MOLECULAR AND CELLULAR BIOLOGY, May 1983, p. 829–838
0270-7306/83/050829-10$02.00/0

Vol. 3, No. 5

Localization of the Adenovirus E1A$_a$ Protein, a Positive-Acting Transcriptional Factor, in Infected Cells Infected Cells

LAWRENCE T. FELDMAN AND JOSEPH R. NEVINS*
The Rockefeller University, New York, New York 10021

Received 28 December 1982/Accepted 18 February 1983

The function of the adenovirus E1A$_a$ protein (the product of the 13S E1A mRNA) during a productive viral infection is to activate transcription of the six early viral transcription units. To study the mechanism of action of this protein, a peptide which was 13 amino acids long and had a sequence unique to the protein product of the adenovirus 13S E1A mRNA (pE1A$_a$) was coupled to keyhole limpet hemocyanin and used to raise an antibody in rabbits. The resulting antiserum was specific to this protein and did not react with the protein product of the 12S E1A mRNA, which shares considerable sequence with the E1A$_a$ protein. This antiserum was used to probe for the E1A$_a$ protein in situ by indirect immunofluorescence and in extracts of infected HeLa cells. We found that the protein was associated with large cellular structures both in the nucleus and in the cytoplasm. The nuclear form of the protein was analyzed further and was found to purify with the nuclear matrix.

Studies on the mechanisms controlling eucaryotic gene expression are most often limited by the inability to identify and study the factors mediating regulation. The use of appropriate viral systems affords the opportunity to overcome these limitations. The expression of adenovirus genes during the early phase of a viral infection is strictly regulated at multiple levels of RNA biogenesis (33, 37, 50). Much of the regulation of early viral gene expression occurs at the level of transcription and is mediated through the action of specific viral gene products (32, 34, 35). Perhaps the most dramatic example of control of adenovirus gene expression is the dependence of early viral transcription on the action of the E1A gene product (3, 18, 32). Although the precise mechanism by which the E1A protein activates early viral transcription remains to be determined, the following lines of experimentation indicate an indirect action for the E1A protein. (i) The early adenovirus genes can be activated in the absence of E1A function through the inhibition of cellular protein synthesis (32). This finding has led to the suggestion that there may be a negative controlling element in the cells that are the target of the E1A protein. There is also evidence that protein synthesis inhibition may have a posttranscriptional effect and may indicate that the E1A gene has more than one function (19). (ii) Early viral genes can be transcribed in HeLa cells in the absence of E1A function by using a high multiplicity of input virus for infection (32, 40, 41). However, this E1A-independent expression is host range dependent since certain human cell lines do not allow early viral gene expression in the absence of E1A function even at high multiplicities of infection (M. Imperiale, H.-T. Kao, L. Feldman, J. Nevins, and S. Strickland, manuscript in preparation). (iii) Early adenovirus genes can be activated in the absence of E1A function through the action of a heterologous activator of transcription, the herpesvirus immediate early gene product (10). In fact, herpes-mediated activation of early adenovirus genes is more efficient than E1A-mediated activation, arguing against a direct mechanism of activation. Taken together, all of these findings strongly suggest that the activation of the early adenovirus genes is mediated through an alteration of host cells. In view of this, our approach has been to determine the fate of the E1A$_a$ protein, in terms of its interaction with host cell components, during a productive infection.

The E1A transcription unit encodes three mRNAs which have overlapping sequence contents and encode proteins that share amino acid sequences (4, 7, 20, 36, 44). However, only the protein product of the largest RNA species (a 13S mRNA) is responsible for positive transcriptional control (29, 38). We took advantage of the fact that this protein contains sequences that are not present in the other E1A proteins to produce a specific antibody. This was accomplished by raising an antiserum in rabbits to a synthetic peptide having a sequence from the unique re-

gion. We used this antibody as a probe for the E1A protein to determine the fate of this protein during a productive infection. Our results indicate that the E1A protein is probably associated with the cellular structure commonly called the nuclear matrix.

MATERIALS AND METHODS

Cells and virus. HeLa cells were maintained in suspension culture in Joklik modified minimal essential medium containing 5% fetal calf serum. The growth and preparation of wild-type adenovirus type 5 (Ad5) and mutants H5*ts*125 and *hr*-1 have been described previously (31, 47).

Infection of cells and preparation of mRNA. The procedures used for infection of cells and preparation of cytoplasmic RNA have been described previously (31). For isolation of large quantities of early viral RNA, cycloheximide was added at 1 h postinfection at a concentration of 25 μg/ml, and the RNA was extracted at 12 h postinfection.

E1A-specific peptide. The DNA sequence in Ad5 from nucleotide 1,031 to nucleotide 1,072, which is specific for the 13S mRNA, encodes the following amino acid sequence: His-Tyr-His-Arg-Arg-Asn-Thr-Gly-Asp-Pro-Asp-Ile-Met-Cys (46). This peptide was synthesized commercially (Peninsula Labs, Inc.) and was coupled to keyhole limpet hemocyanin (KLH) via esterification of the C-terminal cysteine residue as described by Liu et al. (26). Briefly, the ester was dissolved in dimethyl formamide and mixed with KLH (10 mg/ml) for 30 min. The activated KLH was purified on a Sephadex G-25 column and then reacted with the E1A peptide for 4 h. Coupling was monitored separately by using ^{125}I-labeled peptide. The resulting peptide-KLH conjugate possessed a molar ratio of peptide to KLH of approximately 6:1.

Immunization. The KLH-coupled peptide (200 μg) was mixed with an equal volume of Freund complete adjuvant and injected into rabbits subcutaneously at multiple sites. After 3 weeks, each rabbit was boosted, and subsequently the rabbits were boosted every 2 to 3 weeks with the protein conjugate (100 μg) in Freund incomplete adjuvant. Serum was collected 10 days after each booster injection beginning after the second injection. Activity was assayed by measuring immunoprecipitation of ^{125}I-labeled peptide.

Selection of E1A mRNA and in vitro translation. Cytoplasmic polyadenylic acid-containing RNA was selected by hybridization to nitrocellulose filters bearing an E1A plasmid (0 to 4.5 map units), using previously described procedures (28, 49). The selected E1A-specific mRNA was translated in rabbit reticulocyte extracts (Bethesda Research Laboratories).

