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Recruitment of CBP/p300, TATA-Binding Protein, and S8 to Distinct Regions at the N Terminus of Adenovirus E1A

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The N-terminal region of the adenovirus (Ad) 12S E1A gene product targets several cellular proteins that are essential for the induction of S phase, cellular immortalization, cellular transformation, transcriptional repression, and transcriptional activation. The precise binding sites for these proteins, however, remain to be resolved. We therefore undertook an extensive site-directed mutagenesis approach to generate specific point mutants and to precisely map the binding sites for CBP, p300, TATA-binding protein (TBP), S4, S8, hGcn5, P/CAF, and Ran within the first 30 amino acids of the Ad5 12S E1A protein. We determined that although common residues within the N-terminal region can form partial binding sites for these proteins, point mutants were also generated that could discriminate between binding sites. These data indicate that AdE1A can target each of these proteins individually through distinct binding sites. It was evident, however, that the mutation of specific hydrophobic residues typically had the greatest effect upon AdE1A's ability to bind individual partners. Indeed, the mutation of L at positions 19 and 20 eliminated the ability of AdE1A to interact with any of the N-terminal binding proteins studied here. Interestingly, although TBP and S8 or CBP/p300 can exist as functional complexes, RNA interference revealed that the recruitment of either TBP, S8, or CBP/p300 to AdE1A was not dependent upon the expression of the other proteins. These data further indicate that AdE1A can target individual partner proteins in vivo and that it does not necessarily recruit these proteins indirectly as components of larger macromolecular complexes. Finally, we took advantage of the fine-mapping data to ascertain which proteins were targeted during the transformation process. Consistent with previous studies, CBP/p300 was found to be targeted by AdE1A during this process, although our data suggest that binding to other N-terminal proteins is also important for transformation.

Adenovirus (Ad) E1A expression is essential for Ad replication and Ad-mediated transformation (34). AdE1A is expressed from two major splice variant transcripts, 12S and 13S, that give rise to protein products of 243 and 289 amino acids, respectively (in adenovirus types 2 and 5 [Ad2/5]). The protein products of 12S and 13S differ only in the presence of conserved region 3 (CR3), which functions to transactivate a number of cellular and viral genes (22). AdE1A can cooperate with AdE1B or activated *ras* genes to transform both human (6) and rodent (33) cells by targeting a limited number of cellular proteins through the N-terminal region, CR1, CR2, and CR4 (3, 18, 26, 44). The N-terminal region and elements in CR1 are required for binding the transcriptional coactivator proteins p300 and CBP (1, 15). The elimination of CBP/p300 binding to AdE1A dramatically reduces the ability of AdE1A to transform cells in culture (21, 42, 44). Mutagenesis studies have indicated that residues which are absolutely conserved between serotypes, i.e., R2 and L20 (Ad5) and L19 (Ad12), are essential for mediating the AdE1A interaction with CBP/p300 in vivo (28, 42). Distinct elements at the N terminus of AdE1A also appear to target chromatin remodeling of p400- and TRRAP-containing complexes during AdE1A-mediated transformation; however, mutation of the conserved residue R2 does not affect this association (11, 17). CR1 and CR2 define structural elements that target the tumor suppressor gene product pRb (43). Deletion of the CR2 LXCXE motif that defines the minimal requirement for pRb binding similarly reduces the ability of AdE1A to cooperate in transformation (16, 21). The contribution of the C-terminal CR4 domain to the transformation process is context dependent. Exon 2, encompassing the whole C-terminal region, suppresses AdE1A/*ras*-mediated transformation, primarily through a conserved PXDLS motif that targets the transcriptional corepressor molecule CtBP, and deletion of the PXDLS motif enhances E1A/*ras*-mediated transformation (4). In contrast, the C-terminal region enhances E1A/E1B-mediated transformation (14) through the targeting of AdE1A to the nucleus and the binding of CtBP.

The N-terminal region of AdE1A is only weakly conserved among Ad serotypes (2, 23) (Fig. 1). Interestingly, however, the AdE1A N-terminal regions from different Ad serotypes appear to bind to a similar set of cellular proteins, presumably to perform similar functions during infection and transformation (3, 18). In this context, secondary structure predictions suggest that the N-terminal regions of all known serotypes will form an α-helix (2). The ability of the N-terminal region to enhance AdE1A-mediated cellular transformation, through the targeting of CBP/p300 and potentially other N-terminal binding pro-

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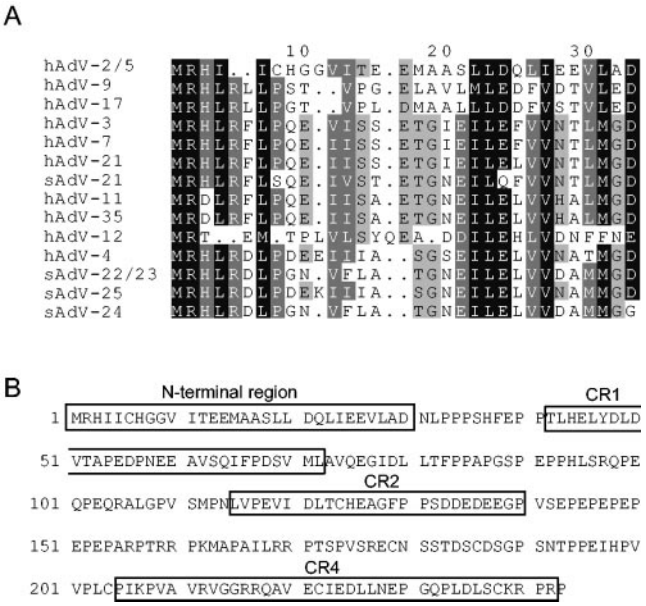

FIG. 1. (A) Comparative sequence alignment at the N terminus of AdE1A. The N termini of AdE1A proteins from the indicated human and simian serotypes were aligned with CLUSTAL W and imported into GeneDoc for shading of conserved residues with the BLOSUM 35 matrix. Other than the initiating M residue, only R2 and L20 (in Ad2/5) within the first 30 amino acids are absolutely conserved among serotypes. (B) Amino acid sequence of the Ad2/5 243-residue E1A protein. Boxed areas define conserved regions within the 12S gene product as well as the 30 amino acids at the N terminus under investigation in this study.

teins, resides primarily in its ability to promote quiescent G_0 cells to enter the cell cycle, progress into S phase, and initiate cellular DNA synthesis (20). In addition to this function, and in the absence of cooperating oncogenes, the N-terminal region of AdE1A will, in conjunction with CR1 and CR2, up-regulate the levels of the tumor suppressor gene product p53 to induce apoptosis (29). It is thought that AdE1A achieves this by specifically targeting 19S proteasomal ATPases 54 and 58 through the N-terminal region, CBP/p300 through the N terminus and CR1, pRb through CR1 and CR2, and the 19S proteasomal non-ATPase S2 through CR2 (8, 13, 19, 31, 41, 46).

Other functions of AdE1A also map to this N-terminal region. For instance, in conjunction with CR1, the N-terminal region can actively repress the transcription of a subset of genes involved in promoting cellular proliferation or cellular differentiation programs. It has been proposed that AdE1A represses transcription by binding both CBP/p300 and TATA-binding protein (TBP) (5, 26, 37–39); the association of AdE1A with CBP/p300 sequesters histone acetyltransferase activity, while the association with TBP interferes with the formation of TBP-TATA box complexes (5). Ad2/5 residues C6 and L20 are key to defining the in vivo AdE1A association with both CBP/p300 and TBP, and thus, the AdE1A-mediated transcriptional repression (5). In conjunction with CR1, the N-terminal region binds P/CAF independently of CBP/p300, sequestering P/CAF-associated histone acetyltransferase activity away from P/CAF-regulated promoters (32). AdE1A is thought to disrupt the CBP/p300 association with P/CAF by binding to CBP/p300 (45). Reports indicate that human Gcn5, a component of the SAGA acetyltransferase complex which is homologous to the C-terminal two-thirds of P/CAF, also binds to the N-terminal region of AdE1A (24, 35, 36). The N-terminal region of AdE1A also induces centrosome amplification in several rodent cell lines through targeting of the small GTPase Ran and inhibition of its nucleotide exchange function (12). Additionally, the N terminus of Ad5 12S E1A will bind the thyroid receptor β1 (TRβ1) to stimulate TR-dependent transcription (30) and the TFIIF component Rap30, presumably to affect specific host cell transcription programs (27).

