Immortalization of Rat Embryo Fibroblasts by an Adenovirus 2 Mutant Expressing a Single Functional E1a Protein

DAVID R. HURWITZ AND G. CHINNADURAI*

Institute for Molecular Virology, St. Louis University Medical Center, St. Louis, Missouri 63110

Received 5 November 1984/Accepted 14 January 1985

Expression of the adenovirus E1a and E1b genes is required for transformation of nonpermissive rodent cells. Differential splicing of the E1a precursor RNA molecules results in the production of two early mRNAs, 13S and 12S, which encode a 289-amino-acid-residue (289R) and 243R protein, respectively. Previously we constructed a mutant virus, *dl231*, which can only produce normal 289R protein from the E1a gene. In this report we demonstrate that *dl231* induced focal transformation of primary rat embryo fibroblasts at 20% of the level of wild-type virus. *dl231* transformants were immortalized and produced normal levels of E1a 13S and E1b mRNAs but only minute levels of defective E1a 12S mRNA. These transformants only minimally expressed the transformation phenotype and were similar to untransformed cells. Unlike wild-type transformants, they had a more fibroblastic morphology, were contact inhibited, grew to only low saturation density, and were limited in their ability to grow in an anchorage-independent manner in soft agar. We conclude that the 289R E1a protein can mediate immortalization of primary cells and that the 243R E1a protein is required to elicit the full transformation phenotype.

Cell transformation is a multistep process affecting at least two major aspects of cellular activity. Primary cells must become established and immortalized as well as express the transformation phenotype, which includes morphological and growth-characteristic changes. The transformation of rat cells by adenovirus reflects these two components.

Transformation of baby rat kidney cells by transfection of adenoviral DNA fragments has indicated that although the viral E1a and E1b genes, located at the extreme left end of the genome, are both required for complete transformation (36, 37), the E1a gene alone is capable of partially transforming these primary cells (18). These E1a-transformed cells are fibroblastic and resemble untransformed cells. It appears therefore that the E1a gene is involved in the immortalization process whereas the E1b gene is required for the morphological and growth characteristics associated with cell transformation. It has been shown that E1b by itself lacks the immortalizing or transforming function (36). This is consistent with experiments involving the E1a and E1b genes and other viral and cellular oncogenes (21, 27, 30). The E1a, *myc*, and polyoma large-T antigen oncogenes are assigned to a functional class involved in establishment and immortalization which complements a second class of genes, including E1b, Ha-*ras*, and middle-T antigen, involved in the induction of the transformation phenotype. Full transformation requires the expression of a member of each class of genes.

Recent results indicate that the E1a gene itself has pleiotropic activity in the transformation process due at least in part to its multiencoding nature. During viral productive infection of human cells, the E1a gene encodes three mRNAs: 13S, 12S, and 9S (4, 8, 33). The 9S mRNA does not appear to be involved in transformation, as it has never been detected in transformed cell lines (15, 32). In contrast, the 13S and 12S mRNAs are expressed in transformed cell lines (15, 32, 41). We and others (18a, 23, 42) have recently shown that the 12S-mRNA-encoded, 243-amino-acid-residue (243R) protein is required for the complete transformation of estab-

lished cell lines. In particular, cells transformed by a 12S-defective viral mutant do not grow in an anchorage-independent manner in soft agar, a growth characteristic of cells fully transformed by wild-type (wt) virus. In this report we show that an adenovirus mutant (*dl231*) expressing only functional 289R protein encoded by the E1a 13S mRNA and E1b mRNA was capable of immortalizing primary rat cells. The cells transformed by *dl231* had a limited ability to manifest morphological and growth characteristics of transformation and therefore to provide an opportunity to dissect the immortalization function from the transformation functions encoded within the adenovirus genome.

MATERIALS AND METHODS

Cells. Rat embryo fibroblasts (REF) were prepared from 2-week-old Wistar/Furth rat embryos. Embryos were removed, minced, and disaggregated in 0.25% trypsin in phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} . Cells were washed and plated in Dulbecco modified Eagle medium containing 10% fetal calf serum. In general REF cells in passage 3 were used for transformation studies.