Immunoprecipitation. Immunoprecipitation of ^{35}S-labeled proteins synthesized in vitro was carried out as described by Anderson and Blobel (Methods Enzymol., in press). Briefly, the translation mixture (25 μl) was made 1% in sodium dodecyl sulfate (SDS), heated to 100°C for 4 min, and then diluted 10-fold. The $E1A_a$ peptide antiserum was added (10 μl), and the preparation was incubated at 4°C for 12 h. Antigen-antibody complexes were selected with protein A-Sepharose, washed, and eluted by boiling in sample buffer. Proteins were then separated in a 10% acrylamide–SDS gel and visualized by fluorography.

Preparation of protein extracts and immunoblot analysis. Protein extracts were prepared as described previously (10). Proteins from these extracts or direct samples of glycerol gradient fractions or nuclear matrix fractions were boiled in SDS sample buffer (0.125 M Tris [pH 6.8], 5% [vol/vol] 2-mercaptoethanol, 3% [wt/vol] SDS) (22) and separated in 10% acrylamide–SDS gels. The proteins were transferred electrophoretically to nitrocellulose filters and probed with antibody as described previously (10). The E1A-specific antiserum or antiserum to the 72-kilodalton (kd) DNA-binding protein was used at a dilution of 1:1,000 in RIPA buffer (0.05 M Tris [pH 7.4], 0.15 M NaCl, 1% [vol/vol] Triton X-100, 1% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS).

Preparation of the nuclear matrix. The procedure used for nuclear matrix isolation generally was that described by Fisher et al. (11). Ad5-infected HeLa cells were washed twice in phosphate-buffered saline (PBS) and then suspended in isohipH buffer (0.01 M Tris [pH 8.4], 0.15 M NaCl, 0.0015 M $MgCl_2$) containing 0.5% Nonidet P-40 and kept on ice for 5 min. The nuclei were pelleted by centrifugation at 2,000 rpm for 3 min and washed in 1% Triton X-100 in STM buffer (0.05 M Tris [pH 7.4], 0.25 M sucrose, 0.005 M $MgCl_2$). The nuclei were suspended in STM buffer and incubated with DNase I (25 μg/ml) at 37°C for 15 min. The nuclei were centrifuged at 2,000 rpm for 5 min and washed in LM buffer (0.01 M Tris [pH 7.4], 0.2 mM $MgCl_2$). The nuclear pellet was then suspended in STM buffer, made 2 M in NaCl, and kept on ice for 10 min. The samples were then centrifuged at 10,000 rpm for 15 min, washed in LM buffer, and sedimented again as described above. The resulting pellet was termed the nuclear matrix and was suspended in SDS sample buffer for immunoblot analysis.

Glycerol gradient sedimentation. Nuclei prepared as described above were suspended in RIPA buffer on ice and sonicated for 10 s. Nuclear and cytoplasmic extracts were then mixed 1:1 in RIPA buffer, and the resulting preparation was loaded onto 10 to 30% glycerol gradients in RIPA buffer and centrifuged for 8 h at 47,000 rpm in an SW56 rotor. Fractions were collected, and equal portions from each fraction were mixed with SDS sample buffer and used for an immunoblot analysis.

Indirect immunofluoresence. HeLa cells were grown on glass cover slips and then infected with Ad5 (2,000 particles per cell). The cells were incubated for 12 h in the presence of cytosine arabinoside (25 μg/ml) to maintain early conditions. The cover slips were washed in PBS and then fixed in acetone at room temperature for 10 min. After drying, the cover slips were incubated for 45 min at 37°C with 50 μl of antiserum diluted 1:30 in PBS. After washing in PBS, 50 μl of a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G was added, and the preparation was incubated for an additional 45 min. The cover slips were then washed in PBS, dried, and mounted. Fluorescence was observed with a Leitz Ortholux fluorescent microscope at a magnification of ×630 with oil immersion.

RESULTS

E1A transcription unit and products. The adenovirus E1A region encodes three overlapping

mRNAs that share 5′ and 3′ sequences (4, 7, 20, 44) (Fig. 1A). (The small 9S mRNA is produced only at late times in infections and thus was not important in our studies.) Splicing to form the two largest mRNAs maintains a single reading frame such that the two proteins share an amino acid sequence (36). The proteins encoded by the 12S and 13S mRNAs have predicted molecular weights of 26,000 and 32,000, respectively, based upon the nucleic acid sequence (36, 46). However, when E1A proteins synthesized in vitro are analyzed by SDS gel electrophoresis, four proteins having apparent molecular weights of 40,000 to 60,000 are often observed (9, 12–14, 16, 27, 42). The reason for the discrepancy between the observed molecular weights and the predicted values is not clear, but it may be due to the acidic nature of the proteins, which results in anomalous migration in acrylamide gels, and to post-translational cleavage in the common C-terminal region of each of the primary translation products. Under our conditions of electrophoresis, the major products of the 13S and 12S E1A mRNAs migrated as 46- and 42-kd polypeptides, respectively. In this paper, we refer to the 13S product as pE1A$_a$ and to the 12S product as pE1A$_b$.

Mutants in the E1A region have provided the basis for hypothesizing the involvement of this gene in transcriptional regulation (3, 15, 18). One such mutant, *hr*-1, has been shown to have a single-base deletion at nucleotide 1,055 (38). As described above, this mutant affects only the 13S E1A mRNA; thus, the 12S RNA product is not responsible for transcriptional activation. Furthermore, a mutation that eliminates production of the 12S mRNA without affecting the 13S mRNA has no effect on the transcriptional regulation phenotype (29). Thus, it is the E1A$_a$ protein that is responsible for the activation of early viral transcription during a productive infection.

Production of an E1A$_a$-specific antibody. Since the aim of our study was to investigate the possible interaction of the adenovirus E1A$_a$ protein with cellular components, we required a means to assay for the protein specifically. Clearly, an antibody would have been the reagent of choice, but no antibody with the required specificity was available. In particular, we required an antibody with specificity for the E1A protein produced from the 13S mRNA (the E1A$_a$ protein). Because the same reading frame is utilized for each mRNA, the proteins produced from the E1A region share an amino acid sequence; thus, the difference between the 289-amino acid protein (pE1A$_a$) and the 243-amino acid protein (pE1A$_b$) is the presence of an additional 46 amino acids in the middle of pE1A$_a$. Therefore, since the protein product of the 13S mRNA (pE1A$_a$) is responsible for early viral gene activation and because this protein contains the unique internal amino acid sequence, we chose a 13-amino acid sequence within this unique region to make an antibody. This sequence (Fig. 1B) was chosen based on its hydrophilicity and thus the probability that it is an antigenic site (17, 24). A synthetic peptide was synthesized, coupled to KLH through the terminal cysteine residue (26, 45), and used to raise an antibody in rabbits according to the immunization schedule described above.

