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Interferon-stimulated transcription: Isolation of an inducible gene and identification of its regulatory region

(transfection/adenoviral vectors/cell surface signals)

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ABSTRACT **A human gene (termed *ISG-54K*) that is induced from near undetectable levels to high transcriptional activity by α- and β-interferons has been cloned. The genomic structure and nucleotide sequence of the coding region were determined and the RNA initiation site was identified. The 5′ portion of the gene was fused with a heterologous gene lacking an active promoter in recombinant plasmid and adenoviral vectors. These fusion genes were used to assess the activity of the *ISG-54K* promoter in response to interferon. RNA was formed in HeLa cells from recombinant plasmids only in response to interferon. Furthermore, in human diploid fibroblasts, infection with the recombinant adenovirus vector resulted in a 50-fold increase in specific RNA in response to interferon, followed by a subsequent decrease, imitating the natural regulated transcriptional cycle of the endogenous gene.**

The establishment and regulation of many cell functions both during development and in adult life are mediated by either cell–cell or cell–matrix contacts or by peptide ligands binding to specific cell-surface receptors (1–3). Communication to the cell nucleus of signals initiated at the cell surface in all these cases could involve similar mechanisms. Changes in specific gene transcription in response to cell-surface receptor binding has been widely assumed to occur and has now been documented in several cases (4–8). Occupation of receptors is often accompanied by changes in the quantity of, or modification of, intracellular compounds referred to as "second messengers"—e.g., cyclic AMP concentration, calcium ion flux, or extent of specific protein phosphorylation (8). How specific nuclear responses are brought about by such a limited number of intracellular signals (if indeed they are) is not known. Interferons (IFNs) are a family of proteins that exert potent biological activities by binding to specific cell-surface receptors to induce antiviral, growth modulatory, and immunomodulatory conditions (9). Secondary to IFN binding, a newly synthesized, limited set of proteins appears in treated cells (10). At least some of the genes encoding these proteins are activated at the level of transcription by IFN binding (4, 5) and a few hours later are deactivated by a process requiring protein synthesis (11). To approach the molecular basis for transcriptional control mediated by such a cell-surface signal, we have studied two cDNAs complementary to mRNAs subject to transcriptional control by α- or β-IFN. To understand the increased transcription of these RNA sequences more fully, we have selected and characterized genomic DNA clones complementary to one of the two mRNAs reported earlier. Here we report the genomic structure, the nucleotide sequence, and the predicted amino acid sequence encoded by the gene, which we call *ISG-54K* to indicate *i*nterferon *s*timulation of a *g*ene encoding a 54-kDa protein. The presumptive RNA initiation site of the mRNA has been identified, allowing construction of recombinant plasmid and viral vectors bearing the 5′ flanking portion of the gene. When delivered into human cells, vectors carrying the human DNA corresponding to approximately −800 base pairs (bp) to +288 bp relative to the initiation site express stable RNA in an interferon-dependent manner. This DNA segment appears to contain all the sequence information necessary for the proper regulation of *ISG-54K*.

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MATERIALS AND METHODS

HeLa cells (clone S3) were obtained from American Type Culture Collection, and human fibroblasts (FS2) were a kind gift of E. Knight (DuPont). Recombinant human α_A-IFN was generously supplied by S. Pestka (Roche Institute). Human genomic libraries in λ-CH4A and λ-EMBL4 were gifts from T. Maniatis (Harvard University) and N. Cowan (New York University), respectively. Libraries were screened by hybridization using conventional procedures (12), and positive clones were sequenced from sequential *Exo* III-generated deletion subclones (13, 14).

In vitro transcription reactions were performed as described by Dignam *et al.* (15). A modification of the SP6 quantitative nuclease protection assay (16) using RNase T2 was used to assess RNA expression in normal and transfected cells. Cells were transfected by using the DEAE-dextran method as described by Lopata *et al.* (17). Recombinant adenoviral vectors were constructed as described (18). Details of the cloning steps involved in the construction of plasmid and viral vectors are available upon request.

