A 100-kD HeLa cell octamer binding protein (OBP100) interacts differently with two separate octamer-related sequences within the SV40 enhancer

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Numerous eukaryotic upstream promoter and enhancer regions contain a functional octamer sequence ATGCAAAT. We have examined the interactions between an octamer binding protein isolated from HeLa cells and the SV40 and immunoglobulin heavy-chain (IgH) gene enhancers. A partially purified octamer binding activity forms a single complex with the IgH enhancer octamer in a gel retardation assay, but two complexes with a SV40 enhancer fragment containing a single 72-bp element. By using point mutants and both dimethyl sulfate and diethyl pyrocarbonate modification interference assays, we show that the SV40 complexes result from binding of a factor to the octamer-related sequence ATGCAAAG (Octa1) and to an adjacent previously unidentified octamer-related sequence ATGCATCT (Octa2). The base-specific interactions with Octa1 and Octa2 differ; chemical modifications over a 10-bp sequence TATGCAAAGC affect Octa1 binding whereas Octa2 binding is affected by modifications spanning a 13-bp sequence ATGCATCTCAATT in which the octamer-like sequence is not centered. The octamer binding activity has been purified extensively by a DNA affinity precipitation procedure and SDS-polyacrylamide gel electrophoresis. The purified protein, OBP100, has an apparent molecular weight of 100 kD and binds both SV40 Octa1 and Octa2, as well as the IgH enhancer. The distinct interactions of OBP100 with the differently sized Octa1 and Octa2 binding sites suggest remarkably flexible sequence recognition between OBP100 and its binding sites.

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Eukaryotic RNA polymerase II promoters frequently appear as a patchwork of *cis*-acting elements that are recognized by a combination of *trans*-acting factors. Such complexity is exemplified by the SV40 early promoter. This prototypical promoter contains, in addition to one or more TATA-like elements (Takahashi et al. 1986), upstream elements in the form of six GC boxes that can interact with the transcription factor Sp1 (Dynan and Tjian 1983a, b; Briggs et al. 1986), and multiple enhancer elements within or upstream of a 72-bp tandem repeat (Herr and Clarke 1986; Zenke et al. 1986). The SV40 enhancer region contains at least three functional elements, A, B, and C, that were defined genetically by the patterns of enhancer duplication observed in revertants of SV40 enhancer point mutants isolated in the African green monkey kidney cell line CV-1 (Herr and Gluzman 1985; Herr and Clarke 1986). These elements are functionally redundant in CV-1 cells because duplication of any one element can compensate for loss of function in the remaining two elements (Herr and Gluzman 1985; Clarke and Herr 1987).

The A, B, and C elements can be functionally differentiated, however, because synthetic enhancers composed of multiple oligomerized copies of each of these three elements (Ondek et al. 1987) or related sequences (Schirm et al. 1987) exhibit different cell-specific enhancer activities. The different activities are consistent with the interaction of these elements with different nuclear factors (Davidson et al. 1986; Bohmann et al. 1987; Johnson et al. 1987; Mitchell et al. 1987). Indeed the SV40 enhancer region contains numerous binding sites for distinct nuclear factors (Davidson et al. 1986; Wildeman et al. 1986; Bohmann et al. 1987; Lee et al. 1987a; Piette and Yaniv 1987). Recently some of these factors have been purified extensively from the human HeLa cell line (Angel et al. 1987; Lee et al. 1987b; Mitchell et al. 1987) or from rat liver (Johnson et al. 1987). How these factors and multiple enhancer elements combine to promote transcription is poorly understood.

Factors that interact with the SV40 early promoter also bind to other mammalian promoters [for review, see

McKnight and Tjian 1986). For example, an activity identified by Bohmann et al. (1987) in a partially purified HeLa cell nuclear extract binds to the SV40 enhancer, a U2 small nuclear RNA gene promoter, and the immunoglobulin heavy chain (IgH) gene enhancer. The binding sites all contain a sequence similar to the 8-bp sequence ATGCAAAT. This octamer sequence was first characterized within an extremely well-conserved 13-bp sequence upstream of the TATA box of H2B histone genes (Harvey et al. 1982) and subsequently within immunoglobulin gene upstream promoter and enhancer regions (Falkner and Zachau 1984; Parslow et al. 1984). Although this sequence is commonly referred to as the octamer element, in some studies the region of sequence conservation has been extended to the decamer sequence ATGCAAATNA (Falkner and Zachau 1984; Falkner et al. 1986). The nuclear factor NF-A1, found in all cell lines tested including HeLa cells, binds to the octamer sequence (Singh et al. 1986; Staudt et al. 1986; see also Sive and Roeder 1986; Bohmann et al. 1987). A potentially related factor involved in adenovirus DNA replication has also been identified that interacts with an octamer-like sequence and is called either NFIII (Prujn et al. 1986) or ORP-C (Rosenfeld et al. 1987).

We have identified and purified a 100-kD octamer binding protein from HeLa cells (OBP100) that binds to two adjacent but different octamer-related sequences within the SV40 enhancer. The resemblance to the octamer consensus at one of these sites has been noted previously (Falkner and Zachau 1984) and lies within the SV40 enhancer B element; the octamer relation of the second site has not been noted previously. Characterization of the base-specific interactions at these two sites by chemical modification interference and gel retardation analyses indicates that this octamer binding protein associates differently with each site.

Results

Identification of a HeLa cell factor(s) interacting with the B element of the SV40 enhancer

A diagram of the SV40 early promoter showing the location of the A, B, and C enhancer elements is shown in Figure 1A. These three elements can each enhance transcription in HeLa cells (Ondek et al. 1987). To assay for SV40 enhancer binding proteins, HeLa cell extracts were prepared and fractionated by heparin-agarose chromatography. DNA binding activities were detected by the gel retardation assay (Fried and Crothers 1981; Garner and Revzin 1981; Carthew et al. 1985), using a 99-bp *PvuII*–*Bam*HI restriction fragment (from pAO; Zenke et al. 1986) that spans one copy of each of the A, B, and C enhancer elements (Fig. 1A). Figure 1B shows the results of such an assay using an 0.1–0.2 M KCl heparin-agarose fraction of a HeLa whole-cell extract. Two major complexes, CI and CII, are readily detectable with the SV40 enhancer probe (lane 2) and are absent with a nonspecific pUC119-derived probe (lane 1). Both of these complexes disappear upon addition of excess unlabeled SV40 probe (lanes 3–5) indicating a specific interaction with the

SV40 enhancer. To determine the sequences responsible for the retardation activity, competition experiments were performed with separate fragments containing six tandem copies of either the A, B, or C SV40 enhancer elements. Addition of the A or C element fragments has little effect on complex formation (Fig. 1B; lanes 6–8 and 12–14), whereas addition of increasing amounts of the B element fragment (6XB17) first abolishes complex CII (lane 9) and then complex CI (lanes 10 and 11). These results suggest one or more specific interactions with the SV40 B element.

