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Structure and expression of cDNA and genes for human interferon-beta-2, a distinct species inducible by growth-stimulatory cytokines

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Induced human fibroblasts produce several mRNAs encoding interferon (IFN) activity. We previously cloned cDNA for a 1.3-kb RNA designated IFN-β2 and distinct from the 0.9-kb IFN-β1 mRNA. *In vitro* transcription–translation mapping of the full-length IFN-β2 cDNA sequence, shows that it encodes a 23.7-kd protein of 212 amino acids. This cDNA, fused to the SV40 early gene promoter, was transfected and amplified in Chinese hamster ovary cells and clones were obtained which constitutively produce human interferon activity. Two IFN-β2 genomic clones were isolated and their expression in hamster and mouse cells also produces biologically active rIFN-β2. Specific immunoassays show that IFN-β2 secreted by DNA-transformed rodent cells is a processed 21-kd protein, whose activity is cross-neutralized by antibodies to human IFN-β1 but not to IFN-α or γ. The immunoassay also demonstrates the induction of IFN-β2 secretion by fibroblasts in response to growth-regulatory cytokines, such as interleukin-1 and tumor necrosis factor. The function of this IFN-β2 as an autoregulatory inhibitor of cell growth is discussed.

Key words: interferon-β2/cytokines/growth regulation/gene expression

Introduction

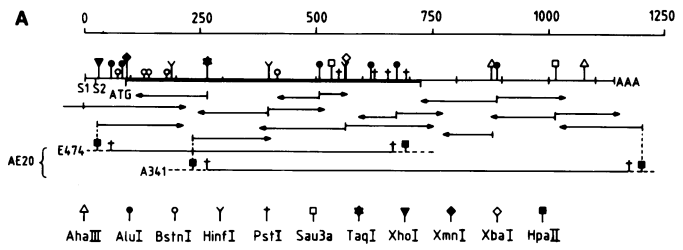
The major part of the interferon (IFN) activity produced by human fibroblasts after IFN priming, and sequential induction by poly(rI)(rC), cycloheximide (CHX)–actinomycin D treatment, is due to the 20-kd glycoprotein IFN-β1, which is encoded by a 0.9-kb RNA transcribed from an intron-less gene on the short arm of chromosome 9 (Knight *et al.*, 1980; Taniguchi *et al.*, 1980; Derynck *et al.*, 1980; Mory *et al.*, 1981; Trent *et al.*, 1982). However, when RNA from such human cells is more fully analyzed, one detects additional mRNAs which yield IFN activity upon translation in frog oocytes (Weissenbach *et al.*, 1980; Sehgal and Sagar, 1980; Sehgal, 1982). We have previously isolated cDNA clones for such an induced 1.3-kb RNA, which is transcribed *in vitro* into a protein of 23–26 kd, and shown that RNA selected by hybridization to these cDNAs produces IFN anti-viral activity when injected into oocytes (Weissenbach *et al.*, 1980). Since this activity was neutralized by antibodies against IFN-β1, the translation product of the 1.3-kb RNA was designated IFN-β2. However, the IFN-β2 cDNA clones revealed only limited regions of homology to the sequence of IFN-β1, and two human genomic DNA clones hybridizing to IFN-β2 cDNA were found to contain large introns, in contrast to the IFN-β1 and IFN-α genes (Revel, 1983; Zilberstein *et al.*, 1985). Furthermore, the IFN-β2 genes were also not located on chromosome 9, at least one of them having been recently mapped to human chromosome 7 (Sehgal *et al.*, 1986). The protein encoded by the 1.3-kb RNA appeared, therefore, to represent a novel and distinct species of the human type I interferons (Revel, 1983; Sehgal, 1982).

In the present work, we establish the structure and biological activity of human IFN-β2 by expression of a full-length cDNA under the control of the SV40 early promoter in stably transfected Chinese hamster ovary (CHO) cells. We show that the IFN-β2 protein produced constitutively by these cells has anti-viral activity on human cells which is neutralized by anti-IFN-β1 antibodies. Such recombinant rHuIFN-β2, produced from the cDNA or from the two genes, activates expression of (2′-5′) oligo(A) synthetase or other typical IFN-induced genes and, as demonstrated elsewhere (Zilberstein *et al.*, 1985), does not act by inducing another IFN molecule. Since the sequence of the IFN-β2 protein is not known, *in vitro* transcription–translation mapping of the cDNA was used to verify the protein-coding sequence. An immunoassay specific for IFN-β2 was developed and demonstrates that this protein is secreted by human cells in response to growth-stimulatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α, conditions under which IFN-β2 RNA but not IFN-β1 is induced (Content *et al.*, 1985; Kohase *et al.*, 1986). IFN-β2 may, therefore, be the first cloned representative of the group of autocrine IFN-β molecules, differing from IFN-β1, which we have found to be secreted by cells undergoing growth transitions and differentiation (Friedman-Einat *et al.*, 1982; Revel, 1983; Yarden *et al.*, 1984).

Results

Structure of IFN-β2 cDNA and protein

Restriction mapping indicated that the cDNA clones A341 and E474 previously isolated (Weissenbach *et al.*, 1980), correspond to two overlapping segments of the 1.3-kb IFN-β2 RNA (Figure 1A). The two cDNAs were recombined through their *Xba*I site to form AE20 cDNA (Figure 1A) and sequenced. A typical polyadenylation signal was found at the 3′ end in front of the poly(A) tail (Figure 1B). Analysis of cDNAs extending furthest in 5′, showed a unique *Xho*I site (J.Weissenbach *et al.*, unpublished), which was used to map the 5′ end of the 1.3-kb RNA on genomic clones isolated from a λ-phage library of partially *Eco*RI-digested human DNA (Mory *et al.*, 1981). Two different genomic clones were obtained, IFA-2 (IFN-β2a gene) and IFA-11 (IFN-β2b gene) (Figure 2). Only IFA-2 hybridized over the whole length to AE20 cDNA (IFN-β2a cDNA). The *Xho*I site at the 5′ end of the cDNA was localized in a 3.5-kb *Eco*RI fragment of IFA-2 (Figure 2) which was subcloned (A132) and sequenced in part (Figure 3A). S1 nuclease analysis with RNA from human fibroblasts induced to produce IFN, indicated two RNA starts in the A132 sequence: a weaker start (S-1) and a stronger region



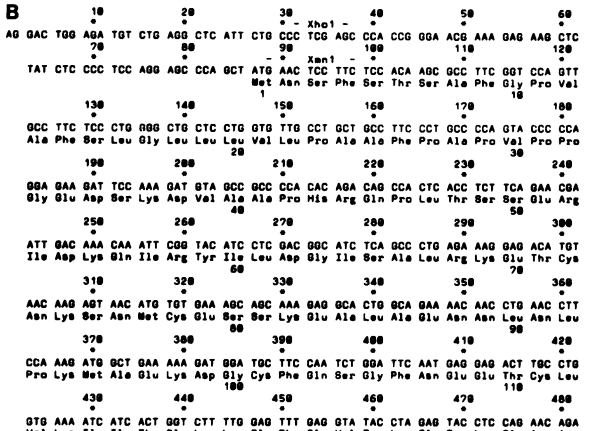


Fig. 1. (A) Restriction map and sequencing strategy of IFN-β2a cDNA clones AE20, E474 and A341. The pBR sequences are shown as dotted lines. S1 and S2 are the two RNA start sites determined from genomic clone A132 (Figure 3). (*B*) Sequence of IFN-β2a cDNA numbered from the S-1 start.

