Transcription factors induced by interferons α and γ

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ABSTRACT

Factors induced by interferons (IFNs) bind to the IFN-stimulated response elements (ISREs) of many genes. In human cells treated with type I (α, β) IFN, factor E is induced in about 1 min and factor M after about 1 hr. Factor G is induced after about 1 hr in cells treated with type II (γ) IFN. G and M have very similar positions in bandshift assays, sensitivities to cycloheximide, footprints on an ISRE and relative affinities for different ISREs. Four different patterns of expression were observed in different cell lines: E,M and G strongly induced; M and G strongly but E weakly; only E and M induced; only E induced. Transcription in response to IFN α is initiated by E and probably maintained by M since, in fibroblasts, M is present maximally when transcription is most active and declines together with transcription. In Bristol-8 cells, where induction of M is not detected, transcription is still induced by IFN α and still declines in its continued presence, suggesting that M is not essential for either process. A variant ISRE with two G-to-C mutations binds E especially weakly but M and G strongly. The mutations don't change the response of a reporter gene to IFN γ but do abolish its response to IFN α, suggesting that the binding of M is not sufficient for the latter. G probably acts positively, since its appearance correlates well with induction of transcription by IFN γ. A 39-bp ISRE from the 9-27 gene binds E much better than M or G. Conversely, a 39-bp ISRE from the 6-16 gene binds M and G much better than E. Different patterns of expression of E, M, and G and different affinities of these factors for alternative ISREs may play a part in modulating the relative responses of genes to type I and type II IFNs *in vivo*.

INTRODUCTION

Treatment of cells with interferons (IFNs) can lead to establishment of an anti-viral state, inhibition of cell growth, and induction of differentiation (reviewed in ref.1). Type I (α, β) IFNs bind to one or more related receptors (2-4), while type II (γ) IFN binds to a unique receptor (5). Upon binding, a signal passes from the membrane to the nucleus, causing transcription of a set of genes to be initiated (6,7). Several IFN-induced genes have been cloned and comparisons of their upstream sequences have revealed interferon-stimulated response elements (ISREs), which confer inducibility by type I and type II IFNs (8). The core ISREs, 13 base pairs (bp) long, are highly conserved (9) but there is no obvious homology in the sequences immediately flanking them. We (9-11) and others (12-15) have shown that ISREs can bind to a number of factors present constitutively in untreated cells or induced in cells treated with IFN. The following factors, originally observed in different laboratories, are likely to be the same, although this is not yet directly proven. For convenience, we use single-letter abbreviations. C is a constitutive factor(s) that binds to ISREs, also called ISGF1 (13,15) and C1-C2 (ourselves and ref.12). E is a factor induced rapidly by IFN α, also called ISGF3 (13,15) or C3 (12). M is a factor induced in 1-2 hr by IFN α, also called ISGF2 (13,15) or C1-C2 (12). G is a factor induced in 1-2 hr by IFN γ, also called ISGF2 (16) or IBP-1 (17).

The recent work of Pine *et al.* (16) has partially clarified the relationship between M and G since both factors seem to be encoded by the IRF-1 gene, previously identified by Miyamoto *et al.* (18). Yet M and G may still be functionally distinct due to post-translational modification or association with other induced factors, and evidence presented here gives support to such possibilities. C is different from M and G (16), despite its very similar binding characteristics (see below).

The role of E as the induced factor responsible for initiating transcription in response to IFN α is well established (10,11,15) but the functions of M, G, and C are much less clear. M was proposed previously to be an IFN-induced negative regulator of transcription (15), a conclusion not in agreement with evidence presented here. We have examined the kinetics of expression of E, M, and G in different cells and correlated these with the kinetics of transcription of IFN-regulated genes. We also show that C, M, and G have very similar affinities for native, mutant and hybrid ISREs and that the relative affinities of E for these ISREs can be very different. A mutant ISRE with high affinity for M and G and low affinity for E is unchanged in its transcriptional response to IFN γ but has lost the response to IFN α characteristic of the wild-type ISRE. We now conclude that M is unlikely to have any negative effect and that it is neither necessary nor sufficient to initiate transcription in response to

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IFN α. Its most likely role is to help maintain transcription that has been initiated by E.

MATERIALS AND METHODS

Treatment of cells with IFN

Bristol-8 B cells (19) and Daudi cells (20) were grown as spinner cultures in RPMI medium, 2% bicarbonate and 5% fetal calf serum. HeLa cells (kindly provided by Dr. H. Land, Imperial Cancer Research Fund) were grown either in suspension spinner cultures or as monolayers in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Human foreskin fibroblast (HFF), HT1080 (human fibrosarcoma) and W138-SV (SV40 transformed human lung fibroblast) cells were also grown as monolayers as above. Cells were treated with 1000 IU/ml of a highly purified mixture of human IFN α's (specific activity 10^8 IU/mg protein), supplied by Dr. K. Fantes, Wellcome Research Laboratories (21). Human recombinant IFN γ was used at 500 units/ml and was a gift of Dr. G.R. Adolph, Ernst-Boehringer-Institut fur Arzneimittel-Forschung, Vienna, Austria.

