

# Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control

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**Human  $\alpha$ - and  $\beta$ -interferons (IFNs) stimulate rapid but transient increases in transcription from a set of previously quiescent genes. Protein synthesis is not required for initial stimulation, but duration of the response is limited to a few hours by a process requiring synthesis of new proteins. An IFN-stimulated response element (ISRE) was identified 5' to an inducible gene by deletion analysis and point mutagenesis, and sequence comparisons with other promoters defined the consensus element YAGTTTC(A/T)YTTYCC. Two classes of IFN-inducible nuclear factors were found that bind to the ISRE. The most rapidly induced factor appeared without new protein synthesis, whereas a second factor required active protein synthesis for its appearance and maintenance. The kinetics of appearance and loss of these binding activities correlate with the activation and repression of IFN-stimulated genes. These different IFN-activated or induced factors may bind sequentially to the same essential promoter element to first increase and then repress transcription.**

[*Key Words*: Transcriptional control; interferon; nuclear factors; promoter elements]

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It is now well established that individual peptides bound to specific cell-surface receptors cause prompt changes in the transcription of specific sets of genes. Type I (Friedman et al. 1984; Larner et al. 1984; Kelly et al. 1985) and type II interferons (Luster et al. 1985), platelet-derived growth factor (PDGF; Greenberg and Ziff 1984; Treisman 1985; Lau and Nathans 1987) and epidermal growth factor (EGF; Murdoch et al. 1982) have all been demonstrated by appropriate nuclear assays to increase transcription of groups of genes. In contrast to steroid hormones, which bind directly to transcription factor receptors, the basis by which polypeptide-generated signals from the cell surface reach the nucleus to cause transcriptional changes is not understood. The signal from the cell surface might either modify existing transcription factors or cause the synthesis of new proteins that are involved in transcription or are transcription factors themselves. Aside from one report on induction in A431 cells but not in HeLa cells of a factor that binds to the *c-fos* enhancer (Prywes and Roeder 1986), no transcription factors that vary in response to cell-surface signals have yet been identified and isolated.

We have previously identified several genes whose transcription in human cells is dependent upon treatment with type I ( $\alpha$ - or  $\beta$ -) interferons (IFNs) (Larner et al. 1984, 1986). This group of genes is referred to as interferon-stimulated genes (ISGs). The genomic 5'-flanking regions of two such genes, the ISG54 and ISG15

genes (so named because they encode proteins of approximately 54 and 15 kD, respectively) have been identified and shown in recombinant plasmid and adenovirus vectors to be capable of directing IFN-stimulated transcription in transfected cells (Levy et al. 1986; Reich et al. 1987). The response of ISGs to IFN is not merely a positive one because the immediate transcriptional increase brought on by IFN treatment is soon followed by a regulated decrease in transcription (Larner et al. 1984, 1986). The positive limb of this cycle does not require protein synthesis, but the return to and the maintenance of a low transcription rate of ISGs does require ongoing protein synthesis. These observations suggest that transcriptional activation by IFN is effected through pre-existing cellular components but is limited by proteins newly formed during the IFN response.

In this paper, we identify a sequence in the 5'-flanking region of the ISG54 gene that can direct IFN-stimulated transcription. This sequence contains an element, the interferon stimulated response element (ISRE), that was found to match a homolog present in many different ISG promoters. Mutations within the ISRE of the ISG54 gene eliminated transcriptional activity.

In the nucleus of untreated cells, there is at least one factor that specifically recognized the ISRE. However, in IFN-treated cells, two other factors appear. The factor that is first induced is not affected by inhibition of protein synthesis and is a candidate for a positive-acting

transcription factor that is modified in response to IFN. The later appearing factor does not appear in cycloheximide-treated cells and is a candidate for a negative-acting IFN-induced transcription factor. By competition experiments and through the use of the mutant ISRE sequence in DNA-binding assays, we conclude that the same oligonucleotide region is bound by both the putative positive and negative factors.

## Results

### *ISG54 minimal inducible promoter*

Recombinant plasmids containing either 3 kb or 800 bp of 5'-flanking sequence plus the first 288 bp of ISG54 fused to a reporter template (adenovirus E1B intron and 3' exon) were shown previously to be equally capable of directing IFN-stimulated transcription (Levy et al. 1986). To more accurately localize the sequences necessary for IFN-stimulated transcriptional induction of ISG54, additional 5'-deletion mutants were prepared and used in transient transfection assays. The constructs used in Figure 1A contained either 800, 171, 122, or 40 nucleotides of 5'-flanking sequence, only 55 nucleotides of the first exon, and none of the cellular intron. Expression of the E1B reporter sequence was scored by protection against nuclease digestion of a labeled antisense RNA (Fig. 1A, lanes 1–6 and 11–12). These plasmids containing 122 nucleotides or more of 5'-flanking region gave equivalent levels of uninduced, basal expression and exhibited an equal increase in expression in response to IFN treatment (lanes 1–6). However, the construct containing only 40 nucleotides of 5'-flanking sequence did not give a transcription signal (lanes 11–12); therefore we concentrated on sequences between –40 and –122 as important for ISG expression. The sequence of the 400 5'-flanking nucleotides of ISG54 has been submitted to GenBank. It contains a TATA box at –30 and a CCAAT box at –110.

### *Protein-binding site identified by DNase I footprinting*

Because the region from –122 to +55 conferred IFN sensitivity to recombinant plasmids, we assumed that there might be an ISRE specific for IFN-induced nuclear factors within this region. We performed DNase I footprint experiments (Galas and Schmitz 1978), using crude nuclear extracts from normal and IFN-treated HeLa cells (Fig. 1B). Binding reactions contained end-labeled DNA fragments from the promoter region (–122 to +55, asymmetrically labeled in either of the two strands) and 100 µg of nuclear proteins. After incubation, the samples were digested with DNase I, and the deproteinized DNA was subjected to denaturing acrylamide gel electrophoresis.

