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Differential binding of interferon-induced factors to an oligonucleotide that mediates transcriptional activation

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ABSTRACT

Type I interferons elicit a number of biological responses by rapidly and transiently stimulating the transcriptional expression of a specific set of genes. The promoters of the inducible genes contain an enhancer element which is required for transcriptional activation. A specific oligonucleotide of 18 residues is sufficient for transcriptional induction when positioned in a heterologous promoter. Previous studies have identified three protein factors which can bind to the interferon stimulated enhancer. We show here that the binding of one of the interferon-induced factors requires specific nucleotides flanking the minimum recognition site for the other two factors. The distinct interaction of this factor with the response element is of significant importance since this factor is the sole candidate for a primary transcriptional activator of interferon-induced genes.

INTRODUCTION

Interferons were first identified by their ability to confer cellular resistance to various viral infections (1). Later these polypeptide hormones were found to exhibit additional biological activities including inhibition of cellular proliferation and the activation of a variety of immune cells (2,3). Type I interferons include both alpha (leucocyte) and beta (fibroblast) interferon. They exert their effects by binding to a specific cell surface receptor and, subsequently, transducing a signal to activate the transcription of a set of interferon stimulated genes (ISGs) (4,5). The coordinate transcriptional control of these genes is dependent upon the presence of a consensus enhancer sequence, the interferon stimulated response element (ISRE) (7). Mutational analyses have demonstrated a requirement of the ISRE for the transcriptional response to interferon (6−11). In this paper we demonstrate that the ISRE is sufficient for transcriptional induction by interferon at the proper start site when positioned in a heterologous gene (herpes virus thymidine kinase) and, in addition, more precisely demark the functional boundary of the element.

The molecular mechanism by which interferon transduces a signal to the nucleus to activate a specific set of genes is not defined. Previous studies have demonstrated the presence of three specific ISRE binding factors by a gel retardation assay (7,8,12). These factors are referred to as interferon stimulated gene factors (ISGFs: previously identified as B1, B2 and B3 (7)). One of the factors (ISGF-1) preexists in untreated cells whereas two additional factors (ISGF-2 and ISGF-3) appear only after interferon treatment (7). Transcriptional activation by interferon does not require new protein synthesis (13), nor does the appearance of ISGF-3 (7), suggesting the response results from modification of this preexisting transcription factor. With the use of methylation interference footprint analyses as well as DNA binding experiments with mutant ISRE sequences, we show that

this factor recognizes unique nucleotides which flank the binding site of the other two factors. The recognition site of ISGF-3 and its correlation with interferon stimulated gene expression distinguish it from related binding factors.

MATERIALS AND METHODS

Cell cultures

HeLa cell cultures (CCL2) were obtained from the American Cell Type Culture Collection and were maintained in Dulbecco's modified Eagle's media with 10% fetal bovine serum. Human fibroblast cultures (FS2) were a gift from E. Knight, Jr. (E.I. duPont deNemours & Co.). Recombinant human alpha-A interferon was generously supplied by P. Sorter (Hoffman-LaRoche) and used at a concentration of 1,000 U/ml.

Transfections

HeLa cells were transiently transfected by the addition of calcium phosphate/DNA coprecipiates for 4 hours (14). 40 μg of test plasmid and 10 μg of control plasmid (SVβglobin,15) were added to 150 mm plates. Monolayers were washed with media and incubated for 18−24 hours. Cultures were then trypsinized, divided into duplicate plates and allowed to proliferate for 2 days. One of the duplicate cultures was then treated with interferon for 4 hours while the other was left untreated. The cytoplasmic mRNA was isolated by NP-40 lysis and phenol extraction (16). Polyadenylated RNA was isolated by oligo (dT) chromatography (17).

RNA analysis

The −108 deletion mutation was introduced into a recombinant plasmid ISG15-E1B (the 5′ region of the ISG15 to +44 fused to the 3′ portion of the adenovirus E1B gene) with BAL-31 exonuclease as previously described (6). RNA transcripts initiating at the proper start site were quantitated by hybridization with an antisense radiolabeled RNA probe protecting 272 nucleotides of the 5′ end of the RNA (6,18).

