Identification and purification of EBP1: a HeLa cell protein that binds to a region overlapping the ‘core’ of the SV40 enhancer

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The SV40 enhancer consists of multiple DNA sequence motifs that are recognized by a variety of trans-acting factors. Using DNase I protection and a gel electrophoresis DNA-binding assay, we identified a HeLa cell protein (EBP1) that binds to the ‘core’ region of the SV40 enhancer. A short double-stranded synthetic oligonucleotide containing the binding site for EBP1 was used to assay for EBP1 activity and to purify a 57,000-m.w. polypeptide by recognition site affinity chromatography. Bromodeoxyuracil cross-linking identified a 60,000-m.w. species as the polypeptide responsible for the DNA-binding activity. Analysis of the DNA sequences required for EBP1 binding indicated that EBP1 could be distinguished from a number of recently characterized proteins (EBP20, AP-2, and AP-3) by its binding to a variety of mutant templates. Correlation of the in vivo transcriptional activity of wild-type and mutated enhancers with EBP1 binding indicates that this protein may be important for SV40 enhancer activity because mutations that abolish EBP1 binding also have a severe deleterious effect on transcription.

[Key Words: SV40 enhancer; DNA-binding protein; transcription factor; affinity chromatography]

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Transcriptional control in eukaryotes has been shown to be modulated by the interaction of sequence-specific DNA-binding proteins with their cis-acting recognition sites [for review, see Jones et al. 1988]. These target DNA sequences can be located close to the 5’ end of a gene as part of the conventionally defined promoter. If the target sequences are part of an enhancer, considerably more flexibility is exhibited in the positioning of control sequences: The hallmark of enhancer sequences is that they can activate transcription of linked genes in a position- and orientation-independent manner with respect to the responding promoter. Although some enhancers are active in a variety of cell types, an increasing number have been identified that are functional only in specific cell types. The immunoglobulin heavy-chain and insulin gene enhancers are examples in this category [Banerji et al. 1983; Gillies et al. 1983; Ohlsson and Edlund 1986]. Another class of enhancers, exemplified by those present in the mouse mammary tumor virus long terminal repeat [MMTV LTR, Chandler et al. 1983], c-fos gene [Treisman 1985], and human β-interferon gene [Goodbourn et al. 1985], are only active in response to an appropriate inducer.

al. 1981; Moreau et al. 1981] is regarded as the prototype enhancer. Although it is active in a number of cell types, it also demonstrates distinct cell-type specificities [Schirm et al. 1987], and its activity is stimulated in cells treated with phorbol esters [Chiu et al. 1987]. The flexible response of the SV40 enhancer appears to result from its modular construction in which multiple sequence motifs act in concert to generate enhancer activity [Herr and Clark 1986; Zenke et al. 1986]. Each of these sequence motifs represents the binding site for at least one trans-acting factor [Wildeman et al. 1986], and it appears to be the interaction of the trans-acting factors with the transcriptional machinery that is responsible for enhancer function. These sequence motifs display cell-type specificities in vivo [Nomiyama et al. 1987; Ondek et al. 1987] and bind proteins in vitro that are cell-type specific [Davidson et al. 1986; Xiao et al. 1987a]. The multiplicity of protein-binding sites within the SV40 enhancer creates a situation in which gene expression can be controlled by the binding of different combinations of interacting transcription factors. In this respect, it has also been demonstrated that cellular proteins can bind to overlapping motifs within the SV40 enhancer [Xiao et al. 1987b], thus giving rise to the possibility that transcriptional activity may not only be modulated by the binding of a particular factor but also by the ability of the bound factor 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 in the uninduced state, a repressor bound to the enhancer excludes the binding of a positive transcription factor. After induction, the positive transcription factor gains access to its recognition site in the enhancer and stimulates transcription (Goodbourn et al. 1986; Zinn and Maniatis 1986).

Purification of transcription factors that interact with the SV40 enhancer has defined a number of proteins with interesting properties. Transcription factor AP-1 interacts with phorbol-ester-inducible elements within the SV40 enhancer [Angel et al. 1987; Lee et al. 1987] and stimulates transcription in a reconstituted in vitro system [Lee et al. 1987]. Likewise, the transcription factor AP-2 binds to different phorbol-ester-responsive elements in the SV40 enhancer and activates transcription in vitro [Imagawa et al. 1987; Mitchell et al. 1987]. Two proteins, EBP20 [Johnson et al. 1987] and AP-3 [Mitchell et al. 1987], have been shown to bind in the ‘core’ region of the SV40 enhancer, which mutational studies indicate is important for enhancer function [Laimins et al. 1982; Weiher et al. 1983; Zenke et al. 1986]. Here, we describe the purification, from HeLa cells, of a protein that binds to this region of the SV40 enhancer but is distinct from the previously described EBP20 [Johnson et al. 1987] or AP-2 and AP-3 [Mitchell et al. 1987]. Mutations in the recognition site of this protein, EBPI, which abolish binding, have been shown previously to have a severe deleterious effect on enhancer-mediated transcription (Zenke et al. 1986; Nomiyama et al. 1987; Ondek et al. 1987).

