# Differential response of the human 6-16 and 9-27 genes to α and γ interferons

Andrew M.Ackrill, Laurence E.Reid+, Christopher S.Gilbert, Dirk R.Gewert§, Andrew C.G.Porterφ, Andrew R.Lewin, George R.Stark and Ian M.Kerr*
Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

Received October 1, 1990; Revised and Accepted January 7, 1990

## ABSTRACT

**9-27 mRNA is expressed to a high level in response to both α and γ interferons. In contrast, 6-16 mRNA is expressed well in response to α but very poorly in response to γ interferon in human cells. The factors governing these different levels of expression were investigated. For both genes the major effect of both interferons is on transcription. A transcriptional bias in the 6-16 promoter/enhancer accounts in large part for the differential response of 6-16 to the two interferons. No single DNA element appears responsible; the smaller the 5′ region analysed the lower the absolute activity and the smaller the differential response to α and γ interferons observed. Both the 6-16 and 9-27 mRNAs are very stable and no effect of the interferons on stability was detected. Nor was any direct evidence obtained for preferential processing of the 9-27 mRNA. Nevertheless, differentials between the transcription and accumulation of mature mRNAs, particularly for 6-16 mRNA in response to γ interferon, suggest that post-transcriptional control(s) must additionally operate. The 9-27 5′ promoter/enhancer is much less active than that from 6-16 when placed 5′ of a marker gene, despite the similar response of the two genes to α interferon.**

## INTRODUCTION

A number of interferon-inducible genes have been isolated. Some respond predominantly to either α (eg 1–4) or γ (eg 5,6) interferon (IFN); others can respond well to both (eg 7,8). All of the predominantly α IFN-responsive genes characterised in detail have highly homologous interferon-stimulable response elements (ISREs) in their 5′ flanking sequence which are both necessary and sufficient to confer an IFN response (1–4). The ISREs include the sequence GGAAAN(N)GAAAC. Of those responding to both α and γ IFNs, the 9-27 and 2–5A synthetase genes at least have ISREs which also have this sequence (8–10). Consistent with this the 6-16 (GGGAAAATGAAACT) and the 9-27 (GGAAATAGAAACT) ISREs can each confer α and γ IFN inducibility upon a marker gene (8). Each can also interact in band shift assays with the α and γ IFN-inducible protein factors likely to be involved in the control of transcription (11,12). The 6-16 ISRE can, therefore, confer responsiveness either predominantly to α or to both α and γ IFNs depending on its context in the endogenous gene or marker constructs (8). Here we have analysed the basis for this context effect upon the transcriptional activity of the 6-16 gene and have investigated whether the IFN-inducible accumulation of the 6-16 and 9-27 mRNAs is to any extent under post-transcriptional control.

## MATERIALS AND METHODS

### Growth of cells, transfections, CAT assays and IFN treatment

These were carried out as described previously (1). A highly purified mixture of human α IFNs (Wellferon, $1.5\times10^8$ IU/mg protein) was from Wellcome Research Laboratories, Beckenham, Kent, UK (13). Recombinant human γ IFN ($4\times10^7$ IU/mg protein) was supplied by Dr. G. Adolf, Ernst Boehringer Institut fur Arzneimittelforschung, Vienna, Austria. HeLa and HPRT$^-$ HT1080 (human fibrosarcoma) cells, kindly provided by Drs. Hartmut Land and Peter Goodfellow, Imperial Cancer Research Fund, respectively, were grown as monolayers in Dulbecco's modified Eagle medium with 10% foetal calf serum. For stable transfectants pools of at least 50 drug-resistant colonies were generated by cotransfecting 1 μg of pSV2hyg or pSV2neo and selecting in medium containing 250 μg/ml hygromycin B or 400 μg/ml G418, respectively.

### RNA extraction and northern and RNase protection analysis of RNAs

All cytoplasmic RNA samples were prepared by the phenol-chloroform method and analysed by northern transfer as described previously (1). DNA probes were labelled by random priming (14) to specific activities of approximately $10^9$ cpm/ μg and were used at concentrations greater than $10^6$ cpm/ml. RNase protection was performed (15) using probes to yield protected fragments of 190 bp for 6-16 and 350 for tk- β-globin (see below, both kindly provided by Dr Stephen Goodbourn, Imperial Cancer

* To whom correspondence should be addressed

Present addresses: +Editorial Office, Cell, 50 Church Street, Cambridge, MA 02138, USA, §Wellcome Biotech, Langley Court, Beckenham BR3 3BS, Kent and φBiochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Research Fund) and a 130 bp fragment for γ-actin. A tk-9-27 fusion probe was used to yield protected fragments of 210 bp for the tk-9-27 fusion mRNA and 160 bp for the endogenous 9-27 mRNA. Probes were labelled to an activity of $2\times10^6$ cpm per μg of input DNA and 300,000 cpm of each probe and 10 μg of cytoplasmic RNA were used in each assay.

### Nuclear run-on assays

The method used was that of Rodaway *et al.* (16). Briefly, nuclei were resuspended in glutamate buffer (125 mM potassium glutamate, 10 mM HEPES, pH 8, 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM EGTA, 40% glycerol) before freezing at −70°C. Elongation (15 min at 37°C) was carried out in 50 μl of glutamate buffer containing 160 μCi [ $\alpha^{32}$P] UTP (800 Ci/mmol), 1.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 10 mM creatine phosphate and 50 μg/ml creatine kinase. Labelled RNA was isolated by phenol extraction followed by two ammonium acetate precipitation steps. The RNA was denatured in 0.3 M NaOH for 10 min before hybridisation to 5 μg of plasmid DNA containing either human actin cDNA, the −603 to +437 5′ region of the 6-16 gene (Figs.1 and 5) or a unique 295 bp 3′ fragment of 9-27 cDNA. Values were corrected for the difference in size of the hybridising regions (437 and 295 bp for 6-16 and 9-27, respectively). Choice of these plasmids was governed in part by the presence of a partial Alu repeat homology in the 3′ untranslated region of the 6-16 cDNA (17) and the fact that the remainder of the 9− cDNA sequence is highly homologous to that of other members of the 1−8 gene family (A.R.L., L.E.R. and I.M.K., unpublished). pGem1 DNA (5 μg) served as a negative control.

