carried out during the last two years suggest that this may indeed be the case in the human IFN-$\alpha$ and $\beta$ systems. We shall first review our experiments in the IFN-$\beta$ system and then turn to a consideration of our IFN-$\alpha$ experiments.

## III PROBLEMS WITH A SUCROSE GRADIENT

In 1978 we reported that polyadenylated human IFN-$\beta$ mRNA derived from poly(I).poly(C)-induced diploid fibroblasts (FS-4 cells) appeared to sediment in sucrose gradients at approximately 12 *s* (Sehgal *et al.*, 1978a). We therefore inferred that this molecule has a length of approximately 850–900 nucleotides inclusive of the 3′ terminal poly(A). At about this time we were engaged in a collaborative project with Dr Hermona Soreq, then a post-doctoral fellow with Dr James Darnell at Rockefeller, in which we trimmed IFN-$\beta$ mRNA prepartaions from the 3′ end using the enzyme polynucleotide phosphorylase (PNPase). This enzyme had been shown by Dr Soreq to preferentially digest the poly(A) tail of globin and other mRNA species in a processive manner at 4° in high $P_i$ (Soreq *et al.*, 1974; reviewed in Littauer *et al.*, 1980). We soon showed that IFN-$\beta$ mRNA preparations that had been deadenylated using PNPase were translationally active in *Xenopus* oocytes (Sehgal *et al.*, 1978b). Dr Soreq's mastery of the PNPase reaction was such that it could be shown that when mRNA preparations were incubated with a molar excess of PNPase at 4° for 20 min, the entire poly(A) tail was removed and when such a reaction mixture was then warmed to 37° the mixed sequence RNA internal to the poly(A) was digested in a synchronous manner ($\pm$ approximately 10 nucleotides) for the next several minutes (reviewed in Littauer *et al.*, 1980). We therefore proceeded to digest IFN-$\beta$ mRNA preparations with PNPase (60 *s* at 37°) internal to the poly(A) under conditions that should have reduced IFN-$\beta$ mRNA length from one of approximately 900 nucleotides to approximately 500–600 nucleotides. This reaction did not lead to any appreciable change in either the translational activity of IFN-$\beta$ or, to our surprise, to any clear shift in sucrose gradient sedimentation. This result could be interpreted in one of two ways: either the PNPase did not work or that the RNA length estimates based on sucrose gradient sedimentation analyses and the resolving power of the gradient technique were just not good enough. In the belief that electrophoresis of RNA through denaturing gels may permit a better resolution we, with the help of Anurag Sagar, a Graduate Fellow at Rockefeller, proceeded to set

up and refine procedures for the electrophoresis of translationally active IFN mRNA first through agarose-7 M urea tube gels and subsequently through agarose-10mM $CH_3HgOH$ gels (Sehgal and Sagar, 1980; Sehgal, 1981). A seminar by Dr Michel Revel at The Rockefeller University in February 1980 describing the detection of "14 *s*" and not the usual "10–12 *s*" human IFN-$\beta$ mRNA species (Weissenbach *et al.*, 1980) helped accelerate the pace of our gel experiments. Within 10 days after Dr Revel's seminar we had the pleasure of letting him know that we had resolved two species of human IFN-$\beta$ mRNA of length 1300 ("14 *s*") and 900 ("11 *s*") nucleotides by gel electrophoresis (Fig. 1). We then went on to show that PNPase did indeed work correctly and that IFN-$\beta$ mRNA species which had extensive (100–200 nucleotides) deletions in their 3′-noncoding sequence were not only translationally active but just as functionally stable as the natural polyadenylated mRNA species (Soreq *et al.*, 1981).

## IV HUMAN $\beta$ INTERFERONS

### A Multiple Human IFN-$\beta$ mRNAs

The IFNs in the oocyte translation products derived from the two individual mRNA species shown in Fig. 1 were found to be completely neutralized by

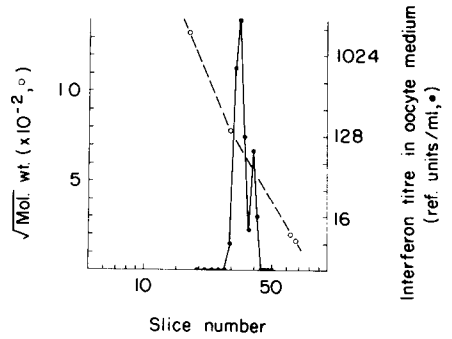


Fig. 1 Electrophoresis of a human fibroblast IFN mRNA preparation through an agarose-$CH_3HgOH$ gel. Cellular polyadenylated RNA (45 $\mu$g) obtained from induced FS-4 cells was mixed with $^{32}$P-labelled marker RNA and was made 10 mM in $CH_3HgOH$ and the RNA electrophoresed through a 1·75% agarose-10 mM $CH_3HgOH$ cylindrical gel (0·6 × 11 cm) in borate buffer at room temperature (4 mA per tube; 3·5 v cm$^{-1}$) until the dye was close to the bottom of the tube gel (5 h). After electrophoresis the gel was placed in 100 ml of a solution containing 100 mM $\beta$-mercaptoethanol and 0·01 M Tris-HCl, pH 7·4, for 40 min on a rotary shaker at room temperature. The gel was then sliced manually, the locations of marker 28 *s*, 18 *s*, 5 *s* and 4 *s* RNAs (O) monitored by Cerenkov counting, RNA in appropriate regions of the gel eluted (the elution buffer contained 1 mM dithiothrietol), dissolved in 2 $\mu$l distilled water and assayed for IFN mRNA activity (●) using the *Xenopus* oocyte translation assay. From Sehgal and Sagar (1980).

an excess of (1) an anti-IFN-$\beta$ antiserum that had been raised by Dr Jan Vilček and colleagues against partially purified human IFN-$\beta$, and (2) an anti-IFN-$\beta$ antiserum that had been raised by Dr Y. H. Tan against homogeneous human IFN-$\beta$ whose *N*-terminal amino acid sequence was identical to that reported by Knight *et al.* (1980). Thus the two polyadenylated mRNA species, which by this time had been detected in the cytoplasm of induced FS-4 cells, coded for human $\beta$ interferons.