RESULTS

Isolation of *ISG-54K* Genomic Clones. To begin the isolation of the *ISG-54K* gene, the partial cDNA clone, which contains the 3′ end of an IFN-induced mRNA (previously called IFN-IND1; ref. 4) was used to screen a human partial *Eco*RI bacteriophage λ library. A clone (λ-1) complementary to the cDNA was selected and characterized for cleavage sites of the restriction endonucleases *Eco*RI and *Hin*dIII (Fig. 1*A*). A 561-bp *Eco*RI fragment from the left end of λ-1 was found by RNA blot analysis to hybridize the previously identified 2.8-kilobase (kb) RNA (4) from IFN-treated cells (not shown). The direction of transcription across the gene segment was determined by RNA blot hybridization (19) using strand-specific probes derived from this restriction fragment. Strand-specific DNA probes were produced by 3′-end-labeling either the *Eco*RI sites that define this fragment or the internal *Hin*dIII site. Of the four resulting end-labeled *Eco*RI/*Hin*dIII strands purified by electrophoresis, only the shorter (135 bp) strand labeled at the left *Eco*RI site and the longer (426 bp) strand 3′-end-labeled at the *Hin*dIII site

Abbreviations: IFN, interferon; bp, base pair(s); kb, kilobase(s); nt, nucleotide(s).

