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Morphological Transformation of Established Rodent Cell Lines by High-Level Expression of the Adenovirus Type 2 Ela Gene

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When a strong promoter derived from the mouse metallothionein gene was substituted for the homologous adenovirus type 2 Ela promoter, leading to enhanced levels of Ela RNAs and proteins in cells transfected with the chimeric gene, the Ela gene alone was able to induce in established cell lines alterations in cellular morphology and growth properties similar to those produced by the combined action of Ela and Elb genes. The qualitative effects of Ela gene expression upon cellular properties thus depend on the level of expression of the Ela gene. Furthermore, Ela may be the primary transforming gene of adenoviruses, since it produced many of the characteristics of transformed cells that had previously been attributed to Elb.

The transformation of cells in vitro by oncogenes has been considered a two-step process in which primary cells are first immortalized (by genes such as *myc* or polyoma large T) to produce established cell lines of indefinite proliferative capacity and then further transformed (by genes such as *ras* or polyoma middle T) to produce cells of altered morphology that are less susceptible to arrest of growth by contact inhibition and other factors (10, 13, 19). Additional factors determine the degree to which these fully transformed cells are tumorigenic in vivo. Application of this model to the transformation of rodent cells in culture by human adenoviruses (Ad) identifies Ela gene products as necessary and sufficient for the immortalization step (10, 19). Elb gene products were thought to be necessary for full transformation although the results of more recent experiments in which Elb genes were expressed in established cell lines in the absence of Ela imply that Elb is not sufficient for the second step (23). Moreover, the morphology and other properties of cells transformed by the entire E1 region depend in part on which of two different Ad serotypes is used as a source of Ela genes (24). Thus the assignment of immortalization and morphological transformation functions to the Ela and Elb regions, respectively, may not be as simple as supposed. The situation is further complicated by the multiplicity of E1 proteins produced by the use of alternative splice sites in both Ela and Elb genes and the use of two different initiation codons in Elb (see Fig. 1 for details).

We have constructed a series of plasmids as a means of studying the effects upon transforming function of the expression in cells of various amounts of particular Ad type 2 (Ad2) E1 proteins. These plasmids were transfected into established lines of contact-inhibited rodent cells to determine the potential of these gene products to morphologically transform already immortalized cells. We report here that the level of expression of Ela gene products has profound effects on cellular properties: when expressed at high levels, Ela proteins alone are able to induce many of the attributes of morphological transformation that were previously assigned to Elb proteins.

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MATERIALS AND METHODS

Construction of plasmids. All plasmids used are derivatives of pBR322 and were constructed by standard methods. The Ad and metallothionein (MT) sequences inserted into each plasmid are shown in Fig. 1 and described in its legend.

Transformation assays. Transfection of plasmid DNAs into NIH 3T3 cells was performed by the calcium phosphate-DNA coprecipitation method of van der Eb and Graham (25). To each 60-mm dish containing 3×10^5 cells seeded the previous day was added a precipitate formed from approximately 1 to 1.5 μg of plasmid DNA (amounts were adjusted for size so that equimolar amounts of each plasmid were used) and 10 μg of calf thymus carrier DNA. To assay for focus formation, cultures were maintained for 3 to 4 weeks in Dulbecco modified Eagle medium (DME) plus 10% fetal bovine serum before fixing and staining.

In some experiments G418-resistant transfected cells were isolated after transfection as above with addition of 50 ng of the plasmid pko.neo (26) to each transfection mixture and subsequent selection by the addition of 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml to the culture medium. Each G418-resistant population consisted of the cells pooled from two such transfected dishes and contained a few dozen clones.

To measure anchorage-independent growth, 5×10^3 cells from each population were seeded in 0.37% agar over a 0.90% agar base; both layers contained DME plus 10% fetal bovine serum. Liquid medium (DME plus 10% fetal bovine serum) overlaying the agar was changed weekly.

