Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor

Richard Eckner, Mark E. Ewen, David Newsome, Mike Gerdes,[1] James A. DeCaprio, Jeanne Bentley Lawrence,[1] and David M. Livingston

Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115 USA; [1]Department of Cell Biology, University of Massachusetts, Medical Center, Worcester, Massachusetts 01655 USA

The growth-controlling functions of the adenovirus E1A oncoprotein depend on its ability to interact with a set of cellular proteins. Among these are the retinoblastoma protein, p107, p130, and p300. We have isolated a cDNA encoding full-length human p300 and mapped the chromosomal location of the gene to chromosome 22q13. p300 contains three cysteine- and histidine-rich regions of which the most carboxy-terminal region interacts specifically with E1A. In its center, p300 contains a bromodomain, a hallmark of certain transcriptional coactivators. We have examined the ability of p300 to overcome the repressive effect of E1A on the SV40 enhancer. We show that p300 molecules lacking an intact E1A-binding site can bypass E1A repression and restore to a significant extent the activity of the SV40 enhancer, even in the presence of high levels of E1A protein. These results imply that p300 may function as a transcriptional adaptor protein for certain complex transcriptional regulatory elements.

[*Key Words*: p300; transcriptional adaptor protein; E1A-binding protein; cell cycle regulatory protein]

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The adenovirus E1A polypeptides are multifunctional proteins that are involved in a wide variety of cellular processes ranging from transcriptional activation and repression to immortalization, blockade of differentiation, and stimulation of DNA synthesis. E1A proteins are synthesized from two differently spliced transcripts to give rise to the 12S and the 13S E1A mRNA products. Comparison of the sequence of E1A proteins from different adenovirus serotypes indicates that the 12S and 13S polypeptides harbor two conserved regions (named CR1 and CR2) that are common to these two proteins, whereas the third conserved region (CR3) is unique to the 13S product. All three conserved regions serve as binding sites for cellular proteins whose interaction with E1A is essential for its many activities. The modular structure of E1A has led to the assignment of the various E1A biological activities to specific conserved regions and to the cellular proteins associated with them (for review, see Moran and Mathews 1987; Dyson and Harlow 1992; Nevins 1992).

The E1A regions required for control of cell growth, differentiation blockade, and transformation comprise the nonconserved amino terminus together with CR1 and CR2 (Lillie et al. 1986; Moran et al. 1986; Smith and Ziff 1988; Subramanian et al. 1988; Whyte et al. 1988, 1989; Jelsma et al. 1989). It can be subdivided into two active sites, each of which can stimulate DNA synthesis on its own (Lillie et al. 1987; Zerler et al. 1987; Howe et al. 1990). The first active site consists of CR2 and the amino-terminal part of CR1. Among the proteins interacting with this part of E1A are pRB (the product of the retinoblastoma gene) and the related proteins p107 and p130. All of them utilize a common domain, termed the pocket, for E1A binding. The second active site consists of the amino terminus of E1A and the carboxy-terminal half of CR1. It binds, at a minimum, to a cellular 300-kD protein called p300 (for review, see Moran 1993). p300 binding can be abolished selectively by mutating the E1A amino terminus or the carboxy-terminal part of CR1 without affecting binding of E1A to any other of its associated protein (Wang et al. 1993). Mutations in either the amino-terminal half of CR1 or in CR2 regularly affect the binding of more than one associated protein (Dyson et al. 1992), and it is therefore difficult to link certain biological functions of E1A to the binding of any single member of the RB family of proteins.

Aside from stimulating S-phase entry of quiescent cells, the region of E1A interacting with p300 is also responsible for repressing a number of transcriptional enhancers and promoters. The first identified targets for E1A repression were the viral enhancers of SV40 and polyoma virus and the enhancer element controlling

transcription of the E1A gene itself (Borelli et al. 1984; Velcich and Ziff 1985). In addition to this group of viral enhancers, a second class of enhancers and promoters that drive transcription of tissue-specific cellular genes associated with the terminal differentiation state of a cell were found to be repressed by E1A (Hen et al. 1985; Stein and Ziff 1987; Webster et al. 1988). Because oncogenic transformation generally inhibits cellular differentiation, the repressive action of E1A on this class of genes may be particularly relevant to the mechanism of how E1A and a cooperating oncogene transform primary rodent cells. Subsequent genetic studies further refined and strengthened the correlation between the repressive activity of E1A and the ability of E1A to bind p300 (Rochette-Egly et al. 1990; Stein et al. 1990; Wang et al. 1993). p300 is therefore a good candidate for mediating this activity of E1A. Recently, in support of a role of p300 in transcription, biochemical evidence has been presented suggesting that p300 is a component of TATA-binding protein (TBP) complexes (Abraham et al. 1993).

In this report we describe the molecular cloning, structural analysis, and initial functional characterization of p300. Our results show that p300 has the structural and functional properties of a transcriptional adaptor molecule that seems to be required for the activity of certain enhancers.

Results

Isolation of cDNAs encoding p300

Microgram quantities of p300 were isolated from 293 cells by applying the anti-E1A immunopurification procedure used previously to purify p107, another E1A associated protein (Ewen et al. 1991). Three different mice were successfully immunized with gel band purified p300, and the resulting antisera specifically recognized p300, as determined by peptide mapping experiments (data not shown). One of the three antisera was used to screen 1.2 million plaques from a 293 cell cDNA library (Ewen et al. 1991). Two positive phage clones were obtained. Plaques of both clones also reacted strongly with the two other antisera, while a nonrecombinant control phage gave no signal (data not shown). The two phages contained overlapping inserts (2-B5 and 2-G2 in Fig. 1A). The subsequent sequential screening of four different cDNA libraries resulted in the recovery of cDNAs that together spanned ~9 kb (see Fig. 1A). We have performed Northern analyses under high stringency conditions with all of the cDNA fragments depicted in Figure 1A and found that they all hybridize to a transcript of ~9 kb in size. In keeping with the ubiquitous expression of the p300 protein (Yaciuk and Moran 1991), we detected the 9-kb mRNA in all eight cell lines examined (see Materials and methods). Figure 1B shows an example of such a Northern blot with cytoplasmic RNA from 293 and Akata cells (lanes 1,2) and poly(A)-selected WERI-RB27 RNA (lane 3). We frequently observed a signal just below the 28S rRNA. This signal was absent when polyadenylated RNA from WERI-RB27 cells was analyzed (lane 3) but was present when total RNA from the same cell line was probed (data not shown). Thus, it likely represents a nonspecific hybridization signal caused by 28S rRNA.

Preliminary experiments indicated that the proteins synthesized by the N24 and N28 cDNA inserts bound E1A. To investigate whether this region of the presumed p300 cDNA could interact with E1A in vivo, in a manner similar to endogenous p300, we transfected U-2 OS cells with a plasmid designated CMV 5.3 ATG. This plasmid contains a consensus ATG translation initiation codon followed by the carboxy-terminal 5.3 kb of the p300

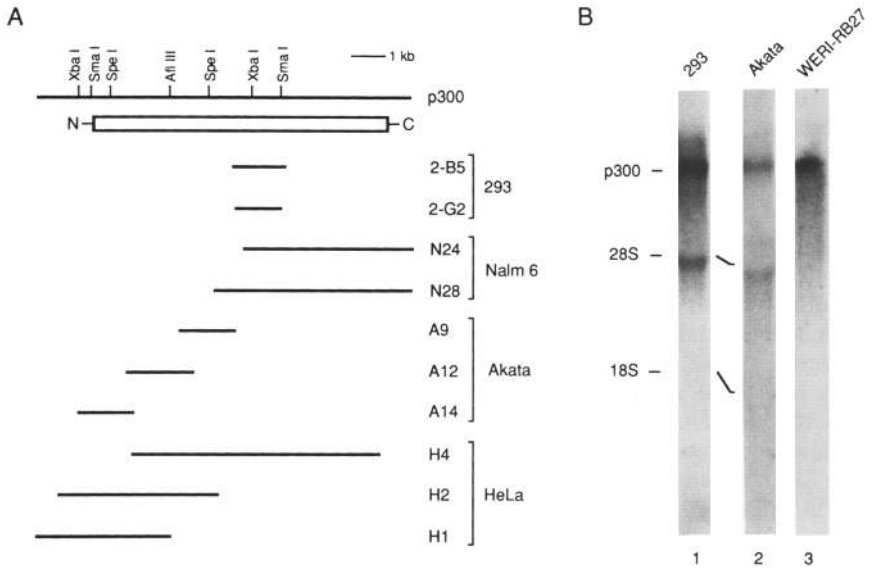

Figure 1. Schematic representation of the isolated p300 cDNA fragments and Northern analysis of the p300 mRNA. (*A*) The assembled p300 cDNA with key restriction sites and the position of the open reading frame is shown (*top*). (*Below*) The most relevant of the obtained cDNA fragments are depicted in the order in which they were isolated (from *top* to *bottom*). The library from which the fragments were derived is indicated (*right*). The Akata cDNA library was screened three times; the other three libraries were each probed once. The HeLa library (Xiao et al. 1991) was a gift of P. Chambon (Institute de Chimie Biologique, Strasbourg, France). (*B*) Northern blot with RNA from three different human cell lines. Cytoplasmic RNA from 293 cells (embryonic kidney cells transformed by the early region of adenovirus) was probed with the 2-B5 insert (lane *1*). Cytoplasmic RNA from Akata cells (a Burkitt-lymphoma cell line) and poly(A)-selected RNA from WERI–RB27 (an RB-derived cell line) were each hybridized with the A12 cDNA fragment (lanes *2*,*3*). Hybridization and washing of the blots were performed under high stringency conditions. The positions of the 18S and 28S rRNAs are indicated (*left*).

