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Sequence requirement for specific interaction of an enhancer binding protein (EBP1) with DNA

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ABSTRACT

Short DNA sequence motifs have been identified in viral and cellular enhancers which represent the binding sites for a variety of *trans*- acting factors. One such HeLa cell factor, EBP1, has been purified and shown to bind to sequences in the SV40 enhancer. The PRDII element in the human β-interferon gene regulatory element (IRE) shows strong sequence similarity to the EBP1 binding site in the SV40 enhancer. We demonstrate here that EBP1 binds to its sites in the SV40 enhancer and IRE in a similar manner, making base specific contacts over one complete turn of the DNA double helix. Mutational analysis of the EBP1 sites in the IRE and SV40 enhancer has identified the DNA sequence requirements necessary for specific EBP1/DNA complex formation. In addition, 34 DNA sequences related to the EBP1 binding site were analysed for their ability to bind EBP1. Sequences constituting high affinity binding sites possess the sequence 5′-GG$(N)_6$CC-3′. Single base pair changes in the region between the conserved Gs and Cs can generally be tolerated although it is clear that these intervening bases contribute to binding affinity. Mutations in the recognition site which could lead to gross structural changes in the DNA abolish EBP1 binding.

INTRODUCTION

Transcriptional control elements are generally grouped into two distinct classes of *cis*-acting regulatory sequences, promoters and enhancers, which can overlap both physically and functionally (1). Within eukaryotic promoters, DNA sequences responsible for defining the RNA start site and the rate of initiation of transcription have been identified (2; 3; 4) with efficient transcription being dependent on the correct spatial arrangement of the various promoter elements (5; 6; 7). Considerably more flexibility is exhibited with enhancer sequences in that they are able to activate transcription of linked genes essentially independent of position or orientation with respect to the responding promoter. This enhancing effect was first identified in nucleotide sequences present in the small DNA tumour virus Simian Virus 40 (SV40) (8; 9) and since then the SV40 enhancer, which serves as the prototype enhancer element, has been the subject of intense investigation.

Mutational analysis has defined short DNA segments, or motifs, within the SV40 enhancer which, though having little enhancing effect in isolation, when multimerised, or combined with other motifs, can act in concert to generate enhancer activity (10; 11; 12; 13). It appears that each of these DNA sequence motifs represents the binding site for at least one *trans*- acting factor (14) and it is likely to be the interaction between various *trans*-acting factors with the transcriptional machinery that is responsible for enhancer function.

Although the SV40 enhancer has been shown to be active in a variety of cell types, it also demonstrates distinct cell type specificities (15). Activation of transcription by individual DNA sequence motifs is cell type specific *in vivo* (16; 17; 13) and this is paralleled by cell type specificity in the binding of cellular factors to these motifs *in vivo* (18; 19).

Nomiyama and co-workers (17) have proposed that the activity of an enhancer, in a given cell type, results from the nature of its constituent motifs as well as the presence or absence of the appropriate specific *trans*- acting factors in that cell type. Thus in complex enhancers, such as that present in SV40, a situation exists whereby gene expression may be controlled by the binding of different combinations of interacting factors . Fromental and coworkers (13) have investigated the cell-type specificity of individual SV40 enhancer motifs, the effects of spacing between motifs and the synergism between combinations of motifs. Their results showed the existence of three distinct classes of sequence motifs which interact with different classes of cell-type specific enhancer binding factors, but which in isolation do not demonstrate enhancer activity. This demonstrates three distinct levels of functional organisation the first of which is the DNA sequence motif itself which represents the binding site for a particular class (A, B, C or D) of *trans*- acting factor. The second level of organisation comprises of the minimum enhancer element which when oligomerised, or combined with other enhancer elements, acts synergistically to generate enhancer activity. The third level exists when a single minimal enhancer element is oligomerised or combined with two or more similar elements—a situation which is apparent in the SV40 enhancer (13) and the Immunoglobulin heavy chain gene enhancer (20; 21). In one category (functional level 1, class A), a HeLa cell factor, TEF-1 (22), binds specifically to two apparently unrelated sequence motifs (GT-IIC and Sph) in the SV40 enhancer. This factor binds cooperatively to DNA templates containing tandem repeats of its cognate motifs which correlates with the ability of the tandem repeats to generate enhancer activity *in vivo*.

Transcriptional activation in response to extracellular stimuli is now a well established phenomenon. The cellular transcription factor AP-1 interacts with DNA sequences common to both SV40 and the human metallothionein II_A ($hMTII_A$) gene enhancers (23), both of which are inducible in cells treated with the tumour promoter 12-O-tetradecanoyl-phorbol-14 acetate (TPA). It is thought that AP-1 is involved in mediating both the basal level of transcription and specific response to induction by TPA from these enhancer elements (24). In mammalian cells, transcription of several genes can be rapidly and transiently activated following serum stimulation. A short DNA sequence element, the serum response element (SRE), has been identified which binds the serum response factor (SRF) and mediates transcriptional activation of c-*fos* and cytoskeletal actin genes (25; 26). The precise role of the SRE in c-*fos* transcription has not been defined but it is probable that activation of c-*fos* gene expression involves an increase in the DNA binding properties of SRF and modification of its ability to activate transcription. In addition, exposure of cells to elevated temperatures leads to transcriptional activation of the heat shock genes, again mediated by a short DNA sequence, the heat shock element (HSE), which is the binding site for the heat shock transcription factor (HSTF) (27; 28; 29). HSTF has been shown to be regulated differently in yeast and HeLa cells. In HeLa cells, binding activity is detectable only after heat shock whereas in yeast cells, the HSTF appears to bind constitutively to DNA and is thought to activate transcription after heat-induced phosphorylation (28).

Transcriptional activity may also be modulated by the ability of bound proteins to exclude binding of other antagonistically acting factors. An example of this type is provided by the inducible enhancer element of the human β-interferon gene. It is thought that prior to induction a repressor protein bound to the interferon gene regulatory element (IRE) excludes the binding of a transcriptional activator (30). Mutational analysis has indicated that the IRE contains a constitutive transcription element, PRDII, that is prevented from functioning in uninduced mouse C127 cells by an adjacent or overlapping negative regulatory

element. The PRDII region is related to the SV40 enhancer 'core' sequence (31; 32) and contributes significantly to the high basal level of gene expression when the negative regulatory element is deleted. The inability to observe 'down' effects on transcription in uninduced cells when this region is mutagenised suggests that access of transcription factors to this element may be blocked by negative regulatory factors (33). We have recently purified a HeLa cell factor, EBP1, which binds to a site in the SV40 enhancer which overlaps binding sites for the transcriptional activator proteins AP-2 and AP-3 and which may exclude the binding of AP-2 and AP-3 or *vise versa* (34; 35).

Given the complexity of the situation in which a large number of proteins have the potential to interact with the same stretch of DNA, it is important to define precisely the recognition sequences for each of these proteins. In this study we demonstrate that EBP1 binds to the region of the IRE which shows strong sequence similarity to the EBP1 site in the SV40 enhancer. The interaction of EBP1 with its recognition site on the IRE was analysed by nuclease protection and by a variety of chemical probing techniques. Results from these studies indicate that EBP1 contacts its cognate binding sites in both the IRE and SV40 enhancer in a similar manner over one complete turn of the DNA double helix.