Analysis of RNA from transfected cells. Total cellular RNA was isolated from populations of G418-resistant cells transfected with various region E1 plasmids, essentially by the method of Favaloro et al. (6), with buffers containing 10 mM vanadyl ribonucleoside complex. A hybridization probe to detect the alternative 5′ exons of Ela mRNAs was labeled by digesting the plasmid pMTEla (Fig. 1) at the 5′ end of the Ela gene with *Bam*HI, removing approximately 40 nucleotides from the 3′ end of the complementary strand with exonuclease III, and filling in the ends with the Klenow fragment of DNA polymerase by using low-specific-activity [^{32}P]dCTP (300 Ci/mmol), followed by digestion with *Eco*RI and agarose gel electrophoresis to isolate the labeled DNA fragment. Hybridizations (6) were performed at 54°C, fol-

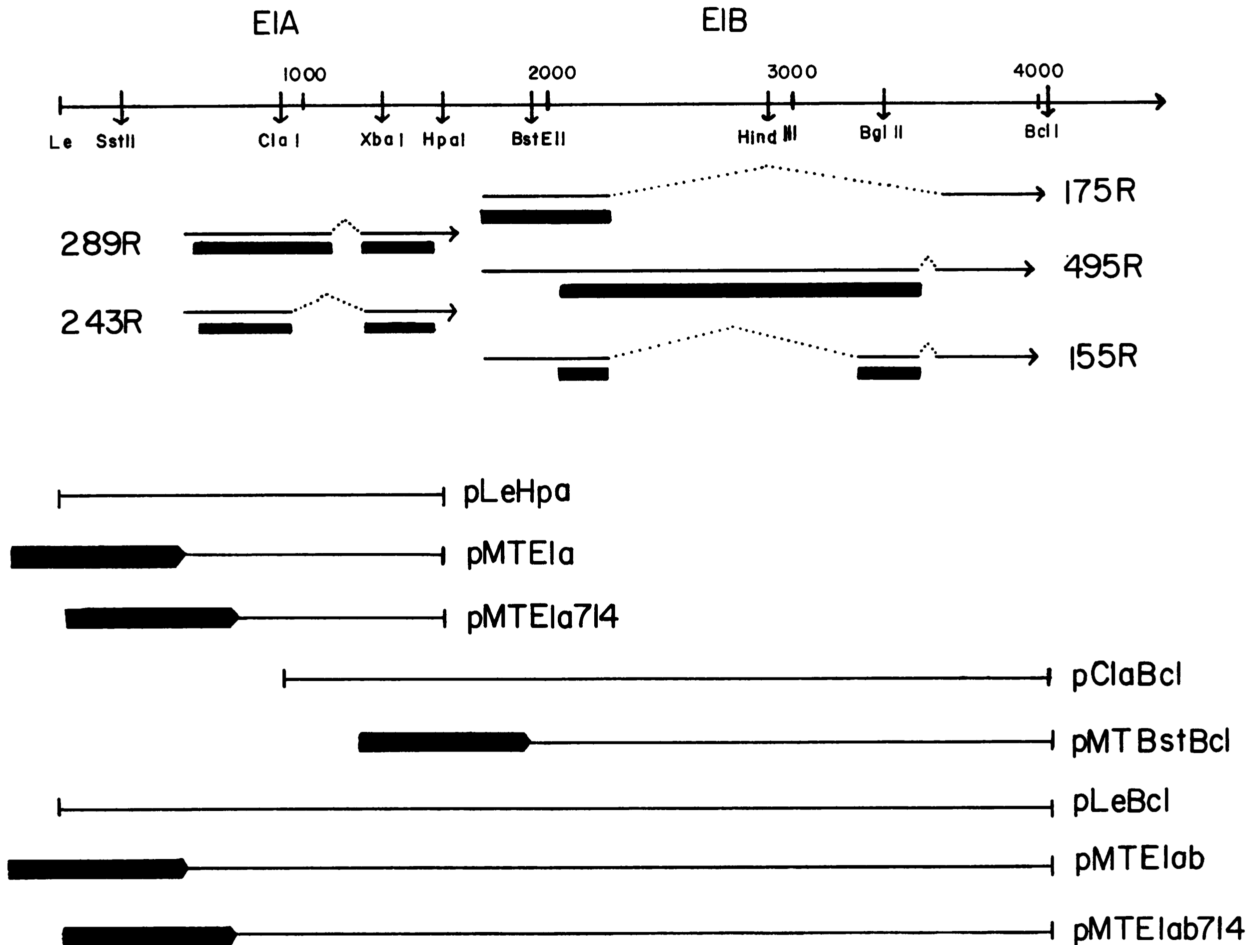

FIG. 1. Map of adenovirus region E1 DNA, mRNAs, proteins and plasmids. The line at the top represents approximately 4.5 kilobases of DNA from the left end (Le) of the Ad genome. Map coordinates in nucleotides (8) are indicated above the line for positions marked by small hatch marks. Down-pointing arrows indicate sites of restriction enzyme cleavage for enzymes identified below the line. mRNAs are indicated by thin lines; intron sequences removed by splicing from each of the mRNAs are indicated by dotted lines. All E1 mRNAs are transcribed from left to right on this map. The translated regions for each of the five E1 proteins are shown by thick lines. Each protein is designated according to its number of amino acid residues (R), as suggested in reference 1. Although the Elb 175R protein is shown associated with the smallest of the Elb mRNAs, it can be translated from all Elb mRNAs shown. Only the major, well-characterized mRNAs and proteins are shown; other Ela and Elb RNA and protein species are believed to exist in vivo. The bottom portion of the figure shows the extent of Ad DNA sequences found in the plasmids used in these experiments. MT gene promoter-regulatory sequences are indicated by the thick arrow pointing to the right; this segment is not drawn to scale with the viral sequences. pLeHpa contains viral DNA sequences derived from Ad5 (by using pLA1 [22]) between the left end of the viral genome and the *Sst*II site (nucleotide 353 of the Ad2 sequence) and from Ad2 between N353 and the *Hpa*I site (N1596) and thus codes for Ad2 Ela proteins under Ad control. pMTEla contains approximately 1.7 kilobases of mouse metallothionein-I DNA (21) located from −1,700 to −12 relative to the MT cap