Protection against DNase digestion was detected on both strands of the DNA (Fig. 1B). The most prominent protection was in the region between –85 and –105 on both strands. Specifically, no protection nearer to the RNA start site could be detected. With equal amounts of extract there was greater protection by extracts of in-

duced cells. The region of greatest protection was 5–10 bases downstream of the CCAAT box motif. These initial experiments thus directed our attention to the –80 to –100 region as an important region in regulation of ISG54.

### *Mutations define functional importance of the ISRE, a conserved DNA element*

To test the functional importance of the individual nucleotides within this DNA region, mutations were introduced into a plasmid construction that contained the –122 to +55 sequence of ISG54 fused to the E1B reporter gene. Mutations were directed to nucleotides protected from DNase attack in the footprint experiment. In mutant M157, the thymidines at position –98 and –97 were changed to adenine and guanine residues, respectively. In mutant M158, the sequence T-T-C at position –91 to –89 was changed to A-G-T.

Each of these mutant plasmids was used in transient transfection assays in comparison with a construction containing wild-type sequence. Little or no mRNA was transcribed from either mutant plasmid, regardless of whether transfected cells were treated with interferon or not (Fig. 1A, lanes 7–10), indicating that the same sequence functions in both basal and induced transcrip-

**Figure 1.** ISG54 Minimal inducible promoter. (A) Promoter mutations abrogate expression transfected cells. HeLa cells were transfected with fusion constructs containing 800 bp (lanes 1 and 2), 171 bp (lanes 3 and 4), 122 bp (lanes 5 and 6), or 40 bp (lanes 11 and 12) of wild-type ISG54 promoter sequence or with mutated –122-bp constructs containing 2 nucleotide changes (lanes 7 and 8) or 3 nucleotide changes (lanes 9 and 10) from wild-type sequence, as described in the text. RNA from transfected cells untreated (odd-numbered lanes) or treated with IFN (even-numbered lanes) was assayed for expression of the transfected plasmid. Endogenous ISG54 and cotransfected pRSVcat were assayed for expression as controls (not shown). (B) DNase I footprint of protein binding to the ISG54 promoter. DNA fragments from –122 to +55 of ISG54 were uniquely labeled at the 5' end for the nontranscribed strand (*left*) or at the 3' end for the transcribed strand (*right*). Footprinting reactions were performed as described in Materials and methods. Control reactions contained no cell protein, whereas the other reactions contained 60 µg of protein from whole cell extracts of untreated (IFN –) or IFN-stimulated (IFN +) HeLa cells. The sequences of the protected region are indicated with numbering relative to the cap site. (C) Sequence similarities between the ISG54 protein-binding domain and promoters of other IFN-stimulated genes. Bases identical to the derived consensus ISRE (*bottom*) are shown in boldface type, and differences are shown in italics. The numbers indicate the number of matches in each row or column, and the arrows indicate the polarity with respect to the start of RNA transcription. The sequences are from 5'-flanking regions of ISG54 (this paper), ISG15 (Reich et al. 1987), human clone 6-16 (G. Stark, pers. comm.), mouse gene 202 (Samanta et al. 1986), HLA-A (Koller and Orr 1985), mouse class I histocompatibility (Moore et al. 1982; Kimura et al. 1986; Korber et al. 1985) or Tla (Fisher et al. 1987) genes, and human complement factor B (Wu et al. 1987).

