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Enhancer-like interferon responsive sequences of the human and murine (2′ – 5′) oligoadenylate synthetase gene promoters

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The human (2′ – 5′) oligo(A) synthetase gene contains two independent *cis*-acting DNA elements, A and B, which act as transcriptional enhancers. Element A alone is not activated by IFN treatment. Element B alone confers IFN-inducibility to the herpes tk promoter. Two murine (2′ – 5′) oligo(A) synthetase genes were isolated and their promoter sequences show high conservation of element A and B. A synthetic oligonucleotide, containing 16 bp of the human element B, or 14 bp of the homologue murine element B, was linked to a TK-CAT construct. These oligonucleotides were shown to be sufficient to activate the TK promoter in the presence of IFN. When multiple repeats of the interferon-responsive sequence (E-IRS) were cloned in 5′ of the TK promoter, the activation ratio was increased. *In vitro*, specific binding of nuclear protein(s) is observed to the radiolabelled synthetic human E-IRS. This binding is competed by the addition of cold synthetic mouse E-IRS or fragments of genomic DNA containing the E-IRS.

Key words: (2′ – 5′) oligo(A) synthetase/gene/human/murine

Introduction

Binding of interferons (IFNs) to their cell surface receptors is followed by the rapid transcriptional activation of a number of cellular genes which may be the mediators of the various biological effects of IFNs (Revel and Chebath, 1986; Friedman *et al.*, 1984; Larner *et al.*, 1984; Hannigan and Williams, 1986; Staeheli *et al.*, 1986). The promoter region of these genes was shown in several cases to contain the information for IFN-inducible transcriptional activity (Samanta *et al.*, 1986; Levy *et al.*, 1986; Vogel *et al.*, 1986; Israel *et al.*, 1986; Chebath *et al.*, 1987a). A conserved DNA sequence of ~28 nt found in the promoter region of most human and murine MHC genes and in the metallothionein gene (Friedman and Stark, 1985) was recently shown to be involved in the response of two murine MHC class I genes to type I IFN (Israel *et al.*, 1987; Sugita *et al.*, 1987). If this conserved region contains an interferon-responsive sequence (IRS) which is the target of IFN action, it would be expected to be present and functional in other IFN-activated genes, even though the entire MHC consensus sequence is not conserved (Revel and Chebath, 1986). In addition several IFN-activated genes show larger increases in their mRNA and protein products than the MHC genes, suggesting that different regulatory elements acting on or after transcription, determine the final level of their products in untreated and IFN-treated cells (Revel and Chebath, 1986; Friedman *et al.*, 1984; Larner *et al.*, 1984).

The human (2′ – 5′) oligo(A) synthetase, E gene (Benech *et al.*, 1985), is highly activated by various IFNs. We previously defined a 5′-flanking DNA segment of the E gene which can drive the IFN-induced transcription and expression of the bacterial chloramphenicol acetyl transferase (CAT) gene in transfected human cells (Benech *et al.*, 1987; Chebath *et al.*, 1987a). Deletions indicated that the active segment comprises 72 bp immediately upstream of multiple RNA start sites, in a region devoid of the consensus TATA and CAAT transcriptional signals (Dynan and Tjian, 1985). An additional repressor-like element was identified by competition experiments and probably constributes to the 100-fold induction ratio of the (2′ – 5′) oligo(A) synthetase (Benech *et al.*, 1987).

The 72 bp fragment contains two enhancers, one in the 5′ part, called element A, which seems to function as a constitutive enhancer and another defined as the last 16 bp in 3′ and designated element B or E-IRS which seems to be the specific target of IFN-β_1 action (Benech *et al.*, 1987). Both elements A and B could be inverted and/or inserted at more than 1 kb from the promoter and keep their activity. As an evaluation of the conservation of the IFN regulatory system between species, we have now investigated whether the regulatory elements A and B exist in the murine (2′ – 5′) oligo(A) synthetase gene 5′ sequences. We have analyzed the sequence and function of the 5′ regions of two of these murine genes recently isolated in our laboratory. Comparison with the human gene showed that the elements A and B are very conserved, and the homologous murine fragment is functionally similar to the human one. The smallest region of homology between the human and mouse E gene IFN-regulated sequences (E-IRS) and the H2 kd IRS (Israel *et al.*, 1986), or the consensus IRS previously defined by sequence homology programs (Friedman and Stark, 1985), was chosen to prepare synthetic oligonucleotides corresponding to the human or murine version of the (2′ – 5′) oligo(A) synthetase E-IRS. These oligonucleotides, cloned in 5′ of the TK promoter, render its transcription IFN-inducible while a mutated one is inactive. The murine and human version of the E-IRS both perform the same function and have the same activity in mouse and human cells. A hexamer of the E-IRS is 5-fold more active than the basic unit. *In vitro*, the oligonucleotides bearing murine or human IRS are able to form a specific complex with nuclear proteins, and seem to compete for binding to the same factor(s).

Results

Comparison of the human and mouse interferon-responsive sequences

As probe for the isolation of the murine (2′ – 5′) oligo(A) synthetase gene, we used cDNA clone L3 (Figure 1A), isolated by cross-hybridization to the human full length