### Plasmids

The whole gene constructs (Fig.4) and plasmids A,B and C (Fig.5) have been described previously (1). Plasmids D and E (Fig.5) were generated by converting the *Hae*III and *NspB*II sites at −282 and −174, respectively, of the 6-16 5′ flanking region to *Bam*HI sites and cloning the *Bam*HI fragments (−282 to −40 and −174 to −40) into $pA_{10}CAT_2$ (18) linearised at the unique *Bgl*II site. To construct pSVG6-16 a fragment corresponding to the entire body of the 6-16 gene, from the *Pst*I site (replaced with *Bgl*II linkers) at −95 just 3′ of the two ISREs (Fig.1) to the *Xho*I site approximately 100 bp into the 3′ flanking region, was cloned into pGem2. PSV2CAT (19) was digested with *Pvu*II and ligated to *Sal*I linkers. The internal *Nco*I site was converted to *Bgl*II by *Nco*I cleavage, filling in with the Klenow fragment of DNA polymerase and ligation to *Bgl*II linkers. Ligation of the *Sal*I to *Bgl*II fragment, equivalent to nucleotides 37 to 272 of the SV40 promoter, into the 6-16 plasmid yielded pSVG6-16 (Fig.6A). For pSVtk9-27 (Fig.7A) a 342 nucleotide SV40 early promoter fragment (270 to 5171 on the circular map) fused to a 162-nucleotide (−105 to +57) fragment of the tk promoter was placed 5′ of the entire coding region of the 9-27 gene in the form of the *Bsp*MII to *Pst*I fragment (The *BspM*II site is in the 5′ non-coding region of the first exon and the *Pst*I site is in the 3′ flanking sequence, Fig.1 in 8). To generate the 6-16 driving 9-27 construct (pAI, Fig.8A) the *Bgl*II to *Bam*H1 (−603 to −40, Fig.1 and 5) 5′ flanking region fragment from 6-16 engineered 5′ of the *Bam*HI (−39) to *Bgl*II (+57) tk promoter fragment from pBLCAT2 (20) was placed 5′ of the *Bsp*MII to *Pst*I entire 9-27 coding region fragment (as above) in pUC19. The 6-16 driving β-globin construct was generated by insertion of the *Bgl*II to *Bam*HI (−603 to −40) 6-16 5′ flanking region fragment into the *Bam*HI site (−105 of the tk promoter) of a tk β-globin plasmid (254/8 kindly provided by Dr. Stephen Goodbourn, Imperial Cancer Research Fund).

## RESULTS

A diagram indicating the major features of the 5′ regions of the 6-16 and 9-27 genes is given in Fig.1. The sequence of each has been published (Fig. 2 in references 1 and 8, EMBl and GenBank database accession numbers Y00828 and J04164 for 6-16 and 9-27, respectively). In HeLa and HT1080 cells 6-16 mRNA

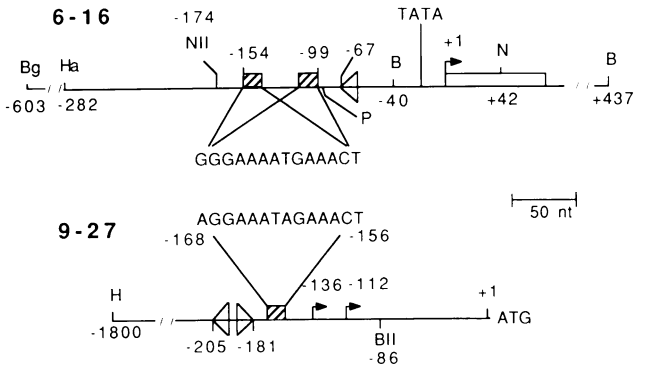



**Figure 1.** Diagrams of the 5′ regions of the 6-16 and 9-27 genes showing the main features: ISRE (hatched boxes); CAAT boxes (open triangles); major transcription sites (filled arrows) and restriction enzyme sites relevant to the construction of plasmids (Bg, *Bgl*II; B, *Bam*HI; BII, *Bsp*MII; H, *Hin*dIII; Ha, *Hae*III; N, *Nci*I; NII, *Nsp*BII; P, *Pst*I). For 6-16, the numbering is from the major transcription start site (17); for 9-27, because of the heterogeneity of transcription start sites, it is from the ATG corresponding to the translation start site(8).

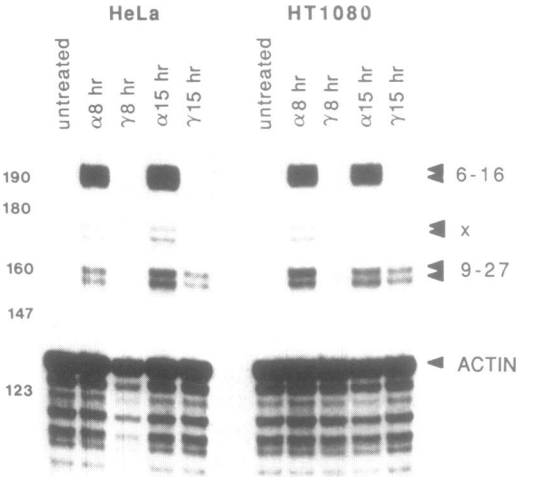



**Figure 2.** Accumulation of 6-16 and 9-27 mRNAs in response to α or γ IFN in human HeLa and HT1080 cells. Cells were treated for the times indicated with 1000 IU/ml of either α or γ IFN, RNA was extracted and the amounts of the 6-16 and 9-27 mRNAs assayed by RNase protection with γ-actin mRNA as an internal standard. The positions to which markers, of known length in nucleotides, migrated are indicated to the left. The radioactive probes should yield protected fragments of 190, 160 and 130 bp for 6-16, 9-27 and actin mRNAs respectively. The doublets for the 6-16 and heterogeneity (most obvious in Fig.8) for the 9-27 signals presumably reflect the major and minor (17) and heterogeneous (8) transcription start sites for these genes. The minor bands at X are by-products routinely obtained with the 6-16 probe−they represent < 5% of the product and were not included in the quantitation.

is highly expressed in response to α but not γ IFN, whereas 9-27 mRNA is highly expressed in response to both (Fig.2). Similar results have been obtained with other human cell lines (21). The frequency with which cDNAs to the 6-16 and 9-27 mRNAs were obtained suggests that each of these mRNAs when optimally expressed in response to α IFN represents about 0.1% of total mRNA (22 and our own unpublished data). In several cell lines the ratio of mature 6-16 mRNA expressed in response to α compared to γ IFN has always been in excess of twenty-fold and on occasion has been at least 100-fold, determined by dilution of the RNA from the IFN-treated cell to a level at which it gave the same signal intensity as that obtained with RNA from control cells (22). Frequently no signal is detectable with or without γ IFN treatment, even after longer exposure of the autoradiograph. This was the case for the experiment shown (Fig.2). For 9-27 an α/ γ ratio of less than three is routinely observed at the time of maximum accumulation of the mRNAs (Figs. 2,7, and reference 8 and Fig. 2 in reference 8). As the response to γ IFN is slower (Fig. 2 and below) a higher ratio is observed at early times after addition of the IFNs. Overall it appears that 6-16 mRNA accumulates much more efficiently in response to α than to γ IFN in human cells. This is not the case for 9-27. It was of interest to determine whether these differences seen at the level of mature mRNA are entirely attributable to an effect on transcription.