Results

Detection of cellular proteins that interact with the SV40 enhancer

The SV40 enhancer consists of the two 72-bp repeats and additional DNA sequences that extend to the Pvull site [Fig. 1A]. In our studies, a plasmid (pUC1X72), derived from pMKD 231 [Everett et al. 1983], with a single 72-bp element, has been used as a source of DNA containing SV40 enhancer sequences. Cellular proteins that bind specifically to the SV40 enhancer were detected by a gel electrophoresis DNA-binding assay [Fried and Crothers 1981; Garner and Revzin 1981]. A 173-bp α2P-labeled BamHI–EcoRI fragment derived from pUC1X72 was incubated with a nuclear extract from HeLa cells that had been partially purified by DEAE–Sepharose chromatography. Interactions between nonspecific DNA-binding proteins and the labeled probe were minimized by the inclusion, in the binding reaction, of a large excess of an equimolar mixture of poly[d(G-C)] and poly[d(A-T)]. Native polyacrylamide gel electrophoresis of the binding reactions resolved free DNA from three distinct DNA–protein complexes labeled a, b, and c in Figure 1B. To determine the sequence specificity of the proteins responsible for the formation of the different DNA–protein complexes, double-stranded oligonucleotides that spanned a number of the motifs recognized in the SV40 enhancer were synthesized. SV1 contains the GT-I and part of the TC-II motifs, whereas SV2 contains the GT-II motif and SV3 the Sph-I, Sph-II, octamer, and part of the P motif [for nomenclature, see Xiao et al. 1987a; for review, see Jones et al. 1988]. Competition analysis, in which an excess of the unlabeled double-stranded oligonucleotide is included in the binding reaction, indicated that the formation of complex a was diminished in the presence of SV1 and, to a lesser extent, by SV2, whereas formation of complex b was unaffected by the presence of SV1, SV2, or SV3 [Fig. 1B]. Formation of complex c was abolished by inclusion of the SV1 double-stranded synthetic oligonucleotide in the binding reaction but was unaffected by the inclusion of the SV2 and SV3 double-stranded synthetic oligonucleotides. The origin of complex c was investigated further by repeating these experiments with a shorter α2P-labeled DNA fragment of 97 bp, which extends from the BamHI to the Pvull site. Only one DNA–protein complex was resolved by polyacrylamide gel electrophoresis after incubation of the labeled probe with the nuclear extract [Fig. 1C]. Equivalence of this single DNA–protein complex with complex c observed previously was suggested by a similar electrophoretic mobility and by the fact that formation of the complex was competed for by inclusion of the double-stranded SV1 oligonucleotide [Fig. 1C].

DNase protection analysis of nuclear proteins binding to the SV40 enhancer

To identify the binding sites of the nuclear proteins on the SV40 genome, DNase I protection experiments [Galas and Schmitz 1978] were carried out. Using a α2P-labeled EcoRI–BamHI fragment as probe, two areas of the SV40 enhancer were protected from DNase I digestion in the presence of HeLa cell nuclear protein [Fig. 2]. The area from position 233 to 252 contains the GT-I motif, whereas the region from 262 to 290 contains the GT-II motif. The addition of an excess of the unlabeled SV1 double-stranded synthetic oligonucleotide to the binding reaction abolished protection between positions 233 and 252 but had no effect in the region from 262 to 290. Conversely, addition of an excess of unlabeled SV2 double-stranded synthetic oligonucleotide reduced the protection over the GT-II motif, whereas the footprint over the GT-I motif was unaltered. The pattern of DNase I protection was not altered substantially in the presence of excess SV3 double-stranded synthetic oligonucleotide [Fig. 2]. To investigate the requirements for sequence-specific binding in the GT-I region, competition assays in which double-stranded oligonucleotides corresponding to SV1 with specific base pair alterations were included in the binding reactions as above. Alteration of the two Gs at positions 244 and 245 to Cs (SV1.M1) abolished the ability of the double-stranded oligonucleotide to compete for binding of the nuclear factors, whereas changing A241 to T (SV1.M2) did not alter the ability of the double-stranded oligonucleotide to compete for binding of the nuclear factors [Fig. 2].
Purification of a cellular protein that binds to the SV40 enhancer

The factor that binds to the GT-I motif in the SV40 enhancer could also bind to the SV1 double-stranded synthetic oligonucleotide (Figs. 1 and 2). Thus, as a means of following purification of the GT-I binding fraction, we used $^{32}$P-labeled SV1 double-stranded oligonucleotide as probe in the gel electrophoresis DNA-binding assay [Fig. 3]. HeLa cells (40 g wet weight) were fractionated into nuclei and cytosol, and the nuclei extracted with 0.35 M NaCl. The nuclear extract, which contained the bulk of the binding activity, was freed of nucleic acids by passage over DEAE-Sepharose and, after reduction of the NaCl concentration was applied to a second DEAE-Sepharose column. Bound proteins were eluted with a linear gradient from 0.05 to 0.4 M NaCl, and individual fractions were monitored for DNA-binding activity.

Figure 1. Detection of enhancer-binding proteins in HeLa cell nuclear extracts. (A) Organization of the SV40 transcriptional control region. Relevant restriction enzyme cleavage sites and important elements of the promoter and enhancer present in plasmid pMKD 231 are indicated. Shown below is the DNA sequence of the enhancer region. DNA sequences present in the double-stranded synthetic oligonucleotides SV1, SV2, and SV3 are also indicated. Probes labeled with $^{32}$P containing the 72-bp enhancer element, EcoRI-BamHI- [B] or PvuII-BamHI- [C] digested pUC19X72, were incubated with 2 μg nuclear extract (partially purified over DEAE-Sepharose) in the standard gel electrophoresis DNA-binding assay, as described in Materials and methods (lanes 0 contained no nuclear extract). Also included in the reaction mixture was 25 (lanes 1), 50 (lanes 2), or 100 (lanes 3) ng of unlabeled double-stranded synthetic oligonucleotides SV1, SV2, or SV3.
Using this procedure, it was possible to resolve two distinct species that bound to the SV1 double-stranded oligonucleotide [Fig. 3]. The two species appear to have identical sequence specificities, and bromodeoxyuracil cross-linking experiments indicate that the cross-linked polypeptide is of identical molecular weight in each case [data not shown]. Fractions containing the two binding species were pooled and purified further by recognition site affinity chromatography [Kadonaga and Tjian 1986; Rosenfeld and Kelly 1986]. The pooled DEAE-Sepharose fractions were mixed with poly[d(A-T)] and poly [d(G-C)], as nonspecific competitor DNAs, and applied to a DNA affinity resin composed of tandemly ligated SV1 double-stranded oligonucleotide coupled to cyanogen-bromide-activated Sepharose-4B [Pharmacia]. Whereas the bulk of the proteins in the DEAE fractions flow through the affinity column, the SV1-binding activity was specifically retained on the column and could be eluted by raising the NaCl concentration to 1.0 M (Fig. 4A). Two further rounds of affinity chromatography resulted in a preparation in which the specific binding activity had increased by 15,000-fold when compared to the crude nuclear extract [Table 1]. Samples from various steps in the purification procedure were precipitated with trichloroacetic acid [TCA] and analyzed by SDS–polyacrylamide gel electrophoresis followed by silver staining. The third-round affinity fraction contained predominantly a 57,000-m.w. polypeptide (Fig. 4B). From 40 grams of HeLa cells we obtained 8 μg of purified protein with 13.4% recovery of specific DNA-binding activity (Table 1). Because the purified protein bound to the SV1 region of the SV40 enhancer, we have designated this protein EBPl.