Figure 2A shows the SV40 enhancer sequences in the region of the B element. The 18-bp SV40 sequence reiterated in the 6XB17 fragment is bracketed; this sequence contains an imperfect 9-bp repeat that has been referred to as the SphI and SphII motifs (Zenke et al. 1986). The tandem Sph motifs form a sequence with a 7- out of 8-base match with the octamer sequence (ATGCAAAG; Falkner and Zachau 1984). This sequence is referred to here as SV40 Octa1. Flanking the Octa1 element is a second octamer-related sequence ATGCATCT. This octamer-like sequence has not been noted previously and we refer to it as SV40 Octa2 (Fig. 2A). We show below that the two complexes, CI and CII, are the result of independent factor interactions with Octa1 and Octa2. Complex CI results from binding of a single factor to either site and complex CII is the result of binding to both sites.

Complexes CI and CII result from independent factor interactions at adjacent sites within the SV40 enhancer

The activity of the SV40 enhancer B element is abolished when a pair of point mutations called *dpm2* are introduced into the element (Herr and Gluzman 1985; Ondek et al. 1987; see Fig. 2A). This double point mutation alters the SphI and Octa2 motifs but does not affect the SphII or Octa1 motifs. Figure 2B shows the effects of these point mutations on complex formation with the partially purified HeLa cell fraction. The *dpm2* mutations reduce the level of complex CII formation but not the level of complex CI (compare lanes 1 and 2). To examine the effects of an analogous set of mutations in the SphII/Octa1 motifs, we created the *dpm8* mutations shown in Figure 2A. [These mutations also abolish the activity of the B element (unpubl.; see Discussion).] The *dpm8* mutations affect complex CII formation more severely than the *dpm2* mutations do but still have only a weak effect on complex CI formation (lane 3). That these two sets of mutations probably affect different binding sites is shown by the lack of complex formation with the double mutant *dpm28* (lane 4). The combined effects of these mutations in the repeated motifs suggest that these motifs constitute two adjacent recognition sites, complex CI being the result of binding to either one of the two sites and complex CII the result of binding at both sites. Consistent with this hypothesis is the fact that increasing the amount of extract used in the gel retardation assay increases complex CII formation with concomitant decrease in complex CI (data not shown).

This interpretation predicts that with the *dpm2* and *dpm8* mutants the binding site involved in complex CI formation will differ. To test this hypothesis we exam-

ined the DNA-protein interactions in complexes CI and CII in detail by assaying the effects of DNA modification by dimethyl sulfate (DMS) and diethyl pyrocarbonate

Figure 1. Identification of a HeLa cell SV40 B element binding activity. (A) Early promoter region of SV40 with one copy of the 72-bp element. Represented from right to left are the early transcription start sites by the wavy arrow, the origin (*ori*) of replication, the A/T-rich TATA sequence and the 21-bp repeats with Sp1 binding sites shown as black boxes. Below the open rectangle representing the 72-bp element is shown the position of the A, B, and C enhancer elements by the stippled boxes. The position of the *PvuII* and *BamHI* (Zenke et al. 1986) restriction sites used to prepare fragments for gel retardation are indicated. (B) Binding assays were performed using a heparin-agarose fractionated (0.1–0.2 M KCl) HeLa whole-cell extract together with the labeled SV40 *PvuII*–*BamHI* fragment (lanes 2–14) or a pUC *PvuII*–*BamHI* fragment of similar size (lane 1) and were analyzed by electrophoresis through 6% polyacrylamide gels in Tris-glycine buffer (see Methods). The free probe (F), and the two retarded complexes CI and CII are indicated. The complexes were competed with 1-, 10-, and 100-fold molar amount of unlabeled *PvuII*–*BamHI* SV40 enhancer fragment (lanes 3–5) or fragments containing the A (lanes 6–8), B (lanes 9–11) or C (lanes 12–14) enhancer elements (note that the A, B, and C element fragments contain six reiterated sites per fragment).

Figure 2. Double point mutations affect the B element binding activity. (A) The nucleotide sequence surrounding the B enhancer element is shown. The left 22 bp correspond to the B element as identified previously (Herr and Clarke 1986) and the 18-bp SV40 sequence created by concatenation of the 17-bp B17 oligonucleotide in 6XB17 is shown by the bracket above the sequence. The base changes corresponding to *dpm2* and *dpm8* are displayed above the sequence. Below the sequence the two Sph repeats are identified by the arrows, with the one base mismatch between the two repeats indicated by the Xs. The two adjacent octamer related sequences Octa1 and Octa2 are bracketed; the Xs indicate the bases differing from the consensus ATGCAAAT. (B) The wild-type (lane 1), and *dpm2* (lane 2), *dpm8* (lane 3), and *dpm28* (lane 4) mutants were assayed by gel retardation of the corresponding SV40 *PvuII*–*Bam*HI fragment as described in Fig. 1. Lane 5 contains a 208-bp *Bam*HI–*PvuII* pUC119 fragment.

(DEPC) on complex formation with the wild-type and mutant SV40 enhancer fragments.