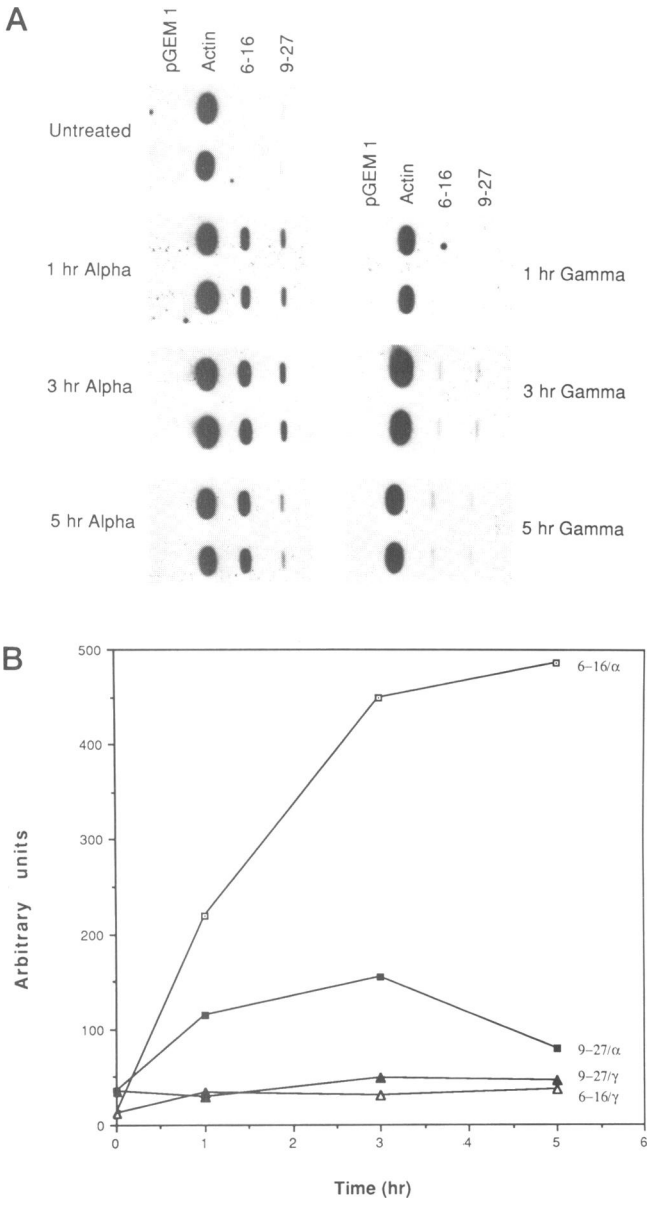



**Figure 3.** Transcriptional response of HT1080 cells to α or γ IFN. Cells were treated for the times indicated with 1000 IU/ml of either α or γ IFN, nuclei were isolated and transcriptional activity was estimated by run-on assay. **A.** Autoradiograph of the run-on assay. **B.** Quantitation of the data was by laser densitometry of autoradiographs. Signals in the linear range of response were corrected against γ-actin as internal standard. The decrease in the reponse of 9-27 but not 6-16 to α IFN at 5hr was not reproducible. The α/ γ ratios for accumulation of mature mRNAs assayed in parallel at 8 and 14 hr were >20 for 6-16 (for which no γ IFN response was detectable) and <3 for 9-27. In eight additional run-on assays with nuclei from HeLa and HT1080 cells the α/ γ ratios for transcription were ten- to twenty-fold for 6-16 and three- to five-fold for 9-27.

## Transcriptional response of the 6-16 and 9-27 genes to α and γ IFNs

Quantitation (Fig.3B) of the autoradiographic data (Fig.3A) from nuclear run-on assays routinely yielded a much greater transcriptional response to α than γ IFN for 6-16 (e.g. eighteen-fold in Fig.3). For 9-27 the α/ γ ratio for transcription is much lower (three-fold, Fig.3). In additional experiments transcription in response to α or γ IFN in HeLa and HT1080 cells was monitored for longer periods (up to 14 hr) with similar results (data not shown). Curiously, for γ IFN, despite the much greater accumulation of mature 9-27 than 6-16 mRNA, transcription of the two genes was always similar (within a factor of two, e.g. Fig.3), a point to which we will return below. It can be concluded that for 6-16 the α/ γ ratio for transcription is high. This is not the case for the 6-16 ISRE in the context of a marker gene for which an α/ γ ratio of less than two is routinely obtained (8). One of several possibilities is, therefore, that the 6-16 gene might contain in its 5′ flanking sequence a negative element effective in preventing the γ but not the α IFN response.

## Absence of a γ IFN specific negative element in the 5′ flanking region of the 6-16 gene

A series of deletions were made (1) to analyse the contribution of sequences between −603 and −154 (just 5′ of the ISREs,

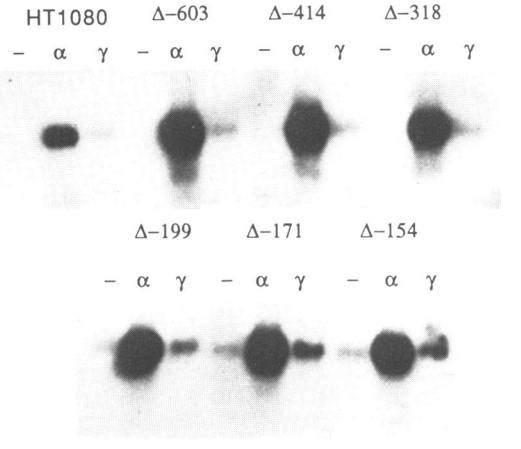
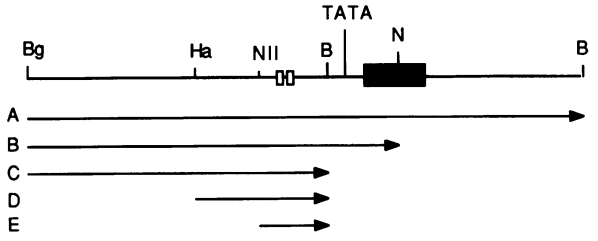



**Figure 4.** Accumulation of 6-16 mRNA in response to α or γ IFN from a series of 6-16 whole-gene deletion constructs stably transfected into HT1080 cells. The cells were incubated with either α or γ IFNs (500 IU/ml for 24hr) as indicated. RNA was extracted and subjected to northern analysis. The deletion constructs are described in Fig.4 of (1). The 3′ limit of each deletion relative to the 6-16 transcription initiation site (Fig.1) is given in bp by the ' Δ-603', for example, notation in the figure.

Figs.1 and 5) to the regulation of the 6-16 gene by the IFNs. The 6-16 mRNA produced in response to α or γ IFN in HT1080 cells stably transfected with the deletion constructs was analysed. The signal from the transfected genes is superimposed on that from the endogenous 6-16 (Fig.4). There is evidence for an increase in the constitutive level of expression as one deletes in from −603 to −154. Making allowance for this there is no evidence for a major increase in the response to γ IFN such as might have been expected had this region contained a strong γ-specific negative element.