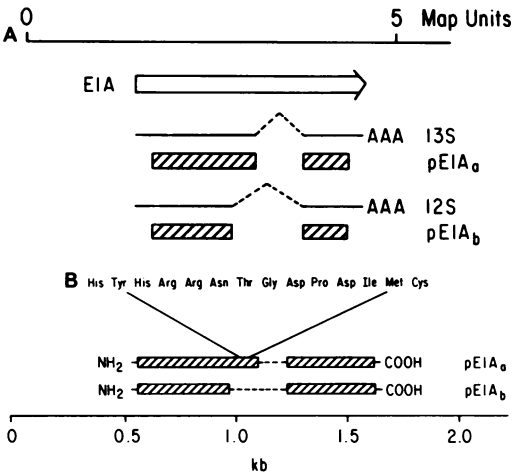

FIG. 1. Schematic representation of the E1A transcription unit and gene products. (A) Genomic positions of the 13S and 12S mRNAs and the major protein products. We refer to the 289-amino acid product of the 13S mRNA as pE1A$_a$ and the 243-amino acid product of the 12S RNA as pE1A$_b$. (B) Sequence of the pE1A$_a$ unique-sequence peptide used for immunization. This sequence represents amino acid residues 158 to 171 of the 289-amino acid E1A$_a$ protein. kb, Kilobase.

All of our attempts to detect the E1A$_a$ protein in extracts of infected cells by immunoprecipitation were unsuccessful. This appeared to be due to low production of the protein in infected cells and, as shown below, the nature of the protein in infected cells. Thus, to detect the E1A$_a$ protein in cellular extracts, we had to rely on biochemical fractionation followed by assay for the presence of the protein in various fractions by a solid-state immunoblot technique. In the initial experiments we utilized immunoblots and proteins from Ad5 H5*ts*125-infected 293 cells maintained at 41°C. The 293 cells contain and express the E1A region constitutively (1). Furthermore, such conditions of infection, where there is a nonfunctional 72-kd DNA-binding protein, result in an increased half-life of the early viral mRNAs (2) and therefore allow greater accumulation of E1A protein per cell. Antiserum was

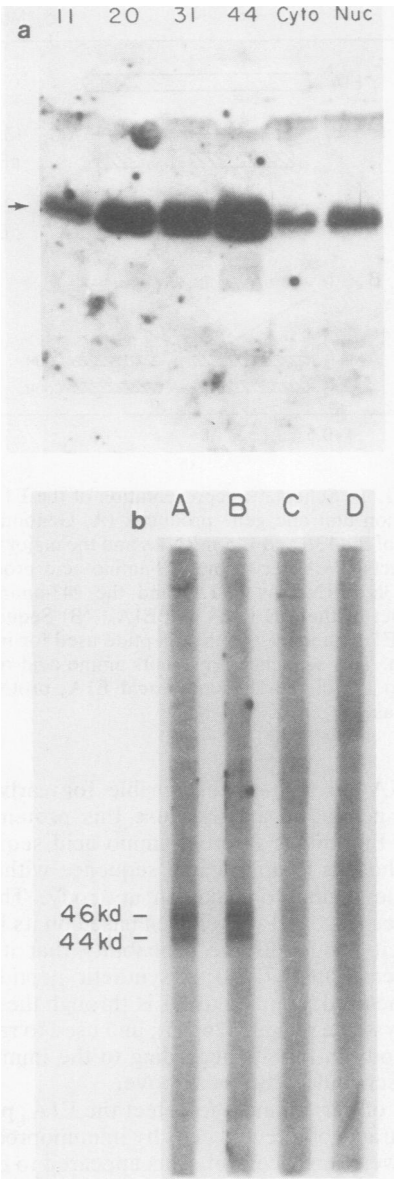

FIG. 2. Assay of the $E1A_a$ peptide antiserum for reactivity with the $E1A_a$ protein from Ad5-infected cells. (a) Proteins prepared from Ad5 H5*ts*125-infected 293 cells at different times postinfection were separated in a 10% acrylamide–SDS gel and transferred to nitrocellulose. The protein blot was then incubated with the peptide antiserum, and this was followed by incubation with ^{125}I-labeled protein A, as described in the text. In addition, a portion of the 20-h sample was separated into nuclei (Nuc) and cytoplasm (Cyto) before the extracts were prepared. The numbers at the top indicate times postinfection (in hours). (b) A protein extract from H5*ts*125-infected 293 cells (24 h postinfection) was used to prepare a protein blot. Individual strips of the blot were then incubated either with the $E1A_a$ peptide serum alone (lane A), with the peptide serum plus 10 ng of the peptide per ml (lane B), with 100 ng of the peptide per ml (lane C), or with normal rabbit serum (lane D). All preparations were then probed with ^{125}I-labeled protein A. The major band migrated as a 46-kd polypeptide.

allowed to react with the protein blot, and then specific antigen-antibody complexes were detected with ^{125}I-labeled protein A. As shown in Fig. 2a, a protein having an apparent molecular weight of 46,000 and minor amounts of a 44-kd protein were detected by using the peptide antiserum. The protein was evident at 11 h postinfection and increased in abundance thereafter. An analysis of extracts of nuclei and cytoplasm revealed that the protein was present in both fractions, with slightly more in the nuclei than in the cytoplasm. That this reactivity to the 46-kd protein was due to specificity induced by the peptide was shown by another experiment (Fig. 2b). An extract from cells infected for 24 h was blotted onto nitrocellulose and probed with the antiserum in the absence (Fig. 2b, lane A) or in the presence (lanes B and C) of increasing concentrations of the peptide. In this sample, there were significant amounts of the $pE1A_a$-related protein, which migrated in our gels at 44 kd. As Fig. 2b shows, the addition of 100 ng of the peptide per ml to the antibody reaction mixture completely blocked the formation of specific complexes.