To identify DNA sequences required for EBP1 binding, a total of 34 naturally occurring or mutated DNA sequences were analysed for their ability to bind EBP1 and at each position in the sequence the frequency at which each base occurs determined. Within the 10 bp recognition site all sequences which engage in high affinity interactions with EBP1 have guanines at positions 1 and 2 and cytosines at positions 9 and 10. In addition, a strong preference for purines in positions 1 to 6 on one strand and in positions 7 to 10 on the opposite strand was observed.

MATERIALS AND METHODS

Plasmids and labelled fragments

Plasmid pUC1X72 contains the SV40 enhancer with one copy of the 72 bp repeat element, inserted between the *Eco* RI and *Bam* HI sites of pUC13 (34). [^{32}P]-labelled DNA was prepared by digestion of pUC1X72 with *Bam* HI and *Pvu* II followed by 3'-end labelling using [alpha-^{32}P]dATP (Amersham; specific activity 3000 Ci/mMol), unlabelled dCTP, dTTP, dGTP and the large Klenow fragment of *E. coli* DNA polymerase I. 5'-end labelled fragments were prepared by dephosphorylating *Bam* HI-cleaved pUC1X72, labelling with [gamma-^{32}P]ATP (Amersham; specific activity 5000 Ci/mmol) and polynucleotide kinase, followed by secondary cleavage with *Pvu* II. Fragments of 97 bp, containing one copy of the SV40 enhancer element were purified on 6% polyacrylamide gels and electroeluted (36).

The pA.. series of plasmids (10) contain triple point mutations throughout the SV40 enhancer region. The plasmids contain the following sequence alterations at the positions indicated:

pA11	5'-TGT-3'	(249–247)
pA12	5'-GTT-3'	(246–244)
pA13	5'-CCC-3'	(243–241)
pA14	5'-TGA-3'	(240–238)
pA15	5'-AAA-3'	(237–235)
pA16	5'-CTT-3'	(234–232)

Plasmids pA11-pA16 were generously provided by P. Chambon, CNRS, Strasbourg, France. Labelled fragments were prepared as described for pUC1X72.

Plasmid pIF10 contains the human β-interferon gene plus upstream regulatory sequences

extending to position −77 relative to the mRNA start site. The β-interferon gene regulatory element (IRE) is located between sequences −77 and −36 (30). A series of pIF10 mutants were constructed containing single point mutations in the IRE (33). These plasmids were a kind gift from S. Goodbourn, ICRF, Lincoln's Inn Fields, London. The nucleotide changes incorporated into these mutants are described in Figure 4. Labelled fragments containing the IRE were generated by digesting the plasmids with *Bgl* II and *Nco* I then 3'-end labelled as described above.

Plasmid pSPIRE was generated by replacing the small region between the *Bam* HI and *Bgl* II sites of pSP64 with the *Bam* HI/*Bgl* II IRE fragment (30). Prior to labelling, pSPIRE was cleaved with *Hind* III then 3'-end labelled or 5'-end labelled as described above. Secondary cleavage with *Bgl* II was then carried out and labelled fragments purified as described above.

Oligonucleotides were synthesised on an Applied Biosystems Model 381A DNA-Synthesizer. The DNA sequences of the top strands of the various oligonucleotides used in this study are shown below.

SV1	5'-GATCTAGGGTGTGGAAAGTCCCG-3'
SV1.M1	5'-GATCTAGGGTGTCCAAAGTCCCG-3'
SV1.M3	5'-GATCTAGGGTGTGGAAAGTGGCCG-3'
SVUP	5'-GATCTTGAGGCGGAAAGAACCAGCTG-3'
SYM	5'-GATCCTGGGGAAATTCCCCAT-3'
IRE	5'-GATCAAAGTGGGAAATTCCTCTG-3'
NF-kB	5'-GATCCTCGGAAAGTCCCCA-3'
H2TF1	5'-GATCCTGGGGAATCCCCA-3'
HIV	5'-GATCCCTGGAAAGTCCCCAGCGGAAAGTCCCTT-3'
HIV-L	5'-GATCCGCGGAAAGTCCCTA-3'
HIV-R	5'-GATCCCTGGAAATCCCCA-3'

Complementary, single stranded, synthetic oligonucleotides were annealed by first heating to 100°C in 0.1 M NaCl, 10 mM Tris.HCl, pH 8.0, 1 mM EDTA, followed by slow cooling to 16°C. The resultant double stranded oligonucleotides, which contained 5'-GATC overhangs, were 3'-end labelled with [alpha-^{32}P]dATP and isolated as described above.

Purification of EBP1 from HeLa cells

EBP1 was purified from nuclear extracts of HeLa cells by ion exchange chromatography over DEAE-Sepharose, followed by three successive applications to a recognition site affinity matrix as described previously (34).

Gel electrophoresis DNA binding assay

Assays contained 0.1−0.5 ng (~10,000 c.p.m.) labelled probe. 0.5 μg of unlabelled carrier DNA (equimolar amounts of poly[d(A-T):poly[d(G-C)]), 25 mM HEPES.NaOH pH 7.5, 1 mM DTT 1 mM EDTA, 10% glycerol, 0.05% NP40 and 1−2 μl EBP1 in a final reaction volume of 20 μl. Binding was allowed to proceed to equilibrium and reaction products fractionation by electrophoresis on 6% polyacrylamide gels. Gels were fixed in 10% acetic acid, dried and exposed to X-ray film at −70°C with an intensifying screen (34).

DNase I protection

Binding reactions contained 0.1−0.5 ng labelled probe (~10,000 c.p.m.), 0.5 μg of unlabelled carrier DNA (equimolar amounts of poly[d(A-T):poly[d(G-C)]), 25 mM HEPES.NaOH pH 7.5, 1 mM DTT 1 mM EDTA, 10% glycerol, 0.05% NP40 and various amounts of affinity purified EBP1 protein fractions, in a final reaction volume of 100 μl. Binding was allowed to proceed for 20 minutes at 20°C after which time the products were digested with 0.5 units of DNase I for 60 seconds at 20°C. An equal volume of 0.6 M NaOAc, 20 mM EDTA was then added. DNA was extracted, denatured at 100°C

for 2 minutes, cooled on ice and the cleavage products fractionated on denaturing 6% polyacrylamide gels as described previously (34). Gels were fixed in 10% acetic acid, baked dry onto glass plates (37) and exposed to X-ray film (Hyperfilm MP; Amersham) at −70°C in the presence of an intensifying screen. Sequence markers were prepared by subjecting end-labelled fragments to G+A or C+T specific reactions (38).

Methylation protection

Binding reactions were set up as described for 'DNase I protection'. After binding had reached equilibrium, the products were treated with 0.5 μl dimethylsulphate (DMS) for 2 minutes at 20°C. DNA was extracted (39) and treated to the 'G greater than A' cleavage method of Maxam and Gilbert (38). Cleavage products were fractionated and visualised as described in 'DNAse I protection'.

