

Characterization of Monoclonal Antibodies Raised against p300: both p300 and CBP Are Present in Intracellular TBP Complexes

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The amino terminus of the adenovirus E1A protein is involved in E1A transforming functions, repression of tissue-specific gene expression, and E1A-mediated enhancer repression. These N-terminal functions are associated with the ability of this region of E1A to bind to p300 and CBP, two closely related cellular proteins thought to function as transcriptional adaptor molecules. Here we describe the characterization of a panel of 11 monoclonal antibodies raised against E1A-affinity-purified 300-kDa proteins. The panel can be divided into two groups based on immunoprecipitation patterns. The first group consists of five p300/CBP-cross-reactive and two p300-specific monoclonal antibodies, all of which immunoprecipitate p300 and/or CBP without associated cellular proteins. In contrast, the second group immunoprecipitates p300 or both p300 and CBP in association with a complex of at least seven other cellular proteins. Taking advantage of the specificities of these monoclonal antibodies, we have identified both p300 and CBP in *in vivo* complexes with TBP, a finding consistent with a role for both p300 and CBP in promoting interactions between upstream promoter elements and the basal transcription apparatus.

The adenovirus early region 1A gene (E1A) encodes two major nuclear phosphoproteins of 243 and 289 amino acids which have profound effects on cell growth and differentiation (14, 22, 28–30). These effects are mediated by the ability of the E1A proteins to directly bind and subvert the function of important cellular regulatory molecules (reviewed in references 5 and 21). The E1A proteins bind to the retinoblastoma susceptibility gene product, pRB, and to pRB-related proteins p107 and p130 (31; reviewed in references 4 and 7). Proteins of the pRB family are known to form complexes with the E2F family of transcriptional activators, thus affecting expression of E2F-responsive genes (reviewed in references 18 and 23). The E1A proteins bind through an independent N-terminal binding site to an approximately 300-kDa cellular phosphoprotein, p300 (9, 29, 32). The E1A amino terminus is involved in E1A transforming functions, repression of tissue-specific gene expression, and E1A-mediated enhancer repression (reviewed in references 5 and 21). E1A mutants that do not bind the pRB protein family but retain p300 binding can activate *G*₁ gene expression and entry into S phase in primary rodent cells (14, 28–30). This *G*₁ gene activation does not involve the induction of E2F activity (24, 30), indicating that p300 regulates gene expression by alternative mechanisms.

The deduced amino acid sequence of p300 (8) predicts a protein closely related to CREB-binding protein (CBP) (6). CBP is an integral component of the cyclic AMP-dependent signal transduction pathway, functioning at least in part through its ability to bind to and coactivate the cyclic AMP response element-binding protein CREB (6, 16). A comparison of the amino acid sequences of the human versions of p300 and CBP (2) indicates that they are 63% identical overall. Greater similarity is observed in specific regions, including the region encompassing the E1A binding site (a comparison of p300 and CBP structures can be seen in Fig. 1). The overall sequence similarity and, in particular, the presence of specific

highly conserved regions in both proteins suggest that p300 and CBP have similar functions.

To facilitate a detailed analysis of the functions of the p300/CBP family of proteins, we have raised a series of monoclonal antibodies directed against the 300-kDa protein species affinity purified from E1A complexes. From a panel of 11 independent hybridomas we have identified 4 as p300 specific and 7 as p300/CBP cross-reactive.

We have previously identified p300 *in vivo* as a component of complexes with the TATA-binding protein (TBP) (1). However, the relationship now known between p300 and CBP indicates that these products would not have been distinguished in that study. The potential significance of this interaction has increased since CBP was identified as a coactivator of CREB that may bridge a physical contact between CREB and the basal transcription complex. The possible interaction between CBP and basal transcription complex proteins was suggested by the ability of TFIIB fusion variants to bind CBP fragments translated *in vitro* (16). The physiological significance of this interaction is not known, and the presence of CBP versus p300 has not been distinguished in intracellular TBP complexes, an important distinction given the close relationship between p300 and CBP. The monoclonal antibody mapping studies reported here have enabled us to demonstrate the presence of both p300 and CBP in complex with TBP *in vivo*.