To demonstrate that the peptide-specific antiserum in fact detected only the adenovirus $E1A_a$ protein, the antibody was also tested for its ability to immunoprecipitate E1A proteins synthesized in vitro. Translation products of E1A mRNAs labeled with [^{35}S]methionine were immunoprecipitated with peptide antiserum or with the preimmune serum. The precipitated proteins and the total translation products were then analyzed in an SDS-acrylamide gel and visualized by fluorography (Fig. 3). An analysis of the E1A-directed translation products revealed two major proteins having molecular weights of 46,000 and 42,000, as well as minor proteins having molecular weights of 44,000 and 40,000. As discussed above, the proteins having molecular weights of 46,000 and 44,000 (labeled $pE1A_a$ and $pE1A_{a'}$, respectively) were products of the E1A 13S mRNA, and the proteins having molecular weights of 42,000 and 40,000 were products of the 12S mRNA. The peptide antibody precipitated only the largest doublet of the four E1A proteins (Fig. 3, lane 2), and there was no reaction when the normal rabbit serum was used.

In addition, protein extracts were prepared from wild-type Ad5-infected cells and *hr*-1-infected cells; proteins were separated by acrylamide-SDS gel electrophoresis and transferred to nitrocellulose. Samples were then probed

with either the E1A peptide antiserum or antiserum to the E2 gene product, the 72-kd DNA-binding protein. As shown in Fig. 4A, the E1A-specific serum detected proteins only from the wild-type Ad5 infection, not from the *hr*-1 infection. The results obtained when the antiserum to the 72-kd protein (the viral E2 gene product) was used clearly show that early gene expression could be detected in the high-multiplicity *hr*-1 infection (Fig. 4B); thus, there must also have been expression of the 12S E1A RNA in these cells, as has been shown previously (3, 37). Therefore, the E1A peptide serum detects only the 13S products which were absent from the *hr*-1 infection. The results of this experiment and the results presented in Fig. 2 and 3 demonstrate the high degree of specificity of this antibody.

Immunofluorescent localization of the E1A$_a$ protein. As a means of probing for the intracellular distribution of the E1A$_a$ protein, we utilized the peptide antiserum and indirect immunofluorescence to assay for the protein in fixed infected cells. HeLa cells grown on cover slips were infected with Ad5 and at 12 h postinfection were incubated with the peptide antiserum, followed by a fluorescent second antibody. The Ad5-infected cells showed nuclear staining and some diffuse cytoplasmic staining (Fig. 5), and therefore we concluded that the E1A$_a$ protein is present predominantly in the nucleus. Furthermore, an examination of cells infected with mutant *hr*-1 and uninfected cells that had been incubated with the antibody as described above revealed no staining (data not shown), as expected from the results shown in Fig. 4.

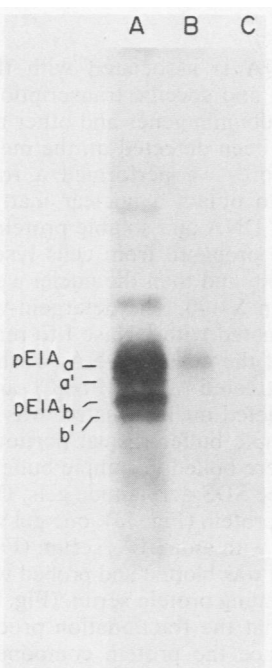

FIG. 3. Specificity of the E1A$_a$ peptide antiserum in immunoprecipitation of E1A proteins synthesized in vitro. E1A proteins were synthesized in a rabbit reticulocyte lysate programmed with E1A-specific mRNA that was selected by hybridization (lane A). The primary products of the 13S and 12S E1A mRNAs are labeled pE1A$_a$ and pE1A$_b$, respectively; the related proteins are labeled a' and b', respectively. The translation products were then precipitated with either the E1A$_a$ peptide serum (lane B) or normal rabbit serum (lane C).

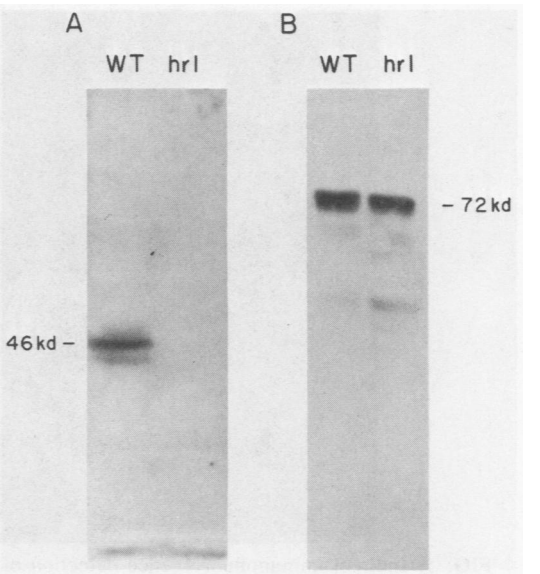

FIG. 4. Specificity of the E1A$_a$ antiserum in the detection of E1A proteins from wild-type Ad5 (WT) and *hr*-1 (hr1) infections. HeLa cells were infected with either wild-type Ad5 or *hr*-1 at a multiplicity of 10^4 particles per cell. Protein extracts were prepared from each preparation at 13 h postinfection, and duplicate immunoblots were prepared. One blot was probed with the E1A$_a$ antiserum (A), and an identical blot was probed with antiserum to the 72-kd DNA-binding protein (B).

Sedimentation analysis of cytoplasmic and nuclear E1A$_a$ proteins. Since it appeared that the peptide-induced antiserum possessed the specificity required for detection of the E1A$_a$ protein, the antibody was used as a probe for studying the size and location of the specific E1A protein. In these experiments, we examined the E1A protein after 12 h of wild-type Ad5 infection in the continuous presence of cytosine arabinoside (to maintain the early phase of infection). Cytoplasmic and nuclear extracts were prepared and analyzed by glycerol gradient sedimentation. By doing this we hoped to measure the size of the native E1A protein and to determine whether, in fact, any of the protein was complexed with

