The EMBO Journal Vol.2 No.3 pp.325–332, 1983

Sequences involved in the regulated expression of the human interferon-β_1 gene in recombinant SV40 DNA vectors replicating in monkey cells

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Communicated by Y. Groner
Received on 11 November 1982; revised on 7 January 1983

The human genomic *Eco*RI fragment of 1.83 kb containing the interferon (IFN) gene IFN-β_1 with 285 nucleotides of 5′-flanking sequences was transfected into monkey kidney CV-1 cells as part of an SV40-pML2 vector. Induction of the monkey cells to produce IFN led to a rapid accumulation of IFN-β_1 RNA whose 5′ ends were identical to the IFN-β_1 mRNA of human fibroblasts. This induction occurred with all recombinants tested. Expression from the SV40 late promoter was also seen in non-induced cells. We conclude that the regulation of the IFN-β_1 gene is retained in the replicating episomal SV40 vectors with high copy number, even when the gene is being transcribed from an external promoter. When the 5′-flanking sequences were deleted to leave only 40 bp before the presumed cap site of the IFN-β_1 gene, inducible formation of IFN-RNA with authentic 5′ ends could still be demonstrated. However, inducibility and expression depended on the position of the deleted IFN-β_1 gene in the vector. We conclude that the sequences around the TATAA box and cap site on the IFN gene are involved in the regulation of its expression. Regulated short-term expression of the human IFN-β_1 gene in SV40 vectors provides a defined system in which the structures required to maintain the regulation and the influence of known external transcription signals can be examined.

Key words: gene expression/interferon induction/regulatory sequences/SV40 vector

Introduction

The human interferon (IFN) type I genes form a family of closely regulated genes which are not expressed unless cells have been exposed to double-stranded (ds) RNA, viruses or other IFN inducers (reviewed by Gordon and Minks, 1981). The genes coding for several leucocyte IFN-α species (for review, see Weissman, 1981) and for the major fibroblast IFN-β_1 species (Lawn *et al.*, 1981; Degrave *et al.*, 1981; Ohno and Taniguchi, 1981; Gross *et al.*, 1981) have been isolated and sequenced. These genes lack introns and can be expressed in bacteria (Nagata *et al.*, 1980; Mory *et al.*, 1981). When transferred into heterologous cells by the gene co-transformation procedure (Wigler *et al.*, 1979), several human IFN genes were shown to retain their inducibility by IFN inducers in the stable mouse cell clones isolated (Ohno and Taniguchi, 1982; Mantei and Weissmann, 1982; Reyes *et al.*, 1982; Hauser *et al.*, 1982; Canaani and Berg, 1982). The use of stable co-transformants suffers, however, from the drawback that the time required to select transformants may allow DNA rearrangements and unknown interactions with host genes, and that the genes may be integrated in different sites of the host genome in each transformant (Scangos and Ruddle, 1981). Regulated expression of the IFN genes was also shown by stable transformation with an extrachromosomal vector such as bovine papillomavirus (Zinn *et al.*, 1982; Mitrani-Rosenbaum *et al.*, 1982).

A more direct way of studying cloned genes is by transient expression after transfection as part of an episomal replicating vector such as SV40 (Mulligan *et al.*, 1979; Hamer and Leder, 1979; Mellon *et al.*, 1981).

Genes introduced in SV40-plasmid vectors are efficiently expressed, but regulation is often not apparent (Elder *et al.*, 1981; Gluzman, 1982). Recently, cadmium regulation of the metallothionein gene in SV40 vectors has been demonstrated (Hamer and Walling, 1982). Gheysen *et al.* (1982) have expressed human IFN-β_1 cDNA linked to the SV40 late promoter, but did not study regulation. We demonstrate here that the IFN-β_1 gene retains its regulation by ds RNA when replicating in multiple copies in an SV40 vector transfected into monkey cells. The DNA sequences involved in expression and regulation of the IFN-β_1 gene were studied by comparing deleted constructions and investigating the role of known transcription elements present on the vector.

Results

Inducible expression of the human IFN-β_1 gene on a replicating episomal SV40 vector

The SV40 vector (pMSV1472) was constructed (Figure 1) by cloning a 3056 bp long fragment of SV40 DNA, through *Eco*RI linkers, in *Escherichia coli* plasmid pML2 which allows the replication of SV40 DNA in eukaryotic cells (Lusky and Botchan, 1981). The viral DNA fragment starts at the single *Hpa*II site of SV40 at 0.726 map units (Tooze, 1980), just downstream from the major late mRNA starts (Elder *et al.*, 1981), it contains the SV40 origin of replication (0.67 map units), the entire early region and ends at 0.143 map units (*Bam*HI site). The human IFN-β_1 gene was isolated as a 1.83-kb *Eco*RI fragment, subcloned in pBR322 from the DNA of lambda Charon 4A genomic clone IFN-C15 (Mory *et al.*, 1981), which had been derived from a human adult DNA library. This 1.83-kb fragment (Figure 2) contains a 5′-flanking region of 357 bp upstream from the initiator ATG. The 5′ end of IFN-β_1 mRNA from human fibroblasts (presumed cap site) has been estimated to map ~70 nucleotides upstream from the ATG codon (Ohno and Taniguchi, 1981; Degrave *et al.*, 1981). The IFN-β_1 gene fragment also contains a 700 bp long 3′-flanking region following the polyadenylation site. In the first experiments, the entire 1.83-kb IFN-β_1 *Eco*RI fragment was ligated to the SV40-pML2 vector, giving rise to four different orientations (pSVIF-R series, Figure 1A). In another construction (pSVIF-K series), the *Bam*HI-cut pML2 DNA was inserted through *Kpn*I linkers in the *Kpn*I site of SV40, 50 bp upstream from the *Hpa*II site (Figure 1B). The recombinant pSVIF-R and K plasmids were cloned in *E. coli* and used to transfect the monkey kidney cell line CV-1, by calcium phosphate co-precipitation (Wigler *et al.*, 1977), with an efficiency of 10–20%, as estimated at