(S-2) located respectively 32 and 10 nucleotides upstream of the *Xho*I site (Figure 3A,B). Sequencing of a primer elongation reaction (not shown) identified the S-2 start as in Figure 3A. These two starts were seen under the various conditions [poly(rI)(rC), cycloheximide or both; Figure 3B] previously shown to induce the 1.3-kb IFN-β2 RNA (Weissenbach *et al.*, 1980; Content *et al.*, 1982). Two potential TATA boxes were found located 30 bp upstream of the S-1 and S-2 RNA starts, respectively (Figure 3A). The second TATA box overlaps with a polyadenylation signal AATAAA. The activity of this presumed IFA-2 gene promoter in transfectants was previously reported (Chernajovsky *et al.*, 1984). From the S-1 site, the complete length of the reconstituted IFN-β2a cDNA is 1140 bp (Figure 1B), which would leave a poly(A) tail of 150–200 residues in the 1.3-kb RNA. Comparison of the cDNA and IFA-2 genomic sequences

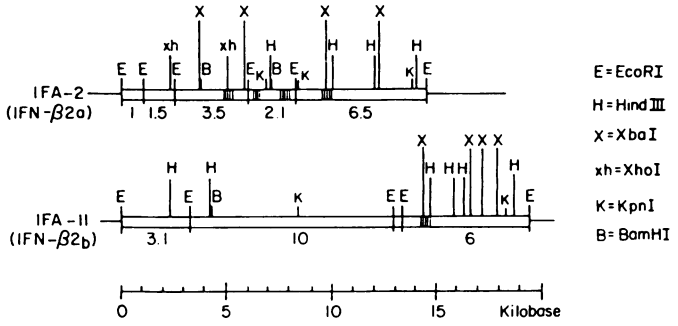


Fig. 2. Genomic clones IFA-2 and IFA-11. The size of *Eco*RI fragments is indicated (kb). The hatched areas in IFA-2 indicate the approximate location of exons for IFN-β2a cDNA: the 3.5-kb *Eco*RI fragment hybridized to E474 but not A341 cDNA; the 2.1-kb *Eco*RI segment hybridized both left and right of its *Bam*HI site to the 5′ *Pst*I fragment of A341 (Figure 1A). The left *Xba*I–*Hin*dIII region of the 6.5-kb IFA-2 fragment and of the 6-kb from IFA-11, both hybridized to the most 3′ *Pst* fragment of A341, and their sequence was the same as in the cDNA. The rest of IFA-11 showed no strong hybridization to A341 cDNA.

showed that the first exon ends 70 bp downstream from the *Xho*I site, and contains two potential initiator ATG codons (Figure 3A).

A transcription–translation system using the T7 promoter of the pGEM-1 vector, confirmed (Figure 4) that the IFN-β2a cDNA encodes the same 23–26 kd protein as produced by poly(A)$^+$ RNA from induced human fibroblasts and which is immunoprecipitated by antibodies to crude fibroblast IFN preparations (Weissenbach *et al.*, 1980) and by the IFN-β2-specific R-antibodies (see below) (Figure 4, lane 1). Transcription–translation mapping was then used to delimit the coding region of IFN-β2a cDNA by comparing a series of deletions (Figure 4), which established that the 23–26 kd protein begins between *Xho*I (residue 32) and *Xmn*I (residue 93), identifying the ATG at position 87 as the functional initiation codon. Similarly, the termination codon was located between the *Mst*II (residue 672) and *Ban*I sites (residue 710) (Figure 4). The protein sequence of IFN-β2a starts, therefore, in the first exon of the cDNA (which encodes the first six amino acids) and predicts a primary translation product of 212 amino acids, with a calculated mol. wt of 23 716 daltons. Two possible glycosylation sites are present at amino acids 73 and 172. A hydropathy plot (Kyte and Doolittle, 1982) indicated that the first 30 amino acids form a strongly hydrophobic region, which is probably processed during secretion (see below). The precise site of processing remains to be determined.

Constitutive expression of IFN-β2a cDNA and gene in CHO cells

The reconstituted IFN-β2a cDNA was cloned in a plasmid expression vector downstream from the SV40 early gene promoter and preceding the T-ag splicing region and polyadenylation site (Figure 5A). This pSVCIFβ2 plasmid construct was co-transfected into dihydrofolate reductase (DHFR)$^-$ CHO cells together with a plasmid containing mouse DHFR cDNA fused to the SV40 promoter, to allow selection and amplification (Kaufman and Sharp, 1982). Several CHO cell clones growing in nucleoside-free medium were isolated (e.g. B-131, B-132) and further selected by treatment with methotrexate (250 and 500 nM). Medium from these CHO–SVIFCβ2 cultures, collected 24 h after medium replacement, was assayed for human IFN activity on monolayers of human diploid fibroblasts FS11 by measuring inhibition of vesicular stomatitis virus (VSV) cytopathic effect. Staining of the infected cell monolayers (Figure