cDNA. It encodes a 200-kD protein [visible in Fig. 2, lane 14, as a hemagglutinin (HA)–epitope-tagged version] that can be well separated from endogenous p300 in SDS–polyacrylamide gels. The U-2 OS cell line is derived from an osteosarcoma, expresses a wild-type RB protein (Huang et al. 1988), and was chosen because it can be transfected well. One day after transfection, the cells were infected with a series of adenoviruses harboring wild-type or mutant E1A genes. Ten hours later, the cells were labeled with [^{35}S]methionine and lysed, and the extract of each dish was immunoprecipitated with the anti-E1A monoclonal antibody (mAb) M73 (Harlow et al. 1985), or with the mAb pAb 419, which served as a control. Figure 2 illustrates that whenever the endogenous p300 was coprecipitated, the 200-kD protein encoded by the 5.3 ATG construct was also present in the immunoprecipitate. This is the case for wild-type E1A (lane 2) and for the E1A mutation NCdl, carrying a deletion between CR1 and CR2 (lane 6). In contrast, E1A proteins, with either a point mutation in amino acid 2 (pm563, lane 4) or bearing a deletion of CR 1 (dl646, lane 10), failed to associate with either endogenous p300 or the exogenous 200-kD protein. The high molecular weight protein in lane 4 is the E1A-associated p400 protein described earlier (Howe and Bayley 1992). No E1A-associated proteins were detected when U-2 OS cells were infected with the dl312 virus, which does not synthesize E1A owing to a large deletion in the early region of this virus (Fig. 2, lane 8). Similarly, no protein in the size range of 200 kD was coprecipitated from lysates of cells transfected with the expression vector lacking an insert (lane 12). This experiment demonstrates that the transfected cDNA encodes a protein exhibiting the same genetics of E1A binding as the endogenous p300 protein.

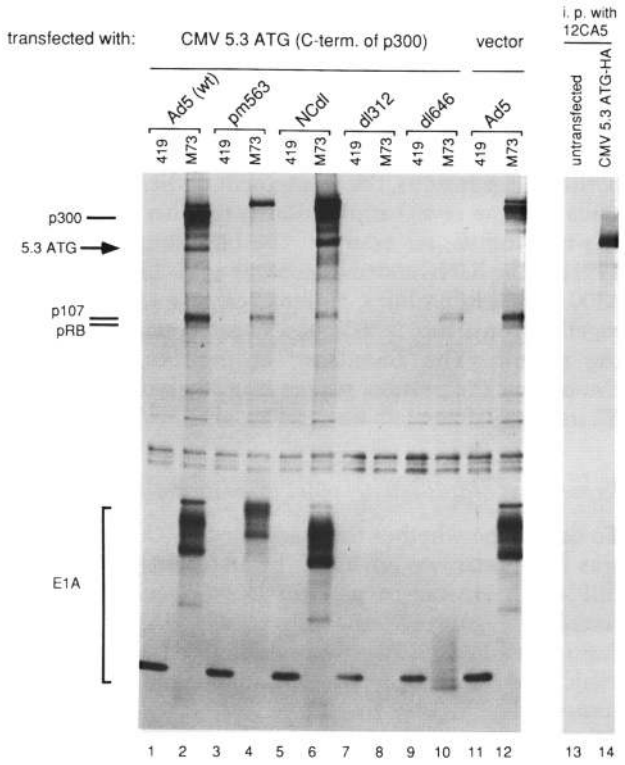

Figure 2. Analysis of the in vivo E1A-binding characteristics of a carboxy-terminal 200-kD fragment of p300. U-2 OS cells were transiently transfected with the CMV 5.3 ATG plasmid directing the expression of a 200-kD carboxy-terminal fragment of p300. One day after transfection, cells analyzed in lanes *1–10* were trypsinized, pooled, and reseeded on five 10-cm dishes, to equalize for transfection efficiencies. Cells were then infected with the adenovirus species indicated (*top*). Ten hours later, they were labeled with [^{35}S]methionine. A lysate of each dish was split in half and immunoprecipitated with pAb 419 (control) or the anti-E1A mAb M73. Adenovirus 5 (Ad5) encodes wild-type E1A proteins; pm563 encodes E1A proteins with a point mutation in amino acid 2; NCdl encodes E1A proteins lacking amino acids 86–120 (these residues lie between CR1 and CR2). The dl312 virus is deleted for the E1A gene, and dl646 encodes E1A proteins missing amino acids 30–85. Lanes *11* and *12* show the immunoprecipitation pattern of cells transfected with the expression vector lacking an insert, followed by infection with wild-type Ad5. In lanes *13* and *14*, lysates of untransfected U-2 OS cells and of U-2 OS cells transfected with a HA-tagged version of the 5.3 ATG plasmid, respectively, were incubated with the anti-HA mAb 12CA5. The positions of E1A and its associated proteins are noted (*left*).

Chromosomal location of the p300 gene

It is conceivable that p300 could be encoded by a tumor suppressor gene based on the precedent of another E1A-associated protein, the RB protein. Therefore, it was of interest to map its chromosomal location to determine whether it maps near a cytogenetic location known or suspected to harbor a tumor suppressor gene. The 4.5-kb N28 cDNA fragment (see Fig. 1A) was used for fluorescence in situ hybridization (FISH) to metaphase chromosome preparations from normal human lymphocytes. Cells (~90%) showed label, with label at a single chromosomal site on a homologous pair of E-group chromosomes. DAPI staining of chromosomes initially indicated that this was human chromosome 22, and the distinction from chromosome 21 was confirmed by simultaneous two-color hybridization of a second sequence known to map on chromosome 21, which clearly mapped to a different chromosome than the p300 gene. As summarized in Figure 3, DAPI-banding analysis of BrdU-incorporated chromosomes in >20 metaphase spreads showed that the p300 gene consistently localized in band 22q13, predominantly in the distal half of that chromosome band corresponding to 22q13.2–q13.3. The recently identified neurofibromatosis 2 gene (Rouleau et al. 1993; Trofatter et al. 1993) is localized proximal to p300 at 22q12. Chromosomal deletions, including the 22q13 region, have been detected in certain types of colon cancer (Okamoto et al. 1988) and gliomas (Jenkins et al. 1989). Further analysis is under way to investigate a potential involvement of p300 in these cases.

Sequence of p300

The sequence of the assembled p300 cDNA and the predicted open reading frame are shown in Figure 4A. The

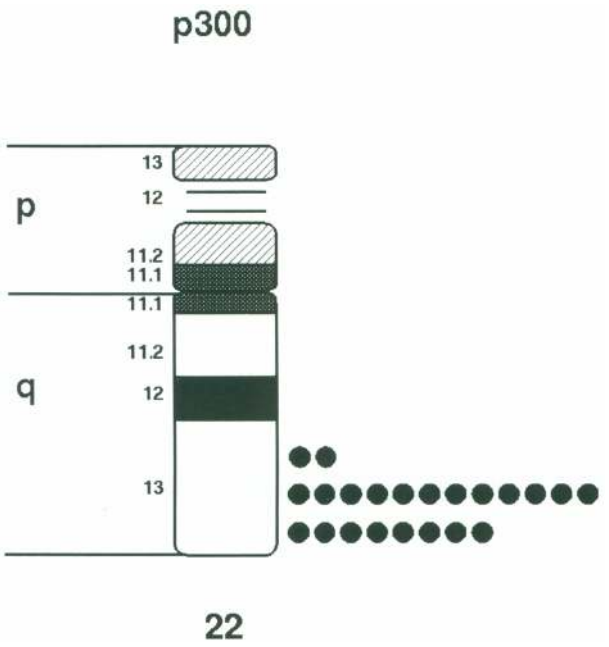

Figure 3. Chromosomal location of the p300 gene. Shown is an ideogram revealing the location of p300 gene signals on chromosome 22, as scored based on BrdU-enhanced DAPI-banding patterns of 22 different images. Although band q13.2 was not visible in these preparations, the localization of the gene to the distal half of the 22q13 band supports its finer localization to 22q13.2–q13.3.

p300 mRNA contains a remarkably long 5′-untranslated region (1.2 kb). The 5′-terminal 1 kb is rich in CpG dinucleotides, indicative of the 5′ ends of many constitutively transcribed housekeeping genes (Bird 1986). The p300 mRNA shares this feature with the pRB and p107 messengers. The translation initiation codon of p300 is preceded by an in-frame stop codon located 45 nucleotides farther upstream. The sequence context of the initiator AUG matches well with the consensus sequence flanking translational start codons (Kozak 1991). The open reading frame of the cDNA encompasses 2414 amino acids and predicts a protein with a molecular mass of 264.236 kD.

Data base searches with the p300 protein sequence revealed that p300 has in its central region a bromodomain (see Fig. 4B). This 65-amino-acid domain of unknown function is conserved from yeast to man (Haynes et al. 1992; Tamkun et al. 1992), and has been found in several proteins implicated as global activators of transcription. Included in this group of polypeptides are the human (Sekiguchi et al. 1991; Hisitake et al. 1993; Ruppert et al. 1993) and *Drosophila* (Kokubo et al. 1993; Weinzierl et al. 1993) 250-kD TBP-associated factors ($TAF_{II}250$/CCG1), each of which contains two bromodomains. As noted by Haynes et al. (1992), the second part of the bromodomain is likely to form two short amphipathic α-helices followed by reverse turns (see Fig. 4B). In keeping with the amphipathic character of this region, the second helix of the p300 bromodomain contains on the hydrophobic side of its surface a heptad repeat of 3 tryptophan residues (Fig. 4B).