An oligonucleotide corresponding to 18 nucleotides of the ISRE (underlined in fig. 1) was cloned with Bam HI linkers into the −109 position of the herpesvirus thymidine kinase gene (6,19). The oligonucleotide was cloned in one, two, three or seven copies (TK-1 through TK-7). An antisense TK RNA probe was employed to score the proper transcriptional start site and protected a fragment of 204 nucleotides (6).

DNA-protein binding assays

Nuclear cell extracts were prepared from fibroblast cells using a previously described technique (20) with the omission of $MgCl_2$ and the addition of 200 μM spermine and 500 μM spermidine to the extraction buffers A and B. The gel retardation assays were performed essentially as described (7,21). The binding reactions contained 2 ng of end labeled ISG15 promoter fragment (−166/−39 with polylinker) and 1−2 μg protein extract in 20 mM HEPES pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 4 μg poly (dIdC):poly (dIdC), 0.5 μg non-specific plasmid DNA and 4% Ficoll. Complexes were formed at 20°C for 30 minutes and 5μl of a 15 μl reaction volume was resolved on low ionic strength 4.5% acrylamide gels electrophoresed at 4°C.

For the methylation interference studies the DNA probe was partially methylated with dimethylsulfate (DMS) as described (22) before using it in a preparative scale (10-fold) binding reaction. After electrophoresis the wet gel was autoradiographed at 4°C. Complexes corresponding to ISGF-1, ISGF-2, ISGF-3 as well as free DNA probe were eluted out of the gel into 0.3 M NH_4Acetate, 1 mM EDTA and 0.1 % SDS. The eluate was

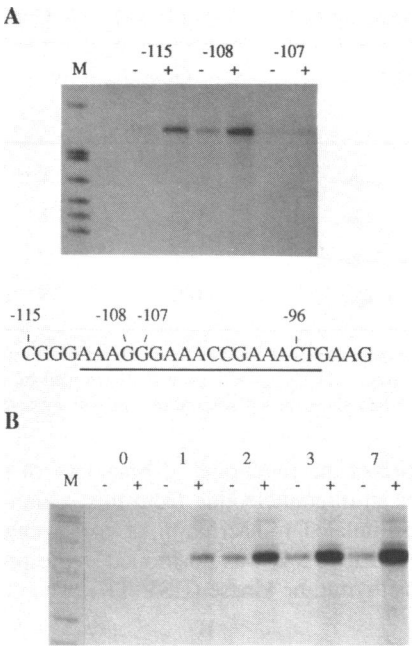

Figure 1: Transient expression analyses. A) HeLa Cl2 cells were transfected with recombinant plasmids containing various upstream regions of the ISG15 (−115/−108/−107) fused to the 3′ region of the adenovirus type 5 E1B gene (6). 72 hours after transfection one set of cultures was treated with interferon for 4 hours (+) while the other set remained untreated (−). The antisense RNA probe used to quantitate the ISG15-E1B RNA protects a fragment of 272 nucleotides (6). B) Effect of the ISG15 oligonucleotide (−111/−94) on the expression of the herpesvirus TK gene. Plasmids containing 0, 1, 2, 3 or 7 copies of the oligonucleotide (sequence underlined) were transfected into HeLa cells and treated with IFN as described above. The antisense RNA probe used to quantitate the TK RNA protects a fragment of 204 nucleotides (6).

extracted with phenol, ethanol precipitated and purified on an ion affinity matrix (elutip; S&S). The isolated DNA was cleaved at methylated residues with 0.1 M NaOH or 1M piperidine and electrophoresed on urea/acrylamide gels (22).