Generation of the hybridomas. p300 was obtained from 293 cells (which constitutively express the E1A products [10] by selective elution with high-detergent buffer from E1A complexes immobilized with E1A-specific monoclonal antibody M73 (12) as described previously (30, 33). Fractions thus enriched for p300 were used directly to immunize female BALB/c mice. Mice with a high-titer antibody response to p300 were sacrificed to obtain splenocytes for hybridoma production by standard procedures (13). Two p300-positive hybridoma cell lines were isolated from a mouse inoculated with p300 prepared as described in reference 33. Nine additional p300-positive lines were generated in a separate fusion from a mouse injected with p300 protein subjected to the additional purification steps described in reference 30. The heavy- and light-

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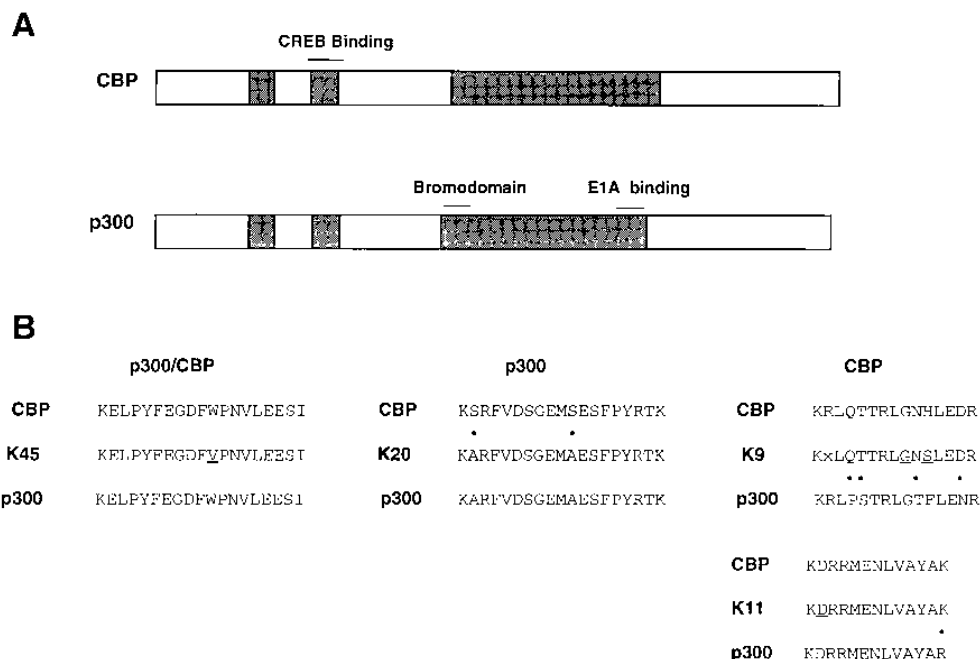


FIG. 1. Both p300-specific and CBP-specific peptide sequences are recovered from the 300-kDa protein fraction purified from E1A complexes. (A) A linear comparison of the p300 and CBP protein sequence. Regions of greater than 80% identity are indicated by shading. The location of the CREB binding site, the E1A binding site, and the bromodomain in p300 and CBP are indicated by lines above the bars. (B) The sequences of several peptides (K45, K20, K9, and K11) obtained from the E1A-affinity-purified p300 population are compared with the amino acid sequences of p300 and CBP as deduced from their cDNA sequences. The presence of the initial lysine (K) residue in each peptide was not determined by sequencing but is inferred from the nature of the enzyme digest, which cuts almost exclusively at lysine residues. Uncertain readings in the peptide sequencing cycle are indicated by underlining. An unreadable cycle is indicated by an X. Mismatches are indicated as dots between residues. Uncertain readings or unreadable residues were not counted as mismatches. The K45 sequence originates from a region where p300 and CBP are identical. K20 represents a sequence across a region where CBP and p300 differ by 2 residues out of 19; K20 corresponds to the p300 sequence exactly. K9 and K11 represent sequences across regions where p300 and CBP differ by 5 residues out of 15 or 1 residue out of 13, respectively. In both cases the peptides correspond to the CBP sequence.

chain components of the antibody panel are listed in Table 1. To confirm that each of the antibodies recognizes authentic E1A-associated proteins, we tested the reactivity by immunoprecipitation of each antibody against the actual p300 population eluted from E1A complexes. The results (not shown) indicate that each antibody is indeed reactive against this affinity-purified material.

