Overlapping sites for constitutive and induced DNA binding factors involved in interferon-stimulated transcription

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A 14 bp interferon (IFN)-stimulated response element (ISRE) from 6-16, a human gene regulated by α-IFN, confers IFN inducibility on a heterologous thymidine kinase promoter. A 39 bp double-stranded oligonucleotide corresponding to a 5' region of 6-16 which includes the ISRE competes for factors required for gene expression by α-IFN in transfected cells and a single base change (A-11 to C) within the ISRE (GGGAAAATGA-AACT) abolishes this competition. Band-shift assays performed with whole-cell extracts and the 39 bp oligonucleotide reveal specific complexes formed by rapidly induced and constitutive factors, both of which fail to bind to the A-11 to C oligonucleotide. A detailed footprinting analysis reveals that these two types of factors bind to overlapping sites within the ISRE, but in very different ways. These data were used to design oligonucleotides which decreased the formation of the inducible complex without affecting the constitutive one. Changes at the 5' margin of the ISRE and upstream of it markedly decrease formation of the induced but not the constitutive complex and also abolish the ability of the 39 bp sequence to function as an inducible enhancer with the thymidine kinase promoter. Thus, induction of 6-16 transcription in IFN-treated cells is likely to be stimulated by binding of the induced factor to the ISRE and upstream sequences, while the subsequent suppression of transcription may involve competition for the ISRE by the other class of factors.
Key words: band shifts/CAT assays/competitions/footprints/ IFN-stimulated response element

Introduction

Cytokines such as α-IFN, γ-IFN (Luster *et al.*, 1985; Luster and Ravetch, 1987; Fan *et al.*, 1988), the interleukins (Reed *et al.*, 1985) and tumour necrosis factors (Defillipi *et al.*, 1987) may act to increase transcription through similar signal transduction mechanisms, which in turn may be related to the mechanisms that mediate transcriptional induction by mitogenic peptides such as PDGF (Greenberg and Ziff, 1984; Treisman, 1985; Lau and Nathans, 1987) and

bombesin (Rozengurt and Sinnett-Smith, 1987). At present, little is known about any of these pathways. Several DNA binding proteins have been isolated which recognize sequences known to mediate inducible transcriptional responses (for review, see Maniatis *et al.*, 1987) and some of these stimulate transcription in cell free systems (Imagawa *et al.*, 1987; Treisman, 1988). Some sequence-specific binding proteins are present both before and after induction, while others appear in an active form only after induction (Prywes and Roeder, 1986; Sen and Baltimore, 1986).

A number of IFN-responsive genes have been cloned and characterized (for reviews, see Revel and Chebath, 1986; Pestka *et al.*, 1987) and comparative sequence analyses, together with transfection experiments, have defined IFN-stimulated response elements (ISREs) in the promoters of these genes. The 13 or 14 bp ISREs are highly homologous [consensus G/AGGAAAN(N)GAAACT] and promoter fragments containing them bind to IFN-induced and constitutive factors *in vitro* (Cohen *et al.*, 1988; Levy *et al.*, 1988; Porter *et al.*, 1988; Rutherford *et al.*, 1988; Shirayoshi *et al.*, 1988). In many IFN-responsive cells, a DNA binding protein appears early (Levy *et al.*, 1988), as soon as 30 s after treatment with α-IFN (Dale *et al.*, 1989), without the need for new protein synthesis (Levy *et al.*, 1988). Additional IFN-induced factors appear much more slowly and induction of these late factors does require protein synthesis (Levy *et al.*, 1988; our unpublished observations). A detailed understanding of how ISREs bind to these various factors should help to reveal how stimulation and suppression of transcription is achieved (Friedman *et al.*, 1984; Larner *et al.*, 1986).

The 6-16 gene (Porter *et al.*, 1988) can encode a 12 kd hydrophobic protein of unknown function. It is induced well from a very low basal level by α,β-IFNs in a process which does not usually require new protein synthesis (for an exception, see Kusari and Sen, 1987). We now report the results of experiments designed to analyse in detail the complexes formed by the binding of proteins present in extracts of IFN-treated and untreated cells to the ISRE of 6-16. Data from these footprinting experiments was then used to design a mutant ISRE which distinguishes between the two types of factors *in vitro*. We then relate this differential binding to IFN-induced transcription in cells transfected with plasmids having the ISREs upstream of a reporter gene.

Results

Effect of the 6-16 ISRE on induction of a reporter gene by α-IFN

The region -89 to -168 upstream of the 6-16 gene contains a tandem duplication in which the 39 and 41 bp repeated sequences differ only by a CT dinucleotide at -138, -139 (Figure 1A and Porter *et al.*, 1988). Each repeat unit includes an ISRE and the 5' limit of a subregion, one copy of which is absolutely required for induction, was localized within the
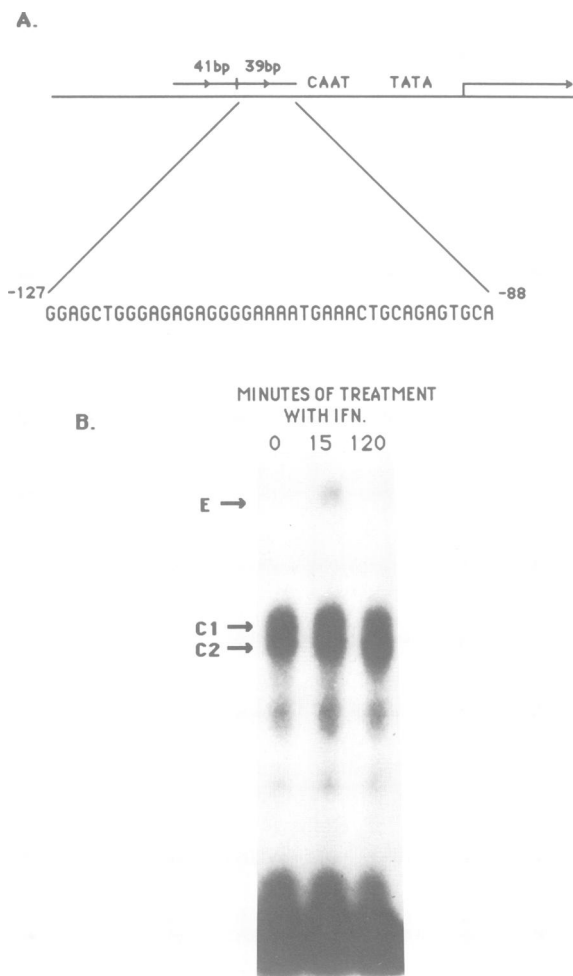
A.

