JOURNAL OF VIROLOGY, Nov. 1991, p. 5848–5859
0022-538X/91/115848-12$02.00/0
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Vol. 65, No. 11

Adenovirus E1A Is Associated with a Serine/Threonine Protein Kinase

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Received 10 June 1991/Accepted 27 July 1991

The adenovirus E1A proteins form stable protein complexes with a number of cellular proteins, including cyclin A and the product of the retinoblastoma susceptibility gene. We have been interested in learning about the function of proteins associated with E1A and therefore looked for an enzymatic activity present in E1A complexes. We found a serine/threonine kinase activity that phosphorylates two proteins bound to E1A, the 107- and 130-kDa (107K and 130K) proteins. The kinase also phosphorylates histone H1 added as an exogenous substrate. The kinase activity is cell cycle regulated, being most active in S and G_2/M-phase cells. The timing of phosphorylation of the 107K protein in vitro correlates with the phosphorylation pattern of the 107K protein in vivo. A variety of genetic and immunochemical approaches indicate that the activity is probably not due to the E1A-associated 300K, 130K, 107K, or pRB protein. Although we have not established the identity of the kinase, we present evidence that the kinase activity is consistent with phosphorylation by $p34^{cdc2}$ or a related kinase.

The E1A proteins of adenovirus possess multiple functions that play important roles in regulating the early events of the adenovirus life cycle. One function of the E1A proteins that has been studied extensively is their ability to immortalize primary rodent cells in culture (29). When E1A is introduced into primary rodent cells, they overcome senescence and become established cell lines. When E1A is introduced along with the adenovirus E1B gene or another cooperating oncogene, such as *ras*, the recipient cells become fully transformed (43, 47). The E1A proteins have a number of other well-characterized functions, including the ability to transactivate the other early viral genes as well as several cellular genes (for a review, see reference 1), to inhibit the expression of certain enhancer-linked genes (for a review, see reference 1), to stimulate cellular DNA synthesis (4, 31, 44), to induce the production of an epithelial cell growth factor (42), and to inhibit the growth-suppressing properties of transforming growth factor β (38).

The mechanism by which the E1A proteins function is still unclear. However, it is known that E1A forms stable complexes with a number of cellular proteins, the most prominent of which have relative molecular masses of 300, 130, 107, 105, and 60 KDa (25, 51). The regions of E1A that are necessary for binding to all of these proteins correspond with the regions of E1A essential for its transforming function (Fig. 1A) (20, 50), suggesting that transformation by E1A may occur via interaction with these proteins.

Two of the proteins that associate with E1A have recently been identified. One of these proteins, the 105-kDa (105K) protein, has been demonstrated to be the product of the retinoblastoma susceptibility gene (RB-1) (48). RB-1 is a prototypic tumor suppressor gene or anti-oncogene, as the loss of expression of its protein product, pRB, has been correlated with the formation of retinoblastomas and other tumors (16–18, 26–28, 33, 45, 46, 48, 52). It is believed that the growth-inhibitory function of pRB is abrogated by the binding of E1A. Similar types of complexes have been reported for transforming proteins of other DNA tumor viruses, including the large T antigens of simian virus 40 (SV40) or other polyomavirus-type viruses and the E7 proteins of human papillomaviruses (9, 11, 13, 37).

The second E1A-associated protein that has been identified recently is the 60K protein. This protein has been shown to be human cyclin A (40). Cyclins are a class of proteins that associate with the major cell cycle-regulating kinase, $p34^{cdc2}$. Cyclins act as a regulatory subunit of this kinase, while $p34^{cdc2}$ is the catalytic subunit (for a review, see reference 41). $p34^{cdc2}$ is highly conserved and plays an important role in regulating cell cycle progression in all eukaryotes. The association of cyclins with $p34^{cdc2}$ is believed to help determine both the substrate specificity and timing of the kinase activity. Two forms of the kinase have been identified in mammalian cells: a $p34^{cdc2}$-cyclin B complex, which is maximally active during mitosis, and a $p34^{cdc2}$-cyclin A complex, which is most active during interphase (21, 39, 40).

Little is known about the other abundant E1A-associated cellular proteins. The 130K and 107K proteins are nuclear phosphoproteins that bind to regions on E1A similar to those to which pRB binds (20, 50). pRB and 107K also bind to similar regions on SV40 large T antigen (12, 14). pRB and 107K have several other characteristics in common. SV40 large T antigen associates preferentially with the underphosphorylated forms of both pRB and 107K. Furthermore, a monoclonal antibody raised against pRB directly recognizes the 107K protein (30). This finding suggests that pRB and 107K share a common structural element. Despite these similarities, pRB and 107K are not products of the same gene. The proteins have different proteolytic patterns, most antibodies raised against pRB do not recognize 107K, and most convincingly, 107K is detected in cells that contain a homozygous deletion of the RB-1 gene.

We have been interested in studying the functions of

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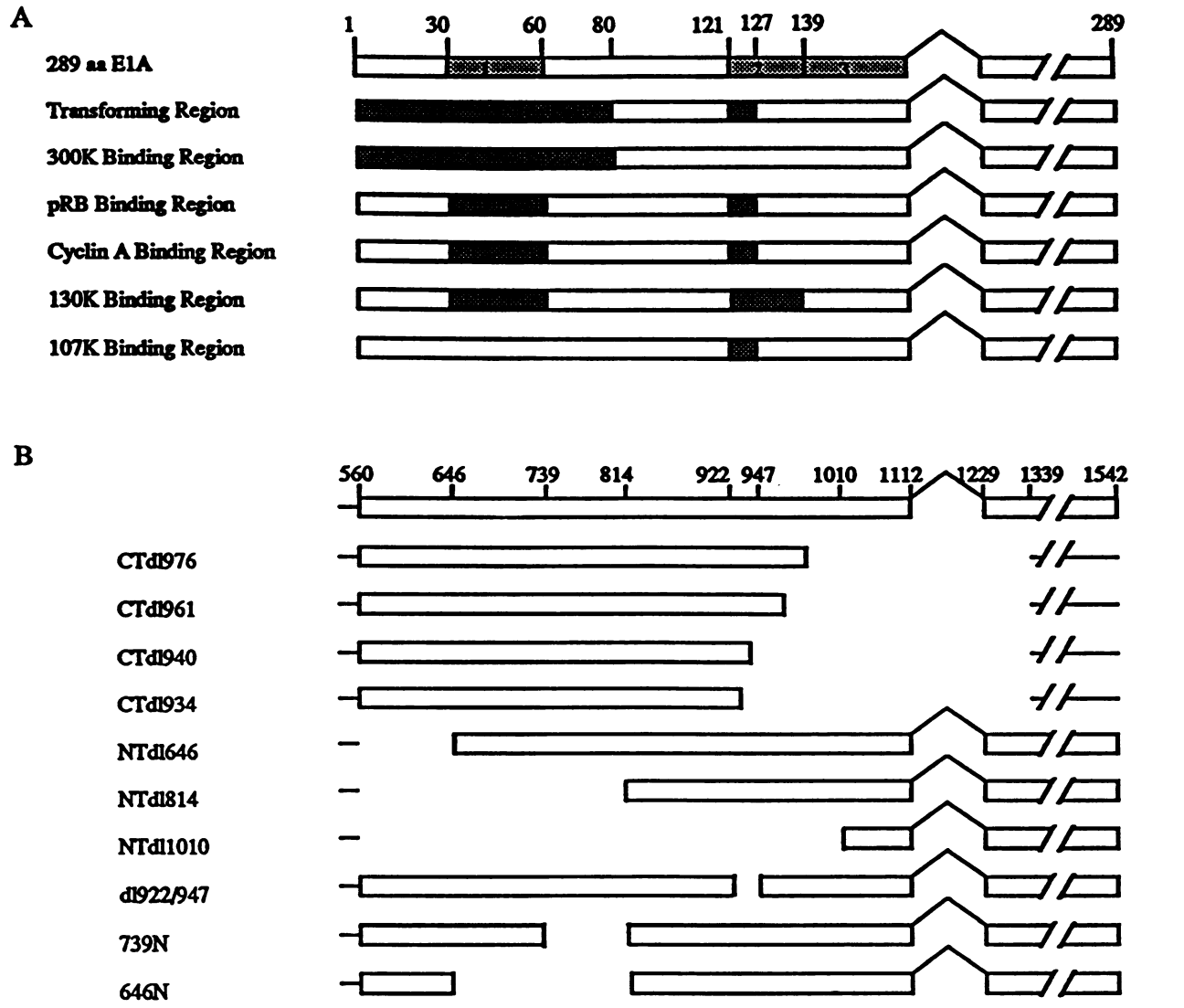


