JOURNAL OF VIROLOGY, June 1988, p. 1948–1955
0022-538X/88/061948-08$02.00/0

Vol. 62, No. 6

Heterogeneity of Adenovirus Type 5 E1A Proteins: Multiple Serine Phosphorylations Induce Slow-Migrating Electrophoretic Variants but Do Not Affect E1A-Induced Transcriptional Activation or Transformation

J. D. RICHTER,[1]* J. M. SLAVICEK,[1] J. F. SCHNEIDER,[2] AND N. C. JONES[2]

Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545,[1] and Gene Regulation Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England[2]

Received 4 November 1987/Accepted 22 February 1988

The 289-amino-acid product encoded by the adenovirus E1A 13S mRNA has several pleiotropic activities, including transcriptional activation, transcriptional repression, and when acting in concert with certain oncogene products, cell transformation. In all cell types in which E1A has been introduced (except bacteria), E1A protein is extensively posttranslationally modified to yield several isoelectric and molecular weight variants. The most striking variant is one that has a retarded mobility, by about M_r = 2,000, in sodium dodecyl sulfate gels. We have investigated the nature of this modification and have assessed its importance for E1A activity. Phosphorylation is responsible for the altered mobility of E1A, since acid phosphatase treatment eliminates the higher apparent molecular weight products. By using several E1A deletion mutants, we show that at least two seryl residues, residing between residues 86 and 120 and 224 and 289, are the sites of phosphorylation and that each phosphorylation can independently induce the mobility shift. However, E1A mutants lacking these seryl residues transcriptionally activate the adenovirus E3 and E2A promoters and transform baby rat kidney cells to near wild-type levels.

The proteins encoded by the early region 1A (E1A) of the human adenoviruses play a central role in the ability of the virus to replicate efficiently and to transform certain primary cells. The major role of the E1A proteins in viral replication is to transcriptionally activate the viral early genes (2, 3, 12; for a review, see reference 20), ensuring efficient synthesis of the early products that are necessary for DNA replication. This activity does not seem to be important for transformation, however, since mutants have been isolated that can transcriptionally activate but not transform. Conversely, other mutants have been shown to transform but not activate transcription (8, 15, 25). A second regulatory activity of the E1A proteins is transcriptional repression, specifically those genes controlled by certain *cis*-acting enhancer elements; the E1A proteins appear to interfere with enhancer-mediated transcriptional activation (5, 11, 34). This activity may play an important role in transformation, since many mutants that have lost the ability to transform primary cells have also lost the ability to repress enhancers (15, 25). The mechanisms by which these proteins regulate gene expression is still obscure, although the available evidence suggests that these proteins may modulate the activity of cellular transcriptional factors (for a review, see reference 20). No enzymatic activity associated with the proteins has so far been described.

At early times of infection, two mRNAs are synthesized from the E1A region, 12S and 13S in size, that arise from a common precursor by differential RNA splicing (4, 7, 14, 22). These messages encode two related proteins of 243 and 289 amino acids, respectively, that are identical, except for an additional 46 amino acids encoded by the 13S message. The primary translation products of the 12S and 13S mRNAs migrate with apparent molecular masses of approximately 42 and 45 kilodaltons (kDa), respectively, which are significantly different from the molecular masses (26 and 32 kDa) deduced from the known primary sequences. These aberrant migrations in sodium dodecyl sulfate (SDS)-polyacrylamide gels are probably due to the unusually high content of proline and acidic residues. A number of studies have shown that, in vivo, the translation of each message gives rise to a heterogeneous set of products (10, 24, 36). In high-resolution two-dimensional gels, the products of the 12S and 13S messages have been resolved into approximately 60 polypeptide species (10). Pulse-chase experiments have shown that this heterogeneity is due to posttranslational modifications that result in species that are more acidic, and in some cases, slower migrating than the primary translation product (31). In one-dimensional SDS gels, the pattern of E1A mobility is greatly simplified, with two forms of product from each message being resolved. The two forms differ in apparent molecular mass by approximately 2 kDa, the faster-migrating species having the same mobility as the primary translation product. What is the nature of the posttranslational modifications that give rise to this heterogeneity? The only known modification of E1A is multiple serine phosphorylation (16, 17, 30, 32, 36), suggesting that the heterogeneity could be caused by differential phosphorylation.

We have been investigating the nature and functional significance of the E1A posttranslational modifications in greater detail. In particular, we have concentrated on the modifications that give rise to the increase in apparent molecular weight that is readily detected in one-dimensional gels. It appears that phosphorylation is the sole type of modification present, since the heterogeneity of the E1A variants can be removed by treatment with potato acid phosphatase. These results suggest that the prominent migratory shift must be due to one or more specific phosphorylations that somehow induce a conformational change in the

* Corresponding author.

protein. As a result of this change, mobility in SDS gels is retarded. We show in this report that phosphorylation in one of two different regions of the protein can independently result in this conformational change. The biological activities of many proteins are modulated by phosphorylation. However, this does not appear to be the case with the phosphorylations that give rise to the migratory shift of E1A, since neither of the regions involved is critical for E1A to transcriptionally activate the viral early promoter or transform baby rat kidney cells.