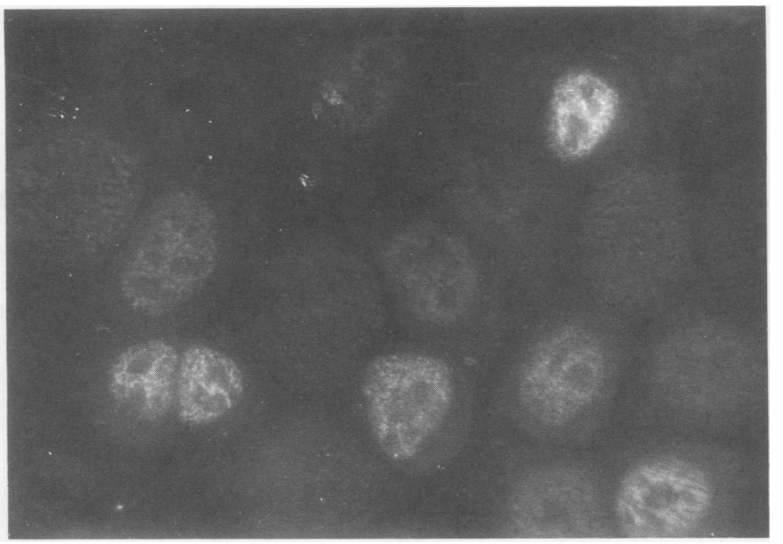

FIG. 5. Indirect immunofluorescence detection of the $E1A_a$ protein. HeLa cells on cover slips that were infected with Ad5 for 12 h were fixed in acetone and incubated with the $E1A_a$ peptide serum. They were then stained with fluorescein isothiocyanate-conjugated goat anti-rabbit serum and examined with a Leitz microscope at a magnification of ×630.

cellular proteins. For example, in a similar fashion it has been shown that when simian virus 40 T antigen is analyzed in crude cell extracts, it sediments as a discrete entity at 8S and also as a complex with a 53-kd cellular protein at 12S (6, 21, 23, 25, 43). It has also been shown that $pp60^{src}$ exists in a free form and as a complex with two cellular proteins (5). Figure 6A shows that the majority of the cytoplasmic $E1A_a$ protein and almost all of the nuclear $E1A_a$ protein were recovered in the pellet under these conditions of centrifugation. As a control for nonspecific aggregation, the same samples were assayed with antiserum specific to the 72-kd DNA-binding protein (Fig. 6B). We found that the great majority of both the cytoplasmic 72-kd protein and the nuclear 72-kd protein was soluble and sedimented near the top of the gradient. Thus, it appears that the majority of the $E1A_a$ protein, either in the cytoplasm or in the nucleus, is associated in a rather large complex and is insoluble.

Intranuclear localization of the $E1A_a$ protein. Because the immunofluorescence data clearly indicated a nuclear localization for the $E1A_a$ protein and given the function of transcriptional activation for the protein, we further investigated the location of the protein within the nucleus. It seemed that the results shown in Fig. 6 could most likely be explained by binding of the $E1A_a$ protein to a nuclear matrix-like structure, particularly since DNase treatment of nuclear extracts before loading onto the glycerol gradient did not alter sedimentation (data not shown). It has been shown previously that adenovirus DNA is associated with the nuclear matrix (48), and specific transcription units for induced ovalbumin genes and other mammalian genes have been detected on the matrix (8, 30, 39). Therefore, we performed a fractionation procedure to obtain a nuclear matrix fraction free of both DNA and soluble proteins. Briefly, nuclei were prepared from cells lysed in 0.5% Nonidet P-40, and then the nuclei were washed in 1% Triton X-100. The detergent-washed nuclei were treated with DNase I to remove more than 99% of the cellular DNA, washed in low-salt buffer, treated with 2 M NaCl, and washed, and the pelleted nuclear matrix was suspended in SDS sample buffer. Equal portions from all fractions were boiled in sample buffer and loaded onto three SDS-acrylamide gels. One gel was stained for protein (Fig. 7a), one gel was blotted and probed with anti-$E1A_a$ serum (Fig. 7b), and the third gel was blotted and probed with anti-72-kd DNA-binding protein serum (Fig. 7c). Figure 7a shows that the fractionation procedure was effective since the protein components of the various fractions are largely distinct. In addition, it is clear that the matrix proteins were quite different from either the cytoplasmic soluble proteins or the nuclear soluble proteins. When each subfraction was analyzed for the $E1A_a$ protein, the great majority was found in the matrix and not in the DNase or 2 M NaCl supernatant. These results agree with the sedimentation data in Fig. 5 and explain why the $E1A_a$ protein is found mostly in the pellet. Furthermore, the results for the 72-kd DNA-

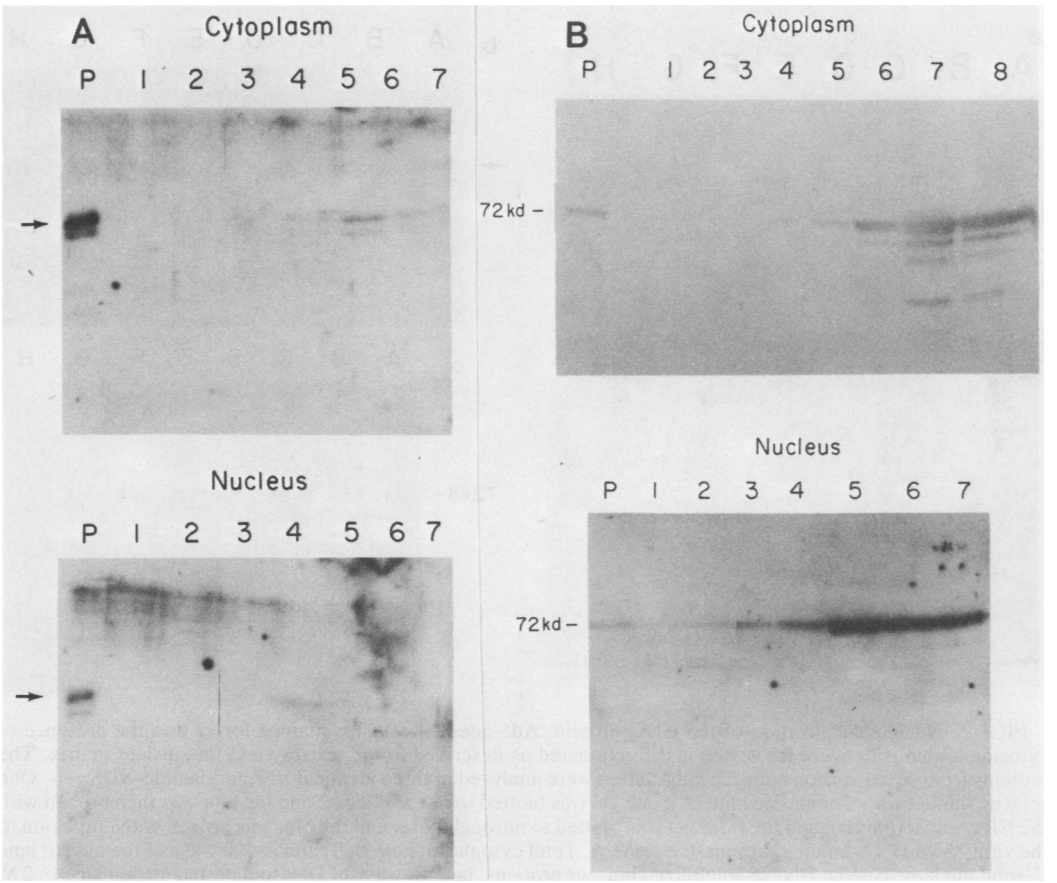

FIG. 6. Glycerol gradient sedimentation analysis of the E1A$_a$ protein. Nuclear and cytoplasmic extracts were prepared from Ad5-infected HeLa cells and centrifuged in glycerol gradients as described in the text. Fractions were collected, and samples of each fraction, as well as the pelleted material, were analyzed by SDS-acrylamide gel electrophoresis. Protein blots were probed with the E1A$_a$ peptide antiserum (A) and with antiserum against the 72-kd DNA-binding protein (B).

