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DNA-Binding Properties of the E1A-Associated 300-Kilodalton Protein

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One of the major E1A-associated cellular proteins is a 300-kDa product (p300) that binds to the N-terminal region of the E1A products. The p300 binding site is distinct from sequences involved in binding the retinoblastoma product and other E1A-associated cellular products such as p60-cyclin A and p107. p300 binding to E1A is linked genetically to the enhancer repression function of E1A and to other E1A-mediated gene-regulating functions as well as to the transforming functions of E1A. However, the biochemical properties of p300 have not yet been characterized. We report here that p300 has an intrinsic DNA-binding activity and shows a preferential affinity for specific DNA sequences. The sequences selectively bound by p300 are related to those of a series of enhancer elements that are recognized by NF-κB. The direct physical interaction of p300 with enhancer elements provides a biochemical basis for the genetic evidence linking the E1A-mediated enhancer repression function with the p300-binding activity of E1A.

The ability of DNA tumor virus transforming proteins such as the adenovirus type 5 (Ad5) E1A gene products to induce unrestricted proliferation in normal resting rodent cells is linked genetically with the ability of these tumor virus gene products to bind to specific cellular proteins (14, 73). A direct or indirect consequence of these specific associations is the alteration of gene expression in the host cells, so that the pattern of gene expression becomes that of proliferating cells rather than of resting cells.

The E1A proteins have three regions of conserved amino acid sequence and three independent active sites which can be impaired selectively by mutations localizing to the N terminus, conserved region 2, or conserved region 3, respectively. The active sites involving the N terminus and region 2 are together sufficient for E1A transforming functions (for a review, see reference 9). Either of these two active sites is sufficient to activate G1 gene expression and DNA synthesis in quiescent host cells, but both are required for full mitogenic activity (28, 30, 70).

In addition to the changes in cellular gene expression that specifically accompany the induction of proliferation in quiescent host cells, an E1A-mediated enhancer repression function has also been reported. The enhancer repression function is linked with host cell growth-regulating functions in E1A; region 2 may contribute to this function, and the amino-terminal region generally plays a crucial role (15, 30, 37, 39, 51, 53, 58, 59, 67).

The E1A products do not appear to mediate these changes in gene expression directly but rather through their association with specific host cell proteins. Presumably, binding to specific cellular proteins has the eventual outcome of altering the composition of cellular transcription factor complexes, thus changing the pattern of cellular gene transcription. Indeed, two of the E1A-associated proteins have recently been shown to be components of such complexes (1, 5, 6, 10, 11, 43). These are the p60 product of the cyclin A gene (p60-cyclin A) and the p105 product of the retinoblastoma tumor susceptibility gene (p105RB), each of which is involved in E2F transcription factor complexes. Both of these cellular products interact with E1A via region 2, which is required for the ability of E1A to activate E2F-dependent transcription (14, 20, 47, 49, 72, 73).

The only cellular product known to date to associate with the E1A products in a manner dependent on the N-terminal region is a 300-kDa product (p300). The p300 binding site is distinct from sequences involved in binding p105RB and other region 2-associated cellular products such as p60-cyclin A and p107 (14, 16, 20, 73). p300 is a ubiquitously expressed nuclear phosphoprotein that is actively phosphorylated in all phases of the cell cycle but shows an additional phosphatase-sensitive modification specific to M-phase enriched cell populations (75). Because p300 binding to E1A is linked with E1A-mediated gene-regulating activities, including the E1A repression function, and in light of the precedent that other E1A-associated proteins are components of transcription factor complexes, we investigated the possibility that p300 has DNA-binding properties. We show here that p300 does, in fact, have DNA-binding activity and a preferential affinity for specific sequences previously identified as enhancer-binding protein target sites. The direct physical interaction of p300 with enhancer elements provides a biochemical basis for the genetic evidence linking the E1A-mediated enhancer repression function with the p300-binding activity of E1A.

MATERIALS AND METHODS

Cells. Monolayer cultures of HeLa and 293 (21) cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 100 μg of penicillin and streptomycin per ml.

Immunoaffinity purification of p300. Immunoprecipitation of 293 cell extracts with monoclonal antibody series M73 (23) was performed as described previously (75). After six washes of the immunocomplexes, including protein A-Sepharose beads (Pharmacia), with lysis buffer, p300 was selectively eluted from the immunocomplexes by incubation in RIPA buffer at 37°C for 15 min and then dialyzed against DNA binding buffer (20 mM Tris-HCl [pH 7.4], 10% glycerol, 50 mM KCl, 0.1 mM dithiothreitol). In our experiments, we could typically recover about 50 ng of p300 from

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10^7 cells. Quantitation of the dried gel shown in Fig. 1 was accomplished by using a Molecular Dynamics Phosphoimager.

Nitrocellulose filter binding assay. Nitrocellulose filter binding assays were carried out essentially as described previously (50). Nitrocellulose filters were activated in 0.5 N KOH and neutralized by soaking in 0.1 M Tris buffer (pH 7.5). Before the binding experiments, each filter was soaked in DNA binding buffer (20 mM Tris-HCl [pH 7.4], 10% glycerol, 50 mM KCl, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) for 30 min. Each reaction mixture contained 10^5 cpm of *Hin*fI, *Hin*dIII, *Eco*RI-digested ^{32}P-end-labeled 293 cell sheared genomic DNA (10 ng), and various amounts of bovine serum albumin (BSA), histone H1, and immunoaffinity-purified p300 in DNA binding buffer. The mixture was incubated at 37°C for 30 min and then filtered through nitrocellulose filters. The filters were washed with 2 ml of DNA binding buffer; bound DNA was detected by autoradiography.

DNA-cellulose chromatography. Cells that had been labeled with [^{35}S]methionine in methionine-free medium for 2 h were lysed in 200 μl of lysis buffer (25 mM Tris-HCl [pH 7.4], 50 mM NaCl, 0.2% Nonidet P-40, 0.5% deoxycholic acid, 200 KIU of aprotinin per ml, 0.02% sodium dodecyl sulfate [SDS]). The lysate was clarified by centrifugation at 16,000 × *g* for 15 min at 4°C. The recovered supernatant was diluted with 10-fold-excess loading buffer (10 mM potassium phosphate [pH 6.2], 0.5% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 100 KIU of aprotinin per ml). The diluted extract was then applied to a column of single-stranded calf thymus DNA cellulose (bed volume was approximately 1 ml; Pharmacia) equilibrated with loading buffer containing 50 mM NaCl. The column was then washed with equilibrating buffer and eluted with elution buffer increasing stepwise in NaCl concentrations from 100 mM to 1 M. Samples of the flowthrough and each fraction were then analyzed by immunoprecipitation with M73-E1A beads. M73-bound E1A was prepared by elution of p300 with RIPA buffer from immunocomplexes of 293 cell extracts. These immunocomplexes, although partially saturated with E1A and E1A-associated products other than p300, showed immunoaffinity to E1A, p105RB, p107, and p130 in addition to p300.