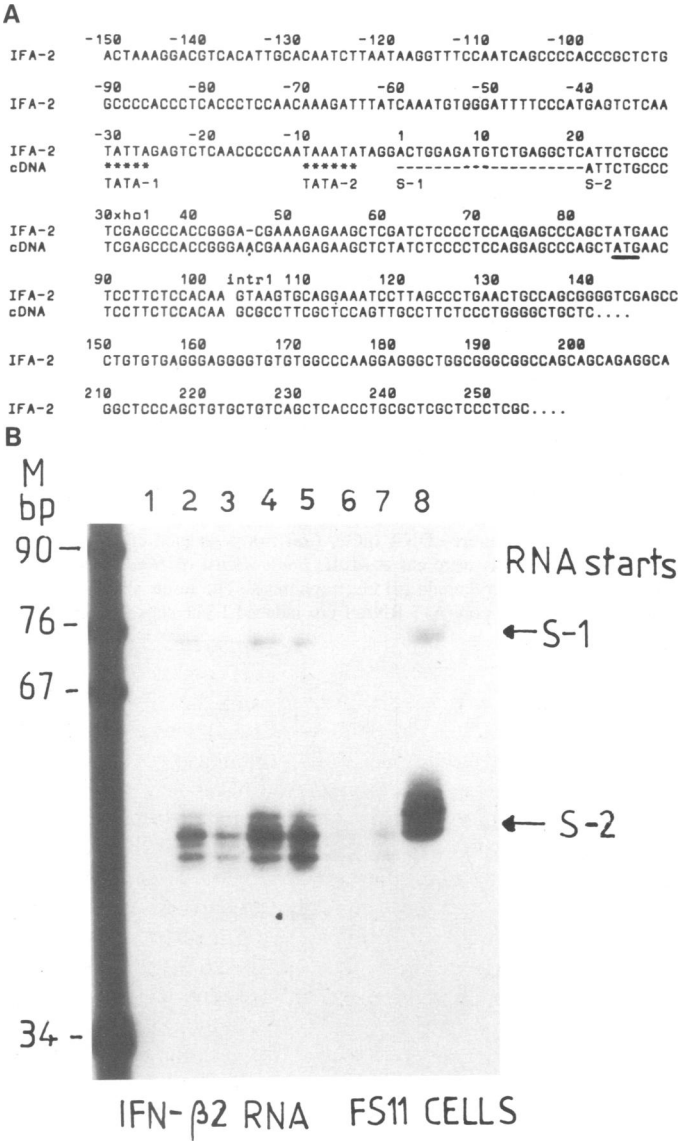


Fig. 3. (**A**) Sequence of IFA-2 genomic subclone A132 (3.5-kb *Eco*RI fragment of IFA-2) around its *Xho*I site. The cDNA sequence is given in comparison, the dotted line being the part deduced from the gene. The two potential TATA boxes are marked by stars. Initiator codon is underlined; intr1 is the first intron. (**B**) S1 nuclease mapping of IFN-β2 transcripts on the IFA-2 gene. A 560 bp long *Bst*NI fragment of A132 DNA was 5′ labeled with T4 polynucleotide kinase and the separated anti-sense strand labeled at position 73 in (**A**) was hybridized to 50 μg total FS11 cell RNA, as in Maroteaux *et al.* (1983). Protected fragments after digestion with 300 units S1 nuclease, electrophoresis in 8% polyacrylamide−8.3 M urea gels and autoradiography are shown for RNA from FS11 fibroblasts treated as follows: **lane 1**: priming with 200 U/ml IFN-β1 for 16 h; **lane 2**: priming and poly(rI)(rC) 50 μg/ml for 3.5 h; **lane 3**: priming and cycloheximide (CHX) 50 μg/ml, 3.5 h; **lane 4**: priming poly(rI)(rC) and CHX; **lane 5**: priming and CHX 6.5 h; **lane 6**: poly(rI)(rC) 3.5 h; **lane 7**: CHX 3.5 h; **lane 8**: poly(rI)(rC) plus CHX.

5B) demonstrates that the CHO−SVCIFβ2 transformants produce anti-viral activity on the human cells, while control CHO cells transformed by pSVDHFR DNA alone had no such activity. The IFN titers were calculated by comparison with an IFN-β1 standard, and progressively increased as the cells were selected for resistance to higher doses of methotrexate (Table I), reflecting probably the amplification of the IFN-β2 cDNA sequences together with the transfected DHFR gene (Kaufman and Sharp, 1982; Chernajovsky *et al.*, 1984). After concentration of the medium from cultures in 1% serum of such cells constitutively producing rIFN-β2, solutions with titers of 5000 international units of IFN per ml were routinely obtained. The recombinant IFN-β2 produced in this way was as efficient as pure rIFN-β1 (Chernajovsky *et al.*, 1984) for inducing the (2′−5′) oligo(A) synthetase in cultures of FS11 fibroblasts when equal anti-viral units were compared (Figure 5C). One anti-viral unit per ml of rIFN-β2 produced a significant increase in this typical IFN-induced enzyme. However, calculation of the specific activity (units/mg protein) of IFN-β2 indicate that it is lower than that of IFN-β1 by a factor of 50−100 (Weissenbach *et al.*, 1980; Zilberstein *et al.*, 1985).

Another series of CHO cells constitutively producing rHuIFN-β2, was obtained by transformation with the entire IFN-β2a gene. A plasmid construct pSVIFA-2-I was made by fusing to the SV40 early promoter, a 4.8-kb segment of the IFA-2 cloned DNA (Figure 2), from the *Xho*I site in the *Eco*RI 3.5-kb fragment to the first *Hin*dIII site of the 6.5-kb *Eco*RI fragment. The gene is thereby fused after its RNA start (Figure 3A) to the T-ag transcription unit. CHO DHFR$^+$ clones co-transformed as above, by this plasmid and a DHFR gene, produced human IFN activity. Table II shows that one of these clones, CHO SI-15, constitutively produced 100 U/ml without methotrexate amplification. The anti-viral activity of IFN-β2a expressed from the cDNA and the gene was specific for human cells (Table II). Similar titers were obtained on human Wish cells as on FS11 (not shown), and with picornavirus Mengo as with VSV (Table II).

Immunological characterisation of rIFN-β2

We have obtained IFN-β2-specific rabbit antisera R and S (Zilberstein *et al.*, 1985) by using as antigen a minor fraction of crude human fibroblast IFN preparations, separated from IFN-β1 because it does not adsorb to Cibacron Blue Sepharose, and then purified on DEAE-cellulose (see Materials and methods). This fraction had been found to contain native IFN-β2 because it competes with the 23−26 kd *in vitro* translation product of IFN-β2 mRNA for the same antibody binding sites (Figure 6B, lane 3). The specificity of the R-antibodies was further demonstrated by their inability to immunoprecipitate IFN-β1 (Zilberstein *et al.*, 1985) and by the fact that excess unlabeled IFN-β1 does not compete against recognition of the IFN-β2 mRNA translation product by these antibodies (Figure 6B, lane 2). This antiserum R immunoprecipitates from the culture medium of CHO−SVCIFβ2 cells, metabolically labeled by [^{35}S]methionine, a 21-kd protein not seen in CHO control cells (Figure 6A), whose size is similar to that of the protein it recognizes in the culture medium of induced human FS11 fibroblasts (Zilberstein *et al.*, 1985). This confirms that the CHO cells transformed by IFN-β2 cDNA, express and secrete the IFN-β2 protein. The smaller size of the IFN-β2 cDNA product secreted by the cells, as compared with that made *in vitro* (Figure 4; Figure 6 A versus B), suggests that a part of the polypeptide chain is cleaved during processing which may also involve glycosylation at two sites in the molecule (Figure 1B). For the same number of anti-viral IFN units added, we found that the CHO rIFN-β2 gives a competition curve parallel to that of FS11 cell-produced IFN-β2, indicating similar specific activities (not shown). The R-antibodies in the immunocompetition assay of Figure 6B provide a specific immunoasay of IFN-β2, sensitive to 1 unit of this interferon.

With the rHuIFN-β2 produced by either clone B-131 or SI-15, we could confirm that anti-IFN-β1 antibodies including a monoclonal antibody against IFN-β1 (Novick *et al.*, 1983b),