Methylation interference

To ~10 ng of end-labeled DNA and 0.5 μg unlabeled poly[d(A-T)]: poly[d(C-G)] in 200 μl of 50 mM sodium cacodylate pH8.0, 1 μl DMS was added and incubation continued for 10 minutes at 20°C. The reaction was terminated by the addition of 50 μl 1.5 M sodium acetate, 1.0 M β-mercaptoethanol containing 4 μg unlabelled poly[d(A-T)]: poly[d(G-C)] and the DNA isolated by ethanol precipitation. Methylated DNA was resuspended in binding buffer and binding reactions carried out as described above. Specific DNA−protein complexes were separated from free DNA in 6% polyacrylamide gels and bound and free DNA isolated as described previously (39). DNA was solubilized in 100 μl of 2 mM EDTA pH 8.0 and heated at 90°C for 15 minutes in a sealed tube. NaOH was added to a final concentration of 100 mM and incubation at 90°C continued for a further 15 minutes. The solution was neutralised by the addition of acetic acid to 100 mM, sodium acetate to 400 mM and the DNA precipitated with ethanol. DNA cleavage products were fractionated on 6% polyacrylamide gels as described in 'DNAse I protection'.

Orthophenanthroline/copper (OP/Cu^+) chemical nuclease footprinting

Binding reactions containing ~10 ng labelled probe, 0.5 μg unlabelled poly[d(A-T)]: poly[d(C-G)] and affinity purified EBP1, were carried as described for the gel electrophoresis DNA binding assay. Specific DNA−protein complexes were separated from free DNA in 6% native polyacrylamide gels as described above. 'Footprinting' reactions using OP/Cu^+ were then carried out within the gel matrix essentially as described by Kuwabara and Sigman (40). Bands corresponding to bound or free DNA were excised from the gels and the DNA eluted as described in 'Methylation interference'. Reaction products were fractionated and visualised as described in 'DNAse I protection'.

RESULTS

Binding of EBP1 to the human β-interferon gene regulatory element (IRE)

The *cis*- acting DNA sequences required for induction of the human β-interferon gene have been identified as a 42 bp region (the IRE), located at position −77 to −36 with respect to the transcriptional start site (30). Within this region is a DNA sequence similar to that present in the 'core' region of the SV40 enhancer. We have recently purified a HeLa cell protein, EBP1, which binds to a DNA sequence overlapping the 'core' region in the SV40 enhancer (34). To determine if it would also bind to the IRE, EBP1, purified by recognition site affinity chromatography, was incubated with a restriction enzyme fragment [^{32}P]-labelled on either the top or bottom strand of the IRE, digested with DNase I, and the cleavage products displayed on a denaturing polyacrylamide gel. On both the top and bottom strand a region of the IRE is protected by EBP1 from DNase I digestion

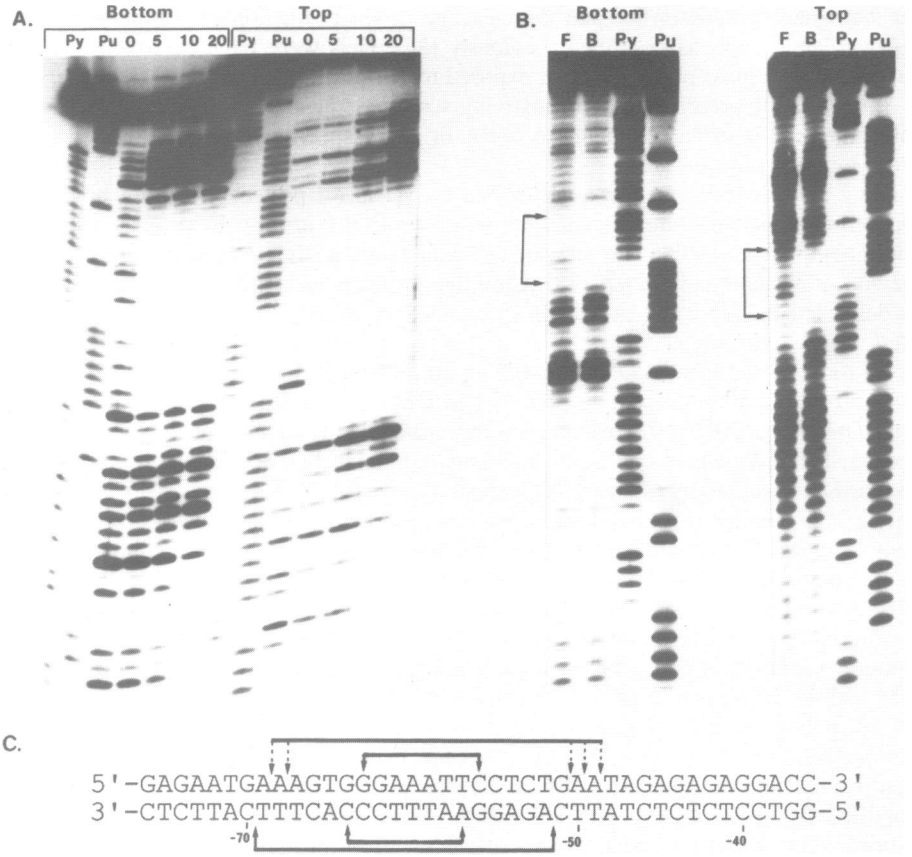


Figure 1. DNase I protection and OP/Cu$^+$ cleavage of the IRE in the presence of EBP1
A. A *Hind* III to *Bgl* II fragment from pSPIRE, was 3'-[^{32}P] labelled (top) or 5'-[^{32}P] labelled (bottom) at the *Hind* III site, incubated with 0, 5, 10 or 20 μl of affinity purified EBP1 and digested with DNase I as described in 'Materials and Methods'. DNA was isolated, fractionated by electrophoresis in 8% denaturing polyacrylamide gels and the cleavage products visualised by autoradiography. C+T (Py) and G+A (Pu) specific cleavage reactions of the labelled fragment were electrophoresed in parallel as markers.
B. Labelled fragments (described in **A**) were incubated with 20 μl of affinity purified EBP1 and, after binding had reached equilibrium, free DNA (F) was separated from EBP1−DNA complexes (B) by electrophoresis in a native polyacrylamide gel. Cleavage with OP/Cu$^+$ was carried out within the acrylamide matrix and DNA eluted as described in 'Materials and Methods'. DNA was fractionated, and products visualised as described in **A**. Deoxyribose residues within the bracketed region are protected from cleavage by OP/Cu$^+$.
C. DNA sequence of the IRE containing the EBP1 binding site. Phosphate bonds within the large brackets are protected from DNase I cleavage (broken lines indicate uncertainty in locating the boundary of protection since DNAse I does not cleave DNA in these regions). Deoxyribose residues within the small bracketed regions are protected from OP/Cu$^+$ cleavage in the presence of EBP1.