PCR-assisted DNA binding selection from random oligonucleotides. Immunoaffinity-purified p300 (10 μg) was coupled to CNBr-activated Sepharose (Pharmacia) essentially as described previously (48). A mixture of 50-base-length oligonucleotides, in which the middle 20 bases consisted of random nucleotides generated by incorporating each of the four nucleotides at equimolar concentration, was converted to double-stranded oligonucleotides by polymerase chain reaction (PCR), using ^{32}P-end-labeled primers 1 (5′-GTAC CCGGGGATCCT-3′) and 2 (5′-TTGCATGCCTGCAGG-3′). Approximately 100 ng of polyacrylamide gel-purified double-stranded oligonucleotides was suspended in 100 μl of buffer B (50 mM Tris-HCl [pH 8.0], 1 mM dithiothreitol, 10 μg of gelatin per ml) and applied to the p300-Sepharose column. Nonspecifically bound oligonucleotides were washed out with lower-concentration buffer B plus NaCl washes (up to 100 mM), and the p300-bound oligonucleotides were eluted with buffer B plus 300 mM NaCl. In the first round of selection, approximately 4% of total oligonucleotide-associated counts per minute remained in the column after it had been washed with 50 mM NaCl buffer. The eluted double-stranded oligonucleotides were PCR amplified by using primers 1 and 2, and the amplified products were reapplied to the p300-Sepharose columns. After four sequential cycles of elution, amplification, and reapplication, the amount of bound oligonucleotides increased to 21% of the total oligonucleotides applied and did not increase further in an additional round. The double-stranded oligonucleotides from the fifth 300 mM fraction were digested with *Bam*HI and *Pst*I and cloned into a *Bam*HI-*Pst*I-digested pUC118 vector. DNA sequencing of oligonucleotide inserts was performed with a Taq Track sequencing system (Promega), using double-stranded miniprep DNA templates and reverse primer.

UV cross-linking. UV cross-linking was done essentially according to the procedure of Wu et al. (74). Cloned F593 DNA fragments used for UV cross-linking (see Fig. 5) were made by PCR, using universal and reverse primer in the presence of [^{32}P]dATP, [^{32}P]dCTP, [^{32}P]dGTP, and 5-bromo-2′-deoxyuridine triphosphate (Sigma). Oligonucleotide DNA for UV cross-linking (see Fig. 6 and 7) were made by PCR, using primer 3 (5′-CTGCTGGGGAGCC-3′) and primer 4 (5′-TGCAGTTAGGGTG-3′) in the presence of [^{32}P]dATP, [^{32}P]dCTP, [^{32}P]dGTP, and 5-bromo-2′-deoxyuridine triphosphate (Sigma). The sequence of the plus strand of each oligonucleotide is as follows:

RAN, 5′-CTGCTGGGGAGCCNNNNNNNNNNNNNCACCCTAACTGCA-3′
NFκB, 5′-CTGCTGGGGAGCCTGGGGACTTTCCACACCCTAACTGCA-3′
H2TF1, 5′-CTGCTGGGGAGCCTGGGGATTCCCCACACCCTAACTGCA-3′
F593, 5′-CTGCTGGGGAGCCTAGGGACTGCATCCACCCTAACTGCA-3′
H2TF·DM, 5′-CTGCTGGGGAGCCTGCGGATTCCCGACACCCTAACTGCA-3′
E2F, 5′-CTGCTGGGGAGCCGTTTTCGCGCTTACACCCTAACTGCA-3′
AP1, 5′-CTGCTGGGGAGCCGCATGAGTCAGACCACCCTAACTGCA-3′
70GC, 5′-CTGCTGGGGAGCCGGCGGGTCTCCGTCACCCTAACTGCA-3′

Each double-stranded DNA fragment was purified on 12% polyacrylamide gels. Binding reaction mixtures were prepared as described for nitrocellulose filter binding assays in a total reaction volume of 15 μl, including 1 ng of probes. Once equilibrium was reached, samples were irradiated under a UV lamp (maximum emission wavelength, 7 mW/cm^2) at a distance of 8 cm from the UV source. The mixtures were then treated with 4.0 μg of DNase I (Sigma) for 30 min at 37°C after addition of $CaCl_2$ to a final concentration 5 mM. Reaction products were separated by electrophoresis through an SDS–6.5% polyacrylamide gel, stained with silver nitrate, and analyzed by autoradiography. The competition studies for UV cross-linking were carried out by preincubating the p300 sample for 10 min with different amounts of cold PCR-amplified and polyacrylamide gel-purified DNA fragments prior to the addition of labeled probes, and UV cross-linking was then performed.

RESULTS

Immunoaffinity-purified p300 has DNA-binding activity in a nitrocellulose filter binding assay. To study the DNA-binding ability of p300, we purified p300 from 293 cells, which constitutively express E1A (21), using an E1A immunoaffinity purification method described previously (75). p300 can be eluted selectively from E1A immunocomplexes by incubation in high-detergent buffer and recovered in the supernatant. An aliquot of one such affinity-purified p300 sample, made from ^{35}S-labeled cell extracts and visualized by silver staining, is shown in Fig. 1A (lane 1). Calculation by phosphoimaging of ^{35}S incorporation in each detectable band in the partially purified sample indicated that more than 95% of the p300-enriched sample was p300; there were small amounts of contamination (less than 1% each) with other E1A-associated proteins, such as p105RB, and with the E1A