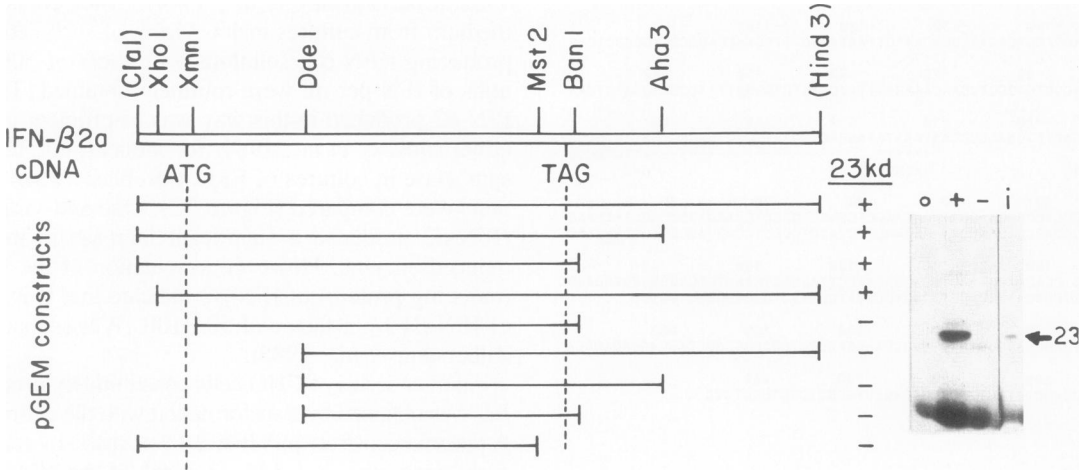


Fig. 4. Transcription−translation mapping of IFN-β2 cDNA. Construction of pGEM-1 plasmids with entire cDNA (pG2, *Cla*I), or 5′-deleted cDNA (pG3, *Xho*I; pGB1, *Xmn*I; pG16, *Dde*I) is detailed in Materials and methods. Before transcription the plasmids were cut at *Mst*II, *Ban*I, *Aha*III or *Hin*dIII as indicated and the synthesis of the 23−26 kd IFN-β2 translation product was measured by SDS−polyacrylamide gel electrophoresis. The insert shows translation without RNA (○), with RNA from pG3-*Hin*dIII-cut (+), with pG2-*Mst*II-cut (−) and with poly(A)$^+$ RNA from induced FS11 cells (i), only the latter being immunoprecipitated with R-antibodies.

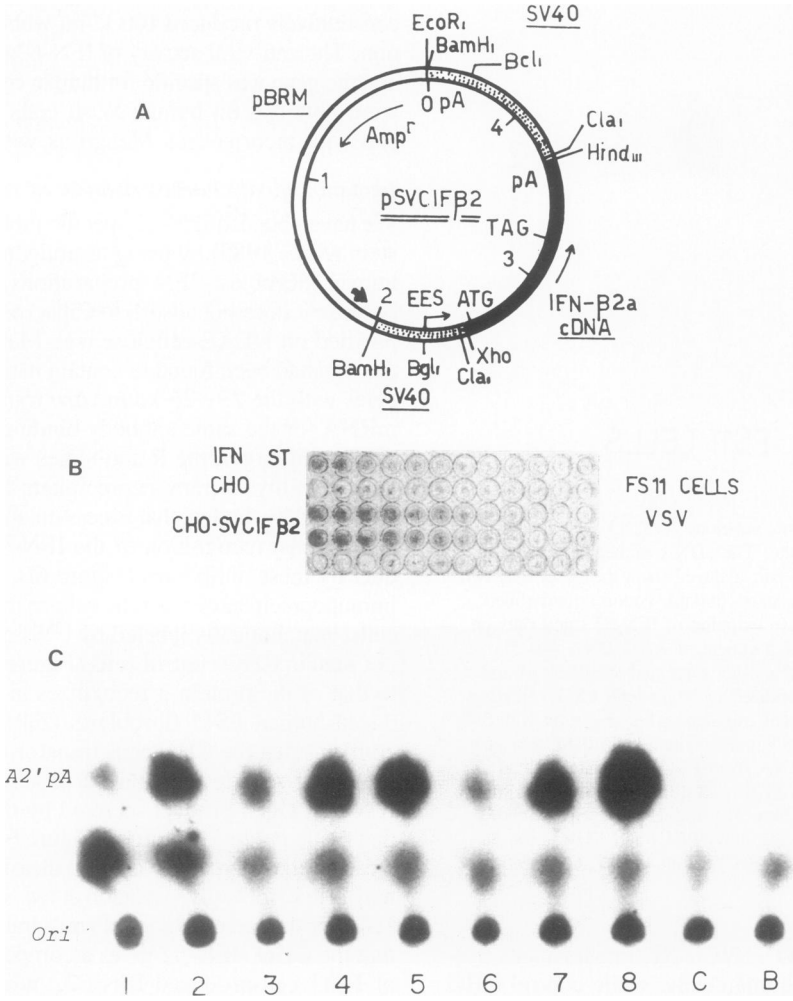


Fig. 5. Constitutive expression of human IFN-β2 in CHO cells. (**A**) Structure of pSVIFβ2 plasmid containing full-length IFN-β2 cDNA. EES is the early early RNA start of the SV40 promoter. pA indicates the polyadenylation sites. (**B**) Inhibition of VSV cytopathic effect by rIFN-β2. Medium from 24 h cultures of CHO B-131 cells in 1% FCS, concentrated 10 times (2000 U/ml) was serially diluted from left to right (rows 3,4). Medium similarly prepared from CHO−DHFR$^+$ cells (row 2) and IFN-β standard (row 1) were used as controls. Microplate stained 48 h after VSV infection. (**C**) (2′-5′) Oligo(A) synthetase activity in extracts of FS11 cells treated 16 h by 1 and 5 U/ml pure IFN-β1 (**lanes 1** and **2**), by 1, 5 and 50 U/ml of rIFN-β2 from B-131-5M cells (**lanes 3−5**) or B-132-5M cells (**lanes 6−8**) or untreated (**lane C**). Synthesis of [^{32}P](2′-5′)ApA (upper spot) is measured by electrophoresis at pH 3.5 of the phosphatased products (Revel *et al.*, 1981). B is reaction without cell extract.

neutralize the anti-viral activity of IFN-β2 (Table III). In the same experiment rIFN-β2, like rIFN-β1, was not neutralized by anti-IFN-α and by anti-IFN-γ antibodies (Table III), justifying its designation as a β-type IFN (Stewart *et al.*, 1980). Atlhough IFN-β1 and β2 have different structures and can each be immunoprecipitated only by specific antibodies, their biological activity appears cross-neutralized. Anti-IFN-β1 antiserum-B which does not immunoprecipitate IFN-β2 (Zilberstein *et al.*, 1985) neutralizes well the anti-viral effect of CHO rHu-IFN-β2 and β1 (Table III); furthermore, antiserum-R, which is IFN-β2-specific (Figure 6B), does not immunoprecipitate IFN-β1 but can neutralize the anti-viral activity of both IFN-β1 and β2 (Zilberstein *et al.*, 1985).

Inducible expression of IFN-β2b gene in mouse L cells

To examine if the IFA-11 genomic clone (Figure 2) contains a functional IFN-β2 gene, we transfected mouse LTK$^-$ cells with the λ-phage clone containing the entire 19-kb IFA-11 insert, using an HSV-TK gene plasmid as selectible marker (Colbere-Garapin *et al.*, 1981). Several LTK$^+$ transformant clones were isolated which produced human IFN activity upon induction by poly(rI)(rC) with cycloheximide−actinomycin D treatment. Clone LI-39 produces under these conditions, ~500 IU of IFN per ml, whereas LTK$^+$ cells lacking the human IFA-11 gene produced no significant IFN activity on human cells (Table I). For these experiments we could not use hamster cells, because hamster IFN cross-reacts more with human cells than does the mouse IFN produced during the induction process (Table II). After passage of the IFN from induced LI-39 through Cibacron Blue−Sepharose, the effluant was free of this mouse IFN (which binds to the column) but still fully active on human cells (Table II). The IFN-β2-specific R-antibodies immunoprecipitated a 21-kd protein from metabolically labeled induced LI-39 cells (not shown). The IFA-11 genomic clone, although differing in structure from IFA-2, clearly contains an active gene which we designate IFN-β2b. The IFN-β2b gene promoter was not yet identified, but we see here that it responds to the superinduction regimen which induces IFN-β1 and β2 in human cells (Vilcek and Havell, 1973; Weissenbach *et al.*, 1980). We previously showed that the IFA-2 gene promoter region transfected in hamster cells, is induced by poly(rI)(rC) but also responds to cycloheximide−actinomycin D treatment by itself (Chernajovsky *et al.*, 1984), in line with the conditions which induce the IFN-β2 RNA in human cells (Figure 3B).