CBP is present in the E1A-associated p300 population. CBP is very similar to p300 across the entire region containing the E1A binding site of p300 (Fig. 1A). In addition, analysis with

CBP-directed antipeptide antibodies suggests that CBP is present in E1A immune complexes (3, 20). Therefore, it is likely that CBP was present in the E1A-affinity-purified material used to raise the p300 antibodies. We have obtained direct information bearing on this question from peptide sequencing of the p300 fraction eluted from E1A complexes. Comparison of these peptide sequences with the amino acid sequence deduced from the p300 and CBP cDNA sequences (6, 8) indicates the presence of both p300-specific and CBP-specific peptides in the eluted material (Fig. 1B). Thus, we conclude that both p300 and CBP were part of the immunogen in the generation of the hybridomas.

The monoclonal antibody panel includes p300-specific and p300/CBP-cross-reactive antibody specificities. To generate a test reagent specific for CBP, we raised rabbit polyclonal antibodies against a synthetic peptide corresponding to the amino-terminal sequence of CBP (6). The antipeptide serum and each of the 11 monoclonal lines were used to immunoprecipitate its target population from metabolically labeled HeLa cells as described previously (30, 33). The immunoprecipitated species were electrophoretically transferred to an Immobilon-P membrane (Millipore) by standard procedures (27) and then screened with the CBP antibody. Membranes were checked by autoradiography to verify transfer prior to antibody screening. Bound antibody was detected with alkaline phosphatase-conjugated secondary antibodies (Promega) according to the manufacturer's instructions. Representative results are shown in Fig. 2A. The CBP antibody reacts positively with the material precipitated by itself and by NM1, NM4, NM5, NM10, and NM11. It does not react with the material precipitated by

TABLE 1. Antibody classification

Antibody	Heavy chain	Light chain	Reacts with:		Suitable for Western blots
			CBP	p300	
Fusion 1					
NM1	G1	λ	+	+	-
NM2	G1	λ	+	+	-
Fusion 2					
NM3	G2a	κ	-	+	-
NM4	G1	κ	+	+	+
NM5	G1	κ	+	+	+
NM6	G2b	κ	-	+	+
NM7	G1	κ	-	+	+
NM8	G2a	κ	-	+	-
NM9	G1	κ	+	+	+
NM10	G1	κ	+	+	+
NM11	G2b	κ	+	+	+

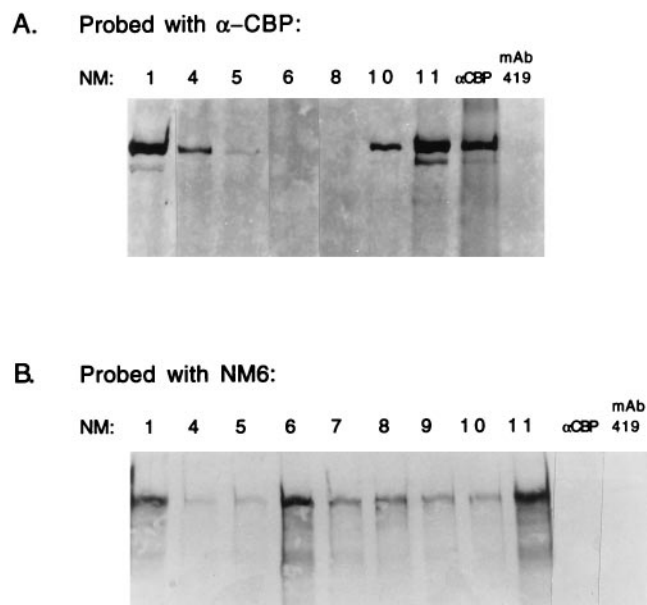


FIG. 2. Identification of p300-specific and CBP-cross-reactive antibodies within the NM antibody panel. 32 P-labeled HeLa cell lysates were immunoprecipitated with representatives of the NM series of antibodies, with CBP-specific serum (α CBP lanes), or with negative control monoclonal antibody (mAb) 419 as indicated. The precipitated proteins were Western blotted and screened with CBP-specific antipeptide antiserum (A) or with NM6 (B). Successful transfer of the immune complexes to the membrane was verified by autoradiography.

NM6 or NM8 or by a control antibody, 419, which recognizes the simian virus 40 T antigen (11), a viral protein not present in these cells. In similar experiments, the CBP antibody reacted with material precipitated by NM2 and NM9 but not by NM3 or NM7. These results indicate that NM3, NM6, NM7, and NM8 recognize p300 but do not react with CBP. These results are summarized in Table 1.