Given the increasing number of cellular proteins that have been found to bind, apparently independently, to the N-terminal region of AdE1A, we determined that it was important to precisely map the binding sites for each of these proteins in the context of the whole 12S gene product. Thus, we undertook a comprehensive analysis of the N-terminal region in order to establish whether any of these proteins shared common binding sites or points of contact and/or whether unique binding sites for these proteins exist. The data presented here establish that although some proteins share common contact residues, it appears that unique binding sites with defined contact points exist for each of these N-terminal binding proteins. These data imply that these proteins can bind independently to the N-terminal region of AdE1A and are not recruited indirectly as larger macromolecular complexes. Indeed, RNA interference (RNAi) studies demonstrated that CBP/p300, TBP, and S8 can all be recruited independently to the N-terminal region. The ability of these N-terminal mutants to cooperate with N-*ras* during the transformation process was also investigated. Consistent with previous studies, AdE1A mutants that were unable to bind CBP/p300 were defective in transformation. However, our data also suggested that AdE1A may additionally target other N-terminal binding proteins to facilitate transformation.

MATERIALS AND METHODS

Cells. Human A549 cells, which were derived from a small-cell lung carcinoma, were grown and maintained in HEPES-buffered Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine and 8% fetal calf serum (FCS). For the generation of A549 cells that stably expressed wild-type (wt) 12S Ad5E1A or 12S Ad5E1A mutants, cells were transfected with appropriate pcDNA3.1-AdE1A constructs. Transfected A549 cells expressing Ad5E1A were selected for growth in the presence of G418 (800 μg/ml). At the appropriate time posttransfection, individual colonies were isolated and AdE1A expression was determined by Western blotting. Clones expressing similar levels of the different AdE1A mutants were used for binding studies. Primary hooded Lister rat embryo fibroblasts (HLREFs) were prepared from 18-day-old embryos and used for transformation assays at passage two.

Plasmids. Ad5 12S E1A and AdE1A mutants were cloned into pcDNA3.1 (Invitrogen) for both in vitro transcription-translation and mammalian expression and into pGEX 4T-1 (Amersham Pharmacia) for bacterial expression. For in vitro transcription-translation coupled reactions, the human p300 fragment 1573-1825 was subcloned into pCITE (Novagen), mouse CBP and mouse P/CAF fragment 310-832 were cloned into pcDNA3.1, and human Gcn5 short was cloned into pBluescript (Stratagene). The sequences were verified by direct sequencing of the sense and antisense strands by use of an ABI Prism 3100 genetic analyzer.

Generation of AdE1A mutants. The pcDNA3.1-Ad5 12S E1A construct was used as a template to generate AdE1A mutants by PCR in accordance with the manufacturer's instructions (QuikChange site-directed mutagenesis kit; Stratagene). Typically, 35-mer DNA oligonucleotides that were complementary to both sense and antisense regions of AdE1A but that incorporated appropriate base changes to generate specific mutants (Alta Bioscience, The University of Birmingham) were synthesized and then utilized for PCRs. The base changes

incorporated into the full-length AdE1A sense sequence (antisense sequences were omitted for clarity) were as follows: R2G, AGA to GGA; I5G, ATC to GGC; C6A, TGC to GCC; H7A, CAC to GCC; G8A, GGA to GCA; V10A, GTT to GCT; I11A, ATT to GCT; T12A, ACC to GCC; E14A, GAA to GCA; A16G, GCC to GGC; S18G, AGT to GCT; L19A, CTT to GCT; the double mutation L1920A, CTT TTG to GCT GCG; L20A, TTG to GCG; D21A, GAC to GCC; L23A, CTG to GCG; I24A, ATC to GCC; E25A, GAA to GCA; E26A, GAG to GCG; V27A, GTA to GCA; L28A, CTG to GCG; A29G, GCT to GGT; and D30A, GAT to GCT. Mutants were validated by direct sequencing of both strands of the AdE1A cDNAs by use of an ABI Prism 3100 genetic analyzer.

Transformation. HLREFs were cultured in HEPES-buffered DMEM supplemented with 2 mM glutamine and 10% FCS. For each experimental condition, plasmid DNA, brought up to 15 μg with heat-denatured salmon sperm DNA, was added to 2×10^6 cells in a final volume of 250 μl of medium. Cells were electroporated by use of a Bio-Rad gene pulser at 960 μF and 220 V in 4-mm-deep cuvettes. Posttransfection cultures were fed every 3 days with a 4:1 mix of Joklic's modified minimum essential medium and HEPES-buffered DMEM supplemented with 8% FCS and 2 mM glutamine. G418 selection (final concentration, 200 μg/ml) was initiated at 18 h postelectroporation and continued until day 14, when transformed foci were counted by low-power microscopy to identify genuine E1A/*ras* transformants.

RNA interference. Purified, annealed, double-stranded 21-mer RNA oligonucleotides with dTdT overhangs were purchased from either Ambion or QIAGEN. The targeted gene sequences were as follows: S8 (nucleotides 485 to 507), 5′ AA GAA GTG ATC GAG CTG CCT GTT 3′; and TBP (nucleotides 606 to 628), 5′ GA GGA TAA GAG AGC CAC GAA CTT 3′. Typically, 5×10^5 A549 cells were transfected with an appropriate small interfering RNA (siRNA) by electroporation (960 μF and 220 V in 4-mm-deep cuvettes). A nonsilencing siRNA with no known homology to any human gene was used as a negative control (QIAGEN).

Antibodies. The anti-E1A monoclonal antibody (MAb) M73, the anti-CtBP1 MAb M1, and the anti-pRb MAb IF8 were all obtained as supernatant fluids from cultures of the relevant expressing hybridoma cell lines. Anti-CBP/p300 polyclonal antibodies (PAb) used for immunoprecipitation were generously provided by Betty Moran (Temple University, Philadelphia, Pa.). For Western blotting, CBP was detected with the rabbit PAb A-22 and p300 was detected with the PAb N-15 (both from Santa Cruz Biotechnology). An anti-S8 PAb was a gift from Wenlan Wang (A. I. duPont Hospital for Children, Wilmington, Del.), and an anti-TBP PAb was kindly provided by Nouria Hernandez (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). The anti-Ran MAb was purchased from Transduction Laboratories.

GST pull-down assay. Wild-type Ad5 12S E1A and 12S E1A mutants cloned into the appropriate pGEX vectors (Amersham Pharmacia) were used to transform competent BL21 RIL cells (Stratagene). Glutathione *S*-transferase (GST) fusion proteins were expressed and purified as described previously (40). Purified proteins were dialyzed extensively against a buffer containing 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1 mM dithiothreitol, and 10% (vol/vol) glycerol and then stored at −80°C. For GST pull-down assays, A549 cell lysates were prepared by solubilization in a buffer containing 50 mM Tris-HCl (pH 7.4), 0.825 M NaCl, and 1% NP-40 and then clarified by sonication and centrifugation. Alternatively, proteins were expressed by in vitro transcription and translation in the presence of L-[α-^{35}S]methionine (Amersham Pharmacia) by use of the TNT-coupled wheat germ system (Promega). Typically, 10 μg of the appropriate GST fusion protein was mixed with either 5 mg of A549 cell lysate or 20 μl of the appropriately L-[α-^{35}S]methionine-labeled protein. GST pull-down assays were then performed as described previously (40). After selective elution with reduced glutathione, the samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then subjected to either Western blot analysis or fluorography (Amersham Pharmacia).

Immunoprecipitation. For immunoprecipitation, cells were washed twice in isotonic saline and then lysed by the addition of 1 ml of a buffer containing 50 mM Tris-HCl (pH 7.4), 0.825 M NaCl, and 1% NP-40. After occasional agitation on ice for 15 min, the lysates were sonicated and cleared by centrifugation. Typically, 5 to 10 μg of antibody was used to immunoprecipitate antigen-containing complexes from 5 mg of protein lysate. Immunoprecipitation was performed as described previously (41). Immunoprecipitates were resuspended in the appropriate sample buffer, boiled for 5 min when necessary, and separated by PAGE.