(Figure 1A). On the bottom strand the region of protection extends from the phosphate bond 3′ of base −51 to the phosphate bond 3′ of base −69. The boundary of the DNase I footprint on the top strand is not so well defined since DNase I cuts poorly in these regions. However, the cleavage patterns are normally staggered by 2 bp, suggesting that

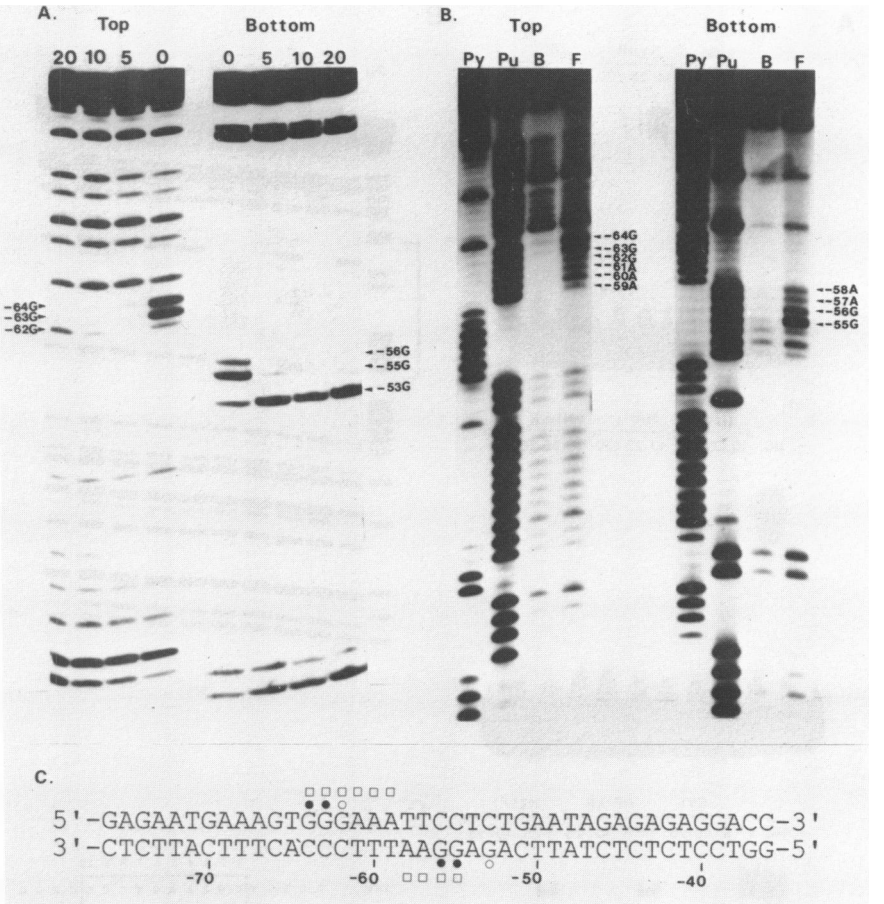


Figure 2. Methylation protection of the EBP1 binding site and methylated bases that interfere with EBP1 binding

A. A *Hind* III to *Bgl* II fragment from pSPIRE, 3'-[^{32}P] labelled (top) or 5'-[^{32}P] labelled (bottom) at the *Hind* III site, was incubated with 0, 5, 10 or 20 μl of affinity purified EBP1 and treated with DMS as described in 'Materials and Methods'. DNA was cleaved at modified guanine residues by treatment with piperidine and reaction products fractionated by electrophoresis in 8% denaturing polyacrylamide gels. Cleavage products were visualised by autoradiography. Positions of bases with altered sensitivity to DMS in the presence of EBP1 are indicated by arrows and the corresponding IRE nucleotide numbers.

B. The labelled fragments described in **A** were treated with DMS and the methylated DNA incubated with 20 μl of affinity purified EBP1. Free DNA (F) and EBP1−DNA complexes (B) were separated by electrophoresis in a native polyacrylamide gel. DNA was eluted from the gel and cleaved at modified purine bases by treatment with NaOH. Cleavage products were fractionated and visualised as described in **A**. C+T (Py) and G+A (Pu) specific cleavage reactions of the labelled fragments were electrophoresed in parallel as markers.

C. DNA sequence of the IRE containing the EBP1 binding site. Filled circles represent guanines protected from DMS methylation in the presence of EBP1 with open circles depicting guanines hypersensitive to DMS methylation. Open squares represent DMS modified bases which interfere with EBP1 binding.

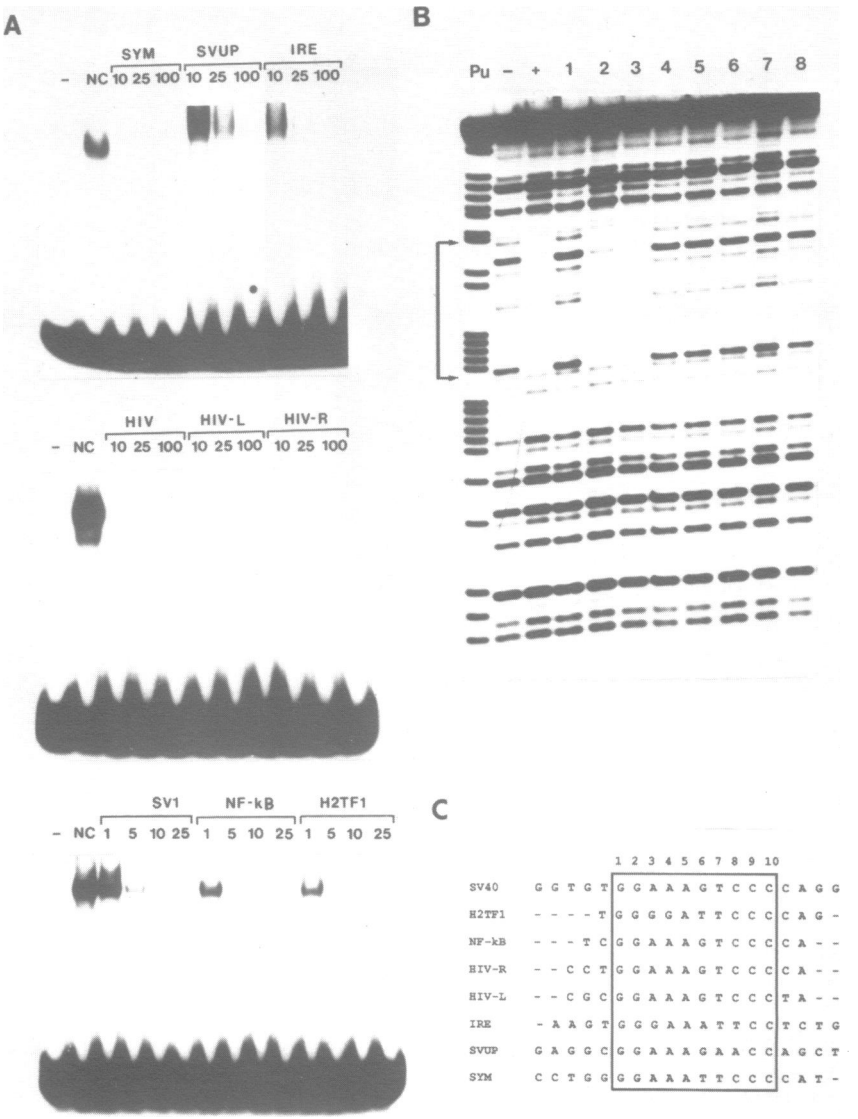


Figure 3. Competition analysis of EBP1 binding

A. Reactions contained 0.1 ng [^{32}P]-labelled double stranded SV1 oligonucleotide, 0.5 μg of unlabelled poly[d(A-T)]: poly[d(G-C)] and 2 μl of affinity purified EBP1 (dashes represent reactions which did not contain any EBP1). In addition reactions contained 0 (NC), 1, 5, 10, 25 or 100 ng of unlabelled double stranded SV1, SVUP, IRE, SYM, HIV, HIV-L, HIV-R, NF-kB or H2TFI synthetic oligonucleotides, the sequences of which are listed in 'Materials and Methods'. Binding reactions were fractionated on 6% polyacrylamide gels and products visualised by autoradiography.

