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Regulated expression of an extrachromosomal human β-interferon gene in mouse cells

(interferon induction/double-stranded RNA/bovine papilloma virus vector/DNA-mediated gene transfer/transcript mapping)

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ABSTRACT **β (fibroblast)-interferon mRNA and protein are induced by the synthetic double-stranded RNA poly(I)·poly(C) in cultured human fibroblasts. To study the mechanism of this induction, we have isolated a human β-interferon gene and inserted it in a vector plasmid containing DNA of the bovine papilloma virus. After removal of bacterial plasmid sequences, the bovine papilloma virus·β-interferon recombinant was used to morphologically transform mouse fibroblasts. Analysis of DNA from the transformed cell lines indicated that this recombinant is propagated as a stable multicopy extrachromosomal element. Human β-interferon mRNA and protein are inducible by poly(I)·poly(C) in all of these cell lines, and the mRNA is indistinguishable from β-interferon mRNA synthesized by induced human cells.**

Interferons are a heterogeneous group of secreted proteins that protect cells against viral infection. Human α (leukocyte)-interferons are encoded in a multigene family (1), but only one human β (fibroblast)-interferon gene has been isolated (2–6). Interferon activity can be induced *in vivo* or in cell culture by viruses, viral RNA, or the synthetic double-stranded RNA poly(I)·poly(C). In human cells, poly(I)·poly(C) induces only β-interferon, whereas viral infection may induce α-interferon, β-interferon, or a mixture of the two, depending on the cell type (for review, see ref. 7). β-interferon mRNA and protein are detectable 1–3 hr after poly(I)·poly(C) addition, and induction of the mRNA does not require protein synthesis (7, 8).

To study the mechanism of interferon gene regulation, we have isolated a human β-interferon gene and introduced it into C127 mouse fibroblast cells, which produce high levels of mouse interferon activity after poly(I)·poly(C) treatment. The mouse interferon mRNA and protein are both distinguishable from the products of the human gene, since human β-interferon gene DNA does not hybridize to mouse sequences (9) and mouse interferon does not protect human cells against viral infection (7). The ability to independently assay mouse and human interferon provides a useful internal control for induction experiments.

The human β-interferon gene was introduced into mouse cells by using the DNA of bovine papilloma virus (BPV) as a vector (10, 11). BPV is a small DNA virus that grows in bovine epithelial cells and is capable of morphologically transforming fibroblasts of several species. A 5.5-kilobase pair (kbp) DNA fragment comprising 69% of the BPV genome (69% BPV) is sufficient for morphological transformation (12). Recombinant plasmids containing 69% BPV also transform fibroblasts if certain bacterial plasmid sequences that inhibit transformation are removed by restriction enzyme digestion (12). These recombinant plasmids are propagated as multicopy extrachromosomal circular DNA in transformed cells (10). The plasmids within cells of an individual transformed line have identical structures, so each copy of an isolated gene linked to 69% BPV is flanked by the same DNA sequences and may therefore be expressed at the same level.

MATERIALS AND METHODS

Cells and DNA Transformation. C127 mouse fibroblasts were transfected with pBPVIF DNA as described (13). One transformed focus was picked per plate and grown in mass culture.

Interferon Induction and Assay. Mouse cells were incubated 3 to 4 days after reaching confluence without medium change and then induced with poly(I)·poly(C) (50 μg/ml) in the presence of DEAE-dextran (100 μg/ml) and cycloheximide (50 μg/ml), followed by treatment with actinomycin D (1 μg/ml) as described by Havell and Vilcek (14). MG63 cells were induced similarly except that DEAE-dextran was omitted and the poly(I)·poly(C) concentration was increased to 100 μg/ml. Uninduced control cells were treated exactly the same as induced cells except that poly(I)· poly(C) was omitted.

Interferon was assayed by cytopathic effect inhibition using vesicular stomatitis virus (10 plaque-forming units per cell; obtained from A. Huang) as the challenge virus (7). There is a twofold error inherent in this assay, because interferon was serially diluted in steps of two. Human interferon activity was titered on human WISH cells and mouse activity was titered on C127 cells, using human and mouse international reference interferons as standards.