PAGE and Western blot analysis. Samples which had been solubilized in a solution containing 9 M urea, 50 mM Tris-HCl (pH 7.4), and 0.15 M β-mercaptoethanol were sonicated and cleared by centrifugation. Protein concentrations were determined by the Bradford assay (Bio-Rad). Fifty-microgram protein samples and immunoprecipitates were separated in 12% polyacrylamide gels run either in the presence of 0.1 M Tris, 0.1 M Bicine, and 0.1% SDS or in the presence of 7 M urea, 93 mM Tris, and 15 mM glycine but in the absence of SDS. The separated proteins were electroblotted onto nitrocellulose filters (Gelman Sciences) and hybridized with the appropriate antibodies. Antigens were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia).

RESULTS

Alignment of N-terminal regions from different Ad serotypes. In addition to the initiating M1 residue and R2 and L20 (numbered with respect to Ad2/5) that are absolutely conserved between serotypes, there are several other highly conserved residues in E1A proteins (Fig. 1A), as determined by a sequence analysis. In particular, hydrophobic amino acids at positions 4, 5, 10, 11, 19, 20, 23, 24, 27, and 28 are conserved among the majority of serotypes. Acidic residues at positions 14, 21, and 30 are also highly conserved. There are other residues within this region that are similar, yet these are not uniformly conserved (Fig. 1A). We thus undertook an extensive mutational analysis across this region to assess the contribution of individual amino acids to mediating interactions with known cellular binding partners. The relationship between the N-terminal region and the rest of the Ad2/5 243-residue E1A protein is depicted schematically in Fig. 1B.

Binding of N-terminal AdE1A mutants to partner proteins in vitro. AdE1A N-terminal mutant sequences, in the context of the whole 12S gene, were verified in pGEX. GST-AdE1A fusion proteins were expressed and purified as described in Materials and Methods. Prior to examining the binding capacities of N-terminal binding proteins, we assessed the integrity of the whole proteins by determining the binding capacities of the mutants for pRb and CtBP1, which bind CR1/CR2 and the C terminus, respectively. As shown in Fig. 2, all of the N-terminal mutants bound pRb and CtBP1 as efficiently as wt AdE1A, demonstrating their validity for use in this binding study. We subsequently determined the affinities of the mutants for bona fide N-terminal binding proteins. Initially, we assessed the ability of L-[α-^{35}S]methionine-labeled CBP, p300, P/CAF, and Gcn5 acetyltransferases to bind the N-terminal mutants in vitro. The levels of AdE1A binding to CBP and p300 were, perhaps not surprisingly, very similar (Fig. 3A). The H7A, G8A, E25A, V27A, and D30A mutants bound CBP and p300 with high affinities, while the AdE1A I11A, T12A, A16G, L19A, L20A, L23A, and I24A mutants and the double mutant L1920A all had severely compromised abilities of binding these proteins (Fig. 3A). Furthermore, it appeared that E14A and A29G bound full-length CBP more strongly than the C/H3 fragment of p300 (Fig. 3A). N-terminal AdE1A mutants showed a similar binding pattern with hGcn5 and P/CAF, although subtle differences were apparent (Fig. 3B). The AdE1A H7A, G8A, and D21A mutants bound hGcn5 and P/CAF with the same affinity as wt AdE1A (Fig. 3B). The I5G, I11A, A16G, L19A, L1920A, L20A, L23A, I24A, and L28A mutants, however, had little or no affinity for these proteins. The C6A, V10A, and V27A mutants had much higher affinities for hGcn5 than for P/CAF (Fig. 3B), while the E14A, E25A, E26A, A29G, and D30A mutants bound P/CAF more efficiently than hGcn5 (Fig. 3B).

We next investigated the in vitro binding capacities of the N-terminal AdE1A mutants for TBP and S8. The results from these experiments indicated that the L19A, L1920A, L20A,

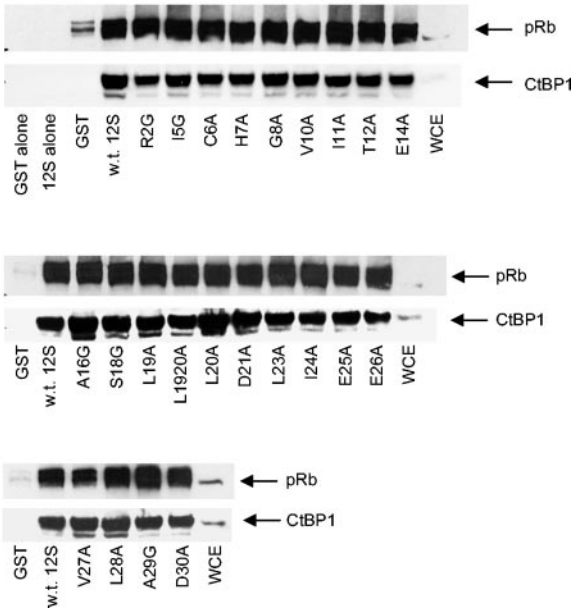

FIG. 2. In vitro binding capacities of N-terminal AdE1A mutants for pRb and CtBP1. Ten micrograms of the appropriate GST-AdE1A fusion protein was incubated with 5 mg of A549 cell lysate. AdE1A-interacting proteins were precipitated with glutathione-Sepharose and selectively eluted with glutathione (see Materials and Methods). Proteins were subsequently separated by SDS-PAGE and transferred to nitrocellulose. The membranes were then Western blotted for pRb and CtBP1 to assess the binding capacities of these proteins for different N-terminal AdE1A mutants.

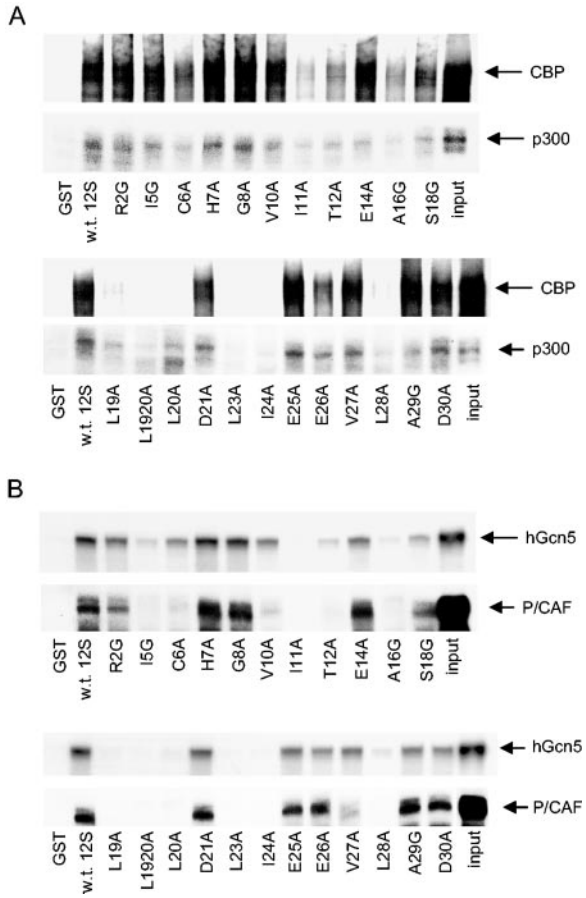

FIG. 3. In vitro binding capacities of N-terminal AdE1A mutants for the acetyltransferases CBP, p300, hGcn5, and P/CAF. Ten micrograms of the appropriate GST-AdE1A fusion protein was incubated with 20 μl of L-[α-^{35}S]methionine-labeled CBP, p300, hGcn5, or P/CAF. Bound proteins were precipitated with glutathione-Sepharose and selectively eluted with glutathione (see Materials and Methods). Proteins were subsequently separated by SDS-PAGE and subjected to fluorography (Amersham Pharmacia). The gels were dried and then subjected to autoradiography. (A) Ability of CBP and p300 to bind N-terminal AdE1A mutants. (B) Ability of AdE1A mutants to bind hGcn5 and P/CAF.

L23A, and I24A mutants had little or no capacity to bind either TBP or S8 (Fig. 4A). Additional data suggested that there were differences in the abilities of certain AdE1A mutants to bind S8 and TBP: the L28A and D30A mutants retained their binding capacity for TBP but could not bind S8, while the R2G and E25A mutants displayed reduced affinities for TBP, but not S8 (Fig. 4A). Additionally, the I5G, C6A, I11A, and T12A mutants had reduced capacities for binding to both S8 and TBP (Fig. 4A). We also determined the ability of these mutants to bind Ran. Interestingly, Ran displayed a very different binding pattern from those of the other N-terminal binding proteins studied here. It was apparent that residues L19 to E26 defined a major binding site for Ran: a mutation at any residue in this region severely disrupted binding (Fig. 4B). Residues L28 and D30 also form part of the Ran binding site. The AdE1A I5G, C6A, T12A, and E14A mutants all displayed modest reductions in binding capacity (Fig. 4B). For ease of comparison, the in vitro binding potentials of N-terminal AdE1A mutants for CBP/p30, P/CAF, hGcn5, S4/S8, TBP, and Ran are summarized in Table 1. Taken in their entirety, these data indicate that although common contact points exist, the N-terminal region targets individual partner proteins through unique binding sites, suggesting that AdE1A can bind to each of these proteins separately.