**The differential expression of 6-16 mRNA in response to α and γ IFNs does not appear to be attributable to a single response element**

DNA fragments from the 5′ region of the 6-16 gene (Fig. 5, Top) were cloned upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene in pSV0CAT or $pA_{10}CAT_2$ (18,19). Both vectors lack enhancers and express minimal CAT activity. pSV0CAT has no promoter elements 5′ of the CAT gene whereas $pA_{10}CAT_2$ has a 200 bp region of the SV40 early promoter including a TATA box between the cloning site and the CAT gene (18,19). The response of each of the constructs to α or γ IFN was assayed in transient transfection experiments. For constructs C, D and E the three- to five-fold α/ γ ratio observed (Fig.5, bottom) was greater than the less than two-fold




| Insert | Vector | IFN | CAT Activity (cpm) | α/γ |
|---|---|---|---|---|
| A | pSVOCAT | – | 420 | |
| | | α | 11670 | 11.5 |
| | | γ | 1003 | |
| B | " | – | 263 | |
| | | α | 5684 | 5.1 |
| | | γ | 1132 | |
| C | pA10CAT2 | – | 180 | |
| | | α | 2960 | 3.4 |
| | | γ | 880 | |
| D | " | – | 120 | |
| | | α | 1620 | 4.0 |
| | | γ | 420 | |
| E | " | – | 90 | |
| | | α | 1100 | 4.5 |
| | | γ | 260 | |

**Figure 5.** Design and analysis of 6-16 CAT constructs. **Top.** Diagram of the 1046bp large *Bgl*II/*Bam*HI fragment of the 5′ promoter region of 6-16 (Fig.1 and, for sequence, Fig.2 of reference 1) including the ISREs (open boxes, not to scale), first exon (solid box), TATA box and sites for the enzymes *Bgl*II (Bg), *Bam*HI (B), *Hae*III (Ha), *Nci*I (N) and *Nsp*BII (NII) which were used to generate fragments (arrows) cloned into the vectors pSV0CAT or $pA_{10}CAT_2$ (Methods). **Bottom.** CAT activity in lysates from HeLa cells transiently transfected with each of the above constructs. Cells were treated with 1000 IU/ml of either α or γ IFNs starting 17 hr after transfection and CAT activity was assayed after a further 40 hr by which time no further increase in enzymic activity was routinely observed. Each number represents the average for duplicate analyses which agreed to within 10%. Similar results have been obtained on multiple occasions.

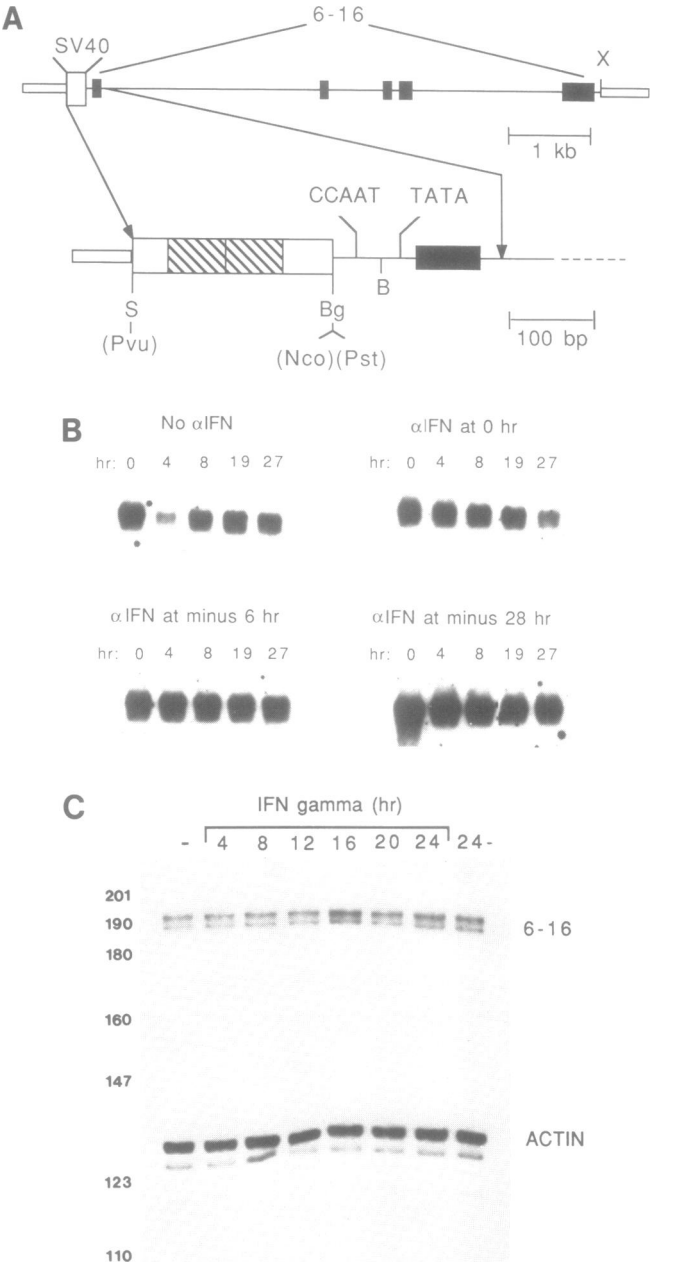



**Figure 6.** Stability of 6-16 mRNA expressed constitutively from an SV40 promoter in the presence or absence of α or γ IFN. **A.** Diagram of the pSVG6-16 construct. The 6-16 gene from nucleotide −95 (just 3′ of the ISREs in the 5′ flanking region, Fig.1) through to the *Xho*I site 100 bp 3′ of the polyA addition site was fused to a 235-bp fragment (nucleotides 272 to 35 on the circular map) of the SV40 promoter (open box). The positions of the 6-16 exons (filled boxes) CAAT and TATA boxes are shown together with the 72 bp repeats of SV40 (hatched boxes). Restriction enzyme sites: *Xho*I, X; *Sal*I, S; *Bgl*II, Bg and *Bam*HI, B. (Pst), (Pvu) and (Nco) represent sites destroyed in cloning. **B.** Stability of 6-16 mRNA in the presence of α IFN. Stably transfected human HT1080 cells were incubated with or without 500 IU/ml of α IFN for zero, 6 or 28 hr before the addition of actinomycin D (3 μg/ml, Boehringer). At the subsequent times indicated RNA was extracted and analysed by northern transfer. The increased levels of 6-16 mRNA at the zero time points (0) for the 6 and 28 hr IFN-pretreated cells reflects RNA expressed from the endogenous gene. The actinomycin treatment inhibited by >99% the incorporation of [$^3$H] uridine into RNA. **C.** Stability of 6-16 mRNA in the presence of γ IFN. Stable transfectants were incubated with or without 1000 IU/ml of γ IFN for the times indicated. The constitutively expressed levels of 6-16 mRNA were analysed by RNase protection with γ-actin mRNA as an internal control. In a separate experiment (not shown) incubation with γ IFN was continued to 32, 40 and 48 hr without any detectable change in the level of 6-16 mRNA.