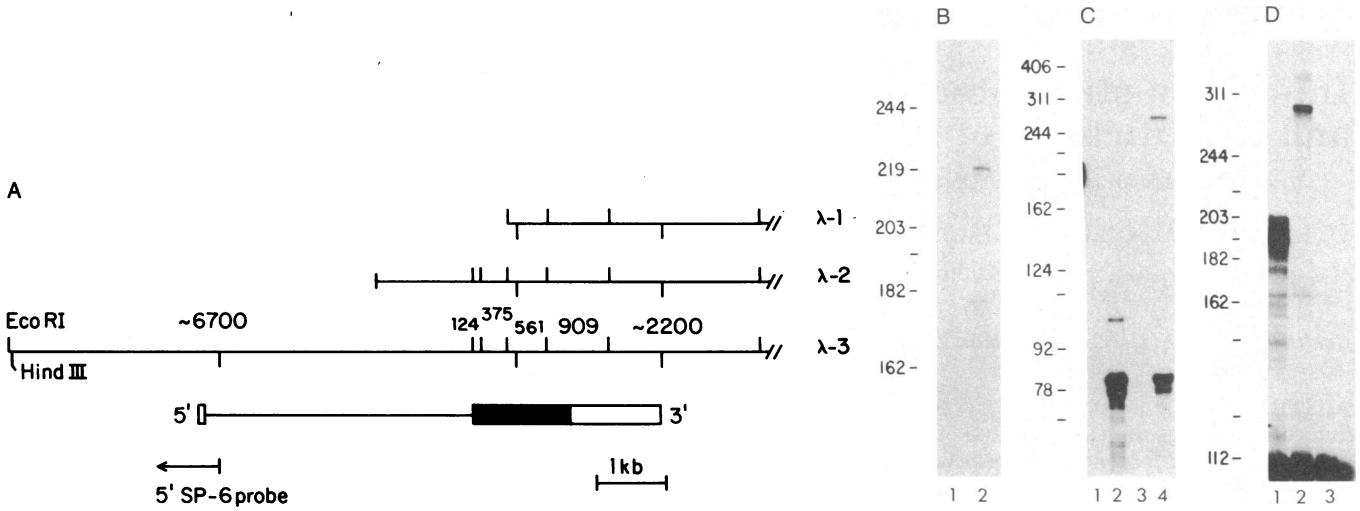

FIG. 1. Genomic structure of *ISG-54K*. (*A*) Partial restriction maps (*Eco*RI, above line; *Hin*dIII, below line) of the human DNA inserts of three λ clones are presented above a diagram of the *ISG-54K* transcription unit. Sizes of *Eco*RI fragments that are described in the text are indicated. Boxed regions are exons connected by a single intervening sequence. Shading represents the longest open reading frame. (*B*) The 5′ end-labeled 124-bp *Eco*RI fragment of λ-2 was extended by reverse transcriptase, using cytoplasmic RNA from uninduced (lane 1) or IFN-treated (lane 2) HeLa cells as templates, and products were analyzed by denaturing gel electrophoresis (12). (*C*) The first exon and RNA initiation site were mapped by nuclease protection analysis using the SP6 probe indicated in *A*. Labeled riboprobe was hybridized with 10 μg of cytoplasmic RNA from uninduced (lane 1) or IFN-treated (lane 2) human fibroblasts or with 5 μg of nuclear RNA from the same control (lane 3) or IFN-treated (lane 4) fibroblasts used in lanes 1 and 2. (*D*) RNA transcription reactions with a HeLa cell nuclear extract were programmed with a *Pst* I/*Hin*dIII restriction fragment (nt 4972–6231 of adenovirus 2) containing the major late promoter (lane 1), with the leftmost 3-kb *Hin*dIII restriction fragment from λ-3 (lane 2), or with no added DNA (lane 3), and the labeled products were separated by gel electrophoresis. Size markers in nt, derived from coelectrophoresed labeled *Hpa* II restriction fragments of pBR322, are indicated to the left of each autoradiogram.

hybridized to mRNA from IFN-stimulated cells, while the opposite strands failed to do so. This result indicated that the 5′ to 3′ orientation for transcription proceeds left to right as displayed in Fig. 1*A*.

Further genomic clones were obtained by screening a library derived from partially *Mbo* I-digested human DNA with the 135-bp *Eco*RI/*Hin*dIII fragment of λ-1. The structure of λ-2, selected by this screening, is illustrated in Fig. 1*A*. The human DNA insert of λ-2 overlapped λ-1 and extended in the 5′ direction by 1.9 kb. This additional DNA contained 375-bp and 124-bp *Eco*RI fragments that hybridized only to mRNA from IFN-stimulated cells. The neighboring 1.4-kb terminal fragment did not hybridize to human mRNA (not shown). Thus, the 124-bp *Eco*RI fragment was the most 5′ portion of λ-2 that was complementary to mRNA. This fragment, end-labeled at its 5′ termini, was hybridized to cellular RNA and extended with reverse transcriptase. A band of ≈220 nucleotides (nt) was detected only when the 124-nt primer was hybridized to RNA from IFN-stimulated cells (Fig. 1*B*, lane 2). Thus, ≈90 nt of the 5′ end of the ISG-54K mRNA was not encoded in λ-2. Using the 1.4-kb terminal restriction fragment of λ-2 to rescreen the partial *Eco*RI λ library, a clone was selected (λ-3 in Fig. 1*A*) that proved to contain (in addition to the sequences contained in λ-1 and λ-2) a 6.7-kb *Eco*RI fragment that included the sequence of the 1.4-kb probe. This 6.7-kb *Eco*RI fragment contained sequences that hybridized to the 2.8-kb RNA from IFN-treated cells as well as the labeled product generated by primer extension of the 124-bp *Eco*RI fragment, indicating that λ-3 contained the entire *ISG-54K* transcription unit, including a small 5′ exon missing from the previous clones.

