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.

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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 sites 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* (Nomiya 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

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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, EBP1, 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 *PvuII* 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 ³²P-labeled *Bam*HI–*Eco*RI fragment derived from pUC1X72 was incubated with a nuclear extract from HeLa cells that had been partially purified by DEAE–Sephacrose 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 ³²P-labeled DNA fragment of 97 bp, which extends from the *Bam*HI to the *PvuII* 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 ³²P-labeled *Eco*RI–*Bam*HI 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-Sephrose and, after reduction of the NaCl concentration was applied to a second DEAE-Sephrose 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 pUC1X72, were incubated with 2 μg nuclear extract (partially purified over DEAE-Sephrose) in the standard gel electrophoresis DNA-binding assay, as described in Materials and Methods (lanes - 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.

Figure 2. DNase I protection of the SV40 enhancer by partially purified enhancer-binding factors. A *Bam*HI–*Eco*RI fragment from pUC1X72, 5'-end labeled at the *Bam*HI site, was incubated with 40 μ g of nuclear extract (DEAE–Sephacrose fraction) and digested with DNase I, as described in Materials and methods (lane – contained no nuclear extract). Included in the reaction mixture was 100 ng of unlabeled double-stranded oligonucleotides, SV1, SV1.M1, SV1.M2, SV2, or SV3. After treatment with DNase I, reaction products were electrophoresed in a 6% denaturing polyacrylamide gel, and the cleavage products visualized by autoradiography. Oligonucleotides SV1.M1 and SV1.M2 are derivatives of SV1 in which cytosines 244 and 245 have been altered to guanines (SV1.M1) and thymine 241 has been altered to adenine (SV1.M2).

Table 1. Purification of EBP1 from HeLa cell nuclear extracts

Fraction	Protein (mg)	Units ^a	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

^a 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.

^b Estimate based on comparative silver staining.

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–Sephacrose 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 μ g 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 EBP1.

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

Figure 3. Fractionation of HeLa nuclear extract. Dialyzed nuclear extract was loaded onto a DEAE-Sephacel CL-6B column, and bound proteins eluted with a linear gradient of 0.05–0.4 M NaCl. Fractions were incubated with ^{32}P -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-Sephacel CL-6B column; (L, F2, W) column load, flow through, and 0.05 M NaCl wash from the second DEAE-Sephacel 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 mo-

lecular 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 EBP1 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 NF1 or NF1B 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 EBP1. (A) Poly[d(G-C)] and poly[d(A-T)] were added to the DEAE-Sephacel fractions containing EBP1 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 EBP1 activity. (L) Load; (FT) flowthrough; (W) wash; (1–8) fractions from 1 M NaCl elution. (B) Fractions from each stage of the EBP1 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-Sephacel fractions; (1, 2, and 3) first, second, and third-round affinity column eluates; (M) protein molecular weight standards.

Figure 5. Bromodeoxyuracil cross-linking of EBP1 to its binding site on the SV40 genome. A bromodeoxyuridine-substituted, ^{32}P -labeled double-stranded DNA fragment containing the EBP1-binding site was prepared as described in Materials and methods. Probe DNA was incubated with affinity-purified EBP1 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, EBP1 was allowed to bind in the presence of 0.1 μg SV1, 0.5 μg SV1, 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 EBP1

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 EBP1, 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 SV1 and SV1.M2 oligonucleotides can compete for binding of the factor, whereas the SV1.M1 oligonucleotide is incapable of competing (Fig. 6).

Interaction of the purified factor with ^{32}P -labeled SV1 double-stranded oligonucleotide was monitored in the gel electrophoresis DNA-binding assay. Whereas unlabeled SV1 oligonucleotide could compete efficiently for the binding of the purified factor, the SV1.M1 oligonucleotide with G $\rightarrow$ C changes at positions 244 and 245

Figure 6. DNase I protection of the SV40 enhancer by affinity-purified EBP1. The *Bam*HI-*Pvu*II fragment from pUC1X72, 3' ^{32}P labeled at the *Bam*HI site, was incubated with 0, 5, 10, or 20 μl of affinity-purified EBP1, as described in Materials and methods. Competition assays were carried out in which 10 μl of EBP1 was incubated with labeled probe in the presence of 500 ng of unlabeled SV1, SV1.M1, and SV1.M2 double-stranded oligonucleotide. After treatment with DNase I, reaction products were electrophoresed in a 6% denaturing polyacrylamide gel. C + T- and A + G-specific cleavage reactions of the labeled fragment were electrophoresed in parallel as markers.