Preparation and Analysis of Cellular DNA. Total cellular DNA was extracted from cells as described by Wigler *et al.* (15). Low M_r DNA was prepared as described by Hirt (16). Gel electrophoresis and blot hybridization were carried out by standard methods (17, 18).

Preparation and Blotting Analysis of RNA. Cells were induced by poly(I)·poly(C) for RNA preparation as described in *Results*. Uninduced cells were treated exactly the same except that poly(I)·poly(C) was omitted. In all cases, parallel plates were incubated 24 hr in medium after induction and assayed for mouse and human interferon activities. Total cytoplasmic RNA was prepared as described by Favaloro *et al.* (19). The RNA was subjected to electrophoresis through formaldehyde/agarose gels and transferred to nitrocellulose as described (20). The probes used to map the higher molecular weight interferon RNAs were the 735-base pair (bp) *Pvu* I/*Eco*RI fragment of pBR322 and the 3-kbp *Hin*dIII/*Eco*RI fragment of BPV (see Fig. 2). To observe these RNAs, total cytoplasmic RNA was extracted from cells with and without induction at subconfluence, confluence, and 1.5 days after reaching confluence.

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Abbreviations: BPV, bovine papilloma virus; kbp, kilobase pair(s); bp, base pair(s).

Nuclease S1 Analysis of RNA. A 200-nucleotide 5′-end-labeled single-stranded *Ava* II/*Hin*fI fragment spanning the 5′ end of the interferon gene was purified (see Fig. 4). Five femtomoles of this DNA fragment was hybridized with 20–40 μg of total cytoplasmic RNA (>10-fold DNA excess) at 30°C overnight, treated with nuclease S1 as described (13), and subjected to electrophoresis at 1,000 V through thin 50% (wt/vol) urea/6% polyacrylamide gels.

RESULTS

Isolation of a Human β-Interferon Gene. We isolated the human β-interferon gene by recombination and selection *in vivo*. In this procedure (B. Seed, personal communication), a small plasmid containing a suppressor tRNA gene, πVX, is used to select genetic recombinants between a cloned probe sequence and a homologous sequence present on a λ phage bearing multiple amber mutations. Only those phage that incorporate the suppressor tRNA gene via homologous recombination with the probe sequence (Fig. 1) can grow on sup^0 cells. A 370-bp *Pst* I/*Bgl* II coding sequence fragment from TpIF319, a β-interferon cDNA clone (21), was inserted into πVX to create the plasmid πIF. (TpIF319 was kindly provided by Tadatsugu Taniguchi.) At least five independent phage were isolated from a Charon 4A library of human genomic DNA (22) by recombination with πIF, and all of these contained several kilobase pairs