In a converse experiment, the NM6 antibody was used to screen the same panel of immunoprecipitates. Representative results are shown in Fig. 2B. The NM6 antibody reacts with the material precipitated by itself and by NM1, NM4, NM5, NM7, NM8, NM9, NM10, and NM11 but not with the material precipitated by the CBP antibody or by the 419 control antibody. In additional experiments, the NM6 antibody also reacted with NM2 and NM3. The inability to recognize the material precipitated by the CBP antibody confirms that NM6 is indeed specific for p300. The reactivity of the entire panel of NM series immune precipitates with NM6 indicates that the entire panel of monoclonal antibodies recognizes p300. Conversely, the inability to recognize the material precipitated by NM6 and NM8 confirms the specificity of the CBP antipeptide serum. Thus, of the 11 lines that we have generated, all produce antibodies that react with p300, 4 are specific for p300 (NM3, NM6, NM7, and NM8), and 7 cross-react with CBP (NM1, NM2, NM4, NM5, NM9, NM10, and NM11). We have also determined that each of these antibodies except NM1, NM2, NM3, and NM8 can be used in Western blots. These results are summarized in Table 1.

Identification of p300 and CBP in TBP complexes. The monoclonal antibody panel makes it possible now to distinguish p300 from CBP in immune assays. Because both p300 and CBP appear to act as transcriptional adaptor molecules, an analysis of their ability to bind to other cellular proteins is essential to understanding their functions. CBP was cloned as a CREB-interactive product and has been detected in intra-

cellular complexes with CREB (6). Peptide fragments of CBP have also been shown to bind to a glutathione *S*-transferase-TFIIB full-length fusion protein *in vitro* (16). This interaction with a basal transcription factor suggests that one role of CBP is to convey an upstream transcription signal to the basal transcription machinery. p300 may act similarly. p300 can bind to CREB *in vitro* and in a HeLa two-hybrid system (19, 20), and we have shown that p300 is present in intracellular complexes with TBP (1). However, in the latter study the immune reagents used would not have distinguished between p300 and CBP. The *in vivo* analyses reveal physiological associations which cannot be determined from *in vitro* binding assays. Therefore, it is important to determine whether the intracellular complexes include both p300 and CBP or only one of these products. Accordingly, we have reexamined TBP immune complexes with p300-specific and CBP-specific antibodies.

A panel of TBP-specific monoclonal antibodies reveals TBP in association with a number of cellular proteins (26). Different subsets of TBP-interactive proteins are coprecipitated depending on the site of binding of the antibody within TBP. At least two independent monoclonal antibodies from this panel, SL8 and SL27, coprecipitate a protein band that comigrates with p300 and CBP as detected by NM1 immunoprecipitations (1). In the present study, we have precipitated immune complexes from isotopically labeled HeLa cells with each of these antibodies and with p300/CBP-reactive antibody NM1. The proteins in the immune complexes were examined by Western analysis using either p300-specific antibody NM6 or the N-

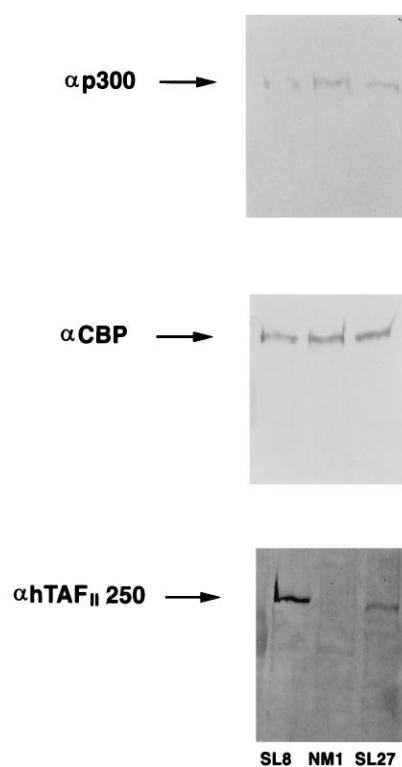


FIG. 3. Association of both p300 and CBP with TBP *in vivo*. HeLa cell lysates were immunoprecipitated with NM1 or with the TBP-specific antibodies SL8 and SL27 as indicated. The immunocomplex proteins were Western blotted and screened with p300-specific antibody NM6, the CBP-specific antiserum described in the legend to Fig. 2, or TAF_{II}250-specific monoclonal antibodies as indicated. Equivalent transfer of the immune complexes to the membrane was verified by autoradiography.