**Identification of the 57,000-m.w. polypeptide (EBP1) as the specific DNA-binding species**

To determine that the 57,000-m.w. polypeptide [EBP1] contained the activity responsible for binding to the SV1 oligonucleotide, we cross-linked bound protein to bromodeoxyuracil-substituted, 32P-labeled DNA [Ogata and Gilbert 1977]. The double-stranded SV1 oligonucleotide was first inserted into M13mp8, and single-stranded phage DNA prepared. A complementary strand was synthesized containing [32P]dGMP and bromodeoxyuracil, as a substitute for thymidine. Restriction enzyme cleavage released a small labeled fragment that was incubated with affinity-purified protein fractions under

**Table 1. Purification of EBPl from HeLa cell nuclear extracts**

| Fraction                  | Protein (mg) | Unitsa | Yield (%) | Specific activity (units/mg/protein) | -Fold purification |
|---------------------------|--------------|--------|-----------|-------------------------------------|-------------------|
| Crude nuclear extract     | 861          | 84,378 | 100       | 98                                  | 1                 |
| DEAE pool                 | 77           | 36,400 | 43        | 473                                 | 5                 |
| First ds SV1 pool         | 1.3          | 25,480 | 30        | 19,600                              | 210               |
| Second ds SV1 pool        | 0.08         | 19,110 | 22.6      | 238,875                             | 2,560             |
| Third ds SV1 pool         | 0.008        | 11,275 | 13.4      | 1,409,375                           | 15,150            |

* One unit of activity represents the retention of 15 fmole specific DNA bound in the DNA–protein complexes in the gel electrophoresis DNA-binding assay.

* Estimate based on comparative silver staining.

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Figure 3. Fractionation of HeLa nuclear extract. Dialyzed nuclear extract was loaded onto a DEAE-Sepharose CL-6B column, and bound proteins eluted with a linear gradient of 0.05–0.4 M NaCl. Fractions were incubated with \(^{32P}\)-labeled SV1 double-stranded synthetic oligonucleotide under standard conditions [Materials and methods], and DNA–protein complexes resolved on 6% polyacrylamide gels. Gels were either dried down and autoradiographed [A] or enhancer-protein-binding activity quantified by excising bound species from the gels and determining the radioactivity present by Cerenkov counting [B]. Protein concentration [■] was determined by the method of Bradford [1976]. Fractions sampled are indicated below each panel. [C] Cytoplasmic extract; [E] nuclear extract; [F1] material passing through the first DEAE-Sepharose CL-6B column; [L, F2, W] column load, flow through, and 0.05 M NaCl wash from the second DEAE-Sepharose CL-6B column, respectively.

conditions similar to those employed in the gel electrophoresis DNA-binding assay. Once equilibration had been reached, the samples were irradiated with ultraviolet (UV) light, digested with nucleases, and analyzed by autoradiography after SDS–polyacrylamide gel electrophoresis. When the products of the cross-linking reactions were analyzed without nuclease treatment, it was evident that the labeled oligonucleotide had been cross-linked to a polypeptide present in the binding reaction (Fig. 5). Treatment of the cross-linked products with DNase I and micrococcal nuclease, prior to gel electrophoresis, decreased the apparent molecular weight of the labeled species and identified a polypeptide with a molecular weight of ~60,000 (Fig. 5). The labeled species accumulated with increased irradiation (Fig. 5), but no cross-linking was observed if bovine serum albumin was substituted for EBPI in the DNA-binding reaction [data not shown]. Addition of an excess of unlabeled SV1 oligonucleotide to the binding reaction abolished cross-linking, whereas inclusion of double-stranded oligonucleotides containing the binding sites for NFI or NFIII had little effect (Fig. 5). These data indicate that a polypeptide of ~60,000 is responsible for specific binding to the GT-I region of the SV40 enhancer and is the major component present in our most highly purified preparations.

Figure 4. Recognition site affinity chromatography of EBPI. [A] Poly[d(G-C)] and poly[d(A-T)] were added to the DEAE-Sepharose fractions containing EBPI activity and passed over an affinity matrix composed of tandemly ligated SV1 oligonucleotides covalently linked to cyanogen-bromide-activated Sepharose 4B. Bound proteins were eluted with 1.0 M NaCl, and fractions assayed for EBPI activity. [L] Load; [FT] flowthrough; [W] wash; [1–8] fractions from 1 M NaCl elution. [B] Fractions from each stage of the EBPI purification were precipitated with TCA and analyzed by SDS–polyacrylamide gel electrophoresis and silver staining. Equivalent amounts [28 units] of each affinity column eluate were applied to each lane. The molecular weights of the marker proteins are indicated. [D] DEAE-Sepharose fractions, [1, 2, and 3] first, second, and third-round affinity column eluates; [M] protein molecular weight standards.
Figure 5. Bromodeoxyuracil cross-linking of EBPI to its binding site on the SV40 genome. A bromodeoxyuridine-substituted, $^{32}$P-labeled double-stranded DNA fragment containing the EBPI-binding site was prepared as described in Materials and methods. Probe DNA was incubated with affinity-purified EBPI under conditions employed in the gel electrophoresis DNA-binding assay. Once equilibrium was reached, samples were irradiated with UV light, followed by treatment with DNase I and micrococcal nuclease, as indicated. Reaction products were fractionated on 10% SDS-polyacrylamide gels and visualized by autoradiography. The positions of the 66,000- and 45,000- m.w. protein standards are indicated by the arrows. UV irradiation, in the absence of competitor, was for 0, 5, 10, and 20 min. In the competition analysis, EBPI was allowed to bind in the presence of 0.1 μg SVI, 0.5 μg SVI, 0.5 μg NFI or 0.5 μg NFIII double-stranded oligonucleotides prior to UV irradiation for 10 min. Reaction products that were UV irradiated for 10 min without subsequent DNase I or micrococcal nuclease digestion are also shown (–MNase, DNase).