In general, modification of bases that normally form a contact with a bound protein will disrupt complex formation. These sites are thus underrepresented in the complexed probes relative to the free DNA (Siebenlist and Gilbert 1980). Figure 3A shows the results of methylation interference at guanines with the wild-type, *dpm2*, and *dpm8* *PvuII*–*Bam*HI restriction fragments labeled on either strand at the *Bam*HI site. The end-labeled DNAs were modified with DMS and used in a preparative gel retardation assay. The resulting free and complexed DNAs were eluted from the gel and cleaved at sites of modification by treatment with piperidine (Maxam and Gilbert 1980), and the modified sites were displayed by fractionation on a denaturing polyacrylamide gel. The effect of DMS modification is not readily apparent on complex CI formation of the wild-type probe (compare the pattern of the free DNAs in lanes 1 and 8 to the CI DNAs shown in lanes 2 and 9). Complex CII formation, however, is clearly affected by modifica-

Figure 3. Methylation and carbethoxylation interference analyses indicate different interactions of the HeLa cell binding factor with two adjacent octamer-related sequences. (A) The *PvuII*–*Bam*HI wild type, *dpm2*, and *dpm8* containing probes either 3' (early mRNA sense strand) or 5' (late mRNA sense strand) end-labeled at the *Bam*HI site were treated with DMS and used in a preparative gel retardation assay with a heparin-agarose purified whole-cell extract fraction. Free (F) and complexed (CI and CII) DNAs were recovered from the gel and cleaved with piperidine and equivalent cpm of each sample were loaded onto a 16% polyacrylamide sequencing gel (see Methods). The position of the octamer related sequences Octa1 and Octa2 are shown by brackets. The G residues referred to in the text are labeled. (B) The *PvuII*–*Bam*HI wild-type and *dpm2* probes either 3' or 5' end-labeled at the *Bam*HI site were treated with DEPC and used in a binding assay as in A (see Methods). An A + G marker lane prepared by treatment of the wild-type probe with formic acid (Maxam and Gilbert 1980) shows the location of purines. The DEPC modification generates an A > G pattern. The two adjacent Octa1 and Octa2 sequences are shown by the brackets and the A residues spanning this region are labeled. The changes in the upper regions of lanes 2–4 and 8–10 (and also A, lane 3) are due to nuclease activity during the gel retardation assay because the bands in complexes CI and CII appear in the absence of piperidine-induced cleavage of the modified DNA. (C) Summary of the DMS and DEPC modification interference data. The SV40 enhancer sequence (nucleotides 185–220) surrounding and including the Octa1 and Octa2 binding sites is shown at the top of the panel. The guanine and adenine residues whose methylation or carbethoxylation, respectively, interferes with complex I formation in the *dpm2* mutant are marked by dots and those that interfere in the *dpm8* mutant are marked by triangles. The nucleotides affected by the *dpm8* and *dpm2* mutations are shown by the bars in the Octa1 and Octa2 sequences, respectively. The effects of guanine modification by DEPC generally parallel the methylation effects but are less easily interpreted than the DMS modifications because of the weaker reactivity of DEPC with guanines. The large symbols indicate a three-fold or greater reduction in band intensity while the smaller symbols are about a two- to threefold reduction in band intensity. The lower portion of the panel compares the interferences at equivalent bases between the Octa1 and Octa2 binding sites. Positions of sequence differences between the two binding sites are indicated by the dashes.

Figure 3. (See facing page for legend.)

tion of guanines G201 and G210 on the early strand (lane 3) and G200, G204, and G209 on the late strand (lane 10). These results are consistent with the interpretation that complex CI is the result of binding to either of two sites—when one site is adversely modified complex CI can still form by association with the unmodified site. Because complex CII results from binding to both sites, modification of either site affects complex CII formation, thus the clear appearance of interfering modifications.

These interpretations predict that complex CI formation by the *dpm2* and *dpm8* mutants will be disrupted by modifications within the remaining wild-type site. Consistent with this prediction complex CI formation is inhibited with the *dpm2* probe by modification of residues G210 (lane 5), G204, and G209 (lane 12) in the unmutated site, but not by modification of residue G201 which lies between the *dpm2* mutations (see lane 5). Furthermore, the *dpm8* mutations have the opposite pattern as methylation of residues G201 (lane 7) and G200 (lane 14) prevents complex CI formation but modified residues G204 and G210, the latter of which lies between the *dpm8* mutations, do not affect complex CI formation. The patterns generated by the *dpm2* and *dpm8* mutants in complex CI are complementary subsets of the interferences observed with the wild-type probe in complex CII. These results show that complex CI can form by independent interaction of a factor(s) with either of the two sites affected by *dpm2* and *dpm8* and that complex CII results from simultaneous interaction at both sites.

To examine these interactions in greater detail, we have performed interference experiments with DNA modified by diethyl pyrocarbonate. Diethyl pyrocarbonate modifies the N7 position of free purines; the likely site of modification in single- and double-stranded DNA (see Herr 1985). Diethyl pyrocarbonate modifies adenines preferentially, generating an A-greater-than-G pattern. Figure 3B shows the results of a diethyl pyrocarbonate interference assay using the wild-type and *dpm2* *PvuII*–*Bam*HI fragments. Consistent with the results of the methylation interference assay, modification of the wild-type fragment does not readily affect complex CI formation (compare lanes 2 and 3, and 8 and 9). A number of modifications affect CII formation (lanes 4 and 10), however, including adenines A191 and A192 on the late strand and A193 and A194 on the early strand. These latter residues lie downstream of the Octa2 sequence (see Fig. 3C), indicating interaction over an extended region that overlaps the binding site of the enhancer binding protein AP-1 (Angel et al. 1987; Lee et al. 1987b). As in the methylation interference assay, the *dpm2* mutations (lanes 6 and 12) and, in a separate experiment not shown, the *dpm8* mutations cause interferences to appear in complex CI over the unmutated site.

The effects of DMS modification of guanines and DEPC modifications of adenines are summarized in Figure 3C. The sites of interference with wild-type complex CII formation are the sum of the sites of interfer-

ence observed in complex CI formation with the *dpm2* mutant (identified by the solid circles) and the *dpm8* mutant (identified by the solid triangles). The interference patterns suggest that the *dpm2*- and *dpm8*-specific complexes result from interactions with the Octa1 and Octa2 motifs, respectively, and not the Sph motifs (compare with Fig. 2A). These Octa1 and Octa2 interactions differ, however. First, Octa1 binding is interfered with by modifications over a 10-bp region (nucleotides 204–213), whereas Octa2 binding is affected by modifications over a 13-bp sequence (nucleotides 191–203) in which the octamer-related sequence is off-center. Second, as shown at the bottom of Figure 3C, modification of equivalent positions in the Octa1 and Octa2 binding sites has different effects. Modification of A212 and G204 affects Octa1 interactions but modification of A203 has a weak effect and modification of G195 has little or no effect on Octa2 binding. In contrast, modification of residue A194 affects Octa2 interactions but A203 modification has little effect on Octa1 interaction (compare lanes 5 and 6, Fig. 3B). These different base-specific interactions are not due to the close proximity of the two binding sites because each interaction was analyzed separately with the *dpm2* and *dpm8* mutants. These separate interactions probably result from binding of the same factor (see below).