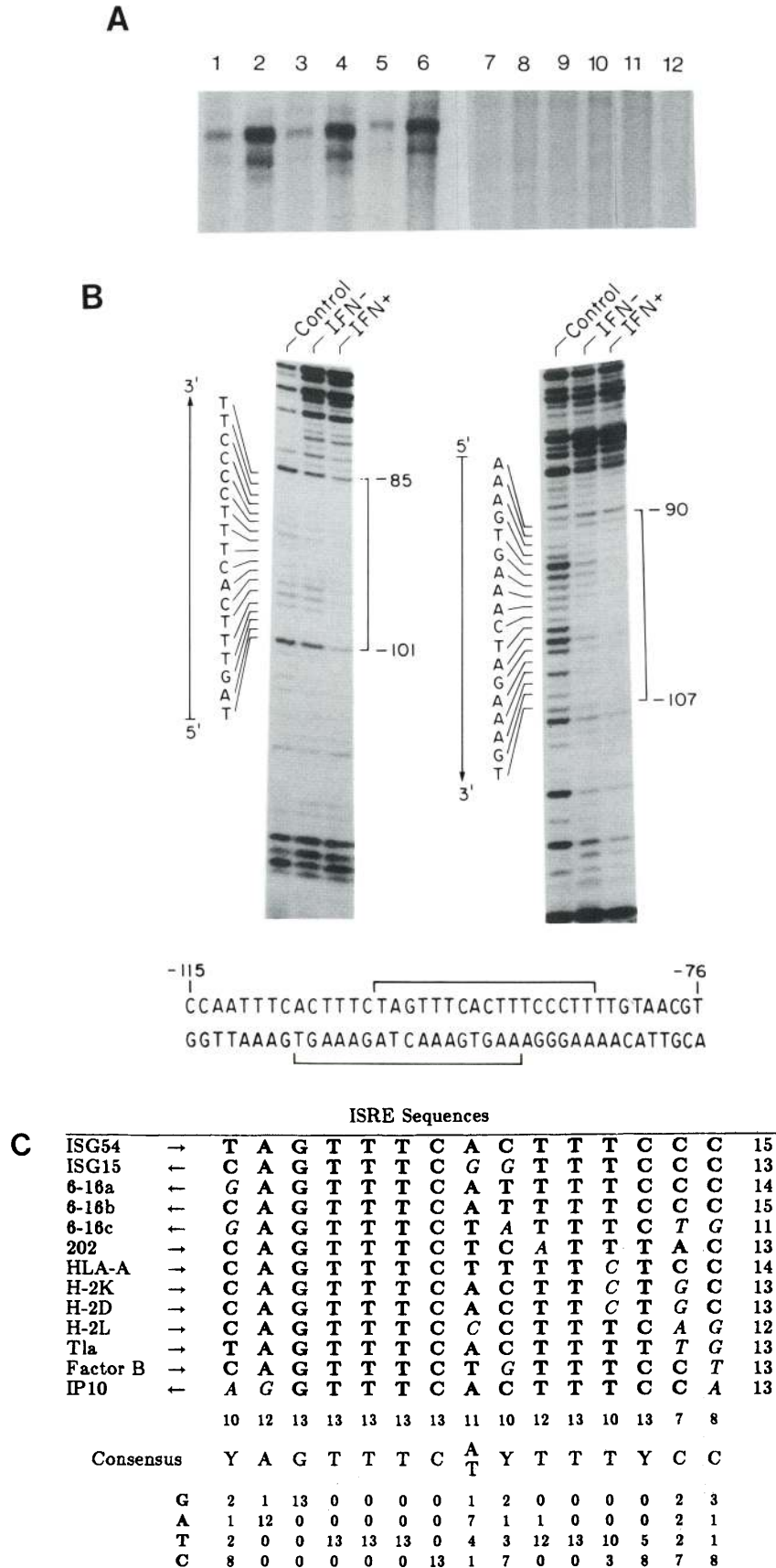


Figure 1. (See facing page for legend.)

tion. Upon prolonged exposure of autoradiograms, a weak, properly initiated RNA was detectable for M157 (not shown). M158, however, gave no detectable signal.

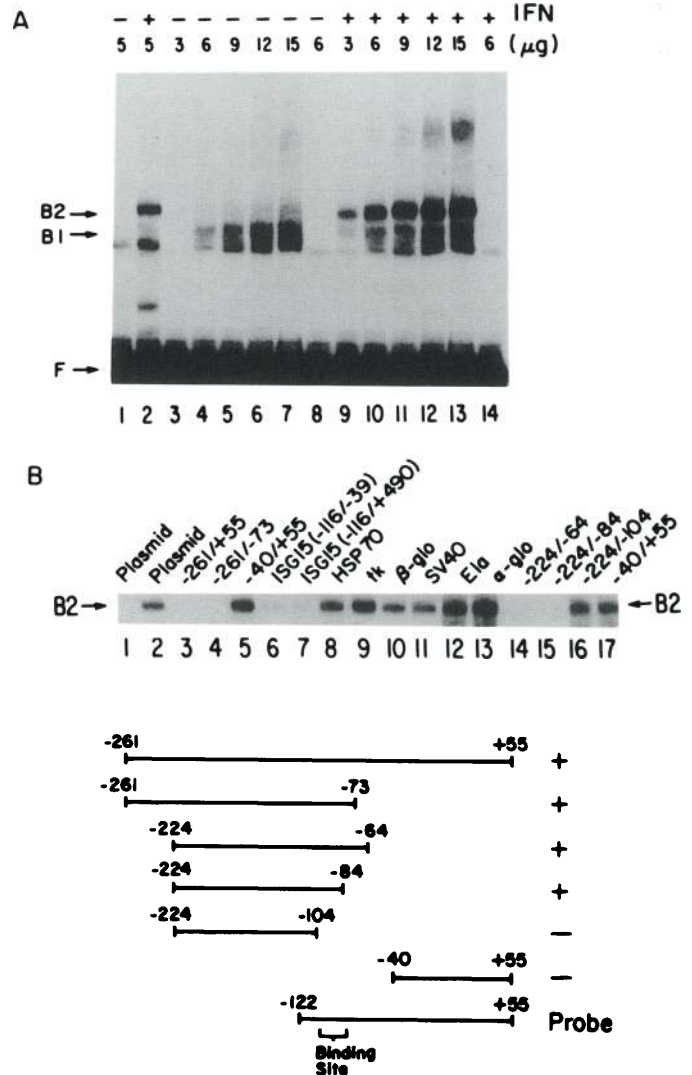
The definition in the 5'-flanking region of crucial nucleotides for expression of the ISG54 gene led us to compare the sequence of this gene with other genes induced by  $\alpha$ -IFN. First, a strong homology was noted with the other gene, ISG15, which we have cloned and examined (Reich et al. 1987). The homologous sequence in ISG15 was found within the 115 5' flanking nucleotides that were shown to be necessary for transcriptional activation. This similar sequence was in the opposite orientation, consistent with the enhancerlike capacity of the ISG15 5'-flanking sequence (Reich et al. 1987). Other genes known to be strongly induced by IFN, such as 6-16 (Porter et al. 1988), C202 (Samanta et al. 1986), and H-2 (Israel et al. 1986; Vogel et al. 1986), also contain a strong homology limited to about 15 nucleotides. This region of homology is much smaller, but it is contained within the sequence predicted previously by Friedman and Stark (1985) on the basis of sequence comparison alone for genes whose sequences were known (metallothionine and histocompatibility genes) but whose IFN-responsive regions had not been identified. By comparison of the ISG54 and ISG15 homology with a collection of 5'-flanking sequences from genes known to respond to IFN, we were able to derive a consensus motif, YAGTTTC(A/T)YTTTTYCC (Fig. 1C). However, the functionally important regions of some of these genes have yet to be described.

*Several distinct nuclear factors bind the ISRE*

If a specific sequence within the -122 to -40 region of ISG54 is responsible for the regulated cycle of increase and subsequent decrease in ISG transcription following IFN treatment, protein extracts from IFN-treated cells might be expected to contain multiple proteins that bind within this region. The gel-shift or gel-retardation assay first described by Fried and Crothers (1981) and Garner and Revzin (1981) was used to search for such proteins.