DNA-binding specificity of purified EBPI

DNase I protection experiments were employed to define the binding site of the purified protein on the SV40 enhancer. A 3' $^{32}$P-labeled DNA fragment containing SV40 enhancer sequences was incubated with the purified preparations of EBPI, subjected to DNase I digestion, and the cleavage products were displayed. The purified protein protects the region from 230 to 250 on the SV40 enhancer from cleavage with DNase I [Fig. 6]. Protection against DNase I cleavage of a similar region between positions 233 and 252 on the opposite strand [Fig. 2] was observed with partially purified nuclear extracts, indicating that the protein we have purified is probably responsible for the binding activity observed in the nuclear extract. This conclusion was strengthened by the observations that the SVI and SVI.M2 oligonucleotides can compete for binding of the factor, whereas the SVI.M1 oligonucleotide is incapable of competing [Fig. 6].

Interaction of the purified factor with $^{32}$P-labeled SVI double-stranded oligonucleotide was monitored in the gel electrophoresis DNA-binding assay. Whereas unlabeled SVI oligonucleotide could compete efficiently for the binding of the purified factor, the SVI.M1 oligonucleotide with G → C changes at positions 244 and 245 was unable to do so. This was also true of the SVI.M3 oligonucleotide, which contained C → G alterations at position 237 and 238 [Fig. 7A]. Zenke et al. [1986] constructed a series of clustered point mutants in the SV40 enhancer that have been used extensively to study enhancer function. We therefore examined the binding of EBPI to three of these scanning point mutants, pA12, pA15, and pA16, in the gel electrophoresis DNA-binding assay. Affinity-purified EBPI was incubated with $^{32}$P-labeled DNA fragments containing one copy of the wild-type or mutated SV40 enhancer and DNA–protein complexes resolved by native polyacrylamide gel electrophoresis. Whereas the mutation present in pA16 has no effect on EBPI binding, the mutations present in p12 and pA15 reduce EBPI binding to an undetectable level [Fig. 7B]. Thus, the purified protein makes sequence-specific DNA contacts within the GT-I motif, at positions 244 and 245, and in the TC-II motif, at positions 237 and 238 [Fig. 7C].
Figure 7. DNA-binding specificity of affinity-purified EBPl. (A) Competition analysis of EBPl binding. Reactions contained 0.5 ng of $^3^P$-labeled, double-stranded SV1 oligonucleotide, 1.0 µg of unlabeled poly[d(A-T)] : poly[d(C-G)], and 1.0 µl of affinity-purified EBPl (lane — contained no EBPl). In addition, reactions contained 25 (lanes 1), 50 (lanes 2), or 100 (lanes 3) ng of unlabeled, double-stranded SV1, SV1.M1, or SV1.M3 oligonucleotides. Reaction products were analyzed on 6% polyacrylamide gels. (B) Binding of purified EBPl to pA mutants. DNA fragments containing wild-type and mutant enhancers were excised by BamHI and PvuII digestion and 3'-end labeled with $^3^P$ at the BamHI site. Labeled DNA was incubated with EBPl (as described in A), and DNA-protein complexes resolved on a 6% polyacrylamide gel. DNA templates utilized are indicated above each lane; (—) EBPl was omitted from the binding reactions. (C) DNA sequence of the GT-I and TC-II motifs and summary of EBPl binding to mutated DNAs. Bases altered in the mutants are indicated; dashes represent unchanged bases.

Discussion

We describe here the purification and DNA-binding specificity of a 57,000-m.w. HeLa cell nuclear protein, EBPl, which binds to the SV40 enhancer. The region of the enhancer protected from DNase I cleavage extends from position 230 to position 250, which includes the GT-I motif and spans the core sequence (5' - GTGG - G - 3'), identified by comparison of DNA sequences from a number of different enhancers (Laimins et al. 1982; Weiher et al. 1983). However, this sequence alone does not constitute the binding site for EBPl because the SV2 double-stranded oligonucleotide, which contains this sequence and spans the GT-II motif, fails to compete for EBPl binding (Figs. 1 and 2). Although this sequence is clearly important, mutation of the Gs at positions 244 and 245 abolishes binding (Figs. 1, 2, 6, and 7). Additional sequences are required to constitute a functional binding site for EBPl. These additional sequences are identified by the mutations in the SV1.M3 oligonucleotide; Cs at positions 237 and 238 are changed to Gs, with the result that the double-stranded oligonucleotide can no longer be bound by EBPl (Fig. 7). This extension of the binding site past the GT-I motif and into the TC-II motif differentiates this factor from a number of previously identified proteins that bind to the SV40 enhancer (Barrett et al. 1987; Johnson et al. 1987; Xiao et al. 1987a). Purification of two factors, AP-2 and AP-3, from HeLa cells, which bind close to this region, have been described recently (Imagawa et al. 1987; Mitchell et al. 1987). EBPl can be distinguished from both of these factors by comparing the ability of the different proteins to bind to mutated templates. The plasmid pA12 contains a mutated SV40 enhancer element in which the sequence 5' - TGG - 3' (246 - 244) is altered to 5' - GTT - 3' (Zenke et al. 1986). AP-2 binds normally to this fragment (Mitchell et al. 1987), whereas EBPl binding is abolished (Fig. 7), thus indicating that EBPl and AP-2 have distinct recognition sites. EBPl binding is also abolished by the mutation present in pA15 (Fig. 7), in which 5' - CCC - 3' (237 - 235) is altered to 5' - AAA - 3' (Zenke et al. 1986), whereas AP-3 binds normally to this template (Mitchell et al. 1987). This finding therefore distinguishes EBPl from AP-3. We have recently established the importance of these mutated bases in a detailed contact point analysis of EBPl binding to the SV40 enhancer and have shown that the Gs present on one strand at positions 244 and 245 and on the opposite strand at positions 237 and 238 are in close contact with the EBPl protein (L. Clark, J. Nicholson, and R.T. Hay, in prep.).