B. A *Bam* HI to *Pvu* II fragment from pUC1X72, was 5'-[^{32}P] labelled at the *Bam* HI site, incubated with 0.5 μg of unlabelled poly[d(A-T)]:poly[d(G-C)], 20 μl of affinity purified EBP1 (+) and digested with DNAse I as

described in 'Materials and Methods'. Also included in the reactions were 100 ng of unlabelled double stranded SV1 (1), SV1.M1 (2), SV1.M2 (3), NF-kB (4), H2TF1 (5), HIV (6), HIV-L (7) and HIV-R (8) oligonucleotides. DNA was isolated, fractionated by electrophoresis in 8% denaturing polyacrylamide gels and the cleavage products visualised by autoradiography. G+A (Pu) specific cleavage reactions of the labelled fragment were electrophoresed in parallel as markers.

C. DNA sequences which compete for binding of EBP1. Sequences thought to be important for EBP1 are boxed.

the protected region extends from the phosphate bond 3′ of position −50 to the bond 3′ of position −68.

To determine which deoxyribose residues in the DNA backbone are in the proximity of bound EBP1, we have subjected to the EBP1−DNA complex to cleavage by hydroxyl radicals generated by the 1,10 orthophenanthroline/copper ion (OP/Cu^+) complex. Restriction enzyme fragments, [^{32}P]-labelled on either the top or bottom strand of the IRE were incubated with purified EBP1 and EBP1−DNA complexes resolved from free DNA by electrophoresis in a non-denaturing polyacrylamide gel. OP/Cu^+ cleavage was carried out *in situ* (40), DNA extracted from the gel and the cleaved products fractionated by electrophoresis on denaturing polyacrylamide gels. On each strand a total of eight deoxyribose residues are protected from OP/Cu^+ cleavage by EBP1. The region of protection extends from the deoxyribose residue attached to base −57 to that attached to base −64 on the bottom strand, and from −56 to −63 on the top strand. A summary of the DNase I and OP/Cu^+ protection data is presented in Figure 1C.

Base specific contacts of EBP1 on the IRE

To establish which purine bases within the IRE are in close contact with EBP1, methylation protection and interference experiments were performed. EBP1 was incubated with a restriction enzyme fragment containing the IRE, [^{32}P]-labelled on either the top or bottom strand, and the resulting DNA−protein complexes exposed to dimethylsulphate (DMS). Guanine residues methylated at the N-7 position were identified by treating with piperidine to cleave the DNA at the point of modification. On the top strand, G residues −63 and −64 are protected by EBP1 from modification by DMS. The G residue at −62 demonstrates the unusual property of being protected from methylation at low concentrations of EBP1, but at high concentrations of protein appears to be hypermethylated (Figure 2A). On the bottom strand −55G and −56G are protected from methylation by EBP1 whereas the reactivity of DMS to the G residue at −53 is increased by the presence of EBP1 (Figure 2A). In a complementary series of experiments the ability of methylated purine bases to interfere with binding of EBP1 was examined. A restriction enzyme fragment containing the IRE, [^{32}P]-labelled on either the top or the bottom strand, was treated with DMS to generate less than one methylated base per fragment. Partially methylated DNA fragments were incubated with EBP1 and DNA−protein complexes resolved from free DNA in a native polyacrylamide gel. DNA present in the bound and free fractions was eluted from the gel and treated with NaOH to cleave N-7 methylated G residues and N-3 methylated A residues at the point of modification. Comparison of the bound and free DNA on a denaturing polyacrylamide gel reveals which purine bases interfere with EBP1 binding when methylated. On the top strand the six consecutive purine bases, −59, −60 and −61A, and −62, −63 and −64G, all interfere with EBP1 binding when methylated (Figure 2B). A similar situation is apparent on the bottom strand where the four consecutive purine bases −55 and −56G, and −57 and −58A, all interfere with binding when methylated (Figure 2B). These data are summarised in Figure 2C and indicate that all purine bases in the 10 base pairs from −55 to −64 interfere with EBP1 binding when methylated. As expected the G residues that were protected by EBP1 from methylation interfered with

binding when methylated as did −62G which was hypermethylated at high concentrations of EBP1. Guanine residue −53, which demonstrated an increased reactivity towards DMS in the presence of EBP1, did not interfere with EBP1 binding when methylated.

EBP1 binds to a variety of viral and cellular enhancers

DNA sequences similar to the recognition site of EBP1 in the SV40 enhancer and the IRE are also present in a variety of other viral and cellular transcriptional control regions (Figure 3C), including the human immunodeficiency virus (HIV) enhancer (41), mouse immunoglobulin kappa chain enhancer (42) and mouse class 1 H2 major histocompatibility gene enhancer (43). An additional sequence is also located on the 'late' side of the SV40 enhancer. To determine if these DNA sequences also represented binding sites for EBP1, double stranded synthetic oligonucleotides containing these sites were assessed for their ability to compete with a labelled probe for binding of EBP1. EBP1 was incubated with a [^{32}P]-labelled restriction enzyme fragment derived from the SV40 enhancer and binding monitored by DNase I footprinting. In the presence of EBP1 the region between 232 and 251 on the SV40 enhancer is protected from digestion by EBP1 (Figure 3B). Binding to the labelled probe is, as we have previously noted (34), eliminated by inclusion of an excess of double stranded oligonucleotide containing the EBP1 binding site in the SV40 enhancer (SV1) but not by oligonucleotides containing mutations within the EBP1 binding site (SV1.M1, SV1.M3). Inclusion of double stranded oligonucleotides containing the sequences from the immunoglobulin kappa chain enhancer (NF-kB), the mouse class 1 H2 major histocompatibility gene enhancer (H2TF1) all competed for binding of the labelled SV40 fragment (Figure 3B). Two tandem copies of potential EBP1 binding sites are present in the HIV enhancer and it is apparent that each sequence separately (HIV-R, HIV-L) as well as the two together (HIV) can compete for binding (Figure 3B).

The relative affinity of the different sequences (Figure 3C) for EBP1 was determined in a gel electrophoresis DNA binding assay by addition of increasing amounts of each double stranded oligonucleotide to a binding reaction containing [^{32}P]-labelled SV1 oligonucleotide and purified EBP1. The EBP1 binding sequence present in the immunoglobulin kappa chain enhancer, class 1 H2 genes, the HIV enhancer and a synthetic symmetrical sequence (Sym) all compete for binding of EBP1 with similar affinities (Figure 3B). The IRE sequence also competes for binding of EBP1, but a ten-fold higher concentration of this binding site is required to give a similar level of competition to that obtained with SV1. Competition by the additional SV40 sequence on the late side of the enhancer (SVUP) only takes place at very high concentrations of oligonucleotide, indicating a low affinity for EBP1. Comparison of the different binding sites for EBP1 indicates that each contains a conserved 10 bp sequence (Figure 3C).