RESULTS

Transient transfection analyses

The promoter region of one of the interferon stimulated genes, ISG15 (encoding a protein product of 15 kD, 6), was employed to determine more precisely the functional 5′ boundary of the ISRE. Deletion mutations were generated to within a few nucleotides of the ISRE and analyzed for transcriptional induction by interferon after transient transfection of HeLa cells. RNA corresponding to the correct transcriptional start site of the transfected gene was measured by hybridization with an antisense RNA probe (6). Figure 1A displays the results of RNA synthesized from transfected plasmids containing 115, 108 or 107 nucleotides upstream of the transcriptional start site. The −108 deletion construct retained the full potential for induction after interferon treatment. However, removal of the guanine residue at position −108 significantly reduced the transcriptional stimulation by interferon (−107).

TABLE 1. Design and Expression of ISG15 (-111/-94) - TK Constructs.

ISG15 - TK Constructs	Gene Activity -IFN	Gene Activity +IFN	Induction Ratio (+/-)
TK - 1 →	0.2	0.8	4.0
TK - 2 ← →	0.7	4.8	6.9
TK - 3 ← ← →	0.7	6.5	9.3
TK - 7 (←)	0.6	7.9	13.2

The ISG15−TK plasmids used in the transfection assay of figure 1B are presented with the number and orientation of oligonucleotide insertions (arrows). The gene activity was determined by a densitometric scanning of the autoradiogram which was preflashed and exposed without an intensifying screen.

This nucleotide thereby marks the functional 5′ boundary of the response element.

In order to test whether an oligonucleotide from this region of the promoter is alone sufficient for interferon stimulated transcription, a corresponding 18 nucleotide DNA sequence from ISG15 (−111 to −94) was positioned in the promoter of a heterologous gene (herpes simplex virus thymidine kinase (HSV TK). The oligonucleotide was cloned

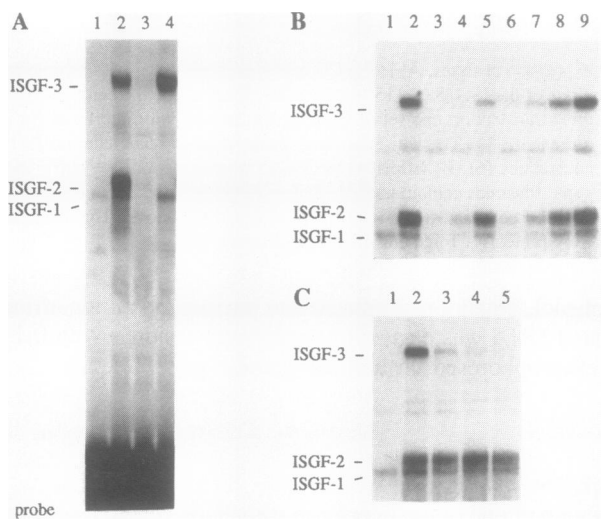

Figure 2: Gel retardation assays of nuclear factors that bind to the ISRE within the ISG15 promoter. A) Nuclear extracts were prepared from cells that were untreated (lanes 1), treated with interferon for 90 minutes (lanes 2,3) or treated with interferon in the continual presence of 20μg/ml anisomycin (lane 4). Complexes in lane 3 were formed in the presence of 100ng competing ISG15 oligonucleotide (−111/−94 with BamHI single stranded ends). B) Nuclear extracts from untreated cells (lane 1) or interferon treated cells (lanes 2−9) were mixed with labeled probe in the absence (lanes 1,2) or presence of various competing plasmids. Lanes 3−5 contained decreasing amounts of ISG15 −108 plasmid DNA as competitor (100-, 50- and 10-fold excess of probe respectively). Lanes 6−8 contained decreasing amount of the −107 plasmid DNA (100-, 50- and 10-fold excess respectively). Lane 9 contained 100-fold excess of −96 plasmid DNA. C) Lane 1 represents complexes formed with untreated cell extracts. Lanes 2−5 represent complexes formed with interferon treated cell extracts in buffers containing increasing salt concentrations (50mM, 100mM, 150mM and 200mM KCl respectively).