MATERIALS AND METHODS

Plasmid constructions. The plasmid Sp13s contains the E1A 13S coding sequence in the Sp6 promoter-containing vector Sp65pA (a gift from A. Jacobson). The *Sac*II-*Hpa*I fragment from JN20 (9), containing the entire 13S coding sequence, the 5'-noncoding sequence, and some 5'-nontranscribed sequence, was cloned into the *Sma*I site of Sp65pA. During this construction, the *Sac*II site was recreated. The plasmids SpX, SpXX, and SpNX were all derived from Sp13s. For the construction of SpX, the Sp13s plasmid was cleaved with *Xba*I, which cut once within the E1A coding sequence and once in the polylinker sequence 3' to the E1A insert. The ends generated by this digestion were filled in with DNA polymerase, and the treated DNA was subsequently religated. This resulted in deletion of the E1A sequence downstream of nucleotide 1340 and an insertion at the point of the deletion of an in-frame TAG termination codon. Similarly, SpXX was constructed by cleaving Sp13s DNA with *Xma*I and *Xba*I, filling in the ends and religating; SpNX was constructed by cleaving with *Nae*I and *Xba*I before filling-in the ends and religating. The SpRX plasmid was constructed from an intermediate plasmid SpXL3, which contains the *Sac*II-*Hpa*I fragment from the linker-scanning mutant pSVXL3 (28) (gift from E. Ziff) inserted into Sp65A. The plasmid SpXL3 was then cleaved with *Eco*RI and *Xho*I to remove all the E1A sequences upstream of nucleotide position 750, the ends were filled in, and the resulting DNA was religated. For the construction of Sp105NX, the E1A containing the *Pst*I-*Eco*RI fragment of pSVXLI05 (28) (gift from E. Ziff) was first moved to pUC8 and cut at *Nae*I at position 815 of the E1A sequence, and a *Bgl*II linker was added (10-mer). This intermediate was then cut with *Xho*I and *Bgl*II, filled in, and religated. The *Sst*II-*Hpa*I fragment of the resulting plasmid was then inserted into Sp65pA to give Sp105NX. The plasmid SpNC was constructed by removing base pairs between the *Nae*I (nucleotide 813) and filled-in *Cla*I sites of pJN20 (9). The *Sst*II-*Hpa*I fragment of this intermediate was cloned into Sp65pA. An intermediate plasmid containing the deletion present in SpCS was first constructed by cutting pPM957 (25) with *Cla*I and *Sal*I, filling in the ends, and religating. The *Sst*II-*Xma*I fragment from this intermediate was then cloned into the *Sst*II-*Xma*I sites of Sp13s, where it replaced the wild-type sequence. The plasmids Sp5/3X and SpNCX were constructed by digesting Sp5/3 and SpNC DNAs with *Xba*I, filling in, and religating.

The plasmids pCE, GCE-R, and GNC have been described previously (25). GNC-R was constructed from GNC, by digesting with *Xba*I, filling in with a Klenow fragment, and religating.

In vitro transcription and translation. In vitro transcription reactions were carried out essentially as described by Melton et al. (18). Briefly, DNA templates (1 mg/ml), linearized at the *Hin*dIII site, were transcribed in a solution consisting of 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 500 μM of UTP, ATP, and CTP, 50 μM GTP, 500 μM GpppG, 10 mM dithiothreitol, and RNAsin (0.5 U/μl). A typical reaction volume was 50 μl, 1 U of SP6 RNA polymerase was added per microgram of DNA template, and incubation proceeded at 37°C for 1 h. Following synthesis, the DNA template was digested with RNase-free DNase (50 μg/ml). After 15 min at 37°C, the RNA was extracted with phenol-chloroform and precipitated by the addition of potassium acetate to 0.5 M and 2 volumes of ethanol.

Translation of the RNA was in a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega Biotec). Typically, approximately 1 μg of in vitro-synthesized RNA was added to 35 μl of nuclease-treated lysate, together with a mixture containing each of the amino acids at 50 μM (except methionine) and [^{35}S]methionine at 10 μCi/ml. Incubation was at 30°C for various lengths of time. Samples were removed from the translation reaction, mixed with SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels.

Potato acid phosphatase treatment of proteins. Samples from an in vitro translation reaction were diluted in 5× PEM (0.5 M PIPES [piperazine-*N*, *N'*-bis (2-ethanesulfonic acid] [pH 6.6], 5 mM EGTA [ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*,*N'*-tetraacetic acid], 5 mM $MgSO_4$). Potato acid phosphatase (Sigma Ltd.) was added at various concentrations, and incubation proceeded at 37°C for 15 min. The samples were then processed for analysis by SDS-PAGE.

Inhibition of phosphatase. Reticulocyte-synthesized [^{35}S]-methionine-labeled E1A (both modified and unmodified) was incubated with 5 U of potato acid phosphatase plus 50 mM sodium phosphate. Following a 1-h incubation at 30°C, the products were boiled in SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Rephosphorylation. [^{35}S]methionine-labeled reticulocyte-synthesized E1A was dephosphorylated with potato acid phosphatase. E1A was then immunoselected with biotinylated anti-E1A and Streptavidin and eluted with 0.2 M glycine (pH 2.5). The eluted material (about 5% of the counts per minute originally bound by the beads) was then added to 50 μl of fresh reticulocyte lysate supplemented with all amino acids. This mixture was incubated for 1 h at 30°C, and an aliquot was removed for analysis by SDS-PAGE and fluorography.

Two-dimensional gel analysis of E1A. HeLa cells (3×10^5 cells per 100-mm-diameter dish) were infected with adenovirus type 5 at a multiplicity of infection of 40 and cultured for 16 h in Dulbecco modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum. The cells were then washed with methionine-free DME with 10% fetal bovine serum. The cells were cultured for an additional 1.5 h in 5 ml of 50-μCi [^{35}S]methionine (specific activity, >1,000 Ci/mmol; Amersham Corp.) in methionine-free DME with 10% fetal bovine serum, centrifuged, lysed in RIPA (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS) and the E1A protein was immunoprecipitated with rabbit anti-E1A immunoglobulin G and protein A-Sepharose essentially as described previously (6). The Sepharose beads were suspended in lysis buffer (9.5 M urea, 2% Nonidet P-40, 2% ampholines [pH 3 through 10], and 5% β-mercaptoethanol), and E1A was resolved by isoelectric focusing in the first dimension and SDS-PAGE (10% polyacrylamide) in the second dimension (21). The E1A spots were visualized by fluorography. Wild-type E1A proteins synthesized in a rabbit reticulocyte lysate were resolved in an identical manner.