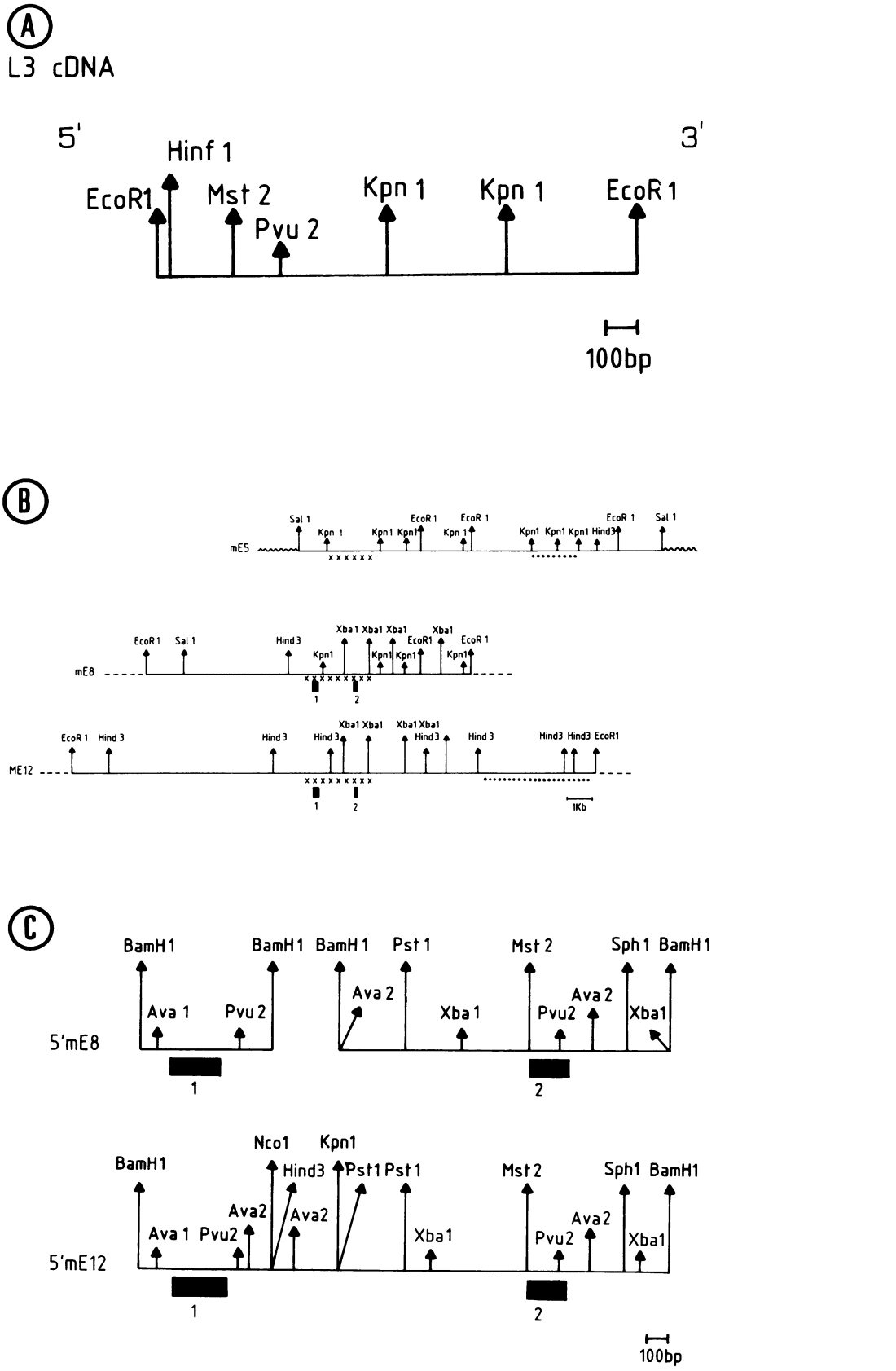

Fig. 1. Murine (2′−5′) oligo(A) synthetase cDNA and genes. **A**: Restriction map of the L3 cDNA. **B**: Restriction maps of the overlapping ME-5 and ME-8 genomic clones and of the distinct ME-12 gene. Crosses indicate hybridization with the *Eco*RI−*Pvu*II 5′ part of L3 cDNA. Dots indicate hybridization only with the entire L3 cDNA. **C**: Detail of genes ME-8 and ME-12 in the region of the first and second exons (black squares). Sequence in Figure 2 is from the leftmost *Bam*HI site.