The role of EBPl in transcriptional activation of the SV40 genome is suggested by correlating the in vivo activity of wild-type and mutated enhancers with EBPl binding. Tandemly ligated copies of an oligonucleotide containing the EBPl-binding site can function as a tran-
scriptional enhancer when inserted into an appropriate expression vector [Ondek et al. 1987; R.M. Pollock, L. Clark, and R.T. Hay, unpubl.]. Mutation of the two Gs at positions 244 and 245 abolished the enhancer activity of the ligated oligonucleotides [Ondek et al. 1987] and also abolished binding of EBPl (Figs. 1, 2, 6, and 7). Nomiyama et al. [1987] have examined the activity of the various enhancer motifs with the presence of trans-acting factors. One cell-type-specific protein, GT-1C (probably equivalent to AP-3), present in HeLa but not in MCFII cells, binds to the GT-I motif in the SV40 enhancer [Xiao et al. 1987a]. An additional cell-type-specific protein, TC-IIA, and a ubiquitous protein, TC-IIB, are reported to bind to the TC-II motif [Nomiyama et al. 1987]. TC-IIA and TC-IIB appear to have very similar recognition sites to that of EBPl. The mutation present in pA12 (described above) abolishes binding of all of these proteins and has a severe deleterious effect on transcription in all of the cell lines tested [Nomiyama et al. 1987]. Mutations in the GT-I motif (pAlO and pAll), which eliminate GT-1C binding but have no effect on the binding of TC-IIA, TC-IIB, or EBPl, have no effect in MCFII cells where GT-1C is absent. In HeLa cells, mutations pAlO and pAll have only a minimal effect on enhancer activity, whereas mutation pAl5, which abolishes binding of TC-IIA, TC-IIB, and EBPl, reduces transcription in vivo to 40–50% of wild type. Because binding of GT-I C (AP-3) and EBPl (TC-IIB) is eliminated by pA12, these proteins may compete for the same binding site in HeLa cells. The minimal effect of mutations pAlO and pAll in HeLa cells suggests that EBPl (TC-IIB) is bound to the template in preference to GT-1C. Elimination of EBPl (TC-IIB) binding by mutant pA15 permits the binding of GT-1C (AP-3), which also appears to stimulate transcription, because a template that contains both the pAlO and pA15 mutations gives only 5% enhancer activity in HeLa cells [Nomiyama et al. 1987]. These data are summarized in Figure 8, which shows the disposition on the SV40 enhancer of the transcription factors AP-2, AP-3 [Mitchell et al. 1987], and EBPl.

The binding site for EBPl clearly overlaps the binding sites for both AP-2 and AP-3, as discussed above, and binding of EBPl may exclude binding of AP-2 and AP-3, or vice versa. This poses the question of which proteins are bound to the enhancer in vivo in cell types other than HeLa. Answering this question is important because it has been shown that the regions bound by AP-3 and EBPl can act to mediate the transcriptional response to phorbol esters [Chiu et al. 1987]. Thus, the multimerized C element described by Ondek et al. [1987], which is equivalent to the SV1 oligonucleotide used in this study, can act as a phorbol-ester-responsive element [Chiu et al. 1987]. In addition, mutation of the two Gs at positions 244 and 245 destroys the ability of the multimerized oligonucleotides to act as a phorbol-ester-inducible element [Chiu et al. 1987] and, as we have shown here, abolishes the ability of EBPl to recognize its binding site. The binding site for EBPl is similar to that described for the inducible factor NF-kB [Sen and Baltimore 1986a,b] and the apparently ubiquitous factor H2TFI or KBFI [Baldwin and Sharp 1987, 1988; Israel et al. 1987; Yano et al. 1987]. These proteins may constitute a family of related, but distinguishable, proteins that recognize similar DNA sequences. Synthetic oligonucleotides containing the NF-kB- and H2TFI-binding sites compete for EBPl binding with similar efficiency to the SV1 oligonucleotide [L. Clark and R.T. Hay, in prep.]. However, EBPl can be distinguished from NF-kB and H2TFI/KBFI by a number of criteria. NF-kB binds to a site in the immunoglobulin μ light-chain enhancer [Sen and Baltimore 1986a] but unlike EBPl, which is constitutively produced in HeLa cells, NF-kB binding activity is normally restricted to B cells and its activity is only apparent in other cell types after stimulation with agents such as bacterial lipopolysaccharide and phorbol esters. This induction of NF-kB-binding activity is evident even when the stimulation is carried out in the presence of inhibitors of protein synthesis. Therefore, it appears that prior to induction, NF-kB exists in the cell in an inactive form that is converted to an active form by a post-translational modification undefined as yet [Sen and Baltimore 1986b]. We have also found that NF-kB, induced in mouse B cells, binds to the SV1 oligonucleotide, but the DNA–protein complex formed has an increased electrophoretic mobility when compared to the EBPl/SV1 complex [L. Clark, G. McGarvie, W. Cushley, and R.T. Hay, unpubl.].
The distinguishing features of H2TFI/KBFI are its high affinity for the sequence

\[-TGGGGATTCCCCA-\]
\[-ACCCTAGGGGT-\]