FIG. 1. Diagram of the E1A protein and structure of E1A mutants. (A) The 289R protein. Conserved regions 1, 2, and 3 are indicated. Numbers at the top refer to amino acid (aa) residues within the E1A protein. Regions important for transformation and binding to the major E1A-associated cellular proteins are represented by shaded boxes. (B) Structure of each of the E1A mutants used in this study is illustrated. The numbers at the top refer to nucleotides within the adenovirus genome. The open boxes and straight lines represent translated and untranslated regions of the mRNA, respectively.

cellular proteins that associate with E1A. For this reason, we looked for an enzymatic activity present in the E1A complexes. We found an associated serine/threonine kinase activity that phosphorylates both the 107K and 130K proteins as well as exogenous histone H1. The activity of the kinase is cell cycle regulated and is probably not due to the 300K, 130K, 107K, or pRB protein. The identity of this associated kinase remains unknown, but the properties of the kinase activity are consistent with phosphorylation by $p34^{cdc2}$ or a related kinase.

MATERIALS AND METHODS

Cells and viruses. Cells were grown in Dulbecco modified Eagle medium with 100 μg each of penicillin and streptomycin per ml (DMEM) and supplemented with 10% fetal bovine serum. HeLa and 293 cells were obtained from the Cold Spring Harbor Laboratory Cell Culture Facility and were maintained by R. Whittaker. The Retro-3B cell line, a HeLa cell derivative that stably expresses the E1A 12S protein, was generated and maintained by C. McCall. The E1A mutant viruses used in this study have been described previously (49, 50).

Infections. Monolayer cultures of HeLa cells in 100-mm-diameter dishes at approximately 50% confluency were infected in duplicate with 100 PFU of adenovirus serotype 5 or recombinant adenoviruses per cell. Infection was carried out for 1 h in DMEM, after which the cells were incubated in fresh DMEM containing 2% fetal bovine serum. At 12 to 14 h postinfection, one set of infected cells was labeled with 300 to 500 μCi of [^{35}S]methionine (Trans ^{35}S-label; ICN Radiochemicals). Lysates of labeled and unlabeled cells were prepared at 16 to 18 h postinfection.

Cell labeling and immunoprecipitations. Monolayer cultures of uninfected 293 cells or infected HeLa cells were

labeled with 300 to 500 μCi of [^{35}S]methionine (Trans ^{35}S-label). Labeling was performed in 1 ml of DMEM without methionine for 4 h. Cells were lysed as previously described (25) in a 1-ml solution of E1A lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 250 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 50 mM sodium fluoride). Immunoprecipitations were performed as previously described (23, 25), using the anti-E1A antibody M58 or M73 (24) or PAb416, which recognizes SV40 large T antigen (22). The XZ panel of monoclonal antibodies were raised against the E1A and T-antigen binding regions of pRB (30). Protein complexes were resolved on a sodium dodecyl sulfate (SDS)–7% polyacrylamide gel (23, 32) and detected by fluorography (2, 23).

Kinase assays. Immune complexes prepared by using unlabeled cell lysates were incubated in a 50-μl reaction mix containing 50 mM HEPES (pH 7.0), 5 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM dithiothreitol, 5 μCi of [γ-^{32}P]ATP, and 50 μg of histone H1 (Boehringer Mannheim) per ml where indicated and incubated for 30 min at room temperature. The reaction was terminated by the addition of 2× sample buffer (32). The phosphorylated products were resolved on an SDS–8% polyacrylamide gel (23, 32) and autoradiographed in the presence of an intensifying screen.

Partial proteolytic mapping. The 107K protein was labeled in vivo with 1 mCi of $^{32}P_i$ for 4 h and immunoprecipitated or labeled in vitro in the immune complex kinase reaction. The proteins were separated on an SDS–6% polyacrylamide gel. Labeled protein was located by autoradiography, excised from the dried gel, and treated with 0.1 or 0.5 μg of *Staphylococcus aureus* V8 protease by the method of Cleveland et al. (7). The partial proteolytic fragments were resolved on an SDS–15% polyacrylamide gel and autoradiographed in the presence of an intensifying screen.

Glycerol gradients. Lysates from five 100-mm-diameter dishes of unlabeled 293 cells or 293 cells labeled with 1 mCi of [^{35}S]methionine per plate were loaded on a 20 to 40% glycerol gradient prepared in 50 mM HEPES (pH 7.0)–250 mM NaCl–5 mM EDTA–0.5% Nonidet P-40. The gradients were centrifuged at 40,000 rpm for 24 h at 4°C in an SW41 rotor; 0.5-ml fractions were collected from each gradient by needle puncture from the bottom of the tube. The fractions were diluted with E1A lysis buffer, split in half, and immunoprecipitated with the anti-E1A antibody M73 or with PAb416 antibody as a negative control. The ^{35}S-labeled proteins and the products of the kinase reaction were resolved on 7 and 8%, respectively, polyacrylamide gels containing SDS. Cold molecular weight standards were run on parallel gradients and detected by Coomassie staining of the SDS-polyacrylamide gel.

Phosphoamino acid analysis. Proteins phosphorylated in the immune complex kinase reaction were detected by autoradiography, excised from the dried gel, and prepared for analysis essentially as described previously (5). The phosphoamino acids were separated by electrophoresis in pH 3.5 buffer (pyridine, glacial acetic acid, H_2O, 10:100:1,890 [vol/vol/vol]) for 1 h at 800 V and visualized by autoradiography. Cold phosphoamino acid standards (5 μg) were run next to the phosphorylated samples and were detected by ninhydrin staining.