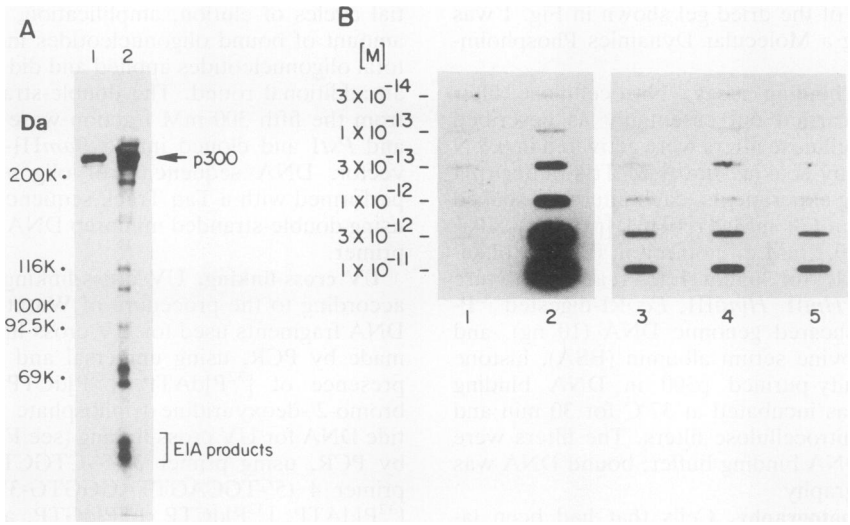

FIG. 1. (A) Immunoaffinity purification of p300. Protein extracts from 293 cells were immunoprecipitated with E1A-specific monoclonal antibody series M73 (23) and fractionated by electrophoresis through an SDS–6.5% polyacrylamide gel (lane 2). p300 was eluted from whole M73 immunocomplexes by incubation in RIPA buffer at 37°C for 15 min (lane 1). The separated proteins were detected by silver staining. Protein molecular weight standards are indicated on the left, and the positions of p300 and the E1A products are indicated on the right. (B) Nitrocellulose filter binding assay with p300. Nitrocellulose filter binding assays were done as described in Materials and Methods. Various amounts of BSA (row 1), histone H1 (row 2), and three independent immunoaffinity-purified p300 samples (rows 3 to 5) were mixed with ^{32}P-end-labeled 293 cell genomic DNA.

products. After dialysis with DNA binding buffer, this and additional independent samples prepared in the same manner were used for the following assays.

The DNA-binding ability of p300 was first evaluated in a nitrocellulose filter binding assay. Three independent p300 samples were tested; BSA and histone H1 protein were included as negative and positive controls, respectively. Autoradiography of these filters (Fig. 1B) showed no prominent signal with BSA (row 1) and a concentration-dependent signal with histone H1 (row 2). With each of the p300 samples, we detected a positive signal at protein concentrations greater than 3×10^{-12} M (rows 3 to 5). The p300 sample shown in Fig. 1A is analyzed in row 3.

p300 binds specifically to DNA-cellulose. The affinity-purified p300 samples contain trace amounts of products such as p105RB, which is known to have DNA-binding activity in vitro (27, 38). Therefore, to verify that p300 itself has DNA-binding activity, we applied ^{35}S-labeled HeLa cell lysates to a DNA-cellulose column and visualized the E1A-binding proteins in each eluted fraction by affinity precipitation with unlabeled M73-bound E1A (Fig. 2A). Of the total trichloroacetic acid-precipitable counts applied to the column, only 12% were retained after the low-salt wash. In contrast, 64% of recovered p300 was eluted in the column-bound fractions, indicating that p300 has specific DNA-binding activity. p300 was maximally eluted in the 200 mM salt fraction. Peptide digest analysis using V8 protease confirmed that the DNA-cellulose-bound 300-kDa species is authentic p300 defined by E1A association (not shown).

To determine whether the presence of the E1A products in cell lysates affects the ability of p300 to bind to DNA, we applied ^{35}S-labeled 293 cell lysates to a DNA-cellulose column as described above. 293 cells express the Ad5 early region 1 (E1A and E1B) products constitutively at high levels (21). Analysis with p300-specific polyclonal antiserum indicates that most or all p300 in 293 cell lysates is associated with the E1A products (75). The elution pattern of p300 from 100 to 300 mM was essentially the same in 293 cells as in HeLa cells (Fig. 2B), implying that the DNA-binding ability of p300 is not severely affected in the presence of E1A. The elution pattern of p300 from 100 to 300 mM was also essentially the same in total cell lysates from several other cell lines, including WI-38, IMR-90, and primary BRK cells (additional data not shown), implying that the DNA-binding activity of p300 is similar in either normal or transformed cells.

To determine whether the DNA-binding ability of p300 is altered when the presence of other E1A-associated proteins and cellular factors is reduced, an affinity-purified p300 sample, prepared as described for Fig. 1A, was applied to a DNA-cellulose column. Affinity-purified p300 remained able to bind to DNA-cellulose (Fig. 2C) despite the dramatically reduced presence of other cellular proteins. Moreover, while 60% of the p300 was retained on the column, more than 70% of the contaminating p105RB and p107 bands eluted in the flowthrough. The flowthrough of the majority of the region 2-associated products is also clearly apparent in Fig. 2B. In addition, we have performed DNA-cellulose chromatography with lysates of Y79 cells, a retinoblastoma-derived line (41) which does not express p105RB. The elution pattern of p300 from this cell line (not shown) was essentially the same as that from HeLa cells, confirming that p105RB is not required for the DNA-binding ability of p300.

When affinity-purified p300 was applied to DNA-cellulose columns, the p300 elution pattern shifted reproducibly to a higher concentration of NaCl compared with the profile seen when p300 was applied as a component of total cell extracts (Fig. 2C versus 2A and B). If p300 has preferential affinity for specific DNA sequences for which other cellular DNA-binding proteins compete, as suggested by the data presented below, this enhanced binding after partial purification might result from reduced competition for preferred binding sites. Similar results have been reported for other specific DNA-binding proteins (33, 55). Another consideration con-