Identification of ISG-54K RNA Initiation Site and Promoter. Exon 1 of *ISG-54K* was mapped using labeled RNA probes generated following subcloning of various restriction fragments of the 6.7-kb *Eco*RI fragment into appropriate SP6 bacterial vectors (16). These probes were analyzed for exon complementarity by protection from RNase T2 digestion following hybridization of IFN-stimulated RNA. Labeled RNA transcribed leftward from a *Hin*dIII site in the 6.7-kb *Eco*RI fragment (5′ SP-6 probe in Fig. 1*A*) was protected by mRNA over a stretch of ≈80 nt (Fig. 1*C*, lanes 1 and 2). A similar analysis applied to nuclear RNA identified the RNA initiation site and position of exon 1 on the map. Nuclear RNA from IFN-treated cells, which should contain unspliced RNA transcripts for *ISG-54K*, was found to protect an ≈300-nt portion of the probe in addition to the 80-nt fragment protected by cytoplasmic RNA (Fig. 1*C*, lane 4). Nuclear RNA from untreated cells failed to protect any portion of the probe (lane 3). Thus, the ≈80-nt exon 1 must begin ≈300 nt upstream from the *Hin*dIII site and ≈2.75 kb from the 5′ end of the leftmost *Eco*RI fragment of λ-3 (Fig. 1*A*).

The position of exon 1 indicated that λ-3 contained ≈2.75 kb upstream of the RNA initiation site and therefore should contain the *ISG-54K* promoter. Evidence that a promoter lay within the 5′ 3-kb *Hin*dIII fragment of λ-3 was obtained from RNA polymerase II *in vitro* transcription reactions with HeLa cell nuclear extracts (Fig. 1*D*). A control linearized template containing the adenovirus type 2 major late promoter (15) stimulated formation of a 197-nt labeled RNA (lane 1), indicating correct initiation on this template. Reactions performed in the absence of added DNA generated no specific RNA transcripts (lane 3). When the 3-kb *Hin*dIII fragment of λ-3 was used as template, a labeled RNA of ≈300 nt was generated (lane 2). The 300-nt RNA produced *in vitro* corresponds in size to the 300-nt fragment of the SP6 probe protected by nuclear RNA in the RNase T2 experiment (see Fig. 1*C*, lane 4).

Nucleotide Sequence of *ISG-54K*. The nucleotide and predicted amino acid sequences of ISG-54K are presented in Fig. 2. The sequence TATATATA, which contains the TATA motif important in determining the start site of transcription by RNA polymerase II (20), occurs 33 nt prior to the presumed initiation site (labeled +1 in Fig. 2). An adenosine residue preceded by a cytidine is considered favorable for RNA polymerase II initiation. The sequence AG/GT, a splice donor consensus (21), occurs at positions 79–82, marking the presumptive 3′ end of exon 1. The probable splice acceptor site AG/TG preceded by a stretch of 9 pyrimidine residues