In vitro binding studies presented within this report (Fig. 3 and 4) and elsewhere (5, 27) suggest that AdE1A mutants synthesized in bacteria differ significantly from AdE1A mutants expressed in mammalian cells in their capacity to bind CBP/p300 (42). Indeed, both Ad5 and Ad12 E1A R2G mutants retained substantial affinities for CBP and p300 when expressed in vitro, whereas the same mutants were unable to bind CBP and p300 when expressed in vivo. This suggests that either the posttranslational modification of AdE1A or an associated protein or an AdE1A interaction with other partner proteins affect the AdE1A association with partner proteins such as CBP/p300 in vivo. Therefore, we decided to generate A549 cell lines that stably expressed a subset of these N-terminal mutants, in the context of full-length 12S Ad5E1A and in the absence of any peptide or protein tags (see Materials and Methods), to establish further the critical requirement for N-terminal residues in the binding of CBP/p300, TBP, and S8. When expressed in vivo, all of the AdE1A mutants retained the same capacity to bind pRb as wt AdE1A (Table 2), verifying their functional integrity.

AdE1A N-terminal residues important for binding CBP/p300, S8, and TBP in vivo. Immunoprecipitation of CBP/p300 from A549 cells expressing the various AdE1A N-terminal

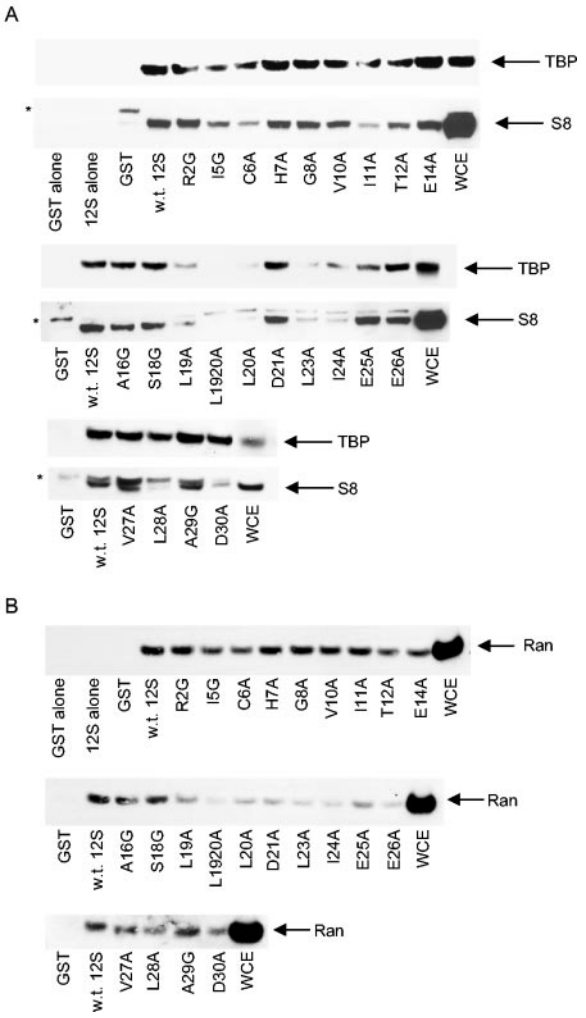

FIG. 4. In vitro binding capacities of N-terminal AdE1A mutants for TBP, S8, and Ran. Ten micrograms of the appropriate GST-AdE1A fusion protein was incubated with 5 mg of A549 cell lysate. AdE1A-interacting proteins were precipitated with glutathione-Sepharose and selectively eluted with glutathione (see Materials and Methods). Proteins were subsequently separated by SDS-PAGE and transferred to nitrocellulose. The membranes were then Western blotted for TBP and S8 (A) or Ran (B) to assess their relative affinities for different N-terminal AdE1A mutants. *, nonspecific band detected with the S8 Ab.

mutants and Western blotting for bound AdE1A revealed that the AdE1A R2G, I5G, and L1920A mutants could not bind CBP/p300 in vivo (Fig. 5A). Similar analyses revealed that the C6A, L19A, L20A, L23A, and L28A mutants had substantially reduced affinities for CBP/p300 in vivo (Fig. 5A). Of the 16 N-terminal mutants tested, only the H7A, E14A, V27A, and D30A mutants retained a wt AdE1A capacity to bind CBP/p300 (Fig. 5A). Using an identical approach, we investigated the ability of these same mutants to bind the proteasomal ATPase S8. Interestingly, there were significant differences in the pattern of S8 binding compared to that of CBP/p300 binding over the same region (Fig. 5B). For instance, in contrast to the case in CBP/p300 binding studies, the AdE1A R2G and I5G mutants retained significant capacities for binding S8. The AdE1A L1920A, L20A, L23A, and L28A mutants were, however, unable to bind S8, while the E14A and I24A mutants retained wt AdE1A binding in vivo. Similarly, the AdE1A C6A, H7A, I11A, S18G, and V27A mutants all retained the capacity to bind S8 (Fig. 5B). TBP binding to these AdE1A mutants was also investigated in vivo (Fig. 5C). Akin to the results of the S8 binding studies, the AdE1A L1920A, L20A, and L23A mutants were unable to bind TBP. Interestingly, and in sharp contrast to the results of the S8 binding studies, the AdE1A L28A mutant bound TBP as well as the wt (cf. Fig. 5B and C). The I5G, H7A, E14A, and V27A mutants also bound TBP as well as wt AdE1A. However, these studies demonstrated that the AdE1A R2G, C6A, and I24A mutants all had severely compromised abilities to bind TBP in vivo (Fig. 5C). To illustrate further differences in the binding specificities of particular AdE1A mutants, we reexamined the ability of the I5G and L28A mutants to bind CBP/p300, S8, and TBP. Further immunoprecipitation studies confirmed that these mutants had significant differential binding potentials for each of these proteins (Fig. 5D). The I5G mutant, for instance, bound TBP as well as wt AdE1A, had a reduced affinity for binding S8, and did not bind CBP/p300. Similarly, the L28A mutant bound TBP much like wt AdE1A, had a very low affinity for S8, and had a reduced binding capacity for CBP/p300 (Fig. 5D). It is thus apparent that although common contact points within the N-terminal region exist for CBP/p300, TBP, and S8, there are also a number of residues within this region that define distinct contact points for each of these proteins, suggesting that there is not one common binding site. These data are summarized in Table 2.

Effect of mutation on the proposed secondary structure of AdE1A. Jpred consensus secondary structure predictions (9) suggested that a β-pleated sheet extends from residues H3 to C6 and that an α-helix extends from residues I11 to A29 at the N terminus of the wild-type Ad5 12S E1A protein (Fig. 6A). These predictions also suggest that the R-to-G mutation at position 2 has only modest effects on the formation of the proposed β-pleated sheet. Interestingly, however, the mutation of I to G at position 5 is proposed to severely disrupt the β-pleated-sheet structure, and moreover, α-helical formation at its extreme N terminus (Fig. 6A). Furthermore, mutations of residues C6, H7, and G8 to A all have a dramatic effect on the proposed secondary structure at the N terminus. Indeed, predictions suggest that the β-pleated sheet present in the wt AdE1A protein reverts to an α-helical structure after these substitutions (Fig. 6A). Perhaps more surprisingly, the substitution of residues within the proposed α-helical structure, on the whole, had very little effect upon the proposed formation of the α-helix (Fig. 6A). Indeed, the replacement of the large hydrophobic residues L19, L20, V23, I24, V27, and L28 with smaller, nonpolar A residues did not affect α-helix formation (Fig. 6A), and the replacement of the acidic residues D21, E25, and D30 with A similarly had no effect on the proposed α-helix formation (Fig. 6A). These findings address the ability of AdE1A mutants to bind partner proteins (see Discussion). The spatial organization of amino acids comprising the proposed α-helix at the N terminus of wt Ad5 E1A is depicted schematically as a helical wheel (Fig. 6B). Interestingly, the hydrophobic, nonpolar residues A16, L20, L23, and V27 are suggested to lie in close proximity on the same side of the helix (Fig. 6B).