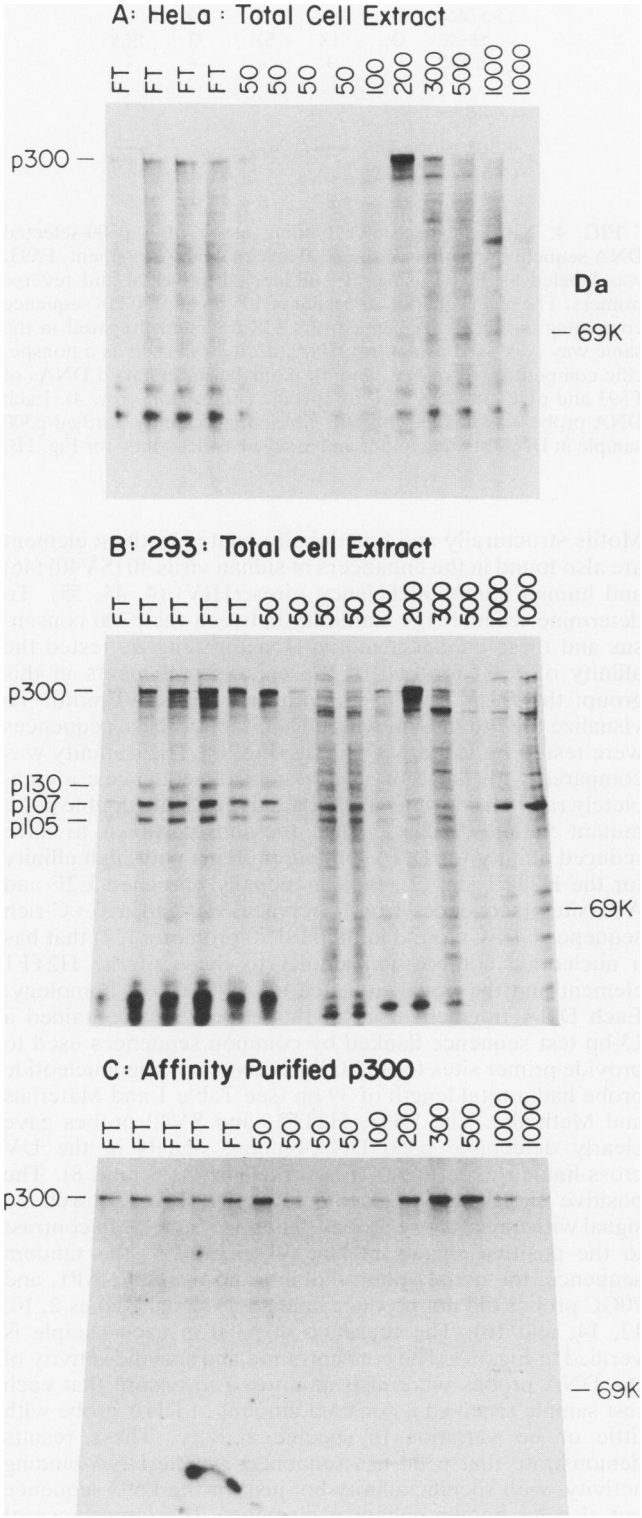

FIG. 2. (A) DNA-cellulose chromatography of p300 in a total HeLa cell extract. A ^{35}S-labeled HeLa cell lysate was applied to a calf thymus DNA-cellulose column. Fractions were immunoprecipitated with M73-E1A beads, separated in an SDS–6.5% polyacrylamide gel, and visualized by autoradiography. (B) DNA-cellulose chromatography of p300 in a total 293 cell extract. A ^{35}S-labeled 293 cell lysate was applied to a calf thymus DNA-cellulose column. Fractions were analyzed as described for panel A. (C) DNA-cellulose chromatography of p300 immunoaffinity purified from 293 cells. p300 purified from ^{35}S-labeled 293 cells as shown in Fig. 1A was diluted with loading buffer, passed through a 1-ml column of denatured DNA-cellulose, and eluted as described for panel A. The eluted fractions were separated in an SDS–6.5% polyacrylamide gel, and the recovered ^{35}S-labeled species were visualized by autoradiography. FT, flowthrough.

cerns the properties of the fraction of p300 that does not bind to the DNA-cellulose columns. Consistently, in both total cell extracts and affinity-purified preparations, only about 60% of p300 is retained on the columns. The 40% of p300 recovered in the flowthrough fractions may represent a specific subfraction of p300 that is less able to bind DNA, because when flowthrough fractions were reapplied to a fresh column, approximately 73% of the detectable p300 was again recovered in the flowthrough fractions (not shown). It is possible that a portion of p300 loses its DNA-binding activity nonspecifically during purification. However, we are also investigating the possibility that DNA-binding activity is a property of a specific subfraction of p300. One possibility is that the ability to bind DNA correlates with specific posttranslationally modified forms of p300, some of which vary specifically during the cell cycle (75).

Considering that other DNA-binding proteins with affinity to E1A elute preferentially in different fractions than p300, and that the affinity of p300 for the DNA-cellulose column is not reduced despite greater than 90% purification away from other E1A-associated proteins and cellular products, it is unlikely that the DNA-binding activity of p300 is directly dependent on the presence of other E1A-associated proteins or other cellular factors. However, the existence of a cellular factor that specifically copurifies with p300 cannot be ruled out, as discussed further below.

p300 shows sequence selectivity in its DNA-binding activity. To characterize p300 DNA-binding activity further, we determined whether DNA fragments that have higher than random affinity to p300 can be selected. The PCR-amplification strategy that we used has been described previously (7, 35) (details are provided in Materials and Methods). During four rounds of PCR-amplified selection, the percentage of an originally random oligonucleotide population that remained bound to Sepharose-immobilized p300 increased progressively from 4 to 22% (not shown) and did not increase further in an additional round. The progressive increase in binding activity indicated that selection did indeed occur and that maximal binding activity had been achieved by the fourth round. After the fifth round, oligonucleotides that were preferentially retained by p300 were cloned; 16 independent clones were isolated and sequenced. The retained sequences were not random. Two cloned inserts (F549 and F555) had an identical 6-bp sequence, and each of the other clones showed homology to that sequence. The deduced consensus sequence is 5′-GGGAGTG-3′ (5′-CACTCCC-3′) (Fig. 3).

The affinity of the cloned selected sequences to p300 samples was tested in a nitrocellulose filter binding assay. Each gave a stronger signal than did vector sequence alone (not shown). p300 preference for an individual clone, F593, over pUC118 vector sequence is shown in Fig. 4. Row 1 shows background signal detected in the absence of protein. In the presence of an equal amount of the nonspecific competitor, dI-dC (row 2), the pUC118 sequence is barely detectable above background, while the F593 probe gives a strong signal. A fivefold excess of dI-dC reduces the pUC118 signal to background but does not affect the F593 signal (row

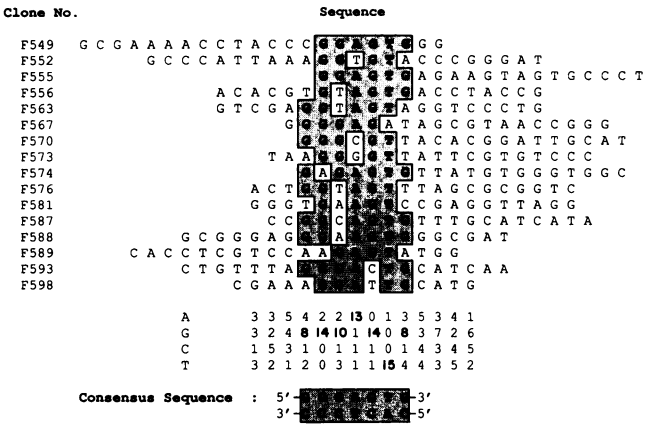

FIG. 3. Sequences of p300-bound DNA fragments. p300-bound oligonucleotides were cloned and sequenced as described in Materials and Methods. The frequency of the nucleotides appearing at each position is summarized below the sequences. The consensus sequence shown in boldface represents the nucleotides that appear with high frequency.