Could the coding regions of the two RNA molecules be identical or closely-related? This question was answered by preparing a Northern blot of an IFN-$\beta$ mRNA preparation that had been verified to contain predominantly the 1·3 kb mRNA and hybridizing the blot with a $^{32}$P-labelled DNA insert derived from Dr T. Taniguchi's cDNA clone (and obtained from him) under rather relaxed hybridization and washing conditions. This DNA probe hybridized exclusively with RNA of length 0·9 kb. Thus the Knight–Taniguchi sequence corresponded to the 0·9 kb mRNA while the longer 1·3 kb mRNA did not cross-hybridize the DNA probe even though it coded for human IFN which could be completely neutralized by antiserum raised against the Knight protein! These data led us to designate the IFN product of the 0·9 kb mRNA as IFN-$\beta_1$ and that of the 1·3 kb mRNA as IFN-$\beta_2$ (Sehgal and Sagar, 1980). These data allow one to infer that there exist at least two distinct human $\beta$ interferons and that the corresponding nucleic acids do not cross-hybridize even though both proteins are neutralized by an anti-IFN-$\beta_1$ antiserum. (More recent experiments carried out in collaboration with Dr Y. H. Tan do reveal that it takes 2–5-fold more anti-IFN-$\beta_1$ antiserum to neutralize IFN-$\beta_2$ than the homologous IFN-$\beta_1$.) These experiments led us to realize that screening a human DNA gene bank with an IFN-$\beta_1$ cDNA probe will not necessarily lead to the detection of all human IFN-$\beta$ genes.

With the agarose-$CH_3HgOH$ gel procedure in hand we then screened a large number of IFN mRNA preparations for their mRNA species composition (Sagar *et al.*, 1982). Two further observations emerged from these experiments.

1. The 0·9 kb and 1·3 kb mRNAs were expressed in a grossly non-coordinate manner in different FS-4-derived cell strains. Cells derived from a batch of FS-4 cells obtained from Dr Jan Vilček in 1973 predominantly expressed IFN-$\beta_2$ mRNA while those derived from a second batch obtained from Dr Vilček in the fall of 1975 mainly expressed IFN-$\beta_1$.
2. Two additional IFN-$\beta$ mRNA species of approximate length 1·8 and 0·6–0·7 kb were observed in several preparations of poly(I).poly(C)-induced FS-4 cell mRNA. For convenience these were designated IFN-$\beta_3$ and $\beta_4$ mRNAs respectively. The picture that emerged from these experiments was that there should exist at least two distinct

human IFN-$\beta$ genes and at least four mRNAs species derived therefrom. Further experiments, reviewed in the next section, helped increase this list to at least three human IFN-$\beta$ genes.

## B Multiple Human IFN-$\beta$ Genes

Genetic experiments using somatic cell hybrids of the human–rodent variety had been used in the mid-seventies to obtain data suggestive of the presence of independent loci for human IFN-$\beta$ on human chromosomes 2, 5 and 9. However the results of these experiments had been controversial. On the one hand, Tan *et al.* (1974) and Slate and Ruddle (1979, 1980) obtained evidence for the involvement of chromosomes 2 and 5 and later of 9 in IFN-$\beta$ production while on the other hand Meager and his colleagues (Meager *et al.*, 1979a, b) concluded that chromosome 9 alone was involved in IFN-$\beta$ production. The latter investigators were unable to find clear evidence to implicate chromosomes 2 or 5 although low levels of human IFN production were observed in somatic cell hybrids lacking chromosome 9. When the single IFN-$\beta$ cDNA clone corresponding to the Knight–Taniguchi sequence (IFN-$\beta_1$) was localized to chromosome 9 alone (the grapevine had picked up this result in the summer of 1980), additional questions arose about the correctness of the early somatic cell hybrid experiments.

With the discovery that translationally active human IFN-$\beta$ mRNAs are heterogenous in RNA length, that at least one of these ($\beta_2$, of length 1·3 kb) does not cross-hybridize $\beta_1$ cDNA probe (Sehgal and Sagar, 1980) and the realization that the expression of these mRNAs can be consistently non-coordinate (Sehgal *et al.*, 1981a; Sagar *et al.*, 1982) it was apparent that we had the theoretical elements for the resolution of the chromosomal localization controversy in hand. The somatic cell hybrids analysed by Meager *et al.* (1979a, b) may have consistently expressed mainly IFN-$\beta_1$ derived from chromosome 9 while those analysed by Tan *et al.* (1974) and Slate and Ruddle (1979, 1980) may have expressed additional IFN-$\beta$ mRNAs derived from other chromosomes. These ideas surfaced at the First International Interferon Congress arranged by Dr Bill Stewart in Washington in November 1980. Dr Doris Slate was in attendance during this discussion. It was in Washington that Doris made contact with us and we decided to carry out gel analyses of IFN mRNA preparations derived from poly(I).poly(C)-induced human–mouse somatic cell hybrids. This repesents at least one tangible project that was catalysed by that meeting in November 1980 and by April 1981 (the Rotterdam meeting) we had the situation well in hand (Sehgal *et al.*, 1981a).

A series of human–mouse somatic cell hybrids that had been extensively investigated in the past for human interferon prodution and whose karyotype had been characterized were selected for study (Table I). These hybrids