Mutational analysis of the EBP1 binding site in the IRE

Comparison of the naturally occurring binding sites for EBP1, and chemical probing with dimethylsulphate, indicates which base pairs within the recognition site are important for EBP1 binding. To evaluate the contribution of individual base pairs we have made use of a series of point mutants (Figure 4B) which span the EBP1 binding site in the IRE (33). [^{32}P]-labelled restriction enzyme fragments containing the mutated bases were incubated with EBP1, and free DNA separated from DNA−protein complexes on a native polyacrylamide gel. It is apparent from the autoradiograph shown in Figure 4A that mutations within the EBP1 binding site have widely differing effects on EBP1 binding. Mutations −65C, −60G and −57C have an negligable effect on EBP1 binding, unlike mutations −64A, −63A, −62A, −56T and −55T which have a severe deleterious effect

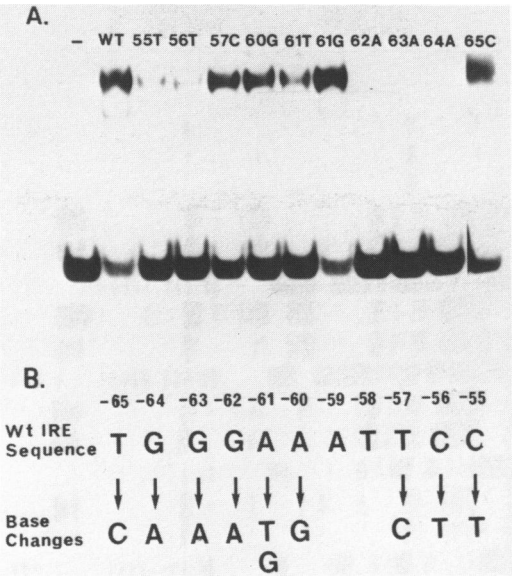


Figure 4. Mutational analysis of the EBP1 binding site present in the human β-interferon gene regulatory element (IRE)

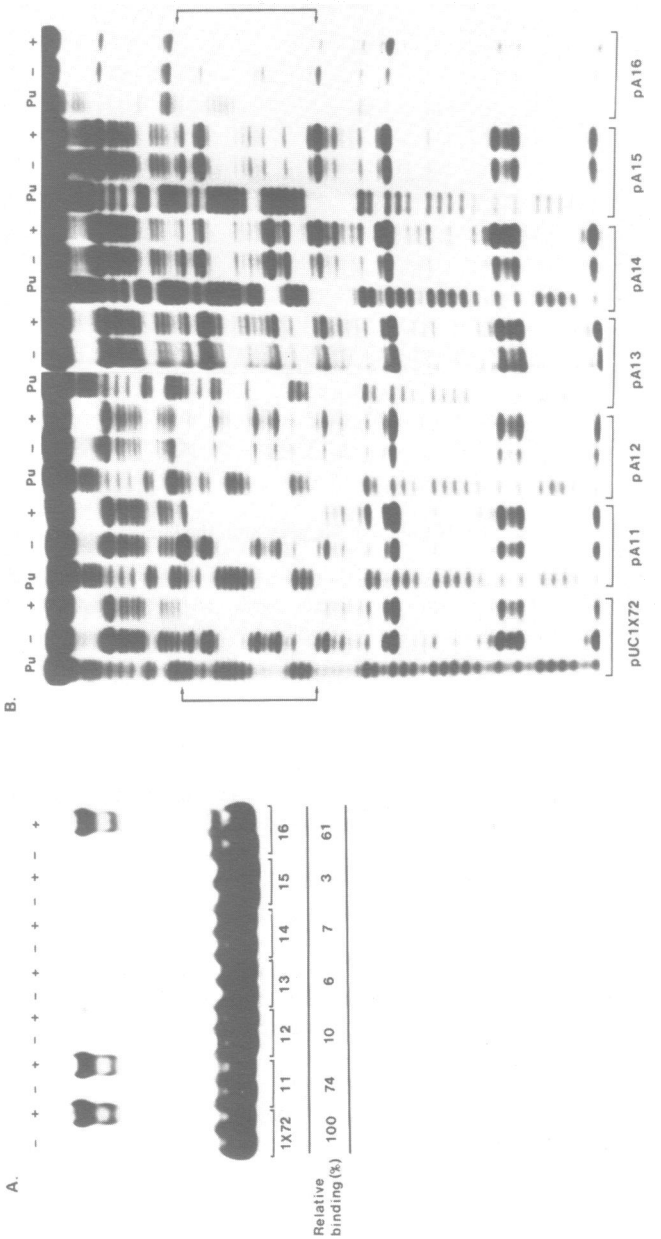
A. [^{32}P]-labelled *Bgl* II to *Nco* I fragments were isolated from the pIF10 series of plasmids, which contain single point mutations throughout the IRE (Goodbourn and Maniatis, 1988), and [^{32}P]-labelled at the *Bgl* II site. Labelled fragments were incubated with 0.5 μg of unlabelled poly[d(A-T)]: poly [d(G-C)] and 2 μl of affinity purified EBP1 in the standard gel electrophoresis DNA binding assay. Binding reactions were fractionated on 6% polyacrylamide gels and products visualised by autoradiography. The position of the mutation is indicated above the corresponding lane. No EBP1 was present in the reaction represented by the dash.

B. Nucleotide changes incorporated in the pIF10 mutants used in this study. The wild type (wt) sequence and its position in the IRE is shown above, with base pair changes in mutants shown below.

on EBP1 binding. Changes at position −61 are of interest in that an A to T change reduces binding whereas an A to G change increases binding above the wild type level. Thus a subset of the predicted base-pairs within the binding site, that are predicted from chemical modification experiments to be in close contact with EBP1 (−64, −63, −62, −56 and −55), have also been shown by mutational analysis to be critical residues in recognition (Figures 2A and 4A).

Mutational analysis of the EBP1 binding site in the SV40 enhancer

Although bases critical to EBP1 binding were identified by analysis of single base pair mutations, it was not clear to what extent the nature of sequences between these residues contribute to EBP1 binding since their mutation did not have a severe deleterious effect on binding (Figure 4). To address this question we made use of a series of clustered triple point mutations within the SV40 enhancer (10). We have previously shown that mutations pA12 and pA15, which alter the critical residues mentioned above, have a severe deleterious effect on EBP1 binding (34). The ability of EBP1 to bind to the full set of pA mutants that span the binding site was therefore determined by the gel electrophoresis DNA binding assay and by DNase I footprinting. [^{32}P]-labelled restriction enzyme fragments harbouring the various mutations were incubated with EBP1 and free DNA separated from DNA–protein complexes by electrophoresis in a native polyacrylamide gel. The extent of binding


A.
- + - + - + - + - + - +
1X72 11 12 13 14 15 16
Relative binding (%) 100 74 10 6 7 3 61
B.
Pu - + Pu - + Pu - + Pu - + Pu - + Pu - +
pUC1X72 pA11 pA12 pA13 pA14 pA15 pA16

was quantified by determining the [^{32}P]-radioactivity in the bound fraction using liquid scintillation counting. Examination of Figure 5A indicates that mutations pA11 and 16 have only a marginal effect on EBP1 binding whereas mutations pA12, 13, 14 and 15 reduce binding by at least an order of magnitude. These results were confirmed by DNase I footprinting which also indicates that EBP1 binding to pA11 and 16 was unaltered by mutations which lie on the boundary of the EBP1 recognition site (Figure 5B).