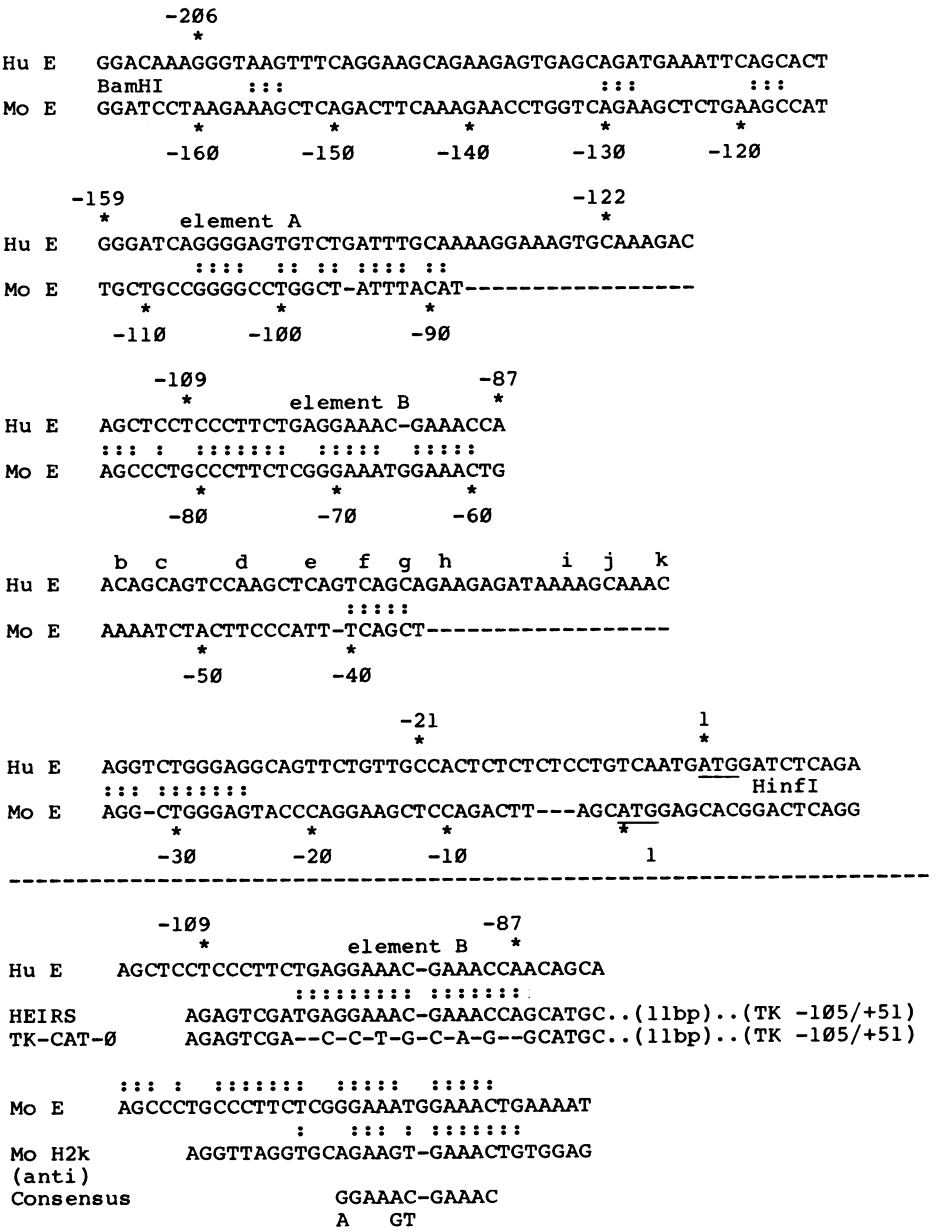

Fig. 2. 5′ flanking sequences of the (2′–5′) oligo A synthetase genes. The human E gene (Hu E) is numbered from the initiator ATG codon (Benech *et al.*, 1985). Letters b to k designate the multiple RNA start sites mapped previously (Benech *et al.*, 1987). The weak start site a at −96 is not shown. The murine ME-12 gene (Me-E) *Bam*HI–*Hin*fI (Figure 3) is aligned for maximum homology with the human gene. The ATG codon of the mouse cDNA (Ichii *et al.*, 1986) is underlined. **Bottom**: the antisense strand of the mouse H2-K gene promoter IRS region (Israel *et al.*, 1986) is compared to human element B. TK-CAT-O shows the sequence of part of the polylinker in pGEM-TK-CAT-O preceding the TK promoter (see Materials and methods), in comparison to hE-IRS containing the synthetic hexadecamer element B inserted between the *Sal*I and *Sph*I blunt-ended sites of the polylinker.

cDNA 9–21 (Benech *et al.*, 1985) from a λgt11 cDNA library from mouse L929 cells treated by murine IFN-α,β (E.Coccia, G.B.Rossi, J.Chebath, unpublished). The sequence of this murine cDNA was found to be the same as that determined by Ichii *et al.* (1986) and included 20 bp of the 5′ untranslated region. With this probe three genomic clones were purified from BALB/c mouse genomic libraries and mapped by restriction analysis and hybridization to either the *Eco*RI–*Pvu*II 5′ fragment or the *Kpn*I–*Eco*RI 3′ fragment of the L3 cDNA (Figure 1B). Clone ME-12 represented a complete gene while clones ME-8 and ME-5 appear to be overlaps of a second murine (2′–5′) oligo(A) synthetase gene differing from ME-12. Analysis of additional genomic clones revealed still other forms of the gene. A Southern electrophoretic blot of mouse genomic DNA hybridized to L3 cDNA showd six bands of 21, 12, 6, 2.4, 1.9 and 1.1 kb (not shown). Four of the bands can be accounted for by ME-5/ME-8 and ME-12 and the other bands probably originate from one or more additional genes.

The 5′ end of the L3 cDNA was mapped on the two mouse genes ME-8 and ME-12 and the localization of the first and second exon was determined by partial sequencing (Figure 1C, black squares and Figure 2). The 600 bp segments containing exon 1 between two *Bam*HI sites (ME-8), or between a *Bam*HI and a *Hin*dIII site (ME-12), (Figure 1) were cloned in the multiple cloning site of the PGEM TK CAT-O vector

(Figure 3), in front of the TK promoter. Alternatively, 175 bp fragments starting at the *Bam*HI site in 5′ and ending after the ATG at a *Hin*fI site (Figure 2) were inserted in the same vector. The chimeric construct DNAs were transfected into monkey COS-7 cells, or murine NIH/3T3 cells challenged respectively with human β_1 IFN or mouse α/β IFN. As shown in Table I, the CAT activity expressed from all the constructs is inducible by IFN. The IFN-induced expression is on average 5-fold higher than the constitutive one. The ratio of induction is similar in the monkey and murine

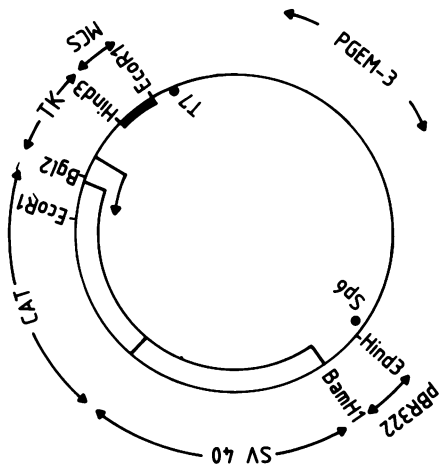

Fig. 3. Restriction map of the pGEM TK CAT-O vector. The TK promoter, the coding sequence of the CAT and the SV40 DNA Tag intron and polyadenylation site are cloned in the pGEM-3 vector (see Materials and methods). TK = The *Bam*HI−*Bgl*II fragment of the pBLCAT-2 vector (Klein-Hitpass *et al.*, 1986) representing the −105/+51 fragment of the herpes virus thymidine kinase was cloned in the *Bgl*II site of the small polylinker preceding the CAT gene. The other cloned DNA fragments were described before (see Materials and methods, and Benech *et al.*, 1987). The main transcription start site and orientation of transcription is shown by an arrow pointing from the position +1 of the TK promoter. The oligonucleotide hE-IRS, mE-IRS or mutated IRS (see Materials and methods) were cloned between the *Sal*I and *Sph*I (rendered blunt end) sites of the multiple cloning site (MCS) of the pGEM-3.

cells. The 600 bp and 170 bp segments have the same ability to make the TK promoter inducible by IFN.

The sequence of the 170 bp ME-12 region upstream of the initiator ATG and 5′ untranslated cDNA segment is shown in Figure 2, in comparison to the human gene.