Band-shift assays

Whole cell extracts were prepared by the method of Zimarino and Wu (22). Cells treated with IFN for various lengths of time were collected by centrifugation, frozen immediately in either liquid N_2 or dry ice, and stored at −70°C. All extracts were dialyzed against binding buffer (10 mM HEPES, pH 7.9, 0.1 mM EGTA, 75 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonylfluoride, 5% glycerol) for 2 h in a microdialyzer. In a few experiments, we supplemented this buffer with increasing concentrations of $MgCl_2$, up to 10 mM, with no significant changes in binding activity, using extracts of Bristol-8, HeLa and W138 SV cells. Band shift assays were performed essentially as described by Dale *et al.* (10). The final concentration of NaCl in binding reactions was 75 mM for all dialyzed extracts. Competition assays were performed by adding 2 μl of double-stranded competitor DNA in 75 mM NaCl to the probe mixture before adding the extracts.

Cleavage with copper orthophenanthroline

Binding reactions and preparative band-shifts were performed as described before (10). Whole cell extracts were usually used, but in some experiments we used fractions from a heparin-agarose column, kindly provided by John Parrington, Imperial Cancer Research Fund. Cleavage in the agarose gel was performed as described earlier (10).

Oligonucleotides

Probes used in band-shift and competition assays were purified by high performance liquid chromatography. Their sequences are:

6-16 wt:
GAGCTGGGAGAGAGGGGAAAATGAAACTGCAGAGTGCAC

6-16 Double early mutant:
GAGCTGGCAGAGAGGCGAAAATGAAACTGCAGAGTGCAC

6-16 Core mutant:
GAGCTGGGAGAGAGGGGACAATGACACTGCAGAGTGCAC

9-27 wt:
TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAATACA

6-9-6:
GAGCTGGGAGAGAGGGGAAATAGAAACTGCAGAGTGCAC

9-6-9:
TTTACAAACAGCAGGAAAATGAAACTTAAGAGAAATACA

H-2K^b:
AGGTTAGGTGCAGAAGTGAAACTGTGGAGATGGGGAATCC

HEX4:
AAGTGAAAGTGAAAGTGAAAGTGA

Nuclear run-on assays

The method used was that of Rodaway *et al.* (23). 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 [α^{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 hybridization.

RESULTS

Different patterns of induction of E, M and G in different cells

All three factors are induced well in the fibroblast cell line W138-SV (Fig.1) and in HT1080 and human foreskin fibroblasts (data not shown). In cells treated with IFN α, E appears rapidly and then declines; M usually appears within 1−2 hr (Fig. 1). Note that the constitutive factor C, present in untreated cells, appears in a position similar to that of M. In cells treated with IFN γ, G is induced at a rate similar to that of M in IFN α-treated cells, and these two factors migrate at very similar positions in bandshift assays using either agarose (Figs. 1, 5, 6A) or acrylamide gels (not shown). In our experiments, factor G is usually more abundant than M after several hours. Factors C, M and G bind preferentially to the 6-16 probe and E binds preferentially to 9-27. Although the endogenous 6-16 gene responds poorly to IFN γ compared with IFN α (24), the 6-16 ISRE is as efficient as the 9-27 ISRE in conferring a response to IFN γ upon a marker gene. Consistent with this, the 39-bp 6-16 ISRE binds well to factor G (Figs. 1 and 6A). Poor accumulation of 6-16 mRNA in response to IFN γ reflects a

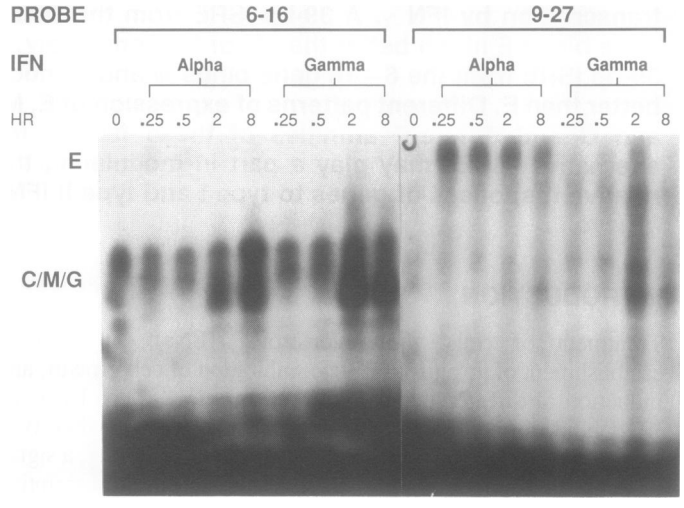

Figure 1: Time courses of induction by IFNs α and γ of factors that bind to the 6-16 and 9-27 ISREs in W138-SV cells. The 39-bp probes used correspond to upstream sequences of the human 6-16 or 9-27 genes, with the highly conserved 13-bp core ISREs (GGGAAANNGAAAC) positioned centrally.

Table I. Patterns of induced factors and transcriptional responses for human cells treated with IFNα or IFNγ

Pattern of Response	Induced Factor			Transcriptional response to:	
	E	M	G	IFNa	IFNγ
All factors induced well (W138-SV, HFF, HT1080)	+	+	+	+	+
E induced poorly (HeLa)	±	+	+	+	+
Only E and M induced (Daudi)	+	+	−	+	−
Only E induced (Bristol-8)	+	−	−	+	−

In cells treated with IFN α, E was detected within 1−2 min with the 9-27 probe and M was usually detected within 1−2 hr with the 6-16 probe. G was detected in 1−2 hr in cells treated with IFN γ, with the 6-16 probe. All untreated cells tested have a constitutive factor C that migrates slightly differently from M or G in bandshift assays.