Cell transformation. About 2×10^5 REF cells were plated per 25-cm² flask. Cells were allowed to attach for 2 h and infected with adenovirus 2 wt or *dl231* virus at a multiplicity of 10 PFU per cell. Cells were fed with medium containing calcium and 10% fetal calf serum for 1 day and maintained on calcium-free medium with 10% fetal calf serum thereafter. Foci were counted at 6 weeks postinfection. Cell lines established from individual, transformed foci were maintained on calcium-free medium with 5% fetal calf and 5% calf sera.

Anchorage-independent growth. Established cell lines were separated into individual cells by trypsinization and suspended in 0.3% agarose containing calcium-free medium with 10% fetal calf serum at 10^4 cells per 25-cm² flask. An underlay of 0.5% agarose containing the same medium was used as a support and nutrient pool. Colony formation was analyzed at the end of 2 weeks.

RNA preparation and analysis. Cytoplasmic RNA was extracted from transformed cell lines as previously de-

* Corresponding author.

scribed (7). S1 mapping was performed by the procedure of Berk and Sharp (3) as modified by Weaver and Weissman (39) under conditions of excess DNA, as described elsewhere by Hurwitz and Chinnadurai (18a). Electrophoresis of samples was on 8% polyacrylamide sequencing gels (22). Gels were dried onto filter paper and autoradiographed at -70°C .

RNA dot hybridization analysis of cytoplasmic RNAs was by the procedure of White and Bancroft (40). For each dot, 5 μg of ethanol-precipitated RNA was dissolved in 100 μl of water. To this solution, 60 μl of $20\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)—40 μl of 37% (wt/wt) formaldehyde was added. After heating to 60°C for 15 min and quick chilling in ice water, samples were loaded onto a nitrocellulose filter by using a Bethesda Research Laboratories Hybri-Dot manifold and were washed with $10\times$ SSC. RNA was hybridized directly with nick-translated (^{32}P -labeled) probe as previously described (9). Autoradiography of baked filters was done at -70°C .

RESULTS

Mutant expressing functional 13S mRNA. The adenovirus E1a gene, located between map positions 1.2 and 4.3 of the viral genome, encodes three mRNAs, 13S, 12S, and 9S, all of which are coterminal and are produced by differential splicing of intervening sequences from precursor RNA. When translated, they produced a 289R protein, a 243R protein, and a 55R protein, respectively (Fig. 1). We have constructed a deletion mutant virus, *d1231*, which precisely lacks the E1a 13S intervening sequence and whose primary E1a transcript is colinear with the 13S mRNA (18a). In mutant *d1231* the splice junctions of E1a 13S mRNA were replaced with the corresponding segment from a 13S cDNA clone. A similar mutant has been constructed by others (42). In human cells infected with these mutants or in transient expression assays involving transfection of 13S cDNA constructs, it has been shown that splicing is not required for 13S mRNA biogenesis (18a, 35, 42). Furthermore, it has been shown that the 13S mRNA can serve as a precursor for the 12S and 9S mRNAs (35). However, due to a shift in the 3' RNA splice site junction (acceptor), only aberrant 12S or 9S mRNA can be produced from the abnormal splicing of 13S. Normally, the smaller mRNAs (12S and 9S) are produced from the splicing of the normal precursor molecule colinear with the entire E1a gene in a nonsequential manner. We have shown that mutant *d1231* produces normal amounts of 13S mRNA and greatly reduced amounts of these defective 12S and 9S mRNAs during the productive infection of human cells (18a). Therefore, we have concluded that *d1231* expresses functional 289R protein but is defective in the synthesis of E1a 243R and 55R proteins.