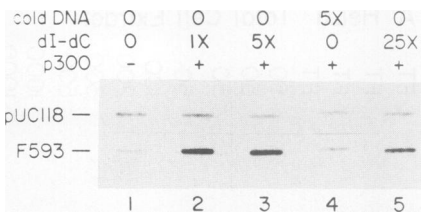

FIG. 4. Nitrocellulose filter binding assay of a p300-selected DNA sequence. A probe of the p300-selected DNA fragment, F593, was labeled by PCR, using ^{32}P-end-labeled universal and reverse primers. The test sequence consisted of 105 bp of pUC118 sequence containing the insert. A labeled pUC118 fragment prepared in the same way was used as control DNA. dI-dC was used as a nonspecific competitor (rows 2, 3, and 5). Nonlabeled amplified DNAs of F593 and pUC118 were used as specific competitors (row 4). Each DNA probe was mixed with 10^{-12} M immunoaffinity-purified p300 sample in DNA binding buffer and assayed as described for Fig. 1B.

3). In contrast, a fivefold excess of cold F593 probe competes completely (row 4). Even a 25-fold excess of nonspecific competitor does not abolish the F593 signal (row 5). The preference of p300 for pUC118 containing the F593 insert over pUC118 alone, or dI-dC, demonstrates that p300 does indeed have a higher specific affinity for selected DNA sequences than for randomly occurring sequences.

Demonstration of p300-intrinsic DNA-binding activity by UV cross-linking. To determine whether the DNA-binding activity is completely intrinsic to p300, the ability of p300 to contact DNA directly was tested in a UV cross-linking experiment with the F593 DNA clone (Fig. 5). When probe DNA was irradiated in the absence of protein (lane 2) or when UV irradiation was omitted (lane 3), no nuclease-resistant labeled species were generated. However, when complete binding reaction mixtures were irradiated and analyzed, a labeled p300 band resulted (lane 4). No labeled species were seen when an equal amount of BSA was substituted for p300 (lane 1), demonstrating that DNA and protein mixtures do not cross-link indiscriminately in these reaction conditions merely because they are placed in close physical proximity. When UV cross-linked binding reaction mixtures were treated with protease K (lane 7), no labeled species were observed, indicating that the labeled species were true protein-DNA photoadducts. Addition of a large excess of unlabeled dI-dC (lane 5) or cold F593 DNA (lane 6) to the binding reaction mixtures blocked formation of the labeled DNA-protein adducts. These data indicate that p300 itself can contact DNA directly.

p300 binds to known enhancer element motifs. Comparison of the F593 sequence or the p300 consensus DNA-binding sequence with previously reported transcription factor-binding sites revealed an intriguing degree of similarity between the p300 consensus site and a series of known enhancer sequence elements typified by the sequence TGGGGATTC CCCA found in the enhancer of the major histocompatibility complex class I gene, *H-2K^b*, which can be bound by a factor designated H2TF1 (4). The core of the conserved sequences in these elements is the sequence GGGANT, and this sequence occurs both in the p300 consensus and in F593 (Table 1). A related element occurs in the κ immunoglobulin gene enhancer, where it is a binding target of NF-κB (55). Motifs structurally and functionally related to the κ element are also found in the enhancers of simian virus 40 (SV40) (46) and human immunodeficiency virus (HIV) (4, 44, 55). To determine whether the similarity between the p300 consensus and these enhancer motifs is significant, we tested the affinity of p300 for two of the enhancer elements in this group: the *H-2K^b* (H2TF1) motif and the SV40 motif. To visualize the interaction with p300 directly, these sequences were tested by UV cross-linking (Fig. 6). Their affinity was compared with those of several control sequences: a completely random sequence (RAN), an H2TF1 site double point mutant variant (H2TF1 · DM) previously shown to have reduced affinity for DNA-binding proteins with high affinity for the H2TF1 site (2), the functionally unrelated E2F and AP1 sites (sequences from reference 32), and a G+C-rich sequence (70GC) found in the HSP70 promoter (22) that has a nucleotide composition similar to those of the H2TF1 element and the F593 sequence but little direct homology. Each DNA fragment used in this experiment contained a 13-bp test sequence flanked by common sequences used to provide primer sites for PCR, such that each oligonucleotide probe had a total length of 39 bp (see Table 1 and Materials and Methods). The F593, H2TF1, and SV40 probes gave clearly detectable p300 DNA-binding signals in the UV cross-linking experiment (Fig. 6B, lanes 4, 6, and 8). The positive signals were specific for p300; BSA showed no signal with any of these probes (lanes 3, 5, and 7). In contrast to the positive signals in lanes 4, 6, and 8, the random sequence, the double point mutant, and the E2F, AP1, and 70GC probes did not produce detectable signals (lanes 2, 10, 12, 14, and 16). The presence of p300 in each sample is verified in Fig. 6A. The concentration and specific activity of the DNA probes were also monitored to ensure that each test sample received a constant amount of DNA probe with little or no variation in specific activity. These results demonstrate that p300 has sequence-specific DNA-binding activity, with specific affinity not just for the F593 sequence but also for known enhancer elements. The importance of the related sequences between F593 and the known enhancer elements is emphasized by a close comparison of their sequences. The only sequences completely conserved between the three probes with strong affinity for p300 is the motif GGGANT. This motif is also part of the p300 consensus sequence. In contrast, the H2TF1 · DM probe differs from its most closely related sequence (the H2TF1 site) at only two positions yet fails to give a detectable DNA-binding

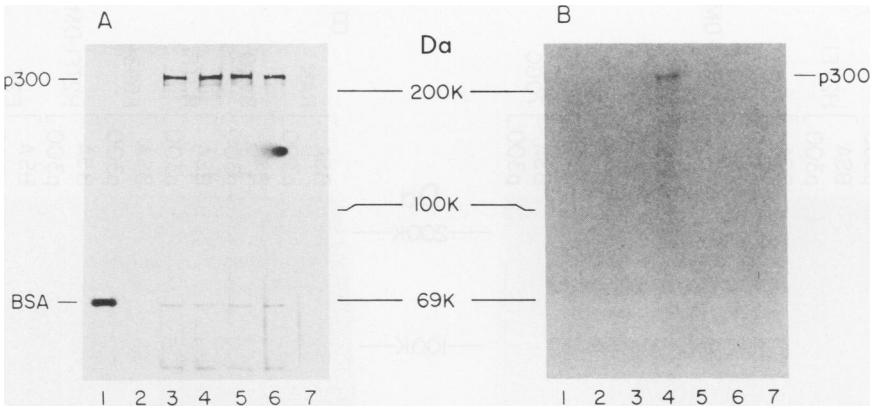

FIG. 5. UV cross-linking of immunoaffinity-purified p300 with DNA. A ^{32}P-labeled F593 DNA fragment was cross-linked to protein samples as described in Materials and Methods. UV-irradiated samples applied to the SDS–6.5% polyacrylamide gel differed as follows: lane 1, 10^{-12} M BSA in place of p300; lane 2, no protein; and lanes 3 to 7, 10^{-12} M p300 with no UV irradiation (lane 3), no competitor DNA (lane 4), 100-fold-excess dI-dC (lane 5), 100-fold-excess nonlabeled F593 DNA (lane 6), or protease K treatment (lane 7). Separated proteins were visualized by silver staining (A) followed by autoradiography (B).

signal in this assay. It is probably a key point that one of the changes in H2TF1 · DM alters the core motif conserved among the p300 consensus, H2TF1, and SV40 probes.