Centrifugal elutriation and flow cytometry. Suspension cultures of Retro-3B cells (approximately 3×10^9) were collected by centrifugation and resuspended in 50 ml of elutriation buffer (phosphate-buffered saline, 1% calf serum, 0.1% glucose, 0.3 mM EDTA). The cells were loaded into the elutriation rotor (Beckman JE-10X) at a buffer flow rate of 130 ml/min and at a rotor speed of 1,550 rpm. Fractions were collected beginning at a buffer flow rate of 170 ml/min, with incremental increases of 20 ml/min for the first six fractions and increases of 10 ml/min for the remaining fractions. The elutriated fractions were then concentrated by centrifugation, and 10^7 cells from each fraction were lysed, immunoprecipitated, assayed for kinase activity or labeled in vivo with 1 mCi of [^{35}S]methionine or 1 mCi of $^{32}P_i$ (2×10^6 cells) for 1 h, and immunoprecipitated. Alternatively, the cells from fractions 3 to 5 were pooled (4.5×10^7 cells) and reinoculated into culture, and at 4-h time intervals 1.8×10^6 cells were labeled with 0.5 mCi of [^{35}S]methionine or 1 mCi of $^{32}P_i$ for 1 h, after which lysates were prepared for immunoprecipitation.

A total of 0.9×10^6 to 2×10^6 cells per fraction were fixed and stained for flow cytometry analysis as previously described (5).

RESULTS

Detection of kinase activity associated with E1A. There are a number of cellular proteins that form stable complexes with E1A and that can be detected by immunoprecipitation of [^{35}S]methionine-labeled proteins with an anti-E1A antibody. The most abundant of these associated proteins (Fig. 2A) include the previously identified pRB and cyclin A as well as several polypeptides that are presently known only by their relative molecular masses of 300, 130, and 107 kDa. Although not seen on this exposure, several less abundant proteins are evident on longer exposures of the gel. To investigate the function of the E1A-associated proteins, we looked for an enzymatic activity associated with the E1A complexes. Originally, immune complexes were tested for an associated ATPase activity, but no ATPase activity was observed in these complexes (data not shown). However, in subsequent experiments, a potent protein kinase activity was detected. When the E1A immune complexes from unlabeled 293 cell lysates were incubated in the presence of [γ-^{32}P]ATP and the products of the reaction were analyzed by gel electrophoresis, two proteins that comigrated with the E1A-associated 107K and 130K proteins became highly phosphorylated (Fig. 2A). On long exposures, weak phosphorylation of the 300K, pRB, and E1A proteins was sometimes detected. We do not know whether these activities are due to contaminating kinases or whether they represent very low levels of authentic associated kinases.

In an early series of experiments, the optimal conditions of the kinase reactions were determined, and it was found that phosphorylation of the 107K and 130K proteins was greater in a reaction mix containing solely Mn^{2+} rather than Mg^{2+} but was best when both cations were included in the kinase assay (data not shown). The reaction was optimal in the absence of NaCl and decreased with increasing concentrations of the salt, although some activity was still detectable at 500 mM NaCl. The kinase was active over a fairly narrow range of pH, with the greatest activity at pH 7.0, less activity at pH 7.5 and 8.0, and virtually no activity at pH 6.0 or 9.0. The optimal conditions for this kinase are described in Materials and Methods. While determining the optimal conditions for this reaction, we noted that the associated kinase activity could phosphorylate added histone H1. In many of the subsequent experiments, histone H1 was added as an exogenous substrate.

To determine whether the proteins phosphorylated in the in vitro kinase reaction corresponded to the previously identified E1A-associated 107K and 130K proteins, partial

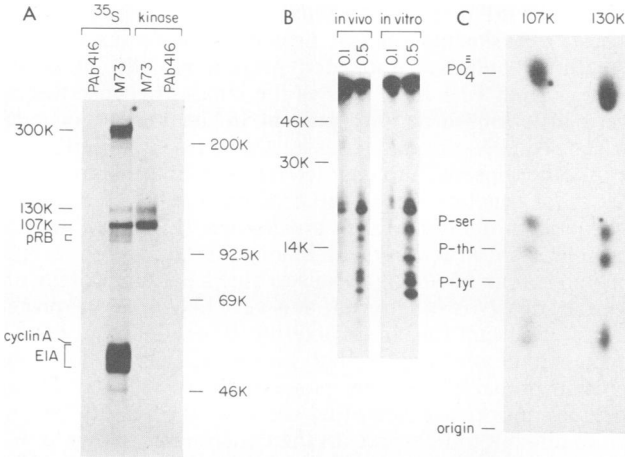


FIG. 2. Phosphorylation of the E1A-associated 107K and 130K proteins in an E1A immune complex kinase reaction. (A) Immunoprecipitation of E1A-containing complexes from 293 cells (which constitutively express E1A). The two lanes on the left show immunoprecipitation from [^{35}S]methionine-labeled 293 cells (0.75 mCi/100-mm dish for 4 h), using PAb416 (negative control antibody) or M73 (anti-E1A antibody). The two lanes on the right show immunoprecipitation with M73 or PAb416 from unlabeled 293 cells followed by incubation in a kinase reaction mix containing [γ-^{32}P]ATP as described in Materials and Methods. Products were separated by electrophoresis on an SDS–7% polyacrylamide gel and detected by fluorography. The positions of migration of E1A and the major complex proteins are indicated at the left, and the sizes of protein molecular weight standards are marked at the right. (B) Partial proteolytic mapping of the 107K protein. The 107K protein labeled in vivo with $^{32}P_i$ or in an in vitro kinase reaction was excised from an SDS–6% polyacrylamide gel, rehydrated, and subjected to digestion with 0.1 or 0.5 μg of *S. aureus* V8 protease as described in Materials and Methods. Digested products were separated by electrophoresis on an SDS–15% polyacrylamide gel. Positions of protein molecular weight standards are shown at the left. (C) Phosphoamino acid analysis. The 107K and 130K proteins labeled during an in vitro kinase reaction were excised from a dried SDS–7% polyacrylamide gel, rehydrated, and hydrolyzed as described in Materials and Methods. The products were separated by one-dimensional thin-layer chromatography. Positions of migration of free phosphate and phosphoamino acid standards are shown at the left. The more slowly migrating spots represent partially hydrolyzed peptides.

proteolytic mapping was performed. The 107K protein bands labeled with $^{32}P_i$ in vivo or labeled with [γ-^{32}P]ATP in vitro were excised from the gel and digested with different concentrations of *S. aureus* V8 protease, an endopeptidase which cleaves after acidic amino acid residues. As seen in Fig. 2B, the patterns of digestion of the 107K protein labeled in vivo and in vitro were similar, although the intensities of several of the partial digested polypeptides varied significantly. We interpret these data to suggest that the in vitro-phosphorylated 107K band is in fact the authentic E1A-associated protein but that the pattern or intensity of the in vitro phosphorylation sites is somewhat different. Similar experiments with the 130K proteins also have demonstrated that this in vitro phosphoprotein is identical to the authentic 130K E1A-associated protein (data not shown).

To determine which amino acids are phosphorylated in vitro by the E1A-associated kinase, we subjected the 107K and 130K proteins to phosphoamino acid analysis. The results in Fig. 2C reveal that these proteins are phosphorylated on serine and threonine residues.

Cell cycle regulation of E1A-associated kinase activity. To determine whether the E1A-associated kinase activity corresponds to changes in the phosphorylation of the 107K protein in vivo, we used the technique of centrifugal elutriation to compare the associated kinase activity throughout the cell cycle. The cell line used for these experiments, Retro-3B, is a derivative of the suspension HeLa cell line S3 which was infected with an amphitropic recombinant retroviral vector that expresses the adenovirus E1A 12S protein (8). The Retro-3B cells express low levels of the E1A protein, and it is difficult to detect in these cells because of its heterogeneous nature; the E1A 12S protein runs as a broad series of bands on a one-dimensional gel and can be seen easily only when expressed at higher levels. We have confirmed by using immunoblots that the E1A 12S protein is expressed in these cells. Nonetheless, 107K can be coprecipitated with the anti-E1A antibody from these cells, while only low levels of the other E1A-associated proteins are detected. We suspect, but have not proven, that 107K can be detected in these immunoprecipitations because it has a higher affinity for E1A than do the other E1A-associated proteins and runs as a discrete band on SDS-polyacrylamide gels.