The SV40 B element binding activity interacts with the immunoglobulin heavy-chain enhancer octamer sequence

To establish the sequence specificity of the SV40 B element binding factor, we performed gel retardation and DMS interference analyses with the IgH enhancer, which contains the canonical octamer sequence ATGCAAAT. Figure 4A shows the results of gel retardation using a 226-bp *Hinfl* fragment from the mouse IgH enhancer (Banerji et al. 1983; Gilles et al. 1983) and the 0.1–0.2 M KCl heparin-agarose fraction; one major complex is formed (lane 2) compared to the two complexes with the SV40 enhancer fragment (lane 1). [A weak IgH complex(es) of lesser mobility is also apparent when most of the probe is complexed—this complex has not been analyzed further.] The major IgH enhancer complex migrates more slowly than the SV40 complex CI probably because of the larger size of the IgH probe. As with the complexes formed by the SV40 enhancer (Fig. 1B, lanes 9–11), formation of the IgH complex is inhibited by the 6XB17 fragment containing multiple copies of the B element (Fig. 4A, lanes 3–5), albeit apparently less efficiently.

We determined the site of IgH enhancer interaction by examining the DMS interference patterns on both strands of the IgH enhancer fragment (Fig. 4B). Only methylation of the G residues contained in the octamer sequence affected complex formation; methylation of the guanine on the coding strand displays a strong interference (compare lanes 2 and 3), whereas the guanine in the noncoding strand displays a weak interference (compare lanes 4 and 5). These results are summarized in

Figure 4. The SV40 Octal and Octa2 binding activity interacts with the IgH enhancer octamer sequence. (A) Gel retardation of the SV40 enhancer *PvuII*–*Bam*HI fragment (lane 1) and a 226-bp IgH enhancer fragment (lanes 2–5) by the heparin-agarose purified HeLa whole-cell extract fraction. Competition is with 1-, 10-, and 100-fold (lanes 3–5, respectively) molar excess of the 6XB17 fragment used in Fig. 1B. (B) Methylation interference analysis of complexed IgH enhancer DNA. The *HinfI* IgH enhancer fragment, cloned into a pUC119-derived vector, was labeled on both strands at the polylinker *HindIII* site as described in Methods. The 3' (coding) or 5' (noncoding) end-labeled fragments were modified with DMS and used in an interference assay (see Methods). (Lane 1), A + G markers of the coding strand; (lanes 2–5), equivalent amounts of free (F) and complex CI (CI) probe from the coding and noncoding strands. The octamer sequence is bracketed. (C) The sequence of the IgH octamer is bracketed and the coding strand G residue that fully interferes and the noncoding strand G residue that partially interferes with complex formation are shown.

Figure 4C. The pattern of partial interference by the noncoding G of the IgH enhancer octamer is similar to that reported for the ubiquitous octamer binding factor NF-A1 (Sen and Baltimore 1986; Staudt et al. 1986), but we do not observe the effects of methylation at the site, 6 bp distal from the octamer sequence on the coding strand, reported by Sen and Baltimore (1986). The data presented here suggest that the activity that binds to the

SV40 enhancer B element is a general octamer binding factor.

The octamer binding activity fractionates in a single size class upon SDS polyacrylamide gel electrophoresis

To determine whether the SV40 Octal and Octa2 factor binding interactions are the result of one or more dif-

ferent factors, the gel retardation activity was purified. In addition to heparin-agarose chromatography, the activity responsible for complexes CI and CII cofractionates during phosphocellulose (0.05–0.25 M NaCl fraction) and S300-Sephacryl filtration chromatography (data not shown). Figure 5 shows that this activity also cofractionates as a single size class when a nuclear extract, partially purified by heparin-agarose chromatography, is fractionated by SDS-polyacrylamide gel electrophoresis. Such a gel was cut into 11 slices and the proteins eluted and renatured as described by Briggs et al. (1986). Each of the 11 fractions was then tested for gel retardation activity with the SV40 enhancer probe. Only fraction 6, containing proteins in the 100–130 kD range, generates complexes CI and/or CII; thus, the gel retardation activity in the heparin-agarose fraction is the result of one or more proteins of similar size. A separate factor with retardation activity is observed in fraction 9 which contains proteins about 50–70 kD in size. This factor has not been studied further and may result from binding at a different site within the SV40 enhancer, another octamer binding protein, or a degradation product of the protein(s) in fraction 6.

Purification of the octamer binding factor OBP100

The octamer binding factor was extensively purified by using a nucleic acid affinity-precipitation procedure (Franza et al. 1987) followed by SDS-polyacrylamide gel electrophoresis. In the DNA affinity procedure a sequence-specific DNA binding protein is resolved from a complex mixture of proteins by selective interaction with a biotinylated DNA fragment in the presence of double-stranded poly(dI-dC) competitor. The protein-DNA complex is purified by binding to streptavidin-agarose beads. After several washes, the bound protein is eluted and can be identified by SDS-polyacrylamide gel electrophoresis. To purify the octamer binding factor we used a nuclear extract purified twice by heparin-agarose chromatography and a fragment containing 14 copies of the B17 oligonucleotide (14XB17; Ondek et al. 1987), a fragment that contains the Octa1 sequence but not the Octa2 sequence (see Fig. 2A).