Further analysis of the p300 amino acid sequence revealed the presence of three cysteine- and histidine-rich domains (Fig. 4A). The most amino-terminal of these domains can be aligned as two zinc fingers. In each of the two putative fingers, two pairs of cysteines are spaced by a 12-amino-acid linker reminiscent of the structure of zinc fingers. However, the two fingers of p300 do not display a number of residues well conserved among various subgroups of zinc finger proteins and, therefore, do not completely conform to the classical zinc finger consensus sequence. Moreover, there are additional cysteine and histidine residues present between the pairs of cysteines highlighted in Figure 4A. Conceivably, they also participate in the coordination of zinc ions. The other two cysteine/histidine-rich regions of p300 are localized in the carboxy-terminal half of the protein (Fig. 4A). In both of these regions, the arrangment of these two amino acids does not reveal any similarity to other known Cys/His-rich motifs, for example, the LIM domain (Li et al. 1991) or the RING motif (Freemont et al. 1991). Overall, p300 is rich in prolines, glutamines, and serines, which together constitute >30% of all amino acid residues of the protein. The abundance of prolines, distributed throughout the protein, makes it unlikely that p300 exhibits many extended α-helical regions.

Expression of full-length p300 in mammalian cells

To determine whether the assembled p300 cDNA clone was functional, we produced, by transient transfection, full-length HA-tagged p300 in U-2 OS cells. As illustrated in Figure 5A, the exogenous, HA-tagged p300 comigrated in an SDS–polyacrylamide gel with the E1A-associated 300-kD protein (cf. lanes 2 and 3). In vitro-translated p300 (lane 1) displayed a slightly faster mobility than in vivo-synthesized p300, suggesting that it lacks certain post-translational modifications.

We then subjected ^{35}S-labeled HA-tagged p300, derived from transfected U-2 OS cells, and authentic, E1A-bound, endogenous 300-kD protein to comparative peptide mapping experiments with *Staphylococcus aureus* V8 protease. The patterns of the two proteins obtained by partial digestion with this enzyme were generally similar (Fig. 5B). There were some minor differences between the HA-tagged, cloned p300 and the E1A-bound 300-kD protein. To rule out a cloning artifact, we reassembled a full-length p300 cDNA using a set of cDNA fragments completely different from that used for the construction of the first p300 cDNA (see Materials and methods). This newly assembled p300 clone encoded a protein again showing the same small deviations seen before from the proteolytic pattern of E1A-bound, endogenous p300 (data not shown). This result argues against a cloning artifact, and suggests that the small differences are attributable to other influences, for example, overproduction (see below) or the presence of an HA tag.

To probe the structural similarity of the cloned p300 and the E1A-associated 300-kD protein by a second ap-

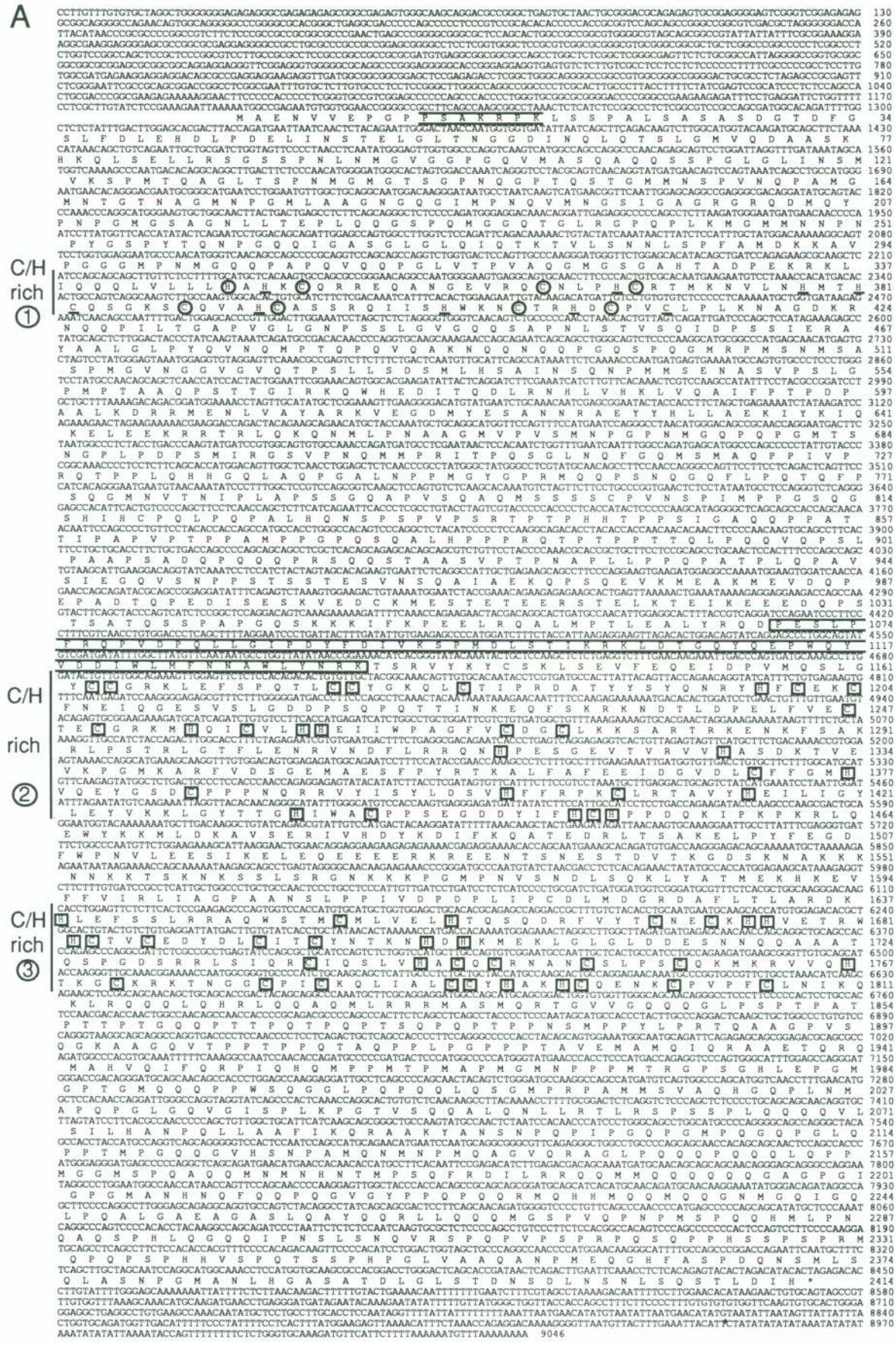

Figure 4. {*See following page for B and legend.*}

proach, we used the chemical agent *N*-chlorosuccinimide (NCS), which cleaves proteins after tryptophan residues. Figure 5C illustrates that this method also resulted in virtually identical cleavage patterns among the cloned and endogenous proteins.

Finally, a series of five monoclonal antibodies raised against *Escherichia coli*-produced p300 protein provided another piece of evidence for the authenticity of the cloned p300 cDNAs. These antibodies recognize different epitopes in the carboxyl terminus of p300, based on the pattern of in vitro translation products recognized by immunoprecipitation with each (data not shown) and based on epitope-mapping studies summarized in Table 1. The major product immunoprecipitated by these monoclonal antibodies from ^{35}S-labeled U-2 OS cell lysates was a 300-kD protein (Fig. 6A) that displayed, in each case, a proteolytic peptide mapping pattern identical to that of the E1A-associated 300-kD protein (Fig. 6B shows the V8 protease map for RW105). In addition, when the monoclonal antibodies directed against p300 were used with lysates prepared from the E1A-transformed 293 cell line, some of the antibodies coprecipitated E1A (data not shown).

Taken together, the results presented above suggest strongly that the isolated cDNAs encode the E1A-associated 300-kD protein.

Determination of the E1A-binding site on p300

We then sought to map the E1A interaction site on p300. To this end, a series of in vitro translation templates with progressive 5′ or 3′ deletions was prepared. The templates were derived from the carboxy-terminal half of p300 shown before (Fig. 2) to contain the binding site for E1A. They are depicted schematically in Figure 7D. ^{35}S-Labeled products from in vitro translation reactions were mixed with cold cell lysates derived from the E1A-expressing cell line 293. The ability of the translation products to associate with E1A was assayed by immunoprecipitation with the anti-E1A antibody M73. Analysis of the 5′ deletion series indicated that proteins beginning at the *Mun*I site can still interact with E1A (Fig. 7A, lane 9), though with decreased efficiency compared with longer proteins (cf. lanes 6–8 with lane 9). This conclusion takes into account the lower specific activity of the shorter translation products as compared with the longer ones (owing to the presence of fewer methionine residues). Once sequences up to the *Aha*II site were removed, translation products no longer bound E1A (Fig. 7A, lane 10). Interpreting these results conservatively, the 5′ border of the p300 region required for efficient interaction with E1A appears to be located between the *Sma*I and *Mun*I sites. Analysis of the 3′ border of the E1A-binding site placed it at the *Pvu*II site (Fig. 7B). Translation products ending at the *Mun*I site were unable to bind E1A (lane 12, Fig. 7B). The p300 sequences defined by this approach overlap the third Cys/His-rich region located between amino acids 1572 and 1818 (see Fig. 7D).