41bp 39bp
CAAT TATA

-127 -88

GGAGCTGGGAGAGAGGGGAAAATGAAACTGCAGAGTGCA

B.

MINUTES OF TREATMENT
WITH IFN.

0 15 120

E →

C1 →
C2 →

Fig. 1. Induction of the E factor in Bristol 8 cells. (**A**) Schematic representation of the regulatory region of 6-16 (Porter *et al.*, 1988). Shown (left to right) are the tandemly duplicated regulatory sequences, each of which includes a copy of the 14 bp ISRE, the inverted CCAAT box, the TATA box, and the transcription start site. The sequence of the 39 bp repeat in the coding (+) strand is shown. The sequence differs from the 41 bp repeat only by the absence of a CT dinucleotide immediately 3' of the ISRE. (**B**) Whole cell extracts from Bristol 8 cells treated with α-IFN for the times shown were incubated with an end-labelled double-stranded oligonucleotide corresponding to the sequence shown and the products were separated by electrophoresis in an agarose gel. The complexes indicated by arrows were competed specifically by unlabelled probe (Figure 2).

ISRE of the downstream (39 bp) copy. To determine whether the 6-16 ISRE was sufficient to confer induction by α-IFN, it was placed in front of a tkCAT reporter gene in the pBLCAT2 construct described by Klock *et al.* (1987). After transient transfection in HeLa cells and treatment with α-IFN for 24 h, an 11.5-fold induction of chloramphenicol acetyl transferase (CAT) activity was observed (Table I, experiment 1), showing that the 6-16 ISRE can mediate a response to α-IFN.

Competition of the IFN response by oligonucleotides

In order to evaluate the function of the 6-16 ISRE more fully, we have employed a transient transfection assay in which a pSV0CAT construct, containing a 1046 bp *Bgl*II−*Bam*HI fragment derived from the 5' end of 6-16 (Table IIA), is used as a reporter (Porter *et al.*, 1988). As shown in Table IIB, mixtures of the reporter plasmid with various amounts

Table I. Transfection experiments with the 14 bp ISRE of 6-16 in a tkCAT construct

A

14 or 39bp elements

CAT

-105 TK

B Construct	Relative CAT activity		Ratio
	−IFN	+IFN	
Experiment 1			
pBLCAT2 control	1	2.3	2.3
14mer in pBLCAT2	7.2	83.4	11.5
Experiment 2			
pBLCAT2 control	1	0.9	0.9
39mer in pBLCAT2	1.8	10	5.5
double early mutant 39mer in pBLCAT2	1.6	1.7	1.1

(A) Map of the ISRE of 6-16, cloned into the *Bam*HI site of pBLCAT2 (Klock *et al.*, 1987).
(B) The constructs or parental vector were transiently transfected into HeLa S3 cells which were then treated for 24 h with α-IFN. Duplicate plates were assayed for CAT activity.

Table II. Competition-transfection experiments

A

6-16

-603 +437 CAT

BglII BamH1 BamH1

B Molar excess of oligonucleotide		CAT activity		Percent of maximum induction
wild-type	Mutant A-11 to C	−IFN	+IFN	
0	30	0.014	0.930	100
5	25	0.012	0.370	46
10	20	0.013	0.070	7.5
30	0	0.013	0.070	7.5

(A) Map of the 1046 bp *Bgl*II−*Bam*HI fragment from 6-16 in pSVOCAT (Porter *et al.*, 1988). The positions of the direct repeat and the transcription start site are indicated schematically.
(B) The construct shown was used as a reporter in co-transfection experiments with wild-type and A-11 to C mutant 39 bp oligonucleotides (Table III). The total amount of oligonucleotide remained constant. After treatment with α-IFN for 24 h, CAT activity was measured as in Table I. The activity in the absence of any oligonucleotide was the same as that measured with a 30-fold excess of the A-11 to C mutant oligonucleotide.

of competitor oligonucleotide [wild-type (wt) 39 bp, Table III] were co-transfected into HeLa cells. The total amount of oligonucleotide was kept constant by including varying amounts of a mutant oligonucleotide (A-11 to C, Table III) which fails to compete in either the co-transfection assay (Table II) or band-shift assay (Table III). A 30-fold excess of the wt 39 bp oligonucleotide reduces induced transcription from the reporter plasmid by 85% after 24 h of treatment with α-IFN. Thus, the 39 bp sequence can bind *trans*-acting factors necessary for induction by α-IFN.

Table III. A summary of competition band-shift experiments

Competitor		M/2[a]	
		Complex E	Complex C1/C2
	1 3 5 7 9 11	5	5
wt 39 bp	G C T G G G A G A G A G G G G A A A A T G A A A C T G C A G A G T G C A C	5	5
wt ISRE in random	G A A T T C G A G C T C G G G A A A A T G A A A C T A A G C T T G C A T G C G	25	5
G-2 to C	G C T G G G A G A G A G G C G A A A A T G A A A C T G C A G A G T G C A C	25	5
G-3 to A[b]	G C T G G G A G A G A G G G A A A A A T G A A A C T G C A G A G T G C A C	25	5
A-5 to C	G C T G G G A G A G A G G G G A C A A T G A A A C T G C A G A G T G C A C	250	250
A-11 to C	G C T G G G A G A G A G G G G A A A A T G A C A C T G C A G A G T G C A C	250	250
GG to CA	G C T G G G A G A G A G G C A A A A A T G A A A C T G C A G A G T G C A C	25	5
4G to CCCA	G C T G G G A G A G A C C C A A A A A T G A A A C T G C A G A G T G C A C	25	5
CT to GC	G C T G G G A G A G A G G G G A A A A T G A A A G C G C A G A G T G C A C	25	5
Double early	G C T G G C A G A G A G G C G A A A A T G A A A C T G C A G A G T G C A C	75	5
wt ISRE	G G G A A A A T G A A A C T	>500	50

Nuclear extracts from Bristol 8 cells were used in competition titrations. To obtain a relative value for the ability of each oligonucleotide to compete, the molar ratios of competitor to reduce the signals by one-half was estimated in several independent experiments in which the mutant oligonucleotides were compared to wt 39 bp directly. Estimates of intensity were made by comparing different exposures of the autoradiograms. Differences in sequence from the wt 39 bp probe are underlined.
[a]Approximate molar excess of competitor required for half competition.
[b]Data shown in Figure 2.