DNA sequence requirement for EBP1 binding

The mutational analysis of binding sites for EBP1 in the IRE and the SV40 enhancer is compiled in Table 1, where DNA sequences which bind EBP1 are compared with those that do not. An invarient feature of all DNA sequences that constitute high affinity binding sites for EBP1 is that they possess the sequence 5'-G-G separated by six base pairs from the complementary sequence 5'-C-C. Alteration of any one of these four bases reduces EBP1 binding considerably. The absence of the two C residues in related naturally occurring sequences from the GTII motif of the SV40 enhancer (SV2) and two regions of the adenovirus type 2 E1a enhancer (enhA, enhB) explain why these sequences fail to bind EBP1 (Table 1). One T residue located between these essential sequences is also conserved but is not absolutely required for binding since the H2TF1 sequence contains a C at this position (Table 1). However, the SVUP sequence represents a low affinity binding site for EBP1 and this may be explained by the absence of the conserved T residue. Base pair changes between the conserved C's and G's can usually be tolerated, although this does not appear to be the case with the −62A mutant or the triple point mutations. We have analysed the binding of EBP1 to 34 different DNA sequences that are related to the binding site for EBP1 in the SV40 enhancer. Nineteen different sequences bound EBP1, and we have used this information to determine the DNA sequence requirement for EBP1 binding (Table 1; Figure 3C). At each position in the sequence the frequency at which each base ocurrs has been determined and is presented graphically in Figure 6. As mentioned above, all sequences which bind EBP1 have G's at positions 1 and 2 and C's at positions 9 and 10. Also apparent is the strong preference for purines in positions 1 to 6 and the equally strong preference for pyrimidines in positions 7 to 10 on the top strand.

DISCUSSION

The PRDII region present in the human β-interferon gene regulatory element (30) is very similar to the EBP1 binding site in the SV40 enhancer. In this study we demonstrate, using

Figure 5. Mutational analysis of the EBP1 binding site on the SV40 enhancer.

A. *Bam* HI to *Pvu* II fragments from pUC1X72 and pA11 to pA16, were 3'-[^{32}P] labelled at the *Bam* HI site, and incubated with 2 μl of affinity purified EBP1, in the presence of 0.5 μg of unlabelled poly[d(A-T)]: poly[d(G-C)], in the standard gel electrophoresis DNA binding assay. Binding reactions were fractionated in a 6% polyacrylamide gel and products visualised by autoradiography. The relative binding of EBP1 to the various templates (as determined by liquid scintillation counting of bound species) is indicated.

B. DNase I protection of mutated SV40 enhancer sequences by EBP1. The [^{32}P]-labelled fragments described in **A** were incubated with 0 (−) or 20 (+) μl of affinity purified EBP1 and treated with DNase I as described in 'Materials and Methods'. DNA was isolated and fractionated in 8% denaturing polyacrylamide gels and the cleavage products visualised by autoradiography. G+A (Pu) specific cleavage reactions of the labelled fragments were electrophoresed in parallel as markers. Sources of labelled fragments are indicated at the foot of the appropriate lanes.

Table 1. Mutational analysis of EBP1 binding sites
DNA sequences of wild type and mutant templates which bind EBP1 (top panel) or those which do not bind EBP1 (lower panel). Nucleotide changes, with respect to the EBP1 recognition site present in the SV40 enhancer, are underlined.

SV1	G T G G A A A G T C C C G
SYM BS	G G G G A A A T T C C C C A
SV1 M2	G T G G A A T G T C C C G
SV1 M4	C T G G A A A G T C C C G
SV pA11	T T G G A A A G T C C C C A
SV pA16	G T G G A A A G T C C C C C
IRE 61G	G T G G G G A A T T C C T C
IRE 65C	G C G G G A A A T T C C T C
IRE 60G	G T G G G A G A T T C C T C
IRE 57C	G T G G G A A A T C C C T C
IRE 54C	G T G G G A A A T T C C C C
IRE 61T	G T G G G T A A T T C C T C
SV pA12	G G T T A A A G T C C C C
IRE 64A	G T A G G A A A T T C C T
IRE 63A	G T G A G A A A T T C C T
SV1 M1	G T C C A A A G T C C C G
IRE 62A	G T G G A A A A T T C C T
SV pA13	G T G G C C C G T C C C C
SV pA14	G T G G A A A T G A C C C
SV1 M3	G T G G A A A G T G G C C
IRE 56T	G T G G G A A A T T T C T
IRE 55T	G T G G G A A A T T C T T
SV pA15	G T G G A A A G T C A A A
SV2	G T G G A A T G T G T G
Enh A	G T G G T A A A A G T G
Enh B	G A G G A A G T G A A A

nuclease protection, chemical probing techniques and mutational analysis, that EBP1 binds to these sites in the IRE and SV40 enhancer (39) in a similar way. DNase I protection studies show that EBP1 occupies a region of 19 bp on each strand of the IRE and that, within this region, a total of eight deoxyribose residues on each strand are protected from hydroxyl radical attack (generated by OP/Cu^+) in the presence of EBP1.

EBP1 protects guanine residues −64, −63, −56 and −55 in the IRE from methylation by DMS. Since the N-7 position of guanine lies in the major groove of B-DNA (44; 45), perturbation of the methylation reaction at guanine suggests a protein contact in the major groove. All guanine residues which are protected from DMS modification also interfere with EBP1 binding when methylated. A total of 10 purine bases within the 10 bp from −55 to −64 of the IRE interfere with EBP1 binding when methylated. On the top strand