To confirm the specific affinity of p300 for known enhancer elements, the affinity of p300 for the H2TF1 probe was evaluated in a specific competition assay in a UV cross-linking experiment (Fig. 7). For this experiment, we prepared ^{32}P-labeled H2TF1 probe DNA and three different types of nonlabeled competitor DNA fragments: random sequence (RAN), H2TF1, and H2TF1 · DM. The ability of each of these unlabeled probes to compete with p300 binding to the H2TF1 site was tested in competitor excess ranging from 1- to 125-fold. The authentic H2TF1 probe competed readily with itself; a fivefold excess of cold competitor left a barely detectable signal (lane 10 versus lane 4). In contrast, it took an approximate 25- to 125-fold excess of the closely related H2TF1 · DM probe (lane 15 and 16) or a 125-fold excess of totally random sequence (lane 8) to compete as well as a 5-fold excess of authentic H2TF1 probe (lane 10). This experiment also demonstrates that BSA does not compete with the UV cross-linking activity of p300 when present in the same reaction mixture (lane 3 versus lane 4).

From this experiment and others described above, we conclude that p300 has a specific affinity for sequences functionally related to known enhancer-binding protein sites. Moreover, the results imply strongly that the specificity of the DNA-binding reaction is intrinsic to p300. Cellular products of approximately 68 and 55 kDa are occasionally detectable in affinity-purified preparations of p300 and can be seen by silver staining in Fig. 5 and 6, but there is no evidence in the corresponding autoradiograms that these proteins cross-link to the DNA. Thus, it is unlikely in the conditions of this reaction that nonspecific DNA cross-linking is occurring, even when there is reason to think that there may be a direct physical association between the non-DNA-binding proteins and p300. Very faintly labeled species migrating at approximately 110 kDa are detectable along with p300 in lane 4 of Fig. 5. These are likely p105RB-related species, often present at low levels in the E1A affinity-purified p300 preparations, and appear to have DNA-binding activity independent of p300.

DISCUSSION

The results presented here demonstrate that the E1A-binding protein p300 has an intrinsic DNA-binding activity that does not appear to depend on the presence of other cellular factors. Moreover, p300 shows a preferential binding affinity for DNA motifs characteristic of known enhancer-binding protein target sites. This finding correlates remarkably with previous reports showing that the E1A products repress the effect of a variety of viral and tissue-specific enhancers in a manner generally dependent on the p300-binding activity of E1A (15, 30, 37, 39, 51, 53, 58, 59, 68). The wide variety of enhancer sequences affected by E1A, coupled with the observation that E1A proteins do not bind directly to these sequences (18, 36), suggested the possibility that E1A mediates its effects by interfering with the normal assembly of transcription complexes that recognize enhancer sequences. The results reported here provide a direct biochemical basis for this model; they complete a chain of demonstrated physical associations that extends from E1A through p300 directly to enhancer target sites.

Distribution of the p300 DNA-binding motif among E1A-responsive enhancers. A major question that arises from these results is the extent to which the motif that p300 binds

TABLE 1. Nuclear factor binding sites

Promoter or enhancer element/binding factor	Sequence
H2-K^b (MHC class I)/H2TF1	TGGGGATTCCCCA
κ light chain/NF-κB	AGGGGACTTTCCG
SV40	TGGGGACTTTCCA
HIV (left)	AAGGGACTTTCCG
HIV (right)	TGGGGACTTTCCA
Beta interferon/PRDII-BF1	GTGGGAAATTCCT
APRE/AGIE-BP1	TTGGGATTTCCCA
F593	TAGGGACTGCATT
p300 consensus	GGGAGTG
70GC	GGCGGGTCTCCGT
H2TF1 double point mutant	TGCGGATTCCCGA
E2F	TTTCGCGC
AP-1	TG(T)AGTCA

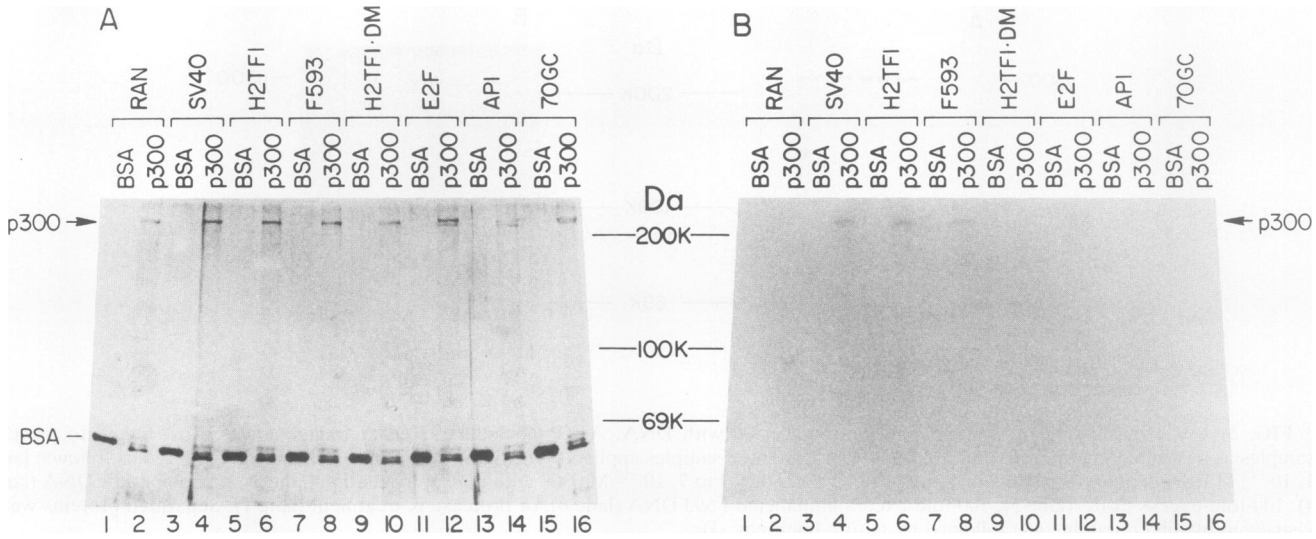

FIG. 6. Specificity of p300 binding to F593, H2TF1, and SV40 probes. A series of ^{32}P-labeled double-stranded oligonucleotides was screened for p300 binding as described in Materials and Methods. Reaction mixtures in odd-numbered lanes contained 10^{-12} M BSA in place of an equimolar amount of p300. The oligonucleotides screened represented random sequence (RAN; lanes 1 and 2), the SV40 motif (SV40; lanes 3 and 4), the H2TF1 motif (H2TF1; lanes 5 and 6), F593 (lanes 7 and 8), a double point mutant of the H2TF1 motif (H2TF1 · DM; lanes 9 and 10), an E2F consensus motif (E2F; lanes 11 and 12), an AP-1 consensus motif (AP-1; lanes 13 and 14), and an HSP70 G+C-rich region (70GC; lanes 15 and 16). Separated proteins were visualized by silver staining (A) followed by autoradiography (B).