ratio routinely obtained with constructs in which the ISRE was placed 5′ of the intact tk promoter in pBLCAT2 (8), but still less than that for the transcription of the endogenous gene (ten- to twenty-fold, Fig.3) or for the CAT construct A (11.5-fold, Fig.5, bottom). No consistent difference was seen between the $\alpha/\gamma$ ratios observed for constructs C, D and E, values of between three- and five-fold being routinely obtained for each. Construct A always yielded a much higher absolute level of CAT activity than did constructs B to E (cf. Fig.5) for which the downward trend from B to E is reproducible. Multiple DNA elements may, therefore, contribute to the overall activity observed. In addition, comparison of the three- to five-fold $\alpha/\gamma$ ratio obtained here for constructs C to E with the values of ten- to twenty-fold for the relative transcriptional activity of the endogenous gene (Fig.3) suggested that sequences between −40 and +437 might contribute significantly to determining the transcriptional response. Obviously interpretation of results based on CAT enzyme activity only is complicated for constructs A and B by the fact that the RNAs produced are 6-16/CAT fusion products and an effect of the 5′-region of the 6-16 RNA on the processing, stability or even translatability of the mRNAs cannot be excluded. It would appear, however, that while a large 6-16 promoter/enhancer fragment (−603 to −40) can increase the $\alpha/\gamma$ ratio from the less than two-fold consistently observed with ISRE constructs to three- to five-fold, additional elements may be required to increase this to the 10- to twenty-fold observed at the level of transcription for the endogenous gene (Fig.3). It is likely that post-transcriptional effects also operate in order to increase the ratio to the twenty- to 100-fold consistently observed for mature 6-16 mRNA. As a first step towards addressing the possible involvement of post-transcriptional control the effects of $\alpha$ and $\gamma$ IFNs on mRNA stability were determined.

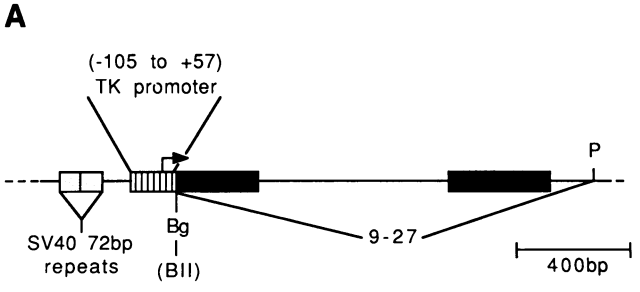
### The stability of 6-16 mRNA in $\alpha$ or $\gamma$ IFN-treated cells

The entire transcribed region of the 6-16 gene was placed 3′ of the SV40 promoter (Fig.6A). The construct was stably transfected into HT1080 cells and the stability of the mRNA assayed in the presence of Actinomycin D with or without prior IFN treatment. The 6-16 mRNA is extremely stable with a half life of >19 hr in the presence of Actinomycin D (Fig.6B). Stability did not appear to be affected by $\alpha$ IFN irrespective of whether the IFN was added at the same time or 6 or 28 hr before the Actinomycin D (Fig. 6B). As the 6-16 mRNA from this construct is intact and correctly initiated, it cannot be distinguished from that of the endogenous gene, hence the higher signals observed for the 6 and 28 hr IFN-treated samples. On the other hand, in the case of $\gamma$ IFN there is virtually no accumulation of mature mRNA from the endogenous 6-16 gene. It was, therefore, possible to look for effects of this IFN on the steady state levels of 6-16 mRNA expressed constitutively from the SV40 promoter without the complication of any contribution from the endogenous gene or the use of Actinomycin D. No significant effect was observed over 24 hr (Fig.6C) or even 48 hr (data not presented). More specifically, there was no obvious reduction in the steady state levels of the mRNA, which would have been expected had treatment with $\gamma$ IFN substantially reduced its stability. Nevertheless the difference in the $\alpha/\gamma$ ratio for 6-16 transcription (ten- to twenty-fold) to that for the accumulation of mature mRNA (twenty- to 100-fold) has been consistently observed in the same experiment.

In short, for 6-16 there appears to be a high $\alpha/\gamma$ ratio at the level of transcription. This cannot entirely account for the even higher ratio consistently observed for mature mRNA (Fig.2). There is no obvious effect of either IFN on mRNA stability (Fig.6). A differential effect of the two IFNs on some other aspect of post-transcriptional control must, therefore, be invoked.

### The stability and processing of 9-27 mRNA in $\alpha$ or $\gamma$ IFN-treated cells

Experiments in which the stability of 9-27 mRNA was assayed in the presence of Actinomycin D added after the IFNs indicated a half life for 9-27 mRNA of >>16 hr (data not shown). To determine stability in the absence or presence of IFN, the transcribed region of the 9-27 gene was placed 3′ of a tk promoter/SV40 enhancer complex (Fig.7A). In this construct, a small (57 nucleotide) fragment from the 5′ untranslated region of the tk gene was fused to the 5′ untranslated region of the 9-27 gene in order to distinguish the products of the transfected and endogenous genes by RNase protection. No obvious effect of either $\alpha$ or $\gamma$ IFN on the steady state levels of the constitutively



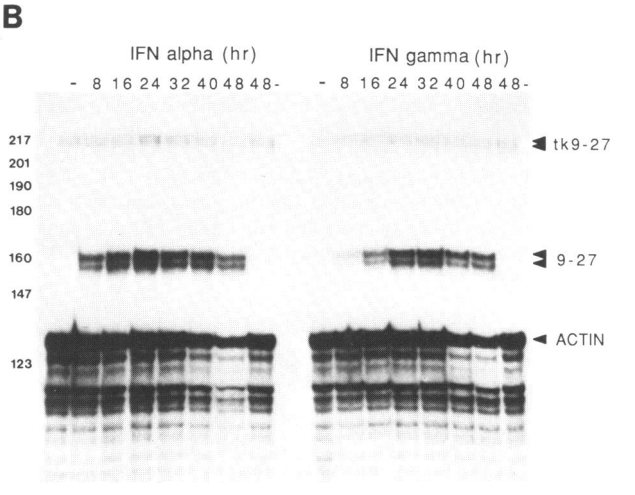




**Figure 7.** Stability of 9-27 mRNA in the presence or absence of $\alpha$ or $\gamma$ IFN. **A.** Diagram of the pSVtk9-27 construct for constitutive expression of 9-27 mRNA tagged with a 57 nucleotide fragment of tkRNA. The 9-27 gene from the *BspM*II site (in 5′ untranslated region DNA) to the *Pst*I site (3′ of the polyA addition site) was placed 3′ of a composite SV40 enhancer/tk promoter. The 9-27 exons are represented by the filled boxes. Restriction enzyme sites: *Bgl*II, B; *Pst*I, P. (BII) is the *BspM*II site destroyed in cloning. **B.** Stably transfected HT1080 cells were incubated with or without 1000 IU/ml of either $\alpha$ or $\gamma$ IFN for the times indicated. The levels of the constitutively expressed tk/9-27 mRNA were assayed by RNAse protection in comparison with that of the 9-27 mRNA (9-27) induced from the endogenous gene and with $\gamma$-actin mRNA as an internal standard. Markers (lengths in nucleotides) are on the left.