In asynchronously growing Retro-3B cells, phosphorylation of 107K and histone H1 is readily apparent, but as described above, the 130K phosphorylation is not detected (Fig. 3A). Elutriated fractions were assayed for DNA content by flow cytometry analysis (Fig. 3B) and for kinase activity (Fig. 3A). The flow cytometry data show that the peak of G_1 cells was in fractions 2 and 3 (approximately 95% of cells), the peak of S-phase cells was in fraction 8 (approximately 30% of cells), and the peak of G_2/M cells was in fraction 10 (approximately 65% of cells). As shown in Fig. 3A, there was no detectable kinase activity in G_1 cells. Phosphorylation of 107K and histone H1 occurs as the cells enter S phase.

This experiment was also performed in another way. G_1-enriched cells were obtained by centrifugal elutriation and reinoculated into culture. At 4-h time points, cell lysates were prepared and assayed for kinase activity. The conclusions were the same as for the previous experiment (data not shown). The G_1 cells contained no detectable kinase activity, while phosphorylation of 107K and histone H1 was observed in S and G_2/M cells. Kinase activity was significantly reduced as the cells reentered the G_1 stage of the cell cycle.

If phosphorylation of 107K by the E1A-associated kinase is biologically relevant, one would expect to observe a change in the phosphorylation pattern of the 107K protein in vivo as cells progress through the cell cycle. It was therefore of interest to examine the synthesis and phosphorylation pattern of 107K in vivo. Retro-3B cells were elutriated, and 107K synthesis and phosphorylation were analyzed by using elutriated fractions (data not shown) or G_1-synchronized cells that were reinoculated into culture. At 4-h time intervals, cells were labeled with [^{35}S]methionine (Fig. 4A) or $^{32}P_i$ (Fig. 4B) for 1 h, lysed, and immunoprecipitated with the anti-E1A or negative control antibody (PAb416). Cells were also prepared for flow cytometry analysis (Fig. 4C). In the experiment shown in Fig. 4, G_1-enriched cells (94% of cells) were reinoculated into culture. The peak of cells in S phase was seen at 12 h (47% of cells), and the peak in G_2/M was seen at 16 h (35% of cells); 73% of the cells reentered G_1 at 24 h. The synthesis of the 107K protein, as measured by [^{35}S]methionine incorporation, was relatively constant throughout the cell cycle (Fig. 4A). The phosphorylation

state of the 107K protein, however, varied as cells traversed the cell cycle (Fig. 4B). While only low levels of phosphorylation were detected in G_1-phase cells, elevated levels of phosphorylated 107K are seen in S and G_2/M cells. Similar results were obtained for elutriated fractions (data not shown).

It is possible that the kinase binds to E1A only at certain points during the cell cycle; alternatively, it may be bound to E1A constitutively and its specific activity changes as a function of the cell cycle. Our experiments do not distinguish between these possibilities. It should also be noted in these experiments that we are examining only the population of 107K that is bound to E1A, which may not be representative of the total population of 107K in the cell. Nevertheless, it appears that the pattern of phosphorylation of 107K in vivo correlates with the timing of phosphorylation of 107K by the kinase in vitro, consistent with the suggestion that the kinase activity detected in the E1A immune complex kinase reaction may at least be one of the kinases that phosphorylates 107K in vivo. Since the 107K protein is phosphorylated in vivo in G_1 at low levels and the E1A-associated kinase does not appear to be active in G_1 cells, it is likely that other kinases, acting at different points in the cell cycle, recognize 107K as a substrate in vivo. This view is consistent with the partial proteolytic mapping data shown in Fig. 2B, in which a unique band is present when 107K is phosphorylated in vivo.

Together, these experiments suggest that the E1A-associated kinase activity that phosphorylates the 107K protein is a subset of the in vivo phosphorylation events seen for the native 107K protein. It is not clear why this activity is retained in the E1A complex.

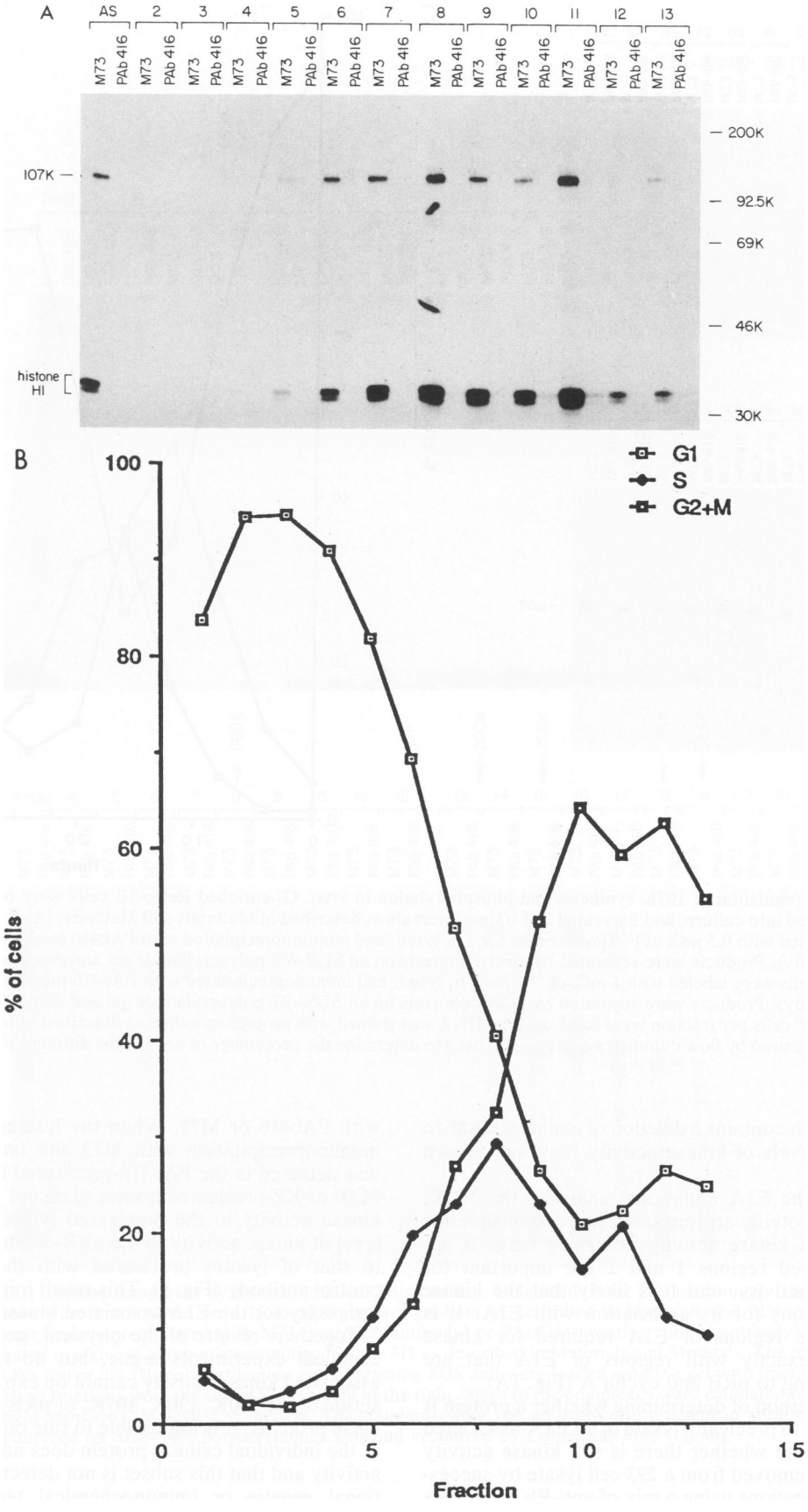
Physical, genetic, and immunochemical characterization of the associated kinase. Of the many proteins that associate with E1A, we wished to identify which protein (or proteins) possessed the kinase activity. We took several approaches to address this issue. One method was to determine the size of the complex that contained kinase activity and compare it with the sizes of the different E1A-associated complexes. Unlabeled and [^{35}S]methionine-labeled 293 cell lysates were run on parallel 20 to 40% glycerol gradients. Fractions were collected and immunoprecipitated with the anti-E1A antibody or the anti-T-antigen antibody, used here as a negative control. Kinase reactions were performed with the unlabeled 293 cell lysate fractions. As shown in Fig. 5A, the majority of the kinase activity, as determined by phosphorylation of 107K, 130K, and histone H1, was present in fractions 12 to 15. In contrast, the fractions containing the peak levels of the 107K and 130K proteins, as evidenced by [^{35}S]methionine labeling of the proteins, ran in fractions 15 to 17 (Fig. 5B). This finding suggests that only a subset of the 107K and 130K proteins bound to E1A can act as substrates for the associated kinase. Also, because the kinase activity sediments more quickly in the gradient than does the bulk of the 107K or 130K protein, these complexes probably represent association with another protein or a major conformational change in the three-dimensional structure of this complex. These experiments do not provide enough resolution to identify a putative associated protein directly. It is also unlikely that E1A itself acts as the kinase, because there is very little kinase activity present in the lower-molecular-weight complexes containing E1A and 107K or 130K.