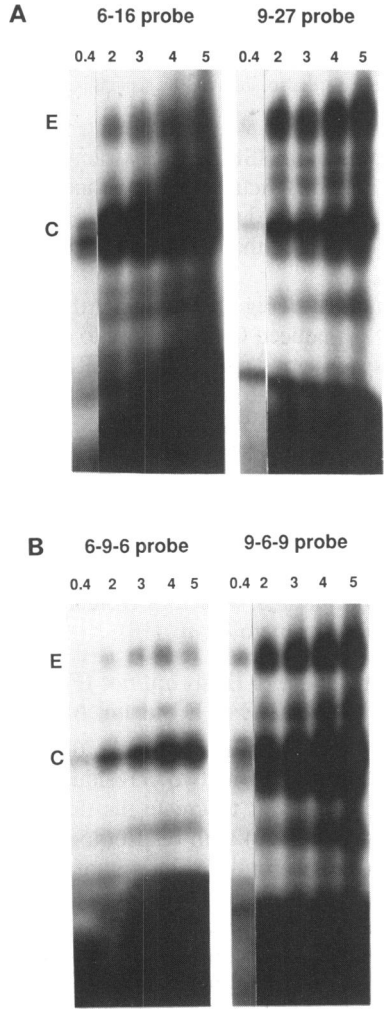

Figure 2: Relative affinities of E and C for wild type and hybrid ISREs. Double-stranded 39-bp probes for (A) wild type 6-16 and 9-27 ISREs and (B) hybrid 6-9-6 and 9-6-9 ISREs were used in band shift assays. The concentration of extract (Bristol-8 cells, IFN α, 30 min) was kept constant at 15 μg/ μl and the probe concentration was varied from 0.4 to 5.0 ng/20 μl.

number of factors not yet defined fully (A.M.A., G.R.S. and I.M.K., unpublished results).

Using both 6-16 and 9-27 probes to allow sensitive detection of all three induced factors, we surveyed several cell lines and found four distinct patterns of expression (Table 1). In addition to the data shown for the fibroblast line W138-SV in Fig. 1, results for HeLa and Bristol-8 cells can be seen in Figs. 6A and 7B, respectively. Although HeLa cells show very poor induction of E, they respond as well to IFN α in transcription assays as do HT1080 cells and better than Bristol-8 cells. However, Levy *et al.* (25) have shown that pretreatment with IFN γ greatly increases the level of E in HeLa cells, with a marked improvement in the transcriptional response to IFN α. Treatment of the lymphoid cell lines Daudi and Bristol-8 with IFN γ does not lead to induction of G. There is also no induction of 9-27 transcription by IFN γ in a run-on assay and no accumulation of 9-27 mRNA in an RNase protection assay (data for Bristol-8 not shown, Daudi not tested). Other lymphocyte cell lines and B and T cells do respond to IFN γ, as judged by accumulation of induced proteins and mRNAs (26−28). In contrast to all the other cells tested, Bristol-8 cells fail to show induction of M in response to IFN α (Fig. 7B), and yet still have a transient transcriptional response. These results suggest that M is not essential either for induction of transcription in response to IFN α or for the decline of such transcription.

Basis of preferential binding of E to 9-27 and C, M, and G to 6-16 ISREs

The natural ISREs as well as the 39-bp hybrid ISREs 6-9-6 (6-16 flanking sequences, 9-27 core) and 9-6-9 (9-27 flanking sequences, 6-16 core) were tested with extracts made from Bristol-8 cells treated with IFN α for 30 min (Fig. 2). These extracts provide only E and C. However, results similar to those for C were obtained with M and G from W138-SV cells (data not shown). The 9-6-9 ISRE binds E, C, M, and G tightly, whereas the 6-9-6 ISRE binds all four factors weakly (Fig. 2B). Therefore tight binding of C,M and G is a property of the 13-bp 6-16 core ISRE, which differs from the 9-27 core ISRE only in the sequence of the two central A and T residues. These residues, hypervariable in the ISREs of a variety of human and mouse genes (8), may help to regulate genes differentially in response to IFNs α and γ. Conversely, tight binding of E, which interacts not only with the two GAAA motifs in the 13-bp core ISRE but also with sequences that flank the core of 6-16 (10) and 9-27 (Fig. 3), is preferentially enhanced by interaction with 9-27 flanking sequences that are not highly conserved in the ISREs of other IFN-regulated genes. Distinctive patterns of induction of E, M, and G in different cells and differential affinities of individual ISREs for these three factors may help to modulate the relative effects of IFNs α and γ in different tissues. Other influences, such as different levels of receptors and constitutive transcription factors, and the presence or absence of dominant negative regulators of transcription, will almost certainly contribute as well.