To probe the relevance of the in vitro-determined E1A-binding site in vivo, we prepared two mutants bearing internal deletions that remove the second half of the above noted Cys/His-rich region. They were inserted into the 5.3 ATG backbone used before for studying the genetics of the interaction between E1A and cloned p300 (Fig. 2) and were termed 5.3 ATG del30 and del33 (see Fig. 7D for schematic drawing). U-2 OS cells were transiently transfected with an expression vector for the 12S E1A product along with the wild type and either one of the internal deletion mutants. All three proteins were produced as HA-tagged 200-kD derivatives. Lysates from ^{35}S-labeled transfected cells were split in half and immunoprecipitated, in parallel, with the anti-HA mAb 12CA5 and with the anti-E1A mAb M73, respectively. The 12CA5 immunoprecipitation was performed to

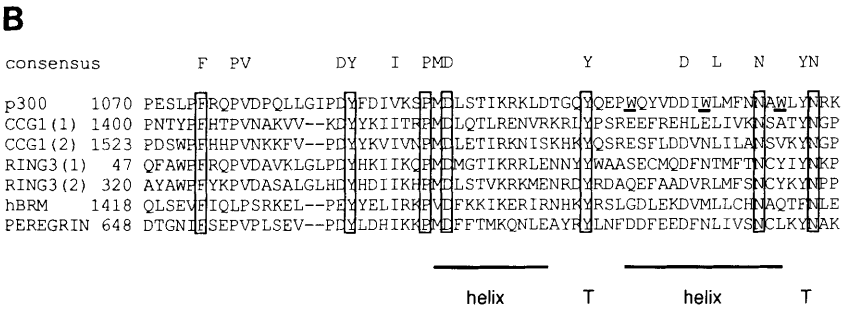

Figure 4. Features of the p300 sequence. (*A*) Nucleotide sequence of the p300 cDNA and its predicted amino acid sequence. The three Cys/His-rich regions are marked C/H rich (*left*). In the first region, the residues of the two putative zinc finger motifs involved in coordinating zinc are circled and the other Cys and His residues are underlined. In the second and third C/H rich region, the Cys and His residues are highlighted with squares. The bromodomain in the middle of p300 is boxed, and the nuclear location signal at the amino terminus (see Fig. 8) is marked with a line above and below it. The GenBank accession number for the p300 sequence is U01877. (*B*) Sequence alignments of human bromodomain proteins. The bromodomains of the five human bromodomain-containing proteins known at the time of submission of this manuscript were aligned. CCG1 (TAF 250) and RING3 each contain two bromodomains. hBRM is a human homolog of the *Drosophila brahma* protein. The consensus sequence is given (*top*): Only amino acid residues that are conserved in all seven bromodomains or that are present in at least six of the seven human bromodomains are listed. In addition, residues that are conserved in all seven domains are marked in the alignment with a box. The three tryptophan residues in the p300 bromodomain that are spaced as a heptad repeat are underlined. The position of the region predicted to form two α-helices followed by reverse turns is indicated (*below*). Data base accession numbers for sequences: CCG1, D90359; RING3, X62083; hBRM, X72889; PEREGRIN, M91585.

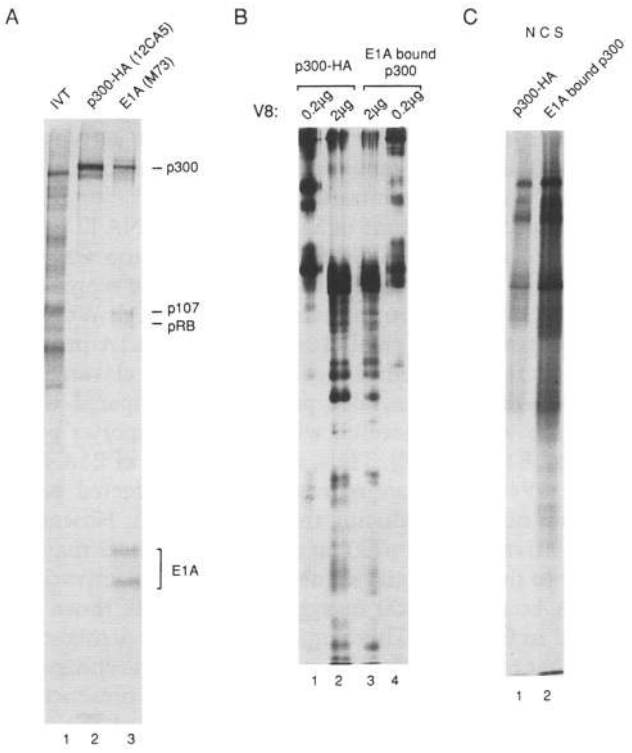

Figure 5. Comparison of the cloned p300 protein with the E1A-bound 300-kD protein. (*A*) The migration of full-length, ^{35}S-labeled p300 generated by in vitro translation (lane *1*) is compared with the migration of HA-tagged, full-length p300 immunoprecipitated with the 12CA5 antibody from lysates of transfected, [^{35}S]methionine labeled U-2 OS cells (lane *2*) and with the mobility of the E1A bound 300-kD protein immunoprecipitated from 293 cells with the anti-E1A mAb M73 (lane *3*). The faint bands visible in lane *2* below p300 represent most likely background bands, as they can also be seen with lysates from untransfected cells (see, e.g., Fig. 7, lane 7). (*B*) SDS–protein–gel-purified, ^{35}S-labeled p300 proteins derived from U-2 OS cells transiently transfected with HA-tagged, cloned p300 (p300–HA) and from 293 cells (E1A bound-p300) were partially digested with *S. aureus* V8 protease, employing the procedure of Cleveland et al. (1977). The amount of V8 protease used is indicated (*top*). (*C*) NCS cleavage map of p300–HA and E1A-bound p300. NCS cleavage was carried out as described (Draetta 1987).

monitor the expression levels of the three different p300 variants. Figure 7C shows that all three p300 versions were present at comparable levels (lanes 1,3,5). However, only the wild-type 200-kD protein bound to E1A (lane 2). At the same time, endogenous p300 coprecipitated efficiently with E1A (see lanes 2,4,6), demonstrating that the failure to detect E1A bound to the two internal deletion mutants was not attributable to immunoprecipitation difficulties. The result from this experiment supports the notion that the third Cys/His-rich region (amino acids 1572–1818) represents the binding site for E1A on p300. It remains to be seen whether this segment of p300 by itself is sufficient to interact with wild-type and mutant E1A proteins in the same way as does full-length p300.

p300 has an amino-terminal nuclear location signal

p300 is a nuclear protein (Yaciuk and Moran 1991). As expected, full-length, HA-tagged p300 was exclusively nuclear, as shown by immunofluorescence in Figure 8A. However, a p300 protein missing 140 amino acids at the amino terminus (termed ΔN-p300) was predominantly, albeit not exclusively, cytoplasmic (Fig. 8B, left), as was the 200-kD carboxy-terminal fragment encoded by the 5.3 ATG chimera (data not shown). Interestingly, in the presence of E1A, all p300 derivatives capable of associating with it, including those that were otherwise cytoplasmic, were again exclusively nuclear (e.g., the 5.3 ATG chimera in Fig. 8B, middle). The rescue of the nuclear transport defect of amino terminally truncated p300 molecules was dependent on their ability to interact with E1A. This is illustrated by the mutant 5.3. ATG del33, which cannot bind E1A (see Fig. 7C) and, hence, can no longer be transported to the nucleus by E1A (Fig. 8B, right). Because E1A mutants unable to interact with p300 also failed to transport amino-terminally truncated versions of p300 to the nucleus efficiently (data not shown), cytoplasmic p300 versions are likely transported to the nucleus as E1A complexes via an E1A-dependent piggyback mechanism. Analysis of the first 140 amino acids of p300 suggested that residues 11–17 represent an amino-terminal nuclear location signal (see Fig. 4A). This sequence represents the only cluster of basic amino acids in this region and fits the consensus sequence for one subtype of nuclear location signals (Dingwall and Laskey 1991). It is likely that p300 harbors a second nuclear targeting signal, because some of the ΔN-p300 molecules were still able to reach the nucleus.

Prominent anti-p300 reactive speckles were visible in transfected cells in the nucleus or distributed throughout the cell, depending on the localization of the relevant p300 species. We suspect that these speckles represent aggregates of p300 that perhaps arise because of the high cysteine content of p300 coupled with its overproduction. This difficulty with transfected p300 could also help to explain why the peptide maps from transfected p300 exhibited small deviations from the pattern observed with endogenous 300-kD protein. In this model, p300 aggregation after overproduction could inhibit its post-translational modification such that the pattern resulting from limited proteolysis is slightly different from that observed with the correctly modified endogenous protein.