A labeled DNA fragment (-122 to +55 of ISG54) was mixed with whole cell extracts prepared in 1.2 M KCl buffer from uninduced cells and from cells induced with IFN for 2 hr. The protein-DNA mixture was subjected to nondenaturing gel electrophoresis and autoradiography (Fig. 2A). Two cell types, a transformed human fibroblast (clone 75.1, ATCC) and HeLa cells, were used for extracts shown in Figure 2. With the HeLa cell extracts (lanes 3-14), two prominent gel-shift bands were noted with extracts from uninduced cells (lanes 3-7); only one of these, denoted B1, was completely competed by a 35-fold molar excess of unlabeled homologous DNA (lane 8), a characteristic of sequence-specific interactions. The other band, migrating slightly faster than B1, was not completely competed by homologous DNA and, therefore, could be due to an abundant, generalized DNA-binding protein. However, extracts from IFN-treated HeLa cells (lanes 9-14) produced a prominent new band (denoted B2) that was not found in uninduced

cells and was also subject to homologous competition (lane 14). The same bands seen in the B1 region using uninduced extracts were also found using extracts of induced cells. The competition with homologous DNA



**Figure 2.** Gel-shift protein-DNA binding assays with whole cell extracts. (A) Gel shifts were performed by mixing the indicated amounts of whole cell extracts from untreated or IFN-treated WI38 fibroblasts (lanes 1 and 2) or HeLa cells (lanes 3-14) with a -122/+55-bp end-labeled probe. Lanes 8 and 14 represent reactions in which unlabeled homologous competitor DNA was present in a 35-fold molar excess. The positions of the unbound probe (F) and the B1 and B2 retarded complexes are indicated. (B) Gel-shift assays were performed using a labeled DNA probe containing sequences from -122 to +55 using whole cell extracts from untreated (lane 1) or IFN-treated cells (lanes 2-17). (Lanes 1-13) Results using WI38 extracts; (lanes 14-17) results from HeLa cell experiments. Nonspecific and specific competitor DNA (0.5  $\mu$ g) included in each reaction is indicated above each lane, as described in the text. Only the band corresponding to the B2 complex is shown. A summary of the competition results is diagrammed below.

also removed B1 entirely but left a trace of the faster moving gel band (lane 14). As a control for extraction efficiency of the treated and untreated cells, both extracts were found to contain the same amount of NF-1 (Nagata et al. 1983; Pruijn et al. 1986), a nuclear factor that binds to sequences in the terminus of adenovirus (not shown).

In addition to showing the induced B2 band, the experiment in Figure 2 shows the effect of adding increasing amounts of the two cell extracts to the binding reactions. Even at the highest concentration of protein from uninduced cells, only a faint signal was detected in the B2 region of the gel, much less distinct than the B2 band produced by fivefold less protein from induced cells (cf. lanes 7 and 9). This B2-like signal from uninduced extracts may not be due to the same protein producing IFN-induced B2 because with high amounts of protein, complexes that migrated slower than B1 and B2 were seen with extracts of induced and, to a much lesser extent, uninduced cells. These slower moving bands could be aggregates due to high protein concentrations exceeding the resolving power of the gel-shift assays.

Extracts from a fibroblast cell line (Fig. 2, lanes 1 and 2) also showed an IFN-induced DNA-binding protein that formed a complex migrating similar to B2 (lane 2). Much less of the B1-like activity was observed in most extracts of either uninduced or induced fibroblasts, but some of the nonspecific protein that formed the band migrating faster than B1 was observed in fibroblasts (lane 1). In addition to the IFN-induced B2 complex, faster migrating IFN-specific gel-shift bands (near the bottom of lane 2) were generally seen in extracts from IFN-treated fibroblast lines but were rarely found using HeLa extracts. These complexes could represent proteolytic degradation of the major IFN-induced binding activity in extracts of those cell lines. In addition to extracts of HeLa cells and fibroblasts, IFN-induced bands like B2 were routinely observed with extracts of IFN-treated human primary fibroblasts and lymphoblastoid (Daudi) cells but not with extracts from untreated cells (not shown).

To determine which sequences within the  $-122$  to  $+55$  region were involved in forming the B2 complex, a series of competition experiments were performed. In these experiments (Fig. 2B),  $\sim 35$ -fold molar excess of various unlabeled DNA segments was incubated with the labeled  $-122$  to  $+55$  DNA probe during the protein-binding reaction. Several significant results came from these experiments (Fig. 2B). First, only DNA segments containing sequences between  $-84$  and  $-104$  were found to compete for protein binding. Thus, the site responsible for the B2 gel-shift band is in the same region as the major footprint region described in Figure 1B. Second, the promoter and transcription factor-binding domains of many viral and cellular genes failed to compete for formation of the B2 band (lanes 8–13). However, the promoter region for the ISG15 gene, an IFN-stimulated gene whose 5' sequence contains the ISRE homology (Fig. 1C), did completely compete for the formation of the B2 complex (Fig. 2B, lanes 6 and 7).

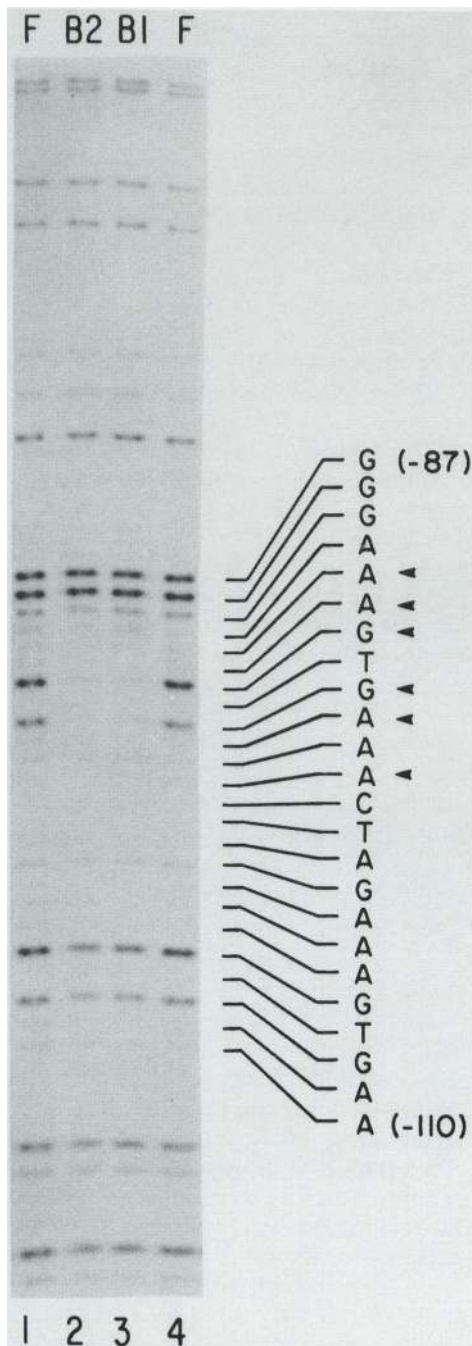
#### *Binding sites detected by methylation interference*