Another approach to characterize the kinase activity is to use viral genetics. Since the binding regions of the major complex proteins on E1A are known (Fig. 1A), we can correlate kinase activity with binding of the different cellular proteins. If an E1A mutant fails to bind a certain protein, and kinase activity is still present, it is very likely that the protein is not important for kinase activity. If, on the other hand, a protein still binds E1A, but kinase activity is lost, that protein probably does not possess kinase activity. If both binding and kinase activities are lost, the results become more difficult to interpret. In the experiments shown in Fig. 6 and 7, HeLa cells were infected with recombinant adenoviruses expressing different E1A mutants (diagrammed in Fig. 1B) and then assayed for the presence of the E1A-associated proteins by [^{35}S]methionine labeling (Fig. 6A and 7A) or for kinase activity (Fig. 6C and 7B).

Analysis of carboxy-terminal mutants is shown in Fig. 6. Mutant CT*dl*976 binds to all of the major E1A-associated proteins and contains a high level of kinase activity. Mutants CT*dl*961 and -940 no longer bind to the 130K protein but still retain high levels of kinase activity. We infer that 130K binding is not essential for kinase activity. Mutant CT*dl*934, which contains a slightly larger deletion, no longer contains kinase activity, and this mutant does not bind to 107K, pRB, or cyclin A. Although mutant CT*dl*934 expresses significantly lower levels of E1A protein than does the wild-type virus, the level of E1A is comparable to that of the CT*dl*940 mutant (Fig. 6B), which still retains kinase activity, suggesting that lack of kinase activity is not due simply to reduced levels of E1A.

The results using amino-terminal and internal deletion mutants are shown in Fig. 7. The smallest N-terminal deletion mutant, NT*dl*646, abolishes 300K binding but not binding to the other major E1A-associated proteins (Fig. 7A). This mutant retains near-wild-type levels of kinase activity (Fig. 7B), strongly suggesting that the 300K protein is not required for kinase activity. The slightly larger N-terminal mutant, NT*dl*814, loses binding to pRB, cyclin A, and 130K but still binds similar levels of the 107K protein. This mutant no longer contains kinase activity. Similarly, while the internal deletion mutants, *dl*739N and *dl*646N, bind equivalent levels of the 107K protein, mutant *dl*739N is positive for kinase activity whereas mutant *dl*646N is negative. Therefore, consistent with the results in Fig. 5, the 107K protein is unlikely to be a kinase that phosphorylates itself, or it at least needs another protein for activation. Mutant NT*dl*1010 fails to bind to any of the major complex proteins and is negative for kinase activity, while mutant *dl*922/947, which contains an internal deletion of conserved region 2, also does not possess kinase activity. Another

FIG. 3. Cell cycle regulation of kinase activity in vitro. Retro-3B cells were centrifugally elutriated as described in Materials and Methods to obtain populations of cells enriched in various stages of the cell cycle. (A) The protein content of each fraction was determined as described by Bradford (3), and equal amounts of protein per fraction were used for immunoprecipitation with M73 (anti-E1A antibody) or PAb416 (negative control antibody) and subsequent kinase assay. Products were separated by electrophoresis on SDS–8% polyacrylamide gels and detected by autoradiography. (B) A total of 2×10^6 cells per fraction were fixed, and the DNA was stained with propidium iodide as described in Materials and Methods. DNA content was measured by flow cytometry analysis and used to determine the percentage of cells in the different phases of the cell cycle.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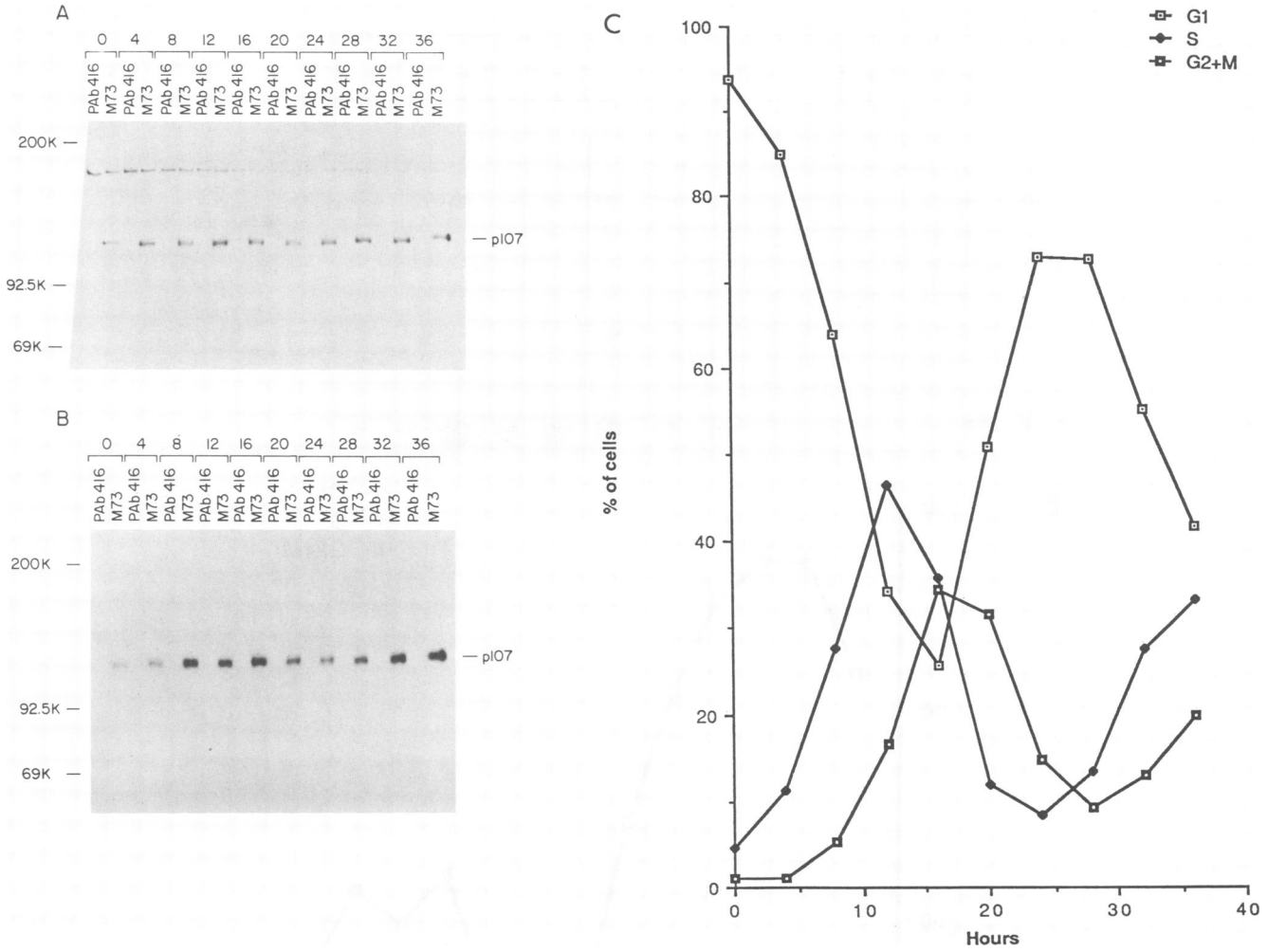