upstream of the transcriptional start site of the HSV TK gene at position −109 in one, two, three or seven copies separated by Bam HI linkers. The ability of the recombinant plasmids to respond to interferon was analyzed in a transient transfection assay (figure 1B). In this transfection experiment the proper transcriptional start site of the TK gene was scored by hybridization with an antisense TK RNA probe (6). The TK gene previously has been shown to express poorly in human cell lines in the absense of a transactivating viral protein (23). However, one copy of the ISG15 oligonucleotide was able to confer interferon inducibility to the TK gene. In addition, increasing the number of ISG15 oligonucleotide copies results in a proportionately increased transcriptional activation by interferon (table 1). Presence of the oligonucleotide(s) was also found to slightly increase the basal gene activity. This observation may reflect the transient activation of ISGs following calcium phosphate transfection (24). It appears therefore that the ISG15 18 base pair oligonucleotide containing the ISRE is sufficient to confer interferon inducibility to an otherwise non-stimulated promoter.

Binding of nuclear factors to the ISRE

Three interferon stimulated gene factors (ISGFs) have been detected that can specifically bind to the ISRE (7,8,12). Two of the ISGFs are induced upon interferon treatment. The induced appearance of ISGF-3 is independent of protein synthesis (as is transcriptional stimulation) whereas the induction of ISGF-2 requires ongoing protein synthesis. This observation is intriguing in light of the fact that the transcriptional response to interferon is not just a positive one, but is soon followed by a regulated decrease in transcription (13). The return to basal transcription requires ongoing protein synthesis as does the appearance of ISGF-2.

A typical gel retardation assay that demonstrates protein complexes with the ISG15 promoter is shown in figure 2A. Nuclear extracts prepared from uninduced cells display the shifted complex corresponding to ISGF-1 (lane 1) whereas induced extracts display the additional DNA-protein complexes of ISGF-2 and ISGF-3 (lane 2) (The lower intensity of ISGF-1 in lane 1 is due to slightly less protein in the extract). The DNA binding of all three factors can be specifically competed with an ISG15 oligonucleotide sequence (−111/ −94 lane 3). If protein synthesis is inhibited during induction by interferon, the only induced factor which appears is ISGF-3 (lane 4).

Since there is a functional distinction in the ISG15 promoter containing 107 or 108 nucleotides upstream of the transcription start site (figure 1A), plasmids containing these deletion mutations were tested for their ability to bind the ISGFs. The deletion mutations were used as specific DNA competitors in a gel retardation assay with radiolabeled ISG15 promoter DNA (figure 2B). A range of DNA concentrations was used to best study the effectiveness of competition of the −108 plasmid (lanes 3−5) and the −107 plasmid (lanes 6−8). At a 100-fold molar excess, the −108 plasmid can effectively compete for binding of the ISGF-1, -2 and -3 (lane3). Surprisingly, the same amount of the −107 plasmid can also compete for all three ISGFs (lane 6). However, as the molar ratio of competing plasmid is decreased to 50- or 10-fold, it becomes evident that the −107 plasmid cannot as efficiently compete for binding to the ISGF-3 (lanes 7 and 8). Although the weaker binding of the −107 mutation plasmid to ISGF-3 is very reproducible and correlates with the weak transcriptional induction of this plasmid by interferon in the transient expression assays, it is not as dramatic an effect as the defect in transcriptional activation. A competing plasmid containing 96 nucleotides upstream of the cap site was also included in this study and clearly cannot compete for the ISGF binding even at 100-fold molar excess (lane 9).