IFN-β2 secretion is induced in human cells by growth-stimulatory cytokines

Induction of mRNA which hybridizes with IFN-β2 cDNA probes but not to IFN-β1 cDNA, has been observed in human cell lines after exposure to IL-1 (Content *et al.*, 1985) and TNF-α (Kohase *et al.*, 1986). The IFN-β2-specific immunocompetition assay with R-antibodies was used to examine if human fibroblast FS11 cells indeed secrete the IFN-β2 protein in response to these two cytokines (Figure 6B). Both IL-1α and TNF-α induced the synthesis and secretion of the IFN-β2 protein, to levels we estimate by the immunoassay to be 10−20 U/ml IFN-β2. Optimal IFN-β2 induction was seen with concentrations of IL-1α of 4 U/ml (0.13 ng/ml), similar to those required for IL-1 induction of prostaglandin E_2, collagenase and hyaluronic acid in human fibroblasts (Korn *et al.*, 1985). The optimal concentra-

Table I. Production of recombinant human IFN-β2 in rodent cells

		Human IFN activity[a] units/ml/24 h
I. Constitutive expression of IFN-β2a cDNA		
CHO−pSVCIFβ2 clones:		
B-132	(no MTX)	60
B-132-5M	(250 nM MTX)[b]	200
B-132-10M	(500 nM MTX)[b]	600
CHO−DHFR$^+$ cells[c]		<4
II. Inducible expression of IFN-β2b gene		
L-IFA-11 clones		
LI-39	Induced[d]	500
LI-39	Non-induced	<4
L-TK$^+$ cells[c]	Induced[d]	<4

[a]Anti-viral activity measured on FS11 cells with VSV as detailed in Materials and methods and Figure 5, with unconcentrated 24 h medium from cultures of 1−2 × 10^6 cells per ml.
[b]Cells selected for resistance to indicated concentrations of methotrexate.
[c]CHO or L cells transformed by selectable DHFR or TK gene marker only.
[d]Induction by poly(rI)(rC), cycloheximide and actinomycin D as in Materials and methods.

Table II. Anti-viral activity of recombinant human human IFN-β2

Transfected human DNA	Origin of IFN		Virus test	IFN activity on	
	Cell clones	Treatment		Human cells (U/ml)	Mouse cells (U/ml)
IFN-β2a cDNA (pSVCIFβ2)	CHO B-131[a]	10× concent.	VSV	2000	n.d.
		10× concent.	Mengo	1600	n.d.
IFN-β2a gene (pSVIFA-2-I)	CHO-SI-15[a]	20× concent.	VSV	2000	6
IFN-β2b gene (IFA-11)	LI-39 (ind.)[b]	Unconcentrated	VSV	800	1000
IFN-β2b gene	LI-39 (ind.)[b]	Blue Seph. unbound	VSV	750	8
None	L-TK$^+$ (ind.)[b]	Unconcentrated	VSV	4	1500
None	Mouse IFN-α,β[c]		VSV	4	50 000
None	Human IFN-β1[c]		VSV	20 000	8

[a]Medium harvested with 1% serum from constitutively producing cells, concentrated and assayed on human FS11 or mouse L-929 cells as in Materials and methods.
[b]Medium from cultures induced by poly(rI)(rC), cycloheximide and actinomycin D as in Materials and methods, assayed directly or after passage through Cibacron-Blue−Sepharose (see Materials and methods).
[c]Standard preparation of NDV-induced mouse L cell IFN and recombinant human IFN-β1 produced in CHO cells.

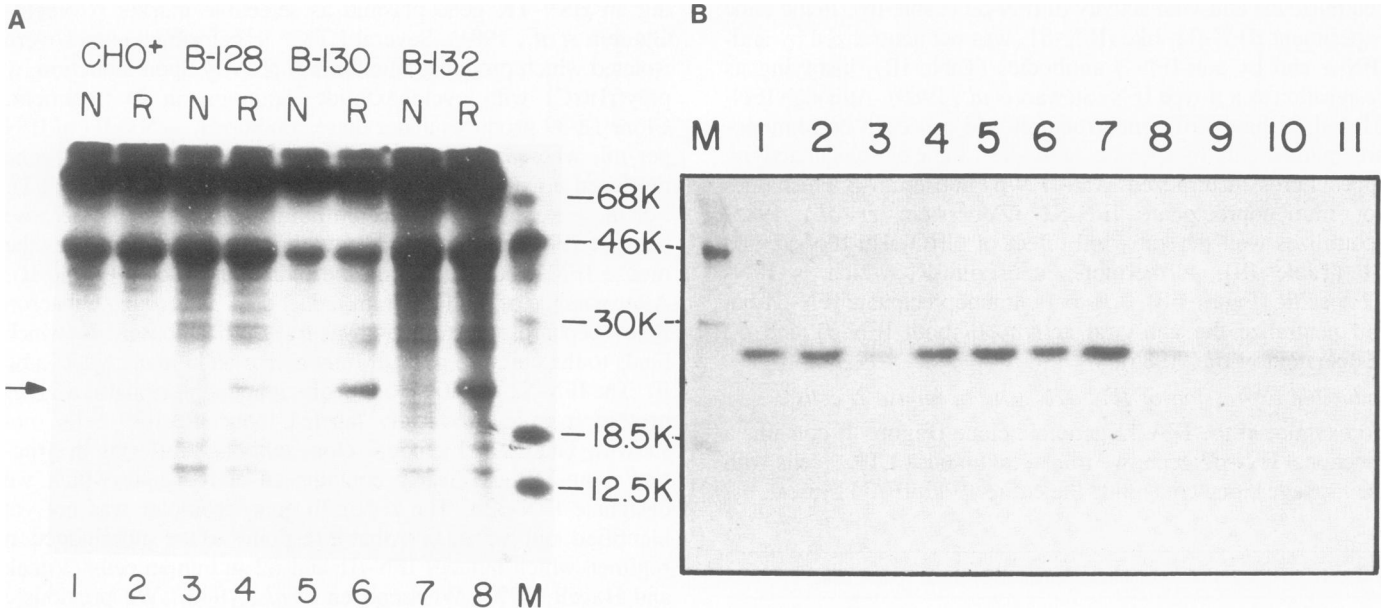


Fig. 6. (**A**) Immunoprecipitation of IFN-β2 secreted by [^{35}S]methionine-labeled CHO−SVCIFβ2 cells B-128, B-130 and B-132, by anti-IFN-β2 serum (R). Normal serum (N) and CHO−DHFR$^+$ cells (**lanes 1,2**) were used as controls. The SDS−polyacrylamide gel electrophoresis shows cell-secreted IFN-β2 (arrow) migrating as a 21−22 kd protein. M = mol. wt markers. (**B**) Immunocompetition assays of IFN-β2. The [^{35}S]methionine-labeled, *in vitro* translation IFN-β2 (26 kd) was immunoprecipitated either without cold competitor (**lane 1**), or: with 100 U rIFN-β1 (**lane 2**), with 1 U IFN-β2 from FS11 cells (**lane 3**), or with respectively 50 and 200 μl medium from untreated FS11 cultures (**lanes 4,6** and **5,7**), FS11 cultures pre-treated for 18 h by 4 U/ml rIL-1α (**lanes 8,9**), and FS11 cultures pre-treated by 200 U/ml nTNF-α (**lanes 10,11**).