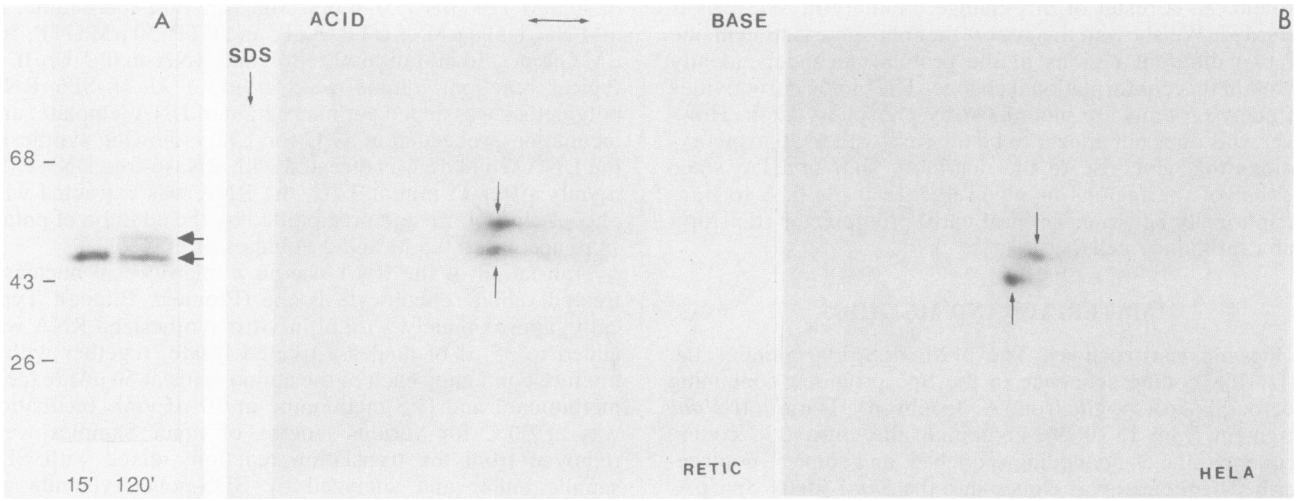

FIG. 1. (A) Modification of the E1A 13S product in a rabbit reticulocyte lysate. In vitro-synthesized 13S RNA was translated in a rabbit reticulocyte lysate for either 15 or 120 min. The radiolabeled proteins were analyzed by SDS-PAGE and autoradiography. The arrows denote the 45- and 47-kDa forms of the E1A protein. (B) Two-dimensional gel analysis of E1A proteins. [^{35}S]methionine-labeled E1A was immunoprecipitated from adenovirus type 5-infected HeLa cells (HELA) and from a reticulocyte lysate (RETIC) primed with Sp6-derived 13S E1A mRNA and resolved by two-dimensional gel electrophoresis. The first isoelectric dimension and the second SDS dimension are denoted. The arrows denote the 45- and 47-kDa forms of E1A.

Oocyte microinjection. Defolliculated stage 6 *Xenopus laevis* oocytes were injected with 30 ng of Sp6-derived mRNA and incubated for 2.5 h in Barth medium at 19°C. The oocytes were subsequently injected with [^{35}S]methionine (0.3 pmol, 1,100 Ci/mol; Amersham) and incubated for 1 h. Some oocytes were injected a second time with radioinert methionine (3,000 pmol) and incubated for an additional 3 h. All oocytes were homogenized in Barth medium plus 0.1% Triton X-100, the yolk was removed by centrifugation, and the supernatant was adjusted to 1× RIPA buffer. The samples were boiled briefly and microfuged, and E1A antiserum generated in rabbits was added to the supernatant. Following 1 to 2 h of incubation, protein A-Sepharose was added, the mixture was incubated for 1 to 2 h, the beads were washed with RIPA buffer, and then E1A was eluted from the beads in SDS gel loading buffer.

Transcriptional activation and transformation assays. Transcriptional activation of the E2ACAT gene (19) and the E3CAT gene (KCAT23; 35) and transformation of primary baby rat kidney cells in cooperation with the activated *ras* gene was measured essentially as described by Schneider et al. (25). HeLa cells were transfected with 5 μg of the chloramphenicol transferase (CAT) gene-containing plasmid plus or minus 10 μg of the E1A-containing plasmid. The cells were harvested 48 h after transfection and assayed by the method of Sleigh (27). In this assay, chloramphenicol transferase enzyme activity is measured by the transfer of ^{14}C-labeled acetyl groups from labeled acetyl coenzyme A to unlabeled chloramphenicol. For the transformation assay, primary baby rat kidney cells (20 to 40% confluent, 60-mm-diameter dish) were transfected with 3 μg of E1A plasmid, 3 μg of EJ6.6 plasmid (26), which contains the H-*ras* oncogene from the EJ/T24 human bladder carcinoma cell line, and 4 μg of pSV2neo (29). On the next day, the cells were passed onto 100-mm-diameter dishes and G418 (200 μg/ml) was added. Colonies were fixed, stained, and counted 3 to 4 weeks later.

RESULTS

Phosphatase treatment of modified E1A protein. To investigate the possibility that the majority or even all the different E1A species result from differential phosphorylation of the primary translation product, we have analyzed the effect of treating modified E1A protein with phosphatase enzymes.

Since the rabbit reticulocyte lysate translates E1A mRNA and modifies the protein product, we have used it as a convenient source of modified protein. The E1A 13S coding sequence was inserted behind the bacteriophage Sp6 promoter (18) in the vector SP65pA (a gift from A. Jacobson). This vector contains not only the Sp6 promoter, followed by a multiple cloning site region, but also a stretch of 46 T residues that are immediately followed by a *Hin*dIII restriction endonuclease cleavage site. The E1A coding sequence was inserted into the multiple cloning region in the orientation that would give the coding strand following transcription from the Sp6 promoter. Before transcription, the vector was cleaved with *Hin*dIII, resulting in a poly(A) tail at the 3′ end of the transcript. The transcription was also carried out in the presence of the cap analog GpppG to ensure that the final message could be efficiently translated in a rabbit reticulocyte lysate and injected *Xenopus* oocytes. Translation of the message for 10 to 15 min gave predominantly a single species of E1A protein that migrated with an apparent size of 45 kDa (Fig. 1A). However, continuation of the translation for 1 to 2 h resulted in a second species, which migrated at 47 kDa. The two species had migrations on two-dimensional gels similar to the predominant species seen in infected HeLa cells (Fig. 1B). It appears therefore that the reticulocyte lysate is capable of efficiently modifying the newly synthesized protein. Modification need not necessarily be linked to translation, however, since the incubation of bacterially synthesized E1A protein (which is unmodified; 45 kDa) in a reticulocyte lysate also results in a new species that migrates at 47 kDa (23).

We initially treated the E1A-containing lysates with a variety of agents that could cleave modifications from the primary protein product. Following alkaline phosphatase treatment, it was clear that some conversion of the 47-kDa species (modified form) to the 45-kDa species (primary translation product) did take place, although it never exceeded more than 50%. However, treatment of the lysates