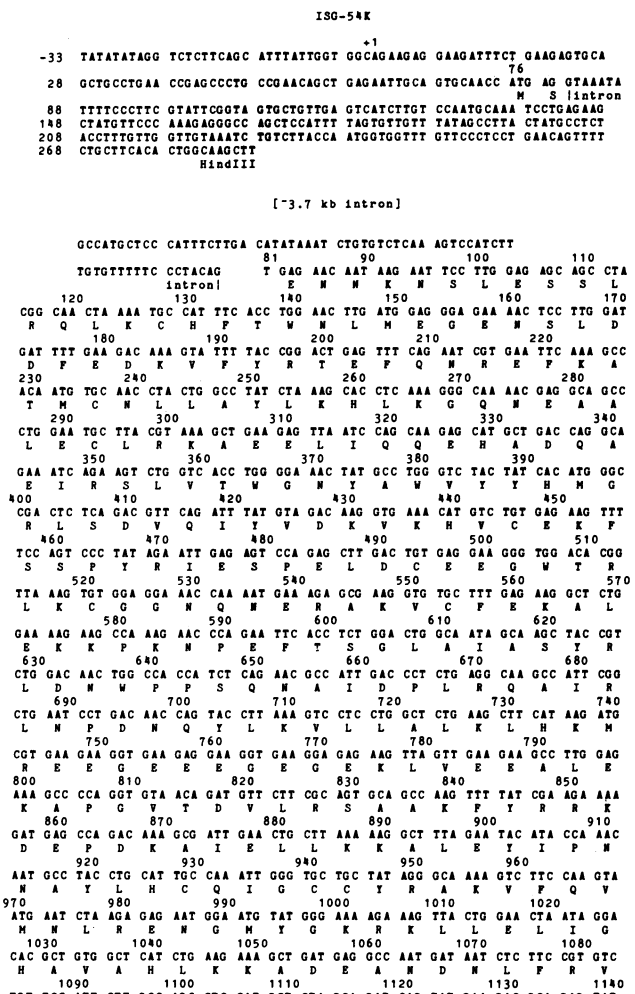

FIG. 2. Nucleotide sequence and translated amino acid sequence (single-letter code) of ISG-54K. Sequence is presented in two sections separated by ≈3.7 kb of intron that has not been totally sequenced. Numbering is from the proposed RNA initiation site with respect to the primary transcript in the first section and from the initiation site with respect to the processed mRNA in the second part. Proposed splice donor and acceptor sites are indicated. The sequence is presented from 33 bp upstream to the first *Hin*dIII site downstream of the gene.

occurs ≈3.7 kb downstream, identifying the likely beginning of exon 2 (position 81 in Fig. 2 *Lower*). This potential splice acceptor site is located 13 nt prior to the *Eco*RI cleavage site marking the boundary between the 1.4-kb terminal fragment and the 124-bp *Eco*RI fragment of λ-2. An SP6 probe complementary to the region from 96 nt upstream of this potential splice site to nt 332 of the mRNA was protected by RNA for ≈250 nt (not shown), reinforcing the assignment of a splice junction at this position.

Exon 2 is presented as a single contiguous sequence. This was justified by the detection of a single 2.7-kb uninterrupted R loop on λ-2 DNA after annealing with IFN-stimulated RNA (not shown). This sequence contains an open reading frame of 470 triplets (positions 82–1491) followed by ≈1120 nt of presumed 3′ untranslated sequence. The hexanucleotide AATAAA is encountered at nt 2576 signaling 3′ end processing. Use of this putative poly(A) site (22) and splicing of exons 1 and 2 would lead to production of a mRNA molecule of the observed 2.8-kb size. The sequence of the cDNA clone used for the initial selection of *ISG-54K* lies just upstream between nt 2221 and 2400. The use of two additional upstream polyadenylylation sites within the 3′ untranslated sequence were also detected in mRNA from IFN-treated HeLa cells by an RNase T2 protection assay mapping to potential processing signals at nt 2308 and 2436 (not shown). The open reading frame at the 5′ end of exon 2 does not begin with a methionine initiator codon, ATG. However, when exons 1 and 2 are fused, as predicted by the splice donor and acceptor sites, a methionine codon from the 3′ end of exon 1 is brought into register with the open reading frame of exon 2, creating the 472-amino acid contiguous open reading frame. Interestingly, a 15-kDa IFN-induced protein (23) is also encoded by a gene in which the second coding exon similarly lacks an AUG and the first short exon provides the initiating methionine codon (N. Reich, B. Evans, E. K. Knight, and J.E.D., unpublished data).

The predicted protein for ISG-54K would contain 472 amino acids and have an approximate molecular mass of 54 kDa. The primary translation product would be very hydrophilic, rich in uncharged polar (29.1%) and charged (33.7%) amino acid residues, with the latter displaying a somewhat clustered distribution. This composition is somewhat reminiscent of the predicted structure for Mx protein, a mouse IFN-inducible virus inhibitory protein (24). In fact, the 14-amino acid sequence from one charged segment of ISG-54K (positions 718–759) is very similar to a 13-amino acid stretch of Mx (residues 73–85), conserving charge and hydrophobicity, as shown:

L A L K L H K M R E E G E E = ISG-54K
L V L K L K K L R – E G E E = Mx

Beyond this limited possible homology, the ISG-54K sequence bears no striking similarity to any protein or DNA sequence contained in the GenBank§ or National Biomedical Research Foundation¶ computer databases.