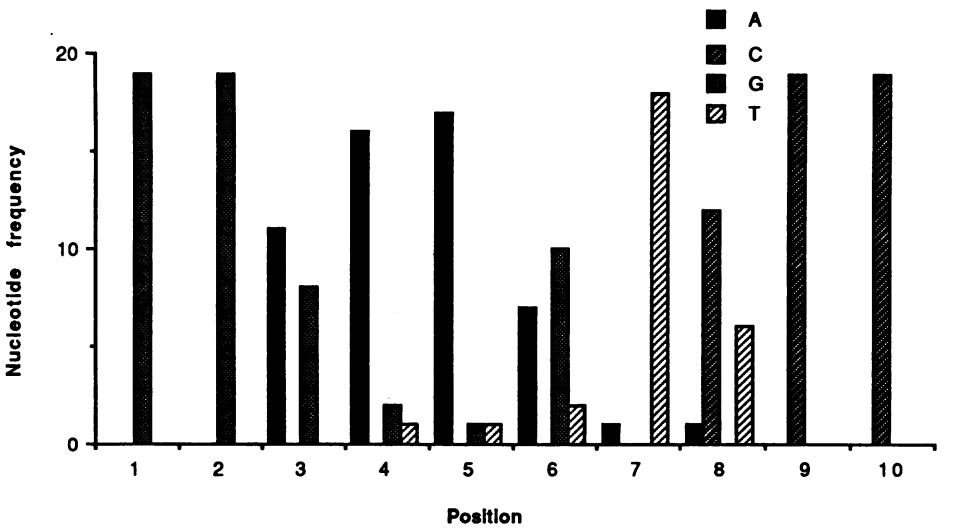


Figure 6. Frequency of base usage within EBP1 binding sites
Nineteen different sequences (both naturally occurring and mutated derivatives) which bound EBP1 were analysed and the frequency with which each base occurs, at each position, determined. A=adenine, C=cytosine, G=guanine, T=thymine.

the six consecutive purine bases −59, −60 and −61A, and −62, −63 and −64G all interfere with binding when methylated (Figure 2B). A similar situation is apparent on the bottom strand where the four consecutive purine bases −55 and −56G, and −57 and −58A also interfere with binding when methylated (Figure 2B). The pattern of specific base and backbone contacts between EBP1 and its recognition site in the IRE is remarkably similar to that previously observed for SV40 (39), indicating that EBP1 interacts with both sequences in a very similar fashion.

Mutational analysis of the EBP1 binding sites in the IRE and SV40 enhancer has identified the DNA sequence requirements necessary for specific EBP1/DNA complex formation. More than thirty DNA sequences related to the binding site for EBP1, including those present in the mouse immunoglobulin kappa light chain gene enhancer, mouse class 1 H2 major histocompatibility gene enhancer, HIV enhancer, IRE, SV40 enhancer and mutated derivatives thereof, were analysed for their ability to bind EBP1. A common feature in all the DNA sequences which bind EBP1 is that they possess the 10 bp sequence 5′-GG$(N)_6$CC-3′, within which every purine base interferes with EBP1 binding when methylated. Single base pair changes, as revealed in studies using mutant IRE templates (Figure 4), between the conserved Gs and Cs can generally be tolerated (Table 1), although it is clear that these bases contribute to binding affinity. This is exemplified at position −61 in the IRE where an A to T change reduces binding, but an A to G change increases binding.

Mutational analysis also reveals that alteration in the spacing between the conserved Gs at positions 1 and 2, and the conserved Cs at positions 9 and 10 (Figure 3C) abolish EBP1 binding. The SV1.M3 double stranded oligonucleotide alters this spacing to 7 bp, whilst the −64A IRE mutant alters it to 5 bp.; both of these are unable to bind EBP1. The ability of EBP1 to bind to the pA series of clustered triple point mutations in the SV40

enhancer was assessed (10). Binding studies revealed that pA11 and 16, which lie on the boundary of the EBP1 recognition site have only a marginal effect on EBP1 binding (Figure 5A and B) whereas mutations pA12, 13, 14 and 15, which lie within the recognition site, and also span the 10 bp region which, when methylated, interferes with EBP1 binding, reduce binding by at least an order of magnitude (Figure 5A). That no changes are tolerated within the EBP1 recognition site in this instance may be due to the inability of the DNA to accomodate the gross structural changes which may arise as a result of the triple point mutations.

The importance of DNA structure in directing high affinity EBP1 binding is suggested by the effect of mutations at −62, in which a G to A alteration abolishes EBP1 binding. This was somewhat unexpected since an adenine is permitted at this position in the EBP1 binding site in the SV40 enhancer (Figure 3C). The −62A mutant creates a situation whereby four consecutive adenine residues are present in the binding site. The unusual structural features of an oligo(dA).oligo(dT) tract and its biological implications have been described by Nelson *et al.*, (46). Their studies revealed that these sequences have an unusually high propeller twist of the bases along their longitudinal axis with a consequent narrowing of the minor groove. This in turn causes the major groove side of each base to point towards the 3'-end of its strand, thereby facilitating the formation of three-centred bifurcated hydrogen bonds. The inability of the −62A DNA template to bind EBP1 may therefore be due to structural changes in the DNA in the region of the four consecutive adenine residues.

The IRE consists of two genetically separable positive regulatory domains, PRDI and PRDII, both of which are required for maximal induction by virus or poly(I).poly(C), and a negative control region, NRDI, which overlaps PRDII (33). The EBP1 binding site in the IRE lies within PRDII. Interactions between the regulatory domains were investigated by analysing the effects of the IRE single base pair mutations on the expression of the β-interferon gene (33). Single point mutations in PRDII have the potential to affect binding of both negative and positive effectors such that the resulting phenotypes may be rather complex. PRDII was identified as being crucial for induction of β-interferon expression since mutations at positions −64 to −55 inclusive are all defective in their ability to respond to agents which activate β-interferon transcription. This effect has been ascribed to the inability of a positive transcription factor to interact with mutant PRDIIs. Examination of the IRE point mutants which alter the conserved guanine and cytosine blocks in the EBP1 binding site, that is −64A, −63A, −56T and −55T, reveals that these mutations reduce EBP1 binding to almost undetectable levels (Figure 4). In addition, the −64A and −56T mutations reduce induced transcription levels to virtually zero, whilst −63A and −55T mutations reduce transcription to approximately half that of wild type levels. Mutant −61G, which increases EBP1 binding to higher than wild type levels, shows an almost two-fold increase in relative transcription level after induction, whereas mutation −62A, which abolishes EBP1 binding and becomes hypermethylated at high concentrations of EBP1, demonstrates a 50% reduction in induced transcription levels. It would therefore appear that mutations in PRDII of the IRE which increase or decrease EBP1 binding, show a corresponding increase or decrease in the relative transcriptional induction of β-interferon mRNA.

The recognition site for EBP1 is similar to that of NF-kB, which binds to DNA sequences present in the inducible immunoglobulin kappa light chain gene enhancer (42). The two factors however, can be differentiated by virtue of their cell type specificities. EBP1 is constitutively expressed in HeLa cells (34) whereas NF-kB is normally only expressed

in mature B cells but can be activated in other cell types, after treatment with cycloheximide, bacterial lipopolysaccharide or phorbol esters (47). This implies that NF-kB is activated from a pre-existing form. Baeuerle and Baltimore (48) have recently demonstrated *in vitro* activation of DNA binding activity of what is believed to be a cytoplasmic precursor of NF-kB in pre-B and HeLa cell extracts. The use of dissociating agents to effect this activation suggests the presence of an inhibitor which blocks translocation to the nucleus and specific DNA binding.

A constitutive factor, H2TFI/KBFI, has been described which has similar recognition specificity as EBP1 and NF-kB (49; 50), and recent cloning of the gene encoding a protein which recognises the same sites as those recognised by EBP1, NF-kB and H2TFI/KBFI, suggests that these proteins may contain a conserved DNA binding domain (51). Isolation of cDNA's corresponding to each of these proteins will be required to establish the relationship between this family of proteins that recognise similar DNA sequences.

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