Competition band-shift assays

Previously, using the band-shift assay, we demonstrated that the downstream (39 bp) unit of the 6-16 tandem repeat binds proteins induced in HeLa cells 2 h after treatment with α-IFN (Porter et al., 1988). More extensive analysis of several different human cell lines has revealed both constitutive and IFN-inducible factors which bind specifically to the wt 39 bp element and not to non-specific oligo-nucleotides of similar length such as TGTCTCACTTTT-GGAAGAGAAACAGTTATAGAGTT (data not shown). Three distinct factors found at different times after treatment with IFN form complexes which migrate at different positions in the band-shift assay. In most cells, an IFN-induced complex (E) appears a few minutes after treatment with α-IFN and migrates relatively slowly. Its amount is reduced after longer times of treatment with IFN (Figure 1). In HeLa cells, additional complexes (M and L) appear after ~2 and 8 h and similar induced complexes are also seen with extracts of WI-38 cells and human foreskin fibro-blasts. The appearance of E does not require new protein synthesis, whereas M and L do not appear when extracts of cells pre-treated with cycloheximide are used (our unpublished data). Since no single cell line studied so far yields extracts which have all three of the inducible activities responsible for forming E, M and L, we decided to charac-terize thoroughly the complexes formed with extracts from a single cell line. We chose Bristol 8 B cells, which contain easily observed levels of E (Figures 1 and 2), together with constitutive complexes of lower mobility that appear either as a broad smear or as two separate bands (C1/C2). In some experiments C2 appears to be induced slightly by α-IFN after 2 h (Figure 1, 120 min). The constitutive C1/C2 complexes in Bristol 8 cells have mobilities similar to those of the induced M and L complexes in HeLa cells and also have similar footprints (data not shown).

To establish which nucleotide residues were important for forming the E and C1/C2 complexes in Bristol 8 cells, labelled wt 39 bp was competed with several different unlabelled mutant oligonucleotides in band-shift assays (Table III). An example of the data is shown in Figure 2.

The molar excess of competitor required to reduce the signal by one-half (defined as M/2) measures the efficiency of competition. Unlabelled wt 39 bp oligonucleotide competed both the E and C1/C2 complexes well (M/2 = 5). The 14 bp ISRE of 6-16 did not compete for forming E, although it did compete for forming C1/C2, indicating that the binding sites in the E and C1/C2 complexes are different. To determine why the 14 bp ISRE oligonucleotide did not compete for forming E, we tested a 39 bp oligonucleotide containing the 14 bp ISRE flanked by random sequences ('wt ISRE in random', Table III). This oligonucleotide was able to compete as effectively as wt 39 bp for C1/C2 but was ~5 times less efficient in competing for E. These results indicate that in part the 14 bp 6-16 ISRE is a poor competitor for E because it is too short, but also that sequences flanking the 6-16 ISRE within the wt 39 bp oligonucleotide probably interact with the E factor(s). This conclusion is also supported by footprinting data shown below. The 14 bp ISRE of 6-16 alone was able to compete for forming C1/C2 but was 10 times less effective than wt 39 bp. However, since 'wt ISRE in random' competed as well as wt 39 bp, we conclude that no specific sequences important for forming C1/C2 can lie outside the 14 bp 6-16 ISRE.

Several single bases or small regions within or near the ISRE of the wt 39 bp oligonucleotide were changed to test the effects on binding. The great sequence specificity for forming both complexes is illustrated by mutant oligonucleo-tide A-11 to C, which did not compete in the transfection experiments (Table II) and competed poorly in band-shift assays (50 times worse than wt 39 bp). Both the E and C1/C2 complexes were also poorly competed by mutant oligo-nucleotide A-5 to C. However, the oligonucleotides G-2 to C and G-3 to A competed almost as well as wt 39 bp for forming C1/C2 but competed ~5 times less well for forming E. To investigate further the basis of the differential effects seen with G-2 to C and G-3 to A, the double mutant GG to CA was tested. This mutant oligonucleotide was still only ~5 times worse than wt 39 bp in its ability to compete for E, suggesting that either G-2 to C or G-3 to A alone can eliminate interaction of a single binding element within the
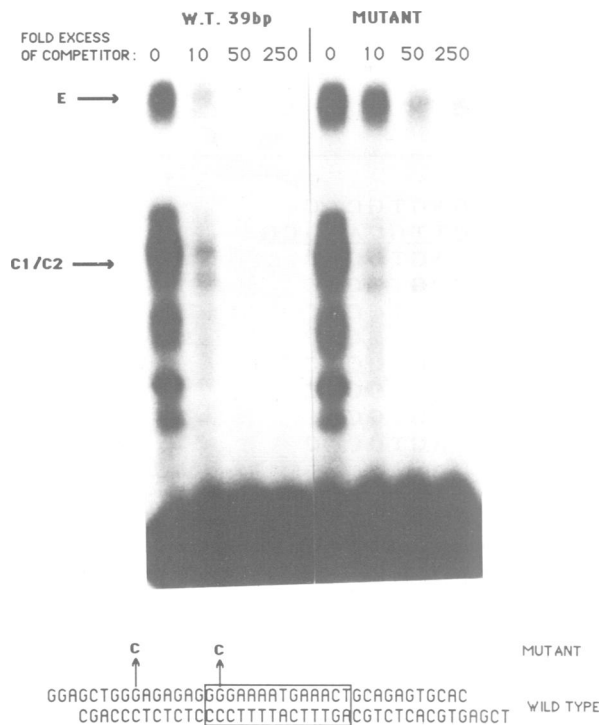
Fig. 2. Competition of the E complex. A dialysed nuclear extract from Bristol 8 cells, made 15 min after treatment with α-IFN, was used in a gel retardation assay with the end-labelled wt 39 bp probe (Table III). The unlabelled competitors double early and wt 39 bp were used. The molar excess of competitor is shown above each track. Specific complexes are indicated with arrows. E, IFN-induced early complex; C1/C2, constitutive complexes; other complexes of lower mobility, observed inconsistently, are probably products of proteolysis.
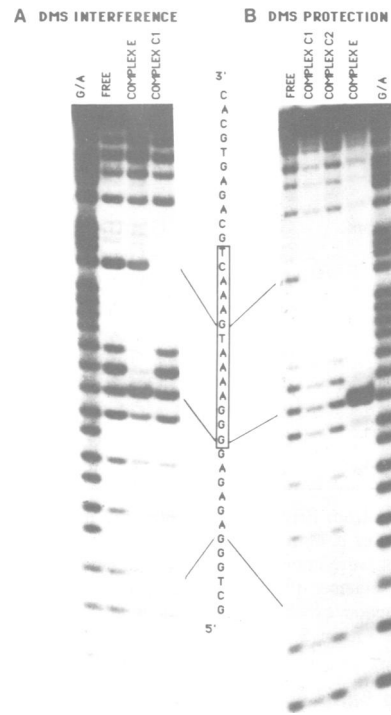
Fig. 3. Methylation interference and DMS protection experiments. End-labelled wt 39 bp oligonucleotide was treated with DMS either before (interference) or after (protection) adding nuclear extracts. After retardation in agarose gels, DNA isolated from each of the separated complexes was cleaved at the modified bases with piperidine. The cleavage products were fractionated in a 15% polyacrylamide gel in 7.5 M urea. The sequence of the 39 bp direct repeat is shown, with the ISRE boxed. (A) A methylation interference experiment and (B) a DMS protection experiment, both with dialysed nuclear extracts made from Bristol 8 cells treated with α-IFN for 15 min. G/A, marker track showing cleavage at G and A residues; free, uncomplexed DNA isolated from the retardation gel. Complexes E, C-1 and C-2 were as in Figure 2.