Footprints of E, M, and G on high-affinity ISREs

Previously, we examined the detailed contacts of E and C with the 6-16 ISRE by using four different techniques: methylation interference and protection, ethylation interference, and footprinting with copper orthophenanthroline (10). We concluded that E and C bound to overlapping regions of the core ISRE but in very different ways and that E had additional contacts with

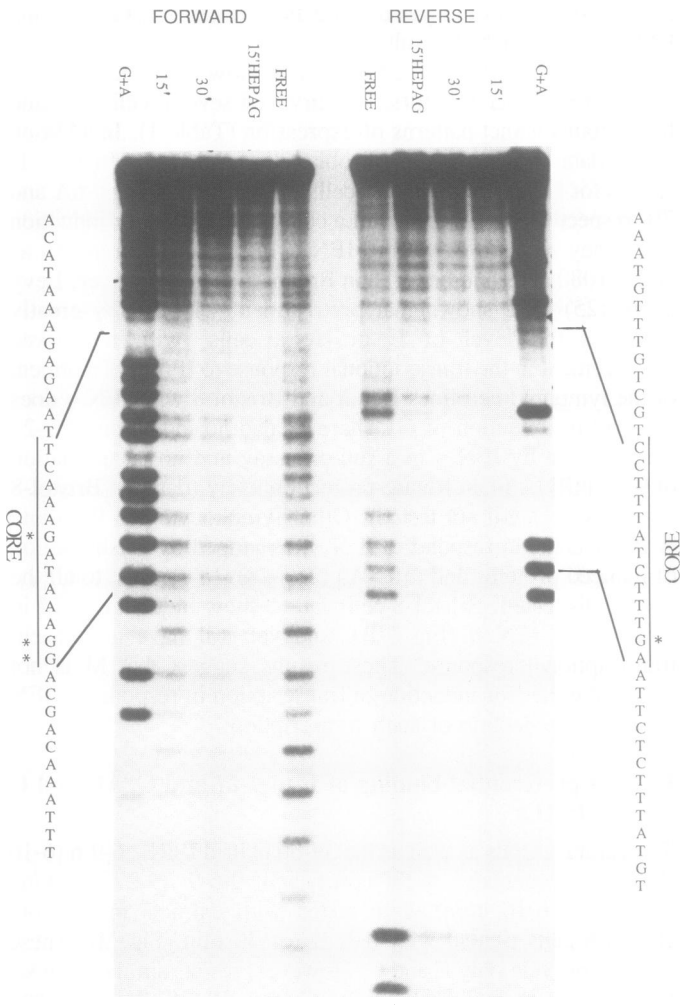

Figure 3: Copper orthophenanthroline footprint of E on both strands of the 9-27 ISRE. The experiments were performed after separation of the complexes in an agarose gel, using whole cell extracts of Bristol-8 cells treated with IFN α for 15 or 30 min. The 15 min extract was also tested after passage through a heparin-agarose column to remove nonspecific DNA binding proteins. Free probe from the same gel was also analyzed as a standard. G residues whose methylation interferes with binding of E are starred (data not shown).

residues upstream of the core. Using mainly the copper orthophenanthroline footprinting technique, we have now analyzed the contacts of E with both strands of the 9-27 ISRE, to which it binds more tightly (Fig. 3) and the contacts of M and G with the 6-16 ISRE (Fig. 4). Binding of E protects flanking sequences on both strands of the 9-27 ISRE from cleavage. The footprint is very asymmetric and extends over most of the 39-base pair region examined. Starred in Fig. 3 are the G residues that interfere with binding of E after reaction with dimethyl sulfate (data not shown). The footprint is very similar to those of E on the 6-16 (10) and ISG54 (15) ISREs. Such a large footprint is consistent with the large size and complex structure of E (25).

The footprints of M (Fig. 4A) and G (Fig. 4B) on the 6-16 ISRE are virtually indistinguishable from each other and from the previously determined footprint of C on the same ISRE (10). Protection is limited to the 13-bp core of the ISRE, and there is also a remarkable enhancement of cleavage at the G and C residues that lie immediately 3′ of the core on the 6-16 coding strand. The same enhancement was observed previously for the complex of C with the 6-16 ISRE (10). These results show that

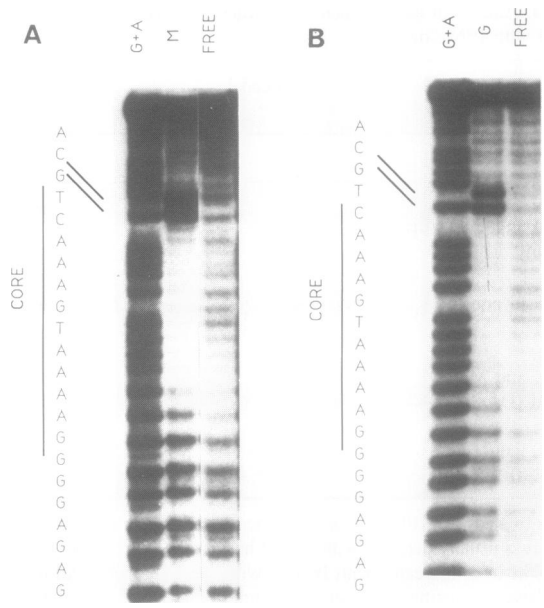

Figure 4: Copper orthophenanthroline footprints of M and G on the coding strand of the 6-16 ISRE. The experiments were performed with M or G complexes after separation in an agarose gel, using whole cell extracts of HeLa cells treated with IFN α for 4 hr (M, Fig. 4A) or with IFN γ for 4 hr (G, Fig. 4B). Free probes from the same gels were also analyzed as standards.

C, M, and G bind to the ISRE in an extraordinarily similar way. Binding does not equal activity since G and M are likely to act positively (see below) and C is not a positive factor. Also, note that C is not encoded by the IRF-1 gene that does encode G and M (16).