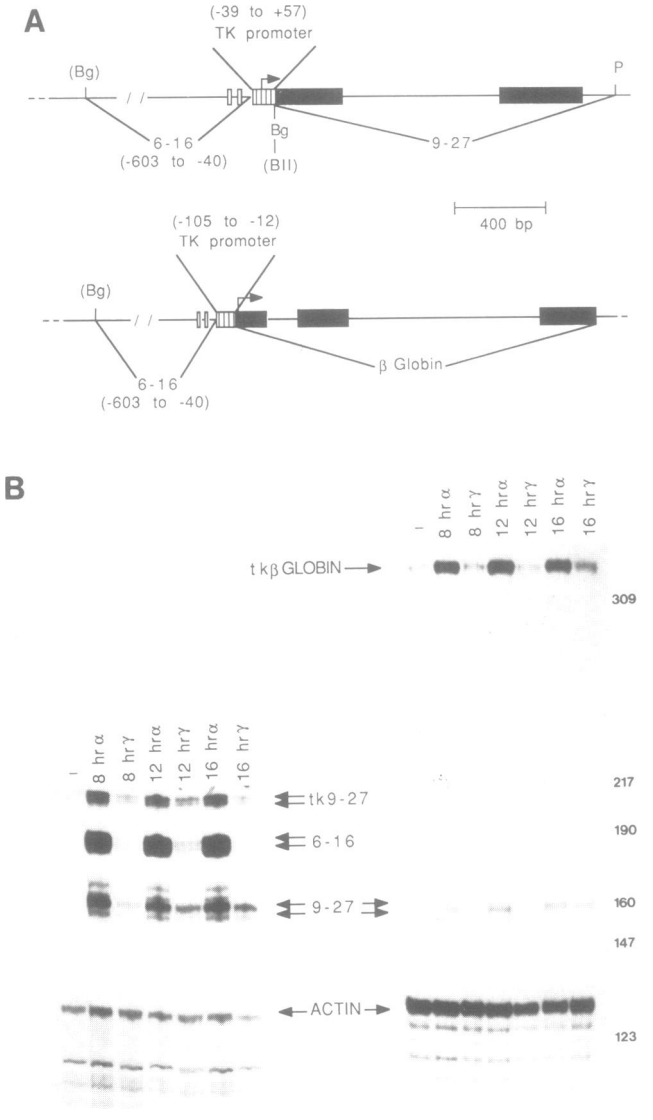



**Figure 8.** Accumulation of 9-27 and β-globin mRNAs from IFN-inducible constructs. **A.** Diagrams of the constructs. The 9-27 gene (*BspM*II/*Pst*I fragment, legend to Fig.7) and a β-globin gene fragment were each placed 3′ of composite 6-16/tk promoters. The 6-16 ISREs are represented by the two open boxes 5′ of the tk promoter and the exons by filled boxes. Restriction enzyme sites: *Bgl*II, Bg; *Pst*I, P. (Bg) and (BII) represent *Bgl*II and *BspM*II sites destroyed in cloning. **B.** Stably transfected HT1080 cells were incubated with or without 1000 IU/ml of α or γ IFN for the times indicated. The levels of tagged 9-27 (tk9-27) and β-globin (tk globin) mRNAs, compared with the endogenous 9-27 and 6-16 mRNAs were analysed by RNase protection with γ-actin mRNA as an internal control. Quantitation to give the values quoted in the text was as in Fig.3. Markers (length in nucleotides) are on the right.

expressed mRNA was observed (Fig.7B). As an extension of this type of experiment it was of interest to determine whether there is any preferential effect of γ IFN on the processing of the 9-27 transcript, particularly in view of the relatively efficient accumulation of the mature 9-27 mRNA in response to γ IFN. The transcribed regions of the 9-27 and β-globin genes were, therefore, placed 3′ of the same IFN-inducible 6-16 promoter fragment (−603 to −40) used to drive the CAT gene in construct C in Fig.5 (Fig.8A). The accumulation of the 9-27 and globin RNAs from the stably transfected constructs was then compared in response to α or γ IFN. The 9-27 mRNA was again tagged with the 57 nucleotide fragment from the 5′ end of the tk gene.

**Table 1.** Activities of the 5 promoter/enhancer regions of the 6-16 and 9-27 genes when placed 5′ of a marker CAT gene

| Promoter | IFN | CAT Activity (cpm) Transient transfection Time of treatment 24 hr | 48 hr | Stable transfection Time of IFN treatment 24 hr | 48 hr |
|---|---|---|---|---|---|
| None[1] | 0 | 75 | – | 130 | 160 |
| | α | 95 | – | 115 | 165 |
| | γ | 85 | – | 105 | 150 |
| 9.27 | 0 | 80 | 90 | 125 | 185 |
| | α | 280 | 460 | 160 | 320 |
| | γ | 120 | 180 | 125 | 180 |
| 6-16 | 0 | 990 | 2150 | 800 | 1250 |
| | α | 25000 | 32000 | 4550 | 11850 |
| | γ | 3000 | 5950 | 1300 | 2100 |

[1]Vector-pSVOCAT-only

HeLa cells transiently, or HT1080 cells stably transfected with the 9-27 and 6-16 constructs were incubated for the indicated times with 1000 IU/ml of either α or γ IFN beginning in the transients 17 hr after transfection. CAT activities assayed in duplicate agreed within 10%. For 6-16 the *Bgl*II to *Nci*I, −603 to +40, fragment was placed 5′ of the CAT gene in pSV0CAT (construct B, Fig.5). For 9-27 the *Spe*I to *BspM*II (−312 to −86) fragment (Fig.1) was placed 5′ of the CAT gene in pCATb′ (equivalent to pSV0CAT but with additional cloning sites) as described previously (Fig.4 in 8). Note in each case that the 3′ termini of the fragments at +40 (6-16) and −86 (9-27) are positioned in regions of the DNA corresponding to the 5′ untranslated region of the mRNA products (the numbering in the case of 9-27 is from the initiator ATG (8 and Fig.1)).