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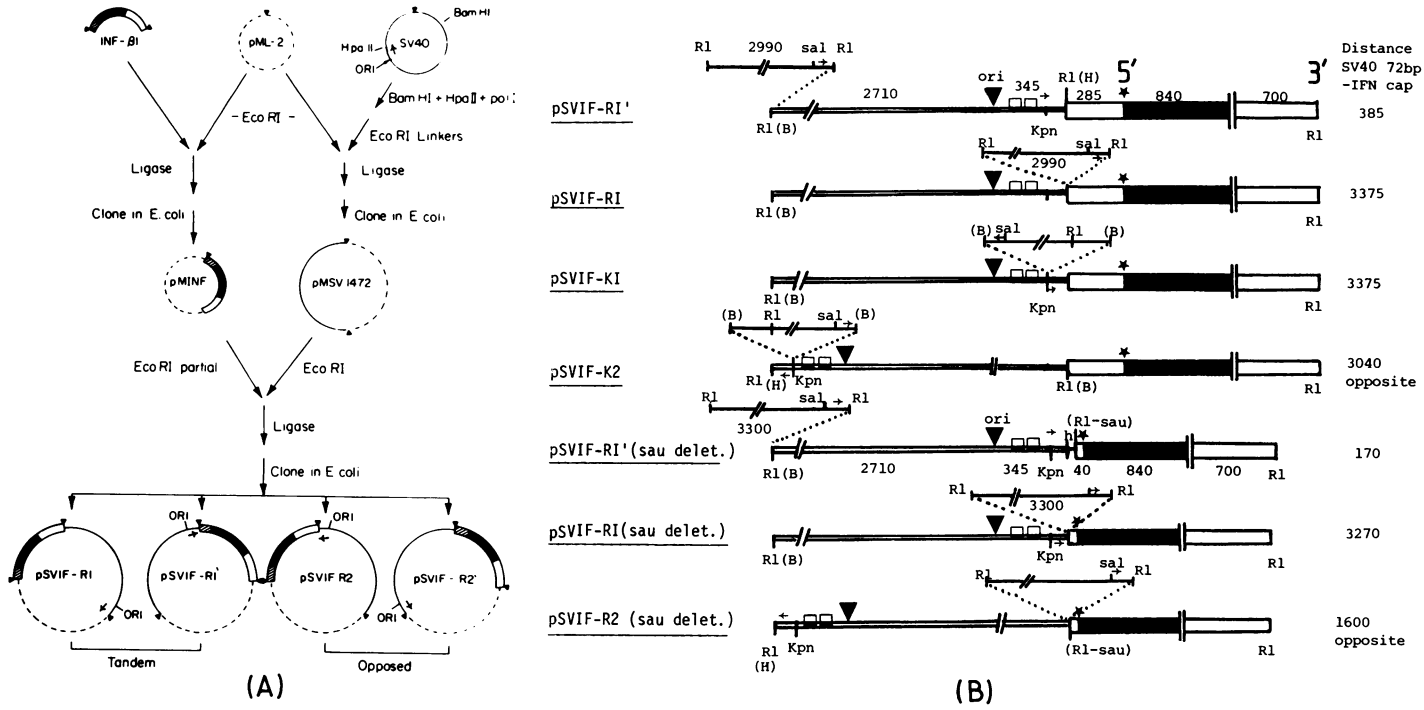

Fig. 1. (A) Construction of the SV40-IFN-β_1 recombinants, pSVIF-R series. The human IFN-β_1 1.83-kb *Eco*RI genomic fragment is shown (double line) with its three regions: hatched areas = 5'-flanking sequences, black area = coding sequences, open areas = 3'-flanking sequences. Plasmid pML2 is shown by dashed lines and SV40 by the solid lines. The origin of DNA replication is shown (ORI). The *Eco*RI sites are shown by black triangles. The arrows indicate the site and orientation of the SV40 late promoter. Construction of the recombinants in the four possible orientations is described in Materials and methods. The tandem orientation refers to the same orientation of SV40 late transcription and of the IFN-β_1 gene. Construction of the pSVIF-K series is described in Materials and methods. **(B)** Structure of the SV40-IFN-β_1 recombinants. The IFN-β_1 gene is shown by boxed line, the transcribed region is in black and the flanking regions white; the star shows the IFN RNA start site (presumed cap site). The SV40 fragment is shown by the double lines; the triangle indicates the origin of replication and the white square the 72-bp repeat. The arrow shows the SV40 late promoter direction. Plasmid sequences are indicated by single lines; for the first four recombinants, pML2 was used; for the three deleted recombinants, pSVOD was used (see Materials and methods). The arrow shows the transcription initiation site of pBR322. Abbreviations: RI = *Eco*RI; B = *Bam*HI; Sau = *Sau*96-1. In pSVIF-RI' (*Sau* deleted), a 26-nucleotide *Cla*I-*Eco*RI fragment of pBR322 connects the SV40 *Hpa*II site (h) to the IFN *Sau*96-1 site (RI-Sau). The sequence around the *Sau*96-1 site is shown in Figure 4.

50 – 60 h by the number of SV40 T-antigen positive cells. Expression of the IFN-β_1 gene was studied 50 h after the beginning of transfection.

The single-stranded (ss) nuclease mapping method (Berk and Sharp, 1977) was used to assay for the presence, in transfected cells, of RNA transcripts initiated at the cap site of the IFN-β_1 gene. As probe, we used a 545-bp DNA fragment of the cloned gene comprising 100 bp from pBR322, the entire 5'-flanking region of IFN-β_1 and part of the coding region into the first *Hinf*I site (Figure 2). The probe was ^{32}P-labeled at the 5' end of the *Hinf*I site, denatured and annealed to total RNA extracted from the transfected cells. The hybrids were digested by either S1 or mung bean nuclease (Green and Roeder, 1980) and analyzed by a polyacrylamide sequencing gel. RNA extracted from human diploid fibroblasts superinduced for IFN production (Weissenbach *et al.*, 1980) protects several fragments of ~160 nucleotides against ss nuclease digestion (Figure 3). By comparison with the sequence in Figure 3, several 5' end nucleotides were identified (arrows, Figure 2 insert). This may be an artifact of the nuclease technique, but as it was observed with both S1 and mung bean nucleases, it may reflect heterogeneity of the 5' ends as found for other mRNAs in which initiation was shown to occur at closely situated capping sites (Canaani *et al.*, 1979; Gidoni *et al.*, 1981).

The first question we investigated was whether the IFN-β_1 gene in the SV40-pML2 vector retains its normal expression and inducibility. Cultures of CV-1 cells transfected for 50 h with pSVIF-R1' DNA (Figure 1) were induced for 4 h with poly(rI)(rC), DEAE-dextran and cycloheximide, or left uninduced. Ss nuclease analysis showed that RNA from the induced transfected cells protected the same 160-nucleotide fragment, as authentic IFN-mRNA from human cells (Figure 2B). When, however, the CV-1 cells transfected for 50 h were not induced, very little, if any, protection of the 160 fragment was seen (Figure 3A, B and Figure 5, lane 1). In cells transfected by the SV40-pML2 vector without the IFN-β_1 gene, induction did not reveal any RNA which protects the 160-nucleotide fragment (Figure 3, lane 10), indicating that the monkey IFN-mRNA, if present, does not interfere in the ss nuclease assay. Four different plasmids containing the IFN-β_1 gene in various orientations (Figure 1) were compared in the experiment of Figure 3. In all of them, IFN-RNA with authentic 5' ends was produced only after induction by poly(rI)(rC). When transfected cells were treated within the last 24 h with cytosine arabinoside (ara C, 20 μg/ml) which inhibits the replication of the SV40 vector, the amount of IFN-RNA was reduced to very low levels indicating that induction did occur on the replicating SV40 IFN-β_1 DNA (experiments not shown).