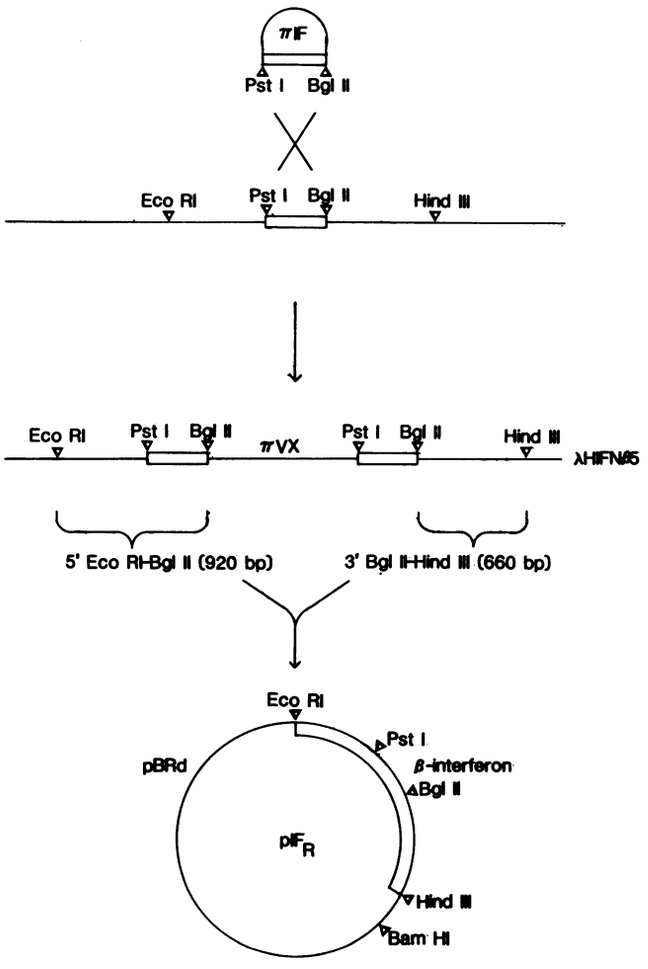

FIG. 1. Isolation and subcloning of the human β-interferon gene. A portion of the insert of λHIFNβ5 is shown. This phage has a structure similar to that of λHIFN121-β isolated by Ohno and Taniguchi (5).

of sequence 5′ and 3′ to the interferon gene. The recombination method appears to select a subpopulation of β-interferon phage from the library, because several other groups (3–5) have observed that >90% of the phage isolated from the same library by hybridization with β-interferon cDNA have inserts that end ≈100 bp upstream from the interferon coding sequence. As shown in Fig. 1, recombination between a β-interferon phage and πIF inserts plasmid sequences into the chromosomal interferon gene and creates a duplication of the cloned interferon cDNA fragment. To subclone the intact chromosomal interferon gene, fragments spanning its 5′ and 3′ ends were purified from recombinant phage DNA and inserted into plasmid pBRd (ref. 13; see Fig. 1). The resulting plasmid, pIFR, contains the β-interferon coding region with 350 bp and 660 bp of 5′ and 3′ flanking sequence, respectively.

The Isolated Human β-Interferon Gene Introduced into Mouse Cells Is Inducible by Poly(I)·Poly(C). To introduce the cloned β-interferon gene into cells in culture, we joined pIFR to the 69% *Hin*dIII/*Bam*HI fragment of BPV DNA to create plasmid pBPVIF (Fig. 2). This plasmid was digested with *Pvu* I/*Sal* I to remove pBR322 sequences that inhibit transformation of mouse cells (12) and introduced into C127 mouse fibroblasts by calcium phosphate precipitation (13). Morphologically transformed foci were observed after 8 days, and all cell lines established from these foci express human interferon activity without induction. Five independent lines (B1, B5, B7, B8, and B9) were tested, and all were found to produce increased levels of human interferon after induction with poly(I)·poly(C) (Table 1). The protocol we found most effective for human interferon induction combines poly(I)·poly(C) treatment with "superinduction" by metabolic inhibitors, which enhance interferon yields from some cell lines (7, 14). High levels of mouse interferon were also induced under these conditions (Table 1).

The β-Interferon Gene Linked to 69% BPV Is Propagated as a Multicopy Circular Extrachromosomal Element. It has been shown that DNA containing 69% BPV can propagate as monomeric, multimeric, or catenated plasmids in transformed C127 cells (10, 11, 13). To examine the state of the pBPVIF DNA in our transformed cell lines, we purified low M_r extrachromosomal DNA from the cells by the procedure of Hirt (16). Low M_r DNA from cells of each line hybridizes to BPV and interferon probes, and the bands observed after restriction enzyme digestion and DNA blotting are consistent with the presence of a circular molecule; i.e., all enzymes that recognize sites in pBPVIF generate only one band per site. Uncut B1 and B8 low M_r DNAs contain multiple species complementary to pBPVIF that migrate with supercoiled and nicked pBPVIF plasmid DNA from bacteria whereas, with uncut B5, B7, and B9 low M_r DNAs, a single band migrating at the gel exclusion limit for linear DNA is observed (Fig. 2). This band is not generated by catenated or circular multimeric DNA, both of which migrate more slowly than excluded linear DNA (ref. 23; Fig. 2). These results suggest that, in B1 and B8 cells, the BPV-interferon plasmid (BPV-IF) replicates primarily as a unit-length circle but, in B5, B7, and B9 cells, it exists as large multimeric species that are broken during low M_r DNA isolation, generating linear fragments migrating at the exclusion limit. Cells from Shope papilloma virus-induced carcinomas also contain such multimeric viral plasmids (24).