Table III. Antibody neutralization of recombinant human IFN-β2

Human IFN	U/ml used	Polyclonal sera		Monoclonal antibodies		
		Anti-IFN-β1 serum B	Normal serum	Anti-IFN-β1 29AB1	Anti-IFN-α 9-3	Anti-IFN-γ 128-4
rIFN-β2						
B-131-5M	33	12 400	<6	400	<6	<6
SI-15	17	13 600	<6	3400	<6	<6
rIFN-β1	25	11 250	<6	900	<6	<6
rIFN-α2	50	<6	<6	<6	10 000	<6
rIFN-γ	25	150	150	300	6	38 000

Serial 2-fold dilutions of the antibodies were made in 96-well microplates and a constant amount of each IFN was added. After 1 h at 30°C, FS11 cells were added and the anti-viral assay continued as in Materials and methods. Neutralization titers were calculated as the product of the dilution of antibodies which inhibits by 50% the IFN protection against viral cytopathic effect, multiplied by the IFN titer used, determined in the same experiment. Antibodies and IFNs are described in Materials and methods. The rIFN-β2 was from concentrated medium of B-131-5M cells (8000 U/ml) or SI-15 cells (2000 U/ml) as in Table II.

tion of TNF-α for IFN-β2 induction (400 U/ml; 40 ng/ml) was higher than that required for the cytolytic effect on sensitive cells (0.1 ng/ml; Wang *et al.*, 1985) and even higher than that giving optimal growth stimulation of diploid fibroblasts (2 ng/ml; Kohase *et al.*, 1986). The onset of IFN-β2 secretion was somewhat faster with TNF-α reaching half maximal levels at ~6 h, versus 8−12 h with IL-1 (not shown).

Discussion

The IFN-β2 cDNA encodes a protein of 212 amino acids (23.7 kd) which is secreted from induced human cells as well as from the hamster cells transfected by the IFN-β2 cDNA as a smaller protein of 21 kd. Hydropathy plots indicate that the N-terminal region of IFN-β2 is hydrophobic from residue 1 to 32 and intracellular processing may involve removal of this region and glycosylation at one or both of the potential sites predicted by the sequence to form the 21-kd mature IFN-β2. Computer analyses reported elsewhere (Revel *et al.*, 1986) indicate that when aligned by their C termini, the IFN-β1 and β2 sequences show homology in their overall hydropathy profiles. Some sequence homology can be seen; in particular, about half of the 37 amino acid positions conserved in all type I human IFNs, are present in IFN-β2 (Figure 7). However, the overall homology is low (~20% at the protein level), and the presence of introns, as well as the different chromosomal gene location, emphasize the evolutionary distance between the IFN-β2 and the type I IFN cluster on chromosome 9 (reviewed in Revel, 1983). Nevertheless, gene and cDNA expression in transfected rodent cells demonstrates that the products of IFN-β2 genes have genuine human IFN activity which is neutralized by polyclonal and monoclonal antibodies to IFN-β1. We have previously discussed and excluded the possibility that IFN-β2 acts by inducing IFN-β1 or another IFN (Zilberstein *et al.*, 1985; Revel *et al.*, 1986). Briefly the evidence is based (i) on the absence of IFN-β1 RNA and

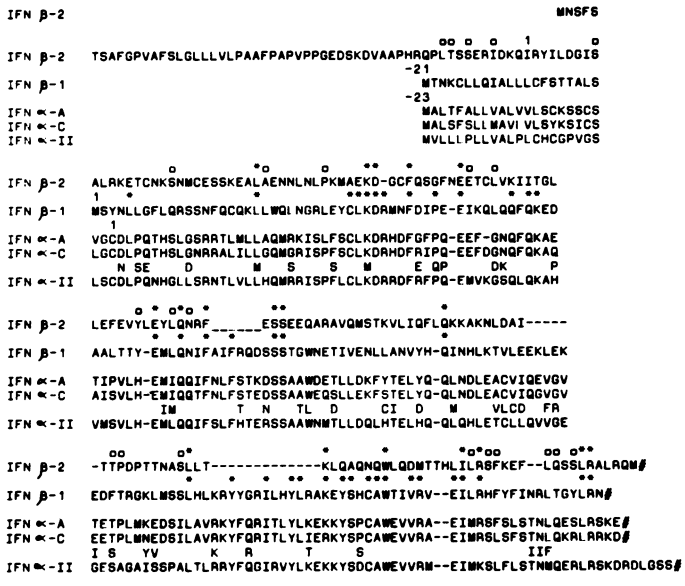


Fig. 7. Comparison of amino acid sequence of IFN-β2 with human type I IFNs. Amino acids conserved in all type I IFNs are marked by stars above the IFN-β1 sequence. Stars above IFN-β2 are positions conserved in all type I IFNs; squares are positions conserved in some IFNs. Sequences are from Weissmann (1981) and Revel (1983). A computer search among IFNs of human and animal origin identified only some homology with Bovine IFN-β1.

protein in IFN-β2-treated human cells, (ii) on the induction of an anti-viral effect in mouse−human hybrids containing only human chromosome 21 (and therefore lacking the IFN-β1 gene, but containing the human type I IFN receptor gene), and (iii) on the induction by rIFN-β2 of (2′−5′) oligo(A) synthetase and HLA mRNAs even in the presence of concentrations of cycloheximide which would block the synthesis of an eventual intermediate protein needed for induction by IFN-β2 of these typical IFN-activated genes (Revel and Chebath, 1986). Therefore, the neutralization of rIFN-β2 by anti-IFN-β1, and the converse neutralization of IFN-β1 by anti-IFN-β2 (Zilberstein *et al.*, 1985) probably reflect some common structural feature, possibly in the active site of the two proteins. Neutralization has been the basis for IFN classification (Stewart *et al.*, 1980). The absence of neutralization by anti-IFN-α or γ indicates that, in this respect as well as in its fibroblast origin, IFN-β2 is closer to IFN-β1 than to the other types of human IFN. However, by its structure and gene organization, it is a distinct species.