Inducibility of the transfected human IFN-β_1 gene could also be demonstrated by measuring the amount of biologically active IFN produced. As induced CV-1 cells produce monkey IFN which is active on human cells (Yakobson *et al.*, 1979) some anti-viral activity was observed in the non-transfected cells. Nevertheless, the IFN activity in the culture

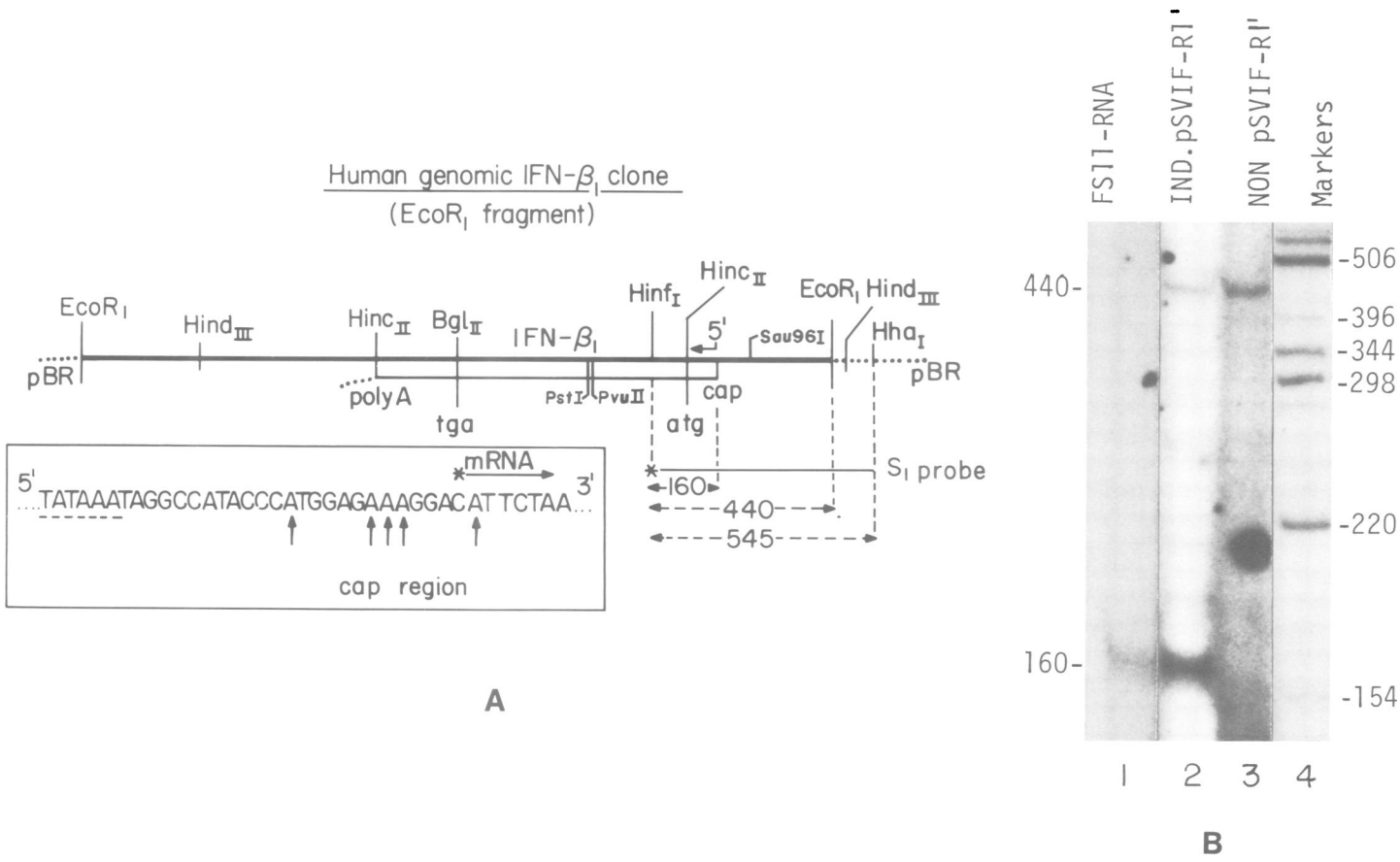

Fig. 2. (A) Restriction map of IFN-β_1 genomic fragment and S_1 probe. The 1.83-kb *Eco*RI fragment is shown in the orientation in which it is inserted in the pBR322 sequences of clone IFN-C631. The box represents the sequences of IFN-β_1 mRNA (Ohno and Taniguchi, 1981). The *Hinf*1-*Hha*I fragment of 545 bp used for ss nuclease analysis is shown as well as the expected fragments protected by cap-initiated authentic IFN-β_1 mRNA (160) and by read-through RNA (440). The insert shows the nucleotide sequence of this fragment around the cap site of IFN-β_1 mRNA, from the gel in Figure 3A. The arrows indicate the 5' ends of IFN-β_1 mRNA deduced from the analysis of Figure 3A. The Hogness box is underlined. The asterisk designates the cap site proposed by Degrave *et al.* (1981). **(B)** Ss nuclease analysis of IFN-β_1 mRNAs in transfected cells. CV-1 cells transfected with pSVIF-RI' DNA were poly(rI)(rC)-induced at 50 h for 4 h (IND) or left uninduced (NON), and RNA was prepared as in Materials and methods. S1 nuclease analysis was carried out as in Figure 3A, a partial *Hinf*1 digest of pBR322 DNA served as markers. RNA from induced cells protected both the 440 and 160 DNA fragments.

medium of CV-1 cells transfected with pSVIF-RI' or pSVIF-K2 plasmids, 24 h after induction, was much higher than with cells transfected by the pMSV1472 vector without the IFN-β_1 gene (Table I). Accumulation of this IFN activity was dependent on poly(rI)(rC) induction. Non-induced cells transfected by pSVIF-RI' showed, in addition, some level of constitutive IFN production, but cells transfected by other recombinant plasmids such as pSVIF-K2, did not produce IFN without induction (Table I). This constitutive expression will be discussed below.