Transformation of primary REF. Earlier results from our laboratory have shown that mutant *d1231* induces partial transformation of established REF (Hurwitz and Chinnadurai, in press) indicating that the 243R protein plays an important role in full transformation. In this report we show that the 289R E1a protein in conjunction with E1b expression could immortalize primary REF. REF cells were prepared from 2-week-old Wistar rat embryos, infected with adenovirus 2 wt or mutant *d1231*, and tested for focal transformation as described elsewhere (18a). The frequency of mutant focus formation was about one-fifth of the wt level. The average number of foci per dish for *d1231*-infected cells was 4.1, whereas the average number of foci per dish for wt-infected cells was 18.9 (there were no foci in the

FIG. 1. Organization of the adenovirus 2 E1a gene. DNA sequence data as determined by Gingeras et al. (12) and Virtanen and Pettersson (38); protein sequence data as determined by Anderson et al. (1). The 9S mRNA is expressed only at late times in productive infection and is shown by a half solid, half open bar. m.p., Map position; b.p., base pairs.

dishes of uninfected control cells). In addition, whereas wt-induced foci were round and dense, *d1231*-induced foci were much less dense and relatively flat. This suggested that *d1231* could induce foci of actively growing cells which were not as fully transformed as those of wt-induced foci. To test whether these cells were indeed immortalized and to examine their transformation characteristics, we established four cell lines from individual wt foci and seven cell lines from individual *d1231* foci.

All cell lines were established and immortalized as judged by their ability to be continually passaged in calcium-free medium and to contain viral DNA sequences as determined by DNA dot blot analysis (data not shown).

Properties of transformed cells. Although there was variability, in general, wt-transformed cells exhibited a typical transformed cuboidal or epithelioid morphology when viewed as individual cells (Fig. 2A). In contrast *d1231*-transformed cells resembled normal untransformed cells in that they were more fibroblastic in morphology (Fig. 2B) although they were not as fully fibroblastic as primary REF cells. In confluent cultures *d1231*-transformed cells appeared less fibroblastic than they appeared as individual cells and did not grow in the parallel orientation characteristic of fibroblasts (Fig. 2D). However, these cells were highly contact inhibited, formed only monolayers, and grew to only low saturation density as opposed to the lack of contact inhibition, formation of multilayered thick mats of cells, and growth to high saturation density of the fully transformed wt cells (Fig. 2C).

All 11 cell lines were tested for anchorage-independent growth in soft agar. Representative results are shown in Fig. 3A and B. Cells transformed by the wt readily formed macroscopic colonies in soft agar within 2 weeks, whereas most of the cell lines transformed by *d1231* could not form visible colonies in that time. Cell lines *d1-1* and *d1-6* formed some relatively small colonies.

FIG. 2. Transformed REF cells. Representative cell morphologies of wt- (A) and *dl231*-transformed (B) cell lines. (C) wt-transformed cells at high saturation density; (D) *dl231*-transformed cells at low saturation density. The scale bar represents 0.1 mm.

These results demonstrated that primary REF cells could be immortalized by mutant *dl231* that expressed a single E1a protein and that these cells exhibited only slight indications of the morphological and growth characteristics of fully transformed cells.

E1a and E1b mRNAs in transformed cell lines. To verify that the morphological and growth differences found between wt- and *dl231*-transformed cells were due to their differences in E1a 12S mRNA, we analyzed the E1a and E1b RNAs in two wt- and two *dl231*-transformed cell lines. E1a cytoplasmic RNAs from wt-transformed cell line 1 (*wt-1*), *wt-2*, *dl-5*, and *dl-6* were subjected to quantitative S1 nuclease mapping analysis (Fig. 4). A ^{32}P -5'-end-labeled DNA probe colinear with E1a 13S mRNA was used to determine the relative concentrations of 13S and 12S mRNAs. All four cell lines produced readily detectable levels of E1a 13S mRNA which protected a labeled DNA fragment of 729 nucleotides. The cytoplasmic RNAs of both wt-transformed cell lines protected a labeled DNA fragment of 115 nucleotides in length. Although both the E1a 12S and 9S mRNAs would protect this DNA fragment, we attributed this protection to 12S mRNA since 9S mRNA has never been detected in any of the adenovirus-transformed cells examined. In sharp contrast, the two *dl231*-transformed cell lines produced only minute amounts of defective E1a 12S mRNA.

The E1b gene was also required for the complete transformation of rodent cells. A semiquantitative RNA dot blot analysis with a DNA probe specific to E1b revealed similar levels of E1b mRNAs in both the wt- and *dl231*-transformed cell lines (Fig. 5), indicating that the inability of the *dl231*-transformed cells to exhibit characteristics associated with

full transformation was not due to a defect in the expression of E1b.