The biological significance of IFN-β2 lies most probably in the fact that it is induced under conditions where IFN-β1 is not induced, as in metabolically stressed cells (Zilberstein *et al.*, 1985). Most important is the observation that TNF induces IFN-β2 in fibroblasts and that the proliferation of these cells which is stimulated by TNF, is further enhanced if anti-IFN-β antibodies are added (Kohase *et al.*, 1986). A number of cell lines undergoing differentiation, have been shown to produce autocrine IFN-β species (defined by antibody neturalization) and, in human U937 cells, these IFNs are responsible for the induction of HLA antigens on these cells (Friedman-Einat *et al.*, 1982; Yarden *et al.*, 1984). In a mouse myeloleukemic cell line induced to differentiate by CSF-1, the growth-arrest of the cells which characterizes terminal differentiation, is abolished by anti-INF-β antibodies (Resnitzky *et al.*, 1986). IL-1 stimulates growth of several cell types (Oppenheim *et al.*, 1986) and the classical growth factor platelet-derived growth factor (PDGF) was found to induce IFN-β RNA in mouse cells (Zullo *et al.*, 1985). Although multiple IFN-β species may be involved (Sehgal, 1982; Revel, 1983), IFN-β2 appears to be one of these autocrine autoregulators of cell growth produced in response to growth factors. This would be in line with the low specific activity of IFN-β2 (which we estimated by comparison of immunoassay and anti-viral activity to be 50−100 times lower than the 3×10^8 U/mg of IFN-β1), since only very small anti-viral activity is seen in cells producing the autocrine IFN-β species (Revel, 1983; Kohase *et al.*, 1986). This could also explain why the hamster cells expressing IFN-β2 cDNA produce less IFN activity than similar CHO cells harbouring the human IFN-β1 gene (Chernajovsky *et al.*, 1984). Interestingly, the IFN-α1 (D) species has also a 100 times lower specific activity on human cells than other IFN-α species (Goren *et al.*, 1983), although it represents a large proportion of the total mass of leukocyte IFN (Weissmann, 1981). It is not excluded that some of the multiple IFN functions will be expressed more efficiently by IFN-β2 than the anti-viral effect. Despite their low concentration, growth-regulatory IFN-β induce HLA and (2′−5′) oligo(A) synthetase more strongly than expected, have more prolonged effects and markedly inhibit the growth of the cells which produce them (Resnitzky *et al.*, 1986; Kohase *et al.*, 1986). The induction of IFN-β2 by IL-1 and TNF suggests that it may play a role as an autocrine mediator of some effects of these cytokines in inflammation and acute phase responses, as well as regulate cell proliferation.

Materials and methods

IFN-β2 expression plasmid constructs

DNA constructions and preparations were made using standard procedures (Maniatis *et al.*, 1982). Isolation of IFN-β2 cDNA clones A341 and E474 was described previously (Weissenbach *et al.*, 1980). The overlapping inserts (Figure 1) in counterclockwise orientation in the *Pst*I site of pBR322, were recombined through their internal *Xba*I site by ligating the *Eco*RI−*Xba*I fragment of E474 to the *Xba*I−*Eco*RI fragment of A341 producing clone AE20. Sequencing was done as outlined in Figure 1A by the methods of Maxam and Gilbert (1980). The genomic clones IFA-2 and IFA-11 were isolated by screening, with *in situ* hybridization to A341 cDNA, a library of human adult blood cell DNA partially cut by *Eco*RI and cloned in λ Charon 4A, as detailed previously (Mory *et al.*, 1981). The *Xho*I−*Bam*HI segment of the IFA-2 phage DNA insert, from coordinate 5.2 kb to 7.1 kb in Figure 2, was fused with a *Cla*I−*Xho*I synthetic adaptor of 26 bp (restoring nucleotides 11−32 of the cDNA in Figure 1B), and the resulting *Cla*I−*Bam*HI segment was purified after subcloning in a *Cla*I/*Bam*HI-cut pBR plasmid. The *Bam*HI−*Hin*dIII segment of IFA-2 DNA from coordinates 7.1 to 10 kb in Figure 2, subcloned in a *Hin*dIII/*Bam*HI-cut pBR, was excised as a *Bam*HI−*Cla*I fragment using the *Cla*I site adjacent to *Hin*dIII in pBR322, and ligated by its *Bam*HI site to the above *Cla*I−*Bam*HI 5′ IFA-2 segment. The *Cla*I−*Cla*I segment now containing the entire 4.8-kb IFN-β2a gene was cloned in the SV40-derived pSVE3 vector, cut by *Hin*dIII as previously detailed (Chernajovsky *et al.*, 1984) and religated with synthetic *Cla*I linkers. The resulting pSVIFA-2-II contains the IFN-β2 gene in the *Cla*I site of this modified pSVE3, oriented as the SV40 early promoter. To obtain pSVCIFβ2 of Figure 5, pSVIFA-2-II was cut with *Xba*I and then partially digested with *Xmn*I to open the *Xmn*I site 60 bp after *Xho*I in the cDNA (Figure 1B) but not the *Xmn*I site in the amp^R gene of pSVE3. The *Xmn*I−*Xba*I segment of AE20 cDNA (92−566 in Figure 1B) was then ligated in the above *Xmn*I/*Xba*I-cut pSVIFA-2-II restoring the uninterrupted IFN-β2a cDNA sequence of Figure 1B but followed by the IFA-2 gene's own polyadenylation site. In the resulting pSVCIFβ2 plasmid (Figure 5) the IFN-β2a sequence follows the SV40 early promoter and the first 60 nucleotides of T-ag mRNA, while it precedes the T-ag mRA splicing region and polyadenylation site. The pSVIFA-2-I expression vector was constructed as pSVIFA-2-II above, but in the first step the *Xho*I site of IFA-2 was fused directly to the *Cla*I site of pBR322 (which is lost), the neighbouring *Eco*RI of pBR322 being fused after filling-in to the *Hin*dIII site of pVE3. Thus, in pSVIFA-2-I, the first 60 nucleotides of the T-ag RNA are linked to the *Xho*I site of the IFN-β2a gene by the 25-bp *Eco*RI−*Cla*I sequence form pBR322.

DNA transfections and assay of rHuIFN-β2 activity

CHO-K1 clone DXB11 cells, lacking dihydrofolate reductase (Urlaub and Chasin, 1980) were grown and used for DNA transfections as detailed before (Cherna-

jovsky *et al.*, 1984). Briefly, 10^6 cells were seeded in 9 cm plates with F-12 medium (Gibco), 10% fetal calf serum (FCS). After 2 days, the cells were transfected with plasmid pSVCIFβ2 DNA, 24 μg, mixed with mpSV2DHFR DNA, 2.5 ug (Subramani *et al.*, 1981) in a 10:1 molar ratio, by the calcium phosphate precipitation procedure. DHFR$^+$ clones were selected in DMEM medium with dialyzed serum, isolated after 12–15 days and subjected to further selection for methotrexate (MTX) resistance as before (Chernajovsky *et al.*, 1984). Cell clones were screened for constitutive IFN-β2 secretion by immunocompetition (see below) and biological assay in the medium of confluent cultures in DMEM, 150 μg/ml proline, 10% FCS, harvested 24 h after medium change. If needed, medium of cultures with 1% dialyzed FCS was concentrated by vacuum dialysis or with polyethylene glycol 20 000. Clone B132 was one of a dozen pSVCIFβ2 DNA transformants producing IFN-β2. Clone SI-15 was isolated from a similar transformation with pSVIFA-2-II DNA. CHO$^+$ clones transformed with the DHFR plasmid alone were used as controls. Interferon antiviral activity was assayed by 2-fold serial dilutions of each medium sample (50 μl) in 96-well microplates (Nunc) into which 4×10^4 human foreskin diploid fibroblasts (below passage 14) were seeded per well in 50 μl MEM, 2% FCS, 0.5% gentamycin. After 16–20 h at 37°C, medium was removed and each well infected by VSV 1 p.f.u./cell in the same medium. The last dilution inhibiting the cytopathic effect was recorded by microscopic observation from 24 to 48 h and by crystal violet staining. The assay was calibrated against the international IFN-β reference standard G-023-901-527 provided by the NIH (Bethesda, MD, USA), and a laboratory standard of rIFN-β1 (Chernajovsky *et al.*, 1984). When Mengo virus was used instead of VSV, the assay was read at 30 h post-infection. IFN activity was also assayed by measuring the induction of (2′-5′) oligo(A) synthetase in human FS11 cells exposed for 16 h to rHuIFN-β2. The enzyme levels were determined in Nonidet P-40 extracts bound to poly(rI)(rC)–agarose beads as previously detailed (Revel *et al.*, 1981).