We then turned to the use of a methylation-interference footprint experiment to determine the precise nucleotides within the ISRE region that interact with constitutive and induced factors. A uniquely end-labeled antisense DNA fragment ( $-122$  to  $+55$ ), partially methylated by dimethylsulfate (DMS) (Maxam and Gilbert 1980), was mixed with IFN-induced HeLa cell extract and a gel-shift experiment performed. Both the constitutive B1 band and IFN-induced B2 band were located by autoradiography, and the labeled DNA was recovered. The recovered DNA was then cleaved at modified bases by piperidine treatment and resolved by gel electrophoresis. If methylation of a particular G or A prevents protein-DNA complex formation, such a methylated guanine or adenine will not be in the gel-shift band, and thus its cleavage product will not appear in the sequence ladder (Fig. 3). This experiment showed that the G residues at  $-95$  and  $-93$  on the antisense strand were crucial for DNA-protein binding in both B1 and B2. The single guanine residue within this region on the opposite strand (position  $-99$ ) was also affected, though less dramatically, in a similar experiment performed with label in the sense strand (not shown). The adenine residues at  $-96$ ,  $-92$ , and  $-91$  on the antisense strand are probably also necessary for contacting binding proteins because these steps of the sequence ladder were depressed somewhat in both recovered complexes. The proteins responsible for forming the B1 and B2 complexes contact the same nucleotides within the ISRE. This finding explains our earlier observation that the ISRE was protected by both induced and uninduced HeLa extracts in DNase footprinting procedures (Fig. 1B).

#### *The nature of the proteins responsible for the B2 complex*

Transcriptional activation of ISG54 (and other genes) by IFN is very prompt (Larner et al. 1984, 1986; Reich et al. 1987) and does not require protein synthesis (Larner et al. 1986; Levy et al. 1986). We therefore tested the time of appearance of B2 in IFN-treated HeLa cells that were also treated or not treated with cycloheximide. In many experiments, two of which are shown in Figure 4, the clear appearance of B2 required at least 1.5 hr of IFN treatment and reached maximal levels only after 2–4 hr. The B2 gel-shift activity then declined at  $\sim 10$  hr. There was a dramatic effect of cycloheximide in blocking the induction of the B2 activity (Fig. 4, lane 11). In addition, protein synthesis was required to maintain a high level of B2. Cells treated with IFN for 2 hr to induce B2 and for an additional 2 hr with cycloheximide plus IFN had much less B2 than cells treated for 4 hr with IFN alone (lane 12). Thus, the B2 activity did not correlate with the expected properties of a positive-acting factor: There was a time lag before its appearance, its appearance was blocked by cycloheximide, and it disappeared after being induced when cells were treated with cycloheximide. All of these characteristics, however, correlate with the decline of IFN-induced transcription and make B2 more

likely to be related to the deinduction, rather than to the induction, of the ISGs.



**Figure 3.** Methylation interference of protein–DNA complex formation identify a single binding site. Preparative binding reactions were prepared using partially methylated  $-122/+55$  DNA probes 3'-end labeled on the transcribed strand and whole cell extracts from IFN-treated HeLa cells. Free probe (lanes 1 and 4) and retarded complexes B2 (lane 2) and B1 (lane 3) were resolved by nondenaturing gel electrophoresis, the DNA was recovered, and the cleavage products following piperidine treatment were separated on an 8% acrylamide–42% urea gel.

#### *IFN-induced factors in low salt nuclear extracts*

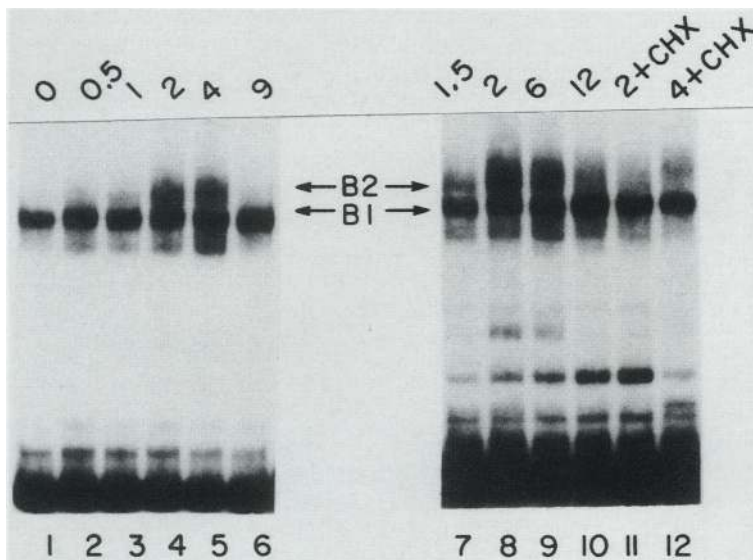
In the DNA–protein binding assays described thus far, extracts of whole cells prepared with 1.2 M KCl were employed. Such extraction conditions were chosen initially in an attempt to ensure release of all DNA-binding proteins. To concentrate nuclear proteins more specifically, isolated nuclei were prepared and proteins extracted in small volumes of buffer containing different salt concentrations (Dignam et al. 1983). No binding activity was detected in cytoplasmic fractions and the previously detected IFN-induced factor responsible for B1 and B2 appeared to be quantitatively recovered by a 0.4 M salt extraction of nuclei.