We have shown before that the regions −159 to −122 (element A) and −109 to −87 (element B) of the human E gene (Figure 2) are essential for the human (2′−5′) oligo(A) synthetase expression (Benech *et al.*, 1987). In addition, when the human E gene promoter was replaced by the TK promoter, we found that the oligonucleotide bearing the −102/−87 sequence of the human gene was by itself sufficient to render the TK promoter inducible by IFN (Benech *et al.*, 1987). Sequence comparison indicates strong homology between the murine ME-12 (or ME-8) and the human gene in the region of element A (Figure 2) with conservation of the G cluster and of a sequence having in both human and mouse genes seven out of eight nucleotide identity with the ATTTGCAT motif of the immunoglobulin gene enhancer (Falkner and Zachau, 1984). Sequence homology is also very strong in the region of element B of the human gene (Figure 2), but 17 residues between the two elements in the human gene appear to be missing in the mouse gene. Outside these two regions, the similarities between the 5′ flanks of the human and mouse (2′−5′) oligo(A) synthetase genes is limited to a few short sequences (Figure 2). This is contrasting with the high homology in the coding sequences of human and mouse cDNAs reported by Ichii *et al.*, 1986. The 5′ flanking *Bam*HI−*Hin*fI fragment of the second murine gene ME-8 showed only individual nucleotide differences with the ME-12 gene; the sequences homologous to human elements A and B were identical in both mouse genes. When the ME-12 *Bam*HI−*Hin*fI segment shown in Figure 2 was tested in front of the TK promoter and CAT gene, the latter became IFN-inducible (Table I).

The interferon responsive sequence common to the (2′−5′) oligo(A) synthetase and histocompatibility antigens

A homology search with the 28 bp-long consensus IRS sequence found by Friedman and Stark (1985) in front of MHC genes and recently shown to be important for the IFN response of these genes (Israel *et al.*, 1986; Sugita *et al.*, 1987), showed significant sequence conservation with both human and mouse (2′−5′) oligo(A) synthetase sequence in element B. Figure 2 (bottom) shows the homology of the antisense strand of the consensus IRS of the murine H2-K gene (Israel *et al.*, 1986) with the sense strand of the (2′−5′) oligo(A) synthetase genes.

The region showing the best homology between these regulatory sequences was located 3′ of the element B, from −102 to −89 (Figure 2, bottom). This region is represented by the chemically produced hexadecamer TGAGGAAACGAAACCA (−102 to −87) in the human gene which confers IFN-induced expression to the TK-CAT

Table I. Effect of 5′ sequences of two murine (2′−5′) oligo(A) synthetase genes on the stimulation of the CAT gene expression by IFNs

Plasmids pGEMTKCAT	COS-7 cells CAT activity			NIH/3T3 CAT activity		
	−IFN	+IFN	Ratio*	−IFN	+IFN	Ratio*
ME-8 *Bam*HI−*Bam*HI 600 bp	8.4	65.3	7.8	7	55	7.8
ME-8 *Bam*HI−*Hin*fI 170 bp	−	−	−	3.7	11	2.9
ME-12 *Bam*HI−*Hin*dIII 600 bp	1.5	4.9	3.2	2.8	8.4	3
ME-12 *Bam*HI−*Hin*fI 170 bp	2.1	4.3	2.0	8.4	41.5	4.9
pGME TK CAT-O	1	1.2	1.2			

The cells were transfected as described in Materials and methods with the indicated plasmid DNAs. Respectively 2 μg of proteins from COS-7 cells (Gluzman, 1981) or 30 μg of proteins from NIH/3T3 cells were used for CAT assay, for 2 h at 37°C. The numbers express the percentage of acetylated chloramphenicol (normalized relatively to the β-galactosidase expression). Values represent average of duplicates in the same transfection experiments. Student test (*t*-test, Dwass, 1970) applied to the ratio of induction showed a difference significant at the threshold of 1% between the ratios of induction of the pGEM TK CAT-O vector and every other plasmid.

construct (Table II). The synthetic 14 bp sequence CGGGAAATGGAAAC corresponding to the murine element B functions similarly (Table II). The nucleotides GAAAC seem to be the most conserved in the different versions of the IRS sequences found by homology searches (Friedman and Stark, 1975). We also prepared the synthetic oligonucleotide CGGGAAATGGCGTC where the GAAAC was replaced by GCGTC. The latter was inefficient to promote expression from the TK promoter, in the absence or presence of IFN (Table II). The polymer of the human sequence xhE-IRS (see Materials and methods), containing five monomers in tandem and one head to tail, is able to activate the TK promoter 5- to 6-fold more than the hE-IRS or mE-IRS unit in the presence of IFN, without significant alteration of the constitutive expression (Table II). In the experiment shown in Figure 4, we have analyzed by S1 nuclease the abundance and initiation sites of the CAT transcripts, after transfection of the chimeric gene promoters into HeLa cells. Most of the transcripts start at the TK promoter cap site whether in absence or presence of IFN, and a clear induction by IFN is observed for the TK-initiated transcripts originating from E-IRS-containing plasmids (3Δ51 (−206/−87), mE-IRS, xhE-IRS), but not for the transcripts from 3Δ52 (−206/−109). This indicates that the different CAT activities obtained are not due to differential stability of the RNAs or proteins produced. As also observed for the CAT activity (Table II) there is more RNA expressed constitutively from the 3Δ52 plasmid than from the 3Δ51 from which it is derived. This suggests that the IRS deletion has released some inhibition on the constitutive expression.

Specific protein binding to the IRS

To understand the mechanisms which underline the function of the IRS, it is important to establish whether some regulatory protein factor is able to bind to this DNA sequence. The mouse and human IRS function similarly in transfected cells, although they have only nine identical nucleotides out of the 14 nucleotides of the cloned mouse IRS. It is important to establish in addition whether the human and murine version of the IRS are binding the same factor(s). As shown in Figure 4, in order to characterize nucleic acid–protein complex formation *in vitro*, we used electrophoresis mobility shift assays (Fried and Crothers, 1981; Strauss and Varshavsky, 1984). In these assays the DNA fragments bound to proteins migrate through low ionic strength polyacrylamide gels more slowly than free fragments.