Table 1. *Epitopes recognized on p300 by the five monoclonal anti-p300 antibodies*

mAb	Subclass	Epitope (amino acids)
RW102	IgG3	2023–2107
RW105	IgG1	1921–2023
RW109	IgG1	1868–1921
RW128	IgG1	2107–2283
RW144	IgG1	1921–2023

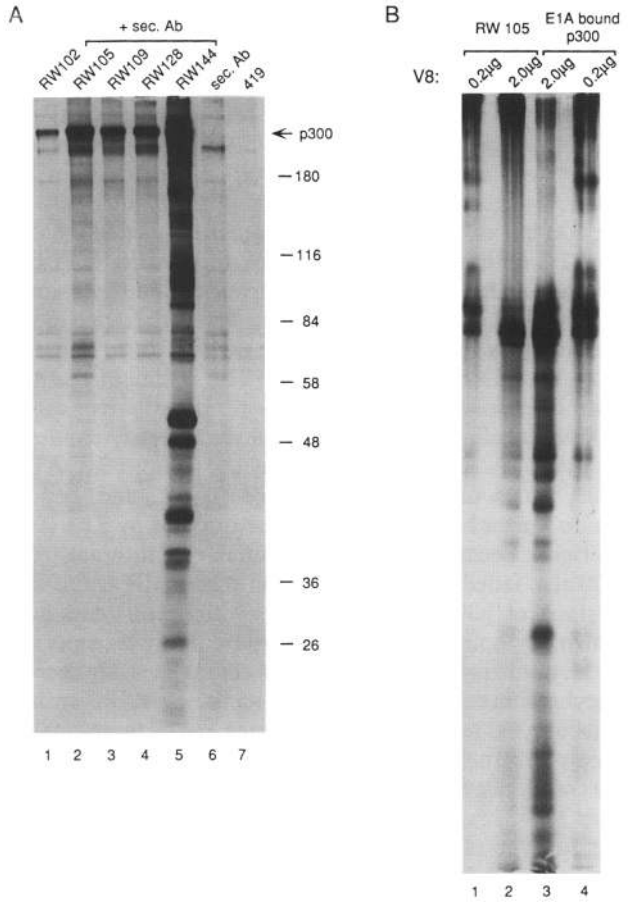

Figure 6. Monoclonal antibodies directed against p300. (*A*) Lysates from [^{35}S]methionine-labeled U-2 OS cells were immunoprecipitated with five different anti-p300 monoclonal antibodies (lanes *1–5*), with a rabbit anti-mouse IgG secondary antibody (lane 6), or with the monoclonal antibody pAb 419, which is directed against SV40 T antigen and which served as a control (lane 7). Four of the five anti-p300 monoclonal antibodies were of the IgG1 isotype and required the use of a secondary antibody to collect immunocomplexes efficiently with protein A–Sepharose (lanes *2–5*). The many bands seen with antibody RW144 likely represent proteins that contain cross-reactive epitopes, because some of these proteins can be detected on a Western blot probed with the same antibody. (*B*) Peptide map comparing p300 from U-2 OS cells brought down with mAb RW105 against E1A-bound p300 from 293 cells. The amount of *S. aureus* V8 protease used is indicated (*top*).

p300 molecules defective for interaction with E1A can bypass E1A-imposed SV40 enhancer repression

Finally, we asked whether p300 could rescue the activity of the SV40 enhancer when it was specifically repressed by E1A. Previous studies had shown that E1A can specifically repress the SV40 enhancer but not its promoter (Borelli et al. 1984; Velcich and Ziff 1985). A luciferase reporter gene driven by the SV40 enhancer/promoter was transfected into U-2 OS cells together with either an expression vector for 12S E1A or a combination of vectors encoding full-length p300 and 12S E1A. In all cases, luciferase activity was normalized to the β-galactosidase activity of a cotransfected *lacZ* gene. Different amounts of E1A expresssion vector were used to elucidate the range of E1A concentrations within which p300 overcame E1A-imposed enhancer repression. The activity of the SV40–luciferase reporter plasmid in the absence of any E1A was given arbitrarily a value of 100.

At low concentrations of E1A-encoding DNA (0.1 and 0.25 μg), suboptimal SV40 enhancer repression was observed, and cotransfected p300 overcame the repressive effect of E1A by about a factor of 2 (Fig. 9A). Western blots carried out in parallel indicated that E1A protein levels were consistently two- to threefold elevated in cells where p300 was also present, as compared with cells that were transfected with only the reporter plasmid and E1A (Fig. 9B). Thus, the mild relief of E1A-mediated SV40 enhancer repression by transfected p300 does not occur by reducing the levels of E1A. However, the positive effect of p300 on the SV40 enhancer may be linked to the functional sequestration (and inactivation) of E1A bound to p300 in aggregates such as those described in Figure 8. This sequestration of E1A might be sufficient to account for the reactivation of the enhancer. Similarly, the elevated levels of E1A in the presence of p300 could be attributed to a stabilization of the normally short-lived E1A molecules in p300–E1A aggregates attributable to shielding from proteolysis. At high concentrations of E1A expression vector (2.5 and 5 μg), the enhancer was completely repressed, and cotransfected p300 was unable to stimulate transcription, indicating that E1A was in excess over p300 under these circumstances.

To circumvent the possible inactivation of E1A, we switched to p300 molecules unable to associate with E1A. For this purpose, the del30 and del33 deletion mutations were introduced into full-length p300. The rationale behind this approach was to sequester the pool of endogenous p300 in E1A complexes and to see whether the exogenous p300, defective for binding E1A, can substitute for the endogenous p300 and restore SV40 enhancer activity. As shown in Figure 9C, both p300del30 and p300del33 efficiently relieved E1A-imposed SV40 enhancer repression, even at the very high concentrations of E1A used in these experiments. On average, about half of the original enhancer activity could be restored. Western blots carried out in parallel confirmed that E1A levels were comparable within these assays, whether or not the p300 internal deletion mutants were present (Fig. 9D). We conclude that p300 can bypass E1A-imposed repression and reactivate the SV40 enhancer, strongly arguing that p300 itself is directly involved in modulating the activity of the SV40 enhancer.

Discussion

We have isolated a cDNA encoding full-length E1A-associated 300-kD protein. The protein encoded by this cDNA interacts with E1A in a manner indistinguishable from the endogenous p300 protein and displays a proteolytic peptide mapping pattern very similar to that of the