DISCUSSION

The ability of adenovirus to fully transform rodent cells lies with its E1a and E1b genes, each of which encodes several mRNAs. The work of van der Eb and his colleagues (18) involving the transformation of primary baby rat kidney cells by DNA transfection demonstrated that the E1a gene is important for the immortalization of primary cells but that E1b is required for morphological transformation. We and others (18a, 23, 42) have shown that the E1a-12S-mRNA-encoded 243R protein is also important in conferring the morphological and growth characteristics associated with full transformation in established rat cell lines. Our present report demonstrates that the E1a 289R protein encoded by the 13S mRNA, in conjunction with E1b expression, possesses the capacity to immortalize primary cells but has minimal effect on the other characteristics of transformation for which the 243R E1a protein is required.

Our mutant virus, *dl231*, lacks the E1a 13S intervening sequence (18a) and is therefore incapable of producing normal 12S or 9S mRNAs (35). Aberrant 12S or 9S mRNAs may be produced from the abnormal splicing of the 13S mRNA. The S1 nuclease analysis of the two *dl231*-transformed cell lines revealed only minute amounts of this aberrant splicing. In sharp contrast, both of the wt-transformed cell lines produced large amounts of 12S or 9S mRNAs or both, which by this analysis would be indistinguishable. However, as E1a 9S mRNA has never been identified in any adenovirus-transformed cell line examined,

we believe that the wt-transformed cells produce E1a 12S mRNA but not 9S mRNA. All wt- and *dl*231-transformed cell lines examined produce readily detectable quantities of E1a 13S mRNAs.

The fact that our *dl*231-transformed cell lines produce such extremely low levels of aberrant 12S or 9S mRNAs or both suggests that the E1a RNA splice site acceptor of *dl*231 has only very weak acceptor activity. The only major difference between this acceptor sequence and the consensus acceptor sequence (24) is a T in the first nucleotide position of the downstream exon as opposed to the G of the consensus in that position. This only weak acceptor activity was also found in *dl*231 productively infected human cells (18a). In that case cycloheximide poisoning was able to increase the strength of the *dl*231 acceptor to wt levels. We have not yet eliminated other possible causes of the difference in levels of mRNAs produced by wt and *dl*231 virus.

The E1b gene has also been shown to be important in the transformation process as determined by analysis of virus-transformed cells (10, 11), DNA transfection (13, 37), and the use of viral mutants (6, 14, 17, 19). RNA dot blot analysis revealed that the two wt- and two *dl*231-transformed cell

FIG. 3. Colonies of transformed REF cells on soft agar. Anchorage-independent growth of transformed REF cell lines was determined by assaying colony formation in 0.3% agarose containing growth media over an underlay of 0.5% agarose containing growth media. wt-transformed cell line wt-1 colonies (A) and *dl*-5 colonies (B) are shown. Photos of living colonies were taken on a Nikon inverted-stage microscope. The scale bar represents 1 mm. The right side of the figure is a quantitation of the sizes of the colony diameters from all transformed cell lines; wt-1 through wt-4 (A through D, respectively) and *dl*-1 through *dl*-7 (E through K, respectively). % Colonies, Numbers of colonies which have grown to a particular size range divided by the total numbers of cells times 100. Individual cells which have not divided are not considered as colonies and therefore are not represented. The size ranges of colonies are represented as follows: 1, 0.06 to 0.1 mm; 2, 0.11 to 0.15 mm; 3, 0.16 to 0.2 mm; 4, 0.21 to 0.25 mm; 5, 0.26 to 0.3 mm; 6, 0.31 to 0.35 mm; 7, 0.36 to 0.4 mm; 8, 0.41 to 0.45 mm; 9, 0.46 to 0.5 mm; and 10, 0.51 to 0.55 mm. Individual cells are smaller than 0.05 mm. Quantitation was by photographic analysis.