When the radiolabelled fragment FI, corresponding to the human E-IRS [−102 to −87 of the human (2′–5′) oligo(A) synthetase gene] (Figure 4) was reacted with different cell extracts, a major complex RII was observed. Specific binding was demonstrated by competition with excess of cold hE-IRS (see e.g. lanes A2 or B6), or mE-IRS (see e.g. lanes A3 or B7). On the contrary, an excess of the −83 to +82 human genomic fragment (5Δ24) which does not contain the IRS was much less efficient to compete for the formation of the radioactive RII (see e.g. lanes A4 or B8). The human

Table II. Effect of the element B of the human and mouse E genes on the IFN-induced expression of the herpes TK promoter fused to the CAT gene

Plasmids pGEMTKCAT	CAT activity		CAT activity
	−IFN	+IFN	Ratios
h3Δ51 (−206/−87)	1.7	17.8	10.5
h3Δ52 (−206/−109)	10.1	10.3	1.0
hE-IRS (−102/−87) TGAGGAAACGAAACCA	5.1	15.6	3.0
mE-IRS (−73/−59) CGGGAAATGGAAACT	4.2	13.7	3.2
mE-IRS mutated CGGGAAATGGCGTCT	1.2	0.9	0.8
xhE-IRS	4.5	92.5	20.5
pGEM TK CAT-O	1.0	1.3	1.3

HeLa cells were used in transient transfection experiments. Cell extracts, 30 μg of proteins, were incubated overnight to determine the CAT activity. The values represent average of triplicates performed in two different transfections. Student test (Dwass, 1970) showed that differences in the ratios of induction between each IRS-bearing plasmids and either the 3Δ52 factor or control pGEM TK CAT-O is significant at the threshold of 1%.

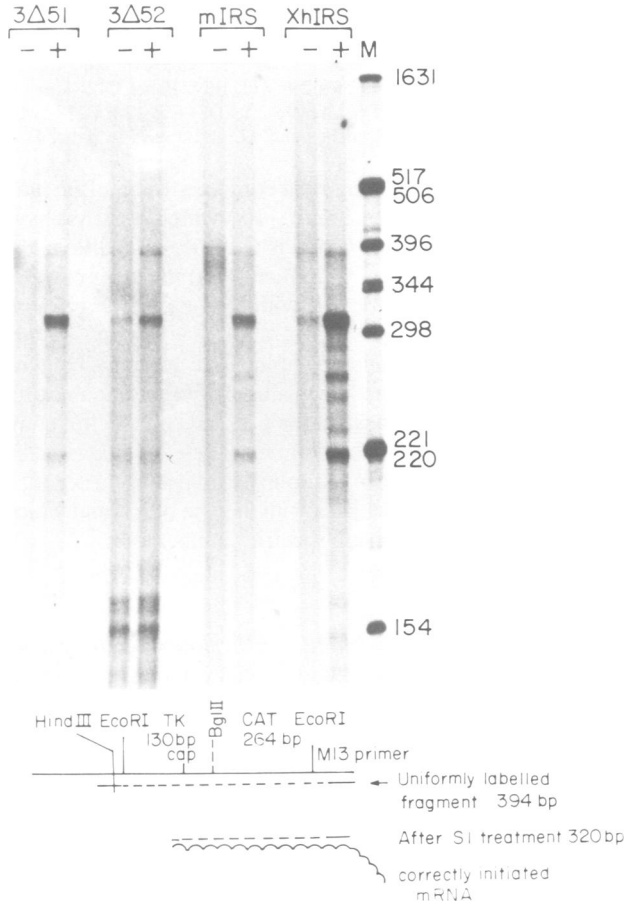

Fig. 4. Mapping of the 5′ end of the transcript derived from transfected chimeric genes. **Bottom**: The ^{32}P-labelled probe used for determining the transcription start site by the S1 nuclease assays and the fragments resulting from correctly initiated transcripts are shown. The probe was initiated at the M13 universal primer, cut with *Hin*dIII and separated from the template by electrophoresis on a denaturing gel. **Upper part:** Total cellular RNA was extracted from HeLa cells after transfection and treatment (+), or not (−) with βIFN. As indicated at the top, the human 3Δ51 fragment (−206/−87), the 3Δ52 (−206/−109), the mouse IRS oligonucleotide (mIRS), the polymer of the human IRS (xhIRS) cloned in the pGEM TK CAT-O were transfected in the cells. The sizes of the S1-nuclease resistant complexes were analyzed on a denaturing polyacrylamide gel (Materials and methods). The arrow points at the most intense signal, corresponding to the transcripts initiated at the TK cap site. M = size marker, end-labelled with ^{32}P (pBR322 *Hin*fI). Sizes are in nucleotides.