TABLE 1. Interaction of N-terminal binding proteins with AdE1A mutants in vitro[a]

Mutant	Interaction with protein						Transformation relative to 12S (%)
	CBP/p300	P/CAF	hGCN5	S4/S8	TBP	Ran	
12S	++++	++++	++++	++++	++++	++++	100
R2G	+++	+++	+++	++++	++	++++	11.5
I5G	+++/++	−	+	++	++	++	4.5
C6A	+	+	++	+	++	++	40
H7A	++++	++++	++++	++++	++++	++++	98.7
G8A	++++	++++	++++	++++	++++	++++	80.4
V10A	++	+	++	+++	+++	++++	83
I11A	+	−	−	+	+	++++	35.5
T12A	++++/++	+	+	++	++	++	56
E14A	++	++++	+++	++++	++++	++	70.5
A16G	+	−	+	+++	++++	+++	30.9
S18G	++	++	++	+++	++++	+++	112
L19A	+	−	−	+	+	+	38
L1920A	−	−	−	−	−	−	15
L20A	−/+	−	−	−	+	+	46
D21A	+++	++++	++++	++++	++++	+	57
L23A	−	−	−	+	+	+	38
I24A	−	−	−	+	+	+	37.6
E25A	++++	++++	+++	+++	++	++	112
E26A	++	++++	++	+++	++++	+	121
V27A	+++	+	+++	+++	++++	+++	74.7
L28A	−	−	−	−	+++	++	45
A29G	++++/++	++++	+++	++++	++++	++++	97
D30A	++++	++++	++	+	++++	++	43.5

[a] The ability of CBP/p300, P/CAF, hGcn5, S4/S8, TBP, and Ran to interact with the indicated N-terminal mutants in vitro was assessed by GST-AdE1A pull-down assays (see Materials and Methods). − and +, binding capacity of each mutant relative to wt 12S. −, no appreciable binding; +, 0 to 25% binding; ++, 25 to 50% binding; +++, 50 to 75% binding; ++++, 75 to 100% binding. When CBP and p300 differ in their binding capacities for AdE1A, this is indicated (−/+). Binding capacity was quantified with a BIO-RAD GS-800 calibrated densitometer using the Quantity One program. The data presented are mean percentages of binding from three independent experiments. Binding capacities for each of the mutants tested were highly reproducible. For comparison, the ability of these mutants to cooperate with activated *ras* in the transformation process is also shown.

A17, I24, and L28 are similarly orientated in close proximity, as are the acidic residues E14, D21, and E25 (Fig. 6B). These regions might be important for conferring helical stability as well as for defining distinct protein binding sites.

Effect of N-terminal mutation on AdE1A-mediated transformation. Previous studies have established that the integrity of the N-terminal region is critical for AdE1A to cooperate in the transformation process (3, 18, 26, 42). Indeed, studies have suggested that AdE1A binding to CBP/p300 is crucial for promoting cellular transformation. Since these studies, however, several additional N-terminal binding proteins have been found. The study detailed here has comprehensively resolved the binding sites for a number of these proteins both in vitro and in vivo (Fig. 3 to 5; Tables 1 and 2). We therefore decided to establish the requirement for these N-terminal binding proteins in the transformation process. To study this requirement, we transfected HLREFs with AdE1A mutants and activated T61 N-*ras*, as described in Materials and Methods, and counted genuine AdE1A/*ras* transformants, as opposed to G418-resistant colonies with a normal morphology, 2 weeks later by low-power microscopy (Fig. 7). The results of this study revealed that the H7A, S18G, E25A, E26A, and A29G mutants all cooperated fully, relative to wt AdE1A, with *ras* in the transformation process (Fig. 7). Although the G8A, V10A, E14A, and V27A mutants possessed substantial transforming potentials, they were less efficient than wt AdE1A (Fig. 7). However, there were several AdE1A mutants that displayed significantly reduced transforming capacities relative to wt AdE1A: the transformation potentials of the C6A, A16G,

TABLE 2. In vivo association of CBP/p300, S8, and TBP with the N terminus of AdE1A[a]

Mutant	Association with protein				Transformation relative to 12S (%)
	CBP/p300	S8	TBP	pRb	
12S	++++	++++	++++	++++	100
R2G	−	++	+	++++	11.5
I5G	−	++	++++	++++	4.5
C6A	++	+++	+	++++	40
H7A	++++	+++	++++	++++	98.7
I11A	+++	+++	++	++++	35.5
T12A	+++	+	++	++++	56
E14A	++++	++++	++++	++++	70.5
S18G	+++	+++	+++	++++	112
L19A	+	+	++	++++	38
L1920A	−	−	−	++++	15
L20A	+	−	−	++++	46
L23A	+	−	−	++++	38
I24A	+++	++++	+	++++	37.6
V27A	++++	+++	++++	++++	74.7
L28A	++	−	++++	++++	45
D30A	++++	+	++	++++	43.5

[a] The ability of CBP/p300, S8, and TBP to interact with the indicated N-terminal mutants in vivo was assessed by immunoprecipitation and Western blotting. A549-derived cells stably expressing AdE1A mutants to comparable levels were used for these studies (see Materials and Methods). − and +, binding capacity of each mutant relative to wt 12S. −, no appreciable binding; +, 0 to 25% binding; ++, 25 to 50% binding; +++, 50 to 75% binding; ++++, 75 to 100% binding. Binding capacity was quantified with a BIO-RAD GS-800 calibrated densitometer using the Quantity One program. The data presented are mean percentages of binding from two, and in some instances three, independent experiments. Binding capacities for each of the mutants tested were highly reproducible. The ability of these mutants to cooperate with activated *ras* in the transformation process is also indicated.