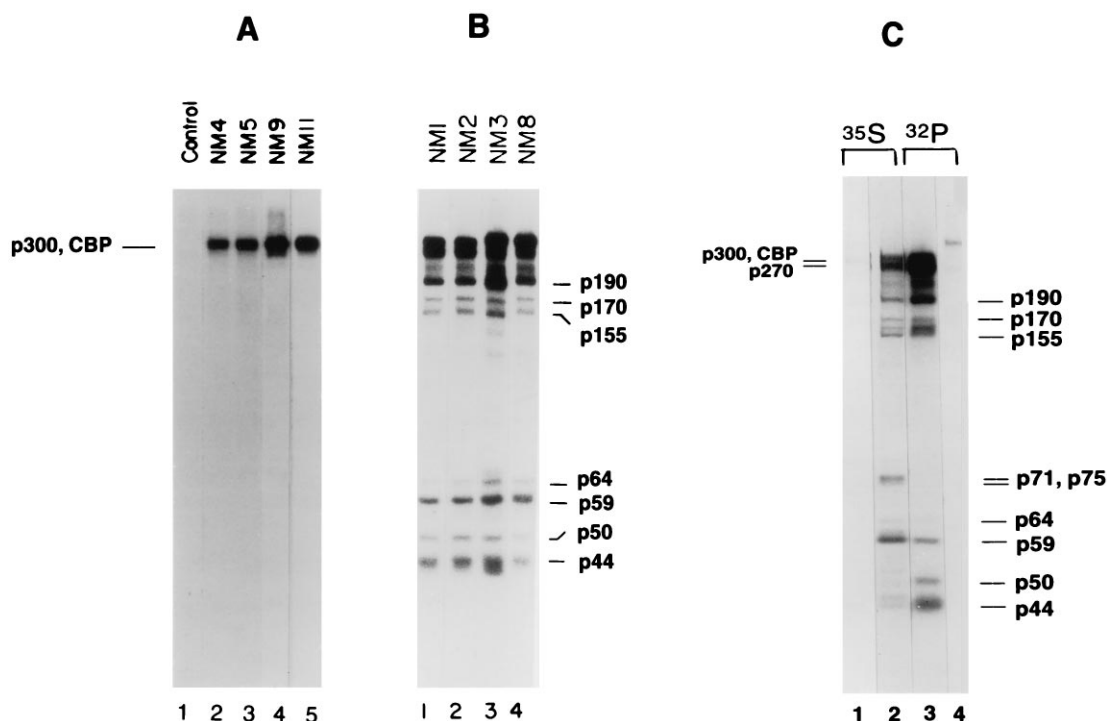


FIG. 4. A subset of monoclonal antibodies raised against p300 coprecipitates a series of cellular proteins. (A) Immunoprecipitation of ^{32}P -labeled HeLa cell lysates with antibodies representative of those which do not coprecipitate associated cellular proteins. (B) Immunoprecipitation of ^{32}P -labeled HeLa cell lysates with the set of antibodies that coprecipitates a series of cellular proteins associated with the p300-related products. The positions of the associated proteins are indicated at the right. (C) ^{35}S - or ^{32}P -labeled WI-38 cell lysates were immunoprecipitated with NM1 (lanes 2 and 3) or with a control monoclonal antibody, 405 (lanes 1 and 4), raised against the simian virus 40 virus T antigen (11), a protein not present in uninfected cells. The positions of the associated proteins are shown on the right. The positions of p300, CBP, and p270 are shown on the left.

terminal CBP-specific antipeptide antibody. Successful transfer of the immune complex proteins was verified by autoradiography. The results of the Western blots (Fig. 3) show clearly that both p300 and CBP are present in the TBP-specific complexes brought down by SL8 and SL27. As a positive control the blots were also probed with a monoclonal antibody, 6B3, which recognizes the better-characterized TBP-associated protein hTAF_{II}250 (25). hTAF_{II}250 is present in both the SL8 and SL27 complexes. However, as expected from the lack of close homology between hTAF_{II}250 and p300 or CBP, the hTAF_{II}250 antibodies are not reactive with the NM1-precipitated products. We interpret the absence of hTAF_{II}250 in immunoprecipitations using the NM series of monoclonal antibodies as evidence for p300, CBP, and hTAF_{II}250 being components of separate TBP complexes *in vivo*.