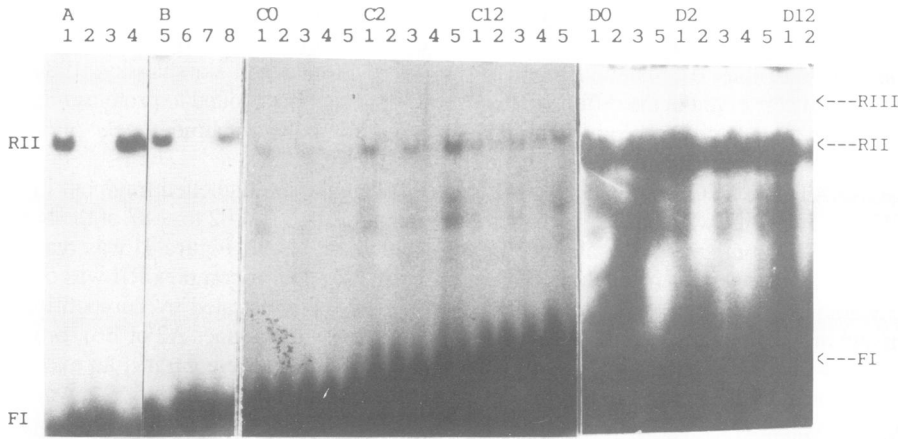

Fig. 5. Gel retardation assay for protein binding to element B. The end-labelled hE-IRS oligonucleotide was used as probe at 5 fmol/assay. Poly dI-dC DNA (50 ng) was added to the proteins 10 min before addition of the probe. **Panel A, lane 1**: 2 μg of proteins from untreated WISH cells. **Panel B, lane 5**: 2 μg of proteins from untreated HeLa cells. Cold competitor DNAs were added in 40-fold excess: **lanes 2** and **6**, hE-IRS; **lanes 3** and **7**, mE-IRS; **lanes 4** and **8**, −83 to +82 (5Δ24) fragment. **Panel C0, lane 1**: 5 μg proteins from untreated Daudi cells. Cold competitor DNA added in: **lane 2**, hE-IRS; **lane 3**, −83 to +82 fragment; **lane 4**, −206 to −87, (3D51); **lane 5**, −206 to −119, (3D52). **Panel C2, lanes 1−5**: same but Daudi cells pretreated for 2 h with IFN. **Panel C12, lanes 1−5**, same but 12 h IFN pretreatment. **Panel D** same as **C** with 10 μg proteins from Daudi cells untreated (D0), treated 2 h (D2) and 12 h (D12) with IFN. Competitors in **lanes 2−5** as in **panel C**. Exposure time was 5 h for the **panels A** and **B**, one overnight **panel C**, and 3 days in **panel D**.

genomic −206 to −87 fragment competes more efficiently for RII formation than −206 to −109 which does not have the E-IRS (see e.g. C2−4 versus C2−5). In addition, the RII complex was formed in higher amounts using extracts of Daudi cells treated for 2 h with IFN-β_1 than with non-treated extracts (compare C0−1 and C2−1 or D0−1 and D2−1). A slower migrating complex RIII, more visible with the 2 h-treated cell extract when a high concentration of protein is used, also disappear in the presence of specific competitors from both mouse and human genes. With Daudi cell extracts we observed also complexes migrating faster than RII, whose intensity correlates with the one of RII and which disappear upon addition of specific competitors.

Discussion

Multiple murine genes for (2′−5′) oligo(A) synthetase

In this work we describe two (2′−5′) oligo(A) synthetase genes ME-12 and ME-5/ME-8 hybridizing to the same murine L3 cDNA, but with clearly different restriction maps. In the genomic libraries screened with L3 cDNA, we found two additional clones ME-3 and ME-23 which are not overlapping ME-12 or ME-5, and account for some of the multiple *Eco*RI restriction fragments detected in Southern genomic blots. It is, therefore, likely that mice have at least two non-allelic (2′−5′) oligo(A) synthetase genes. The existence of multiple (2′−5′) oligo(A) synthetase genes may be related to the multiple enzyme forms (Benech *et al.*, 1985; St Laurent *et al.*, 1983; Chebath *et al.*, 1987b). In human cells, differential splicing of the single gene produces two mRNAs differing only in their 3′ ends (Benech *et al.*, 1985).

Our main objective was to compare the functions and sequences of the 5′-flanking regions of the murine (2′−5′) oligo(A) synthetase genes with the human gene. Both ME-12 and ME-8 showed significant homology to the human gene in the two regions A and B shown by functional assays to be involved in IFN-dependent expression.

IFN-responsive sequences in the human (2′−5′) oligo(A) synthetase gene promoter

The promoter of the human (2′−5′) oligo(A) synthetase gene is characterized by multiple RNA start sites and lacks a TATA box as well as a CAAT box (Chebath *et al.*, 1987a; Benech *et al.*, 1987). Deletion mapping showed that a 72 bp segment immediately preceding these multiple RNA starts is necessary for IFN-activated expression of the (2′−5′) oligo(A) synthetase promoter fused to the CAT gene (Benech *et al.*, 1987). We showed that this segment works as an enhancer which can be placed at distance from the promoter. We also demonstrated that this 72 bp segment contains two independent regulatory elements designated A and B, located respectively near the 5′ and 3′ ends of the 72 bp segment. Both elements have enhancer-like properties of orientation and position independence (Dynan and Tjian, 1985), and do not have to be physically close to each other suggesting that they perform different functions.

A homologous region was found in the two murine (2′−5′) oligo(A) synthetase genes and the alignment in Figure 4 suggests that 17 bp from −132 to −116 are absent in the murine genes and may be dispensable. In both human and murine genes, element A contains an octanucleotide with seven out of eight bases identity to the ATTTGCAT motif of the immunoglobulin gene enhancer (Falkner and Zachau, 1984). An homology of nine out of 15 nucleotides is also seen with the enhancer (CRE) region of H2 genes (Israel *et al.*, 1986; Sugita *et al.*, 1987) and the mouse or human element A, but precise functional mapping of element A remains to be completed. A cluster of four G which in the CRE corresponds to the binding site for a positive transcription factor (may be the Ap2 factor CTGGGGA) (Mitchell *et al.*, 1987; Israel *et al.*, 1987) is conserved in the mouse and human gene. Outside the region A, another homology is found at the sequence TCAGTCAG [−72/−65 in the human 2′−5′ oligo(A) synthetase gene], which is homologous to the Ap1 factor binding site (Lee *et al.*, 1987).

The sequences forming the IFN-responsive element B were identified in this work by the use of synthetic oligonucleotides. Element B had been defined by 3′ deletions as the 22 bp region −109 to −87 of the E gene (Benech *et al.*, 1987). A highly homologous sequence was found in the two murine genes. Since we had observed that element B

Table III. Sequence homology between interferon responsive elements

	References
T G C A G A A G T G A A A C T	H_2K^b, H_2L^d antisense (Kimura *et al.*, 1986)
A G C A G A A G T G A A A C T	Q_{10} antisense (Kimura *et al.*, 1986)
C G G G A A A T G G A A A C T	Murine 2′–5′ A synthetase (this paper)
T G A G G A A A C G A A A C C	Human 2′–5′ A synthetase (this paper)
C G G G A A A G G G A A A C C	ISG15 (−115/−102) (Reich *et al.*, 1987)
A G G G A A A C C G A A A C T	ISG15 (−109/−96) (Reich *et al.*, 1987)
G G G A A A G T G A A A C T	ISG54 (−90/−103) antisense (Reich *et al.*, 1987)
G N G A A A N N G A A A C T	Consensus
A G C	

was orientation independent, a similarity between sequences −102 and −87 in the E gene and a small region of the antisense strand in the MHC gene consensus IRS (Friedman and Stark, 1985) became more meaningful (Figures 2 and 4). Indeed the synthetic oligonucleotide TGAGGAAACGAAACCA corresponding to this region, is sufficient to confer IFN inducible expression to the TK promoter-CAT construct.