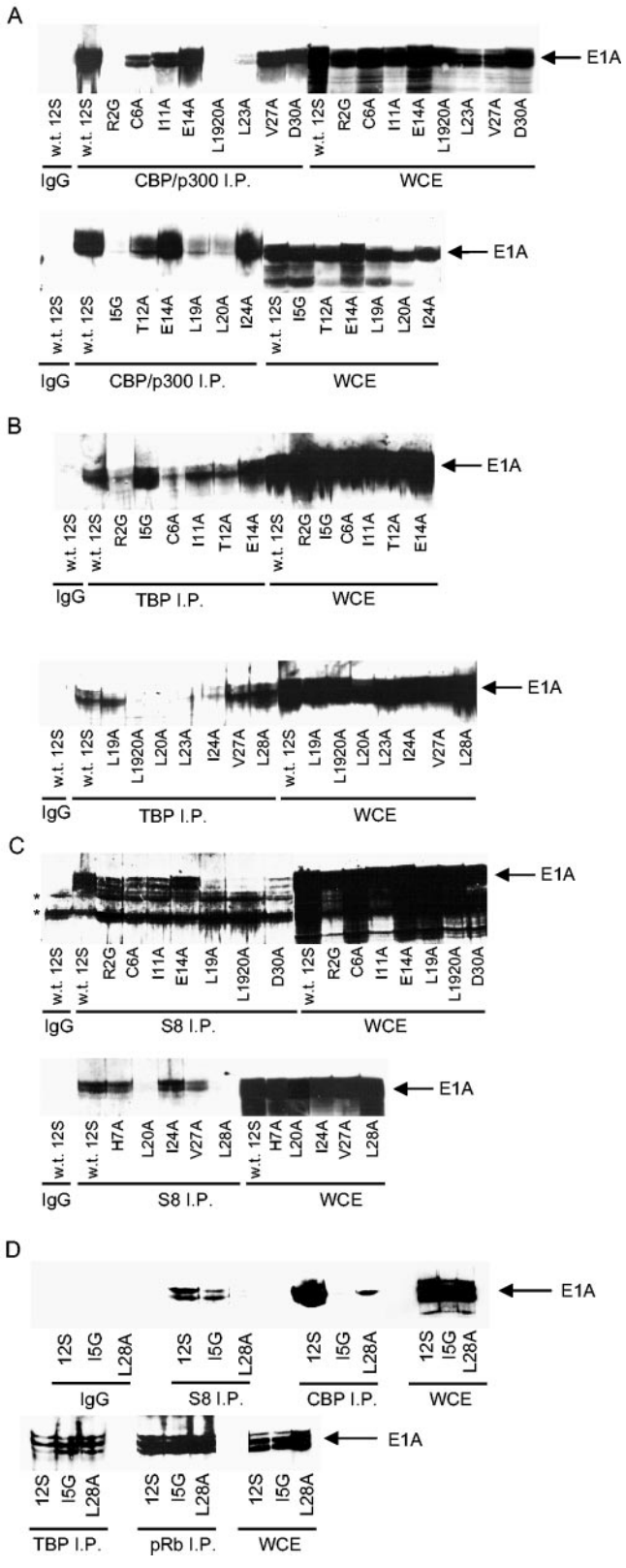

FIG. 5. Binding of CBP/p300, TBP, and S8 to AdE1A N-terminal mutants in vivo. A549-derived cell lines stably expressing AdE1A mutants to similar levels (see Materials and Methods) were isolated in order to establish the binding capacity of each mutant for N-terminal binding proteins in vivo. Five micrograms of a CBP/p300 PAb, 10 μg of a TBP PAb, and 5 μg of an S8 PAb were incubated with 5 mg of the appropriate cell lysate and collected with protein G-Sepharose. Washed immunocomplexes were mixed with a sample buffer lacking SDS, separated in urea gels (see Materials and Methods), and subsequently transferred to nitrocellulose. The membranes were Western blotted for E1A by use of the M73 MAb. The abilities of CBP/p300 (A and D), TBP (B and D), and S8 (C and D) to bind specific AdE1A mutants are shown. *, nonspecific bands. WCE, whole-cell extract; I.P., immunoprecipitate.

L19A, L20A, D21A, L23A, I24A, L28A, and D30A mutants were reduced between 40 and 75% (Fig. 7). Additionally, there were three mutants, R2G, I5G, and L1920A, that had only very modest transforming abilities relative to wt AdE1A (Fig. 7). Tables 1 and 2 specifically relate the transforming potentials of individual N-terminal mutants to their capacity to bind N-terminal binding proteins. The requirement for the N-terminal region for targeting binding partners in order to promote transformation is addressed fully in Discussion.

TBP and S8 bind independently to the N-terminal region of AdE1A. Previous studies have indicated that there is an intimate functional relationship between TBP and S8 (40). The site-directed mutagenesis study presented here clearly indicated that although common residues at the N terminus are involved in binding to both TBP and S8, several distinct contact points for TBP and S8 also exist (Fig. 4A and 5B, C, and D). To determine whether TBP and S8 can bind to AdE1A independent of each other, we used siRNAs complementary to TBP and S8 mRNA sequences to specifically knock out the expression of these genes in A549 cells (Fig. 8A and B). We subsequently assessed the binding capacity of TBP (in the absence of S8) or S8 (in the absence of TBP) for AdE1A. GST pull-down assays revealed that GST-AdE1A retained its maximal capacity, relative to the appropriate controls, to bind TBP from cell lysates when S8 expression was reduced by RNAi (Fig. 8A). GST-AdE1A similarly retained its maximal capacity to bind S8 following the elimination of TBP expression by RNAi (Fig. 8B). To substantiate these findings, we investigated whether AdE1A could form stable complexes in vivo with TBP or S8 in the absence of the other protein. We thus treated 12S AdE1A-expressing A549 cells with the appropriate siRNAs, verified the knockouts by Western blotting, and subsequently performed immunoprecipitation with an anti-TBP or anti-S8 antibody. Western blot analysis of E1A revealed that eliminating S8 expression by RNAi had no effect on the ability of AdE1A to form stable complexes with TBP in vivo (Fig. 8C) and that the elimination of TBP expression by RNAi did not affect AdE1A's ability to interact with S8 in vivo (Fig. 8D). Importantly, the elimination of either S8 or TBP by RNAi did not affect the expression of the other protein or of AdE1A (Fig. 8E). Consistent with previous findings, p53 levels were dramatically increased following the S8 knockout (Fig. 8E) (46) but were only modestly increased upon TBP knockout (Fig. 8E).

CBP/p300 binds to the N-terminal region of AdE1A independently of TBP and S8. Given that CBP and TBP form stable complexes in vivo (10) and that CBP/p300 and S8 both function as coactivators (1, 25, 40), we decided to investigate whether CBP/p300 binding to AdE1A was affected by eliminating the expression of either TBP or S8. Following the