A subset of the p300-reactive antibodies reveals a novel p300/CBP-related species and a series of associated cellular proteins. The members of the NM panel of monoclonal antibodies fall into two very distinct categories when the total cellular immune complex is examined (Fig. 4). NM4, NM5, NM6, NM7, NM9, NM10, and NM11 precipitate a single dominant band that includes either p300 or p300 and CBP, according to the reactivity of the antibody. Representative immunoprecipitations for NM4, NM5, NM9, and NM11 are shown in Fig. 4A. No other cellular proteins are readily detectable in association with the p300/CBP products in the absence of E1A. (Each of these antibodies is capable of coprecipitating E1A with the characteristics that have been reported in detail with NM11 in reference 30.)

Immunoprecipitations done with NM1, NM2, NM3, and NM8 show a very different pattern. A series of cellular prod-

ucts is coprecipitated in addition to p300 and CBP, even in the absence of E1A. Analysis of ^{32}P -labeled cell lysates (Fig. 4B) reveals coprecipitated phosphoprotein species of 190, 170, 155, 64, 59, 50, and 44 kDa. The coprecipitated proteins shown in Fig. 4B represent the phosphoprotein species present in the series. Comparison of ^{35}S -labeled lysates with ^{32}P -labeled lysates (Fig. 4C, lane 2 versus lane 3) reveals a similar series of coprecipitated species. The only prominent species in the ^{35}S -labeled lysate that does not have a phosphoprotein counterpart is a band or doublet migrating at about 71 to 75 kDa.

A close examination of the immune complexes precipitated with the monoclonal antibody panel reveals another characteristic of these complexes, which is the presence in certain of the complexes of a band migrating faster than the p300/CBP band, at a position of about 270 kDa (Fig. 4C and 5A). The species migrating at the p270 position is stable and present in both ^{35}S - and ^{32}P -labeled lysates but is immunoprecipitated only by the NM1, NM2, NM3, and NM8 group and not by the remainder of the panel. Thus, the p270 species is distinct from p300 and CBP in that it is not recognized by all p300/CBP-reactive antibodies. However, p270 is related to p300 at a minimum of two epitopes, because NM1 and NM2 differ from NM3 and NM8 in their p300/CBP-reactive patterns (Fig. 5A and Table 1). The p270 species, which is present in NM1 immunoprecipitates, is not recognized by the hTAF_{II}250-specific antibodies (Fig. 3), so it is distinct from that product as well. We have seen the same pattern of p270 recognition and associated proteins in a variety of cell lines examined, including HeLa, WI-38, 293, and baby rat kidney cells. E1A expression in 293 cells does not dissociate the interactions with the complex proteins.