The mouse homolog, the oligonucleotide CGGGAAATGGAAACT, is as efficient as the human one, even though they have only nine nucleotides in common out of 14 (see Table III) when aligning the 3′ GAAAC sequence.

An excess of the mouse and human IRS oligonucleotides compete for the formation of complexes by the radiolabelled human E-IRS with nuclear proteins *in vitro*. On the contrary, fragments of the human E genes that do not carry the IRS do not decrease the formation of the RII or RIII complexes. These observations indicate that these complexes are sequence specific and that the two IRS versions though apparently different, bind the same protein (or proteins). Indeed the complexes formed by the mE-IRS with the same cell extracts migrate to the same level as the hE-IRS complexes (B.Cohen, in preparation). The comparison of sequences of the IFN responsive segments of various MHC genes (Kimura *et al.*, 1986) or of the ISG 15 and ISG 54 genes (Reich *et al.*, 1987), which show homologies to the mE-IRS or hE-IRS, lead us to the definition of a new consensus sequence (Table III). The GAAAC element of this sequence (nt 9–13) seems to be the most conserved and we could indeed show that mutations of the three A destroys its function.

The importance of the other nucleotides that seem to be highly conserved has still to be tested by functional assay *in vivo* or by protein binding assays *in vitro* using mutated oligonucleotides. The fact that the activity of the cloned E-IRS sequence in the *in vivo* functional assay is multiplied in correlation with the number of copies seems to be a further proof that the IRS itself is the active element to bind the intermediate factor (or factors) and that the adjacent plasmid sequence does not play a major role in its activity *in vivo*. Experiments using the polymer of the IRS as competitor in transient-expression experiments are in progress. They should indicate whether the IRS is the site of binding of a negative or positively acting factor or both.

It is possible that the protein bound to element B in untreated cells is modified as a result of IFN interaction with the cell surface receptors (Revel and Chebath, 1986), or as suggested by some authors (Kushnaryov *et al.*, 1985), after IFN interacts with nuclear receptor molecules. The amount of protein bound to element B may change early after IFN treatment, or the protein could be modified in such a way as to interact with other transcriptional factors. The decreased binding of the protein to element B at late times after IFN, may be related directly to the down regulation of the 2′–5′ oligo(A) synthetase at late times after IFN treatment.

The group of IFN-activated genes does not present a completely coordinated regulation since some of the genes respond to type I IFNs, others mainly to type II IFN-γ and yet others to all types of IFN (see Revel and Chebath, 1986, for review). However, the sequences defined here with type I IFN-β_1 will have to be tested also with the other IFNs. Sugita *et al.* (1987) recently suggested that the IFN-γ induction of MHC genes is not regulated as the response to type I IFNs. Additional regulatory elements which may act post-transcriptionally are likely to function in the IFN-activated genes (Friedman *et al.*, 1984; Larner *et al.*, 1984; Benech *et al.*, 1987). Comparison of the promoter functional sequences and those which are the target of IFN action in different IFN-activated genes may reveal differences sufficient to explain the variable induction patterns of these genes.

Materials and methods

Gene promoter constructs

All the plasmid constructions were derived from the pGEM TK CAT-O vector (Figure 3). The latter was formed by inserting in the *Hin*dIII site of the pGEM 3 (Promega) a *Hin*dIII fragment containing the TK gene promoter (−105/+51) (Coen *et al.*, 1986), the CAT coding sequences, the SV40 T antigen intron and polyadenylation sequences, and the *Bam*HI–*Hin*dIII fragment of pBR322 (Benech *et al.*, 1987). The pGEM TK CAT 3Δ51 and 3Δ52 vectors contain respectively the −206/−87 and −106/−109 fragments of the human E gene obtained by *Bal*31 deletions from 3′ (Benech *et al.*, 1987).

The synthetic double stranded oligonucleotides called hE-IRS, corresponding to the human genomic sequence −102 to −87

tcgaTGAGGAAACGAAACCAg
ACTCCTTTGCTTTGGTc

was cloned into the pGEM TK-CAT-O vector, previously cut with *Sph*I (in the polylinker sequence of the pGEM 3), rendered blunt end and recut with *Sal*I. The insertion of the oligonucleotide was verified by sequencing. As expected, the oligonucleotide was inserted in the same orientation as the TK promoter. The same was done with the double stranded oligonucleotide mouse E-IRS

tcgaCGGGAAATGGAAACTag
GCCCTTTACCTTTGAtc

or the mutated mouse E-IRS

tcgaCGGGAAATGGCGTCTag
GCCCTTTACCGCAGAtc

Another oligonucleotide bearing the human E-IRS with *Bam*HI–*Bgl*II adaptors was cloned in the *Bam*HI site of pGEM-3 after polymerization. Its sequence is

gatcTGAGGAAACGAAACCAg
ACTCCTTTGCTTTGGTcctag

The double-stranded oligonucleotides, 5 μg, were phosphorylated by T4 polynucleotide kinase and precipitated with ethanol. They were ligated overnight at 4°C with T4 DNA ligase (Kadanoga and Tjian, 1986) and mixed with pGEM-3 DNA cut with *Bam*HI and dephosphorylated. One of the cloned plasmids, that showed a 170-bp *Eco*RI−*Hin*dIII segment was sequenced by the dideoxy system (Gemseq K/RT, Promega). It contains one oligonucleotide with 5′ end toward the T7 promoter and five cloned head to tail in the opposite orientation. The *Hin*dIII fragment containing the TK promoter, CAT and SV40 segments (see above) was cloned in the *Hin*dIII site of the MCS of the same constructs.