Restriction enzyme digestion of total cellular DNA or low M_r DNA generates the same hybridizing bands, indicating that all detectable BPV-IF DNA (>0.5 copy per cell) in each line is extrachromosomal. Since analysis of total cellular DNA shows that each line contains 30–60 copies of BPV-IF DNA per cell (data not shown), the apparent variation in copy number between lines observed in the low M_r DNA fraction (Fig. 2) is

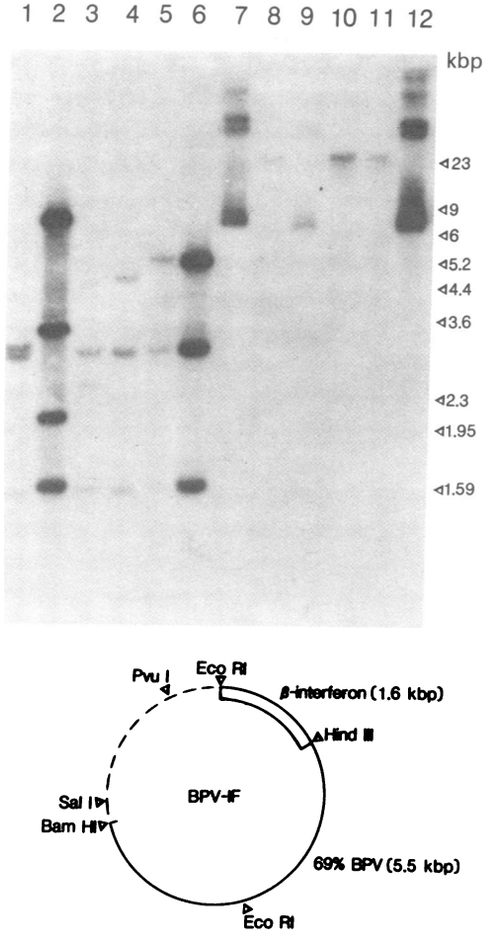

FIG. 2. Analysis of extrachromosomal DNA in BPV-IF-transformed lines. Low M_r extrachromosomal DNA was prepared and subjected to electrophoresis through a 1% ethidium bromide/agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pBPVIF DNA. Each lane contains low M_r DNA from $\approx 2 \times 10^6$ cells. Cell lines used were B7 (lanes 1 and 10), B9 (lanes 3 and 8), B8 (lanes 4 and 9), B5 (lanes 5 and 11), and B1 (lanes 6 and 12). DNAs in lanes 1 and 3–6 were cut with *Eco*RI/*Hin*dIII; those in lanes 8–12 were uncut. Lane 2 contains a set of DNA markers (50 copies per cell equivalent); their sizes are 8, 3.3, 2, and 1.58 kbp. Lane 7 contains uncut pBPVIF (20 copies per cell equivalent); the bands observed are (from top) (*i*) nicked dimer, (*ii* and *iii*) supercoiled dimer and nicked monomer, and (*iv*) supercoiled monomer. Exposure was for 6 hr without an intensifying screen.

probably due to inefficient recovery of large multimers or catenanes.

Previous studies had indicated that extrachromosomal elements containing the 69% BPV fragment often contain insertions of new DNA sequences between the ends of the linear transforming DNA (10, 11). Comparison of the structures of the BPV-IF elements in our transformed cell lines leads to a similar conclusion. *Eco*RI/*Hin*dIII digestion of low M_r DNA from each of the five lines generated three fragments: the original 1.58-kbp interferon fragment, a 3-kbp fragment from BPV, and a third fragment of variable size (Fig. 2). This and other mapping experiments (data not shown) indicate that the BPV-IF plasmids in the B1, B5, and B8 cell lines have incorporated new DNAs of 1.7–2.2, 1.7–2.2, and 1.4–2.0 kbp, respectively, between the ends of input linear DNA, while those in the B7 and B9 lines contain <200 bp of new DNA.