Competition assays (Fig. 5) show that the IFN α-induced M factor and IFN γ-induced G factor bind to different ISREs with very similar specificities and affinities. Results are shown for competitions with a 50-fold excess of each oligonucleotide; similar relative competitions were also observed with a 5-fold excess (data not shown). Mutations of the highly conserved G-AAA motifs of the 13-bp core ISRE abolishes binding to both G and M (Fig. 5) as well as to C (10) but the double early mutant binds well to G, M, and C, as discussed above. The H-2K^b and HEX4 oligonucleotides both compete well, consistent with the identity of G with the IBP-1 factor of Blanar *et al.* (17) and the IRF-1 factor of Miyamoto *et al.* (18).

Expression in response to IFNα but not IFNγ is reduced when binding to the 6-16 ISRE of E but not M or G is weakened

The relatively poor affinity of E for the 6-16 39-bp ISRE can be reduced further by altering two G residues that make specific contacts with the protein, to create the double early mutation studied by Dale *et al.* (10) with respect to the IFN α response. The G-to-C changes are at the 5′ margin of the 13-bp core ISRE and further upstream, as shown in Table II. Since these two G residues do not interact with the C, M, or G factors (ref. 10 and Fig. 4) little effect on the affinity for these three factors is expected, and no reduction in the affinity for C (10), M, or G (Fig. 5) was observed. To test whether the double early ISRE, with its high affinity for M and G and very low affinity for E, would behave differently from the wild-type 6-16 ISRE in response to IFN γ as well as to IFN α, we placed each 39-bp oligonucleotide 5′ of the chloramphenicol acetyltransferase (CAT) gene of *E.coli* and the thymidine kinase promoter of Herpes

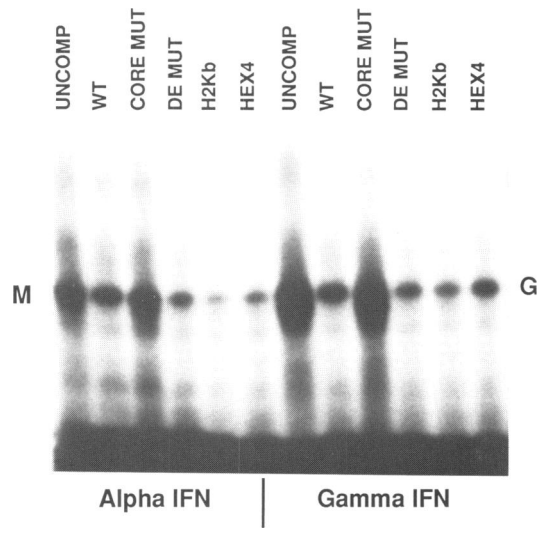

Figure 5: Competition of M or G. Whole cell extracts of HeLa cells, treated with IFN α or IFN γ for 2 hr, were assayed with labeled 39-bp 6-16 ISRE and competed with a 50-fold molar excess of unlabeled oligonucleotides derived from 6-16 (see Materials and Methods for sequence). In CORE MUT the highly conserved GAAA motifs are changed to GACA and in DE MUT two G residues upstream of the core ISRE have been changed to C residues.

Table II. Effect of the double early mutation on response of the 6-16 ISRE to IFNs α or γ

	Data from one experiment (cpm)			Average of 3 experiments (fold induction)	
	No IFN	α	γ	α	γ
6-16	1413	7053	4756	5.5	3.3
double early	823	1071	3238	1.2	3.9

← core →

6-16: GAGCTGGGAGAGAGGGGAAAATGAAACTGCAGAGTGCAC
DE: ———C———————C———————————

Plasmids were constructed by inserting 6-16 wild type or double early (DE) 39-bp oligonucleotides into the CAT expression vector pBLCAT2 (29). Transient transfection and CAT assays were carried out in HeLa cells as previously described (30) but without glycerol shock. IFN treatment was for 30 hr.

simplex virus, as shown in Table II. The responses of the two constructs were then tested after transient transfection into HeLa cells. There was no significant effect of the double early mutation on the ability of the 6-16 ISRE to respond to IFN γ, but the response to IFN α was abolished (Table II). These results are consistent with the idea that G acts positively to stimulate transcription and show that tight binding of M is not sufficient to achieve a good transcriptional response to IFN α.

Similar kinetics of transcription of 9-27 and induction of G in IFNγ-treated cells

The increase in transcription of the 9-27 gene and appearance of G are comparable in HeLa cells after treatment with IFN γ (Fig. 6), indicating that G is likely to be a positively acting transcription factor. Similar observations have been made by Blanar *et al.* (31) using the regulatory element of an MHC Class I gene and by Decker *et al.* (32), using the regulatory element of a gene encoding a guanylate-binding protein. Neither we (Fig. 6) nor Decker *et al.* (32) observed induction of E in response to IFN γ.