A

```
                1       10        20        30
Mutation        MRHIICHGGVITEEMAASLLDQLIEEVLAD
  w.t.      --EEEE----HHHHHHHHHHHHHHHHHHH-
  R2G       ---EEE----HHHHHHHHHHHHHHHHHHH-
  I5G       ---E-----EEHHHHHHHHHHHHHHHHH-
  C6A       --HHHH---HHHHHHHHHHHHHHHHHHH-
  H7A       --HHHH----HHHHHHHHHHHHHHHHHH-
  G8A       --HHHH----HHHHHHHHHHHHHHHHHH-
  V10A      --EEEE----HHHHHHHHHHHHHHHHHH-
  I11A      --EEEE----HHHHHHHHHHHHHHHHHH-
  T12A      --EEEE----HHHHHHHHHHHHHHHHHH-
  E14A      --EEEE---HHHHHHHHHHHHHHHHHH-
  A16G      --EEEE---EEHHHHHHHHHHHHHHHHH-
  S18G      --EEEE---HHHHHHHHHHHHHHHHHH-
  L19A      --EEEE----HHHHHHHHHHHHHHHHHH-
  L1920A    --EEEE---HHHHHHHHHHHHHHHHHH-
  L20A      --EEEE----HHHHHHHHHHHHHHHHHH-
  D21A      --EEEE----HHHHHHHHHHHHHHHHHH-
  V23A      --EEEE----HHHHHHHHHHHHHHHHHH-
  I24A      --EEEE----HHHHHHHHHHHHHHHHHH-
  E25A      --EEEE----HHHHHHHHHHHHHHHHHH-
  V27A      --EEEE----HHHHHHHHHHHHHHHHHH-
  L28A      --EEEE----HHHHHHHHHHHHHHHHHH-
  A29G      --EEEE----HHHHHHHHHHHHHHHHHH-
  D30A      --EEEE----HHHHHHHHHHHHHHHHHH-
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B

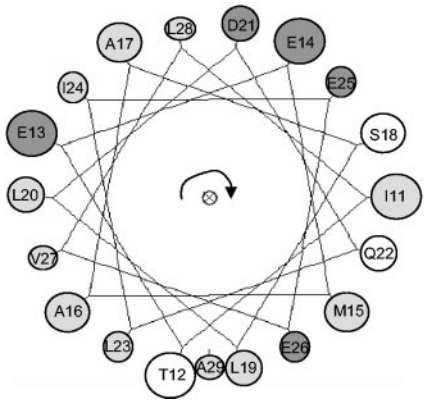

FIG. 6. (A) Secondary structure predictions for the N-terminal region of the Ad2/5 E1A protein by the Jpred consensus method (9). The Jpred program can be found at http://www.compbio.dundee.ac.uk/~www-jpred/. The proposed structure for the first 30 amino acids of wt Ad5 12S E1A is compared with those of the N-terminal mutants used for this study. H, proposed α-helix; E, proposed β-pleated sheet; −, proposed random coil. For wt Ad2/5 E1A, a proposed β-pleated sheet extends from H3 to C6, and a proposed α-helix extends from I11 to A29. (B) Helical wheel depicting the spatial arrangement of amino acids (I11 to A29) comprising the proposed α-helix at the N terminus of wt Ad5 E1A. Residues are represented with shaded circles as follows: white, polar uncharged residues; light gray, nonpolar residues; dark gray, acidic residues. I11, top of the helix; A29, bottom of the helix.

knockout of TBP and S8 in 12S AdE1A-expressing A549 cells by RNAi, we immunoprecipitated CBP and p300 with an anti-CBP/p300 PAb and subjected the immunoprecipitates to PAGE analysis and subsequent Western blotting for AdE1A. Akin to the TBP-S8 scenario, CBP can form stable complexes with AdE1A in the absence of either S8 (Fig. 8F) or TBP (Fig. 8G). Importantly, neither CBP, p300, nor E1A expression was affected by treatment with a siRNA directed against either TBP or S8 (Fig. 8H).

DISCUSSION

Binding sites for N-terminal partner proteins CBP/p300, TBP, P/CAF, and hGcn5. Residues R2 and L20 have previously been reported to comprise part of the binding site in vivo for CBP/p300 (42). Our analyses extend these findings to indicate that I5 and L19 are also required for CBP/p300 binding in vivo (Fig. 5A). In vitro analyses have previously revealed that the mutation of C6 to A eliminates the CBP/p300 association with an AdE1A polypeptide comprising the first 80 amino acids (5). Our in vitro analyses, in the context of the whole 243-residue protein, essentially confirm these findings (Fig. 3A). In contrast, however, our in vivo analyses revealed that the C6A mutant still retains a substantial binding capacity for CBP/p300 (Fig. 5A). In support of the notion that in vitro studies do not fully reflect the situation in vivo, it has been shown previously, and is now confirmed in this report, that the mutation of R2 to G eliminates CBP/p300 binding in vivo but not in vitro (cf. Fig. 3A and 5). It is supposed, therefore, though not yet proven experimentally, that either a posttranslational modification of AdE1A or CBP/p300 or an association with other partner proteins affects their association in vivo. Interestingly, the C6A mutant has also been reported to eliminate the AdE1A association with TBP (5). Our analyses revealed that this mutant retained some capacity to bind TBP in vitro (Fig. 4A) but had a negligible affinity for binding TBP in vivo (Fig. 5B). Given the observation that C6, in the context of the first 80 amino acids, is critical for mediating AdE1A transcriptional repression (5), it should be possible to discern in vivo, by use of the C6A 243-residue mutant protein, the relative contributions of TBP and CBP/p300 to this process. Interestingly, however, C6 is not conserved among any of the other serotypes; instead, P is highly conserved at this position (Fig. 1). Given that other key TBP-interacting residues are conserved between serotypes, it will be of considerable interest to evaluate the relative affinities of other AdE1A species for TBP.

Since P/CAF and hGcn5 share considerable sequence homology, it was not surprising that AdE1A mutants bound these proteins with similar affinities (Fig. 3B). The observable differences in the binding capacities of the two proteins in vitro do suggest, however, that AdE1A can selectively target either P/CAF or hGcn5 in vivo. Given the role of the N-terminal region in transcriptional repression and transformation, it would be interesting to establish, in particular, whether the AdE1A R2G and C6A mutants bind P/CAF and hGcn5 in vivo. Unfortunately, using commercially available antibodies against P/CAF, we have been unable to demonstrate a significant association of endogenous P/CAF with Ad 12S E1A in vivo (M. Rasti and A. Turnell, unpublished data) and thus have not been able to answer this question directly. A previous report demonstrating an AdE1A association with P/CAF in vivo utilized exogenously expressed systems in which hemagglutinin-tagged P/CAF was overexpressed in mammalian cells (32).

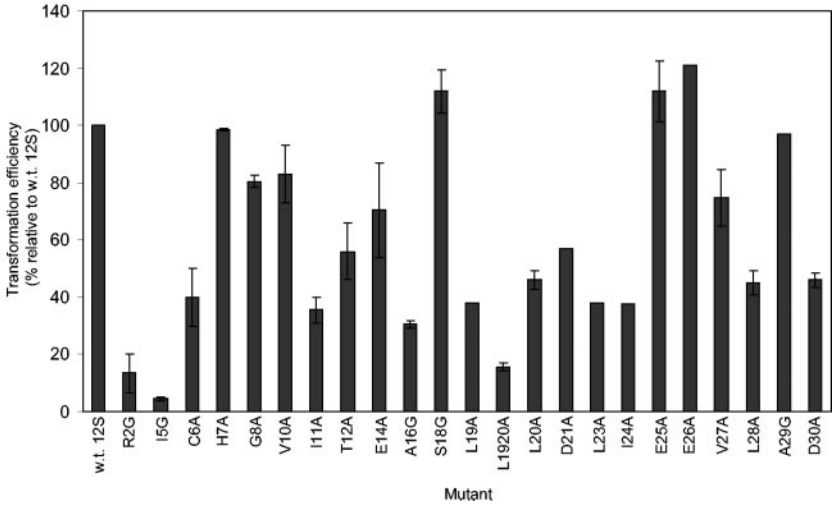

FIG. 7. Transforming potentials of N-terminal AdE1A mutants. HLREFs were transfected by electroporation with wt AdE1A or N-terminal AdE1A mutants in the presence of activated N-*ras* (see Materials and Methods). At 2 weeks posttransfection, bona fide AdE1A/*ras*-transformed colonies were counted by low-power microscopy. The data presented represent the averages of four independent experiments ± standard deviations. The transforming potential of each mutant is expressed relative to the mean ability of wt 12S AdE1A to transform HLREFs in combination with activated N-*ras.*

Binding of S8 and Ran to the N-terminal region of AdE1A. Our in vitro analyses suggested that the binding site on AdE1A for S8 considerably overlaps that for TBP, although the S8 binding site appears to be more extensive than that for TBP (Fig. 4A). Interestingly, differences in S8 and TBP binding to AdE1A were more discernible in vivo, indicating that distinct binding sites do exist for these two proteins (cf. Fig. 5B and C). Our in vitro analyses revealed that the binding site for Ran is very different from those for other N-terminal binding proteins, extending from L19 to E26 (Fig. 4B). The mutation of residues within this region severely affected the ability of AdE1A to interact with Ran. It will be of considerable interest in future studies to investigate whether mutants spanning this region affect both the ability of AdE1A to modulate Ran nucleotide exchange activity, and moreover, the ability to promote centrosome amplification.

Taking all of the binding data into consideration, it is apparent that a number of key residues involved in defining specific binding sites are conserved among Ad serotypes (cf. Tables 1 and 2 with Fig. 1), suggesting that it is highly likely that AdE1A proteins from all serotypes will target all of the known N-terminal binding proteins. In particular, several hydrophobic residues that are well conserved among serotypes (Fig. 1) and comprise part of the proposed α-helical secondary structure (Fig. 6A) also differentially define major contact sites for all of the N-terminal binding proteins studied here. It is therefore tempting to speculate that the conserved secondary structure confers an identity of function among serotypes, with the specificity of interactions being defined by distinct residues. Crucially, mutations of residues within the α-helix are not proposed to affect α-helix formation (Fig. 6B), suggesting that residues within this region define genuine contact points for AdE1A binding proteins and do not merely affect binding through disruption of the α-helix. Interestingly, residues that form part of the proposed β-pleated sheet at the extreme N terminus (Fig. 6A) also define major contact points for AdE1A binding proteins, and mutations in this region disrupt the proposed secondary structure (Fig. 6A). Whether these residues define actual contact points therefore requires clarification. A comparison of the binding data with the proposed organization of the α-helix (Fig. 6B) suggests that binding over this region is extensive, with multiple contact points existing for each binding partner (Fig. 6B). There is no direct correlation between binding capacity and the spatial organization of the amino acids defining the helix.