In several experiments an α/ γ ratio of three- to five-fold was observed for the tk-tagged 9-27 mRNA from the 6-16 promoter construct at the time of optimal mRNA accumulation (*e.g.*, four-fold at 16 hr, Fig.8B). A similar ratio was observed for the β-globin mRNA (five-fold at 16 hr, Fig.8B). This is in good agreement with the ratio observed for the same 6-16 promoter fragment driving the CAT gene (C, Fig.5) when assayed either by enzymic activity (Fig.5) or by RNase protection (data not shown). With the caveat once again that the tagging of the mRNA may have affected the result, these data provide no evidence for any preferential processing of the 9-27 mRNA in response to γ IFN. As the 9-27 and β-globin probes were of similar specific activity, these data also indicate that there is not a major difference in the efficiency of processing of the 9-27 and globin messages in response to either IFN, *i.e.* they provide no evidence to suggest that the processing of an IFN-inducible message is particularly favoured in an IFN-treated cell.

### The 9-27 promoter/enhancer region appears much less active than that from 6-16

In the course of these experiments the activities of a number of analogous 6-16 and 9-27 driving CAT gene constructs were compared on numerous occasions in transient and stable transfection experiments. On every occasion the optimum activity of the 9-27 promoter/enhancer, measured in terms of CAT activity in the presence of α IFN, was ten- to 100-fold less than that from 6-16. A typical set of data for two similar constructs is presented in Table 1. Here, for both 6-16 and 9-27, a 5′ fragment of the gene terminating in the 5′ untranslated region of the first exon was placed 5′ of a promoterless CAT gene. In both transient and stable transfections expression from the 6-16 construct was substantially greater.

## DISCUSSION

Considering first the 6-16 gene, accumulation of mature mRNA to a high level in response to α but not γ IFN (Fig.2) largely reflects the high α/ γ ratio for transcriptional activity (eighteen-

fold, Fig.3). The 6-16 mRNA is very stable and no effect of either $\alpha$ or $\gamma$ IFN on its stability was detected (Fig.6). Nevertheless, as the $\alpha/\gamma$ ratio is even greater (twenty- to 100-fold) at the level of mature mRNA, there must be some form of post-transcriptional control. For example, in the run-on experiments a probe complementary to the 5′ region of the 6-16 message was employed. Attenuation at a point 3′ to this in response to $\gamma$ but not $\alpha$ IFN would produce an increase in the $\alpha/\gamma$ ratio for mature mRNA. Alternatively either IFN could specifically affect the processing of the nascent RNA. The relatively small difference between the ratios observed at the level of transcription and mature message, however, makes further analysis impractical.

No single DNA element appears responsible for the high $\alpha/\gamma$ ratio observed with the endogenous 6-16 promoter compared with the less than two-fold differential seen with ISRE-driven constructs. No evidence was obtained for an upstream negative element capable of inhibiting the response to $\gamma$ but not $\alpha$ IFN (Fig.4). Nor did removal of one of the two ISREs in the 6-16 promoter effect the $\alpha/\gamma$ ratio (ACGP, ARL and IMK, unpublished results). The high $\alpha/\gamma$ ratio is not governed by sequences immediately adjacent to the ISREs. For example, the 39 bp fragment (−89 to −127, Fig.2 in ref.1) from the 6-16 promoter with a centrally positioned ISRE was equivalent to the ISRE alone in conferring a response to both $\alpha$ and $\gamma$ interferons. Consistent with this, the differential response was only partially retained in CAT constructs driven by 5′ flanking promoter fragments varying in length from 564 (−603 to −40) to 135 (−174 to −40) nucleotides (C,D,E, Fig.5). Interestingly, ratios nearer to those obtained with the endogenous gene were observed with construct A (Fig.5) which included the first exon and part of the first intron. This raises the possibility that the 5′ region from −40 to +437 might significantly affect either the activity of the promoter or the processing or stability of the primary transcript. The signal obtained in response to $\gamma$ IFN in run-on transcription assays with material from cells stably transfected with these constructs has, however, proved to be too low to provide conclusive evidence for or against an effect of this section of the DNA on transcription. No attempt has been made to assess any effect of this region of DNA on the processing or stability of the RNA product.

The situation with respect to the 9-27 gene appears more straightforward. The same $\alpha/\gamma$ ratio of two-fold is seen with the mature mRNA (at the time of maximum accumulation) from the endogenous gene, a number of deletion constructs and the ISRE driven CAT constructs (8). In a number of experiments of the type shown in Fig.3, with material from HeLa and HT1080 cells, the $\alpha/\gamma$ ratio for transcription appeared to be, if anything, slightly higher than that for mature message. No direct evidence for a differential effect of $\gamma$ IFN on the processing of 9-27 RNA or for any effect of either IFN on the stability of the mRNA was obtained (Figs. 7 and 8).

Two additional observations remain to be explained. First, the extremely low relative response to $\alpha$ IFN (down ten- to 100-fold) of the 9-27 5′ flanking promoter/enhancer region compared with that from 6-16 when assayed in analogous constructs (Table 1). This has to be reconciled with: (1) The comparable levels to which mature 6-16 and 9-27 mRNAs accumulate in response to this IFN (Fig.2 and in 23). (2) The fact that no such difference was observed on transfecting 9-27 and 6-16 whole gene constructs (L.E.R. and I.M.K., unpublished). Although not strictly quantitative any difference in activity comparable to the ten- to 100-fold consistently observed for the CAT constructs would surely have been detected. (3) The ten- to 100-fold difference seen with the CAT constructs is much larger than the difference in transcriptional activity seen for the endogenous genes in numerous run-on assays (e.g. Fig.3). An obvious possibility is that an element within the transcribed region of the 9-27 gene or 3′ to it contributes to the activity of the endogenous promoter. Preliminary experiments in which, for example, the transcribed region of the 9-27 gene was placed upstream of the 9-27 5′ region driving a marker gene have failed to provide direct evidence for such an enhancer element (L.E.R. and I.M.K., unpublished). A much more detailed analysis will obviously be required before any conclusion can be reached. The composition and arrangement of the 9-27 promoter in terms of known functional elements, specifically, the CCAAT and TATA boxes is very different from that of 6-16 (Fig.1). Lacking a TATA box the activity of the 9-27 promoter might be dependent on an 'initiator' element similar to that described by Smale and Baltimore (23,24). The apparent heterogeneity of transcriptional initiation for 9-27 (8), however, argues against any precise analogy. In addition, as the 9-27/CAT constructs (e.g. Table 1) routinely included sequence downstream of the major transcriptional initiation sites (Fig.1 and in 8) any similar elements should have been included.

Finally it remains unclear why in contrast to the situation with $\alpha$ IFN for which the 6-16 and 9-27 mRNAs appear to accumulate in roughly the same ratio as that in which they are transcribed, this is not the case for $\gamma$ IFN. For $\gamma$ IFN the 6-16 gene appears to be as efficiently transcribed as the 9-27 gene (Fig.3) and yet 6-16 mRNA does not accumulate to comparable levels (Fig.2). Although potential differences in, for example, the specific activities of the 6-16 and 9-27 probes do not permit strict quantitation, any such differences would be the same for the $\alpha$ and $\gamma$ IFNs and cannot therefore account for the anomaly. It is possible that the small single intron 9-27 RNA is intrinsically more efficiently processed than the larger four intron 6-16 RNA, but this would be expected to apply equally to message synthesised in response to either IFN. The data, therefore, suggest that there must be some differential effect of $\alpha$, or more probably $\gamma$, IFN on the processing of one or other of the primary transcripts.