The structures of these DNAs have not changed in 3 months of passaging, except that the B9 line contains a variety of ratios

Table 1. Induction of human and mouse interferon activity and RNA in BPV-IF-transformed mouse cells

Cell line	IFN activity, units/ml		Induced/uninduced human IFN	
	Human	Mouse	Activity	RNA level
B1 induced	384	11,520	2	2
B1 uninduced	192	<6		
B5 induced	192	4,800	3	5
B5 uninduced	72	<6		
B7 induced	768	11,520	16	20
B7 uninduced	48	<6		
B8 induced	384	7,680	4	8
B8 uninduced	96	<6		
B9 induced	192	11,520	8	8
B9 uninduced	24	<6		
β5A induced	<6	4,800	—	—
β5A uninduced	<6	<6		
MG63 induced	7,680	6	—	—
MG63 uninduced	<6	<6		

Interferon (IFN) activity values are from a single typical experiment; human interferon activity was determined in 5 ml of medium per $\approx 10^7$ cells.

of a mixture of two plasmids, one of which retains only 600 bp of the *Eco*RI/*Hin*dIII interferon gene fragment (data not shown). This deleted species generates the faint upper band visible in lane 3 of Fig. 2.

A RNA Identical to Authentic Human Interferon mRNA Is Induced in the BPV-IF-Transformed Cell Lines. To study induction of the cloned human interferon genes in transformed mouse cells, RNA was prepared from cells that were aged 3 days after reaching confluence, treated with poly(I)·poly(C) for 1.5 hr, and incubated 4 hr in cycloheximide-containing medium (8, 14). Total cytoplasmic RNA (19) was subjected to electrophoresis through formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to the human β-interferon probe (20). Induction of a 1-kb RNA migrating with interferon mRNA from poly(I)·poly(C)-treated MG63 human osteosarcoma cells was observed in each line (Fig. 3). We estimate that the induced interferon RNA is 0.02–0.1% of total mRNA or 200–1,000 copies per cell. Mouse C127 cells transformed with a BPV plasmid (β5A) (13) that does not contain the human β-interferon gene did not produce RNA that hybridizes to the human interferon probe, although these cells made high levels of mouse interferon after induction (Fig. 3; Table 1).

The induced human β-interferon mRNA produced in mouse cells has the correct 5′ end, as shown by nuclease S1 analysis. A 200-nucleotide 5′-end-labeled single-stranded DNA fragment spanning the 5′ end of the gene was hybridized to total cytoplasmic RNA and the hybrids were treated with nuclease S1 and subjected to electrophoresis on denaturing polyacrylamide gels (25). A 160-nucleotide doublet band was observed after hybridization to RNA from induced MG63 cells but not after hybridization to sea urchin RNA or RNA from uninduced MG63 cells (Fig. 4). This band corresponds to the 5′ end observed previously in human diploid fibroblasts (5). After hybridization to RNAs from induced cells of each of the BPV-IF-transformed cell lines, the same doublet band was observed. It was also visible after longer exposures when RNA from uninduced cells was used for hybridization. The intensity of the