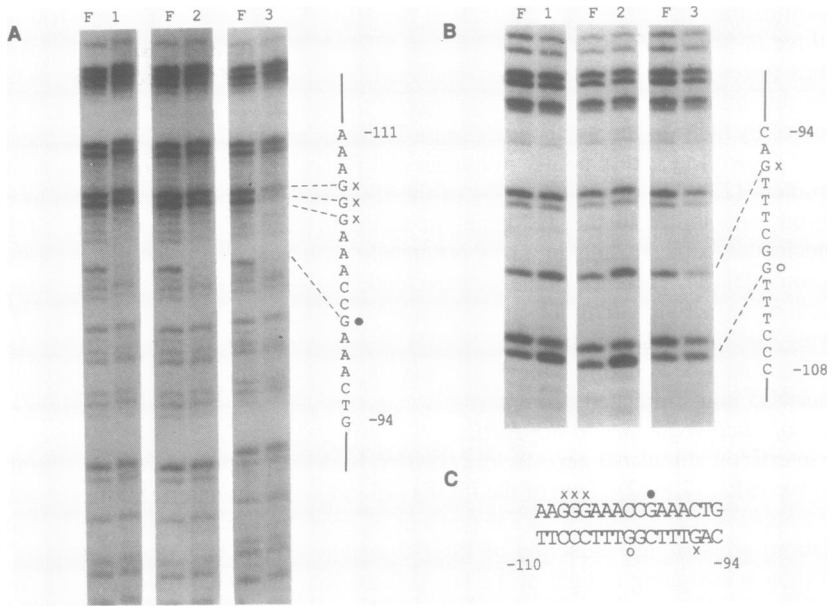

Figure 3: Methylation interference footprint studies of the ISG15 promoter. A) Binding interference with a DNA fragment end-labeled on the noncoding strand: (F) free DNA probe; (1) ISGF-1; (2) ISGF-2; (3) ISGF-3. B) Binding interference with a DNA fragment end-labeled on the coding strand. C) Sites in the ISG15 sequence which, after methylation, interfere with factor binding: (x) specific binding interference of ISGF-3; (●) binding interference of all the ISGFs; (o) enhanced binding of methylated residue by ISGF-1 and −2.

The ISGF-3 is distinct from the ISGF-1 or ISGF-2 in that it is induced in the absence of protein synthesis. In addition to this distinction the ISGF-3 was found to display different binding properties to the ISRE. The ISGF-3/DNA complex appeared unstable in the presence of increasing salt concentrations whereas the binding stabilities of ISGF-1 and -2 were unaffected. The sensitivity of the ISGF-3/DNA complex to higher salt conditions is shown in figure 2C (lanes 2−5). Binding reactions were performed in the presence of increasing concentrations of KCl (50−200mM) and complex formation was analyzed with the mobility shift assay. The specific formation of the ISGF-3 complex was inhibited by increasing KCl concentrations in the binding reaction mixture. Even near isotonic conditions, the binding of the ISGF-3 to the promoter was dramatically more tenuous than ISGF-1 or ISGF-2. This instability was evident with increasing concentrations of a variety of salts ($MgCl_2$, $ZnCl_2$, NaCl of $CaCl_2$) (data not shown). The differential binding of ISGF-3 warranted a more critical analysis of the nucleotide residues required for binding to the ISRE.

In order to determine whether the ISRE-DNA recognition site of ISGF-3 is unique or rather identical to that of ISGF-1 and ISGF-2, DNA footprint analyses were performed employing methylation interference. Earlier studies with another inducible gene, ISG54, showed the recognition binding site of ISGF-1 and ISGF-2 to be similar on one strand of the DNA (7). We show here, however, that ISGF-3 requires specific nucleotides that flank the binding site of the two other factors. Methylation interference assays were performed with a DNA fragment from the ISG15 promoter region containing the ISRE.

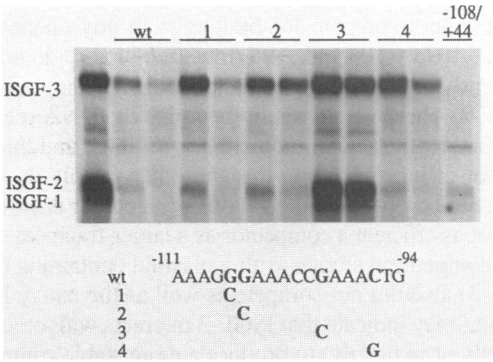

Figure 4: DNA/protein complex formation in the presence of competing oligonucleotides. The left lane displays the shifted complexes formed in the absence of competitor. The following lanes display complexes formed in the presence of 30ng or 150ng of wild type oligonucleotide (wt) or oligonucleotides with substitutions at positions −107 (1), −106 (2), −100 (3) and −96 (4). The double stranded oligonucleotides contained −111/−94 of the ISG15 promoter with single stranded BamH I ends. The far right lane displays complexes formed in the presence of a competing ISG15 fragment of −108/+44 at a molar equivalent of 20ng.