site joined by a *Bam*HI linker to Ad2 DNA extending from a *Hae*III site (N494) located just 5′ to the Ela cap site to the Hpa site (N1569) and thus codes for Ela proteins under MT promoter control. pMTEla714 has the same segment of MT DNA joined to Ela DNA extending from a *Hae*III site at N714 to the *Hpa*I site (N1569); this plasmid may code for N-terminally truncated Ela proteins lacking 70 amino acid residues. pClaBcl contains Ad2 DNA located between *Cla*I (N916) and *Bcl*I (N4036) and codes for all Elb proteins under Elb promoter control. pMTBstBcl contains the 1.7-kilobases MT-I DNA segment joined through a *Bam*HI linker to Ad2 DNA extending from a *Bst*EII site (N1912) to the *Bcl* site (N4036) and should code for the Elb 155R and 495R proteins, but not the Elb 175R protein, under MT promoter control. pLeBcl contains the viral sequences contained in pLeHpa and extends to the *Bcl*I site (N4036) and thus codes for both Ela and Elb proteins under Ad control. pMTElab and pMTElab714 contain the same MT and Ela sequences as pMTEla and pMTEla714, respectively, but extend to N4036 and thus also code for Elb proteins.

lowed by S1 nuclease digestion at 37°C. Each assay contained 30 μg of cellular RNA or 2 μg of RNA from HeLa cells infected with Ad2 and 5 ng (5,000 cpm) of the DNA probes. S1 nuclease-digested hybrids were analyzed by electrophoresis on 6% polyacrylamide–8 M urea gels following denaturing by boiling in 90% formamide.

Analysis of protein from transfected cells. An antiserum to the Ela 289R and 243R proteins was prepared essentially as described by Yee et al. (27); for immunization a peptide homologous to the C-terminal five residues of the Ela proteins was used. Rabbit antiserum was affinity purified as described previously (1). A more detailed characterization of this antiserum will be presented elsewhere (R. C. Schmitt, M. L. Fahnestock, and J. B. Lewis, manuscript in preparation). Whole-cell extracts were prepared and used for immune precipitation as previously described (14).

Southern blot analysis of DNA from transfected cells. A 15-μg portion of DNA from each population of transfected

cells was digested with *Eco*RI and *Hin*dIII. For comparison, amounts of transfected plasmid DNA equivalent to 10 molecules per cell, assuming an average DNA content of 10 pg per NIH 3T3 cell, were mixed with 15 μg of calf thymus DNA before digestion with *Eco*RI and *Hin*dIII. Agarose gel electrophoresis, transfer to nitrocellulose, nick translation, hybridization, and washing conditions were as described by Brinster et al. (4).