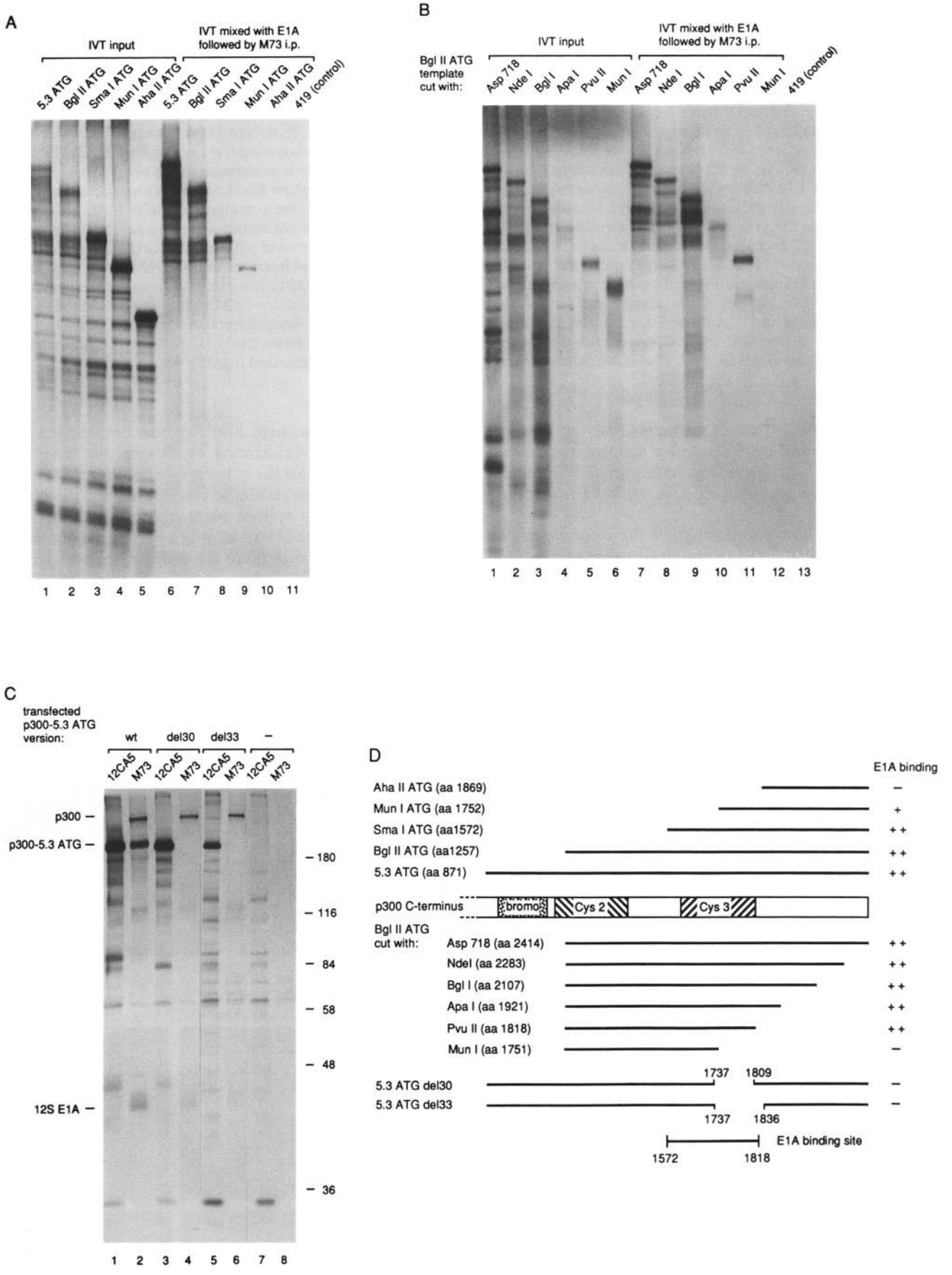

Figure 7. *(See following page for legend.)*

Figure 7. Determination of the E1A-binding site on p300. (*A*) A series of DNA templates for in vitro transcription/translation with progressive 5′ deletions was prepared by fusing in-frame ATG translation initiation codons to restriction enzyme sites (*top*) located in the carboxy-terminal part of the p300 cDNA. In this way, templates with progressive 5′ deletions were generated. All templates were linearized with *Asp*718, which cuts in the polylinker downstream of the p300 cDNA. Ten microliters of ^{35}S-labeled in vitro translation products from these templates (lanes *1–5* shows 1 μl of each of the translates) were mixed with cold extract of ~4×10^6 293 cells (as a source for E1A), followed by immunoprecipitation with the anti-E1A mAb M73 (lanes *6–10*). In lane *11*, as a control, the translation products directed by the 5.3 ATG template were mixed with 293 cell lysate and then immunoprecipitated with pAb 419. (*B*) The *Bgl*II ATG template was cleaved with the restriction enzymes denoted (*top*), to generate p300 protein fragments with progressive carboxy-terminal deletions. E1A-binding assays were performed as described above (*A*). Lanes *1–6* show the input of the translates, and lanes *7–12* shows the translation products recovered after mixing with cold 293 extract and immunoprecipitation with M73. In lane *13*, translation products from the *Bgl*II ATG template linearized with *Asp*718 were mixed with 293 extract and immunoprecipitated with pAb 419 (control). (*C*) Two internal deletion mutants removing part of the E1A-binding region of p300 (termed del30 and del33) were generated in the context of the 5.3 ATG plasmid, which encodes a 200-kD carboxy-terminal fragment of p300. HA-tagged versions of the wild-type 5.3 ATG plasmid and of the two deletion mutants were transfected into U-2 OS cells together with an expression vector coding for the 12S E1A product. Cells were labeled with [^{35}S]methionine and extracted, and one-half of the lysate of each dish was immunoprecipitated with 12CA5 (anti-HA monoclonal antibody). The other half was immunoprecipitated with M73. In lanes 7 and 8, lysates from untransfected U-2 OS cells were immunoprecipitated with 12CA5 and M73, respectively. (*D*) Schematic representation of the deletion mutants used in *A–C*. The E1A-binding properties of each of them is summarized (*right*).

E1A-bound 300-kD protein. Monoclonal antibodies raised against cloned p300 recognize endogenous, cellular p300 and are capable of coprecipitating E1A. Taken together, these results indicate that we have isolated a p300 cDNA.

An important question is whether the cloned species is the only cellular 300-kD protein capable of binding E1A. If there are two or more different p300 species, the peptide maps of cloned p300 might have contained just a subset of the bands seen with the endogenous, E1A-bound p300. Because this was not the case, it appears unlikely that two or more distinct E1A-bound p300 proteins exist. Moreover, in our screening for p300 cDNAs and during our Northern blot experiments, carried out under stringent hybridization conditions, we have not obtained evidence for the existence of another p300 transcript. However, our results do not rule out the existence of an alternatively spliced p300 transcript(s), differing from the one isolated by us in only a very small region(s). It is also possible that there are cellular proteins structurally related to p300 that are, however, unable to interact with E1A or are much less abundant than p300.

Silencing of the SV40 enhancer by E1A can be reversed by p300 molecules lacking part of their E1A-binding domain. In effect, these p300 versions render the SV40 enhancer partially resistant (or immune) to the inhibitory

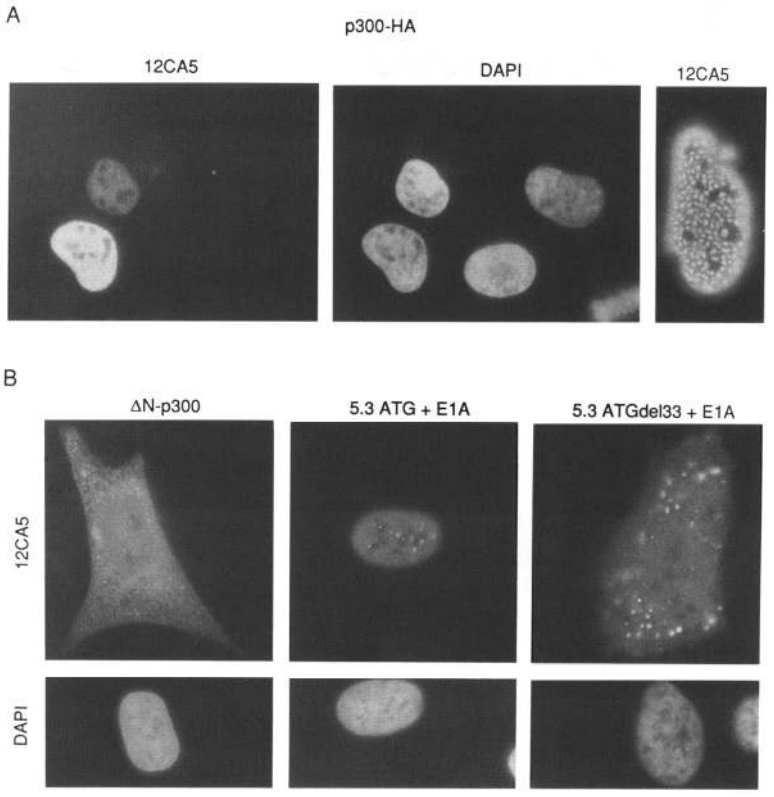

Figure 8. Immunofluorescence analysis of the subcellular location of full-length and truncated p300 molecules. (*A*) U-2 OS cells transiently transfected with a vector encoding HA-tagged full-length p300 were fixed and stained with 12CA5 anti-HA antibody followed by rhodamine-conjugated rabbit anti-mouse secondary antibody (*left*). The same section on the *left* is shown in the *middle* after DAPI staining. (*Right*) An enlarged picture of a nucleus from transfected U-2 OS cells to visualize the speckles that became apparent after overproduction of p300. (*B*) (*Top*) The intracellular distribution in U-2 OS cells of the HA-tagged p300 version indicated above each of the three panels. The primary and secondary antibodies were as described in *A*. Note that for the cytoplasmic p300 derivatives, speckles are now visible in the cytoplasm. (*Bottom*) The DAPI staining patterns of the cells displayed above.

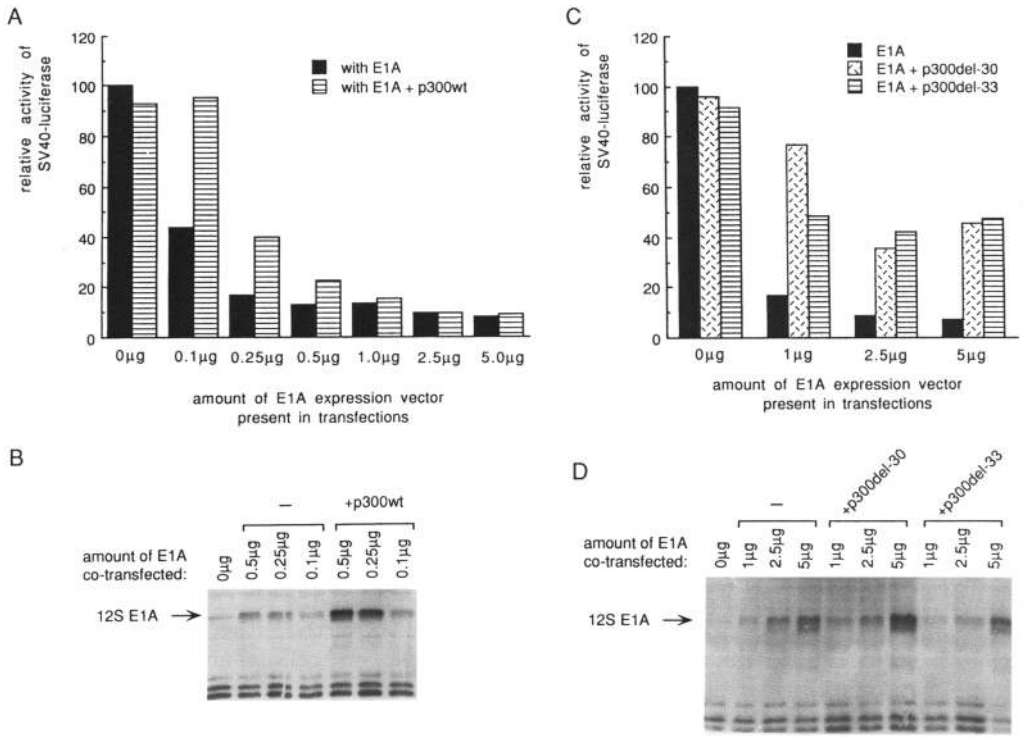

Figure 9. Analysis of the role of p300 in the *trans*-activation potential of the SV40 enhancer. (*A*) A luciferase reporter plasmid driven by the SV40 enhancer/promoter (5 µg), a plasmid encoding β-galactosidase (1 µg, internal standard), and increasing amounts of an expression vector for 12S E1A (see numbers below each pair of bars) were transfected into U-2 OS cells either in combination with 8 µg of pBluescript carrier DNA (solid bars) or in combination with 8 µg of an expression vector encoding full-length p300 (bars with horizontal lines). All luciferase values were normalized to each other based on the respective β-galactosidase activity. The luciferase activity obtained with the reporter alone was set to 100, and all other activities were expressed relative to this sample. The data represent the average of four independent experiments. (*B*) Western blot showing expression levels of E1A in the transfection experiments carried out in *A*. One-sixth of the cells from the 10-cm dishes transfected for the experiment described in *A* were subjected to Western blotting. The membrane was probed with the anti-E1A mAb M73. The amount of E1A expression plasmid present in the transfection mixture is indicated (*top*). (*C*) U-2 OS cells were transfected as described in *A*, except that instead of the expression vector for wild-type p300, the same vector encoding either deletion mutant p300del30 (stippled bars) or deletion mutant p300del33 (lined bars) was used. The bars represent the average luciferase activity from five independent experiments. (*D*) Western blot visualizing the expression levels of the E1A protein in the transfection experiments performed in *C*.

effect of E1A, even at very high intracellular concentrations. Our experiments provide direct evidence that p300 is an activator of enhancer-dependent transcription. In the absence of E1A, the SV40 enhancer is fully active, and exogenous p300 is unable to further stimulate its activity. This suggests that p300 is not a limiting factor for enhancer function under normal circumstances. Another implication of our results is that the E1A-binding domain of p300 is dispensable for its transcriptional activation function, at least in the context of the SV40 enhancer. This is remarkable because deletions in the E1A-binding domain of the RB family of proteins (the pocket) completly abrogate the activity of this group of proteins (Shew et al. 1990; Zhu et al. 1993).