M is likely to act positively

Levy *et al.* (15) have proposed that ISGF2 (M in the present paper) is a negatively acting factor, mediating the down-regulation of transcription observed by many workers several hours after cells are treated with IFN α. However, since G almost certainly acts positively, and since M and G are very similar, it seemed possible that M would also act positively. To investigate the function of M, we compared induction by IFN α of E and M with induction of transcription of the 6-16 gene in two cell lines with different characteristics. In HFF cells, both E and M are induced well by IFN α (bandshift data not shown, but see Fig. 1 for similar results with the fibroblast cell line W138-SV). When the time course of transcriptional induction is compared with changes in the relative amounts of E and M in these cells (Fig. 7A), it is apparent that transcription is nearly maximal when the amount of M is maximal, strongly arguing against the possibility that M acts to suppress transcription. Furthermore, transcription persists at a high rate long after the level of E has declined and correlates best with the sum of E and M, consistent with the idea that both factors act positively. In Bristol-8 cells, E is induced well but there is no detectable induction of M by IFN α (Fig. 7B). Transcription still declines after several hours (Fig. 7C), so M is not likely to be obligatory either for induction or shut-off of transcription. Transcription declines significantly more rapidly in Bristol-8 cells (Fig. 7D) than in HFF cells (Fig. 7A), a result most consistent with the idea that M funtions to maintain the transcription first induced by E in IFN α-treated cells. Shut-off may occur when the levels of E and M have both declined and may not require the action of an induced negative factor that binds to DNA. Induction of transcription of 6-16 is relatively poor in Bristol-8 compared to other cell lines (compare Fig. 7A with 7D) and similar results have been obtained with other IFN-induced genes (data not shown). It may be that this is due to the lack of M.

DISCUSSION

Variation in induction of E, M, and G in different cells and in their affinities for different ISREs

As shown in Table I, we observed four patterns of induction. HeLa cells have low levels of E due to a deficit in one of its subunits, E γ. Pretreatment of the cells with IFN γ to increase E γ leads to an increase of active E after exposure to IFN α, a property of other cells lines as well (25). Daudi and Bristol-8 cells apparently lack a response to IFN γ completely; there is no induction of G, no induction of gene expression, and no ability to achieve an antiviral state upon treatment with IFN γ (data not shown). The basis of this unresponsive state is unclear.

Mutations in ISREs have been shown to affect the affinities for different classes of factors in two ways. Mutations in the 13-bp core that disrupt the two GAAA motifs affect the binding of all factors, whereas some mutations at the edge of the core or outside it interfere with the binding of E selectively (10, 13–15). The double early mutation provides a good example of the latter situation, and the experiment described in Table II shows that reduced binding of E abolishes the response to IFN α *in vivo*, while the response to IFN γ is retained, as expected since the affinity for G is not decreased (Fig. 5). It is important to note that M is not sufficient to induce this construct to respond to IFN α, suggesting that M and G may be functionally different.

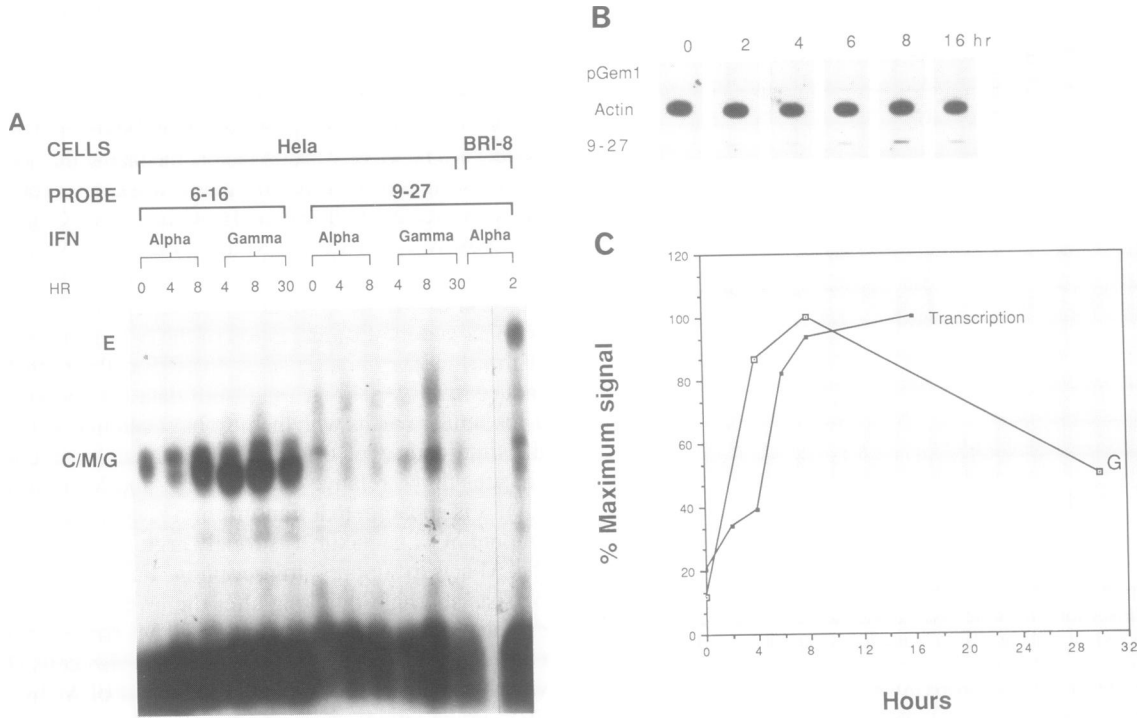

Figure 6: Induction of G and transcription of the 9-27 gene by IFN γ. A. Bandshift assays. HeLa cells were treated with IFN γ or IFN α and whole cell extracts were examined using 6-16 and 9-27 probes. Also shown is an assay with an extract of IFN α-treated Bristol-8 cells, to mark the position of E. B. Nuclear run-on analysis of IFN γ-treated HeLa cells. Nuclei were incubated with [α^{32}P]UTP and the isolated RNA was hybridized to 5 μg each of pGem1 (Promega Biotech), human β-actin plasmid and 9-27 plasmid. C. Composite plot of the bandshift and run-on data.