binding protein (Fig. 7c) clearly show that the fractionation procedure was effective since less than 5% of the nuclear DNA-binding protein was found in the matrix, whereas virtually 100% of the E1A$_a$ protein fractionated with the nuclear matrix. At the present time, however, we cannot exclude the possibility that the E1A$_a$ protein simply copurifies with the nuclear matrix rather than being an integral component of the matrix. Of course, this is also the case for any of the identified matrix proteins and stems largely from the very nature of the matrix and its isolation; the matrix represents the nuclear residium that is resistant to detergent, DNase, and salt extraction. Whether the various protein components that are obtained are actually associated in cells has not been proven yet.

DISCUSSION

The expression of the adenovirus genome, particularly during the early phase of infection, represents one of the best-characterized systems of eucaryotic gene regulation. One of the factors responsible for this regulation is the largest protein product of the E1A gene, a protein that is required for the transcription of the other viral genes (3, 18, 32) and possibly for post-transcriptional activation (19). Clearly, this system represents a unique opportunity for the study of positive control of transcription as not only are the genes that are subject to control available, but also the protein responsible for the control has been identified. The major obstacles to understanding the mechanism by which this protein activates viral gene expression have been the lack of an assay or probe for the protein and the unavailability of the protein in a pure state. To overcome these problems, we produced an antiserum against a synthetic peptide derived from E1A sequences. The antibody that was obtained had the desired specificity; it detected only the E1A protein that was the product of the 13S mRNA.

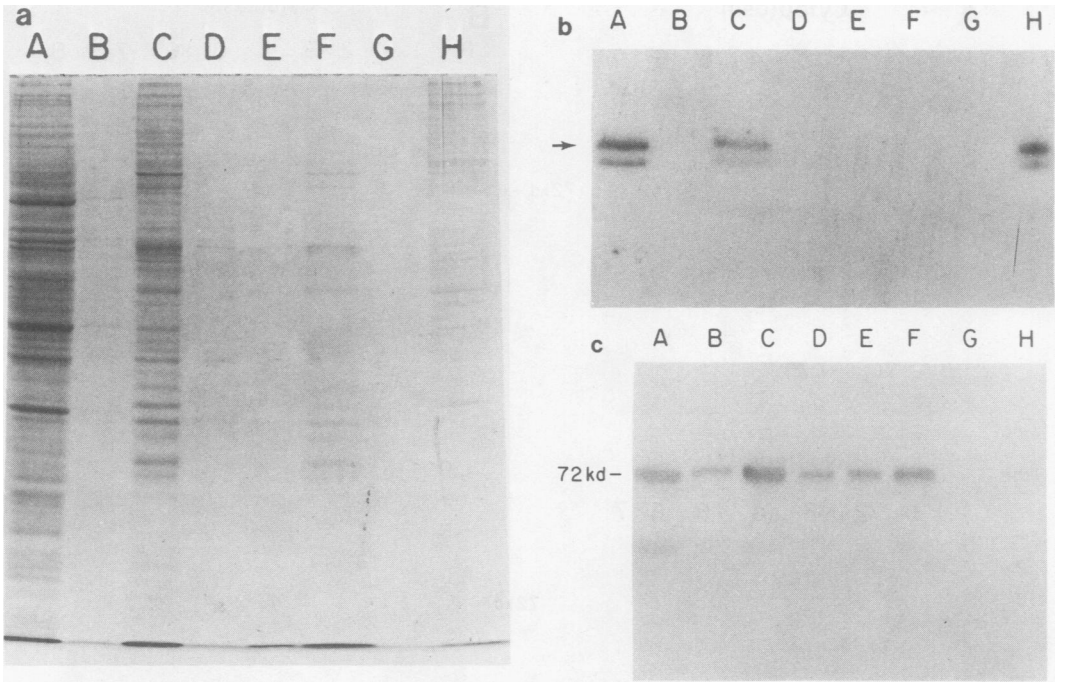

FIG. 7. Nuclear localization of the E1A$_a$ protein. Ad5-infected cells maintained for 12 h in the presence of cytosine arabinoside were harvested and fractionated as described in the text to yield the nuclear matrix. The proteins from equal portions of each subfraction were analyzed in three identical 10% acrylamide–SDS gels. One gel was stained with Coomassie blue (A); one gel was blotted to nitrocellulose, and the blot was then probed with the E1A$_a$ antiserum (B); and the third gel was blotted to nitrocellulose, and the blot was probed with antiserum to the viral 72-kd DNA-binding protein (C). Lane A, Total cytoplasm; lane B, Triton X-100 wash of the nuclei; lane C, total nucleus; lane D, DNase-solubilized nuclear proteins; lane E, wash of DNase-digested nuclei; lane F, 2 M NaCl-solubilized nuclear proteins; lane G, wash of 2 M NaCl-treated nuclei; lane H, nuclear matrix.

In this case an antiserum of high specificity was engineered to eliminate reactivity with proteins having shared sequences. This is an especially important factor when one wishes to localize the protein inside the cell by indirect means, such as fluorescence.