6-16 ISRE and also that other elements contribute to binding, since some ability to compete remains. A more extensive 4 bp change in this region (4G to CCCA) also reduced the ability to compete for forming E by only ~5-fold, suggesting that other elements within the 39 bp unit contribute separately to forming the E complex. Interestingly, a double mutation at the 3' end of the ISRE region of the wt 39 bp oligonucleotide (CT to GC) also competes poorly for E but not for C1/C2, showing that interactions specific for E occur across the whole ISRE region as well as outside it, as shown by the result with 'wt ISRE in random'.

Data from footprinting experiments (see below) suggested that E factor makes specific contacts with a GGGAGA motif which lies upstream of the ISRE. When a single G of this motif was changed to C, in addition to the G-2 to C change within the ISRE ('double early', Table III and Figure 2), the ability of the oligonucleotide to compete for forming the E complex was reduced by 15-fold, but it was still fully able to compete for C1/C2. This result suggested that part of a separate binding element, distinct from G-1−G-3 of the core, had been identified. When tested in a transient transfection assay, the double early oligonucleotide was inactive compared to wt 39 bp, which mediated a 5.2-fold induction in the same experiment (Table I, experiment 2). Thus, base changes which specifically reduce the stability of the E complex *in vitro* also lead to loss of inducible enhancer function.

Methylation interference and protection assays

Four different footprinting techniques were used to examine the protein−DNA contacts of the different complexes in

detail. Methylation interference and dimethylsulphate (DMS) protection involve reaction of a double-stranded oligomer with DMS. The methylation reactions occur primarily on G residues at position N-7, in the major groove and to a lesser extent on A residues at position N-3, in the minor groove. Both techniques can also give rise to enhancement rather than interference or protection, probably because the bound proteins are stabilized by the alterations in DNA structure following methylation of a particular residue or cause an alteration in structure that favours methylation.

There are clear differences in the patterns of protection and interference for the E complex when compared to the C1/C2 complex (Figure 3A and B). For E, there is a strikingly strong enhancement at residue G-1 with the DMS protection method (Figure 3B), together with protections at G-2 and G-3 and partial protection at G-9. Methylation interference gave qualitatively similar results, although the enhancement at G-1 was not nearly as marked. Both techniques gave only partial interference at G-9. The methylation interference method additionally revealed an effect involving A residues in the minor groove. Methylation of bases A-4−7 on the coding strand interferes with formation of complex E. Interestingly, A-7 is the first one or two variable 'N' residues present in different ISREs, where the consensus sequence is G/AGGAAA-N(N)GAAACT, and base specific contacts might not have been expected. For both E and C1/C2 there is a suggestion
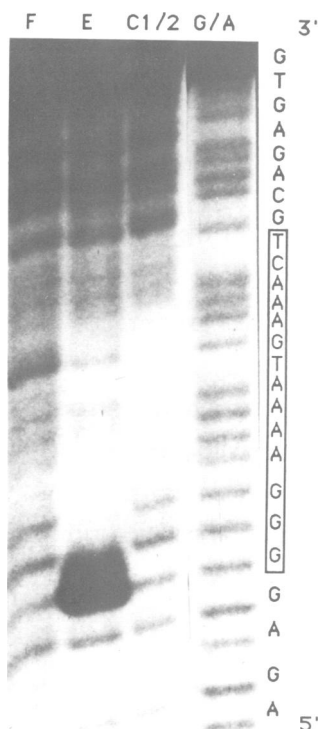
Fig. 4. Ethylation interference. End-labelled wt 39 bp oligonucleotide was treated with ethylnitrosourea before adding a dialysed nuclear extract from Bristol 8 cells treated with α-IFN for 15 min. After retardation in an agarose gel, DNA was isolated from each of the separated complexes and was cleaved at the ethylated phosphates with NaOH. The cleavage products were fractionated in a 15% polyacrylamide gel in 7.5 M urea. The sequence of the 39 bp direct repeat is shown, with the ISRE boxed. F, uncomplexed DNA isolated from the retardation gel; E and C1/C2, complexes as shown in Figure 2; G/A, marker track showing cleavage at G and A residues.

of a footprint on the coding strand upstream of the ISRE region, over the GGGAGA motif.