is present in the various viral and tissue-specific enhancers known to be responsive to E1A regulation. Viral enhancers within the SV40 (8, 67), polyomavirus (8, 26, 66), and HIV (69) genomes are very sensitive to repression by Ad5 E1A, as are the tissue-specific enhancers directing expression of the genes encoding immunoglobulin heavy chain and kappa light chain (25), insulin (60), troponin 1 (15), and cytochrome P-450c (58).

Motifs structurally and functionally related to the H2TF1 and NF-κB motifs have been demonstrated in the kappa light-chain enhancer (4, 55), the SV40 enhancer (46), and the HIV long terminal repeat (44). Moreover, the NF-κB binding site-related motifs in the HIV long terminal repeat appear to be the actual targets for E1A-mediated repression (69). The E1A upstream sequences also contain enhancer elements (24) that are subject to autorepression (13). E1A enhancer

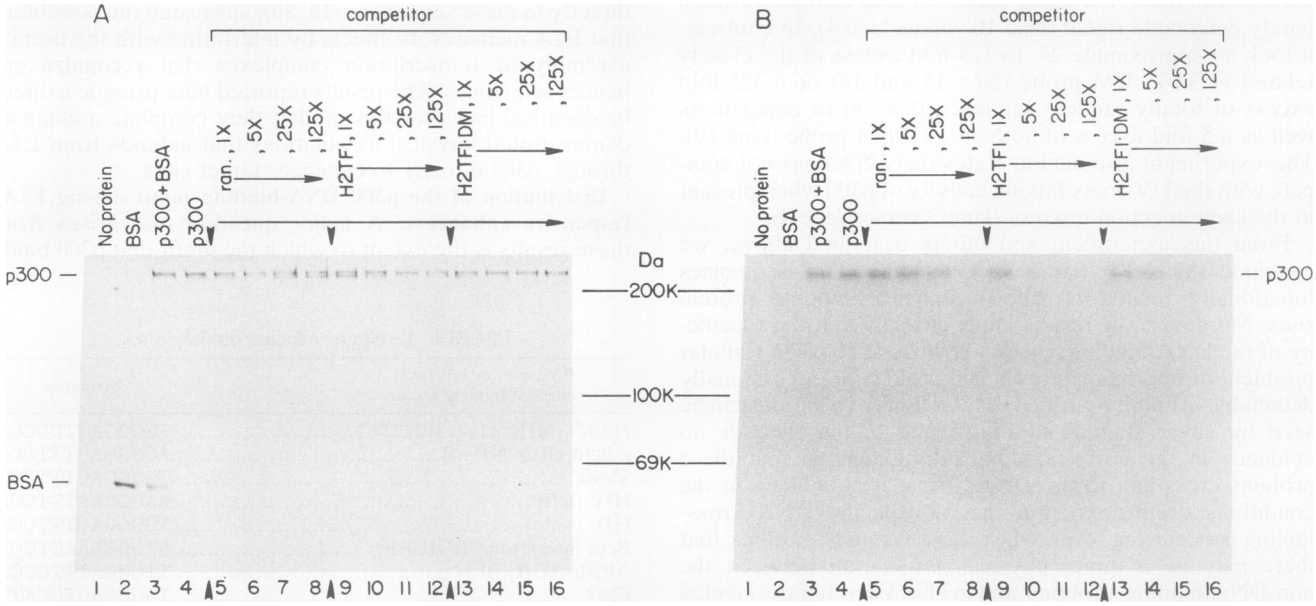

FIG. 7. Specific affinity of p300 for the H2TF1 motif. A ^{32}P-labeled H2TF1 motif DNA fragment was cross-linked with 10^{-12} M p300 as described in Materials and Methods. Reactions applied to the gel differed as follows: lane 1, no protein; lane 2, 10^{-12} M BSA in place of p300; lane 3, 10^{-12} M BSA in addition to p300; lane 4, no competitor DNA; and lanes 5 to 16, 1-, 5-, 25-, and 125-fold excesses of nonlabeled random sequence (RAN; lanes 5 to 8), H2TF1 motif DNA (H2TF1; lanes 9 to 12), or the double point mutant H2TF1 motif (H2TF1 · DM; lanes 13 to 16). Separated proteins were visualized by silver staining (A) followed by autoradiography (B).

activity is dependent on a duplicated element that is conserved among the E1A genes of different adenovirus serotypes (24, 63). An 8-bp consensus sequence for this element, derived from inspection of the enhancers in different serotypes, has the sequence 5′-GGAAGTGA-3′. This sequence is closely related to the p300 consensus recognition site, GGGAGTG (identical nucleotides are underlined). Inspection of other E1A-responsive enhancers reveals sequences similar in some degree to the NF-κB motif, but the role of specific sequences in these enhancers has not been studied in as much detail.

It is also notable that the E1A products of the highly oncogenic adenovirus serotype, Ad12, strongly repress expression of major histocompatibility complex class I genes (19, 54) such as the *H-2K*^{b} gene, whose enhancer contains the prototype of the H2TF1 binding site element. Ad12 E1A-mediated repression of *H-2K*^{b} also appears to be dependent on N-terminal E1A sequences and to be mediated, at least in part, through the H2TF1 binding motif (34). However, it is not yet known whether Ad12 E1A interacts with p300.