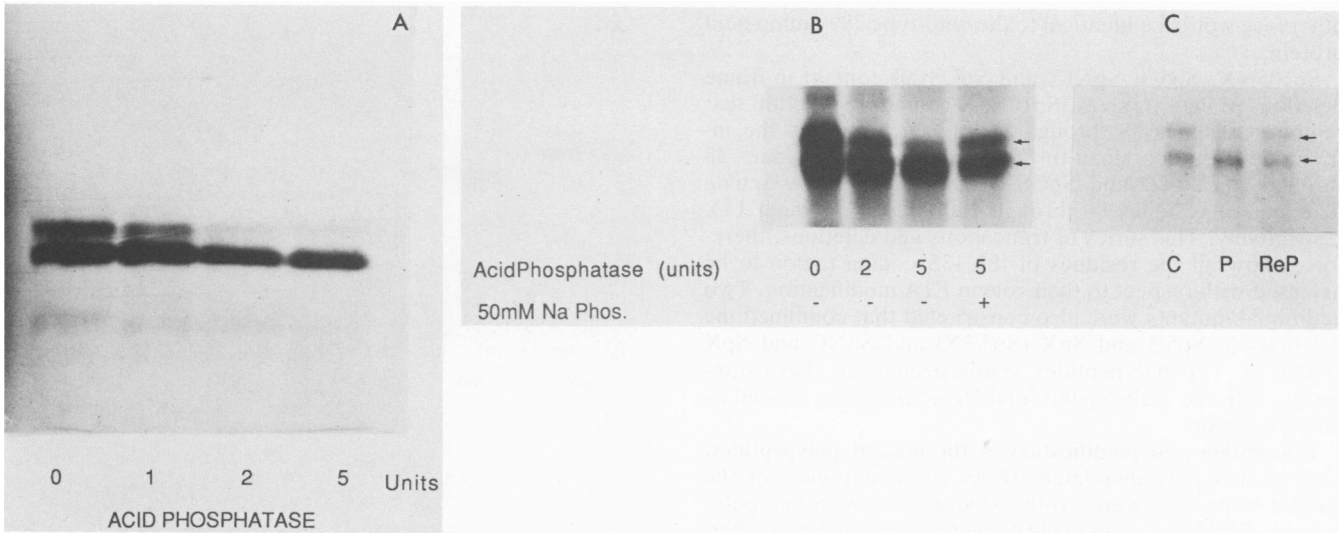

FIG. 2. (A) Potato acid phosphatase treatment of reticulocyte-synthesized E1A 13S mRNA-encoded protein. 13S RNA was translated in a rabbit retinculocyte lysate for 120 min. Aliquots of the lysate were subsequently incubated with 0, 1, 2, or 5 U of potato acid phosphatase at 37°C for 15 min. The phosphatased samples were then analyzed by SDS-PAGE. (B) Inhibition of dephosphorylation by sodium phosphate. Reticulocyte-synthesized and phosphorylated E1A was incubated with 2 or 5 U of potato acid phosphatase or 5 U of potato acid phosphatase plus 50 mM sodium phosphate (Na Phos.). The mixtures were then incubated at 30°C for 1 h, and the products were analyzed as in panel A. (C) Rephosphorylation of E1A. Reticulocyte-synthesized and phosphorylated E1A (lane C) was immunoprecipitated, eluted, and treated with potato acid phosphatase (lane P). A 50-μl quantity of this treated material was then added to a fresh reticulocyte lysate. This mixture was then incubated at 30°C for 1 h before the reaction was terminated by the addition of SDS sample buffer (rephosphorylation [ReP]). The products were analyzed as in panel A. The arrows denote the 45- and 47-kDa forms of the E1A protein.

with increasing amounts of potato acid phosphatase for 15 min resulted in more than 95% of the 47-kDa forms being converted to 45 kDa (Fig. 2A). The conversion of the 47-kDa species to the 45-kDa species could be blocked by adding an excess of sodium phosphate (Fig. 2B). In addition, the dephosphorylated protein (P) could be subsequently remodified (ReP) by incubating with rabbit reticulocyte lysate (Fig. 2C). These results demonstrate that the slower-migrating forms of the E1A protein are predominantly due to protein phosphorylation.

Sp6 vectors containing E1A mutant sequences. The results of the phosphatase experiments suggest that phosphorylation of one or more sites on the E1A protein can result in a conformational change, as manifested by a slower migration in SDS gels. Such a conformational change may be crucial to the biological activity of E1A. To test this possibility, we attempted to identify the regions of E1A containing the residue(s) whose phosphorylation resulted in slower migration. Previous studies involving the microinjection of bacterially synthesized wild-type or mutant E1A proteins into *Xenopus* oocytes suggested that the critical region was located in exon 1, between amino acids 23 and 120 (23). To locate the region more precisely, we introduced several small in-frame deletions or truncations into the E1A sequences present in the Sp6 vector. Following in vitro transcription and translation, the effects of the mutations on E1A modification were assessed.

The different mutants analyzed in this study are shown in Fig. 3. SpX, SpXX, and SpNX all contain deletions at the 3′ end of the coding region and give rise to truncated polypeptides of 223, 150, and 85 amino acids, respectively. Conversely, SpRX has 190 base pairs of the 5′-coding region deleted, thus removing the normal ATG. The next 5′ ATG begins at amino acid 71, which is in-frame with the normal ATG. Thus, the predicted polypeptide from this construction would lack the amino-terminal 70 amino acids but

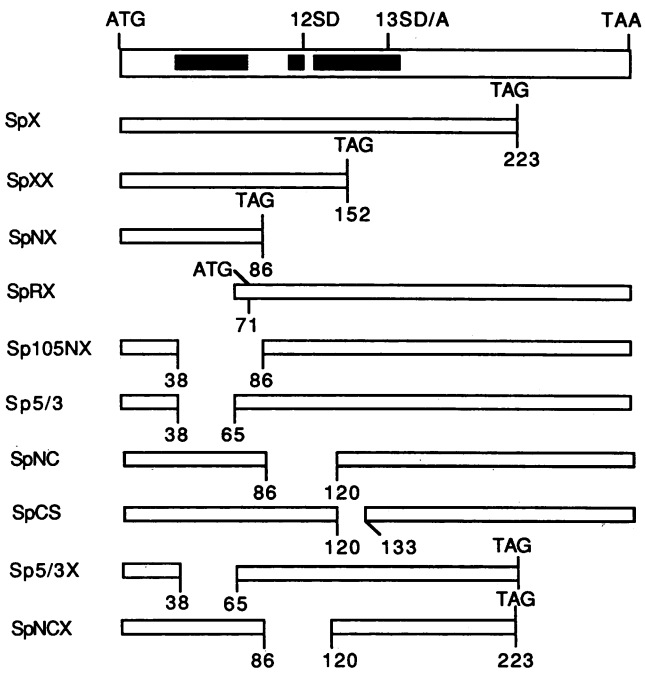

FIG. 3. Description of the mutant E1A genes used in this study. The open box at the top of the figure represents the 13S cDNA sequence that encodes the E1A 289-amino-acid product. The relative positions of the 12S donor (D) and 13S donor/acceptor (D/A) splice junctions are indicated, together with the three regions of the coding sequence highly conserved between the human adenovirus type 5, 7, and 12 serotypes (shaded boxes). Below are indicated the nature of the alterations in each of the mutants studied. The bar denotes the predicted products, and deleted residues are denoted by gaps. All the mutant E1A sequences are present in the vector Sp65pA (see Materials and Methods).

otherwise would be identical to the wild-type 289-amino-acid protein.