C1/C2 and E have very different interference and protection patterns and the strong enhancement at G-1 in E but not in C1/C2 is especially striking. In contrast, C1/C2 and the inducible M and L complexes of HeLa cells, which migrate very similarly in gels, give essentially identical methylation interference footprints (data not shown). There is strong DMS protection over G-9 in C1/C2 and the interference experiment shows that methylation within each stretch of A residues (A-4−7 and A-10−12) blocks binding. On the pyrimidine-rich non-coding strand, no residue is protected, either within or outside the ISRE sequence (data not shown). The two classes of binding to C1/C2 and E, distinguished by the DMS techniques, are summarized in Figure 6.

Ethylation interference

To determine whether the proteins involved in forming the E and C1/C2 complexes also interact with phosphate residues and to see whether any differences might be observed, we used the ethylation interference technique of Siebenlist and Gilbert (1980) as modified by Clark *et al.* (1989). The principle of this method is similar to that of methylation interference. A population of ethylated oligonucleotides with approximately one ethyl group per strand, generated by reaction with ethylnitrosourea, was incubated with nuclear extract from Bristol 8 cells. Any phosphate modification that

interferes with binding will be absent from DNA isolated from the retarded complex (Siebenlist and Gilbert, 1980). Ethylated DNA from the complex is then cleaved with NaOH at the position of modification, giving rise to both 5′ and 3′ phosphates. Because each band in the denaturing polyacrylamide gel represents a binary mixture, the bands are broader than with the DMS methods. The ethylation interference footprints for E and C1/C2 on the coding strands are shown in Figure 4 and the results are summarized in Figure 6. There is interference over sequences within the ISRE region in both E and C1/C2. However, the E footprint is displaced towards the 5′ end and also shows a very large enhancement at residue G-1, the same residue that was enhanced in the DMS protection and methylation interference experiments. Methylation at N-7 of G-1 and ethylation at the phosphate each change the charge by +1, which may be important in stabilizing protein binding. No enhancements at G-1 were seen with the C1/C2 complex. We have not yet been able to obtain reproducible ethylation interference footprints for the non-coding strands of either complex.

Copper orthophenanthroline footprinting

The methylation and ethylation interference/protection techniques can give information about some contacts with the DNA, but they do not give an overall picture of the region covered by the proteins, or any information concerning the pyrimidine bases. To obtain a more complete footprint and to investigate the pyrimidine-rich non-coding strand, we used a method in which cleavage is catalysed by copper orthophenanthroline (CuOP). This method gives a smaller footprint than DNAse, since the reagent is not a large protein molecule which may be prevented sterically from cleaving at residues not in contact with a DNA-binding protein. The small CuOP molecule binds to DNA in the minor groove and, once bound, catalyses the formation of OH radicals which cleave the DNA backbone at or near the site of binding, predominantly at C-1 and C-4 of deoxyribose (Marshall *et al.*, 1981). We have adapted the CuOP technique so that it can be used to treat complexes directly in agarose gels. A partially purified DEAE chromatographic fraction which contained the factors required to form both E and C1/C2 (prepared by John Parrington) was used, since it gave better footprints than crude extracts. The cleavage pattern of naked DNA is not uniform with CuOP, but protection and enhancement of the cleavages can be observed readily (Figure 5). E and C1/C2 give clear but different footprints over the ISRE region on both strands. The most notable feature of either footprint is a strong enhancement of cleavage on the coding strand of C1/C2, just beyond the 3′ boundary of the ISRE region. The enhancement depends on the nature of the flanking DNA sequences, since it is not observed with the oligonucleotide wt ISRE in random (data not shown). This enhancement with CuOP contrasts with the enhancements seen with methylation and ethylation, which occur only in complex E, at residue G-1. The protected regions of both E and C1/C2 are ~10 bp long on each strand, with a displacement of about 2 bp. E and C1/C2 differ in that the footprint on E is displaced towards the 5′ end of the coding strand, whereas the footprint on C1/C2 is displaced towards the 5′ end of the non-coding strand. The two footprints coincide over most of their lengths. Furthermore, there is a weaker but reproducible footprint over the upstream GGGAGA region on the coding strand of E, supporting the suggestion from methylation
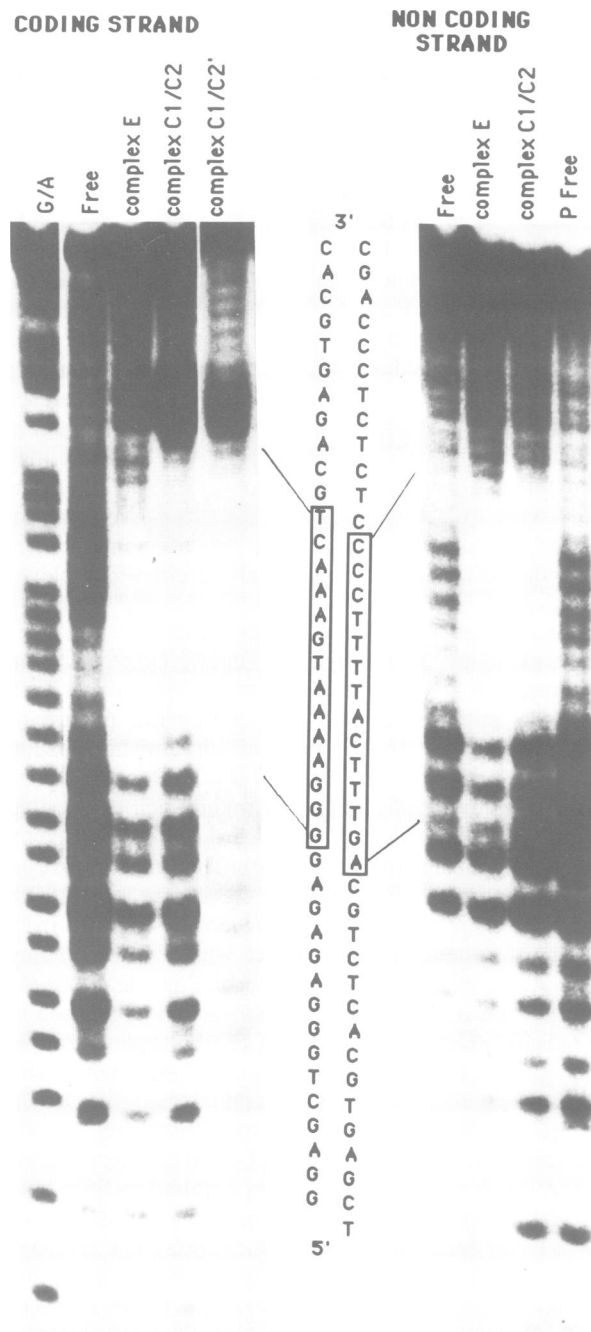
CODING STRAND