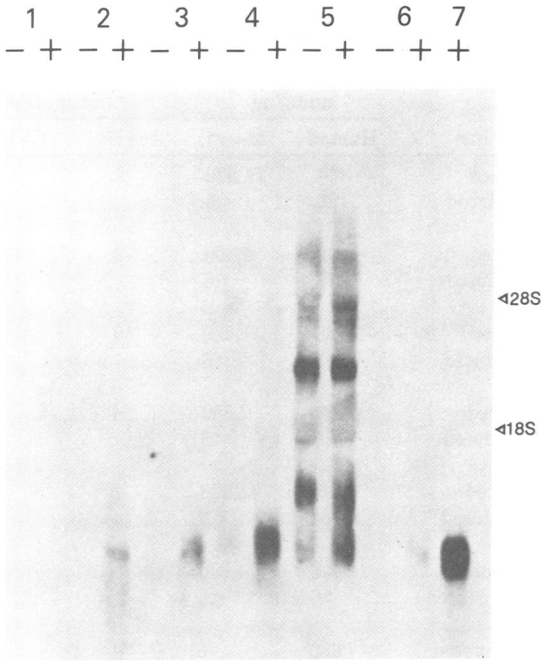

FIG. 3. Blotting analysis of β-interferon RNAs in BPV-IF-transformed lines. Total cytoplasmic RNA (10 μg per lane, except for lanes 2, which contain 3.3 μg each) was subjected to electrophoresis through a 1.5% formaldehyde/agarose gel, transferred to nitrocellulose, and hybridized to the nick-translated 1.58-kbp *Eco*RI/*Hin*dIII interferon fragment. Cell lines used were β5A (lanes 1), B9 (lanes 2), B8 (lanes 3), B7 (lanes 4), B5 (lanes 5), B1 (lanes 6), and MG63 (lanes 7). Positions of 28S and 18S ribosomal RNAs are indicated. Exposure was for 12 hr with an intensifying screen.

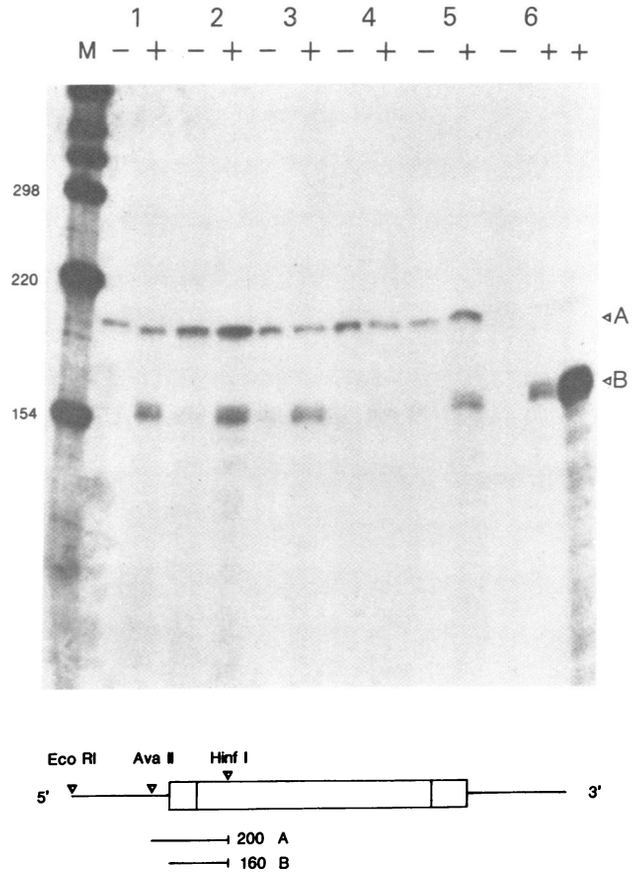

FIG. 4. 5′ nuclease S1 analysis of β-interferon RNAs in BPV-IF-transformed lines. Hybrids between 5 fmol of the 200-nucleotide single-stranded *Ava*/*Hinf*I fragment and total cytoplasmic RNA (20 μg, except where noted) were treated with nuclease S1 and subjected to electrophoresis through a 6% polyacrylamide/urea gel at 1,000 V. Cell lines used were B9 (lanes 1), B8 (lanes 2), B7 (lanes 3), B5 (lanes 4), B1 (lanes 5), and MG63 (lanes 6). Left and right lanes 6 "+" contain 2 μg and 20 μg of induced MG63 RNA, respectively. Lane M contains *Hinf*I-digested labeled pBR322. Exposure was for 6 days with an intensifying screen.

signal observed with 20 μg of RNA from induced MG63 cells (Fig. 4) showed that the DNA probe was in molar excess over interferon RNAs from the BPV-IF-transformed lines, so the data of Fig. 4 could be used to accurately quantitate the levels of these RNAs. Average induction ratios for each line were estimated by densitometry of autoradiograms from several nuclease S1 mapping and RNA blotting experiments (Table 1).