Study of the IFN-β_1 gene with deletions in the 5'-flanking region

The main advantage of the study of gene expression in episomal vectors is that it allows comparison of modified genes under defined conditions of short term expression. The 5'-flanking region of the IFN-β_1 gene in the 1.83-kb fragment contains, before the cap site, ~285 nucleotides in which several potential transcription signals can be recognized (Degrave *et al.*, 1981). As a first step in the investigation of the function of these 5'-flanking sequences in the expression of the gene and its inducibility, we constructed deletions which retain only 40 bp upstream from the cap site. For this purpose, the 5'-proximal *Eco*RI-*Sau*96-1 fragment of the IFN-β_1 gene was excised. This leaves only 10 nucleotides before the TATAA box (Figure 4), but removes the other signals proposed by Degrave *et al.* (1981). The deleted IFN-β_1 gene was introduced into the SV40 vector in three different orientations, corresponding to pSVIF-RI', pSVIF-RI and pSVIF-R2 shown in Figure 1, and were used for transfection experiments in CV-1 cells as above. The results depended on the construction used. In the case of pSVIF-RI' (*Sau*96 deletion), in which the gene was brought very close to the SV40

Table I. IFN production by transfected monkey CV-1 cells

CV-1 cells transfected by:	IFN activity on human cells U/ml	
	Non-Induced	Induced
Vector pMSV1472	4	100
pSVIF-RI'	130	500
pSVIF-K2	4	500
pSVIF-RI' (*Sau*96 deletion)	100	200
pSVIF-R2 (*Sau*96 deletion)	10	200

CV-1 cells transfected for 50 h by the indicated recombinant DNA (see Figure 1B), was induced for 4 h or left uninduced as described in Materials and methods. IFN anti-viral activity in the medium was measured on human diploid fibroblasts FS11 by inhibition of the VSV cytopathic effect, 20 h after induction.

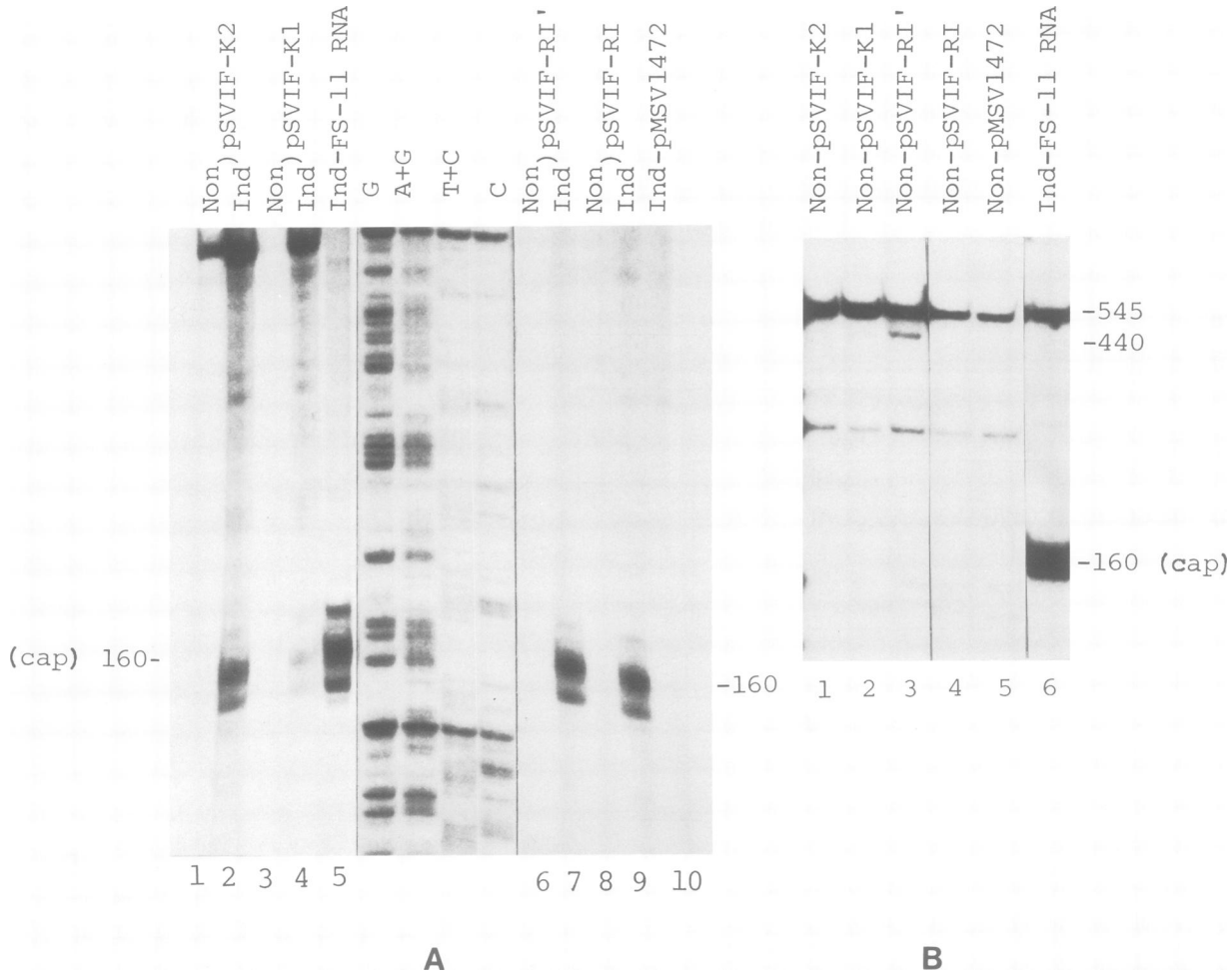

Fig. 3. Ss nuclease analysis of IFN-β_1 RNAs in induced and non-induced transfected CV-1 cells. **(A)** Preparation of the 5′ end labeled [^{32}P]DNA *Hinf*I-*Hha*I probe is described in detail in Materials and methods and Figure 2A. The CV-1 cells transfected for 50 h by the indicated recombinant DNAs (see Figures 1B) were either induced during 4 h from IFN production (IND) or left non-induced (NON). Total CV-1 cell RNA was annealed to the probe and the hybrids were digested with mung bean nuclease and analyzed on a sequencing gel as described in Materials and methods. Each slot represents 50 μg of total RNA hybridized to 2 x 10^4 c.p.m. of the [^{32}P]DNA fragment (10^6 c.p.m./μg DNA). FS11 RNA **(lane 5)** is RNA from the same number of diploid foreskin cells FS11, superinduced to produce IFN-β_1 mRNA (Weissenbach *et al.*, 1980). The [^{32}P]*Hinf*I-*Hha*I probe was subjected to nucleotide-specific degradation (four centre lanes) according to Maxam and Gilbert (1980). This allows the positions of IFN-β_1 5′ ends on the IFN-β_1 gene sequence shown in Figure 2A to be determined. **(B)** RNA was from non-induced transfected CV-1 cells analyzed as in **(A)** with the exception that hybrids were digested with S1 nuclease and the gel electrophoresis was run for a shorter time. RNA from uninduced cells transfected by pSVIF-RI′ protects the 440 bp long fragment corresponding to the entire 5′ flank of the IFN-β_1 gene. (The 300-bp band found with RNA from all clones including pMSV1472 which does not contain the IFN-β_1 gene is probably an artifact of the S1 methods.)