To distinguish which species in the NM1 immunoprecipitate

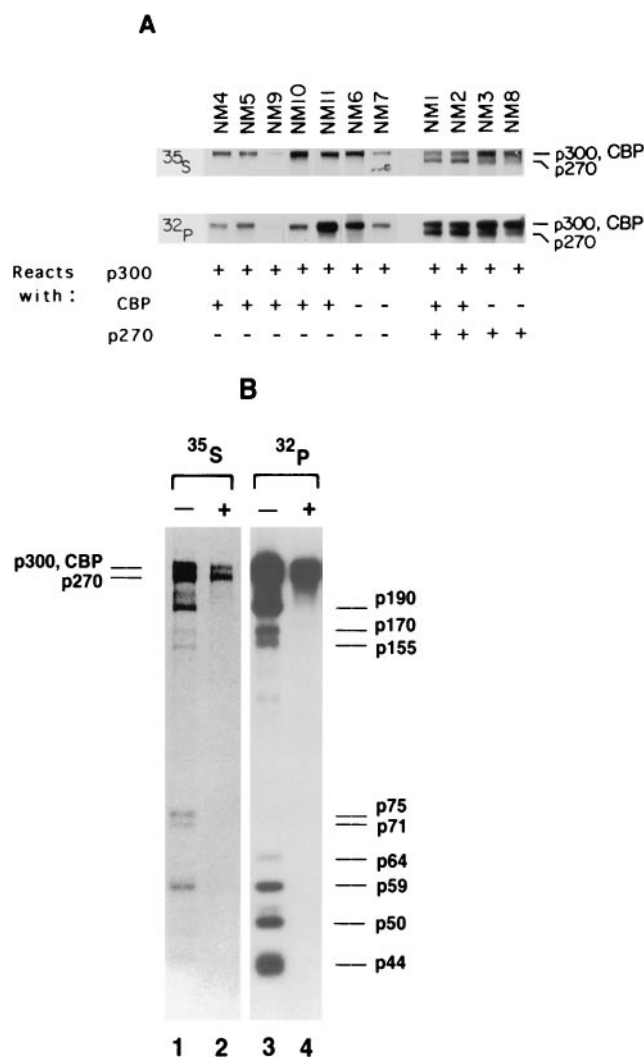


FIG. 5. Detection of a 270-kDa species immunologically related to p300 and CBP. (A) ³⁵S- or ³²P-labeled 293 cell lysates were immunoprecipitated with antibodies in the series NM1 through NM11. A 270-kDa species is detectable under both labeling conditions in precipitations done with NM1, NM2, NM3, and NM8. The positions of p300, CBP, and the 270-kDa species are indicated at the right. (B) ³⁵S- or ³²P-labeled 293 cell lysates were immunoprecipitated with NM1 under nonreducing conditions (-). Half of the immune complex was separated, denatured by boiling in the presence of 2% sodium dodecyl sulfate, and then reprecipitated with fresh NM1 (+). The proteins from each immunoprecipitation were separated by electrophoresis and visualized by autoradiography. The positions of the associated protein species are shown on the right. The positions of p300, CBP, and p270 are shown on the left. The ³⁵S panel is lightly exposed to enhance the resolution of p300, CBP, and p270. Only the bands corresponding to p300, CBP, and p270 are recovered by the antibody after the complex has been denatured.

are recognized directly by the antibody from those that are associated with the antibody targets, we denatured the complexes and reprecipitated them with NM1 (Fig. 5B). To achieve this, immune complexes were first obtained by immunoprecipitation and then divided into two equal portions. One portion was left untreated and placed on ice. The sodium dodecyl sulfate concentration of the other portion was raised to 2%, and the sample was boiled for 10 min. After boiling, samples were spun briefly in a microcentrifuge, and the supernatant was diluted 20-fold in p300 lysis buffer. The samples were then reimmunoprecipitated. Results are shown for both ³⁵S- and ³²P-labeled complexes. The only protein species that are di-

rectly recognized by the antibody are p300, CBP, and p270, confirming that p270 is a p300/CBP-related protein and that the remaining species seen in the NM1 immune complex are associated, not cross-reactive cellular species. The results are the same in both the ³⁵S- and ³²P-labeled complexes. (A diffuse protein band migrating at about 230 kDa can be seen in several of these gels. This band appears to be recognized directly by many of our antibodies. However, it is unstable in pulse-chase experiments. We believe that it represents precursor forms of p300 family members recognized by the antibodies.)

These results indicate that the p300 family of proteins extends to a least one more member, a 270-kDa protein, and that members of this family are present in cells in stable association with a series of proteins whose identification can be expected to shed significant light on the molecular mechanisms of action of the p300 family.

We have previously shown that the phosphoprotein species p64 and p59 visible in the complexes represented by the NM1 immunoprecipitation are identical to species immunoprecipitated by the SL8 and SL27 lines of TBP-specific monoclonal antibodies (1). Here we have extended those observations to demonstrate that intracellular TBP complexes include both p300 and CBP in addition to TAF_{II}250. The detection of these interactions in vivo implies that they are physiologically significant. The detection of p64 and p59 with both the TBP-specific and the p300/CBP-specific antibodies supports the conclusion that this is a stable functional complex. These data are consistent with models suggesting that p300 and CBP can act as bridging molecules between upstream promoter-specific complexes and the basal transcription machinery.

The presence of p64 and p59 in the TBP complexes without the other NM1-coprecipitated proteins suggests that the series of proteins coprecipitated by NM1 represents at least two distinct complexes involving p300/CBP family members. As a step towards understanding the functional significance of these complexes, we have identified the 190-kDa species in the NM1 complex as BRG1, a human homolog of SWI2 (5a). Members of this family, including BRG1, have the structural features of a helicase and exist in cellular complexes with an associated nucleosome disruption activity (15, 17). This finding, together with the recent report of a cellular p300/CBP-associated factor with intrinsic histone acetylase activity (34), suggests that p300/CBP family members are components of specific cellular complexes which mediate gene expression by altering chromatin structure at transcriptionally active sites.

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