Identification of the *ISG-54K* Regulatory Region. Potential regulatory regions of *ISG-54K* were examined by testing recombinant vectors for IFN-stimulated RNA production in human cells. DNA fragments beginning at various distances 5′ to the cap site and ending at the *Hin*dIII site within the intron (203 nt 3′ to exon 1 splice donor site; nt 288 in Fig. 2)

§National Institutes of Health (1986) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Inc., 10 Moulton St., Cambridge, MA 02238), Tape Release No. 44.0.

¶National Biomedical Research Foundation (1985) Protein Sequence Database, Protein Identification Resource (Natl. Biomed. Res. Found., Washington, DC 20007), Release No. 7.0.

were fused to a marker sequence, a portion of the adenovirus type 5 *E1b* gene lacking its own promoter (18). Specific transcription from such a recombinant template can be detected by using a labeled RNA probe complementary to E1b in a RNase T2 protection assay as diagramed in Fig. 3*A*.

Recombinant plasmid vectors containing either 2.75 kb or 800 bp of 5′ flanking DNA were transfected into human HeLa cells as a complex with DEAE-dextran. After 18 hr, transfected cells were treated for an additional 6 hr with IFN-α (500 units/ml), while similarly transfected control cells were left untreated. RNA isolated after these treatments was tested for E1b-specific transcripts in the RNase T2 protection assay. Whether the transfected plasmid contained 2.75 kb or 800 bp of 5′ flanking sequence, IFN-dependent expression of the transfected genes was clear (Fig. 3*A*, compare lanes 1 and 2 with 3 and 4) mimicking the inducibility of the endogenous gene (lanes 5 and 6). Cells transfected with the vector alone or with recombinant vectors containing intron and exon 2 sequence alone failed to produce detectable specific transcripts (not shown). Thus, IFN-stimulated regulatory elements must lie in the 5′ portion of *ISG-54K*.

The normal cycle of IFN-stimulated transcription in euploid fibroblasts involves a rapid initial increase in transcription followed by a return to pretreatment levels within 6–8 hr accompanied by a refractory period lasting 24–48 hr during which cells fail to respond to additional IFN (4, 11). This decay of transcriptional activity appears to be an active process since it can be blocked and reversed by inhibitors of protein synthesis. The second, inhibitory arm of the transcription cycle is more readily detected in normal diploid human fibroblasts than in the aneuploid transformed HeLa cells used for transfection. Due to the inefficiency of transfection of these fibroblasts using naked DNA, the −800 nt ISG-54K:E1b plasmid was incorporated into an adenovirus vector (18). Such viral vectors efficiently infect many cell types and thus serve as a convenient vehicle for enhanced transfer of test DNA sequences into normal cells.

Human FS2 cells were infected with the recombinant adenovirus bearing the −800-bp to +288-bp region of ISG-54K. After 24 hr, a regimen of IFN and cycloheximide addition was begun. At 48 hr postinfection, RNA was isolated from the various treatment groups and analyzed by RNase T2 protection (Fig. 3*C*). Cells that received adenoviruses containing the inactive mouse β-globin promoter produced no detectable E1b RNA regardless of IFN treatment (lane 1). Cells infected with the *ISG-54K* virus and not treated with IFN showed very low levels of E1b RNA (lane 2), while those treated for 3 hr with IFN contained 50-fold higher levels of RNA transcribed from the recombinant gene (lane 3), similar to the level transcribed from the endogenous gene (lanes 6 and 7). However, IFN applied to cells for 24 hr produced substantially less specific RNA than did cells treated for 3 hr (lane 4). This loss of specific transcripts following prolonged exposure to IFN was readily reversed by inhibiting protein synthesis with cycloheximide during the IFN treatment (lane 5). These experiments indicate that the regulatory elements for both the positive and negative arms of the IFN-mediated transcriptional cycle reside within the 5′ portion of *ISG-54K*.