late promoter region (Figure 1), IFN-RNA with authentic 5′ ends appeared in both induced and non-induced cells (Figure 5, lanes 3 and 4) in contrast to the non-deleted gene (Figure 5, lanes 1 and 2) where it was clearly induced. In the case of pSVIF-R2 (*Sau*96 deletion) where the IFN-β_1 gene cap site is oriented opposite ~1600 bp away from the SV40 late promoter region, inducible expression was retained (Figure 5, lanes 5 and 6). In the case of pSVIF-RI (*Sau*96 deletion) where the IFN cap site is >3000 bp away from the SV40 promoter region, expression of IFN-RNA starting at the cap site was markedly reduced and induction was practically lost (Figure 5, lanes 7 and 8). These results should be compared to those of Figure 3, in which the intact 1.83-kb IFN-β_1 gene could be induced in all the orientations studied.

Measurements of IFN anti-viral activity on human cells (Table I), confirmed that the 5′-deleted gene is inducible in pSVIF-R2 (*Sau*96 deletion) and shows some constitutive expression when in pSVIF-RI′ (*Sau*96 deletion). The pSVIF-RI (*Sau*96 deletion) produced much less IFN activity than the two other constructions (not shown).

IFN-RNA transcripts starting outside the gene.

We also studied the formation of RNA transcripts which, by ss nuclease mapping, do not have authentic 5′ ends and are probably initiated at promoters outside the IFN-β_1 gene. In the experiments with the intact 1.83-kb gene, an RNA starting upstream from the gene and spanning the entire 5′-flanking region, would protect a 440-nucleotide fragment of the ss nuclease probe (from *Eco*RI to *Hinf*I, Figure 2). This 440-band was seen (Figure 3B) in non-induced CV-1 cells transfected by pSVIF-RI′ DNA, in which the IFN gene is close to the SV40 late promoter and in the same orientation. This 440-band was, however, not seen in the other construction tested, in which the IFN-β_1 gene is separated from the SV40 promoter by the pML2 sequences, or is in the opposite orientation (Figure 3B). Induction of pSVIF-RI′-transfected cells, had no significant effect on the amount of RNA protection of the 440-fragment (Figure 5, lanes 1 and 2). In induced

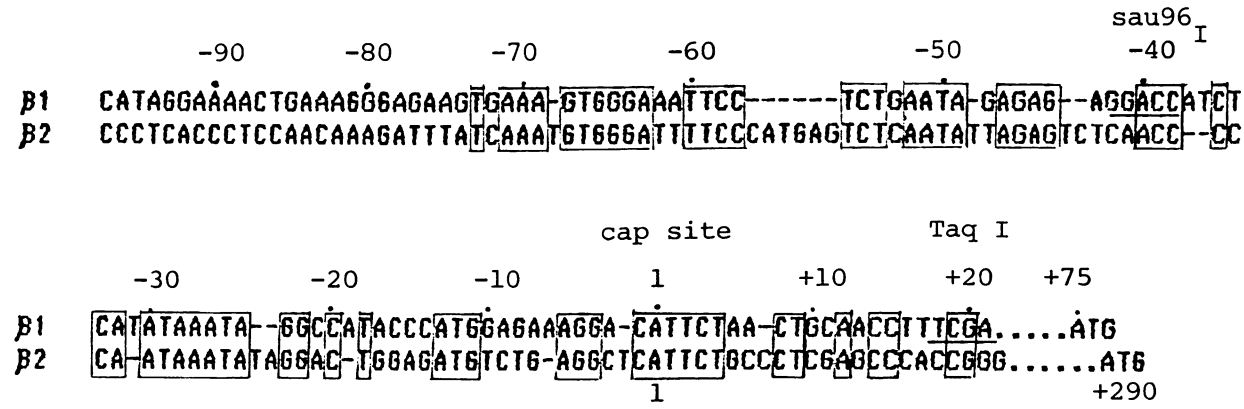

Fig. 4. Nucleotide sequence of the promoter region of IFN-β_1 RNA. The IFN-β_1 gene sequence is compared with the sequence of another poly(rI)(rC)-induced gene of human fibroblasts, IFN-β_2 (Weissenbach *et al.*, 1980; Maroteaux *et al.*, unpublished data). The position of the *Sau*96-1 site used for the deletions in Figures 1B and 5 is shown. The distance of the ATG initiation codon to the cap site is indicated.

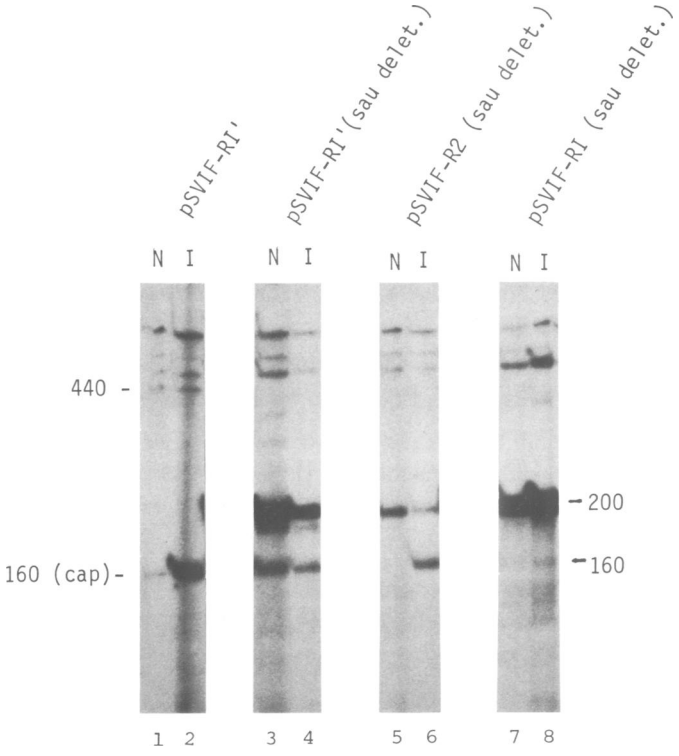

Fig. 5. Ss nuclease analysis of IFN-β_1 RNAs in CV-1 cells transfected by SV40 recombinants with deletion of the 5′ flank of the IFN-β_1 gene. CV-1 cells transfected to the indicated recombinant DNA (see Figure 1B) were induced or left non-induced, and the RNA analysed as in Figure 3A. **Lanes 1, 2,** intact IFN-β_1 gene in pSVIF-RI′. **Lanes 3–8**: IFN-β_1 gene 5′ deleted onto the *Sau*96-1 site (Figure 4) in the various orientations shown in Figure 1B. **Lanes 1,2**: pSVIF-RI′; **lanes 3,4**: pSVIF-RI′ (*Sau* deleted); **lanes 5,6**: pSVIF-R2 (*Sau* deleted); **lanes 7,8**; pSVIF-RI (*Sau* deleted).

cells, the intensity of the 440-band was lower than that of the 160-fragment corresponding to cap-initiated RNA. This transcription from an external promoter in non-induced pSVIF-RI′ transfected cells is in line with the constitutive production of IFN activity by these cells (Table I).