NON CODING STRAND

Fig. 5. Footprints after cleavage with CuOP. A nuclear extract from Bristol 8 cells treated with α-IFN for 15 min was purified partially on a column of DEAE—Sepharose and a fraction containing all the factors required for the specific band-shifts shown in Figure 2 was used. End-labelled wt 39 bp oligonucleotide was mixed with this fraction and the complexes, separated in an agarose gel, were treated with CuOP *in situ* (see Materials and methods). The DNAs were isolated and separated in a 15% polyacrylamide gel in 7.5 M urea. Free, uncomplexed DNA isolated from a separate sample run without protein; free E and C1/C2, as in Figure 2; C1/C2', shorter exposure of the C1/C2 track. We have not discussed the weak apparent protections seen with the CuOP technique because of uncertainty arising from the DNA controls. 'Free' DNA isolated from a track containing protein tends to be cleaved less than free DNA isolated from a track without protein (P free), perhaps because non-specific binding of proteins inibits the cleavage reaction.
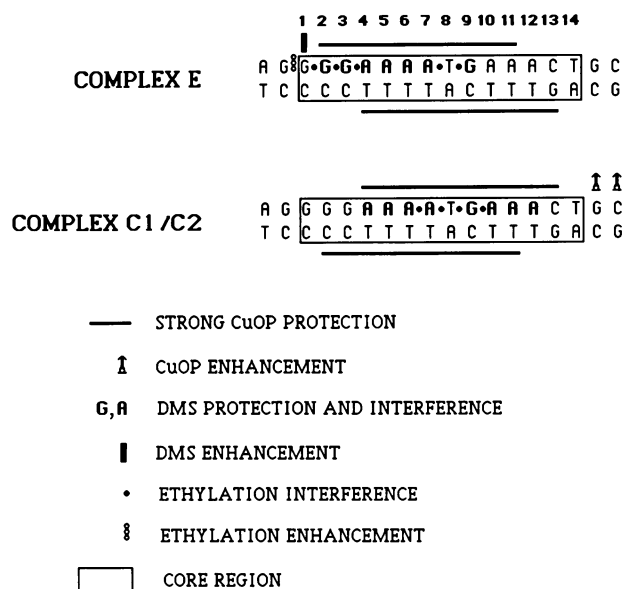
COMPLEX E

COMPLEX C1 /C2

—— STRONG CuOP PROTECTION

↟ CuOP ENHANCEMENT

G,A DMS PROTECTION AND INTERFERENCE

▌ DMS ENHANCEMENT

• ETHYLATION INTERFERENCE

§ ETHYLATION ENHANCEMENT

☐ CORE REGION

Fig. 6. Footprints on the ISRE regions of the E and C1/C2 complexes. The data of Figures 3—5 are summarized. The DMS protection and methylation interference data have been combined, even though data for the A residues was obtained only with the interference method.

interference experiments that these bases may be involved in binding. The CuOP footprint of E extends 2 bp further 3' of the region identified by methylation interference (Figure 6), supporting the competition data (Table III) which indicate that nucleotides in the 3' region of the ISRE are involved in an E-specific contact.

Discussion

The 14 bp ISRE of 6-16, GGGAAAATGAAACT, spans the 5' boundary of sequences required for induction of transcription by α-IFN (Porter *et al.*, 1988) and has extensive homology with the ISREs of several other IFN-inducible genes (Cohen *et al.*, 1988; Levy *et al.*, 1988; Porter *et al.*, 1988; Rutherford *et al.*, 1988). We show here that a single copy of this ISRE can function as an IFN-inducible element in a tkCAT reporter vector (Table I). Cohen *et al.* (1988) have made similar observations with the ISRE of the human 2',5'-oligo A synthetase gene. These results show that the ISREs contain enough functional information to mediate induction of gene expression by α-IFN and raise the possibility that additional sequence elements may contribute as well. Several experiments presented here show that different IFN-induced and constitutive factors bind to overlapping regions of the functionally defined 6-16 ISRE. The factor(s) responsible for forming the E complex *in vitro* are induced by α-IFN in cells rapidly and independently of new protein synthesis, as is 6-16 mRNA (Figures 1 and 2). Furthermore, alterations which dramatically reduce *in vitro* binding of E factor to an oligonucleotide which includes the 6-16 ISRE severely reduce the ability of this sequence to function as an IFN-stimulated element in cells (Table II). These results indicate that the factor(s) involved in forming the E complex *in vitro* are also likely to be involved in inducing transcription in IFN-treated cells.

The factor(s) involved in induction must act positively, since increasing levels of a competitor oligonucleotide cause transcription to be reduced in transfected cells. Similar competition results have been obtained with the human 2',5'-oligo A synthetase gene, using a 40 nucleotide fragment of the promoter region (Rutherford et al., 1988). It is surprising that the E factors do not bind well to the 14 bp ISRE element in competition experiments, even though this element is sufficient to confer inducibility by IFN on the thymidine kinase promoter in a CAT construct. This apparent paradox is explained in two ways. The 14 bp ISRE oligonucleotide is too short to bind well to the E factor, since adding random sequences on both sides improves binding and the double early mutation shows that specific sequences outside the ISRE also contribute to binding. In this regard, it is interesting to note that multiple copies of the 2',5'-oligo A synthetase ISRE function better than a single copy (Cohen et al., 1988) and similar observations have been made for other inducible promoter elements (Bienz and Pelham, 1987; Pierce et al., 1988).