Figure 6A represents a silver-stained gel of proteins recovered after the affinity precipitation procedure. Lane 2 shows the complexity of proteins in the heparin-agarose fraction prior to purification, lane 3 shows the result of the assay using a control fragment containing sequences from the human immunodeficiency virus (HIV) enhancer (Franza et al. 1987), and lane 4 shows those proteins able to bind selectively to the 14XB17 fragment. One major species with an apparent molecular weight of 100 kD is purified by the 14XB17 fragment but not by the HIV enhancer fragment. We refer to this protein as OBP100 (octamer binding protein of 100 kD). The size of OBP100 is consistent with the size class (100–130 kD; fraction 6, Fig. 5) determined by the separation of the heparin-agarose fraction on a SDS-polyacrylamide gel and renaturation of the octamer-specific retardation activity. Other light bands specific to the 14XB17 fragment

Figure 5. SDS-polyacrylamide gel electrophoretic fractionation and renaturation of the SV40 Octa1 and Octa2 binding activity. A HeLa cell nuclear extract from 8 liters of cells was fractionated over a heparin-agarose column, and the active fractions pooled (0.2–0.4 M KCl step) and further purified by SDS-polyacrylamide gel electrophoresis as described in Methods. The gel was cut into 11 slices and the proteins eluted and renatured as described (Briggs et al. 1986; see Methods). The left two lanes show gel retardation with (+) or without (–) the addition of a heparin-agarose purified nuclear extract which indicate the position of SV40 complexes CI and CII. The gel slices (lanes 1–11), from high- to low-molecular-weight fractions are as indicated.

appear in the 50–70 kD size range (Fig. 6A, lane 4); one or more of these bands may be related to the retardation activity seen in fraction 9 of Figure 5.

OBP100 was shown to be responsible for formation of SV40 complexes CI and CII by preparing gel slices from an unstained portion of the gel shown in Figure 6A, onto which were loaded duplicate samples of the HIV and 14XB17 fragment purified samples. Slices of the gel corresponding to the regions of the gel labeled 1–3 in Figure 6A were eluted, and the protein(s) renatured and assayed for gel retardation activity as shown in Figure 6B. None of the three HIV-derived fractions exhibit any gel retarding activity (lanes 3–5) whereas fraction 2 from the 14XB17 sample, which spans the OBP100 band, forms complex CI and a weak complex CII (lane 7). Because only one fraction from the gel-purified heparin-agarose fraction shown in Figure 5 contains complex CI and CII activity and the size class of this fraction (100–130 kD) includes OBP100, it is likely that OBP100 is responsible for the gel-retarding activity observed in the partially purified extracts.

Figure 6. Purification and characterization of OBP100 binding activity. (A) Purification of OBP100. OBP100 was purified by the DNA-affinity precipitation procedure as described (Franza et al. 1987). This experiment was performed with 2 μ g of purified and photobiotinylated 14XB17 restriction fragment, 40 μ g poly(dI-dC) as nonspecific competitor, and 0.5 ml of partially purified OBP100 (the equivalent of 0.5 liters of HeLa cells; see Methods) in 200 mM KCl, 50 mM Tris HCl (pH 7.9), 1 mM EDTA, 1 mM DTT, 12.5 mM $MgCl_2$, 20% glycerol which was diluted with 1 ml of Buffer B (Franza et al. 1987) to adjust the concentration of KCl to 70 mM. After binding to the streptavidin agarose beads the protein–DNA complexes were washed three times with Buffer B adjusted to 50 mM KCl. The HIV fragment is described elsewhere (Franza et al. 1987) and the extraction with this fragment was performed in parallel with the 14XB17 probe. The figure shows a silver-stained 8% SDS-polyacrylamide gel. (Lane 1) BioRad high-molecular weight markers (200 ng of each marker were loaded); (lane 2) 1/250 of the starting material used in the OBP100 purification; (lanes 3 and 4) protein specifically precipitated by the HIV enhancer fragment or 14XB17 fragment, respectively. About 40% of the sample was loaded onto each lane, an amount equivalent to isolation from 200 ml of cells. The 100-kDa protein, OBP100, selectively precipitated using the SV40 B element is indicated by the arrow. The three fractions tested for gel retardation activity are labeled to the right of the figure. (B) SV40 enhancer binding activity correlates with OBP100. Parallel unstained lanes from the gel shown in A loaded with duplicate HIV and 14XB17 precipitated samples were cut blindly into 8-mm slices and the protein(s) eluted, renatured, and the gel retardation assay performed as described in Methods. Subsequent staining of the parallel lanes in A showed that the OBP100 band was cut into two slices (4 and 5) and these both contained OBP100 activity. This result further confirms the identity of OBP100 as the octamer binding protein. In this panel, protein from slices 4 and 5 were combined to produce fraction 2 as shown in A. (Lanes 1 and 2) Probe alone (–) or with (+) a heparin-agarose fraction; (lanes 3–5) fractions 1–3 from the HIV sample; (lanes 6–8) fractions 1–3 obtained with the 14XB17 fragment. These assays were done with 4 μ l of extracted protein. (C) Assay of purified and renatured OBP100 binding activity with wild-type and mutant SV40 enhancer fragments. (Lanes 1 and 2) Complex formation with the wild-type SV40 enhancer with (+) and without (–) heparin-agarose purified extract. The OBP100 from the lower half of fraction 2 (A) was assayed with the wild-type SV40 (lane 3), *dpm2* (lane 4), *dpm8* (lane 5), *dpm28* (lane 6), and 208-bp pUC119 *PvuII*–*Bam*HI fragments. (D) Renatured OBP100 protein binds to the IgH enhancer fragment. (Lanes 1 and 2) Complex formation with (+) and without (–) heparin-agarose purified extract; (lane 3) IgH enhancer fragment with the OBP100 renatured fraction as in C.

To show that OBP100 binds to SV40 Octa1 and Octa2, as well as the IgH octamer sequence, we assayed the gel retardation activity of the highly purified OBP100 eluted from the SDS polyacrylamide gel. Figure 6C shows that complex CI is formed with the wild-type SV40 enhancer fragment (lane 3) and the *dpm2* and *dpm8* mutant fragments (lanes 4 and 5) but not the double mutant *dpm28* (lane 6); as with the heparin-agarose fraction, complex CII forms, albeit more weakly, with the wild-type and *dpm2* mutant probes (lanes 3 and 4). This shows that OBP100 interacts with Octa1 and Octa2 in a manner similar to the heparin-agarose fractions (compare Figs. 2B and 6C). Because OBP100 was purified using a frag-

ment that does not contain Octa2 (14XB17, see Fig. 2A), it is unlikely that the binding to Octa1 and Octa2 is the result of association of two proteins with identical molecular weights but with different sequence-specific DNA binding properties. Consistent with a single polypeptide species interacting with different octamer-related sequences, the purified OBP100 also interacts with the IgH enhancer fragment to form a single complex (Fig. 6D, lane 3). Thus, a single factor, OBP100, has an unusually broad and flexible DNA binding specificity.