With the IFN-β_1 gene deleted in 5′ to the *Sau*96-1 site, RNA initiated upstream from the gene itself would protect a 200-nucleotide fragment of the ss nuclease probe. In cells transfected with the deleted gene, the salient observation was that the intensity of the 200-band was often very high. In pSVIF-RI′ (*Sau*96 deletion), for example, expression from an external promoter was higher (Figure 5, lane 3) than in the non-deleted pSVIF-RI′ (Figure 5, lane 1). In pSVIF-RI (*Sau*96 deletion), the amount of IFN-RNA starting outside the gene was also very high and contrasted with the absence of expression from the cap site (Figure 5, lanes 7 and 8). Moreover, in pSVIF-RI, containing the intact gene, no RNA initiated outside the gene was seen (Figure 3B). Possibly, the deletion increases the probability of RNA polymerase read-through in the IFN gene. In pSVIF-RI, the deletion does not reduce significantly the distance of the gene to the SV40 late promoter (Figure 1B). An RNA initiation site is known to exist near the *Sal*I site of pBR322 and to be sensitive to the SV40 72-bp repeat enhancer (B. Wasylick, personal communication), and since this site is 600 bp from the IFN gene in pSVIF-RI (*Sau*96 deletion), initiation in pBR may be responsible for the strong external expression observed. Although external initiation was high in non-induced cells transfected by pSVIF-RI (*Sau*96 deletion) DNA (Figure 5), no significant IFN activity was produced by these cells (not shown). Some of the RNA detected by the ss nuclease probe may be too short to contain the coding region or may be untranslatable.

Discussion

The human IFN-β_1 gene transfected into monkey cells produces IFN-RNA whose 5′ ends are identical to those of the 0.9-kb IFN-β_1 mRNA from human cells induced by poly(rI)(rC). The RNA 5′ ends map 24–32 nucleotides downstream from a typical 'Goldberg-Hogness' TATAA box in the gene. As monkey RNA does not interfere with the detection of these human IFN-β_1 transcripts by the ss nuclease, we used this method for studying the expression and regulation of the IFN-β_1 gene in the SV40 episomal vector replicating in monkey cells.

The main result of our study is that the IFN-β_1 1.83-kb genomic fragment retains its normal regulation by IFN inducers, even when replicated in multiple copies in monkey CV-1 cells. IFN-RNA with authentic 5′ ends appears when the transfected cells are induced for 4 h by the standard poly(rI)(rC)-dependent procedure for IFN induction. In non-induced CV-1 cells, even 50 h after transfection, very little, if any, such RNA is detected. Inhibition of the replication of the SV40 IFN-β_1 recombinant genomes, by short treatments of the cell with ara C, reduces almost completely the induction of the IFN-β_1 RNA. This confirms that we are actually studying the expression of the episomal vector which has

replicated to numerous copies in the transfected cells. By dot-blot analysis, we estimated that the number of SV40 IFN-β_1 genomes, at the time we induce the CV-1 cells, is of the order of 10^4 copies/cell. As the short-term transfection assay permits the comparison of different cell lines, we chose uncloned CV-1 cells because they showed more clearly the regulation of the IFN-β_1 gene in SV40 vectors than other monkey cells such as BSC-1 or Cos 7, which are known to replicate SV40 DNA more efficiently (E. Winocour, personal communication).

Another advantage of the short-term expression of genes carried in the episomal vector is that the influence of defined external promoters can be studied without the risk of chromosomal rearrangements, which often take place in systems involving genomic integration (Scangos and Ruddle, 1981). In the SV40-IFN-β_1 recombinant pSVIF-RI′ DNA, where the SV40 late promoter region is next to the IFN-β_1 gene and in the same orientation, IFN-RNA initiated outside the IFN-β_1 gene was observed. These transcripts were found even in non-induced CV-1 cells, and IFN biological activity was present in the culture medium of these pSVIF-RI′-transfected cells. Since this was not observed in other recombinant constructions it is likely that, in pSVIF-RI′, the late SV40 promoter serves as initiator site for RNA polymerase molecules which read-through in the IFN gene. Even though the gene was expressed from an external promoter, RNA with authentic 5′ ends was almost absent unless the CV-1 cells were induced. Poly(rI)(rC)-dependent induction is, thus, characterized by the accumulation of RNA which starts at the IFN-β_1 genes cap site, as found also in stable co-transformants (Hauser *et al.*, 1982; Canaani and Berg, 1982). This accumulation may result from an effect of induction on RNA polymerase initiation at this site, or else from an effect on processing or stabilization of RNA with correct 5′ ends.

If induction acts on transcription, deletions of the IFN-β_1 gene 5′-flanking sequences, in which potential transcription signals can be recognized (Degrave *et al.*, 1981) would be expected to alter the induction process. This possibility was verified by constructing deletions that remove most of the 5′-flanking sequences leaving only 10 bp in front of the TATAA box, i.e., 40 bp before the cap site. The deleted constructions behaved differently from the corresponding recombinants containing the 285 bp long flanking sequences. The main difference was that synthesis of IFN-RNA with authentic 5′ ends depended on the construction used. Regulated expression was retained in the deleted variant pSVIF-R2 (*Sau*96 deletion) and cap-initiated IFN-RNA was seen only after poly(rI)(rC)-dependent induction. The sequences from -285 to -40 in the IFN-β_1 gene are, therefore, not indispensable for the induction process. The influence of these sequences became, however, apparent in other constructions: in pSVIF-RI′ (*Sau*96 deletion), the deletion produced an increase in the amount of cap-starting IFN-β_1 RNA formed in non-induced CV-1 cells. This may be due to the removal of some signal sequence, but could also result from the fact that in pSVIF-RI′ (*Sau*96 deletion) the IFN cap site is now very close (<170 bp) to the SV40 promoter region (see Figure 1). An attractive possibility is that the 72-bp repeat enhancer (Banerji *et al.*, 1981) which favors transcription at distance, could be now strong enough to overcome the low cap initiation in non-induced CV-1 cells. Support for this idea comes from the results with pSVIF-RI (*Sau*96 deletion) in which the IFN-β_1 cap site is very far (>3000 bp) from the SV40 promoter region. In this case there was no expression from the genes cap site in either induced or non-induced cells. As the non-deleted corresponding pSVIF-RI DNA was expressed and inducible, the -285 to -40 sequence was seen in this case to contribute to the efficiency of expression. Gross *et al.* (1981) have pointed out the presence in the IFN-β_1 gene of repeats between -270 and -225 and of a palindrome at -205 to -167 before the RNA start site and these features are deleted in our constructions as are the -57, -93 (CAAT box) and -114 transcriptional signal sequences pointed out by Degrave *et al.* (1981). The deleted gene may, therefore, have a weakened transcription capacity, which makes it more dependent on the influence of the SV40 enhancer. In the deleted pSVIF-R2 (*Sau*96 deletion), which is both expressed from its cap site and inducible, the distance of the SV40 promoter region is intermediate to that of the two other constructions (1600 bp), and this distance may just be right for the regulated expression. The distance to the 72-bp repeat enhancer of SV40 could, therefore, be the factor which explains the difference between IFN RNA expression in the three constructions studied. Direct demonstration of this hypothesis by removal of the 72-bp repeat, would require the use of Cos cells (Gluzman, 1981) but these cells (see above) show the IFN induction phenomenon less clearly. In addition to RNAs starting from the cap site of the IFN-β_1 gene, strong expression from RNA initiation sites outside the gene was also seen with the deleted constructions but, as in non-deleted pSVIF-RI′, this external expression was not subject to the poly(rI)(rC)-dependent regulation.