Our initial hope was to use the antibody in immunoprecipitation experiments to coprecipitate the E1A protein with associated cellular components. However, this approach was not successful, probably for two reasons. First, the E1A protein appears to be synthesized in very low amounts during a productive infection. In our experience, the immunoblot technique is considerably more sensitive than immunoprecipitation of in vivo labeled protein, and the use of this technique has allowed us to detect the protein. Second, our results have led us to conclude that the E1A$_a$ protein is not a soluble component of infected cells but rather is part of a rather large and insoluble structure both in the nucleus and in the cytoplasm. In our studies we have ignored the cytoplasmic form of the protein and have concentrated our efforts on the nuclear species. This is simply due to the known information concerning the function of the protein in transcriptional activation. Whether the protein in the cytoplasm is a precursor in transit to the nucleus, or whether the presence of the protein in the cytoplasm indicates that the protein may possess other functions, is not clear. Certainly, the observation that a considerable fraction of the steady-state E1A protein is cytoplasmic warrants further study. The nuclear form does in fact appear to be insoluble due to its association with what is commonly called the nuclear matrix. The definition of the matrix and associated components essentially rests on the property of a protein being resistant to solubilization by detergent, DNase, and high salt concentrations. That the procedure which we used was effective in achieving such a fractionation was indicated by the facts that a distinct viral nuclear protein, the 72-kd DNA-binding protein, was absent from the matrix fraction and that the patterns of stainable proteins were distinct in the various fractions. Because virtually 100% of the nuclear E1A$_a$ protein was recovered in the matrix, it seems reasonable to conclude that the E1A$_a$ protein is not merely a contaminant of the

matrix preparation; however, due to the nature of the nuclear matrix preparation it is difficult at this stage to be sure of this. Final proof that any of the so-called nuclear matrix proteins are actually associated within cells will require direct demonstration (i.e., cross-linking).

What do these results tell us about the mechanism of action of the E1A protein? Several studies have indicated that transcriptionally active genes are associated with the nuclear matrix. Ovalbumin sequences are enriched in matrix-associated DNA in chick oviduct tissue but not in liver tissue (39), and simian virus 40 DNA sequences appear to be matrix associated in virus-transformed cells (30). In this report we describe the first instance of the presence of a positive-acting transcriptionally regulatory protein in the nuclear matrix. In fact, there is evidence for matrix association of adenovirus DNA, but whether this is related to active transcription has not been resolved (48). The most straightforward proposal is the suggestion that the E1A$_a$ protein facilitates the attachment of viral DNA molecules to the matrix, where they are more efficiently transcribed. Alternatively, viral DNA molecules might be associated with the nuclear matrix irrespective of the action of the E1A$_a$ protein but, once associated, they may become available for the activation function. Clearly, a determination of the relationship of viral transcription to the association of DNA with the nuclear matrix is needed.

One final point regarding the implication of these and other results deserves discussion. It is well established that the control of gene expression at the transcriptional level in procaryotic cells involves the interaction of soluble regulatory proteins with appropriate DNA sequences. The fact that the regulatory events can be reproduced in vitro in soluble systems certainly substantiates this notion. However, the same simple situation may not apply in eucaryotic cells. If, in fact, active transcription takes place in association with higher-order protein structures in nuclei, for which there is some evidence (see above), and if transcriptional regulatory proteins are not soluble proteins but rather part of structural elements of the nucleus, as seems to be the case for the adenovirus E1A$_a$ protein, then it may well be that soluble transcription systems for eucaryotic gene regulation will never show correct regulation. Instead, what may be important for the reproduction of regulation as it occurs in cells may be the structure of the nucleus.

ACKNOWLEDGMENTS

We thank Haseeb Ali and Sarah Willis for excellent technical assistance with the experiments. We also thank P. Fisher for helpful discussions concerning the nuclear matrix and M. Imperiale and J. Valinsky for help with the immunofluorescence.

This work was supported by grant MV141 from the American Cancer Society and by Public Health Service grant GM 26765-04 from the National Institutes of Health. L.T.F. is a National Institutes of Health postdoctoral fellow, and J.R.N. is the recipient of Public Health Service Research Career Development Award CA 00666 from the National Institutes of Health.

LITERATURE CITED

1. **Aiello, L., R. Guiloyle, K. Huebner, and R. Weinmann.** 1979. Adenovirus 5 DNA sequences present and RNA sequences transcribed in transformed human embryo kidney cells (HEK-Ad5 or 293). Virology **94**:460–469.
2. **Babich, A., and J. R. Nevins.** 1981. The stability of early adenovirus mRNA is controlled by the viral 72kd DNA-binding protein. Cell **26**:371–379.
3. **Berk, A. J., F. Lee, T. Harrison, J. Williams, and P. A. Sharp.** 1979. 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. **Brugge, J. S., E. Erikson, and R. L. Erikson.** 1981. The specific interaction of the Rous sarcoma virus transforming protein, pp60src, with two cellular proteins. Cell **25**:363–372.
6. **Chang, C., D. T. Simmons, M. A. Martin, and P. T. Mora.** 1979. Identification and partial characterization of new antigens from simian virus 40-transformed mouse cells. J. Virol. **31**:463–471.
7. **Chow, L. T., T. R. Broker, and J. B. Lewis.** 1979. Complex splicing patterns of RNA from the early regions of Ad2. J. Mol. Biol. **134**:265–303.
8. **Cook, P. R., and J. A. Brazell.** 1980. Mapping sequences in loops of nuclear DNA by their progressive detachment from the nuclear cage. Nucleic Acids Res. **8**:2895–2906.
9. **Esche, H., M. B. Mathews, and J. B. Lewis.** 1980. Proteins and messenger RNAs of the transforming region of wild-type and mutant adenovirus. J. Mol. Biol. **142**:399–417.
10. **Feldman, L. T., M. J. Imperiale, and J. R. Nevins.** 1982. Activation of early adenovirus transcription by the herpesvirus immediate early gene: evidence for a common cellular control factor. Proc. Natl. Acad. Sci. U.S.A. **79**:4952–4956.
11. **Fisher, P. A., M. Berrios, and G. Blobel.** 1982. Isolation and characterization of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina, and nuclear pore complexes from embryos of *Drosophila melanogaster*. J. Cell Biol. **92**:674–686.
12. **Green, M., W. S. M. Wold, K. H. Brackman, and M. A. Cartas.** 1979. Identification of families of overlapping polypeptides coded by early transforming gene region I of human adenovirus 2. Virology **97**:225–236.
13. **Halbert, D. N., and H. J. Raskas.** 1982. Tryptic and chymotryptic methionine peptide analysis of the *in vitro* translation products specified by the transforming region of adenovirus type 2. Virology **116**:406–418.
14. **Halbert, D. N., D. J. Spector, and H. J. Raskas.** 1979. In vitro translation products specified by the transforming region of adenovirus type 2. J. Virol. **31**:621–629.
15. **Harrison, T., F. Graham, and J. Williams.** 1977. Host range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology **77**:319–329.
16. **Harter, M. L., and J. B. Lewis.** 1978. Adenovirus type 2 early proteins, synthesized in vivo and in vitro. Identification in infected cells of the 38,000- to 50,000-molecular-weight protein coded by the left end of the adenovirus type 2 genome. J. Virol. **26**:736–749.
17. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. U.S.A. **78**:3824–3828.
18. **Jones, N., and T. Shenk.** 1979. An adenovirus type 5 early gene function regulates expression of other early viral