The activity of the SV40 enhancer was not fully restored in these assays. Three possible reasons can be envisaged. Because it is difficult to express high concentrations of active p300, attributable to the tendency of p300 to form aggregates, we may not have achieved appropriate levels of active p300 for complete reversal of E1A action. Alternatively, E1A could target both p300 and another, as yet unidentified, cellular protein that is required, in concert with p300, for full enhancer activity. If these proteins operate in an additive fashion, establishing plentiful levels of active p300 may not be sufficient to generate full enhancer function. Finally, it is conceivable that the deletion in the E1A-binding domain of p300 somewhat impairs its stimulatory activity on the SV40 enhancer.

The protein sequence of p300 predicts a bromodomain, located in the center of p300, as well as three distinct regions rich in cysteines and histidines. Because many of the proteins containing a bromodomain are implicated as transcriptional adaptors or coactivators, it is likely that p300 functions as a coactivator. In support of such a function was the ability of p300 to partially reactivate the SV40 enhancer when it was silenced by E1A. What is known about the role of the bromodomain in activation of transcription? Experiments carried out with a subclass of bromodomain proteins containing adjacent to this do-

main a region of homology to helicases and DNA-dependent ATPases, namely yeast SNF2 (Laurent et al. 1991) and one of its human homologs, hbrm (Muchardt and Yaniv 1993), indicate that the bromodomain of these two proteins is dispensable for transcriptional activation. In both proteins, this function appears to depend on an intact helicase function that is thought to assist transcription factors in displacing nucleosomes (Winston and Carlson 1992). Another subclass of proteins with bromodomains, represented, for example, by the yeast protein GCN5 (Georgakopoulos and Thireos 1992) and the human $TAF_{II}250$/CCG1, do not contain a helicase-like domain. In the case of GCN5, genetic data indicate that this protein enhances the transactivation function of the transcription factor GCN4. Several lines of experimental evidence point to a key role of $TAF_{II}250$ in transcriptional activation. It performs a central function in the molecular assembly of TFIID (Weinzierl et al. 1993) and is essential for the progression of the G_1 phase of the cell cycle (Sekiguchi et al. 1991), most likely by modulating the activity of a subset of genes required for entry into S phase (Wang and Tjian 1994). Because proteins containing bromodomains have to collaborate with multiple components of the transcription machinery, it is assumed that the bromodomain acts as a surface for protein–protein interactions. Besides the bromodomain, it is noteworthy that cysteine/histidine-rich motifs also occur in a number of other known or suspected coactivator proteins. Among these are the yeast ADA2 protein (Berger et al. 1992), the human CREB binding protein, CBP (Chrivia et al. 1993), *Drosophila* and human trithorax polypeptides (Mazo et al. 1990; Gu et al. 1992; Tkachuk et al. 1992), and the 13S E1A RNA product. Each of these proteins displays a unique spacing of cysteines that are thought to coordinate divalent metal ions. The cysteine-rich motif might serve to provide to these transcription factors an extended and exposed surface, thereby enabling them to interact with multiple proteins at the same time. Our observation that the third cysteine- and histidine-rich domain of p300 interacts with at least one protein, namely E1A, lends support to this notion.

How does p300 stimulate enhancer-controlled transcription? Extensive deletion analyses of the SV40 enhancer have shown that the silencing effect of E1A is not dependent on specific sequences (Rochette-Egly et al. 1990). Hence, it appears that p300 does not activate the enhancer as a classical transcription factor by binding to a specific sequence element and triggering *trans*-activation. Taking the results of Rochette-Egly et al. (1990) and the above noted structural features of p300 into account, it is more likely that p300 functions in a more indirect way, for example, as an adaptor or bridging molecule communicating the activation potential of the enhancer-bound transcription factors to the promoter and to the basal transcription machinery. Given the large number of transcription factors binding to enhancers, steric interference probably precludes that all of them can interact directly with the basal transcription apparatus, especially when one considers that the promoter-bound transcription factors (e.g., Sp1 in the case of SV40) also need to contact the basic transcription factors. Molecules that sense the activation potential of enhancer-bound transcription factors and help in transmitting it to the basal transcription machinery are therefore probably essential. The large size of p300 and its multiple potential domains for protein–protein interactions are certainly compatible with such a model.

It has been reported that p300 has the ability to recognize sequences that resemble NF-κB/H2TF1-like sites (Rikitake and Moran 1992). Such a sequence element is present in the SV40 enhancer. However, other enhancers that are also sensitive to repression by E1A do not contain this motif [e.g., the polyomavirus enhancer (Borelli et al. 1984; Velcich et al. 1986) or the insulin enhancer (Stein and Ziff 1987)]. Furthermore, as pointed out before, various subfragments of the SV40 enhancer that do not encompass the H2TF1-like motif can still be repressed by E1A (Rochette-Egly et al. 1990). On the basis of these considerations, it appears unlikely that p300 acts solely via the H2TF1-like site in stimulating transcription directed either by the SV40 enhancer or by other viral and cellular enhancers that are also the target of E1A-imposed repression. In preliminary experiments, using full-length p300 produced in a baculovirus expression system, we have not detected a specific interaction between radiolabeled SV40 enhancer DNA sequences and p300 (Z. Arany, R. Eckner, and D.M. Livingston, unpubl.). Future studies will have to investigate the mechanism by which p300 is recruited to the SV40 enhancer and the biochemical details of how it exerts its function.

An intriguing correlation between the sensitivity of certain enhancers toward silencing by E1A and the inactivity of the same enhancers in undifferentiated embryonal carcinoma (EC) cells has been noted previously (Borelli et al. 1984; Gorman et al. 1985; Sleigh and Lockett 1985). It has been proposed that a cellular E1A-like activity, present only in undifferentiated EC cells (Imperiale et al. 1984), is responsible for the lack of activity of these enhancers in EC cells. An attractive hypothesis is that this cellular E1A-like activity interacts directly with p300 and negatively regulates its role as transcriptional adaptor protein, perhaps in a manner similar to E1A.

Binding of E1A to p300 is sufficient to induce S-phase entry of quiescent cells. E1A can perform this function without binding to the CR2-associated proteins, albeit less efficiently (for review, see Moran 1993). This finding may mean that p300 is a negative regulator of cell growth that is inactivated by E1A. In addition, E1A represses, via p300 binding, tissue-specific genes associated with the terminal differentiation state of cells (Hen et al. 1985; Stein and Ziff 1987; Webster et al. 1988; Braun et al. 1992; Boulukos and Ziff 1993; Caruso et al. 1993). In light of our results suggesting a role for p300 as a transcriptional adaptor protein, we propose that one part of the cell cycle inhibitory function of p300 is to activate (together with cell type-specific transcription factors) enhancers of genes required for arrest in G_0/G_1

and terminal differentiation. A natural target of adenovirus infections is differentiated epithelial cells lining the respiratory tract. To induce S phase and to replicate its own DNA in these infected cells, it is probably essential for adenovirus to interfere with this growth-repressive role of p300.

Materials and methods

Isolation of p300 cDNAs and Northern analysis

E1A-associated proteins were purified by passing 293 cell extract over columns containing immobilized, anti-E1A mAb M73, as described previously (Ewen et al. 1991). Three different polyclonal mouse sera were raised against gel band-purified p300. To be able to screen a cDNA expression library, the antisera (at a 1:1000 dilution) had to be preadsorbed extensively against nitrocellulose filters containing nonrecombinant λ phage plaques to remove antibodies reacting nonspecifically with *E. coli* proteins. One of these precleared antisera was then used to screen 1.2 million plaques of a 293 cell cDNA library (Ewen et al. 1991) according to standard procedures (Ausubel et al. 1987). Two plaques reacting with the antiserum were identified. Because both plaques also reacted with the other two anti-p300 polyclonal mouse antisera, the inserts of the two phages (2-B5 and 2-G2; see Fig. 1A) were analyzed and found to overlap. Insert 2-B5 was then used to screen a Nalm 6 λ Zap cDNA library (a gift of A. Bernards, Massachusetts General Hospital, Charlestown). The 5′ end of the longest isolated clone (N28) was employed to screen an Akata cell λ gt11 cDNA library (a gift of Jennifer Chen, Dana-Farber Cancer Institute) which resulted in the isolation of clone A9 (among others). The Akata library was rescreened with this clone, and the insert containing the longest extension of the available p300 cDNA (insert A12) was labeled to reprobe the Akata library and to screen a HeLa λ Zap cDNA library (Xiao et al. 1991). This resulted in the isolation of cDNA inserts covering the 5′ end of p300. Hybridization and washing conditions of the filters used for screening libraries and probing Northern blots were as described (Church and Gilbert 1984). RNA, derived from the following cell lines, was examined on Northern blots: Akata, 293, WERI-RB27, HeLa, SV80, U-2 OS, Nalm-6, and Saos2.