A post-transcriptional effect for $\gamma$ IFN has been reported by others (25,26). It is not clear, however, to what extent it is possible to extrapolate from results obtained with one gene to another. For example, the IFN response element(s) for the Class II HLAs is different from that for 6-16 and 9-27 and that for Class I shows only partial homology (discussed in 1). The significance of this is not yet clear. Moreover, a recently isolated mutant defective in the $\gamma$ response for 9-27 has a normal $\gamma$ response for the guanylate binding protein (27,28) and Class I and II HLAs, raising the possibility of further complexity in this response pathway (J.John, R.McKendry, S.Pellegrini, I.M.K. and G.R.S., unpublished).

Overall, for both 9-27 and 6-16 the major effect of the IFNs is on transcription. For 9-27 the activity of the promoter may be increased by an enhancer within the body of the gene. The relative response to $\alpha$ and $\gamma$ IFNs is constant, however, for a whole series of deletion constructs of very different absolute activity. It is reasonable to conclude that for this gene the $\alpha/\gamma$ ratio is governed exclusively by the ISRE. More surprisingly, for 6-16, comparing results for the endogenous gene (Fig. 2) with those for the deletion (Fig.5) and ISRE (8) constructs, the smaller the promoter region fragment present the smaller the differential response to the two IFNs (Fig.5). The $\alpha/\gamma$ ratio may, therefore, be determined by a complex interaction between factors interacting with the ISRE and the remainder of the promoter.

No effect on message stability was observed with either IFN for either gene. On the other hand it is difficult to reconcile the data for transcription with that for accumulation of mature message without invoking an element of post-transcriptional control. If a differential effect of $\alpha$ and $\gamma$ IFNs on the processing of 6-16 RNA does indeed occur it may well affect other RNAs very differently. The actual level of expression obtained with any given gene may therefore be affected not merely by the context of the ISRE in the promoter and by enhancer elements but also by the susceptibility of the transcript to any differential processing. If this is indeed the case the effectiveness of IFN-inducible promoter constructs in yielding high levels of inducible mRNA in response to $\alpha$ or $\gamma$ IFN may prove very variable.

## REFERENCES


1. Porter,A.C.G., Chernajovsky,Y., Dale,T.C., Gilbert,C.S., Stark,G.R. and Kerr,I.M. (1988) *EMBO J.* **7,** 85–92.
2. Reich,N., Evans,B., Levy,D., Fahey,D., Knight,E.,Jr. and Darnell,J.E.,Jr. (1987) *Proc.Natl.Acad.Sci.USA.* **84,** 6394–6398.
3. Levy,D.E., Kessler,D.S., Pine,R., Reich,N. and Darnell,J.E.,Jr. (1988) *Genes & Develop.* **2,** 383–393.
4. Hug,H., Costas,M., Staeheli,P., Aebi,M. and Weissmann,C. (1988) *Mol.Cell.Biol.* **8,** 3065–3079.
5. Luster,A.D. and Ravetch,J.V. (1987) *Mol.Cell.Biol.* **7,** 3723–3731.
6. Boss,J.M. and Strominger,J.L. (1986) *Proc.Natl.Acad.Sci.USA.* **83,** 9139–9143.
7. Benech,P., Merlin,G., Revel,M. and Chebath,J. (1985) *Nucleic Acids Res.* **13,** 1267.
8. Reid,L.E., Brasnett,A.H., Gilbert,C.S., Porter,A.C.G., Gewert,D.R., Stark,G.R. and Kerr,I.M. (1989) *Proc.Natl.Acad.Sci.USA.* **86,** 840–844.
9. Cohen,B., Peretz,D., Vaiman,D., Benech,P. and Chebath,J. (1988) *EMBO J.* **7,** 1411–1419.
10. Rutherford,M.N., Hannigan,G.E. and Williams,B.R.G. (1988) *EMBO J.* **7,** 751–759.
11. Dale,T.C., Rosen,J.M., Guille,M.J., Lewin,A.R., Porter,A.C.G., Kerr,I.M. and Stark,G.R. (1989) *EMBO J.* **8,** 831–839.
12. Dale,T.C., Imam,A.M.A., Kerr,I.M. and Stark,G.R. (1989) *Proc.Natl.Acad.Sci.USA.* **86,** 1203–1207.
13. Allen,G., Fantes,K.H., Burke,D.C. and Morser,T. (1982) *J.Gen.Virol.* **63,** 207–212.
14. Feinberg,A.P. and Vogelstein,B. (1983) *Analyt.Biochem.* **132,** 6–13.
15. Sambrook,J., Fritsch,F.F. and Maniatis,T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor University Press, Cold Spring Harbor.
16. Rodaway,A.R.F., Teahan,C.G., Casimir,C.M., Segal,A.W. and Bentley,D.L. (1990) *Mol.Cell.Biol. In press.*
17. Kelly,J.M., Porter,A.C.G., Chernajovsky,Y., Gilbert,C.S., Stark,G.R. and Kerr,I.M. (1986) *EMBO J.* **5,** 1601–1606.
18. Laimins,L.A., Khoury,G., Gorman,C., Howard,B. and Gruss,P. (1982) *Proc.Natl.Acad.Sci.USA.* **79,** 6453–6457.
19. Gorman,C.M., Moffat,L.F. and Howard,B.H. (1983) *Mol.Cell.Biol.* **2,** 1044–1051.
20. Luckow,B. and Schutz,G. (1987) *Nucleic Acids Res.* **15,** 5490.
21. Kelly,J.M., Gilbert,C.S., Stark,G.R. and Kerr,I.M. (1985) *Eur.J.Biochem.* **153,** 367–371.
22. Friedman,R.L., Manly,S.P., McMahon,M., Kerr,I.M. and Stark,G.R. (1984) *Cell* **38,** 745–755.
23. Smale,S.T. and Baltimore,D. (1989) *Cell* **57,** 103–113.
24. Smale,S.T., Schmidt,M.C., Berk,A.J. and Baltimore,D. (1990) *Proc.Natl.Acad.Sci.USA.* **87,** 4509–4513.
25. Korber,B., Hood,L. and Stroynowski,I. (1987) *Proc.Natl.Acad.Sci.USA.* **84,** 3380–3384.
26. Klar,D. and Hammerling,J. (1989) *EMBO J.* **8,** 475–481.
27. Cheng,Y.S., Becker-Manley,M.F., Nguyen,T.D., Degrado,W.F. and Jonak,G.J. (1986) *J.Inteferon Res.* **6,** 417–427.
28. Decker,T., Lew,D.J., Chang,Y.S.E., Levy,D.E. and Darnell,J.E.,Jr. (1989) *EMBO J.* **8,** 2009–2014.