The DNA was uniquely end-labeled on either extremity and partially methylated by dimethylsulfate (DMS) (22). The modified DNA was incubated with nuclear extracts from interferon treated cells and subjected to gel retardation electrophoresis. The labeled DNA fragments contained in the complex with ISGF-1, -2, and -3 as well as the corresponding free fragments were eluted out of the gel. The recovered DNA was cleaved at modified guanine and/or adenine residues with piperidine or alkali and resolved by gel electrophoresis. If methylation of a nucleotide interfers with the binding of the factor, DNA fragments methylated at that site will not be represented in the shifted complex and those nucleotides will not appear as a cleaved product in the sequencing gel.

Figure 3 presents the results of methylation interference experiments with the ISG15 promoter fragment uniquely end-labeled on the coding or the noncoding DNA strand. Binding interference on the noncoding strand (figure 3A) reveals a guanine residue at −100 which is critical for complex formation with all three factors (ISGF-1, -2 and -3). However, ISGF-3 exhibits a unique recognition of guanine residues at positions −106, −107 and −108: this upstream region was shown to be required for transcriptional activation by interferon (figure 1A). A differential binding of ISGF-3 can also be detected on the coding strand (figure 3B). Although this strand displays very little interaction with the ISGFs, the ISGF-3/DNA complex has a reduced amount of methylated guanine at position −96. In contrast, the ISGF-1 and ISGF-2/DNA complexes have an increased amount of methylated guanine at position −99. The overall pattern of ISGF-3 binding to the ISG15 ISRE is thereby distinct from that of ISGF-1 and ISGF-2. The ISGF-3 recognizes flanking nucleotides that are not involved in ISGF-1 or ISGF-2 DNA complex formation.

The difference in ISRE binding of ISGF-3 in comparison to ISGF-1 and ISGF-2 was also demonstrated in a gel retardation assay with mutated oligonucleotides. Oligonucleotides were prepared that substituted cytosine residues for the guanine residues that were required for DNA−protein binding in the footprint studies (positions −107, −106, −100 and −96 in the ISG15 promoter). These oligonucleotides were tested for their ability to compete with the wild type promoter fragment for binding to the ISGFs in a gel shift assay (figure 4). As predicted by the methylation interference footprint analyses, a substitution mutation

at −100 (mutation 3) cannot compete for binding with any of the factors . In addition, mutations at positions −107, −106 and −96 (mutations 1,2,4) do not compete for binding with ISGF-3 as efficiently as the wild type oligonucleotide, but still compete for binding with ISGF-1 and ISGF-2. These results again show that the DNA recognition site for ISGF -1 and -2 is encompassed by the recognition site for ISGF-3, and that the ISGF-3 requires flanking nucleotides for efficient binding that the other factors do not require. It is also important to note that although the wild type oligonucleotide is sufficient to compete for ISGF-3 binding, it is not as efficient a competitor as a larger fragment of the ISG15 promoter (−108/+44). In fact competition studies with a plasmid containing the ISRE inserted into the TK promoter (TK-3) also did not compete as well as the native ISG15 promoter (data not shown). This finding may indicate that ISGF-3 interacts with other transcription factors on the promoter (directly or indirectly) to produce a more stable complex with the promoter DNA.

DISCUSSION

The expression of type I interferon stimulated genes (ISGs) is regulated through a cisacting DNA sequence, the ISRE (interferon stimulated response element) (6−10). The studies presented here show that an oligonucleotide corresponding to 18 residues of the ISG15 ISRE is sufficient to mediate gene activation by interferon. Although one copy of the oligonucleotide is sufficient to confer interferon inducibility to the herpesvirus TK gene, multiple copies generated a proportionally greater response. A caveat in any study using multimers of an oligonucleotide is the creation of distinct recognition sites. To decrease the influence of this possibility we have separated each ISRE unit with a Bam HI linker. The ability of multiple binding sites to confer greater inducibility to the TK gene may reflect a higher probability of interaction with transactivating factors during the transfection.