was unable to do so. This was also true of the SV1.M3 oligonucleotide, which contained C $\rightarrow$ 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 EBP1 to three of these scanning point mutants, pA12, pA15, and pA16, in the gel electrophoresis DNA-binding assay. Affinity-purified EBP1 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 EBP1 binding, the mutations present in p12 and pA15 reduce EBP1 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 EBP1. (A) Competition analysis of EBP1 binding. Reactions contained 0.5 ng of ^{32} P-labeled, double-stranded SV1 oligonucleotide, 1.0 μ g of unlabeled poly[d(A-T)] : poly[d(C-G)], and 1.0 μ l of affinity-purified EBP1 (lane - contained no EBP1). 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 EBP1 to pA mutants. DNA fragments containing wild-type and mutant enhancers were excised by *Bam*HI and *Pvu*II digestion and 3'-end labeled with ^{32} P at the *Bam*HI site. Labeled DNA was incubated with EBP1 (as described in A), and DNA-protein complexes resolved on a 6% polyacrylamide gel. DNA templates utilized are indicated above each lane; (-) EBP1 was omitted from the binding reactions. (C) DNA sequence of the GT-I and TC-II motifs and summary of EBP1 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, EBP1, 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^A_T^A_T 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 EBP1 because the SV2 double-stranded oligonucleotide, which contains this sequence and spans the GT-II motif, fails to compete for EBP1 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 EBP1. 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 EBP1 (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). EBP1 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 EBP1 binding is abolished (Fig. 7), thus indicating that EBP1 and AP-2 have distinct recognition sites. EBP1 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 EBP1 from AP-3. We have recently established the importance of these mutated bases in a detailed contact point analysis of EBP1 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 EBP1 protein (L. Clark, J. Nicholson, and R.T. Hay, in prep.).

The role of EBP1 in transcriptional activation of the SV40 genome is suggested by correlating the in vivo activity of wild-type and mutated enhancers with EBP1 binding. Tandemly ligated copies of an oligonucleotide containing the EBP1-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 EBP1 (Figs. 1, 2, 6, and 7). Nomiyama et al. (1987) have examined the activity of the pA series of triple point mutants in the SV40 enhancer (Zenke et al. 1986) in a variety of cell types and correlated the activity of the various enhancer motifs with the presence of *trans*-acting factors. One cell-type-specific protein, GT-IC (probably equivalent to AP-3), present in HeLa but not in MCP11 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 EBP1. 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 (pA10 and pA11), which eliminate GT-IC binding but have no effect on the binding of TC-IIA, TC-IIB, or EBP1, have no effect in MCP11 cells where GT-IC is absent. In HeLa cells, mutations pA10 and pA11 have only a minimal effect on enhancer activity, whereas mutation pA15, which abolishes binding of TC-IIA, TC-IIB, and EBP1, reduces transcription *in vivo* to 40–50% of wild type. Because binding of GT-IC (AP-3) and EBP1 (TC-IIB) is eliminated by pA12, these proteins may compete for the same binding site in HeLa cells. The minimal effect of mutations pA10 and pA11 in HeLa cells suggests that EBP1 (TC-IIB) is bound to the template in preference to GT-IC. Elimination of EBP1 (TC-IIB) binding by mutant pA15 permits the binding of GT-IC (AP-3), which also appears to stimulate transcription, because a template that contains both the pA10 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 and EBP1 (Mitchell et al. 1987), and EBP1.

The binding site for EBP1 clearly overlaps the binding sites for both AP-2 and AP-3, as discussed above, and binding of EBP1 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 EBP1 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 EBP1 to recognize its

Figure 8. Disposition of transcription factors AP-2, AP-3, and EBP1 on the SV40 enhancer. The DNase I footprint boundaries for AP-2 and AP-3 on the SV40 enhancer DNA sequence are from Mitchell et al. (1987), and EBP1 is from this study. However, it should be stressed that these boundaries probably represent an overestimate of the region occupied by the DNA-binding proteins, because DNase I is a relatively large molecule and requires an extended binding surface for DNA cleavage (Suck et al. 1988). The effects of mutations in this region on transcription *in vivo* are from Zenke et al. (1986) (with permission). Altered bases in the mutated SV1 oligonucleotides SV1.M1 and SV1.M3, which abolish EBP1 binding, are underlined.