FIG. 4. Cell cycle regulation of 107K synthesis and phosphorylation in vivo. G_1-enriched Retro-3B cells were obtained by centrifugal elutriation, reinoculated into culture, and harvested at 4-h time intervals as described in Materials and Methods. (A) A total of 1.8×10^6 cells per fraction were labeled with 0.5 mCi of [^{35}S]methionine for 1 h, lysed, and immunoprecipitated with PAb416 (negative control antibody) or M73 (anti-E1A antibody). Products were separated by electrophoresis on an SDS–6% polyacrylamide gel and detected by fluorography. (B) A total of 1.8×10^6 cells were labeled with 1 mCi of $^{32}P_i$ for 1 h, lysed, and immunoprecipitated with PAb416 (negative control antibody) or M73 (anti-E1A antibody). Products were separated by electrophoresis on an SDS–6% polyacrylamide gel and detected by autoradiography. (C) A total of 0.9×10^6 cells per fraction were fixed, and the DNA was stained with propidium iodide as described in Materials and Methods. DNA content was measured by flow cytometry analysis and used to determine the percentage of cells in the different phases of the cell cycle.

mutant, NC*dl*, which contains a deletion of amino acids 86 to 120, retains high levels of kinase activity (data not shown [19]).

In summary of the E1A mutational analysis, the 300K, 130K, and 107K proteins are probably not responsible for the E1A-associated kinase activity described here. It appears that conserved regions 1 and 2 are important for associated kinase activity, and it is likely that the kinase requires these regions for its association with E1A. It is interesting that the regions of E1A required for kinase activity coincide exactly with regions of E1A that are necessary for binding to pRB and cyclin A (Fig. 1A).

An alternative method of determining whether a protein is active as a kinase is to preclear a lysate of an E1A-associated protein and determine whether there is still kinase activity present. pRB was removed from a 293 cell lysate by successive immunoprecipitations using a mix of anti-Rb antibodies (XZ series) (30). As a control, lysates were also precleared with PAb416 or M73. When the lysates were analyzed by immunoprecipitation with M73 and immunoblotting, pRB was detected in the PAb416-precleared lysate but not in the M73- or XZ-precleared lysates (data not shown). Analysis of kinase activity in the precleared lysates revealed that the level of kinase activity in the pRB-precleared lysate is equal to that of lysates precleared with the PAb416 negative control antibody (Fig. 8). This result implies that pRB is not necessary for the E1A-associated kinase activity.

Together, results of the physical, genetic, and immunochemical experiments argue, but do not prove, that the associated kinase activity cannot be explained by the simple action of the 300K, 130K, 107K, or pRB protein. For each of these proteins, it is impossible to rule out that a small subset of the individual cellular protein does not carry the catalytic activity and that this subset is not detectable by the conventional genetic or immunochemical techniques described here. However, two other, more likely possibilities exist.