The results from in vitro competition experiments and four footprinting techniques show that the inducible E and constitutive C1/C2 complexes formed in extracts of B cells involve very different but extensively overlapping contacts with the DNA. The C1 and C2 complexes are very similar to one another by the criteria of DMS footprinting, CuOP footprinting, ethylation interference and in vitro competition and they are often not well resolved in band-shift experiments. The proteins which form C1/C2 do not need specific sequences outside the 14 bp core ISRE for optimal binding. Interestingly, the HeLa M and L complexes, induced by α-IFN after ~2 and 8 h, respectively, give essentially the same methylation interference footprints as does the C1/C2 complex. Competition and protein−DNA cross-linking experiments also indicate that the constitutive C1/C2 complexes may be closely related to the inducible HeLa M and L complexes (data not shown). A similar DMS interference footprint has been found for the complex formed between the ISRE of the IFN-induced gene ISG54 and factors present in HeLa cells 2 h after treatment with α-IFN (Levy et al., 1988).

Both runs of A residues within the ISRE are protected from cleavage with CuOP in both the E and C1/C2 complexes. The CuOP protection data are supported by the observation that mutations either at position 5 or at position 11 greatly decrease the ability to form either complex. However, methylation of the downstream stretch of A residues (GGGAAAATGAAACT) interferes with formation of the C1/C2 complexes but not the E complex. Methylation of G-2 or G-3 (GGGAAAATGAAACT) interferes with formation of the E complex but not of C1/C2. This result led us to ask whether mutations at these positions would affect formation of E but not C1/C2. The competition results with the G-2 to C and G-3 to A oligonucleotides (Table III) show that residues G-2 and G-3 are important in forming E but not C1/C2, although these two mutations had only a partial effect. (So far we have not found a mutation that affects C1/C2 but not E.) In an attempt to find a stronger differential effect, the double mutation GG to CA was tested. Surprisingly, GG to CA was no worse in competing for forming the E complex than G-2 to C or G-3 to A (Table III). Furthermore, the more extensive change present in 4G

to CCCA was also no more effective than the single base changes. These results indicate that G-2 and G-3 lie within a single binding domain of the E complex. When this domain is disrupted by either single or multiple changes, its contribution to E factor binding is totally lost. Other independent domains within the 39 bp repeat element must contribute to forming the E complex since specific competition is still seen with high ratios of oligomers which have mutations at the 5' end of the ISRE. One candidate is the upstream motif GGGAGA, which is partially protected in the DMS interference experiment. This sequence differs from the similar 6 bp motif within the ISRE by a single purine-to-purine A-to-G change and is 9 bp upstream of GGGAAAATGAAACT. Results with the double early oligonucleotide, in which this upstream domain is altered in addition to changing G-2, suggest that the domain does contribute to forming the E complex. Repetition of small binding motifs and co-operative binding over these motifs has been observed previously when more than one molecule of protein binds to a region of DNA (SV40 T antigen: McKnight and Tjian, 1986; lambda repressor: Ptashne, 1986) and even when a single protein binds if there is co-operativity between multiple binding units within a single polypeptide (TFIIIA: Fairall et al., 1986). Although the 14 bp ISRE is sufficient to confer IFN inducibility on a heterologous promoter, the efficiency is markedly affected by its context. Reiterated GAAA and GGGAG/AA motifs are found in a number of IFN-inducible genes near the functionally defined ISREs (Reich et al., 1987; Levy et al., 1988; Porter et al., 1988; Rutherford et al., 1988). Further analysis in vivo will be needed to establish whether they play significant roles.

Three-dimensional models (not shown) of the data summarized in Figure 6 confirms that the DNA is contacted in very different ways in the E and C1/C2 complexes. In the C1/C2 complex, the protein probably wraps around the DNA, as shown by protection against methylation of A residues from the direction of the minor groove and by protection against cleavage by CuOP, which requires the reagent to bind in the minor groove. In the E complex, the protein may interact closely with the DNA on only one face of the helix, over the 5' part of the ISRE, making contacts in both the major and minor grooves. The CuOP footprint confirms the contacts in the minor groove over bases 4−7. From the extensive overlap of their binding sites, the E and C1/C2 factors should not be able to bind to the same DNA molecule simultaneously. The E-specific mutations that result in loss of induced transcription in transfected cells strongly suggest that E factor is involved in transcriptional activation but give no information as to the function of C1/C2. Levy et al. (1988) have suggested that similar factors which bind to the ISG54 promoter may be involved in repressing transcription. Thus the relative levels of factor and their affinities would determine whether or not an ISRE was transcriptionally active. Factors which have the same methylation footprint as C1/C2 and which may be involved in repression are induced in HeLa cells by α-IFN. The binding of more than one factor to the same or overlapping sequences has been observed in a number of different systems (Goodbourn et al., 1986; Dorn et al., 1987; Lichtsteiner et al., 1987; Baldwin and Sharp, 1988; Santaro et al., 1988) and gene regulation through mutual factor

exclusion has been proposed to be important in transcriptional regulation both *in vitro* (Monaci *et al.*, 1988) and *in vivo* (Akerblom *et al.*, 1988). The C1/C2 and E factors, partially characterized here, are likely to be important in transcriptional induction and repression of genes regulated by α-IFN. Purification of these factors and cloning of the genes that encode them, together with reconstruction of transcriptional regulation *in vitro*, will be needed to define the mechanisms involved.

Materials and methods

Growth of cells and IFN treatment

HeLa S3 cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Bristol 8 B cells (Brodsky *et al.*, 1979) were grown in spinner culture in RPMI medium, 2% bicarbonate, supplemented with 5% fetal calf serum. Cells were treated with Wellferon, a highly purified mixture of human α-IFNs (10^8 IU/mg protein, Allen *et al.*, 1982) supplied by Dr K.Fantes, Wellcome Research Laboratories. IFN treatment was at 300 IU/ml for the HeLa cells and 1000 IU/ml for the Bristol 8 cells.