Targeting AdE1A binding proteins during AdE1A-mediated transformation. It is well documented that the AdE1A R2G mutant, which fails to bind CBP/p300 in vivo, is also transformation defective (42), implicating CBP/p300 as a major target for AdE1A during transformation. Although our analyses similarly suggest that AdE1A targets CBP/p300 during the transformation process, our in vivo binding studies indicate that there is not a simple relationship between the abilities of AdE1A to bind CBP/p300 and to promote transformation. Consistent with the suggestion that CBP/p300 is the major target of AdE1A during this process, the mutation of residues R2, I5, and L19,20 eliminated both CBP/p300 binding and transforming potential (cf. Fig. 5A and 7). Interestingly, however, the mutation of residues L19, L20, and L23 generated AdE1A mutants that bound CBP/p300 very poorly but whose transforming abilities were only reduced to approximately 40% that of wt AdE1A (cf. Fig. 5A and 7). The transforming activities of the I24 and D30 mutants were similarly reduced to approximately 40% that of wt AdE1A, yet they bound CBP/p300 with a wt affinity (cf. Fig. 5A and 7). In addition, the mutation of residues E14 and V27 produced AdE1A species that bound CBP/p300 as much as the wt yet showed reductions in transforming capacity of between 25 and 30% (cf. Fig. 5A and 7). There was also a situation in which both CBP/p300 binding and transforming ability were unaffected by mutation (H7). Thus, it is perhaps too simplistic to suggest that there is a direct linear correlation between the abilities of AdE1A to

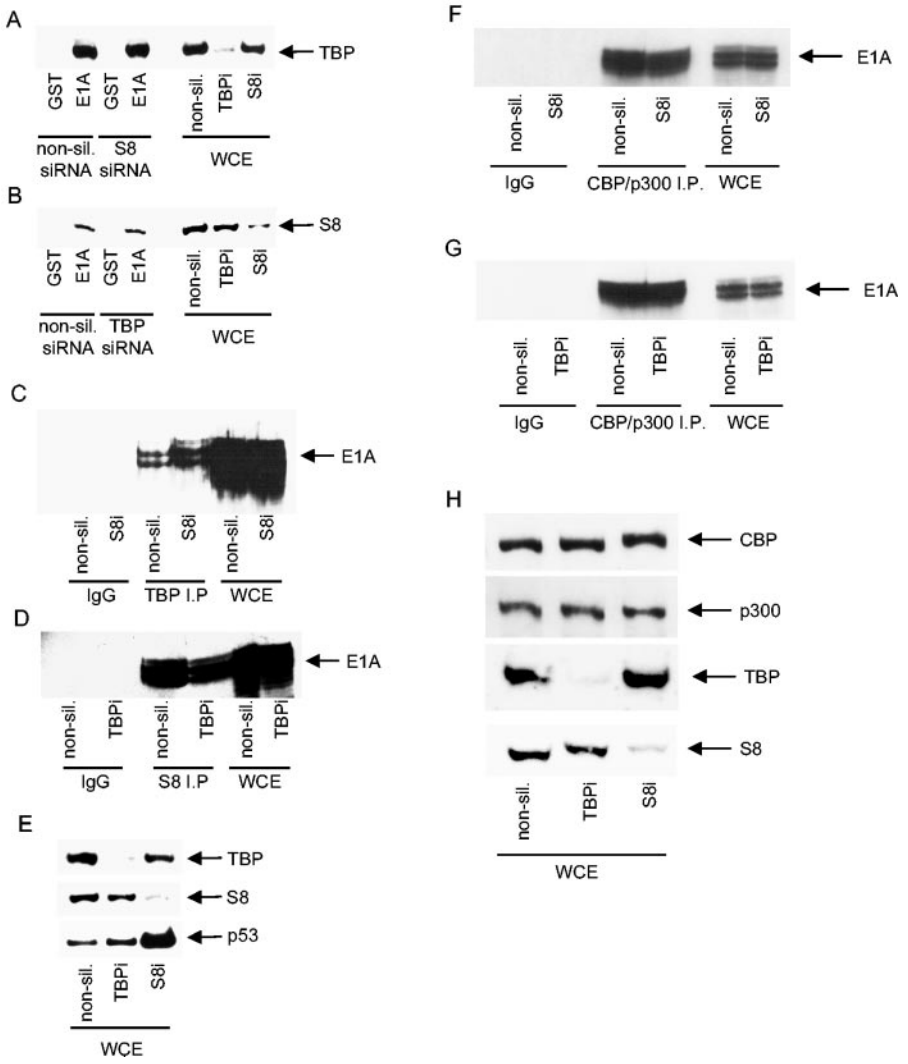

FIG. 8. (A to E) N-terminal region of AdE1A independently targets TBP and S8 in vivo. (A and B) A549 cells were transfected with a siRNA targeted against either TBP or S8. Subsequent GST pull-down assays revealed that TBP and S8 can bind independently to AdE1A in vitro. (C to E) A549 cells were similarly transfected with a siRNA targeted against either TBP or S8. Anti-S8 and anti-TBP immunocomplexes were subsequently precipitated with protein G-Sepharose, separated by PAGE in the presence of urea, and transferred to nitrocellulose (see Materials and Methods). The membranes were then probed for AdE1A by use of the M73 MAb. The data presented indicate that both TBP (C) and S8 (D) associate independently with AdE1A in vivo. Whole-cell extracts separated by SDS-PAGE and transferred to nitrocellulose were probed for their levels of TBP, S8, and p53 (E) to gauge the efficiency of RNAi. (F to H) The N-terminal region of AdE1A targets CBP/p300 in vivo independent of TBP and S8. Anti-CBP/p300 immunocomplexes were precipitated with protein G-Sepharose, subsequently separated by PAGE in the presence of urea, and transferred to nitrocellulose (see Materials and Methods). The membranes were then probed for AdE1A by use of the M73 MAb. The data presented indicate that CBP/p300 can associate, independently of either S8 (F) or TBP (G), with AdE1A in vivo. Whole-cell extracts separated by SDS-PAGE and transferred to nitrocellulose were probed for their levels of CBP, p300, TBP, and S8 (H) to gauge the efficiency of RNAi. WCE, whole-cell extract; nonsil., nonsilencing RNA oligonucleotides. S8i and TBPi refer to situations in which S8 or TBP expression has been abolished by RNAi.

bind CBP/p300 and to promote cellular transformation. These data therefore suggest that other N-terminal binding proteins may also be targeted by AdE1A during the transformation process. In this regard, it will be of considerable interest to determine the affinities of these point mutants in vivo for the AdE1A binding proteins p400 and TRAPP, which have previously been suggested to be targeted by residues 26 to 35 at the N terminus of AdE1A during transformation (11, 17). Moreover, given that CBP/p300, hGcn5, and P/CAF have very similar binding profiles over the N-terminal region in vitro (Fig. 3 and Table 1), it will be important to establish whether this binding pattern is reproducible in vivo. This would establish whether P/CAF and hGcn5 are similarly potential targets for AdE1A during transformation.

The observation that the transformation-defective AdE1A mutants R2G, I5G, and L1920A had variable affinities for S8 and TBP might suggest that these proteins are not major targets during transformation (cf. Fig. 5C and D). Interestingly, however, the AdE1A C6A mutant, which possessed only 40% of the transforming activity of wt AdE1A, had a very weak

affinity for TBP in vivo yet bound S8 and CBP/p300 well (Fig. 5A to C). Another mutant that implicated TBP as a potential target was the I24A mutant. This mutant had a very weak affinity for TBP but bound CBP/p300, in vivo at least, with a wt affinity (Fig. 5A and B). A requirement for S8 binding during transformation is more difficult to discern, however, given the relative in vivo affinities of the AdE1A mutants for S8 (Fig. 5C and Table 2). A consideration of the in vitro Ran binding data again suggested that there is no direct positive correlation between binding and the AdE1A transforming potential. There were several mutations within the proposed Ran binding site that compromised the transforming ability (Fig. 7). However, the E25A and E26A mutants, which comprised part of this site, had wt transforming activities (Fig. 7).

AdE1A independently targets N-terminal binding proteins CBP/p300, S8, and TBP. The fine mapping studies detailed in this report indicate that within the first 30 amino acids of AdE1A, several discrete binding sites exist for AdE1A N-terminal binding proteins (Fig. 2 to 5; Tables 1 and 2). These studies do not preclude the possibility, however, that these proteins may also be recruited to the N-terminal region of AdE1A as components of a larger macromolecular complex. Indeed, previous studies have indicated that TBP can be found in complex with either CBP/p300 or S8, independent of AdE1A. Thus, we attempted to resolve, at least for these three proteins, whether their recruitment in vivo to the N-terminal region of AdE1A was dependent on their association with each other. Using RNAi, we demonstrated that CBP/p300, TBP, and S8 can be recruited to AdE1A independently (Fig. 8), suggesting that AdE1A may target these proteins individually to perform specific functions. These data also suggest that during the course of the viral life cycle or during the cell cycle of an AdE1A-transformed cell line, there exist subpopulations and subcomplexes of AdE1A that perform distinct functions. Indeed, it was established previously that AdE1A can serve as a bridging protein, recruiting both CBP/p300 and pRb to the same complex, to facilitate the CBP/p300-dependent acetylation of pRb (7). Since the function of an AdE1A subcomplex is presumably determined by the cellular proteins in the complex, it will be of considerable interest to determine the macromolecular compositions of AdE1A-S8, AdE1A-TBP, and other AdE1A-containing complexes.

In summary, this report demonstrates that AdE1A can interact with a number of N-terminal binding proteins directly, through discrete, unique binding sites. Our genetic studies further suggest that AdE1A targets several cellular N-terminal binding proteins to promote full transformation. We also demonstrated that AdE1A can form separate complexes with CBP/p300, TBP, and S8 in vivo, providing additional evidence to suggest that there are discrete functions for AdE1A subcomplexes. The composition and function of these subcomplexes will be a major focus of studies in our laboratory in the future.

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