FIG. 4. S1 nuclease analysis of E1a cytoplasmic RNAs from wt- and *dl*231-transformed cell lines. (A) S1-resistant fragments were analyzed on 8% polyacrylamide-urea gels. The DNA probe, the *Sst*II-*Xba*I fragment from the pC831 plasmid (which lacks the E1a 13S IVS) (18a), was ³²P-5'-end labeled at the *Xba*I site. Lane 1, wt-transformed cell line wt-1; lane 2, wt-2; lane 3, *dl*-5; lane 4, *dl*-6. (B) Diagram of the S1 analysis scheme. †, Protected fragments contiguous with the ³²P-5'-end label.

lines examined all produce similar quantities of E1b mRNAs. Therefore, in regard to the expression of the E1a and E1b genes, the only difference between the wt- and *dl*231-transformed cell lines is that *dl*231 lines produce only E1a 13S mRNA and not 12S.

Although the efficiency of transformation by *dl*231 virus is reduced relative to the wt, the presence of normal 289R protein encoded by 13S mRNA and E1b mRNA in the absence of other normal E1a products is sufficient to establish and immortalize primary REF cells. *dl*231-transformed cell lines were easily established from individual foci and appear immortalized as judged by their ability to be continually passaged. However, the presence of functional 289R protein without functional 243R protein is not sufficient to produce the full transformation phenotype.

The cells transformed by *dl*231 resemble untransformed cells to a high degree. Foci formed by *dl*231 are smaller and

FIG. 5. RNA dot hybridization analysis of E1b cytoplasmic RNAs from wt- and *dl231*-transformed cell lines. Cytoplasmic RNAs (lane 1, control [untransformed rat cells]; lane 2, wt-1; lane 3, wt-2; lane 4, *dl*-5; lane 5, *dl*-6) were probed with a ^{32}P -labeled, nick-translated DNA fragment extending from the *Sst*I site (map position, 5.1) to the *Hind*III site (map position, 7.9) of the pC831 plasmid. This probe is specific for E1b RNAs.

flatter, and the resultant cell lines grow more slowly than wt transformants. When viewed as individual cells, the *dl231* cell lines resemble, although they are not identical to, untransformed fibroblasts in morphology. Our *dl231* transformants are less fibroblastic in confluent cultures, but even in confluency they are much less cuboidal than the wt-transformed cells in similar confluency.

Cell lines from *dl231* foci are similar to untransformed cells in their high degree of contact inhibition. They do not grow over each other but form a single layer of cells as opposed to the wt transformants, which form thick mats. In confluency there are few cells which are rounded (thus showing division), whereas wt-transformed cells have a high level of cell division even when confluent. This results in a low saturation density of *dl231* cells in contrast to the high saturation density of wt cell lines. The ability to grow in an anchorage-independent manner in soft agar is a characteristic of full transformation. Clearly the *dl231* cell lines have a greatly diminished capacity for such growth relative to wt cell lines. We conclude that although the E1a-13S-mRNA-encoded 289R protein with E1b is able to immortalize primary cells, such cells are only minimally transformed.

The importance of the E1a 13S mRNA in the immortalization of primary cells is also indicated by the reciprocal experiment with viral mutants in which the E1a 12S mRNA is normal but the 13S mRNA is either abnormal as in mutant *hr1* (14, 28, 29) or absent as in *dl347* (42). In both cases the mutants were able to produce only an abortive transformation of primary rat cells. Practically all individual foci were unable to give rise to clonal cell lines because the *hr1*- and *dl347*-transformed cells gradually stopped dividing. Polyclonal lines were established from entire cultures of *hr1* transformations, and a rare *dl347* focus (1 out of 15) was established as a line. These manifested an abnormal transformation phenotype and were not fully transformed. It is unclear at this time what these abnormal cell lines tell us about the E1a products; however, it is clear from these experiments that lack of a normal E1a 13S mRNA greatly affects the establishment and immortalization of primary cells and is required for the maintenance of these processes.

The E1a 13S mRNA 289R product has been shown to possess a positive regulatory activity. It is responsible for the activation of the other early adenoviral genes (E1b, E2, E3, E4, and L1) during productive infection (2, 20, 25). The activation of cloned DNA constructs of cellular genes (23, 34) has also been a demonstrated activity of 289R protein. In addition, in adenovirus 12, this activity has been shown to be responsible for the suppression of expression of the class I histocompatibility antigens (5, 31). As such our mutant virus, *dl231*, which produces only functional 13S mRNA

289R protein of the E1a gene and is capable of immortalizing primary cells without eliciting the transformation phenotype, may be a powerful tool in the study of the regulation of cellular genes involved in the process of immortalization.