that is found in the enhancers of the mouse H-2 and β2-microglobulin genes and its unique pattern of methylation interference [Baldwin and Sharp 1988; Israel et al. 1987]. As mentioned previously, EBPl binds the SV1 and H2TFI/KBFI sequences with equal affinities, whereas H2TFI/KBFI binds the sequence indicated above with an efficiency 20-fold higher [Baldwin and Sharp 1988]. We have recently carried out a detailed contact point analysis of the interactions of EBPl with its binding site on the SV40 enhancer and have demonstrated that the patterns of methylation interference are distinct from those obtained with H2TFI/KBFI. Methylation interference experiments with H2TFI/KBFI indicate that all of the Gs in the sequence interfere with binding when methylated [Israel et al. 1987; Baldwin and Sharp 1988]. This is in contrast to the pattern obtained with EBPl, where the outermost Gs in the sequence do not interfere with binding when modified [L. Clark, J. Nicholson, and R.T. Hay, in prep.]. An attractive explanation of these differences is that EBPl, NF-κB, and H2TFI/KBFI represent the different modified forms of the same DNA-binding protein. The recent cloning of the gene coding for the DNA-binding domain of a protein that binds to the NF-κB and H2TFI recognition sequences lends support to this idea (Singh et al. 1988).

Although we have no direct evidence for post-translational modification of EBPl, this may account for the observation that two distinct DNA–protein complexes can be detected at early stages of purification. Both complexes have the same DNA-binding specificity and are cross-linked to polypeptides of identical molecular weight (data not shown). After affinity purification, however, only the more slowly migrating complex is detected. This may result from instability of the factor responsible for formation of the faster-migrating DNA–protein complex, modification of the factor during extraction and purification, or removal of associated factors during purification.

The SV40 enhancer, although only ~100 bp long, is an extremely complex transcriptional control unit containing the binding sites for a large number of proteins [Wildeman et al. 1986]. At one level, the enhancer consists of two large domains, which have very little enhancing activity in isolation, but when combined act synergistically to give a large increase in activity [Zenke et al. 1986]. Within each domain, identifiable sequence motifs represent the binding sites for regulatory proteins. In general, single copies of these motifs do not have enhancer activity, but enhancer activity is observed when the motifs are multimerized or combined with other motifs. This suggests that a large nucleoprotein complex is formed in which DNA sequence-specific binding proteins interact with their recognition sites on the genome and with the transcriptional machinery to initiate RNA synthesis directed by RNA polymerase II at the promoter, which may be some distance from the enhancer element.

Understanding the mechanism of enhancer action will require the reconstitution of this process in vitro. Purification of individual transcription factors clearly will be required to attain this goal, and the purification of EBPl is a step in that direction.

Materials and methods

Cell culture

HeLa cells were grown in suspension in Earle’s minimal essential medium (GIBCO), supplemented with 5% newborn calf serum (Seralabs).

Plasmids and labeled fragments

Plasmid pUC1X72 contains the SV40 regulatory region inserted between the EcoRI and BamHI sites of pUC13. The regulatory region was generated from an EcoRI–BamHI fragment of pMKD 231 [Everett et al. 1983], with one copy of the 72-bp repeat element deleted. Plasmid pMKD 231 was a kind gift from R.D. Everett, MRC Institute of Virology, Glasgow, Scotland. Plasmids pA12, pA15, and pA16 (Zenke et al. 1986) were generously provided by P. Chambon, Centre National de la Recherche Scientifique (CNRS), Strasbourg, France. Labeled fragments containing one copy of the 72-bp element were prepared by first cleaving pUC1X72 with BamHI [γ-32P]ATP (Amer­sham; sp. act. 3000 Ci/m mole) and polynucleotide kinase was used to 5'-end label dephosphorylated DNA. [α-32P]ATP [Amersham; sp. act. 3000 Ci/m mole], the other three unlabeled dNTPs, and the large fragment of Escherichia coli DNA polymerase I were used to 3'-end label DNA. Secondary cleavage with PvuII or EcoRI generated fragments of 97 and 173 bp, respectively, both of which contain one copy of the SV40 enhancer element. Fragments were purified on 6% polyacrylamide gels and electroeluted (Hay and DePamphilis 1982). Complementary, synthetic oligonucleotides were annealed by first heating to 100°C in 0.1 M NaCl, 10 mM Tris–HCl (pH 8.0), and 1 mM EDTA, followed by slow cooling to 16°C. The double-stranded oligonucleotides were labeled with [32P]dATP, as described above. Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer, and all contained BamHI–BglIII compatible termini.

Gel electrophoresis DNA-binding assay

Assays contained 0.5–1.0 ng labeled probe (~10,000 cpm), 1 μg of unlabeled DNA (equimolar amounts of poly[d(A-TT)]: poly[d(G-C)]), 20 mM HEPES NaOH (pH 7.5), 1 mM dithiothreitol [DTT], 1 mM EDTA, 10% glycerol, 100 mM NaCl, 0.05% NP-40, and 1–2 μL of protein fraction in a final reaction volume of 20 μL. Binding reactions were allowed to proceed to equilibrium for 20 min at 20°C, after which time 5 μL of 50% glycerol, 25 mM HEPES NaOH (pH 7.5), and 0.1% bromphenol blue were added, and the entire reaction loaded onto 6% [80 : 1 acrylamide / bisacrylamide] polyacrylamide gels containing 0.5 X TBE [50 mM Tris-borate, 0.5 mM EDTA (pH 8.3)]. Electrophoresis was carried out at 200 V for 1 hr. Gels were fixed in 10% acetic acid, dried, and exposed to Fuji RX X-ray film at ~70°C, with an intensifying screen [Dupont]. As a rapid and quantitative means of determining the yield of enhancer-binding activity at each step during the fractionation, bands corresponding to bound DNA were excised from the gels, and their radioactivity was determined by Cerenkov radiation.
counting. One unit of activity represents the retention of 15 fmole specific DNA bound in the DNA–protein complexes in the standard gel electrophoresis DNA-binding assay.