Sp105NX, Sp5/3, SpNC, and SpCS all contain in-frame deletions of various sizes. Sp105NX contains a deletion that removes residues 38 through 86 and encompasses the in-frame deletion contained in Sp5/3, which lacks residues 38 through 65. SpNC and SpCS contain the nonoverlapping deletions of residues 86 through 120 and 121 through 133, respectively. This series of truncations and deletions, therefore, allow all the residues of the 13S coding region to be assessed with respect to their role in E1A modification. Two additional mutants were also constructed that combined the deletions in Sp5/3 and SpX (Sp5/3X) and SpNC and SpX (SpNCX). The polypeptides synthesized from these transcripts lack the carboxy-terminal 66 amino acids, as well as internal regions.

Translation and modification of the mutant polypeptides. Capped and polyadenylated RNAs containing each of the mutant sequences were synthesized in vitro with Sp6 polymerase. The RNAs were subsequently translated in a rabbit reticulocyte lysate, and the protein products were resolved by SDS-PAGE and autoradiography. In most cases, the majority of the primary translation products were synthesized during the initial 15 min of incubation. E1A protein was modified during the subsequent 15- to 120-min period. The degree of modification, as judged by the relative levels of the faster and slower migration species, varied from lysate to lysate but generally was 30 to 50% within the 120-min period. Additional modification could be obtained by incubating the lysate for longer periods of time or addition of fresh lysate.

Translation of SpX, SpXX, and SpRX all gave rise to polypeptides that were subsequently modified to give a slower-migrating species, indicating that modification outside of the amino-terminal 70 residues and carboxy-terminal 139 residues contributed to this migratory shift (Fig. 4A). In contrast, however, no modification of the SpNX primary product was apparent. Clearly, the SpNX product was less stable in the lysates than the other mutant polypeptides, but nevertheless, even on long exposures of the gel, no slower-migrating species were detected. These results suggest that an area critical for the migratory shift was located between residues 71 and 150. Since the 243-amino-acid product of the 12S mRNA is also modified to give slower-migrating species, it is evident that the residues unique to the 13S message, residues 139 to 185, are not necessary, therefore further defining a critical area to between residues 71 and 139.

We expected the series of internal in-frame deletion mutants to define the important region more precisely. The deletion in Sp5/3 removes amino acids 38 to 65, and, not surprisingly, in light of the result with SpRX, the protein encoded by this mutant was modified (Fig. 4B). It was surprising, however, to find that mutants Sp105NX, SpNC, and SpCS likewise gave rise to two species of protein (Fig. 4B), a result in apparent contradiction to those shown in Fig. 4A with the truncation products. The two species formed from the translation of SpNC-generated RNA were not easily resolved and in most gels ran very close to each other. On occasion (Fig. 4C), clear separation of the two species was obtained. The single-deletion or truncation mutants therefore did not define one particular region critical for the migration shift.

Slower-migrating, modified E1A species arising from modifications in at least two regions. One possible explanation for the apparently conflicting results described above is that modification in more than one region results in conformational changes, giving rise to the electrophoretic migration

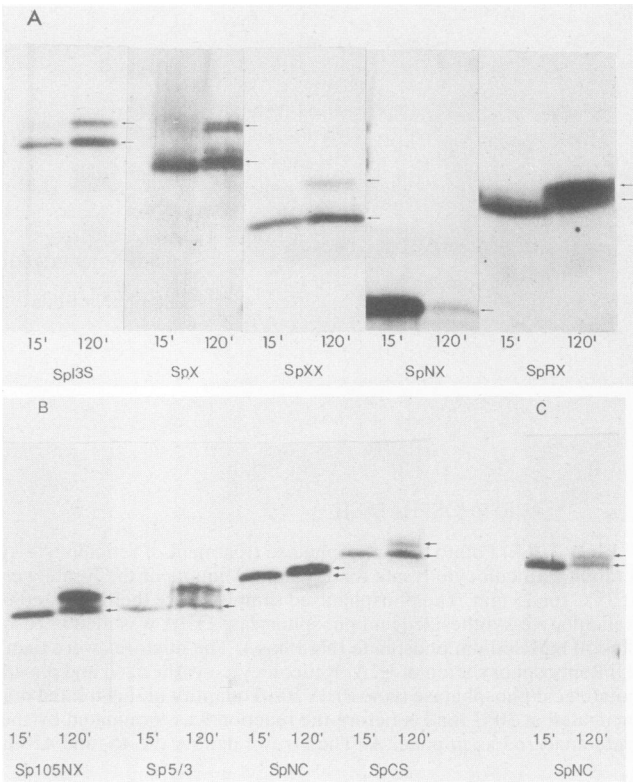

FIG. 4. SDS-PAGE mobility of proteins translated from the mutant E1A RNAs. (A) Sp6-derived mRNA was synthesized in vitro from each of the truncated forms of the E1A gene (Fig. 3) and translated in a rabbit reticulocyte lysate for 15 or 120 min. The translation products were resolved by SDS-PAGE and autoradiographed. (B) RNA was synthesized from the mutant E1A genes containing in-frame deletions and subsequently translated, and the products were analyzed as above. (C) Analysis by SDS-PAGE of the translation products of the SpNC-generated RNA, in which clear separation of the slower- and faster-migrating species was obtained. The arrows denote the modified and unmodified forms of the E1A protein.

shift. Deletion of any individual region would therefore not be sufficient to prevent the shift. If this were the case, one would have to argue from the results shown in Fig. 4, that the N-terminal 86 amino acids did not contain any of these crucial regions, since the product of SpNX was not modified. Furthermore, at least one important region must reside between residues 86 and 152, since the product of SpXX was modified. A likely region would be that between residues 86 and 120, which is deleted in the mutant SpNC, because although a slower-migrating species of the product of this mutant could be detected, the degree of mobility shift appeared to be greatly diminished. A series of double mutants combining certain in-frame deletions and carboxy-terminal truncations were constructed (Fig. 3) and analyzed in the same fashion.