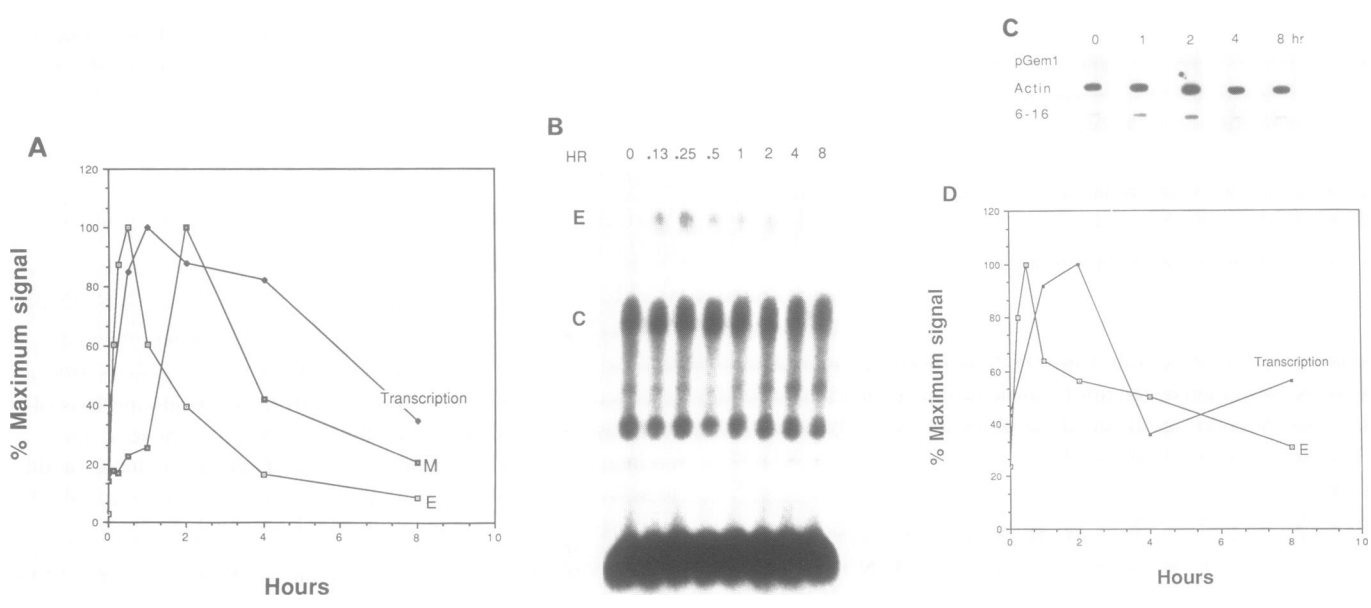

Figure 7: Time courses of transcriptional induction and appearance of factors. A. The induction profiles of E and M from high salt extracts of HFF cells were quantified by densitometry and plotted together with IFN-induced transcription of 6-16 in the same cells. B. DNA binding proteins in Bristol-8 cells treated with IFN α. Bandshift assays were performed with a 6-16 probe. C. Transcription rates in Bristol-8 cells treated with IFN α. The analyses were carried out as described in Fig. 6B. D. Time courses of transcriptional induction and appearance of factors in Bristol-8 cells. The bandshift and run-on data of B and C are plotted.

The highly conserved GAAA motifs of ISREs flank one or two central nucleotides that are not well conserved (8) to give the consensus sequence GAAAN(N)GAAA. These central nucleotides do play a role in binding C, M, and G, since ISREs in which NN=AT (6-16 and 9-6-9) have much higher affinities for these factors than their identical counterparts in which NN=TA (9-27 and 6-9-6, Figs. 1 and 2). Combination of the effects of these NN residues and of sequences flanking the 13-bp

core ISREs can give 4 different types of affinity towards E, compared to C, M, and G: high, high; high, low; low, high; and low, low. It remains to be seen if all four types occur naturally and if they have physiological significance. The results of Lewis *et al.* (33) may bear on this point. In some mouse cell lines, it seems likely that limiting levels of E γ allow expression of only a subset of genes in response to IFN α. Upon pretreatment with IFN γ, which probably induces E γ in these cells as it does in HeLa cells (25,34), the concentration of active E is higher in response to IFN α, now allowing expression of those IFN α-responsive genes which have an intrinsically lower affinity for E.

Factors induced by IFNα

The initial response to IFN α is very rapid, with activation of latent E in the cytoplasm and transport of active E to the nucleus within a minute or two (11,25). The footprint of active E on an ISRE is large, extending well outside the 13-bp core (refs. 10, 14, 15 and Fig. 3). The binding site overlaps that of C, M, and G in the region of the core, but E interacts with the ISRE region very differently from C, M, and G, which bind to a smaller subregion (refs. 10, 14, 15 and Fig. 4). The footprint of E on the tight-binding 9-27 ISRE is very asymmetric, extending both upstream and downstream of the 13-bp core on opposite strands (Fig. 3). These observations raise the possibility that the E α and E γ subunits of E both bind to DNA. Confirmation and details will have to await purification and full analysis of the oligomeric structure of active E.