Discussion

We have described the purification of a 100-kDa octamer

binding protein (OBP100) from HeLa cells and, using both impure fractions and purified OBP100, have characterized its interaction with a canonical octamer sequence in the IgH enhancer and two octamer-related sequences in the SV40 enhancer. OBP100 was purified by three steps: heparin-agarose chromatography, a DNA affinity precipitation procedure (Franza et al. 1987), and SDS-polyacrylamide gel electrophoresis. The greatest purification was attained by the one-step affinity-precipitation procedure (compare lanes 2 and 4 in Fig. 6A). This procedure has been described previously in the purification of proteins that bind to the HIV enhancer (Franza et al. 1987). The efficiency of OBP100 purification may have resulted in part because the heparin-agarose fraction containing the octamer binding activity is relatively devoid of other DNA binding proteins (e.g., Spl elutes at higher salt; Dynan and Tjian 1983a). Assay of the gel-purified OBP100 binding activity relied on renaturation of the eluted protein. Spl (Briggs et al. 1986), EBP20 (Johnson et al. 1987), and AP-1 (Lee et al. 1987b) [but not AP-2 (Mitchell et al. 1987)] DNA binding activities have also been shown to renature successfully. Together, these results indicate that the DNA binding activity of many eukaryotic DNA binding proteins can be renatured after SDS denaturation. Thus, as shown in Figure 5, SDS-polyacrylamide gel electrophoresis of impure preparations can be used to characterize a DNA binding protein prior to extensive purification.

Characterization of the DNA binding specificity of OBP100 with the SV40 enhancer was complicated by the appearance of two complexes and the repeated structure (SphI and SphII motifs) of the B element. Because the region of OBP100 binding is rich in adenines and thymidines, analysis of the binding sites by dimethyl sulfate methylation of guanines in the major groove did not yield a sufficient number of contact points to define clearly the sites of interaction. Therefore, we employed diethyl pyrocarbonate to examine the interactions with adenines (and guanines) in the major groove. The interference patterns with diethyl pyrocarbonate were more extensive and clearly showed an interaction of the B element binding factor with two octamer-related sequences and not the SphI and SphII motifs. Therefore, dimethyl sulfate and diethyl pyrocarbonate are informative in combination because they allow examination of major groove contacts at every base pair. As with any interference assay, the modifications affecting protein binding may not identify actual contact points but rather result from secondary effects such as perturbations of local DNA structure that affect neighboring bases. Potential disadvantages of a diethyl pyrocarbonate interference analysis are that the DNA modifications by this reagent are less well characterized than those of dimethyl sulfate and diethyl pyrocarbonate modifications may affect DNA conformations more severely (see Herr 1985).

The chemical interference patterns with the octamer binding factor resulted in the identification of the similarity between the Octa2 binding site and the octamer consensus sequence. This similarity was not identified by sequence comparisons even though the SV40 early

promoter has been extensively analyzed for sequence similarity to various sequence motifs (e.g., Falkner et al. 1986). The oversight of the resemblance between the Octa2 binding site and the octamer consensus sequence in previous analyses suggests that uncharacterized OBP100 binding sites may exist in other carefully analyzed promoters.

The binding of OBP100 with the wild-type and mutant SV40 enhancers has been assayed by gel retardation with both partially fractionated extracts and the highly purified protein. The chemical interference assays, however, have only been performed with the impure fraction. Nevertheless, the interference patterns probably reflect the binding of OBP100. This is because, first, when the heparin-agarose purified sample is fractionated by SDS-polyacrylamide gel electrophoresis, only proteins corresponding in size to OBP100 produce the octamer binding activity. Second, the OBP100 protein, which appears as a homogeneous band, was purified by interaction with Octa1 (14XB17) but still interacts with both Octa1 and Octa2 as does the heparin-agarose fraction (compare Fig. 2B and 6C). Therefore, the different patterns of chemical interference at Octa1 and Octa2 probably reflect the interactions of one octamer binding protein with these two different but octamer-related sequences. Both the difference in size of the Octa1 and Octa2 binding sites (10 bp and 13 bp, respectively) and the different effects of chemical modification at equivalent bases suggest that the same protein is uniquely interacting with each of these related sequences. A different and more extreme example of a DNA binding protein that recognizes two different sequences is the yeast activator protein HAP1 (Pfeifer et al. 1987). These observations indicate the existence of a class of DNA binding proteins with considerable sequence recognition flexibility.

These results have important implications with respect to the characterization of *cis*-acting control elements. Staudt et al. (1986) have shown that a consensus decamer sequence ATGCAAATNA is sufficient to bind the ubiquitous factor NF-A1, which may be related or identical to OBP100. The related 10 bp of the 13-bp Octamer 2 sequence are not sufficient, however, to create a binding site for OBP100. This conclusion was arrived at because, to assay the activity of Octa1 and Octa2 together, we constructed an oligonucleotide containing both decamer related sequences ATGCAAAGCATG-CATCTCA (the Octa2 decamer is underlined). Consistent with the importance of the additional 3'-terminal ATT sequence in the Octa2 binding site (see Fig. 3C), this 19-bp sequence only interacts with the octamer binding factor at the Octa1 binding site (unpubl.). This result further emphasizes the differences between the Octa1 and Octa2 binding sites because the *dpm2* mutations, which abut the Octa1 binding site, do not affect binding to the Octa1 site but are in an analogous position to the ATT sequence that is required for Octa2 binding (see Figs. 2A and 3C). Therefore, apparently similar octamer elements display different sequence specificities for factor binding. This difference between analo-

gous elements may also extend to other consensus motifs in which small differences in sequence may indicate different requirements for flanking sequences.