A combination of the deletion present in SpNC with the 3′ truncation present in SpX (giving rise to the construct SpNC-X) resulted in a product that was efficiently synthesized but not modified (Fig. 5). Even upon long exposures, no species that migrated slower than the primary translation product were detected. A faint band that migrated faster was evident, but this was present in equal amounts in the 15- and 120-min sample and probably results from a small level of internal translation initiation. These results indicate that

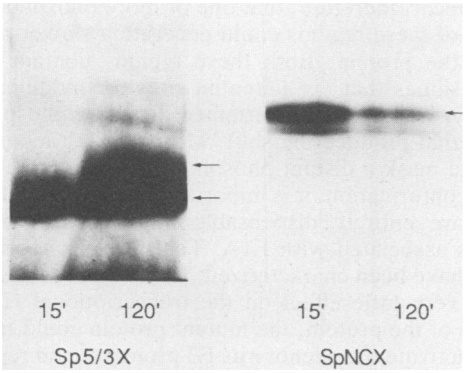

FIG. 5. Analysis of proteins that derive from mutant RNAs containing multiple alterations. RNA was prepared from Sp5/3X and SpNCX and subsequently translated and analyzed as described in the legend to Fig. 1 and as shown in Fig. 3. The arrows denote the major E1A species.

modification within either the 86-to-120 region or the carboxy-terminal 66-amino-acid region can give the shift in electrophoretic migration, and the deletion of both regions is necessary to prevent the migration shift from occurring. Combining the Sp5/3X deletion with the same carboxy-terminal truncation, did not prevent the electrophoretic shift (Fig. 5).

Slower-migrating protein species arising from modification of the same regions in microinjected *Xenopus* oocytes. We have previously shown that E1A modification can also take place in microinjected *Xenopus* oocytes (23). Furthermore, oocyte injection studies with mutant proteins synthesized in *Escherichia coli* and subsequently purified suggested that exon 1 sequences encoding amino acids 23 to 120 were sufficient to give rise to modified, slower-migrating species (23). We investigated whether phosphorylation within the same regions of the E1A protein was responsible for the shift seen in microinjected oocytes. In vitro-synthesized RNA was microinjected into the oocytes together with [^{35}S]methionine, and extracts were prepared 1 and 4 h after [^{35}S]methionine injection. The E1A proteins were immunoprecipitated and analyzed by SDS-PAGE and autoradiographed. Following microinjection of the 13S RNA, the primary translation product (45 kDa) was synthesized within the first hour (Fig. 6). During this time, however, very little modification to the slower-migrating form was observed. However, the modification did take place during the following 3 h, such that at 4 h postinjection, 50% or more of the protein was in the slower-migrating form. Microinjection of both SpNC and SpX RNA resulted in translation products that were subsequently modified. As was the case with the rabbit reticulocyte-mediated modification, the modified form of the SpNC product migrated only slightly slower than the unmodified form (Fig. 6). Microinjection of SpNCX RNA gave rise to a product that was not modified to give a slower form. These results are therefore in complete agreement with those obtained with the reticulocyte lysate.

Phosphorylations that result in the slower-migrating E1A species dispensable for E1A transcriptional activation and transformation. The removal of both the 86-to-120 region and the carboxy-terminal 66-amino-acid region eliminates the phosphorylations that give rise to slower-migrating forms of the E1A protein. We have investigated whether these regions are necessary for the ability of E1A proteins to transcriptionally activate early gene expression and transform primary cells.

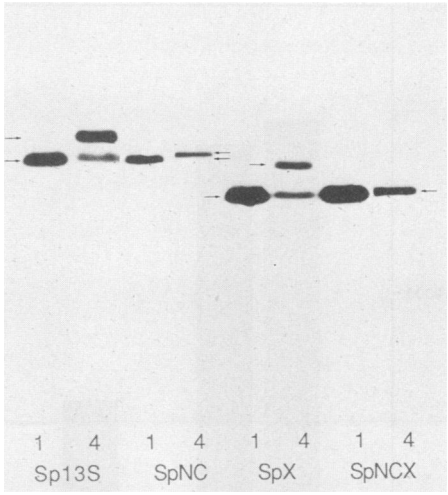

FIG. 6. Analysis of E1A products in mRNA-injected oocytes. Oocytes were injected with wild-type E1A 13S mRNA (Sp13) or various mutants, followed by a radioactive methionine pulse and a radioinert methionine chase for 1 to 4 h. The E1A proteins were immunoprecipitated and analyzed by SDS-PAGE and autoradiography.

Transfection of plasmids containing the E3CAT chimeric gene (35) or the E2ACAT chimeric gene (19) into HeLa cells, either alone or together with E1A-containing plasmids, has frequently been used as a convenient assay for examining the transcriptional activation potential of E1A mutants. We previously showed that individually, the 86-to-120 region and the carboxy-terminal 66-amino-acid region are unnecessary for transcriptional activation (25). The E1A construct GNCR (which is identical to SpNCX, except that the E1A promoter is retained rather than the Sp6 promoter), which lacks both these regions, was also capable of stimulating E2CAT and E3CAT expression (Fig. 7).

The transforming potential of GNCR was tested by measuring its ability to cooperate with the activated *ras* gene to morphologically transform primary baby rat kidney cells. The results of the cooperation assay (Table 1) demonstrate that GNCR can cooperate with wild-type efficiency. Therefore, the simultaneous removal of both the 86-to-120 and the carboxy-terminal regions has no effect on either transcriptional activation or the transformation properties of E1A.

DISCUSSION

Although the two E1A mRNAs that are present early after viral infection each give rise to a single primary translation product, extensive posttranslational modifications produce E1A variants that are usually more acidic and often greater in apparent molecular weight than their parent molecules. On two-dimensional gels, the charge variants observed in the first isoelectric dimension tend to reside in two molecular weight clusters that are detected in the second SDS dimension (10, 36). When the E1A variants are examined by a single SDS dimension, the pattern of proteins is greatly simplified. The E1A proteins fall into two groups, those that comigrate with the primary product and those that migrate about 2 kDa slower. The decreased SDS gel mobility of these latter species in SDS gels probably represents some conformational change induced by a posttranslational modification, the result of which is less SDS binding than the primary products. E1A proteins are known to be extensively phos-