The IFA-11 phage DNA, 4.5 μg, was co-transfected into mouse LTK$^-$ cells (Wigler *et al.*, 1979) with 1.5 μg pAGO DNA containing the HSV-1 TK gene (Colbere-Garapin *et al.*, 1981). Cells transformed by pAGO DNA alone were isolated as LTK$^+$ controls. Confluent monolayers of such clones were treated wtih poly(rI).(rC) 25 μg/ml, DEAE–dextran 500 μg/ml and cycloheximide 50 μg/ml for 8.5 h at 37°C. Actinomycin D was added during the last hour. The cells were thoroughly washed and incubated for 20 h in DMEM, 1% FCS. The media were harvested and assayed for IFN as above.

Transcriptional–translational analysis of IFN-β2 cDNA

The pGEM-1 vector (Promega Biotec) was used to fuse the IFN-β2 cDNA to the T7 RNA polymerase promoter. pG2 was constructed by cloning the pSVCIFβ2 *Cla*I fragment (Figure 5) in the *Acc*I site of pGME-1. For pG3, the *Xho*I–*Hin*dIII fragment of pSVCIFβ2 was cloned, after filling-in the *Xho*I site, in *Sma*I/*Hin*dIII-cut pGEM-1. pG16 was derived from pG2 by cutting-out the 5′ half of the cDNA with *Sma*I and *Xba*I, recutting this 5′ half at the single *Dde*I site and religating after filling-in the *Dde*I site into the *Sma*I/*Xba*I-cut pG2. pGB1 was similarly constructed by cutting at the *Xmn*I site of the cDNA. Plasmid DNAs were prepared and linearized by various restriction enzymes as in Figure 4, and 0.4 μg DNA in H_2O were transcribed in 20 μl reactions containing 4 μl buffer (200 mM Tris-HCl, pH 7.6, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 2 μl of 100 mM dithiothreitol, 0.8 μl RNasin (Promega Biotec), 1 μl each of 2.5 mM ATP, GPT, CTP, UTP and 4 units T7 RNA polymerase (Promega Biotec). After 120 min at 40°C, 0.4 units DNase (RNase-free) were added for 15 min at 37°C and the RNA was extracted with phenol/chloroform and ethanol precipitated. *In vitro* translation was carried out in 12.5 μl reactions containing 3 μl of micrococcal nuclease-treated rabbit reticulocyte lysate (Pelham and Jackson, 1976), 20 μCi [^{35}S]methionine (1000 Ci/mmol, Amersham) with 20 mM Hepes buffer pH 7.6, 68 mM K acetate, 0.05 mM $MgCl_2$, 0.4 mM spermidine, 0.25 mg/ml creatine kinase, 8 mM creatine phosphate, 0.05 mg/ml rabbit liver tRNA, 25 μM each of 19 amino acids minus methionine, and 0.05 μg of *in vitro* transcribed RNA or 0.1 μg poly(A)$^+$ RNA from FS11 cells. After 1 h at 30°C, 1–2 mM unlabeled methionine was added and the samples subjected to SDS–polyacrylamide gel electrophoresis or immunoprecipitation as below.

Immunoassay of IFN-β2

The IFN-β2-specific R-antibodies were obtained by immunizing rabbits with native IFN-β2 which was prepared from crude IFN-β, from primed and superinduced FS11 fibroblasts (Weissenbach *et al.*, 1979; Nir *et al.*, 1985), by fractionation on Cibacron Blue F3G-A Sepharose CL-6B (Pharmacia). The unbound fraction passed four times on Blue Sepharose in phosphate-buffered saline pH 7.4, and which contained ~5% of the IFN activity (IFN-β1 being retained on Blue Speharose) was concentrated on CM–Sephadex, eluted by 0.2 M NaCl and further purified by chromatography on DEAE–Sephadex from which it elutes at 75–150 mM NaCl free of any IFN-β1 (Zilberstein *et al.*, 1985). About 20 μg of this protein fraction (10^6 units IFN/mg) was injected s.c. to rabbits with 50 μg rabbit serum albumin in complete Freund's adjuvant (Difco). Injections were repeated every 3–5 weeks in incomplete Freund's adjuvant and sera, prepared 6–9 days after the third and following injections, were tested for the specific immunoprecipitation of the 23–26 kd *in vitro* translation product of IFN-β2 mRNA. Poly(A)$^+$ RNA was prepared from primed–superinduced FS11 cells (Weissenbach *et al.*, 1979, 1980; Nir *et al.*, 1985) and translated in reticulocyte lystates with [^{35}S] methionine. The translation products were immunoprecipitated with saturating concentrations of R-antiserum or 25 μl non-immune serum per 50 μl reactions, using Protein A–Sepharose CL-4B beads. After transfer of the washed beads to a fresh tube, the proteins eluted by boiling for 5 min in 3% SDS, 2% β-mercaptoethanol were run in 15% polyacrylamide slab gels in SDS followed by autoradiography. For immunocompetition assays, the ^{35}S-labeled *in vitro* translation products were mixed with amounts of R-antibodies 3- to 5-fold below saturation, and the unlabeled samples were added before Protein A–Sepharose. After SDS–polaycrylamide gel electrophoresis, the relative intensity of the 23–26 kd IFN-β2 band was quantitated by scanning the X-ray film in a Gilford spectrophotometer. For immunoprecipitation of cell-produced IFN-β2, cultures were metabolically labeled for 16 h with [^{35}S]methionine (100 μCi/ml, 1000 Ci/mmol) in methionine-free medium, 5% dialyzed FCS.

Neutralizing monoclonal antibodies obtained against human IFN-β1 (Novick *et al.*, 1983b), IFN-α (Novick *et al.*, 1982) and IFN-γ (Novick *et al.*, 1983a) were used. Polyclonal rabbit antiserum B was raised against purified IFN-β1 (Zilberstein *et al.*, 1985). Pure recombinant human IFNs were as described from CHO cells for IFN-β1 (Chernajovsky *et al.*, 1984) and for IFN-γ (Novick *et al.*, 1984), and from *Escherichia coli* for IFN-α2 (gift of Dr C.Weissmann) purified as in Novick *et al.* (1984). Purified recombinant *E. coli*-derived human IL-1α (3×10^7 U/mg) was kindly provided by Drs P.T.Lomedico and A.Stern (Hoffmann-La Roche). Recombinant TNF-α (10^7 U/mg), produced as in Wang *et al.* (1985), was kindly provided by Dr L.S.Lin (Cetus Corporation).

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