Our initial aim in isolating OBP100 was to characterize *trans*-acting factors responsible for the activity of the SV40 enhancer A, B, and C elements. Although OBP100 interacts with the B element and the *dpm2* and *dpm8* mutations that affect its binding also inactivate the B element as defined by the B17 oligonucleotide (Ondek et al. 1987; unpubl.), several lines of evidence suggest that OBP100 is not responsible for the activity of the B element. Two reports have suggested that the octamer-related sequence in Octa1 is a lymphocyte-specific enhancer element (Davidson et al. 1986; Schirm et al. 1987). Our analyses have suggested, however, that the Octa1 binding site is alone insufficient for enhancer function (Ondek et al. 1987). This was shown by the inability of a synthetic B element construct containing the *dpm2* mutations (8XMB17) to activate the human β -globin gene at a distance in a number of cell lines, including HeLa cells and lymphocytes, even though the *dpm2* mutations do not affect the Octa1 sequence nor do they have any significant effect on the binding of OBP100 to the Octa1 site in the SV40 enhancer (see Figs. 2 and 3) or to the B17 oligonucleotide (unpubl.). The Octa1 site alone also does not apparently function as a general upstream promoter element because the 8XMB17 construct is also inactive when positioned about 140 bp upstream of the β -globin initiation site (unpubl.). The Octa1 site is active, however, when it replaces an octamer sequence upstream of a human U2 small nuclear RNA gene (snRNA) and this activity is lost when the Octa1 sequence is mutated (N. Hernandez, pers. comm.). Thus, the single Octa1 element can apparently function in U2 snRNA transcription. We are presently testing whether the combination of Octa1 and Octa2 binding sites together can function as either an enhancer or upstream element.

The *in vivo* significance of the Octa1 and Octa2 OBP100 binding sites is not evident. Compatible with their potential function in the SV40 lytic cycle, a gel retardation activity similar to OBP100 is detectable in an extract from CV-1 cells, the cell line used in our SV40 revertant analyses (unpubl.). This is consistent with the ubiquitous nature of the potentially identical NF-A1 activity (Singh et al. 1986; Staudt et al. 1986). Octamer binding activities, some of which may reflect OBP100, have been implicated in upstream promoter function (e.g., histone H2B; Sive and Roeder 1986), enhancer function (e.g. IgH enhancer; Bohmann et al. 1987; Gerster et al. 1987; Lenardo et al. 1987; Sen and Baltimore 1986), and DNA replication in adenovirus (NFIII or ORP-C; Pruijn et al. 1986; Rosenfeld et al. 1987). The SV40 Octa1 and Octa2 sites could be involved in any one of these functions. It has already become evident that the SV40 enhancer region is not a single functional unit but rather contains multiple enhancer elements (Herr and Clarke 1986; Zenke et al. 1986). Other than an enhancer element(s), the Octa1 and Octa2 sites may be either upstream promoter elements involved in early or

late SV40 transcription, or possibly DNA replication elements because the SV40 enhancer region has been shown to influence SV40 DNA replication (Bergsma et al. 1982; DeLucia et al. 1986; Li et al. 1986).

The involvement of octamer elements in the regulation of cell-cycle-controlled genes such as the histone H2B gene suggests that this factor could be involved in transcription (and possibly replication) during the S phase. If true then, since after infection SV40 T antigen induces the cell to progress into the S phase, the Octa1 and Octa2 sites may serve as control elements after this induction occurs. Both these sites overlap the B element and the Octa2 site overlaps the binding site for the SV40 enhancer binding factor AP-1. Thus, one element of regulation of SV40 enhancer function during the lytic cycle may involve competing interactions between different *trans*-acting factors for overlapping DNA binding sites.

Methods

Construction of probes used for gel retardation

The *EcoRI*–*HindIII* fragment from the plasmid pAO (Zenke et al. 1986), which contains a single copy of the 72-bp repeat and a *Bam*HI site at the junction of the 72-bp and 21-bp repeats (position 103), was cloned into the plasmid vector pUC118 to create pUC118AO. The *dpm2* and *dpm8* mutations were introduced into the pUC118AO clone either singly or in combination (*dpm28*) by oligonucleotide-directed mutagenesis (Zoller and Smith 1984; Kunkel 1985). Fragments for gel retardation were prepared by digestion of the pUC118AO constructs with *Bam*HI and by either 3'- or 5'-end-labeling with DNA polymerase or polynucleotide kinase, respectively, and 32 P-nucleotide triphosphates. The DNA samples were subsequently cleaved with *Pvu*II to release a 99-bp SV40 enhancer fragment. High-specific-activity probes for gel retardation with purified OBP100 were prepared by converting pUC118AO single-stranded DNAs into double-stranded DNA by primer extension in the presence 32 P-labeled nucleotide triphosphates. The inserts were subsequently excised by digestion with *Pvu*II and *Bam*HI. In all cases, labeled fragments were purified by polyacrylamide gel electrophoresis, and eluted by diffusion into 400 μ l of 10 mM Tris (pH 8.0), 0.1 mM EDTA, followed by addition of sodium acetate to 0.3 M, and ethanol precipitation. The level of 32 P incorporation was determined by liquid scintillation counting. Synthetic enhancer competitor fragments were isolated from the plasmids p6XA21, p6XB17, and p6XC17 (Ondek et al. 1987) by digestion with *Hind*III and *Pst*I, and purification by polyacrylamide gel electrophoresis. To prepare an IgH enhancer probe, a 226-bp *Hinf*I fragment from the mouse IgH enhancer (Banerji et al. 1983; Gillies et al. 1983) was 3'-end-labeled as described above. For the DMS interference experiment the fragment was cloned into the *Sph*I site of pBe⁻ (Ondek et al. 1987), to create pB1gH⁺, and subsequently excised as a *Hind*III–*Pst*I fragment. The *Hind*III site was either 3'- or 5'-end-labeled as described above.

Preparation of HeLa cell extracts and heparin-agarose fractionation

Either nuclear (Dignam et al. 1983; Wildeman et al. 1984) or whole-cell extracts (Manley et al. 1983) were prepared as described from HeLa cell spinner cultures grown to a density of $4\text{--}5 \times 10^5$ cells/ml. Crude extracts from 8–32 liters of cells

were desalted on a Biogel P10 column and then fractionated on a 40-ml heparin-agarose column according to Dynan and Tjian (1983a). The heparin-agarose resin was prepared according to Davison et al. (1979). The samples containing octamer binding activity were eluted from the heparin-agarose column by either step elution (0.1–0.2 M KCl) or by a gradient of KCl concentration (0.1 M to 1 M KCl) and the octamer binding activity located by a gel retardation assay. The KCl concentration at which the activity eluted varied (from 0.1 M–0.4 M) depending on the amount of extract loaded onto the column. Peak fractions were combined and gave a protein concentration of approximately 5 mg/ml. Protein concentrations were measured by the BioRad Protein Assay using bovine serum albumin as the standard.