The importance of the ISRE upstream guanine residues in transcriptional activation was demonstrated with transfections of deletion mutations. Transfections with the ISG15 promoter containing 108 nucleotides upstream of the transcriptional start site resulted in optimal induction. However a deletion of one additional guanine nucleotide resulted in a dramatic reduction of the response (−107 mutation). Although the −107 mutation responded poorly to interferon (albeit not completely unresponsive) it was still capable of interacting with the ISRE binding factors. This observation may indicate that it is the quality of the factor-DNA interaction which is not optimal for stimulation of transcription in this mutation. However, complex formation with the ISGF-3 was reduced slightly with the −107 plasmid in competition studies (figure 2B).

Three DNA binding factors have been shown to recognize the ISRE (7,8,12). One of the factors (ISGF-3) is induced by interferon in the absence of protein synthesis and is thereby a candidate for a transcriptional activator. In order to identify which residues of the DNA target are directly involved in the binding of ISGF-3, and to determine whether this target site is identical to that of ISGF-1 and ISGF-2 (7), we performed methylation interference footprint studies (figure 3). Methylation by DMS occurs at the N^7 position of guanine (within the major groove of B-form DNA) and the N^3 position of adenine (minor groove) (22). Since methylation of guanine residues interfered with factor binding, the ISRE target site for all three factors lies within the major groove of the DNA helix. Binding interference is primarily seen on only one strand of the DNA molecule (noncoding). An exception is the −96 guanine nucleotide which is recognized only by ISGF-3. ISGF-3 is also unique in its recognition of upstream guanine nucleotides of the ISRE at positions

−106, −107 and −108. The factors ISGF-1 and ISGF-2 do not interact with these flanking nucleotides and thereby appear to recognize an inner core sequence of the ISRE. Previous studies targeted ISGF-3 involvement in transcriptional activation by interferon (7). We now show that ISGF-3 possesses a distinct DNA recognition site that encompasses the binding site of the other two ISRE factors.

The requirement for upstream guanine nucleotides in the binding of ISGF-3 correlates with the requirement of these nucleotides for transcriptional activation by interferon (figure 1). Point mutations of the ISRE within a different interferon stimulated gene, ISG54, demonstrated a requirement in transfection experiments of nucleotides recognized only by ISGF-3 (11). A 50% reduction in activation was seen with a mutation corresponding to ISG15 position −96, and a reduction to background levels was found with mutations corresponding to positions −107 or −108 (11).

If ISGF-3 is involved in transcriptional activation, what is the function of ISGF-1 and ISGF-2? The transcriptional response to type I interferon is a rapid activation followed by a regulated silencing (13). We have previously hypothesized a role for ISGF-2 in the suppression of transcription following interferon activation (7). The unstable nature of the ISGF-3 DNA binding with higher salt concentrations may be related to the transient nature of transcriptional activation (figure 1C). ISGF-1 and ISGF-2 bind more tenaciously and may have a competitive advantage for binding to the ISRE and result in displacement of the ISGF-3 from the DNA. There is precedent in other systems for distinct transcription factors which can recognize the same DNA sequence, but result in opposite transcriptional effects (25). Alternatively, ISGF-1 and ISGF-2 may also be involved in transcriptional activation.

The consensus sequence of the ISRE shares homology with the positive regulatory element in the promoters of type I interferon genes (7,26). A gene encoding a specific transcriptional activator for type I interferon genes has recently been cloned (IRF-1, 27,28). The DNA binding site for this transcription factor consists of hexamer repeats related to the sequence $(AAGTGA)_n$ (29). The sequence similarity of this element with the inner core region of the consensus ISRE (7) may implicate the involvement of ISGF-1 and ISGF-2 not only in the regulation of interferon-stimulated genes but also in the regulation of interferon genes. The functional role of the ISGFs in regulation of the interferon-stimulated genes and/or interferon genes can only be determined with the future purification, cloning and direct functional analyses.

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