In addition, extracts of nuclei prepared by this protocol using 0.2–0.4 M NaCl also contained proteins that produced additional complexes from treated cells that were not seen in whole cell extract cells (Fig. 5, lanes 4 and 5). Most importantly, one of these bands, denoted B3, has been regularly observed to be maximally induced within 1 hr of treatment. Although the appearance of B3 is a characteristic of all low salt nuclear extracts of IFN-treated cells, its intensity is only approximately one-tenth as great as that of B2. Therefore, exposure times that make B3 easily visible cause overexposure of the B1 and B2 region of the gel-shift pattern.

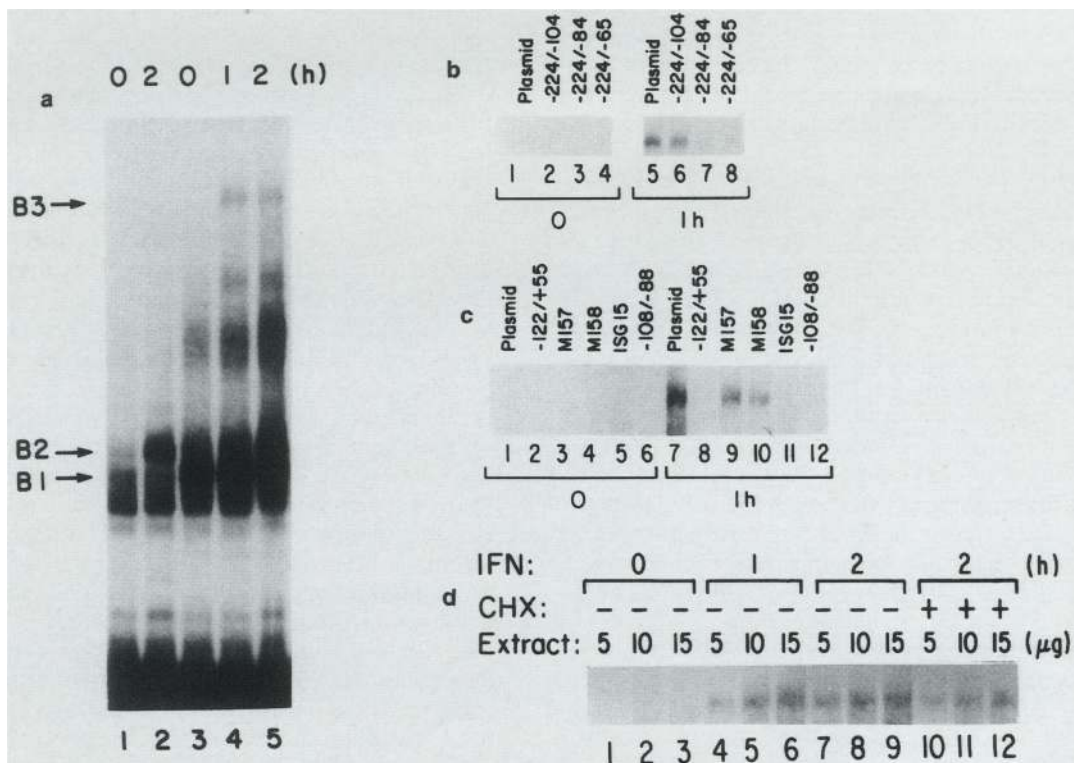
In addition to B3, in all nuclear extracts, there were proteins that produced gel-shift bands between B2 and B3, but the amounts of these bands varied considerably in different extracts. It is possible that the protein(s) responsible for these bands is related to B2. In subsequent experiments where the factor(s) responsible for B2 has been partially purified by heparin–agarose and oligonucleotide affinity columns (R. Pine, D.E. Levy, N. Reich, and J.E. Darnell, unpubl.), the broad gel band that migrates between B2 and the position of B3 becomes more prominent as more protein is used in a gel-shift reaction. (This is also observed with total cell extracts, see Fig. 2, lane 13).

Upon identification of B3 as a more promptly induced gel-shift complex than B2, several additional experiments were carried out to investigate the nature of the B3 complex. The results of these experiments are shown in Figure 5. First, competition experiments with various segments of the ISG54 promoter showed that the same sequences between  $-88$  and  $-104$  that competed for B2 formation also competed B3. The ISG15 promoter also competed both bands, and an oligonucleotide with the sequence  $-84$  to  $-108$  was also effective in competition. However, DNA segments containing the mutant oligonucleotides M157 and M158 competed only very poorly. Thus, the sequences to which B2 and B3 bind overlap considerably and may be identical.

The sensitivity of B3 induction to protein synthesis was tested by treating cells with both IFN and cycloheximide. In contrast to the results shown in Figure 4, that B2 induction is inhibited by cycloheximide treatment, the DNA-binding activity required for forming the B3 complex was induced without protein synthesis.



**Figure 4.** Kinetics of B2 factor induction correspond to transcriptional decline. Gel-shift assays are shown using whole cell extracts from HeLa cells treated with IFN for the indicated times. Experiments shown in lane 11 used extracts from cells treated with IFN and cycloheximide (35  $\mu\text{g}/\text{ml}$ ) for 2 hr, and in lane 12 with IFN for 2 hr followed by IFN plus cycloheximide for an additional 2 hr.