M is likely to be a factor that maintains transcription, in contrast to the earlier suggestion of Levy *et al.* (15) that it might mediate negative regulation of genes containing an ISRE. These workers stated that the appearance of M corresponded with the decline of transcription but did not present coordinate kinetic data for induction of transcription and for the presence of M. According to our results in HFF cells, which have M, and Bristol-8 cells, which do not, transcription is maximal when the level of M is maximal (Fig. 7A) and the rate of transcription declines more rapidly when M is absent than when M is present (Fig. 7D). Furthermore, the decline of transcription occurs perfectly well in Bristol-8 cells in the absence of detectable M. Additional observations are consistent with a role for M as a positive factor that maintains transcription and inconsistent with a role as a negative regulator: We have isolated two mutant cell lines, derived from the 2fTGH cells described by Pellegrini *et al.* (35), in which expression of IFN-regulated genes is constitutive. In both, bandshift assays show that M is present constitutively at a high level (Roslyn McKendry, I.M.K. and G.R.S., unpublished results).

Factors induced by IFNγ

IFN γ regulates the expression of two major classes of genes, those with and those without ISREs. The latter, exemplified by class II MHC genes such as HLA DR α, are regulated by DNA elements different from ISREs that bind to induced factors distinct from G (31, 36, 37). The IRF-1 gene, encoding G, responds rapidly to IFN γ (16) but the factor(s) mediating this response have not yet been defined. It will be interesting to see if induction of IRF-1 by IFN γ is a primary response. Induction of ISRE-containing genes by IFN γ is relatively slow, as expected since the response to G must be secondary. Consistent with this, Blanar *et al.* (17) found that IBP-1 (G) binds to the ISRE of the MHC class I gene H-2K^b and that induction of this factor requires new protein synthesis. G alone might be capable of initiating transcription through an ISRE in response to IFN γ, or other factors might also be involved.

In contrast to M, also encoded by the IRF-1 gene, G persists for a long time in IFN γ-treated cells, prolonging the response: Both Blanar *et al.* (17) and we (Fig. 6) have observed that the levels of G remain high for 24 hr or more after treatment with IFN γ. Decker *et al.* (32) observed that the ISG-54 gene is maintained in a transcriptionally active state in cells treated with IFN α and IFN γ together and that the level of ISGF2 (M and G) remains high in such cells, even though the ISG-54 gene does not respond well to IFN γ alone and is induced only transiently by IFN α. These observations could be accounted for if E were to remove an initial block to transcription so that G could maintain transcription for a long time.

Relationship of C, M, G, IRF-1, and IRF-2

C, M, and G have very similar properties. Especially striking is enhancement of cleavage by copper orthophenanthroline at the same two residues in all 3 ISRE-protein complexes. G and M appear to be encoded by the same gene (16), IRF-1, previously cloned by Miyamoto *et al.* (18). C is likely to be a closely related but different factor (16) and might be related to IRF-2, a repressor present constitutively and also induced by IFN β (38).

The IRF-1 gene is induced by IFN α, possibly through the action of factor E, to yield M and also by IFN γ to yield G. It is also stimulated by several other inducers (18) including, most surprisingly, prolactin (39). Clearly an unmodified IRF-1 gene product can not alone mediate specific responses to such a large number of different signals. The IRF-1 gene product might be modified specifically in response to each different signal or might associate with different induced proteins to give specific responses. More work is obviously needed to understand this potentially complex situation.

There is virtually no information concerning why factors E and M decay in the continued presence of IFN α. If the decay of M were to require new protein synthesis, we can account for the observation of Larner *et al.* (40) that transcription is prolonged when cells are first treated with IFN α and then exposed to cycloheximide. Factor C might help to keep IFN-responsive genes turned off in the absence of IFN by binding to their ISREs. There may be no need for an IFN α-induced negative factor that binds to ISREs since, after both E and M have decayed, factor C may be sufficient to shut off transcription again. The level of C might also increase in response to IFN α: see the observation by Harada *et al.* (38) that IRF-2 is induced by IFN β.

General conclusions

The role of E as the initiator of rapid transcription in response to IFN α is clear. M is likely to be a positively acting factor, induced by IFN α to maintain transcription but not capable of initiating it in the absence of E. G seems to be encoded by the same gene as M but is functionally distinct. Its detailed role in activating genes in response to IFN γ remains to be elucidated fully. The variation in patterns of induction of E, M, and G in different cell lines is probably to be found also in different tissues and seems likely to play a role both in mediating tissue-specific responses to different types of IFNs and in establishing different kinetics of response to IFN α. The differential affinities of natural ISREs for E, based on binding to residues that flank the 13-bp cores, and for C, M, and G, based on binding to the hypervariable NN residues in the center of the core, are independent variables that can also play a role in helping to determine the pattern of

response of a particular gene. The availability and relative concentrations of additional transcription factors, repressors, and extinguishers will surely also contribute to tissue-specific variations in response to the interferons. It is of obvious advantage for an organism to have several ways to regulate the responses of individual genes to the various interferons in different tissues.

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