ACKNOWLEDGMENTS

This research was supported by Public Health Service research grants CA-31719 and CA-33616 from the National Cancer Institute. D.R.H. was supported by a Public Health Service postdoctoral training grant from the National Cancer Institute. G.C. is an established investigator of the American Heart Association.

We thank Maurice Green for critical reading and Ann Bergersen for preparation of the manuscript.

LITERATURE CITED

- Anderson, C. W., R. C. Schmitt, J. E. Smart, and J. B. Lewis. 1984. Early region 1B of adenovirus 2 encodes two coterminal proteins of 495 and 155 amino acid residues. *J. Virol.* **50**:387-396.
- 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.
- Berk, A. J., and P. A. Sharp. 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**:721-732.
- Berk, A. J., and P. A. Sharp. 1978. Structure of the adenovirus 2 early mRNAs. *Cell* **14**:659-711.
- Bernards, R., P. I. Schrier, A. Houweling, J. L. Bos, A. J. van der Eb, M. Zijlstra, and C. J. M. Melief. 1983. Tumorigenicity of cells transformed by adenovirus type 12 by evasion of T-cell immunity. *Nature (London)* **305**:776-779.
- Chinnadurai, G. 1983. Adenovirus 2 *1p*⁺ locus codes for a 19kd tumor antigen that plays an essential role in cell transformation. *Cell* **33**:759-766.
- Chinnadurai, G., H. M. Rho, R. B. Horton, and M. Green. 1976. mRNA from the transforming segment of the adenovirus 2 genome in productively infected and transformed cells. *J. Virol.* **20**:255-263.
- Chow, L. T., T. R. Broker, and J. B. Lewis. 1979. Complex splicing patterns of RNAs from the early region of adenovirus-2. *J. Mol. Biol.* **134**:265-303.
- Derman, E., K. Krauter, L. Walling, C. Weinberger, M. Ray, and J. E. Darnell, Jr. 1981. Transcriptional control in the production of liver-specific mRNAs. *Cell* **23**:731-739.
- Flint, S. J., J. Sambrook, J. F. Williams, and P. A. Sharp. 1976. Viral nucleic acid sequences in transformed cells. IV. A study of the sequences of adenovirus 5 DNA and RNA in four lines of adenovirus 5 transformed rodent cells using specific fragments of the viral genome. *Virology* **72**:456-470.
- Gallimore, P. H., P. A. Sharp, and J. Sambrook. 1974. Viral DNA in transformed cells. II. A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. *J. Mol. Biol.* **89**:49-72.
- Gingeras, T. R., D. Sciaky, R. E. Gelinis, J. Bing-Dong, C. E. Yen, M. M. Kelley, P. A. Bullock, B. L. Parsons, K. E. O'Neill, and R. J. Roberts. 1982. Nucleotide sequences from the adenovirus-2 genome. *J. Biol. Chem.* **257**:13475-13491.
- Graham, F. L., P. J. Abrahams, C. Mulder, H. L. Heijneker, S. O. Warnaar, F. A. J. der Vries, W. Fiers, and A. J. van der Eb. 1974. Studies on *in vitro* transformation by DNA fragments of human adenoviruses and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. **39**:637-650.
- Graham, F. L., T. Harrison, and J. Williams. 1978. Defective transforming capacity of adenovirus type 5 host-range mutants. *Virology* **86**:10-21.
- Green, M., W. S. M. Wold, and W. Büttner. 1981. Integration and transcription of group C human adenovirus sequences in the DNA of five lines of transformed rat cells. *J. Mol. Biol.* **151**:337-366.
- Green, M. R., R. Treisman, and T. Maniatis. 1983. Transcriptional activation of cloned human β -globin genes by viral immediate-early gene products. *Cell* **35**:137-148.
- Ho, Y.-S., R. Galos, and J. Williams. 1982. Isolation of type 5