**Figure 5.** Gel shifts with low salt extracts detect an additional IFN-induced factor correlating with transcriptional stimulation. (a) Gel-shift experiments with the  $-122/+55$ -bp probe used whole cell extracts (lanes 1 and 2) or low salt nuclear extracts (lanes 3–5) made from HeLa cells treated with IFN for the indicated amounts of time. (b) Competition gel-shift experiments with low salt extracts using 35-fold excess of competitor DNA containing the indicated sequences and extracts from untreated HeLa cells (lanes 1–4) or from 1-hr (lanes 5–8) IFN treatments of HeLa cells. (c) Competition experiments using plasmids containing wild-type  $-122/+55$  sequence, point mutations M157 and M158, the ISG15 promoter ( $-116/-39$ ), or a double-stranded synthetic oligonucleotide containing ISG54 sequences from  $-108$  to  $-88$ , as indicated. Low salt nuclear extracts were prepared from untreated (lanes 1–6) or 1-hr (lanes 7–12) IFN-treated HeLa cells. (d) Gel-shift experiments using the indicated amounts of low salt nuclear extracts made from HeLa cells treated with IFN for the indicated amounts of time. For the results shown in lanes 10–12, cycloheximide was present for 2.25 hr, beginning 15 min prior to the 2-hr IFN treatment. For the experiments in b–d, only the band corresponding to B3 complex is shown.

## Discussion

We have shown previously that one of the primary responses of cells to IFN treatment is a two-phase transcriptional cycle (Larner et al. 1984, 1986). Initially, a set of normally quiescent genes is activated to high rates of transcription. This activation occurs within minutes of IFN binding to high affinity cell-surface receptors and shows no requirement for ongoing protein synthesis. Subsequently, transcription falls to near pretreatment levels, controlled by an active process dependent upon continued protein synthesis, suggesting the need for renewal of some protein component that is turning over rapidly (Larner et al. 1986). For ISG54, both phases of the IFN-stimulated transcription cycle can be mediated, at least in part, by DNA sequences at the 5' end of the gene (Levy et al. 1986; D.S. Kessler, D.E. Levy, and J.E. Darnell, unpubl.).

The results presented in this paper lend support to the idea that transcriptional regulation of ISG54, in particular, and coordinate expression of ISGs, in general, involve a specific 5'-flanking sequence, the ISRE, to which several protein factors were demonstrated to bind. There is one constitutive factor (B1 complex) and at least two different factors that are activated by IFN treatment (B2 and B3). The latter two factors possess appropriate characteristics to serve as a positive activator (B3 complex) and as a negatively acting transcriptional factor (B2 complex).

The existence of an ISRE in the ISG54 promoter has been established by a number of different experiments. Deletion analysis delineated the minimal 5'-flanking sequence necessary for IFN induction, and IFN-induced factors were found to bind within this region. Plasmids carrying point mutations in the proposed ISRE no longer directed the IFN-stimulated transcriptional response and no longer bound nuclear factors. Although 800 bp of 5'-flanking sequence are sufficient for both positive and negative transcriptional regulation (Levy et al. 1986), we do not yet have direct evidence that the ISRE alone receives the negative signals or directs the subsequent repression of ISG transcription. Permanently transfected cell lines in which these transcriptional measurements can be made reliably are being constructed for this purpose.

Although a portion of the ISRE showed varying degrees of identity with viral and cellular enhancers, the E1A, SV40 early, and HSP70 and other promoter/enhancer segments failed to compete for binding of any factors with the ISG54 promoter fragment. This result distinguishes the factors described in this paper from those reported by Israel et al. (1987) and Baldwin and Sharp (1987), which were based on binding of proteins to mouse *H-2* and  $\beta_2$ -microglobulin promoters. These previously described binding activities were not detectably modulated by IFN; in addition, they were effectively competed by SV40 early enhancer sequences and mapped to regions of the *H-2* promoter outside the ISRE homology.

One heterologous DNA did compete for binding and did so for all three factors. This was a promoter fragment

from ISG15. The only extended sequence identity between these two IFN-stimulated promoters lies within the ISRE (Fig. 1C; Reich et al. 1987), reinforcing the conclusion that the ISRE is the site for all factor binding. In further experiments, ISG15 promoter fragments have been found to form similar complexes to those described here (N. Reich, R. Pine, D.E. Levy, and J.E. Darnell, unpubl.). We have also found that 5'-flanking segments from the *6-16* gene (Porter et al. 1988) and IP-10 gene (Luster and Ravetch 1987), which contain homologous ISRE sequences (see Fig. 1C), effectively competed for the binding of factors to ISG54 (D. Levy, unpubl.).

### *A model for transcriptional control*

The previous studies on the rise and subsequent fall of transcription of ISGs after IFN treatment and the requirement for protein synthesis for repression but not for induction (Larner et al. 1986) guided the experiments designed to test the possible functions of the IFN-induced factors that gave rise to B2 and B3 complexes. The B3 factor was maximally induced within 60 min and then declined and did not require protein synthesis for induction—characteristics that suggest the B3 complex could be related to ISG activation.

The B2 factor, although strongly induced by IFN, did not appear until about 2 hr after treatment and then only in cells that continued to synthesize new proteins during IFN treatment. Moreover, protein synthesis was also required to maintain the B2 factor at high levels. These are attributes characteristic of the negative phase of the ISG transcription cycle when gene expression declines and is maintained at low levels by a process involving newly synthesized proteins (Larner et al. 1986). Therefore, the B2 complex may be involved in this repression.

Presumably, a component turning over rapidly is part of the B2 complex itself or is required for the formation and persistence of B2. Whether the newly synthesized protein required for B2 appearance results from an IFN-stimulated gene is not known but could be the case. We have recently found that active transcription, as well as protein synthesis, is necessary for B2 induction (D.E. Levy and J.E. Darnell, unpubl.). Repression of transcription could be accomplished by direct competition for binding to the ISRE between an activator (B3) and a repressor (B2), as postulated for the CCAAT replacement factor of sea urchin histone transcription (Barberis et al. 1987). Alternatively, repression could involve interaction of the activator with a second protein, as suggested for *GAL80* repression of the yeast galactose regulon (Johnston et al. 1987; Ma and Ptashne 1987). Thus, a single DNA-binding protein that is modified sequentially could account for all the results. It is even possible that the constitutive B1-binding activity might supply the single sequence specificity observed. Further understanding of the mechanisms underlying the different phases of the IFN-induced transcription cycle will require purification of proteins responsible for this regulation.