Interferon RNAs with Complex Structures Are Transcribed from BPV-IF Elements. Several interferon RNAs larger than the authentic mRNA were detected in both induced and uninduced B5 cells, and the levels of these RNAs were unaffected by poly(I)·poly(C) induction (Fig. 3). In addition, we observed that, when RNA was made from B7 or B9 cells earlier than 2 days after reaching confluence, similar large RNAs hybridizing to the interferon probe were present at the same level in induced and uninduced cells. These RNAs all hybridize to pBR322 DNA upstream of the interferon gene but not to BPV sequences (data not shown). They do not correspond to the transcripts observed by others in 69% BPV-transformed lines, which were present at very low levels and contained BPV sequences (26). Interferon RNAs larger than the authentic mRNA were also detected by nuclease S1 analysis. A band corresponding to protection of the entire hybridization probe was observed with RNA from induced or uninduced cells of each of the lines (Fig. 4), indicating that some of these RNAs are transcribed in the same direction as the interferon gene. The DNA mapping experiments show, however, that the large interferon RNAs cannot be completely contained within the sequences between the *Bam*HI end of the BPV fragment and the 3′ end of the transcribed interferon sequences (see Fig. 2). For example, this distance is <2 kilobases in the B7 and B9 elements, which synthesize RNAs of >6 kilobases. The fact that the three lines that synthesize such RNAs all appear to contain multimeric plasmids (Fig. 2) suggests that they arise from transcription of concatemeric pBR322·interferon·BPV units, followed by splicing out of the BPV sequences.

Since these RNAs contain interferon sequences but are present in equal amounts in induced and uninduced cells, the induction of authentic interferon mRNA observed in this system is likely to be at the level of transcriptional initiation rather than of differential RNA degradation. However, a degradative mechanism that recognizes only interferon RNAs having a correct 5′ end cannot be excluded.

DISCUSSION

In this paper, we present evidence that a cloned human β-interferon gene introduced into mouse cells can be induced by poly(I)·poly(C) and that the mRNA transcribed from this gene is indistinguishable from interferon mRNA in the poly(I)·poly(C)-induced human cells. In human or mouse fibroblasts, however, interferon is induced from <6 units/ml to >5,000 units/ml. This is an induction ratio at least 50-fold higher than that observed for human interferon (2- to 20-fold) in the BPV-IF-transformed mouse cell lines. Mouse interferon induction in these lines is normal (Table 1). Furthermore, if every BPV-IF molecule is capable of being transcribed, the amount of induced

RNA per template is approximately 1/200-fold of that in MG63 human osteosarcoma cells.

In experiments done by other groups, human α (27)- and β (28)-interferon genes introduced into mouse cells by cotransformation with selectable markers were also shown to be inducible at a low level. The low induction ratio observed in all of these experiments could be due to introduction of the human interferon gene into a new chromosomal environment by the process of DNA-mediated gene transfer. It is also possible, however, that a mouse cell is incapable of efficiently regulating the human gene. Finally, the 1,580-bp interferon gene fragment used in these experiments may not contain all the information necessary for high-level expression of the induced gene.

The human β-interferon gene linked to 69% BPV is propagated as a stable multicopy extrachromosomal element in morphologically transformed mouse C127 cells. Each copy of the gene in an individual cell line is therefore flanked by the same DNA sequences. The heterogeneity in the level of induction of human interferon gene expression among different cell lines (Table 1) may be due to the different structures of the BPV-IF elements in these lines (Fig. 2). The use of a new BPV vector that can replicate as a plasmid in both mouse and bacterial cells (13) may eliminate such heterogeneity, because all mouse cell lines made by transformation with this vector contain identical extrachromosomal elements.

The availability of such a BPV shuttle vector (13) in conjunction with the ability to induce human β-interferon gene expression on BPV plasmids should allow a systematic genetic analysis of interferon gene regulation. For example, it may be possible to isolate both plasmid and host cell mutations affecting synthesis of human interferon. Analysis of these mutations may define regulatory sites flanking the interferon gene and cellular regulatory factors controlling its expression.

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