RESULTS

Focus formation with NIH 3T3 cells. Various plasmids (Fig. 1) containing E1a or E1b genes or both were transfected into NIH 3T3 cells, and the foci of transformed cells which overgrew the monolayer were counted and judged for size and density (Table 1; Fig. 2). As expected, a plasmid containing only the E1a gene with its own promoter (pLeHpa) gave rise to a few small, poorly stained foci. Transfection of the E1b gene (pClaBcl) alone gave rise to few, if any, transformants. However, transfection of both E1a and E1b genes together, either on separate plasmids (pLeHpa plus pClaBcl) or on a single plasmid (pLeBcl), yielded many more foci, which were also larger and more densely stained than the few observed with E1a alone. Thus E1a and E1b genes can cooperate to give efficient transformation. The efficiency of this complementation is lower when the two genes are on separate plasmids. This lower efficiency may be due to the loss of interactions between the two genes or their associated sequences when they are physically separated (for example, a possible effect of enhancer elements located in E1a upon expression of E1b), or it may conceivably be due to a lower probability of getting both genes into a single transfected cell.

The results were dramatically different when the E1a (Ad) upstream regulatory sequences were replaced by the mouse

TABLE 1. Transformation assays

Plasmid	Focus formation[a]				Growth in soft agar[b]	
	Expt 1		Expt 2		No. of microscopic colonies	No. of macroscopic colonies
	No. of foci	Size	No. of foci	Size		
pBR322	2	+	0	−	61	0
pLeHpa	15	+	16	+	43	0
pClaBcl	9	+	0	−	52	0
pLeHpa + pClaBcl	79	++	63	++	163	76
pLeBcl	196	++++	65	+++	431	171
pMTE1a	192	+++	52	+++	309	215
pMTE1a + pClaBcl	190	+++	ND	ND	321	135
pMTE1ab	237	++++	77	++++	297	123
pMTE1a714	ND	ND	2	+		
pMTE1ab714	2	+	ND	ND		
pLeHpa + pMTBstBcl	17	+	ND	ND		

[a] NIH 3T3 cell monolayers were transfected as described in Materials and Methods. The extent of transformation was measured both by counting the number of foci and by judging the typical size and density of staining of the foci on each dish. The numbers shown for each experiment are the sum of duplicate dishes; all duplicates were very similar. ND, Not done.

[b] Populations of G418-resistant NIH 3T3 cells that had been cotransfected with the plasmids indicated were assayed for growth in soft agar as described in Materials and Methods. After 5 weeks in culture, all visible colonies of any size were counted (microscopic agar colonies). After an additional 4 weeks, colonies of approximately 1 mm or greater were counted (macroscopic agar colonies); the other (microscopic) colonies were still only marginally visible.

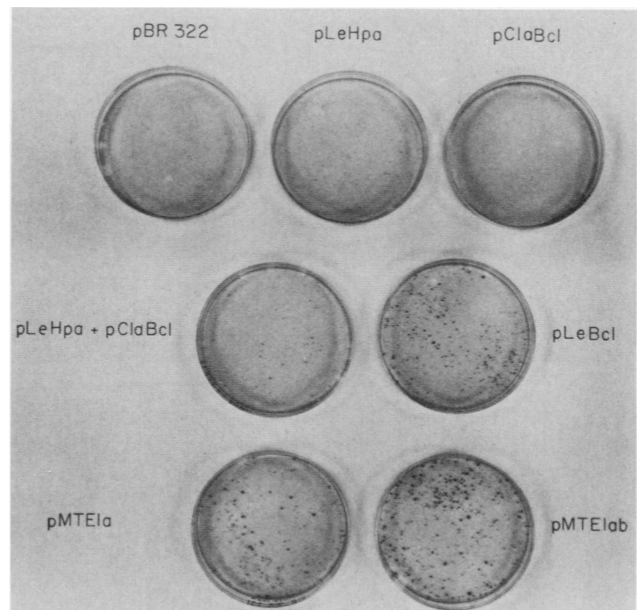

FIG. 2. Focus formation by Ad2 E1 plasmids on NIH 3T3 cells. Transformation assays were performed as described in Materials and Methods.

(MT) upstream regulatory sequences. These experiments did not involve the induction of MT-regulated expression by the addition of metal ions, but instead relied on the level of expression induced by the normal components of serum and medium. The MT promoter seems to be comparatively strong even without induction (4, 15), so that it might promote higher levels of synthesis of E1 products than is found with the Ad promoter. Transfection with either pMTE1ab or pMTE1a gave many foci. pMTE1a was approximately as efficient as AdE1ab (pLeBcl), and the presence of E1b in the MT plasmid had only a marginal effect. Transformation required intact E1a protein, since constructions (pMTE1a714 or pMTE1ab714) in which the MT promoter has been fused to the E1a gene within the coding region and thus can only produce E1a proteins lacking 70 aminoterminal residues, are inactive in the transformation assay. Formally, pMTE1a might be active in transformation because the 1.7-kilobase (kb) region of MT DNA contains an undescribed oncogene. This possibility was ruled out by the inability of plasmids containing the MT promoter linked to fragments of E1b (pMTBstBcl) or to fragments of other genes (E1a or thymidine kinase, data not shown) to complement pLeHpa in the focus assay.