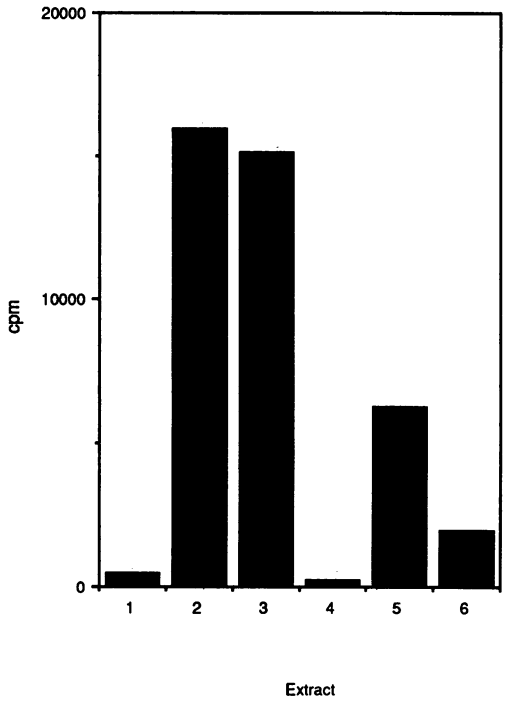

FIG. 7. Relative expression of E3CAT and E2ACAT in HeLa cells transfected with or without E1A alleles. Extracts were prepared from HeLa cells 48 h after transfection with the following: E2ACAT (bar 1), E2ACAT plus pCE (wild-type E1A gene) (bar 2), E2ACAT plus GNC-R (bar 3), E3CAT (bar 4), E3CAT plus pCE (bar 5), and E3CAT plus GNCR (bar 6). A portion of the extract was assayed for chloramphenicol transferase activity by the method of Sleigh (27). The transfer of ^{14}C from [^{14}C]acetyl coenzyme A to unlabeled chloramphenicol is shown. This experiment was performed three times with similar results.

phorylated on serine residues (30, 32, 36), and differential phosphorylation could explain the heterogeneity observed. We show here that phosphorylation is responsible for the mobility shift, since it can be reversed by treatment with potato acid phosphatase. It has also been shown that a mobility shift of the *fos* protein (1) and nucleoplasmin (8) in SDS gels is due to phosphorylation.

To investigate whether such a conformational change was critical for the biological function of the E1A proteins, we first determined which regions of the protein contained the modified residues that caused the electrophoretic mobility shift. Our results indicate that the shift was eliminated when residues 86 to 120 were removed together with the carboxy-terminal 66 amino acids. The removal of any one of these regions, however, was not sufficient to eliminate the shift. It would appear, therefore, that one or more phosphorylations in either of these regions could generate a slower-migrating form of the protein. Both these regions contain multiple serine residues that are potential sites for modification. In this study, we have not determined the exact phosphoserine residues that produce the shift. Moreover, although a deletion could mask a distant phosphorylation site due to alteration of conformation, it is important to emphasize that both regions are entirely dispensable for the main biological functions associated with E1A. The biological activities of mutants have been characterized. Removal of residues 86 to 120 had very little effect on the transcriptional regulatory activities of the protein; the mutant protein could transcriptionally activate the adenovirus E3 promoter and repress the polyoma enhancer with wild-type efficiencies (25). Furthermore, a reconstructed virus containing the mutant gene grew as well as wild-type virus in infected HeLa cells. The mutant could also establish primary baby rat kidney cells and cooperate with the activated *ras* oncogene to morphologically transform these cells with an efficiency similar to that observed with the wild-type gene (25). Clearly, all three activities of E1A are unaffected by removal of these residues. The same is true for a construct that removes the carboxy-terminal 66 amino acids (25). In this report, we show that a mutant that has both regions deleted together is still capable of transcriptional activation and transformation. The efficiency of transcriptional activation is slightly lower than that seen with the wild-type gene, but this may reflect a slight decrease in the stability of the mutant protein (Fig. 5 and 6), rather than a direct involvement of the deleted regions in the transcriptional activation function. Therefore, the phosphorylations that give rise to the SDS mobility shift play little or no role in these biological activities of E1A. Neither region is highly conserved among different adenovirus serotypes, the homology being on the order of 5 to 10%. This contrasts with three separate regions of the protein (CR1-3) that are 50 to 60% conserved (13, 33). The lack of conservation in these regions is consistent with their apparent lack of importance for these major E1A functions.

TABLE 1. Focus formation on primary baby rat kidney cells

Plasmid[a]	Deleted E1A residues	Average no. of foci/plate[b]
pBR322 + EJ6.6		0
pCE[c] + EJ6.6		29
GCE-R + EJ6.6	223–289	40
GNC + EJ6.6	86–120	32
GNCR + EJ6.6	86–120, 223–289	34

[a] The plasmids pCE, GCE-R, and GNC are described in reference 23.
[b] Numbers refer to the average number of foci per plate from three different experiments. Assays are as described in Materials and Methods.
[c] Contains the wild-type E1A gene.

Serine residues within the 86-to-120 region and the carboxy-terminal region have been found to be phosphorylated in vivo (M. L. Trembley, C. J. McGlade, G. E. Gerber, and P. E. Branton, submitted for publication). The serine at position 89 is phosphorylated, and possibly it is this phosphoserine that induces a mobility shift. At least three residues within the carboxy-terminal 66 amino acids have likewise been found to be phosphorylated in vivo. There is at least one serine residue outside of these two regions that is also known to be phosphorylated. Tsukamoto et al. (32) located a major site of phosphorylation to serine 219. However, a mutation that prevented phosphorylation at this site had no effect on the ability of the protein to transcriptionally activate the viral early genes or transform rat embryo fibroblast cells. To date, therefore, there is no evidence to demonstrate that any of the multiple phosphorylations found associated with the E1A proteins are required for activity.

In light of these data, it is not clear why E1A is so heavily phosphorylated. It may be a consequence of the apparent lack of ordered structure of the E1A protein. The protein is rich in proline residues and is predicted to be devoid of extensive ordered secondary structure, a prediction consistent with the observed heat stability of E1A (J. D. Richter and N. C. Jones, unpublished results) and tolerance to relatively large in-frame amino acid deletions (25). This lack of secondary structure may result in many of the serine residues being exposed and available for nonspecific modification.

ACKNOWLEDGMENTS

We thank A. Jacobson for the modified pSP6 vector.

This work was supported by Public Health Service grant CA40189 (to J.D.R.) from the National Institutes of Health. J.D.R. is the recipient of a Cancer Research Scholar Award from the American Cancer Society, Massachusetts Division.