Transfection and competition experiments

HeLa S3 cells were plated at 10^6 cells per 9-cm dish. At 24 h, the cells were transfected by the calcium phosphate method, essentially as described by Spandidos and Wilkie (1984). At 16 h after adding the DNA—calcium phosphate co-precipitate, the cells were treated with 25% glycerol for 1 min, washed with medium and placed in medium containing 300 IU/ml of Wellferon. At 24 h after adding the IFN, the cells were harvested and CAT activity was determined as described by Sleigh (1986) and Porter *et al.* (1988). In experiments to test the function of the ISRE, it was cloned into the *Bam*HI site of the reporter plasmid pBLCAT2 (Klock *et al.*, 1987). In competition experiments, the reporter was the 1046 bp *Bgl*II—*Bam*HI fragment from the 5' end of the 6-16 gene cloned into pSVOCAT (Porter *et al.*, 1988). Amounts of 400 ng of reporter and 75 ng of competing oligomers were used per dish.

Preparation of extracts

Whole cell extracts were prepared from HeLa cells or Bristol 8 B cells as described by Zimarino and Wu (1987). The cells were treated with Wellferon for various times, pelleted quickly by centrifugation, frozen immediately in liquid N_2 and stored at $-70°C$ until assay. Nuclear extracts were prepared by a slight modification of the above procedure (V.Zimarino, personal communication) in which the frozen cell pellets were first thawed in extraction buffer without added salt (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5% glycerol, 0.5 mM phenylmethanesulphonyl fluoride) and spun for 10 s in a microfuge to pellet the nuclei. After washing the pellet twice in the same buffer the nuclei were extracted in one pellet volume of the above buffer plus NaCl (final concentration 0.38 M). The nuclear and whole-cell extracts were then treated in the same way. Where indicated, as a final step, the extracts were dialysed against the above buffer containing 100 mM NaCl for 2 h in a BRL microdialyser.

Binding assay

These assays were carried out essentially as described by Zimarino and Wu (1987). A 10 μl sample of extract (3 mg of protein/ml) was preincubated with 1.3 μl of 5 mg/ml poly(dI·dC)—poly(dI·dC) (Pharmacia) for 10 min at room temperature. A 10 μl aliquot of a mixture of the additional components was then added to bring the final concentrations to 0.5 mg/ml yeast tRNA, 0.5 mg/ml pdN₅ (random pentanucleotides, Pharmacia), 0.25 mg/ml fragmented *Escherichia coli* DNA, 2 mg/ml bovine serum albumin, 10 000 c.p.m. (1 ng end-labelled oligonucleotide probe, 4% Ficoll and 47% extract. The final NaCl concentration was 50 mM in dialysed extracts and in lysates made without added salt and 180 mM in undialysed extracts. The binding reaction was carried out at room temperature for 20 min and the complexes were separated by electrophoresis in a 1% agarose gel in 45 mM Tris—borate buffer, pH 8.0, 0.1 mM EDTA. The gel was blotted onto Whatman DE-81 paper for at least 2 h, followed by autoradiography. Competition assays were performed by adding 1.5 μl of competitor DNA in 75 mM NaCl to the mixture containing the probe, before adding the extract.

DMS interference assays

The probe was prepared by treating 50 ng of HPLC purified, end-labelled wt 39 bp oligonucleotide with 1 μl of DMS for 6 min as described by Maxam and Gilbert (1980). After precipitation with ethanol, the probe was resuspended in 75 mM NaCl before use. In the binding reaction, 200 μl of nuclear extract was incubated with ~5 × 10^5 c.p.m. of DMS-treated probe in a reaction mixture scaled up in proportion to the analytical mixture described above, with a final volume of 400 μl. The band corresponding to each complex was cut from DEAE paper (V.Zimarino, personal communication) and the DNA was eluted with 100 μl of 1.5 M NaCl. After two phenol—chloroform extractions in which the DEAE paper was left at the interface, the NaCl concentration was adjusted to 1 M and 10 μg of tRNA was added. The recovered DNA was precipitated with 2.5 volumes of ethanol and washed three times with 70% ethanol. The pellet was resuspended in 100 μl of piperidine and heated to 90°C for 30 min to cleave the modified bases. After freeze-drying twice from 10 μl of water each time, the samples were separated in a 15% polyacrylamide sequencing gel in 7.5 M urea.

DMS protection assays

A 400 μl reaction mixture with unmodified probe was incubated for 15 min at room temperature. Two μl of DMS was then added for 8 min at room temperature, followed by 1 μl of β-mercaptoethanol. The incubation mixture was then loaded directly onto a 1% agarose gel and treated thereafter as in the methylation interference method.

Cleavage with copper orthophenanthroline

Binding reactions were carried out as described above using a DEAE column fraction that contained all the binding activities seen in the crude extracts. For cleavage *in situ* in the agarose gel, a modification of a previously published procedure was used (Kuwubara and Sigman, 1987). After electrophoresis the gel was soaked for 30 min in 50 mM Tris—HCl, pH 8.0. The buffer was then replaced with 200 ml of fresh 50 mM Tris—HCl, pH 8.0, plus 20 ml of solution A (2 ml of 40 mM 1,10-phenanthroline and 2 ml of 9 mM CuOP diluted to a final volume of 20 ml with water just before use) and 20 ml of solution B (neat β-mercaptopropionic acid, diluted 1:100 with water just before use) for 60 min at room temperature with gentle rocking. To stop the reaction, 40 ml of 28 mM 2,9-dimethyl-1,10-ortho-phenanthroline was added for 30 min. The gel was then rinsed with distilled water and transferred to DEAE paper and the DNA isolated and analysed in a 15% polyacrylamide sequencing gel as described above.

Ethylation interference assays

About 20 ng of end-labelled probe, dissolved in 100 μl of 50 mM sodium cacodylate buffer, pH 8.0, was mixed with 100 μl of ethanol saturated with ethylnitrosourea (Siebenlist and Gilbert, 1980) and incubated at 50°C for 45 min. The ethylated probe was then precipitated twice with 5 μg of pdN₅ carrier, following addition of 20 μl of 3M sodium acetate and 500 μl of ethanol and used in binding reactions as described for methylation interference. After isolating the DNA from each complex as described above, the ethylphosphotriesters were cleaved by dissolving the samples in 100 μl of 2 mM EDTA, pH 8.0 and heating to 90°C for 15 min, then adding NaOH to 100 mM with incubation at 90°C for a further 15 min. The samples were neutralized by adding acetic acid to 100 mM and sodium acetate to 400 mM. After ethanol precipitation, the samples were run on a 15% polyacrylamide sequencing gel.

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Note added in proof

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