Morphological and growth characteristics of E1a transformed cells. The above results suggest that the E1a gene is potentially sufficient for full morphological transformation of established lines of rodent cells and that the MT promoter is formally equivalent to the presence of E1b genes. If so, cells transformed either by E1a plus E1b or by pMTE1a should have similar properties. To further characterize such cells, various E1 plasmids were transfected into NIH 3T3 cells along with a plasmid for resistance to the neomycin derivative G418 (or, not shown, into Rat2 cells along with a thymidine kinase plasmid). Transformation frequencies were judged as the percentage of G418-resistant or thymidine kinase-positive colonies that showed altered morphology, as described below, and increased density, and for fully competent plasmids these frequencies ranged from 30 to 90%.

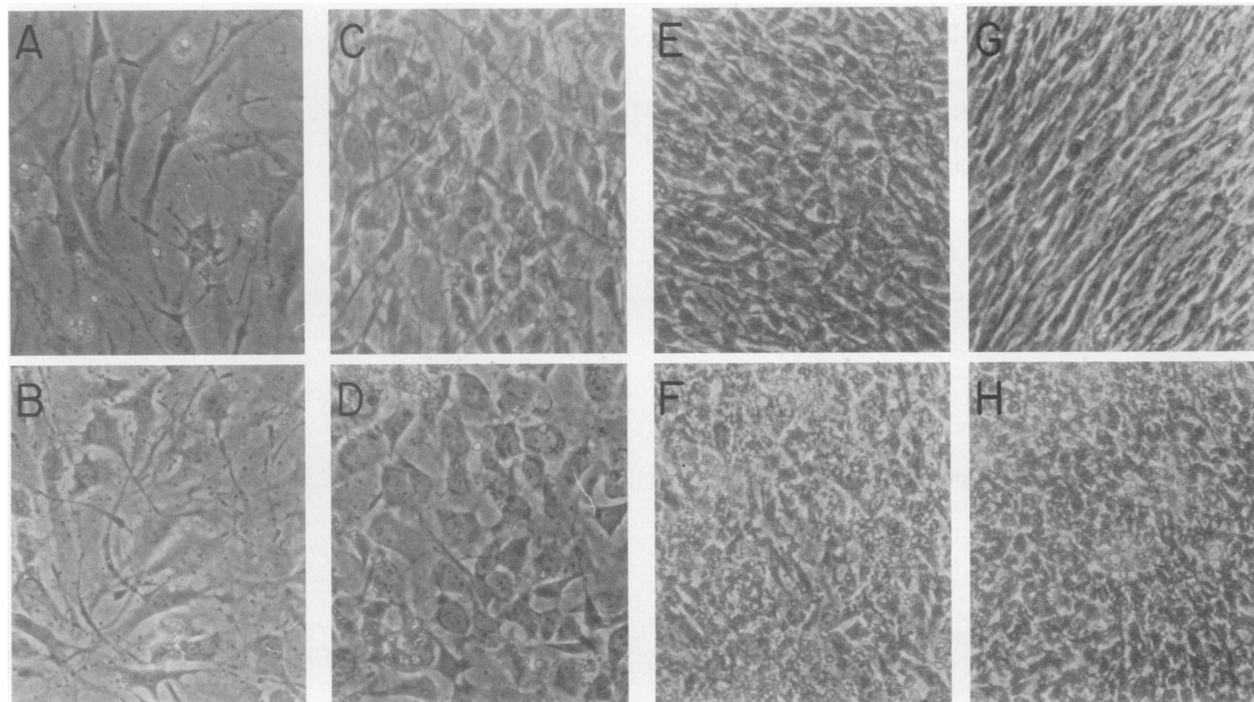

FIG. 3. Morphology of NIH 3T3 cells transfected with Ad2 E1 genes. Representative regions of G418-resistant colonies photographed after transfection of NIH 3T3 cells with pko.neo plus pBR322 (panels A and B), pLeHpa (panels C and D), pMTEla (panels E and F), or pLeBcl (panels G and H).