**UV cross-linking**

Double-stranded SV1 oligonucleotide was cloned into the BamHI site of bacteriophage M13mp8. DNA synthesis, primed by the 17-mer sequencing primer, on single-stranded M13 recombinant DNA was catalyzed by the large fragment of *E. coli* DNA polymerase I in the presence of dTTP, dATP, [α-32P]dGTP, and 5-bromo-2′-deoxyuridine triphosphate (Pharmacia). DNA was digested with EcoRI and PstI, and the small fragment containing the EB1-binding site was purified on 6% polyacrylamide gels and electroeluted (Hay and DePamphilis 1982). Binding reactions were prepared in the same way as for gel electrophoresis DNA-binding assays in a total reaction volume of 50 μl. Once equilibrium was reached, samples were irradiated on a TM-36 Transilluminator (UVP, Inc.)—peak wavelength, 302 nm; peak intensity, 7.0 mW/cm²—the samples were packed on ice to maintain a constant temperature throughout irradiation. Digestion with 0.17 units/μl DNase I (Amersham) and/or 0.07 units/μl micrococcal nuclease (Pharmacia) was then carried out for 30 min at 37°C after addition of MgCl₂ and CaCl₂ to a final concentration of 3 mM. Reaction products were electrophoresed on 10% SDS–polyacrylamide gels and visualized by autoradiography.

**DNase I protection**

Reactions contained 0.5–1.0 ng labeled probe (~10,000 cpm), 1 μg unlabeled carrier DNA, poly[d(A-T)]; poly[d(C-G)], 25 mM HEPES NaOH [pH 7.5], 100 mM NaCl, and various amounts of protein fractions, containing 50% glycerol, 1 mM DTT, 1 mM EDTA, and 0.1% NP-40 in a total reaction volume of 50 μl. After 20 min at 20°C, 0.5 units of DNase I (Amersham) in a final concentration of 4 mM MgCl₂, was added, and the reaction allowed to proceed for 60 sec at 20°C, after which time 60 μl of 0.6 M NaOAc, 20 mM EDTA was added. The DNA was then extracted once with phenol and once with chloroform, ethanol-precipitated, washed with 70% ethanol, dried, and redissolved in 10 μl 98% formamide, 20 mM NaOH, 1 mM EDTA with bromphenol blue and xylene cyanol FF. DNA was denatured at 100°C for 2 min, cooled on ice, and reaction products electrophoresed on 6% polyacrylamide gels containing 50% urea. Gels were fixed in 10% acetic acid, dried, and exposed to X-ray film and third round affinity loads, respectively, and 3 mM n-octyl glucopyranoside (Sigma) was included in affinity chromatography buffers [Treichman 1987]. Protein concentrations were determined by the method of Bradford (1976). Protein was precipitated in 10% TCA, washed with 80% acetone, resuspended in SDS/β-mercaptoethanol, and immersed in a boiling water bath for 3 min prior to electrophoresis in thin [0.3 mm] SDS–polyacrylamide gels and silver staining [Bio-Rad] (Laemmli 1970). Oakley et al. (1980).

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**References**

Angel, P., M. Imagawa, R. Chiu, B. Stein, R.J. Imbra, H.J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* 49: 729–739.

Baldwin, A.S., Jr., and P.A. Sharp. 1987. Binding of a nuclear factor to a regulatory sequence in the promoter of the mouse H-2Kb class I major histocompatibility gene. *Mol. Cell. Biol.* 7: 305–313.

——. 1988. Two transcription factors, NF-kB and H2TFI, interact with a single regulatory sequence in the class I major histocompatibility complex promoter. *Proc. Natl. Acad. Sci.* 85: 723–727.

Banerji, J., L. Olsen, and W. Schaffner. 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. *Cell* 33: 729–740.

Banerji, J., S. Rusconi, and W. Schaffner. 1981. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. *Cell* 27: 299–308.

Barrett, P., L. Clark, and R.T. Hay. 1987. A cellular protein binds to a conserved sequence in the adenovirus type 2 enhancer. *Nucleic Acids Res.* 15: 2719–2735.

Brady, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.

Challberg, M.D. and T.J. Kelly. 1979. Adenovirus DNA replica-
tion in vitro. *Proc. Natl. Acad. Sci.** 76: 655–659.

Chandler, V.L., B.A. Maler, and K.R. Yamamoto. 1983. DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive in vivo. *Cell** 33: 489–499.

Chiu, R., M. Imagawa, R.J. Imbra, J.R. Bockoven, and M. Karin. 1987. Multiple cis- and trans-acting elements mediate the transcriptional response to phorbol esters. *Nature** 329: 648–651.

Davidson, I., C. Fromental, P. Augereau, A. Wildeman, M. Zenke, and P. Chambon. 1986. Cell-type specific protein binding to the enhancer of simian virus 40 in nuclear extracts. *Nature** 323: 544–548.

Everett, R.D., D. Baty, and P. Chambon. 1983. The repeated GC-rich motifs upstream from the TATA box are important elements of the SV40 early promoter. *Nucleic Acids Res.* 11: 2447–2464.

Fried, M. and D.M. Crothers. 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 9: 6505–6525.

Galas, D.J. and A. Schmitz. 1978. DNase footprinting: A simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* 5: 3157–3170.

Garner, M.M. and A. Rezvzin. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions. Applications to components of the E. coli lac operator regulatory system. *Nucleic Acids Res.* 9: 3047–3060.

Gillies, S.D., S.L. Morrison, V.T. Oi, and S. Tonegawa. 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. *Cell* 33: 717–728.

Goodbourn, S., H. Burstein, and T. Maniatis. 1986. The human β-interferon gene enhancer is under negative control. *Cell* 45: 601–610.

Goodbourn, S., K. Zinn, and T. Maniatis. 1985. Human β-interferon gene expression is regulated by an inducible enhancer element. *Cell* 41: 509–520.