Are there sequences in the IFN-β_1 gene that are essential for induction? The inducibility of the -285 to -40 deleted pSVIF-R2 (*Sau*96 deletion) indicates that such sequences must be either within the 40 nucleotides before the cap site or in the transcribed region of the gene. Pitha *et al.* (1982) proposed that induction could occur even when only the coding and 3′-untranslated regions of IFN-β_1 cDNA are present. These experiments were, however, carried out with TK co-transformation and, although very unlikely, it is plausible that the cDNA may have been integrated near one of the host cell poly(rI)(rC)-inducible genes (Raj and Pitha, 1981). Using the present transfection system with SV40 vectors, preliminary experiments have shown that when the entire -285 to $+20$ (*Taq*I site; see Figure 4) region of the IFN-β_1 gene is replaced by a similar fragment from the mouse β-globin, the inducibility of the IFN-β_1 RNA is lost (Maroteaux *et al.*, unpublished data). Together with the other observations reported here, this suggests that critical sequences for regulation may be near the TATAA box and cap site of IFN-β_1. By comparison with the nucleotide sequence of the promoter region of another poly(rI)(rC)-inducible human gene, IFN-β_1 (Weissenbach *et al.*, 1980; Maroteaux *et al.*, unpublished data), it appears that this second gene has extensive homology to IFN-β_1 from -70 to $+20$ (see Figure 4), although the overall structure of the two genes and their coding regions are much more divergent. It will be interesting to investigate whether some of the conserved sequences around the cap site are important for induction. The short-term expression system for the IFN-β_1 gene in SV40 vectors described here is well suited for such studies.

Materials and methods

Construction of IFN recombinants

SV40-pMl2 recombinants. SV40 DNA (strain 776, plaque-purified on BSC-1 cells) was digested with *Bam*HI and *Hpa*II. The 3056-bp fragment con-

taining the origin of replication and the entire early region (Tooze, 1980), was isolated by electrophoresis on 0.7% agarose, eluted and purified on DE-52 DEAE-cellulose. The 5′-protruding ends (0.1 – 0.2 pmol) were filled in with 6 units DNA polymerase I (Boehringer) in 30 μl of 70 mM KPO_4 buffer pH 7, 70 mM NaCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 35 μM of each dXTP for 2 h at 0°C. The ends were further modified by addition of 5′-phosphorylated *Eco*RI linkers (Collaborative Research) in 50-fold molar excess with T4 DNA ligase (Biolabs) in 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP for 12 h at 14°C. This fragment, and pML2 DNA (pBR322 with a 1120 – 2490 deletion; Lusky and Botchan, 1981; Mellon *et al.*, 1981) were both cut with *Eco*RI and ligated together. By transformation of *E. coli* (MM294), the amp-resistant clone pMSV1472 was isolated (Figure 1A).

IFN-β_1-SV40 recombinants, pSVIFR series. The IFN-β_1 1.83-kb *Eco*RI fragment was derived from the previously described lamba Charon 4A IFN-C15 genomic clone (Mory *et al.*, 1981), and subcloned in the *Eco*RI site of pBR322 (clone IFN-C631, Figure 2). The 1.83-kb IFN-β_1 fragment was first recloned in the *Eco*RI site of pML2 in *E. coli* (MM294) to give pMINF (Figure 1A). To open only one *Eco*RI site in pMINF, the DNA was cut with 1 unit/μg DNA of *Eco*RI nuclease for 20 min at 20°C and the linearized plasmids, isolated by electrophoresis on 1.5% agarose, were ligated to the 3-kb SV40 *Eco*RI fragment of pMSV1472. The four possible resulting double recombinants were cloned in *E. coli* and the structure of these pSVIF-RI plasmids (Figure 1A) was verified by restriction mapping.

IFN-β_1-SV40 recombinants, pSVIF-K series. The 1.83-kb IFN-β_1 *Eco*RI fragment was dephosphorylated and ligated to an excess of the 3-kb SV40 *Eco*RI insert of pSV1472. Since the IFN-β_1 fragment could not self-ligate, the major products were the four double recombinants. These DNAs were digested with *Kpn*I and cloned in the *Kpn* site of plasmid pML2-K. This pML2-K was derived from pML2 by cutting with *Bam*HI, filling in with DNA polymerase I, adding *Kpn* linkers and treating with *Kpn*I creating thereby a *Kpn* site at the position of the *Bam*HI site. Among the four recombinants, we were interested only in the two which had the *Kpn* inserts of ~5 kb (pSVIF K1 and K2, Figure 1B). These structures were verified by restriction mapping.

IFN-β_1-SV40 recombinants, pSVOD-IFN. The pSVOD plasmid described by Mellon *et al.* (1981) was cut with *Eco*RI and ligated to an excess of the 1.83-kb IFN-β_1 *Eco*RI fragment. After cloning in *E. coli* (MM294), we isolated recombinants with both orientations of the IFN-β_1 gene.