Gel retardation assay, and DMS and DEPC interference experiments

The protein–DNA complexes were assayed by a modified procedure of Schneider et al. (1986). Heparin-agarose purified extract (usually 1 μ l; 5 μ g protein) was added to a 10- μ l mix containing 5000–10,000 cpm (0.1–0.5 ng) of probe, 10 mM HEPES (pH 7.9), 60 mM KCl, 4 mM MgCl₂, 0.1 mM EDTA, 0.25 mM DTT, 0.1 mg/ml BSA, 10% glycerol, and 1 μ g of poly(dI-dC) (Pharmacia). After incubation at room temperature for 10–15 min the reactions were loaded onto a 6% polyacrylamide gel and the complexes fractionated in 40 mM Tris-glycine (pH 8.5) buffer at 10 V/cm. The gels were dried before autoradiography.

For the methylation interference assays, a portion of the radiolabeled probe was treated with DMS (Maxam and Gilbert 1980) before fragment purification on a polyacrylamide gel as follows. The probe, dissolved in 10 μ l of water, was added to 200 μ l of 50 mM sodium cacodylate (pH 7.1), 1 mM EDTA, and modified by the addition of 1 μ l of a fresh 1 : 40 dilution of DMS in ethanol, followed by incubation at 37°C for 15 min. The reaction was terminated by the addition of 50 μ l of 1.5 M sodium acetate, 100 μ g/ml yeast tRNA and two successive ethanol precipitations. The fragments were then isolated preparatively on a polyacrylamide gel. The binding reaction and gel fractionation were as described above but scaled up 5- to 10-fold with respect to extract and using about 300,000 cpm of modified fragment. Free and complexed probes were eluted from the gel into 400 μ l of 10 mM Tris (pH 8.0), 0.1 mM EDTA, and 0.1% SDS. The eluate was extracted with phenol/chloroform, and ethanol-precipitated in 0.3 M sodium acetate with 10 μ g of carrier tRNA. The samples were resuspended in 200 μ l of 0.3 M sodium acetate, 0.1 mM EDTA and reprecipitated by the addition of 30 μ l of 1% cetyl-trimethyl ammonium bromide (CTAB). The CTAB-precipitated DNAs were reprecipitated twice with ethanol after resuspension in 0.3 M sodium acetate. Precipitation with CTAB removed contaminating polyacrylamide. The samples were ultimately resuspended in 100 μ l of freshly diluted 1 M piperidine and incubated for 30 min at 90°C. The piperidine was removed by multiple rounds of lyophilization. The radioactivity of the dried samples was determined by Cerenkov counting and equal amounts of the free and bound probes were fractionated on 16% denaturing buffer gradient polyacrylamide sequencing gels.

The DEPC interference assays were performed essentially as in the methylation interference assays except for the modification of the probe. Because DEPC does not modify double-stranded DNA efficiently (Herr 1985) the end-labeled DNAs were suspended in 10 μ l of water and boiled for 3 min to denature the DNA. After chilling on ice, the DNAs were diluted in 200 μ l of 50 mM sodium cacodylate (pH 7.1), 1 mM EDTA, to which 4 μ l of DEPC was added. The samples were vortexed well and incubated 37°C for 20 min. Because DEPC is relatively

insoluble in water, the samples were thoroughly remixed half way through the incubation. The DNAs were recovered by two ethanol precipitations and allowed to renature in 10 μ l of 10 mM Tris (pH 8.0), 5 mM MgCl₂ for 10–60 min at ambient temperature before polyacrylamide gel purification.

SDS-polyacrylamide gel fractionation of partially purified extracts and renaturation of DNA binding activity

Heparin-agarose fractionated nuclear or whole-cell extracts from the equivalent of 2–3 liters of HeLa cells were precipitated by acetone (80%), resuspended in SDS loading buffer, denatured by heating at 90°C for 15 min, and loaded into a 4–7-cm-wide slot of a 1-mm-thick 8% preparative SDS polyacrylamide gel. A portion of each side of the slot (about 0.5 cm) was stained to use as a guide for preparing the gel slices. Approximately 1-cm slices across the sample were eluted in 2 ml of 50 mM Tris (pH 8.0), 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl, and 0.1% SDS, recovered by precipitation with 4 volumes of acetone, and renatured according to the procedure of Briggs et al. (1986). The renatured protein was recovered from each fraction in approximately 200 μ l and 5 μ l of each fraction was tested in a gel retardation assay as described above. To determine the size of the proteins within active fractions, the eluted material was refractionated on an analytical SDS-polyacrylamide gel beside molecular weight standards.

Sequence-specific DNA-affinity precipitation procedure

The OBP100 protein was purified by a streptavidin-biotin DNA affinity precipitation procedure with minor modifications (Franza et al. 1987) using a gel-purified and biotinylated 14XB17 *HindIII*–*PstI* fragment (Ondek et al. 1987) and partially purified OBP100 as follows. Fifty milliliters (about 100 mg/ml) of nuclear extract (Dignam et al. 1983) from 1.6×10^{10} cells was dialyzed against 0.1 M TM buffer (Dynan and Tjian 1983a) and fractionated over a 40-ml heparin agarose column in 0.1 M TM. The OBP100 gel retardation activity appeared in the unbound fraction, was pooled (75 ml, about 20 mg/ml), and reloaded onto the 40-ml heparin agarose column in 0.1 M TM. The bound proteins were eluted with a 0.1 M KCl to a 1 M KCl gradient. The OBP100 gel retardation activity eluted between 0.15 M to 0.25 M KCl. Active fractions were pooled (30 ml, 4 mg/ml) and used for DNA affinity purification. The purified proteins were fractionated by SDS-polyacrylamide gel electrophoresis and the proteins eluted and renatured as described above except that 25 μ g/ml bovine serum albumin (Boehringer Mannheim, ultra-pure) was added to the elution buffer and renatured samples were recovered in 100 μ l of 0.1 M TM buffer (Briggs et al. 1986).

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A 100-kD HeLa cell octamer binding protein (OBP100) interacts differently with two separate octamer-related sequences within the SV40 enhancer.

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