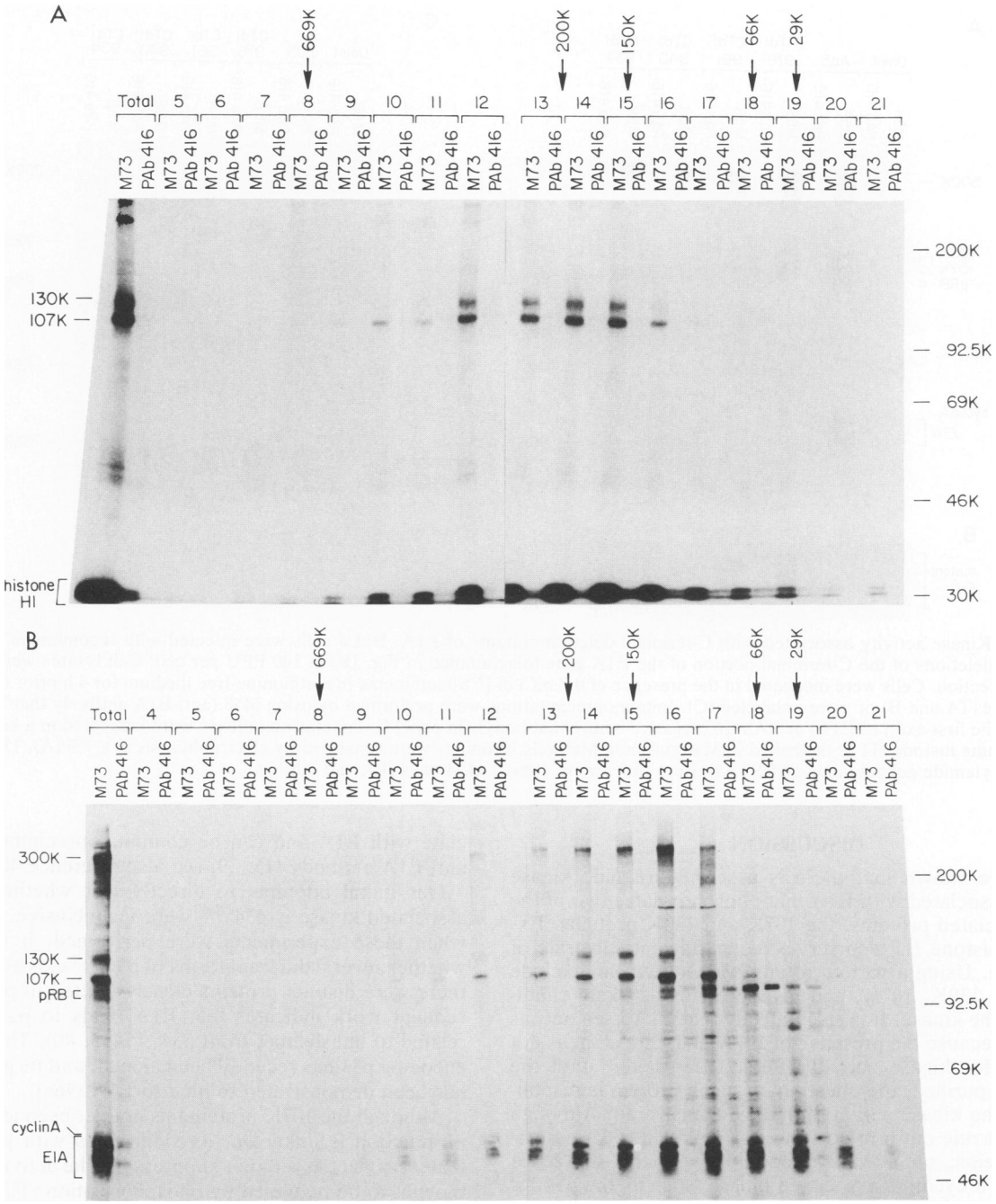

FIG. 5. Sizing of E1A immune complexes by glycerol gradient fractionation. Unlabeled (A) or [^{35}S]methionine-labeled (B) 293 cell lysates from five 100-mm dishes were fractionated on a 15 to 35% glycerol gradient as described in Materials and Methods. Total lysate or the individual fractions were immunoprecipitated with M73 (anti-E1A antibody) or PAb416 (negative control antibody). For panel A, immune complexes were incubated in a kinase assay mix containing histone H1 as described in Materials and Methods. The products were separated by electrophoresis on 8% (A) or 7% (B) polyacrylamide gels containing SDS and were loaded onto the gel with fractions from the bottom of the gradient at the left and fractions from the top of the gradient at the right. Sizes of the complexes were estimated by comparison with cold protein molecular weight standards, indicated at the top. The standards were run on a glycerol gradient in parallel, and aliquots of each fraction were electrophoresed on SDS–10% polyacrylamide gels and visualized by Coomassie blue staining.

The first is that the kinase activity is carried by or is bound to the E1A-associated cyclin A protein. The binding sites on E1A for cyclin A have recently been determined (20) and correspond to the regions needed for detecting the kinase activity. An excellent candidate for this kinase would be the human homolog of the product of the *cdc2* cell cycle gene. This kinase is known to associate with cyclin A and to be regulated in a cell cycle manner. We are currently testing this hypothesis. The other possibility is that the kinase is in fact another associated protein, as yet unidentified or uncharacterized, and that this protein carries the catalytic activity detected here.

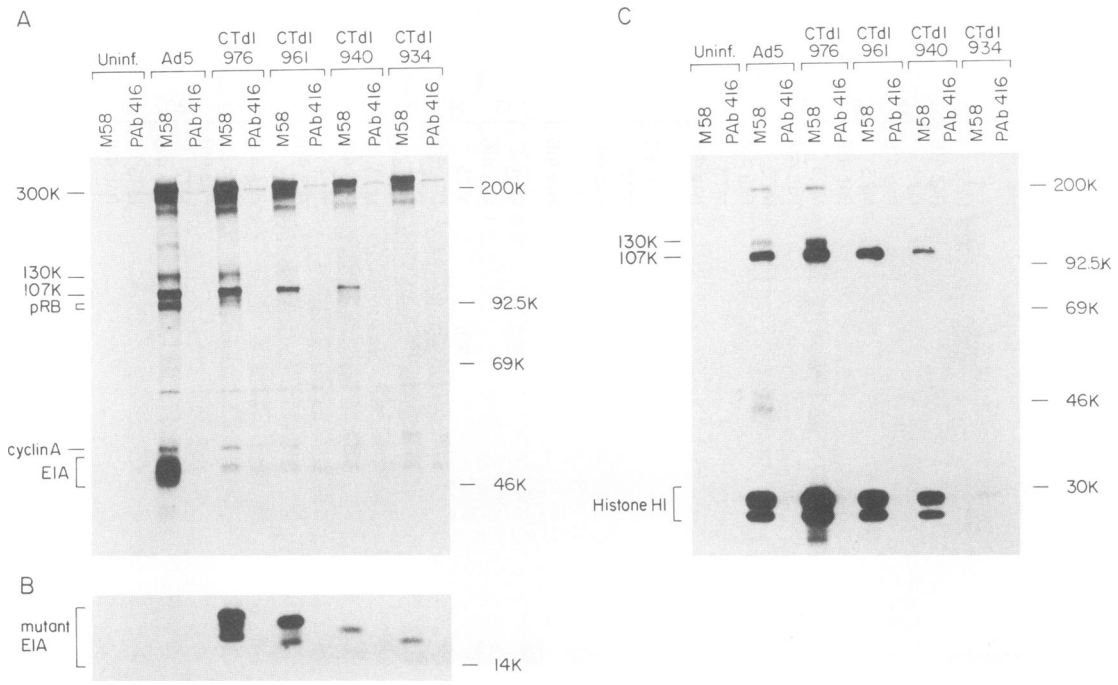

FIG. 6. Kinase activity associated with C-terminal deletion mutants of E1A. HeLa cells were infected with recombinant adenoviruses containing deletions of the C-terminal portion of the E1A gene (diagrammed in Fig. 1B) at 100 PFU per cell. Cell lysates were prepared at 18 h postinfection. Cells were incubated in the presence of 0.8 mCi of [^{35}S]methionine in methionine-free medium for 4 h prior to preparation of the lysates (A and B) or were unlabeled (C). Immunoprecipitations were performed by using M58 (anti-E1A antibody that recognizes an epitope in the first exon of E1A) or PAb416 (negative control antibody). In panel C, immune complexes were incubated in a kinase reaction mix containing histone H1 as described in Materials and Methods. Products were separated by electrophoresis in a 7% (A), 15% (B), or 8% (C) polyacrylamide gel containing SDS. Exposure times were 1 day (A), 4 days (B), and 3 h (C).

DISCUSSION

We have shown that there is a serine/threonine kinase activity associated with E1A that phosphorylates two of the E1A-associated proteins, the 107K and 130K proteins. Exogenous histone H1 also serves as an efficient substrate of the kinase. Using a variety of approaches, we can exclude the 300K, 130K, 107K, and pRB proteins as likely candidates for the kinase. It is also unlikely that E1A itself acts as a kinase because the presence of 107K and E1A alone is not sufficient for kinase activity (Fig. 5). However, until the activity is purified, the question of which protein is responsible for the kinase activity remains unanswered. Although we cannot rule out a role of the less abundant E1A-associated proteins, an attractive possibility of the source of activity is a cyclin A-associated kinase, such as the p34^{cdc2} kinase.