genes. Proc. Natl. Acad. Sci. U.S.A. **76**:3665–3669.
19. **Katze, M. G., H. Persson, and L. Philipson.** 1981. Control of adenovirus early gene expression: posttranscriptional control mediated by both viral and cellular gene products. Mol. Cell. Biol. **1**:807–813.
20. **Kitchingman, G. R., and H. Westphal.** 1980. The structure of adenovirus 2 early nuclear and cytoplasmic RNAs. J. Mol. Biol. **137**:23–48.
21. **Kress, M., F. May, R. Cassingena, and P. May.** 1979. Simian virus 40-transformed cells express new species of protein precipitable by anti-simian virus 40 tumor serum. J. Virol. **31**:472–483.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **277**:680–685.
23. **Lane, D. P., and L. V. Crawford.** 1979. T antigen is bound to a host protein in SV40-transformed cells. Nature (London) **278**:261–263.
24. **Lerner, R. A.** 1982. Tapping the immunological repertoire to produce antibodies of predetermined specificity. Nature (London) **291**:592–596.
25. **Linzer, D. I. H., and A. J. Levine.** 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40 transformed cells and uninfected embryonal carcinoma cells. Cell **17**:43–52.
26. **Liu, F.-T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18**:690–697.
27. **Lupker, J. H., A. Davis, H. Jochemsen, and A. J. van der Eb.** 1981. In vitro synthesis of adenovirus type 5 T antigens. I. Translation of early region 1-specific RNA from lytically infected cells. J. Virol. **37**:524–529.
28. **McGrogan, M., D. J. Spector, C. J. Doldenberg, D. Halbert, and H. J. Raskas.** 1979. Purification of specific adenovirus 2 RNAs by preparative hybridization and selective thermal elution. Nucleic Acids Res. **6**:593–607.
29. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1982. Resolving the functions of overlapping viral genes by site specific mutagenesis at a mRNA splice site. Nature (London) **295**:380–384.
30. **Nelkin, B. D., D. M. Pardoll, and B. Vogelstein.** 1980. Localization of SV40 genes within supercoiled loop domains. Nucleic Acids Res. **8**:5623–5633.
31. **Nevins, J. R.** 1980. Definition and mapping of adenovirus 2 nuclear transcription. Methods Enzymol. **65**:768–785.
32. **Nevins, J. R.** 1981. Mechanism of activation of early viral transcription by the adenovirus E1A gene product. Cell **26**:213–220.
33. **Nevins, J. R.** 1982. Adenovirus gene expression: control at multiple steps of mRNA biogenesis. Cell **28**:1–2.
34. **Nevins, J. R., H. W. Ginsberg, J. M. Blanchard, M. C. Wilson, and J. E. Darnell.** 1979. Regulation of the primary expression of the early adenovirus transcription units. J. Virol. **32**:727–733.
35. **Nevins, J. R., and J. Jensen-Winkler.** 1980. Regulation of early adenovirus transcription: a protein product of early region 2 specifically represses region 4 transcription. Proc. Natl. Acad. Sci. U.S.A. **77**:1893–1897.
36. **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.
37. **Persson, H., and L. Philipson.** 1982. Regulation of adenovirus gene expression. Curr. Top. Microbiol. Immunol. **97**:157–203.
38. **Ricciardi, R. P., R. L. Jones, C. L. Cepko, P. A. Sharp, and B. E. Roberts.** 1981. Expression of early adenovirus genes requires a viral encoded acidic polypeptide. Proc. Natl. Acad. Sci. U.S.A. **78**:6121–6125.
39. **Robinson, S. I., B. D. Nelkin, and B. Vogelstein.** 1982. The ovalbumin gene is associated with the nuclear matrix of chicken oviduct cells. Cell **28**:99–106.
40. **Ross, S. R., A. J. Levine, R. S. Galos, J. Williams, and T. Shenk.** 1980. Early viral proteins in HeLa cells infected with adenovirus type 5 host range mutants. Virology **103**:475–492.
41. **Shenk, T., N. Jones, W. Colby, and D. Fowlkes.** 1979. Functional analysis of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cold Spring Harbor Symp. Quant. Biol. **44**:367–375.
42. **Smart, J. E., J. B. Lewis, M. B. Mathews, M. L. Harter, and C. W. Anderson.** 1981. Adenovirus type 2 early proteins: assignment of the early region 1A proteins synthesized *in vivo* and *in vitro* to specific mRNAs. Virology **112**:703–713.
43. **Smith, A. E., R. Smith, and E. Paucha.** 1979. Characterization of different tumor antigens present in cells transformed by simian virus 40. Cell **18**:335–346.
44. **Spector, D. J., M. McGrogan, and H. J. Raskas.** 1978. Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus genome. J. Mol. Biol. **126**:395–414.
45. **Sutcliffe, J. G., T. M. Shinnick, N. Green, F.-T. Liu, H. L. Niman, and R. A. Lerner.** 1980. Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. Nature (London) **287**:801–805.
46. **Van Ormondt, H., J. Maat, A. de Waard, and A. J. Van der Eb.** 1978. The nucleotide sequence of the transforming HpaI-E fragment of adenovirus type 5 DNA. Gene **4**:309–328.
47. **Wall, R., L. Philipson, and J. E. Darnell, Jr.** 1972. Processing of adenovirus specific nuclear RNA during virus replication. Virology **50**:27–34.
48. **Younghusband, H. B., and K. Maundrell.** 1982. Adenovirus DNA is associated with the nuclear matrix of infected cells. J. Virol. **43**:705–713.
49. **Zeevi, M., J. R. Nevins, and J. E. Darnell.** 1982. Newly formed mRNA lacking polyadenylic acid enters the cytoplasm and the polyribosomes but has a shorter half-life in the absence of polyadenylic acid. Mol. Cell. Biol. **2**:517–525.
50. **Ziff, E. B.** 1980. Transcription and RNA processing by the DNA tumour viruses. Nature (London) **287**:491–499.