LITERATURE CITED

1. **Barber, J. R., and I. M. Verma.** 1987. Modification of *fos* proteins: phosphorylation of c-*fos*, but not v-*fos*, is stimulated by 12-tetradecanoyl-phorbol-13-acetate and serum. Mol. Cell. Biol. **7**:2201–2211.
2. **Berk, A. J.** 1986. Adenovirus promoters and E1a transactivation. Annu. Rev. Genet. **20**:45–79.
3. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. A pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. Cell **17**:935–944.
4. **Berk, A. J., and P. A. Sharp.** 1978. Structure of the Ad2 early mRNAs. Cell **14**:695–711.
5. **Borrelli, E., R. Hen, and P. Chambon.** 1984. Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature (London) **312**:608–612.
6. **Branton, P. E., and D. T. Rowe.** 1985. Stabilities and interrelations of multiple species of human adenovirus type 5 early region 1 proteins in infected and transformed cells. J. Virol. **56**: 633–638.
7. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNAs from the early regions of Ad2. J. Mol. Biol. **134**:265–303.
8. **Cotten, M., L. Sealy, and R. Chalkley.** 1986. Massive phosphorylation distinguishes Xenopus laevis nucleoplasmin isolated from oocytes or unfertilized eggs. Biochemistry **25**:5063–5069.
9. **Haley, K. P., J. Overhauser, L. E. Babiss, H. S. Ginsberg, and N. C. Jones.** 1984. Transformation properties of type 5 adenovirus mutants that differentially express the E1A gene products. Proc. Natl. Acad. Sci. USA **81**:5734–5738.
10. **Harlow, E., B. R. Franza, Jr., and C. Schley.** 1985. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J. Virol. **55**:533–546.
11. **Hen, R., E. Borrelli, and P. Chambon.** 1985. Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 E1A products. Science **230**:1391–1394.
12. **Jones, N. C., and T. E. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral genes. Proc. Natl. Acad. Sci. USA **76**:3665–3669.
13. **Kimelman, D., J. S. Miller, D. Porter, and B. E. Roberts.** 1985. E1a regions of the human adenoviruses and of the highly oncogenic simian adenovirus 7 are closely related. J. Virol. **53**: 399–409.
14. **Kitchingman, G. R., and H. Westphal.** 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. J. Mol. Biol. **137**:23–48.
15. **Lillie, J. W., M. Green, and M. R. Green.** 1986. An adenovirus E1A protein region required for transformation and transcriptional repression. Cell **46**:1043–1051.
16. **Lucher, L. A., D. Kimelman, J. S. Symington, K. H. Brackmann, M. A. Cartas, H. Thornton, and M. Green.** 1984. Identification of adenovirus 12-encoded E1A tumor antigens synthesized in infected and transformed mammalian cells and in *Escherichia coli*. J. Virol. **52**:136–144.
17. **Lucher, L. A., P. M. Loewenstein, and M. Green.** 1985. Phosphorylation in vitro of *Eschericia coli*-produced 235R and 266R tumor antigens encoded by human adenovirus type 12 early transformation region E1A. J. Virol. **56**:183–193.
18. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids. Res. **12**:7035–7056.
19. **Murthy, S. C. S., G. P. Bhat, and B. Thimmappaya.** 1985. Adenovirus induction by the viral pre-early E1A gene, which appears to be sequence independent. Proc. Natl. Acad. Sci. USA **82**:2230–2234.
20. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**:213–220.
21. **O'Farrell, P. H.** 1985. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
22. **Perricaudet, M., G. Akusjarvi, A. Virtanen, and U. Pettersson.** 1979. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature (London) **281**:694–696.
23. **Richter, J. D., P. Young, N. C. Jones, B. Krippl, M. Rosenberg, and B. Ferguson.** 1985. A first exon-encoded domain of E1A sufficient for post-translational modification, nuclear-localization and induction of adenovirus E3 promoter expression in Xenopus oocytes. Proc. Natl. Acad. Sci. USA **82**:8434–8438.
24. **Rowe, D. T., S. P. Yee, J. Otis, F. L. Graham, and P. E. Branton.** 1983. Characterization of human adenovirus type 5 early region 1A polypeptides using anti-tumour sera and an antiserum specific for the carboxy terminus. Virology **127**:253–271.
25. **Schneider, J. F., F. Fisher, C. R. Goding, and N. C. Jones.** 1987. Mutational analysis of the adenovirus E1A gene: the role of transcriptional regulation in transformation. EMBO J. **6**:2053–2060.
26. **Shih, C., and R. A. Weinberg.** 1982. Isolation of a transforming sequence from a human bladder carcinoma cell line. Cell **29**: 161–169.
27. **Sleigh, M. J.** 1986. A nonchromatographic assay for expression of the chloramphenicol acetyl transferase gene in eukaryotic cells. Anal. Biochem. **156**:251–256.
28. **Smith, D. H., D. M. Kegler, and E. B. Ziff.** 1985. Vector expression of adenovirus type 5 E1a proteins: evidence for E1a autoregulation. Mol. Cell. Biol. **5**:2684–2696.
29. **Southern, P. J., and P. Berg.** 1980. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
30. **Spindler, K. R., D. S. E. Rosser, and A. J. Berk.** 1984. Analysis of adenovirus transforming proteins from early regions 1A and 1B with antisera to inducible fusion antigens produced in *Escherichia coli*. J. Virol. **49**:132–141.
31. **Stephens, C., B. R. Franza, Jr., C. Schley, and E. Harlow.** 1986. Heterogeneity of adenovirus E1A proteins is due to post-translational modification of the primary translation products of the 12S and 13S mRNAs, p. 429–434. *In* M. Botchan, T. Grodzicker, and P. A. Sharp (ed.), Cancer cells 4/DNA tumour viruses. Control of gene expression and replication. Cold Spring Harbor Laboratory, New York.
32. **Tsukamoto, A., A. Ponticelli, A. J. Berk, and R. B. Gaynor.** 1986. Genetic mapping of a major site of phosphorylation in adenovirus type 2 E1A proteins. J. Virol. **59**:14–22.
33. **Van Ormondt, H., J. Maat, and R. Dijkema.** 1986. Comparison of nucleotide sequences of the early E1A regions for subgroups A, B, and C of human adenoviruses. Gene **12**:63–76.
34. **Velcich, A., and E. Ziff.** 1985. Adenovirus E1A proteins repress transcription from the SV40 early promoter. Cell **40**:705–716.
35. **Weeks, D. L., and N. C. Jones.** 1983. E1A control of gene expression is mediated by sequences 5′ to the transcriptional starts of the early viral genes. Mol. Cell. Biol. **3**:1222–1234.
36. **Yee, S.-P., D. T. Rowe, M. L. Tremblay, M. McDermott, and P. E. Branton.** 1983. Identification of human adenovirus early region 1 products by using antisera against synthetic peptides corresponding to the predicted carboxy termini. J. Virol. **46**: 1003–1013.