Murine (2′−5′) oligo(A) synthetase genes

The L3 cDNA clone was isolated from a λgt11 cDNA library from mouse L929 cells treated by murine IFN-α,β, using the human E16 cDNA 9−21 (Benech *et al.*, 1985). The L3 cDNA *Eco*RI insert (1.4 kb) was used to isolated ME-8 and ME-12 from a genomic library of Balb/c mouse liver DNA partially digested by *Eco*RI and cloned in the arms of λ Charon 4A (Maniatis *et al.*, 1982). A second library of partially *Sau*3AI digested mouse DNA in EMBL-3 was used to isolate ME-5. Sequencing of the genes was done by the GemSeq K/RT system (Promega Biotech.) on sequential deletions by Exonuclease III (Stratagene) of the genomic subclones in pGEM-3.

To analyze the functional activity of the murine promoter sequences, the *Bam*HI−*Hin*dIII and *Bam*HI−*Hin*fI fragments of the clone ME-12 or the fragments *Bam*HI−*Bam*HI and *Bam*HI−*Hin*fI of the clone ME-8, were cloned in the pGME TK-CAT-O vector.

DNA mediated gene transfer and transient CAT assays

Cells were subcultured 1:4 every other day and plated at $3-4 \times 10^5$ cells/5 cm diameter dish 20 h before transfection. Co-precipitation of supercoiled plasmid DNA and calcium phosphate was as described (Gorman *et al.*, 1982), at a concentration of 10 μg of DNA/ml. As a rule duplicate or triplicate assays were processed as follows: 2 or 3 ml of precipitate were mixed thoroughly by gentle bubbling before distribution into four or six dishes. Precipitates were left on plates for 16 h, the medium was changed and after 6 h, half of the plates were treated with r-IFN-β_1 (Chernajovsky *et al.*, 1984), at 500 units/ml, for 24 h. The cells were harvested and lysed by freeze thawing in 100 μl 0.25 M Tris−HCl (pH 7.8). CAT assays were done as described (Gorman *et al.*, 1982), on various amounts of proteins or extract. For normalization of the results the pCH110 plasmid DNA (Hall *et al.*, 1983) was added to each precipitate at the concentration of 1 μg/ml. The pCH110 plasmid contains the *E.coli LacZ* gene under control of the SV40 early promoter. We used the ratio of CAT and β-galactosidase activities in the same extracts, untreated with IFN, to compare the efficiencies of transfection obtained with different precipitates. In the IFN induced extracts, the β-galactosidase activity was reduced by 15 to 50% relative to the control.

Gel retardation assay for protein binding to DNA

As radiolabelled probes for the DNA binding experiments and gel retardation assays (Fried and Crothers, 1981), we used either the hE-IRS with *Sal*I linker, or the hE-IRS with the *Bam*HI−*Bgl*II linkers. The oligonucleotides were radiolabelled in 5′ with T4 DNA polynucleotide kinase using [γ-^{32}P] ATP and purified by extraction from a non-denaturing polyacrylamide gel.

The unlabelled competitor fragments were the synthetic mE-IRS oligonucleotide or the hE-IRS. The *Xba*I fragments containing the genomic sequences −206 to −87 or −206 to −109, were extracted from the pGEM 3D51-CAT or pGEM 3D52-CAT respectively (Benech *et al.*, 1987). The *Xba*I fragments containing the −85 to +82 genomic sequence of the pGEM 5D24-CAT vector was also used as a non-specific competitor.

Nuclear extracts were prepared from 30 trays of confluent WISH cells. The nuclear fraction was obtained as described (Siebenlist *et al.*, 1984) and extraction made in a buffer containing 0.5 M NaCl, 100 mM Hepes pH 8.0, 25% of glycerol, 0.1 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF and 7 mM 2-mercapthoethanol. The nuclei were stirred for 30 min in ice with a magnetic stirrer. They were then spun for 10 min and the supernatant fractionated into aliquots kept at −70°C.

To obtain nuclear proteins from the lymphoblastoid Daudi cell line or HeLa cells in suspension, we used the technique of Dignam *et al.*, 1983. The cells were grown to a density of $1.5-2 \times 10^6$ cells/ml and treated with IFN-β_1 (250 units/ml) for 2 or 12 h (or left non-treated), before protein extraction.

As a rule, the nuclear extracts (2−10 μg of proteins) were mixed with the non-specific competitor DNA poly dI:dC (50 ng) (Pharmacia) in a final volume of 15 μl for 10 min at room temperature. Radiolabelled probe and cold DNA competitor fragments of known sequence were mixed in the molar proportions of 1/50 before addition to the extracts. Final incubation was done for 30 min, in a volume of 20 μl, in the presence of 10 mM Hepes (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 5% of glycerol. The samples were run on 6% polyacrylamide gels in Tris−Borate−EDTA 1× (Maniatis *et al.*, 1982) (10 Volts/cm, 30 mAmps).

S1 nuclease mapping

The *Eco*RI fragment of the pGEM TK CAT-O vector containing the CAT coding sequence and the TK promoter (Figure 3) was cloned in the polylinker of the Blue-Script vector (Stratagene). Uniformly labelled single-stranded probe (100 000 c.p.m.) was hybridized to 50 μg of total cellular RNA in 30 μl of 40 mM 1,4-piperazinediethane sulfonic acid (Pipes) pH 6.4, 80% (vol/vol) formamide, 0.4 M NaCl, 1 mM EDTA at 45°C for 12 h. Then the heteroduplexes were digested in 300 μl of 40 mM potassium acetate pH 4.8, 300 mM NaCl, 2.5 mM $ZnSO_4$, 20% glycerol containing S1 nuclease at 500 units/ml for 30 min at 37°C. After precipitation with ethanol, the protected fragments were separated on a 5% polyacrylamide 7 M urea gel (Maniatis *et al.*, 1982).

RNA preparation from transfected HeLa cells

The HeLa cells were seeded, 20 h before transfection, on 9 cm diameter dishes. The cells were transfected and treated with IFN in the conditions described above. After 12 h, the cells were lysed and the cellular RNA extracted by hot-phenol (Chebath *et al.*, 1987a).

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