Several lines of evidence are consistent with a role for the cyclin A-p34^{cdc2} kinase in the activity described here. First, both the E1A-associated kinase and cyclin A-p34^{cdc2} recognize histone H1 as a substrate. The E1A-associated kinase also phosphorylates a peptide derived from pRB (data not shown) that has been shown to be phosphorylated in vitro by p34^{cdc2} and contains a site phosphorylated in vivo (34). Second, the genetic analysis indicates that the regions of E1A that are necessary for kinase activity coincide with the regions of E1A that are important for binding to cyclin A (20). Third, the timing of activation of the E1A-associated kinase during the cell cycle is identical to that of the cyclin A-p34^{cdc2} kinase (Fig. 3 and data not shown [18]). Probably most convincing is the recent work demonstrating that a subset of p34^{cdc2} isoforms or closely related proteins associate with E1A and can be coimmunoprecipitated with an anti-E1A antibody (15, 19; see also reference 40).

Our initial attempts to directly test whether the E1A-associated kinase is p34^{cdc2} were inconclusive. At the time when these experiments were performed, it was unclear whether several different forms of p34^{cdc2} existed or whether there were distinct proteins closely related to p34^{cdc2}. Subsequent work indicates that E1A binds to p33, a protein related to but distinct from p34^{cdc2} (15, 40). The *cdk2* gene encoding p33 has recently been cloned, and its gene product has been demonstrated to bind to E1A (46a).

Although the 107K protein has not yet been identified and its function is unknown, its similarities with pRB suggest that it may act as a tumor suppressor. The activity of pRB is thought to be regulated by phosphorylation. The phosphorylation state of pRB is cell cycle dependent (5, 6, 10), and while E1A binds to pRB in its under- and hyperphosphorylated states, SV40 large T antigen associates only with the underphosphorylated form of pRB (36), suggesting that SV40 large T antigen may target the active form of pRB. By analogy with pRB, it is tempting to speculate that phosphorylation of the 107K protein by the E1A-associated kinase may regulate the function of the 107K protein during the cell cycle.

Since the presence of kinase activity correlates with regions of E1A that are necessary (although not sufficient) for transformation by E1A, the association of E1A with a cell cycle-regulated kinase may provide a function important for E1A-mediated transformation. E1A may affect the action of the kinase, perhaps by altering its conformation, stability,

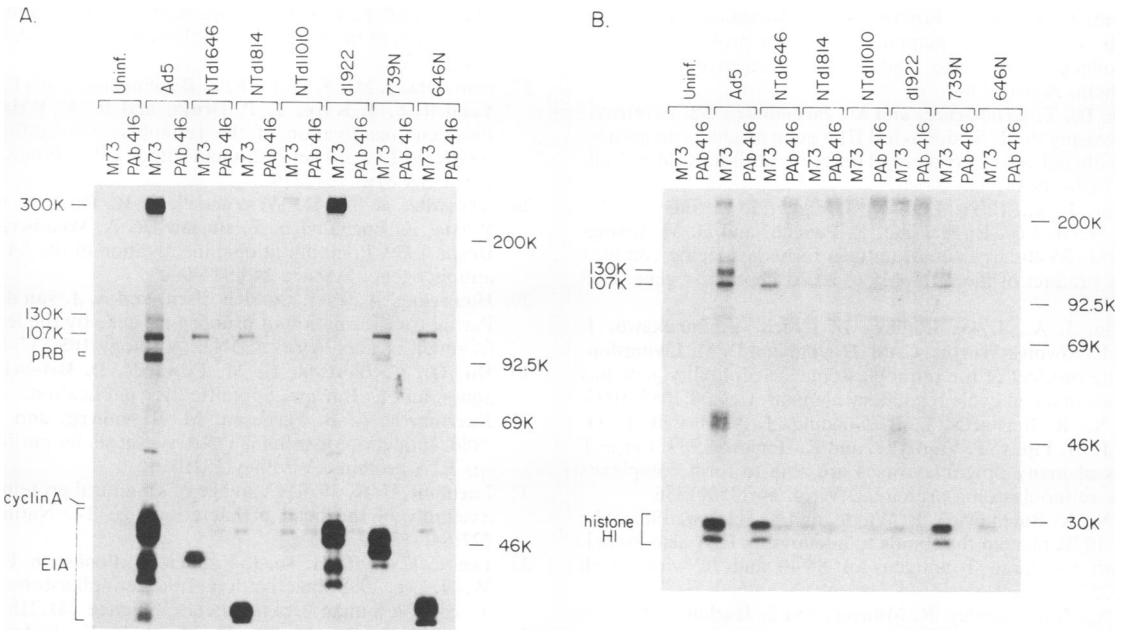

FIG. 7. Kinase activity associated with N-terminal and internal deletion mutants of E1A. HeLa cells were infected with recombinant adenoviruses containing internal deletions or deletions of the N-terminal portion of the E1A gene (diagrammed in Fig. 1B) at 100 PFU per cell. Cell lysates were prepared at 18 h postinfection. Cells were incubated in the presence of 0.3 mCi of [^{35}S]methionine in methionine-free medium for 4 h prior to preparation of the lysates (A) or were unlabeled (B). Immunoprecipitations were performed by using M73 (anti-E1A antibody that recognizes an epitope in the second exon of E1A) or PAb416 (negative control antibody). In panel B, immune complexes were incubated in a kinase reaction mix containing histone H1 as described in Materials and Methods. Products were separated by electrophoresis in a 7% (A) or 8% (B) polyacrylamide gel containing SDS. Exposure times were 24 h (A) and 8 h (B).

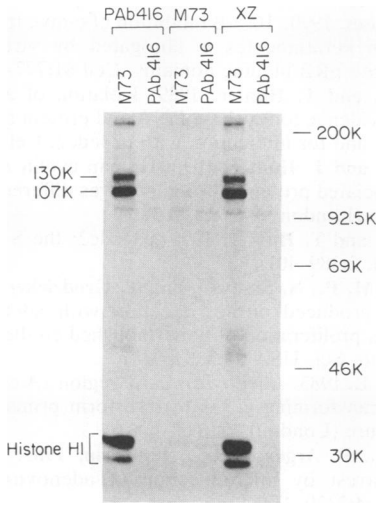

FIG. 8. Preclearing of pRB from a 293 cell lysate. A 293 cell lysate was precleared of pRB by successive immunoprecipitations using a mix of the XZ set of antibodies (anti-Rb antibodies which recognize epitopes in the C-terminal portion of pRB, including the E1A and T-antigen binding regions [30]) until no pRB was detectable by immunoblotting. As controls, lysates were precleared in parallel with M73 (anti-E1A antibody) or PAb416 (negative control antibody) as indicated at the top. The precleared lysates were then immunoprecipitated with M73 or PAb416, and the immune complexes were incubated in a kinase reaction mix containing histone H1.

or subcellular localization. Work is in progress to test these changes.

ACKNOWLEDGMENTS

We thank Peter Whyte and Karen Buchkovich for significant contributions in the early stages of this work. We also thank members of the laboratory for helpful discussions, especially Nick Dyson, Barbara Faha, Antonio Giordano, and Qianjin Hu. We are grateful to Carol McCall for providing the Retro-3B cells and virus stocks and to Pat Burfeind for flow cytometry analysis and technical assistance. We also thank Jim Duffy, Mike Ockler, and Phil Renna for artwork and photography.

This work was supported by NIH grant 13106 (E.H.), NIH training grant CA09311 (C.H.H.), and Damon Runyon-Walter Winchell Cancer Research Fund fellowship DRG-1058 (L.-K.S.).

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