Relationship between p300 and other enhancer-binding proteins with specific affinity for the H2TF1 motif. Another significant question that arises is whether any degree of relationship exists between p300 and the various other enhancer-binding proteins that have demonstrated affinity for H2TF1 binding site-related motifs. There have been many reports about H2TF1 motif-binding proteins. Those in a size range of about 50 to 100 kDa include H2TF1 (3, 4), NF-κB (4, 55, 56), KBF1 (29, 76), TCIIB (46), and EBP-1 (12). At least some of these appear to be distinct gene products, as judged by their tissue distribution and relative affinities for their respective binding sites. Another group is composed of large-molecular-weight proteins. PRDII-BF1 (17), cloned from human osteosarcoma cells on the basis of its affinity to an H2TF1 site-related element in the beta interferon enhancer (Table 1), has been sequenced in its entirety and found to encode a protein of 298 kDa. B-cell libraries, probed with the H2TF1 binding site or related HIV enhancer elements, yielded partial cDNA clones designated MBP-1 (2, 57) and HIV-EP1 (40), respectively, which are identical to PRDII-BF1. A mouse lens cell library, probed with sequences derived from the αA-crystallin enhancer, yielded αA-CRYBPI, the murine homolog of PRDII-BF1 (45). A rat thyroid library probed with sequences derived from the α1-antitrypsin enhancer yielded AT-BP1 and AT-BP2 (42). AT-BP2 appears to be the rat homolog of PRDII-BF1, while AT-BP1 appears to be a related, but distinct, gene product. A clone identical to AT-BP1 was obtained from a rat liver library probed with an H2TF1 site-related element (APRE; Table 1) in the angiotensinogen promoter and designated AGIE-BP1 (52). Antiserum to AGIE-BP1 detects a major protein species of approximately 300 kDa. Thus, there are at least two distinct enhancer-binding proteins in the 300-kDa range that show specific affinity for enhancer motifs related to the H2TF1 element, suggesting that there may be a family of such proteins.

The two distinct 300-kDa products represented by PRDII-BF1 and AGIE-BP1 are either growth regulated or somewhat tissue specific in their expression (2, 17, 42, 52) in patterns that p300 does not appear to share (reference 75 and additional data not published). Moreover, we have determined that p300 does not comigrate with the approximately 300-kDa species immunoprecipitated by antiserum raised against AGIE-BP1 (52) or PRDII-BF1 (17) (data not shown). Thus, p300 is not identical to either of these proteins, although it is possible that their DNA-binding regions are related.

Interestingly, AT-BP1 and AT-BP2, which are believed to be identical to the two distinct 300-kDa proteins cloned on the basis of strong affinity to the H2TF1 binding site or closely related motifs, were cloned on the basis of specific binding to an α1-antitrypsin enhancer probe which contains no sequence motifs directly homologous to the H2TF1 or APRE enhancer elements, although a potentially related site has been proposed (42). Whether or not this proposed site is the actual binding site for AT-BP1 and/or AT-BP2, it seems likely that DNA-binding proteins in this group can bind to a wider diversity of sequences than is represented by the motifs so far recognized as specifically related to the H2TF1 binding site. Similar considerations might apply to p300. The limited p300 binding site consensus obtained from the sensitive PCR selection technique (Fig. 3) may be a very stringent reflection of preferred sequences. It is clear that the F593 clone represents a preferred binding site for p300. Not only does it bind p300 with higher efficiency than do random sequences, it binds with higher efficiency than does the H2TF1 · DM sequence. The ability of p300 to discriminate between the H2TF1 · DM sequence and preferred binding sites (Fig. 6 and 7) argues strongly that the ability of p300 to bind known enhancer motifs is biologically relevant.

Does the E1A N-terminal activity repress at multiple elements? The affinity of p300 for known enhancer motifs related to the NF-κB binding site suggests strongly that transcription complexes at these sites are specific targets of the E1A N-terminally-mediated enhancer repression function. Previously, it has been suggested that E1A repression does not have specific DNA sequence targets because E1A can exert repressive effects at multiple different elements. For example, Enkemann et al. (15) found that E1A represses the expression of reporter genes controlled by several different muscle-specific regulatory elements. In addition, Rochette-Egly et al. (51) tested separately the several different proto-enhancers within the SV40 enhancer and found that E1A could repress each of them, including that which contains the NF-κB-related motif. Thus, there is fairly direct evidence that the E1A products can repress enhancer-mediated transcription dependent on this element, but E1A can apparently also repress transcription dependent on other elements. E1A has also been shown to repress expression of several cellular genes in which the target of repression (enhancer or promoter) was not identified (15, 61, 62, 64, 65, 71, 77).

Several possibilities may explain these observations. In some cases, the repression observed may be a secondary effect, resulting from repression of a product dependent for its expression on a p300 binding motif. It is also possible that p300 binds to additional enhancer motifs since its consensus sequence is more limited than is that of the NF-κB-related motifs. For example, the GT-I (GGGTGTGG) and GT-IIC (GTGGAATGT) enhansons in SV40 (51) show considerable similarity to the p300 consensus. It has been suggested that the NF-κB site actually consists of two half sites (78), each of which can be bound by an NF-κB molecule. In some analogous way, p300 may combine with other factors to promote transcription at multiple elements. Indeed, a factor with such properties would be a likely target for E1A. It is also possible that E1A interacts with other enhancer-related products in addition to p300; there are observed E1A-associated proteins that have not yet been characterized, and E1A may well exert repressive effects through several mechanisms. Regardless of these speculations, the clear

affinity of p300 for identified elements found in both tissue-specific and viral enhancers correlates strikingly with the pattern of enhancer repression observed in E1A studies. Our results suggest strongly that an important component of the E1A transcription repression function is directed, through p300, at specific enhancer elements.

Possible mechanisms of action. It will be important to determine whether E1A association with p300 interferes directly with p300 DNA binding. The DNA-binding activity of p300 on DNA-cellulose columns is not obviously different in 293 cell extracts compared with HeLa cell extracts, so no changes in p300 activity are yet associated with E1A expression. It is possible that E1A affects different aspects of p300 function, such as potential associations between p300 and as yet unidentified cellular factors. Such a possibility is precedented by the manner in which the E1A products affect E2F transcription factor complexes and the increasing recognition of multicomponent transcriptional complexes (for a review, see reference 31). Immunoprecipitations with rabbit polyclonal antiserum raised against p300 show several other proteins in addition to p300 which may be p300-associated products (75). We have begun to characterize a number of these, and it is possible that these proteins include factors that can interact with p300 to modulate its activity.

It has been known for some time that conserved region 3 of E1A is involved in transcriptional activation. With the recent demonstration that E1A region 2 alters the composition of cellular E2F-related transcription factor complexes, it has become clear that at least one of the two transforming domains of E1A also targets transcription factors. The present demonstration that p300 has enhancer site binding activity makes it reasonable to postulate that p300 is also involved in transcriptional regulation and suggests as a general model that each of the E1A active sites may have evolved to alter the activity of specific sets of cellular transcription factors. It has already been suggested (60) that an activity selectively capable of repressing tissue-specific gene expression and cellular differentiation may be advantageous for the ability of E1A to induce host cell proliferative functions. To this end, E1A region 2 may serve primarily to activate latent cell growth potential while the N-terminal active site represses the program signalling terminal differentiation and the cessation of cell growth.

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