DISCUSSION

IFN-stimulated genes provide a particularly attractive system in which to examine how transcription in the cell nucleus is governed through occupation of a cell-surface receptor by its polypeptide ligand. These genes respond to the inductive signal rapidly with an increase in transcriptional initiation of at least 100-fold (4). Since cycloheximide does not block the transcriptional response (11), a previously existing pool of transcription factors is apparently used, possibly after some modification governed by a change in second messenger level. After treatment with IFN for several hours, a decline of transcription rate occurs followed by the establishment of a refractory state (11). These events imply the involvement of negative as well as positive signals that affect transcription of IFN-sensitive genes. In the present work, we have isolated an IFN-stimulated gene and, with the aid of plasmid and adenovirus vectors, identified IFN-stimulated regulatory elements in the 5′ region responsible for both a regulated increase and a decrease in expression. This latter conclusion is based on the experiment with the recombinant adenovirus where the ISG-54K:E1B mRNA is present in large amounts 3 hr after IFN treatment but is greatly reduced after 24 hr of

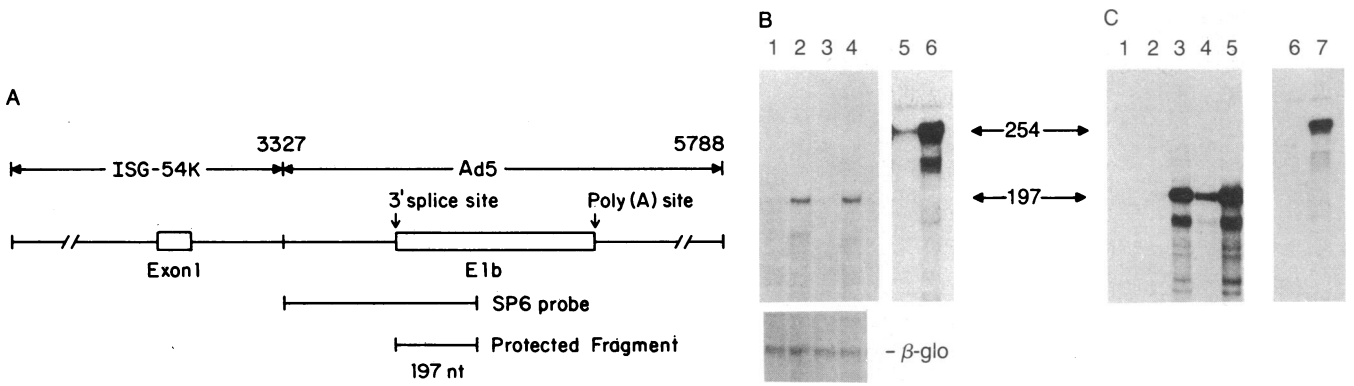

FIG. 3. Expression of the ISG-54K recombinant promoter constructs in recipient cells. (*A*) Schematic representation of E1b fusion vectors. The 5′ portion of *ISG-54K* was fused with the E1B region (nt 3327–5788) of adenovirus type 5 (Ad5). Transcripts initiated at the *ISG-54K* promoter will result in the *ISG-54K* first exon spliced to the E1b second exon. The SP6 probe complementary to the E1b region will be protected by mRNA for 197 nt, as indicated. (*B*) HeLa cells were transfected with E1B fusion vectors containing ≈2.75 kb (lanes 1 and 2) or ≈800 bp (lanes 3 and 4) of ISG-54K 5′ flanking sequence along with a control plasmid, containing the simian virus 40 early region enhancer and expressing the first two exons of mouse β-globin, used to assess transfection efficiency. After 24 hr, cytoplasmic RNA was extracted from cells untreated (lanes 1, 3, and 5) or treated for 6 hr with α-IFN (500 units/ml) (lanes 2, 4, and 6) and was assayed for the expression of E1B sequences (*Upper*) or β-globin (β-glo) sequences (*Lower*). Expression from the endogenous *ISG-54K* was measured with a probe containing 254 nt complementary to exon 2 (lanes 5 and 6). (*C*) Human fibroblasts were infected at a multiplicity of 70 plaque-forming units per cell with recombinant adenoviruses containing either the mouse β-globin promoter (lane 1) or the *ISG-54K* promoter fused to E1B sequences (lanes 2–5). After 48 hr, total cell RNA was prepared from cells untreated (lanes 1 and 2) or treated for 3 hr (lane 3) or for 24 hr (lane 4) with α-IFN (500 units/ml) or for 24 hr with α-IFN (500 units/ml) and cycloheximide (35 μg/ml) (lane 5). All samples were then assayed for E1B expression using 10 μg of extracted RNA. Expression of endogenous *ISG-54K* was measured using 10 μg of RNA from control fibroblasts untreated (lane 6) or IFN-stimulated for 3 hr (lane 7) using the 254-nt exon 2 probe. Positions of the 254-nt exon 2, the 197-nt E1B, and the β-globin protected fragments are indicated.