binding site. The binding site for EBP1 is similar to that described for the inducible factor NF- κ B (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- κ B- and H2TFI-binding sites compete for EBP1 binding with similar efficiency to the SV1 oligonucleotide (L. Clark and R.T. Hay, in prep.). However, EBP1 can be distinguished from NF- κ B and H2TFI/KBFI by a number of criteria. NF- κ B binds to a site in the immunoglobulin κ light-chain enhancer (Sen and Baltimore 1986a) but unlike EBP1, which is constitutively produced in HeLa cells, NF- κ B 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- κ B-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- κ B 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- κ B, 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 EBP1/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–
–ACCCCTAAGGGGT–

that is found in the enhancers of the mouse H-2 and β 2-microglobulin genes and its unique pattern of methylation interference (Baldwin and Sharp 1988; Israel et al. 1987). As mentioned previously, EBP1 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 EBP1 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 EBP1, 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 EBP1, NF- κ B, 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- κ B and H2TFI recognition sequences lends support to this idea (Singh et al. 1988).

Although we have no direct evidence for post-translational modification of EBP1, 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 EBP1 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 - ^{32} P]ATP (Amersham, sp. act. 3000 Ci/mmol) and polynucleotide kinase were used to 5'-end label dephosphorylated DNA. [α - ^{32} P]dATP (Amersham, sp. act. 3000 Ci/mmol), 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 [^{32} P]dATP, as described above. Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer, and all contained *BamHI*–*BglII* compatible termini.

Gel electrophoresis DNA-binding assay

Assays contained 0.5–1.0 ng labeled probe (~10,000 cpm), 1 μ g of unlabeled DNA (equimolar amounts of poly[d(A-T)] : 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 μ l of protein fraction in a final reaction volume of 20 μ l. Binding reactions were allowed to proceed to equilibrium for 20 min at 20°C, after which time 5 μ l of 50% glycerol, 25 mM HEPES NaOH (pH 7.5), and 0.1% bromophenol blue were added, and the entire reaction loaded onto 6% (80 : 1 acrylamide / bisacrylamide) polyacrylamide gels containing 0.5 $\times$ 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 *Bam*HI 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 - ^{32} P]dGTP, and 5-bromo-2'-deoxyuridine triphosphate (Pharmacia). DNA was digested with *Eco*RI and *Pst*I, and the small fragment containing the EBP1-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 μ l. 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/ μ l DNase I (Amersham) and/or 0.07 units/ μ l 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 μ g 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 μ l. 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 μ l 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 μ l 98% formamide, 20 mM NaOH, 1 mM EDTA with bromophenol 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 (Hyperfilm MP; Amersham) at –70°C in the presence of an intensifying screen. Sequence markers were prepared by subjecting end-labeled fragments to G + A- or C + T-specific cleavage reactions (Maxam and Gilbert 1980).

Purification of EBP1 from HeLa nuclear extract

Uninfected HeLa cells (2×10^{10}) were fractionated into nuclei and cytosol, as described previously (Challberg and Kelly 1979). After washing three times with 25 mM HEPES NaOH (pH 7.5), 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM DTT, 0.2 M sucrose, and 1 mM phenylmethylsulfonyl fluoride (PMSF), the nuclei were resuspended in the same buffer containing 0.35 M NaCl and stirred on ice for 30 min. Particulate material was removed by centrifugation at 100,000g for 60 min. The supernatant was adjusted to 0.3 M NaCl and applied to a DEAE-Sephrose CL-6B (Pharmacia) column equilibrated with buffer A [25 mM HEPES NaOH (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 1 mM PMSF, 20% glycerol] containing 0.3 M NaCl. The flowthrough was dialyzed against buffer A containing 50 mM NaCl, and insoluble material was removed by centrifugation at 100,000g for

30 min. The clarified extract was then applied to a second DEAE-Sephrose column equilibrated with buffer A containing 50 mM NaCl. After washing with two column volumes of equilibration buffer, the column was developed with a linear gradient of 50–400 mM NaCl. Fractions containing EBP1 activity were pooled, mixed with carrier DNA [poly[d(A-T)] : poly[d(C-G)]], and subjected to three rounds of recognition site affinity chromatography on DNA affinity columns prepared with the sequence

5'-GATCTAGGGTGTGGAAAGTCCCG-3'
3'-ATCCCACACCTTTCAGGGCCTAG-5'

essentially as described by Kadonaga and Tjian (1986), except that 400, 50, and 5 μ g of carrier was added to the first, second, and third round affinity loads, respectively, and 3 mM *n*-octyl glucopyranoside (Sigma) was included in affinity chromatography buffers (Treisman 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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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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