## Materials and methods

### Cell culture

Cultured cells were maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum and antibiotics and subcultured by trypsinization. HeLa cells (clone S3) and SV40-transformed WI38 human lung fibroblasts (CCL 75.1) were obtained from ATCC, Rockville, Maryland. For IFN treatment, recombinant bacteria-derived  $\alpha$ -IFN, a kind gift of Dr. S. Pestka (Hoffman-La Roche, Nutley, New Jersey), was added to culture media at 500 U/ml. Cycloheximide (Sigma) was used at 35  $\mu$ g/ml.

### Transfections

HeLa cells were transiently transfected by DNA complexed with DEAE-dextran (Sompayrac and Danna 1981; Lopata et al. 1984). Plasmid DNA containing ISG54 sequences (1  $\mu$ g) and a control plasmid pRSVcat (Gorman et al. 1982) (0.8  $\mu$ g) were mixed in 500  $\mu$ l of DME and adjusted to 1 mg/ml DEAE-dextran (Pharmacia). This mixture was overlaid on HeLa cell monolayers in 100-mm culture dishes and incubated at 37°C for 4 hr. Following removal of the DNA mixture, cells were rinsed with 15% glycerol in HEPES-buffered saline and then incubated in medium for 6–12 hr. Subsequently, each transfected culture was trypsinized and divided into two duplicate cultures. After an additional 24-hr incubation period, one dish was treated with IFN, and the second served as control. This procedure minimized fluctuations due to variations in transfection efficiency. Following a 4-hr IFN treatment, cells were harvested and cytoplasmic RNA obtained by NP-40 lysis and phenol extraction (Wilson and Darnell 1981). To assay endogenous and transfected gene expression,  $^{32}$ P-labeled cRNA synthesized *in vitro* (Melton et al. 1984) was hybridized with 10  $\mu$ g of HeLa cell RNA.

### Cell extracts

Nuclear and whole cell extracts were prepared by a modification of the procedure of Dignam et al. (1983). Monolayer cells were harvested by scraping, washed with phosphate-buffered saline (PBS) by centrifugation, and swelled and homogenized in Buffer A [20 mM HEPES (pH 7.0), 10 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), and 0.25 mM phenylmethylsulfonyl fluoride (PMSF)]. For nuclear extracts, nuclei were collected by centrifugation, washed in 0.14 M KCl in Buffer A, and resuspended at  $1 \times 10^8$  nuclei per milliliter in Buffer A. For high salt extracts, nuclei or homogenized cells (for whole cell extracts) were extracted in 1.2 M KCl, 10% glycerol, and 0.1 mM EDTA in Buffer A. Insoluble material was removed by centrifugation at 200,000g, and the clarified supernatants were dialyzed against 100 mM KCl, 20 mM HEPES (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 0.25 mM PMSF, and 10% glycerol. Dialysates were clarified by centrifugation at 10,000g. Low salt extracts were prepared by suspending nuclei directly in 0.21 M KCl, as described by Dignam et al. (1983).

### Protein-DNA binding assays

Gel-shift assays were performed essentially as described by Fried and Crothers (1981). End-labeled DNA fragments (7 fmols) were incubated with 10–15  $\mu$ g of protein in 40 mM KCl, 20 mM HEPES (pH 7.9), 1 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.5 mM DTT, 320  $\mu$ g/ml poly(dIdC) : poly(dIdC), and 4% Ficoll in a final volume of 12.5  $\mu$ l for 20 min. Bound DNA complexes were

separated from free probe on 4% acrylamide gels run in 25 mM Tris-borate and 0.25 mM EDTA.

For DNase I footprinting, DNA-protein binding reactions (as described above) were scaled up to 30  $\mu$ l in the same buffer lacking Ficoll and containing 100  $\mu$ g/ml poly(dIdC) : poly(dIdC). Following a 20-min incubation at room temperature, DNase I was added to 16  $\mu$ g/ml and incubated for 4 min at 30°C. DNase digestion was terminated by the addition of 10 mM EDTA, 0.2% SDS, and 100  $\mu$ g/ml proteinase K. Following 15 min at 60°C, DNA was recovered by phenol-chloroform extraction and ethanol precipitation and was fractionated on denaturing acrylamide-urea gels. Methylation interference experiments were performed as described by Sen and Baltimore (1986).

### *In vitro* mutagenesis

Point mutants were introduced into the ISRE by oligonucleotide-directed DNA repair of gapped heteroduplexes, as described by Morinaga et al. (1984). Heteroduplexes were formed between a plasmid containing an ISG54-E1B fusion gene and a second plasmid containing E1B sequences with no promoter. Two oligonucleotides were designed to produce 2 nucleotide changes (M157) or 3 nucleotide changes (M158) within the ISRE and simultaneously create restriction enzyme recognition sites. Their sequences were 5'-CTTTCTAGAGTCACITTTCCC-3' (M157) and 5'-CTAGTTTCACTAGTCCTTTTGTAACG-3' (M158).

Oligonucleotides were annealed with the gapped heteroduplexes, which were subsequently repaired using DNA polymerase I (Klenow fragment) and nucleotides. Bacterial colonies transformed with the repaired DNA were screened by hybridization (Grunstein and Hogness 1975), using the mutagenic oligonucleotides as labeled probes. Filters were hybridized at 37°C (M157) or 47°C (M158) and washed at 42°C (M157) or 57°C (M158) in 6 $\times$  SSC, and mutated plasmids were isolated and confirmed by restriction analysis and DNA sequencing. Mutants were obtained at a frequency of ~5%.

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