IFN-β_1-SV40 recombinants, Sau96-1 deletions. The 5′ *Eco*RI-*Pst*I fragment of the IFN-β_1 genomic fragment (see Figure 2A) was subcloned in pBR322 digested with *Eco*RI and *Pst*I. From this subclone the *Eco*RI *Sau*96-1 fragment was removed and the recombinant plasmid was religated to obtain the *Pst*I-*Sau*96-1 fragment integrated in *Pst*I-*Eco*RI sites of pBR. From this 5′ end (*Sau* deleted) subclone the *Pvu*II-*Sal*I fragment was isolated and inserted into the pSVOD IFN-β_1 which was cut with *Pvu*II and *Sal*I yielding pSVOD IFN-β_1 (*Sau* deleted). This step restored the ampicillin and tetracycline resistance and removed the SV40 sequences. This pSVOD IFN-β_1 (*Sau*96 deleted) was linearized with *Cla*I and treated with alkaline phosphatase ligated to a pSVIF-RI cut with *Msp*I and *Cla*I. After transformation in *E. coli* MM294 and selection on tetracycline and ampicillin, pSVIF-RI′ (*Sau* deleted) was characterized by restriction enzyme digestion. The pSVOD IFN-β_1 (*Sau*96 deleted) was then cut with *Bgl*II and *Pvu*I, phosphorylated and ligated with pSVIF-RI cut with *Bgl*II and *Pvu*I. After transformation and selection on tetracycline and ampicillin, pSVIF-RI (*Sau* deleted) was characterized by restriction enzyme digestion. The pSVIF-R2 (*Sau* deleted) was obtained by cutting of the pSVIF-RI (*Sau* deleted) with *Eco*RI and religation.

IFN-β_1 RNA synthesis in transfected cells

CV-1 monkey cells were grown in 9 cm dishes in Dulbecco's modified Eagle medium (DMEM Gibco) with 10% fetal calf serum (FCS) and subcultured 1:10. One day later, 4 ml fresh medium was added and 4 h later the cells were transfected by calcium phosphate co-precipitation (Wigler *et al.*, 1977; Banerji *et al.*, 1981). Each recombinant DNA, 10 μg, was dissolved in 0.21 ml of 1 mM Tris-HCl pH 7.9, 0.1 mM EDTA and 30 μl of 2 M $CaCl_2$ was added at 0°C. This solution was slowly added with agitation to 0.24 ml of 0.28 M NaCl, 1.5 mM Na_2HPO_4, 50 mM Hepes buffer pH 7.1 (2 x HBS) at room temperature and left for 15 min. The precipitate was added to the cells for 20 h, then the cells were washed with 0.15 M NaCl, 5 mM KCl, 0.75 mM Na_2HPO_4, 25 mM Tris-HCl pH 7.4 (TBS) and incubated in fresh medium. Cells were trypsinized 36 h after DNA addition, 10% were reseeded in a 35 mm dish for immunofluorescence assay of T-antigen as described by Banerji *et al.* (1981) and the rest of the cells were reseeded in two 9 cm dishes and incubated for another 24 h before RNA extraction. When indicated, the cells were induced for IFN production by incubation with 50 μg/ml poly(rI)(rC), 50 μg/ml DEAE-dextran (0.5 x 10^6 M), 50 μg/ml cycloheximide, for 4 h prior to RNA extraction of IFN. The cells were lysed with 1% SDS, 50 mM sodium acetate buffer pH 5.2, and extracted at 60°C for 15 min with phenol equilibrated in the same buffer. After cooling at −4°C for 15 min and centrifugation at −4°C, the RNA was ethanol precipitated from the soluble phase and dissolved in S1 hybridization buffer.

IFN production in transfected cells

After 4 h of induction, as described above, the cells were washed twice with phosphate-buffered saline (PBS) and then incubated with 0.5 ml/3 x 10^5 cells of DMEM 0.2% FCS and 20 h later the medium was assayed for IFN-β_1 as in Mory *et al.* (1981). The medium to be tested, and an IFN standard (100 U/ml), were diluted serially 2-fold in 50 μl MEM-5% FCS-0.5% gentamicin, and 3 x 10^4 human FS11 fibroblasts in 50 μl were added to each well. After 18 h, medium was removed and the cells infected with 5 x 10^3 p.f.u. vesicular stomatitis virus (VSV) in 0.1 ml MEM-2% FCS. Inhibition of the VSV cytopathic effect was recorded 24 h after infection. The IFN titers were calculated in reference to IFN-β NIH standard 8023-902-527.

Ss nuclease mapping

The IFN-β_1 RNA present in transfected cells was detected by ss nuclease mapping (Berk and Sharp, 1977) modified by Favaloro *et al.* (1980), with S1 nuclease or mung bean nuclease (Green and Roeder, 1980) and 5′ end-labeled DNA probes (Weaver and Weissmann, 1979). The DNA probe was the 545-bp fragment of clone IFN-C631 DNA (Figure 2) obtained by restriction with *Hinf*I and *Hha*I. The *Hinf*I site was 5′-labeled by T4 polynucleotide kinase (PL Biochemicals) and [γ-^{32}P]ATP (>7000 Ci/mmol; New England Nuclear) as described by Maxam and Gilbert (1980). The end-labeled DNA probe denatured at 68°C for 15 min in hybridization buffer was hybridized for 15 h at 42°C, to 1/2 or 1/4 of the total RNA from one 9 cm dish, in 30 μl of S1 hybridization buffer containing 80% formamide, 0.4 M NaCl, 40 mM Pipes buffer pH 6.4, 1 mM EDTA (Casey and Davidson, 1977). After addition of 0.3 ml of 0.2 M NaCl, 1 mM $ZnSO_4$, 30 mM sodium acetate buffer pH 4.6 containing either 125 U of S1 nuclease (BRL or PL Biochemicals) or 125 U of mung bean nuclease (PL Biochemicals), the hybrids were digested for 30 min at 30°C or 42°C. The reaction was stopped with 75 μl of 2.5 M NH_4 acetate pH 5.5, 50 mM EDTA containing 400 μg/ml of *E. coli* tRNA and the protected hybrids were precipitated with 0.4 ml isopropanol. They were then dissolved in sequencing Blue dye and analyzed on a 6% polyacrylamide-8.3 M urea sequencing gel (Maxam and Gilbert, 1980). DNA sequencing was also performed according to Maxam and Gilbert (1980).

Other methods

Restriction endonucleases and T4 DNA ligase were purchased from Boehringer or Biolabs and used as indicated by the suppliers. Cloning in *E. coli* and CsCl plasmid purification were carried out according to usual methods (Bolivar and Backman, 1979; Mory *et al.*, 1981). In some cases, the rapid plasmid purification technique (Horowicz and Burke, 1981) was used.

Acknowledgements

We thank Dr. L. Chen for her help in IFN assays and S.I. Feinstein, Y. Chernajovsky and D. Gidoni for their contributions and discussions. We thank Dr. M. Botchan for gifts of plasmids. The technical assistance of Ms. N. Dafni and P. Federman is acknowledged. This work was supported in part by US-Israel Binational Science Foundation, Jerusalem, Israel, and by InterYeda (Israel).

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