- adenovirus mutants with a cold-sensitive host range phenotype: genetic evidence for an adenovirus transformation maintenance function. *Virology* 122:109-124.
18. Houweling, A., P. J. van den Elsen, and A. J. van der Eb. 1980. Partial transformation of primary rat cells by the left most 4.5% fragment of adenovirus 5 DNA. *Virology* 105:537-550.
 - 18a. Hurwitz, D. R., and G. Chinnadurai. 1985. Evidence that a second tumor antigen coded by adenovirus E1a region is required for efficient cell transformation. *Proc. Natl. Acad. Sci. U.S.A.* 82:163-167.
 19. Jones, N., and T. Shenk. 1979. Isolation of adenovirus type 5 host range mutants defective for transformation of rat embryo cells. *Cell* 17:683-689.
 20. 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.
 21. Land, H., L. F. Parada, and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature (London)* 304:596-602.
 22. Maxam, A., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499-560.
 23. Montell, C., G. Courtois, C. Eng, and A. Berk. 1984. Complete transformation by adenovirus 2 requires both E1a proteins. *Cell* 36:951-961.
 24. Mount, S. M. 1982. A catalogue of splice junction sequences. *Nucleic Acids Res.* 10:459-472.
 25. Nevins, J. R. 1981. Mechanism of activation of early viral transcription by the adenovirus E1a gene product. *Cell* 26:213-220.
 26. Nevins, J. R. 1982. Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1a gene product. *Cell* 29:913-919.
 27. Rassoulzadegan, M., A. Couie, A. Carr, N. Gleichenhau, R. Kamen, and F. Cuzin. 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. *Nature (London)* 300:713-718.
 28. Ricciardi, R. L., 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.
 29. Ruben, M., S. Bacchetti, and F. L. Graham. 1982. Integration and expression of viral DNA in cells transformed by host range mutants of adenovirus type 5. *J. Virol.* 41:674-685.
 30. Ruley, H. E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature (London)* 304:602-606.
 31. Schrier, P. I., R. Bernards, R. T. M. J. Vaessen, A. Houweling, and A. J. van der Eb. 1983. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. *Nature (London)* 305:771-775.
 32. Spector, D. J., D. N. Halbert, and H. J. Raskas. 1980. Regulation of integrated adenovirus sequences during adenovirus infection of transformed cell. *J. Virol.* 36:860-871.
 33. Spector, D. J., M. McGrogan, and H. J. Raskas. 1978. Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus 2 genome. *J. Mol. Biol.* 126:395-414.
 34. Svensson, C., and G. Akusjarvi. 1984. Adenovirus 2 early region 1a stimulates expression of both viral and cellular genes. *EMBO J.* 3:789-794.
 35. Svensson, C., U. Pettersson, and G. Akusjarvi. 1983. Splicing of adenovirus 2 early region 1A mRNAs is non-sequential. *J. Mol. Biol.* 165:475-499.
 36. van den Elsen, P., A. Houweling, and A. van der Eb. 1983. Expression of region E1b of human adenoviruses in the absence of region E1a is not sufficient for complete transformation. *Virology* 128:377-390.
 37. van der Eb, A. J., C. Mulder, F. L. Graham, and A. Houweling. 1977. Transformation with specific fragments of adenovirus DNAs. I. Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. *Gene* 2:115-132.
 38. Virtanen, A., and U. Pettersson. 1983. The molecular structure of the 9S mRNA from early region 1A of adenovirus serotype 2. *J. Mol. Biol.* 165:496-499.
 39. Weaver, R. F., and C. Weissman. 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5' termini of 14S β -globin mRNA precursor and mature 10S β -globin mRNA have identical map coordinates. *Nucleic Acids Res.* 7:1175-1193.
 40. White, B. A., and F. C. Bancroft. 1982. Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. *J. Biol. Chem.* 257:8569-8572.
 41. Wilson, M. C., S. G. Sawicki, M. Salditt-Georgieff, and J. E. Darnell. 1978. Adenovirus type 2 mRNA in transformed cells: map positions and difference in transport time. *J. Virol.* 25:97-103.
 42. Winberg, G., and T. Shenk. 1984. Dissection of overlapping functions within the adenovirus type 5 E1a gene. *EMBO J.* 3:1907-1912.