treatment. In addition, this specific reduction in mRNA abundance is blocked by cycloheximide treatment. Thus, the recombinant gene in an adenovirus vector behaves similarly to the endogenous chromosomal gene. These experiments are important initial steps toward elucidation of the regulatory proteins responsible for the cycle of transcriptional responses to IFN.

In this paper, we have not addressed the issue of whether changes in gene expression from newly introduced DNA result from transcriptional rather than posttranscriptional events. However, we note that removal of sequences between positions −800 and −162 decreases expression from recombinant plasmids by ≈75%, indicating that the IFN response from both the plasmid and adenovirus vectors must be mainly at the transcriptional level (unpublished observations). Furthermore, initial direct assays of transcription in isolated nuclei from recombinant virus-infected cells show IFN-dependent transcriptional activation from the ISG-54K promoter.

Two other cases of transfer of IFN-sensitive genes into cells have recently been reported. Yoshie *et al.* (25) described experiments in which the human *HLA-B7* gene was introduced stably into mouse cells. Of the resulting transfectants, some showed a modest increase in HLA mRNA abundance following IFN treatment, but this response did not appear to be promoter dependent. Kelly *et al.* (29) have placed a 20-kb human fragment containing a gene termed 6-16 stably into mouse cells. Mouse IFN induces the expression of RNA from the human gene, indicating that the factors responsible may cross species barriers. Although expression in this case may be promoter dependent, IFN induction of the endogenous 6-16 gene has not been shown to be regulated at the level of transcription.

We have also stably transfected *ISG-54K* into human cells. In this case, as well as in the cases of the adenoviral and plasmid vectors discussed above, IFN-dependent expression was found. Therefore, no particular chromosomal location or configuration seems necessary to ensure that regulatory elements are receptive to the cell-surface signal.

The nature of the intracellular signals that in response to IFN result in gene-specific transcriptional activation remains quite obscure. Consider, for example, the fact that platelet-derived growth factor (PDGF) and β-IFN, acting through distinct receptors, can both stimulate specific gene transcription in human fibroblasts but of different genes (27). However, PDGF and γ-IFN or β-IFN and γ-IFN, also occupying separate receptors, can stimulate some of the same genes (26, 28). A popular hypothesis has been that different transcriptional factors are activated by different receptor–ligand interactions. The availability of purified DNA that responds appropriately when introduced into ligand-susceptible cells should allow a further definition of the DNA sequences and transcriptional factors responsive to cell-surface signals.

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