Sequencing

The p300 cDNA sequence was established by sequencing overlapping cDNA inserts on both strands. One part of the p300 cDNA fragments was sequenced by the dideoxy-chain termination method using the Sequenase kit 2.0 (U.S. Biochemical Corporation). The other part was sequenced on an Applied Biosystems automated sequencer using a primer walking approach.

Fluorescence in situ hybridization

Methods for chromosome mapping of the p300 gene were essentially as described elsewhere (Lawrence et al. 1988; Johnson et al. 1991) and are outlined only briefly here. A human cDNA probe for p300 (N28) was labeled by nick translation with digoxygenin dUTP (BRL) and detected after hybridization with fluorescein anti-digoxygenin antibody. Elongated metaphase and prometaphase chromosomes were prepared by standard procedures from normal peripheral blood lymphocytes treated with methotrexate for 18 hr, bromodeoxyuridine (BrdU) for 6 hr to enhance banding and, finally, a 10-min colcemid treatment. Samples were hybridized with 5 μg/ml of labeled probe in 50% formamide, and 2× SSC at 37°C. For hybridization with two probes simultaneously, the chromosome 21 marker probe was labeled with biotin and visualized with rhodamine–avidin. Samples were visualized on a Zeiss Axioplan microscope and images captured using a CCD camera connected to a silicon graphics workstation.

Cell culture

All cells were grown at 37°C in a humidified, 10% CO_2-containing atmosphere in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum (Hyclone).

Transfections and adenovirus infections

The human osteosarcoma cell line U-2 OS was transfected by the HEPES–$CaPO_4$ method (Ausubel et al. 1987). The precipitates were left for 12 hr on the cells. Cells were then washed twice with PBS and processed 36 hr later for either immunoprecipitation or luciferase experiments. For adenovirus infections, the respective viruses were added to U-2 OS cells in 3 ml of DMEM without serum at an m.o.i. of 10 and incubated for 1 hr. Subsequently, DMEM containing 10% fetal calf serum was added, and cells were labeled with [^{35}S]methionine 10 hr later.

Cell labeling and immunoprecipitations

Cells were labeled for 4 hr using 0.5 mCi of [^{35}S]methionine per 10-cm dish in DMEM containing 5% dialyzed fetal calf serum. Typically, a 10-cm dish was lysed for 20 min at 4°C in 1 ml of EBC (50 mM Tris-HCl at pH 8.0, 170 mM NaCl, 0.5% NP-40, 50 mM NaF) containing 10 μg/ml of protease inhibitors aprotinin, leupeptin, and PMSF. The lysate was then centrifuged at 14,000*g* for 10 min to pellet debris. Following preclearing for 30 min with 50 μl of a 1:1 slurry of protein A–Sepharose in 4% BSA, the supernatant was incubated on a rocker for 1 hr with 100 μl of the respective monoclonal antibody. Immunocomplexes were collected by adding 25 μl of protein A–Sepharose beads and further rocking for 30 min. Finally, the beads were washed five times with NETN (10 mM Tris-HCl at pH 8.0, 250 mM NaCl, 5 mM EDTA, and 0.5% NP-40). For analysis of immunoprecipitated proteins, the beads were boiled in 1× SDS–sample buffer (2% SDS, 10% glycerol, 62 mM Tris-HCl at pH 6.8, 1% β-mercaptoethanol) and loaded onto SDS–polyacrylamide gels.

p300 expression plasmids

Two full-length p300 cDNAs were assembled in pBluescript (Promega) from two different sets of partial cDNA inserts to minimize chances of cloning artifacts. The first p300 cDNA (pBluescript p300-1) was constructed from inserts N28, A9, and H2 (see Fig. 1A), using suitable internal restriction sites to ligate the three fragments together. The second version of full-length p300 (pBluescript p300-2) was assembled using cDNA fragments N24, H4, and H1. Comparative peptide mapping experiments comfirmed that both clones were identical. To distinguish between endogenous p300 and introduced, full-length, p300 an HA tag was attached to either the carboxyl terminus or the amino terminus of p300. The carboxy-terminal HA tag was generated by ligating the oligonucleotide 5′-CTAGCCCCGGGATGGCCTACCCATACGACGTGCCTGACTACGCCTCCCTCGGATA-3′ and its complementary strand between the *Nhe*I site near the carboxyl terminus of p300 and the *Hin*dIII site in the polylinker of pBluescript. This manipulation removes the last 36-amino-acid residues of p300 and replaces them by 16 residues comprising the HA tag. The amino terminal HA tag was fused to the p300 cDNA by using the oligonu-

cleotide 5′-CCTGGATCCACCATGGCATACCCATACGACGTGCCTGACTACGCCTCCGCCGAGAATGTGGTG-3′ as the upstream primer and 5′-GTAGGACCCTGATTTGGTC-3′ as the downstream primer in a PCR reaction. The 420-bp PCR product was digested with *Bam*HI and *Spe*I and ligated to the *Spe*I site near the amino terminus of p300. This manipulation led to the addition of an in-frame HA tag to the second amino acid residue of p300. The mutant ΔN-p300 was generated by ligating the oligonucleotide 5′-GGCCGCAAGCTTCACCATGGCATACCCATACGACGTGCCTGACTACGCCTCCGGAA-3′ and its complementary strand to the *Spe*I site near the amino terminus of p300. The 5.3 ATG construct was created by ligating the oligonucleotide 5′-CGCGTGATCAGCCACCATGGCCCCACCT-3′ and its complementary strand to the *Bgl*I site at position 3808 of the p300 cDNA sequence. The sequence of the above described four constructs was checked by sequencing. To express the four constructs in mammalian cells, they were transferred as *Not*I–*Hin*dIII fragments from pBluescript to the mammalian expression vector CMVβ, in which the cytomegalovirus (CMV) promoter/enhancer drives the expression of an inserted cDNA. In this vector, the second intron and the polyadenylation signal from the rabbit β-globin gene provide the signals for efficient RNA processing.

The templates for in vitro transcription/translation were constructed by fusing oligonucleotides containing a eukaryotic consensus translation initiation signal to the restriction sites indicated in Figure 7D.

The two internal deletions in the E1A-binding region of p300 (del30 and del33) were generated by resecting *Eco*47 III-cut pBluescript p300-1 DNA with Bal31 followed by digestion with *Hin*dIII to release the carboxy-terminal part of the p300 cDNA. This fragment was then ligated to pBluescript p300-1 cleaved with *Eco*47 III and *Hin*dIII to create plasmids containing full-length p300 with unidirectional Bal 31 deletions. Deletion endpoints were determined by sequencing. For the experiment in Figure 7C, the two internal deletions were introduced into the 5.3 ATG context by transferring a *Bgl*II–*Hin*dIII fragment spanning the deletions from the full-length construct into the 5.3 ATG plasmid cleaved with the same two restriction enzymes. Both constructs were expressed in mammalian cells from the CMVβ expression vehicle.

Production of monoclonal antibodies

A glutathione *S*-transferase (GST)–p300 fusion protein encompassing amino acids 1572–2371 of p300 was injected intraperitoneally into BALB/c mice. Spleen cells of positive animals were fused to NS-I cells according to standard procedures (Harlow and Lane 1988). Supernatants from the resulting hybridoma colonies were screened for their ability to immunoprecipitate ^{35}S-labeled in vitro translation products derived from the *Bgl*II ATG template. The epitopes recognized by the monoclonal anti-p300 antibodies were mapped using a series of in vitro translation products encoded by templates carrying progressive 5′ and 3′ deletions.

Immunofluorescence

U-2 OS cells were seeded on coverslips and transfected with expression vectors encoding HA-tagged p300 versions. They were fixed in 3% paraformaldehyde/2% sucrose in PBS for 10 min at room temperature. Subsequently, cells were washed twice with PBS, followed by permeabilization in ice-cold Triton X-100 buffer (50 mM NaCl, 3 mM $MgCl_2$, 200 mM sucrose, 10 mM HEPES at pH 7.4, 0.5% Triton X-100) for 5 min. Antibody incubations (with anti-HA tag mAb 12CA5) were for 30 min at 37°C, followed by three washes in PBS for 5 min each. Secondary antibodies were rhodamine-conjugated goat anti-mouse IgG (Boehringer Mannheim).

After another three 5-min washes in PBS, slides were incubated for 2 min in a DAPI solution (0.5 μg/ml of DAPI in PBS) to stain DNA, rinsed twice with PBS, and mounted. Microscopy was carried out using a Nikon Microphot SA microscope equipped with a PlanApo 60× oil immersion objective.

SV40 enhancer assays

The plasmid pGL2–control (Promega) was used as a reporter construct. It contains the luciferase gene driven by the SV40 promoter and the SV40 enhancer, which is inserted downstream of the luciferase gene. pGL2–control (5 μg) and pCMV-lacZ (1 μg) were transfected with varying amounts of CMV12SE1A and 8 μg of pBluescript or 8 μg of the indicated p300 expression vector, respectively. It was important not to use too much of the p300 expression vector, because less restoration of SV40 enhancer activity was obtained when higher amounts of this expression plasmid were utilized, probably because of increased p300 aggregate formation. The final DNA concentration in the transfection mixture was between 15 and 20 μg, depending on the amount of E1A expression plasmid included. Cell lysis, β-galactosidase, and luciferase assays were carried out as described (Ausubel et al. 1987). In each experiment, one-sixth of the cells of a transfected dish were lysed directly in SDS–sample buffer, and the E1A levels were monitored by Western blotting. The anti-E1A mAb M73 was utilized as primary antibody. The secondary antibody was a goat anti-mouse antibody conjugated to alkaline phosphatase (Boehringer Mannheim).

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