Hay, R.T. and M.L. DePamphilis. 1982. Initiation of SV40 DNA replication in vivo: Location and structure of δ’ ends of DNA synthesized in the ori region. *Cell* 28: 767–779.

Herr, W. and J. Clarke. 1986. The SV40 enhancer is composed of multiple functional elements that can compensate for one another. *Cell* 45: 461–470.

Imagawa, M., R. Chiu, and M. Karin. 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: Protein kinase C and cAMP. *Cell* 51: 251–260.

Israel, A., A. Kimura, M. Kieran, O. Yano, J. Kanellopoulos, O. Le Bail, and P. Kourilsky. 1987. A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and β2-microglobulin genes. *Proc. Natl. Acad. Sci.* 84: 2653–2657.

Johnson, P.F., W.H. Landschultz, B.J. Graves, and S.L. McKnight. 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev.* 1: 133–146.

Jones, N.C., P.W.J. Rigby, and E.B. Ziff. 1988. Trans-acting protein factors and the regulation of euarkytic transcription: Lessons from studies on DNA tumor viruses. *Genes Dev.* 2: 267–281.

Kadonaga, J.T. and R. Tjian. 1986. Affinity purification of sequence-specific DNA binding proteins. *Proc. Natl. Acad. Sci.* 83: 5889–5893.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Laimins, L.A., G. Khoury, C. Gorman, B. Howard, and P. Gruss. 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. *Proc. Natl. Acad. Sci.* 79: 6453–6457.

Lee, W., P. Mitchel, and R. Tjian. 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49: 741–752.

Maxam, A.M. and W. Gilbert. 1980. Sequencing end labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65: 499–560.

Mitchell, P., C. Wang, and R. Tjian. 1987. Positive and negative regulation of transcription in vitro: Enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* 50: 847–861.

Moreau, P., R. Hen, B. Wasylyk, R. Everett, M.P. Gaub, and P. Chambon. 1981. The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. *Nucleic Acids Res.* 9: 6047–6068.

Nomiyama, H., C. Fromental, J.H. Xiao, and P. Chambon. 1987. Cell-specific activity of the constituent elements of the simian virus 40 enhancer. *Proc. Natl. Acad. Sci.* 84: 7881–7885.

Oakley, B.R., D.R. Kirsch, and N.R. Morris. 1980. A simplified method for the detection of protein-DNA binding specificity. *Anal. Biochem.* 105: 361–363.

Ogata, R.T. and W. Gilbert. 1977. Contacts between the lac repressor and thymines in the lac operator. *Proc. Natl. Acad. Sci.* 74: 4973–4976.

Ohrsson, H. and T. Edlund. 1986. Sequence-specific interactions of nuclear factors with the insulin gene enhancer. *Cell* 45: 35–44.

Ondek, B., A. Shepard, and W. Herr. 1987. Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities. *EMBO J.* 6: 1017–1025.

Rosenfeld, P.J. and T.J. Kelly. 1986. Purification of nuclear factor I by DNA recognition site affinity chromatography. *J. Biol. Chem.* 261: 1398–1408.

Schirm, S., J. Jiricny, and W. Schaffner. 1987. The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity. *Genes Dev.* 1: 65–74.

Seng, R. and D. Baltimore. 1986a. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46: 705–716.

—.—. 1986b. Inducibility of k immunoglobulin enhancer-binding protein NF-kB by a posttranslational mechanism. *Cell* 47: 921–928.

Singh, H., J.H. LeBowitz, A.S. Baldwin, Jr., and P.A. Sharp. 1988. Molecular cloning of an enhancer binding protein: Isolation by screening of an expression library with a recognition DNA site. *Cell* 52: 415–423.

Suck, D., A. Lahm, and C. Oefner. 1988. Structure refined to 2Å of a nicked DNA octanucleotide complex with DNase I. *Nature* 332: 464–468.

Treisman, R. 1985. Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. *Cell* 42: 889–902.

—.—. 1987. Identification and purification of a polypeptide that binds to the c-fos serum response element. *EMBO J.* 6: 2711–2717.

Weiber, H., M. Konig, and P. Gruss. 1983. Multiple point mutations affecting the simian virus 40 enhancer. *Science* 219: 626–631.

Wildeman, A.G., M. Zenke, C. Schatz, M. Wintzerith, T. Grundstrom, H. Matthes, K. Takahashi, and P. Chambon. 1986. Specific protein binding to the simian virus 40 enhancer in vitro. *Mol. Cell Biol.* 6: 2098–2105.

**SV40 enhancer-binding domain**
Clark et al.

Xiao, J.H., I. Davidson, M. Macchi, R. Rosales, M. Vigneron, A. Staub, and P. Chambon. 1987a. In vitro binding of several cell-specific and ubiquitous nuclear proteins to the GT-1 motif of the SV40 enhancer. Genes Dev. 1: 794–807.

Xiao, J.H., I. Davidson, D. Ferrandon, R. Rosales, M. Vigneron, M. Macchi, F. Ruffenach, and P. Chambon. 1987b. One cell-specific and three ubiquitous nuclear proteins bind in vitro to overlapping motifs in the domain B1 of the SV40 enhancer. EMBO J. 6: 3005–3013.

Yano, O., J. Kanellopoulos, M. Kieran, O. Le Bail, A. Israel, and P. Kourilsky. 1987. Purification of KBFI, a common factor binding to both H-2 and β2-microglobulin enhancers. EMBO J. 6: 3317–3324.

Zenke, M., T. Grundstrom, H. Matthes, M. Wintzerith, C. Schatz, A. Wildeman, and P. Chambon. 1986. Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. 5: 387–397.

Zinn, K. and T. Maniatis. 1986. Detection of factors that interact with the human β-interferon regulatory region in vivo by DNase I footprinting. Cell 45: 611–618.
Identification and purification of EBP1: a HeLa cell protein that binds to a region overlapping the 'core' of the SV40 enhancer.

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**References**

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