Such frequencies are typical for cotransfection of any two markers, so that transformation by the Ad2 E1 region is intrinsically efficient, confirming the results of van den Elsen et al. (24). The results of these colony experiments (transformation frequencies not shown) are very similar to those of the focus formation experiments in Table 1. The Ad Ela plasmid alone is not able to efficiently induce transformed colonies, but the Ela and Elb genes together, or Ela under MT control, do transform efficiently.

Visual analysis (Fig. 3) of such colonies shows that cells transfected with pBR322 (panels A and B) or with Elb alone (not shown) maintain the extremely flattened appearance of the parental cells and cease growing when they become a confluent monolayer. Cells transfected with pLeHpa (Ad Ela; panels C and D) maintain some of the morphological characteristics of their parents. These cells continue to grow as a flat, tightly adherent monolayer; however, they are generally somewhat smaller, have a larger nuclear-to-cytoplasmic ratio, and are frequently organized into fairly regular arrays of polygonal or elongated cells. Occasional colonies contain some cells that resemble the cells described below. Cells transfected with pLeBcl (AdElab; panels G and H), pMTEla (panels E and F), and pMTElab (not shown) form a third morphological class. These cells are much smaller, adopt a loosely attached rounded or cylindrical shape, and readily overgrow one another in a highly disorganized manner (panels F and H). Foci containing large numbers of very small cells piled up several layers deep are found scattered throughout dense colonies. When at low density or on the edges of isolated colonies (panels E and G), these cells may take on a more flattened polygonal or elongated shape similar to that seen with the Ad Ela cells, but this morphology is lost in the presence of any crowding.

For further studies, we analyzed populations of cells, containing several dozen independent colonies each, generated by transfection performed by using a low ratio of G418 resistance to E1 plasmids, so that nearly all G418-resistant colonies cotransfected with appropriate plasmids (pLeBcl, pMTEla, pMTElab) were transformed. We used pools of cells selected for drug resistance rather than clonal cell lines to avoid any biases in the selection of particular cells for study and because we hoped that pooled populations would give results close to the average of many lines, avoiding the considerable variation in the level of expression of transfected genes that is often observed among clones. However, as judged by visual morphology, these populations did appear to be fairly homogeneous.

As with the morphology of single colonies, the growth properties of these populations fell into three classes. The growth rate of these cells was not significantly affected by the presence of E1 DNA; in 10% serum all cell types grew with a doubling time of approximately 20 h, while in 1% serum they all grew very poorly. However, as expected from our visual observation, they differed greatly in their maximum cell density. The pBR322 contransfected cells grew to a final density of 3×10^6 to 4×10^6 cells per 60-cm dish, as did the parental NIH 3T3 cells. The population transfected with Ad Ela (pLeHpa) grew to a somewhat higher density, 10×10^6. The Ad Elab (pLeBcl), pMTEla, and pMTElab populations all behaved similarly, growing rapidly to a density of 30×10^6, with slower patchy overgrowth later yielding progressively higher densities so that no final saturation density could be clearly defined.

Anchorage-independent growth in soft agar yielded only two classes of cells (Table 1). At 1 month after plating, the pBR322 and Ad Ela (pLeHpa) populations gave rise to a few microscopic colonies (a few dozen cells each) which failed to grow further. The Ad Elab (pLeBcl), Ad Ela plus Ad Elb, pMTEla, and pMTElab populations all had a 5- to 10-fold-higher cloning efficiency; furthermore, even at 1 month

many of these colonies were significantly larger than those of the first class, and they continued to grow until they were several millimeters in diameter.

We thus found that the morphology and both anchorage-dependent and anchorage-independent growth properties of the NIH 3T3 cells transformed with the pMTE1a gene were very similar to those of cells transformed with the E1a and E1b genes together, while the cells transfected with the Ad E1a plasmid alone were generally affected to a much lesser extent, if at all. That these states may be different points along a single continuum was suggested by observations of rare Ad E1a colonies containing somewhat more transformed properties, and of some colonies in the pMTE1a and Ad E1ab populations with the lower density and flat polygonal morphology characteristic of Ad E1a cells.

Expression of E1a mRNAs and proteins. We investigated the possible relationship between transformation potential and level of E1a gene expression by analyzing both RNA and [^{35}S]methionine-labeled protein from our transfected cell populations. To measure E1a mRNA levels total cellular RNA was isolated and hybridized to a DNA fragment labeled at the 5' end of the E1a gene on the complementary strand; hybrids were then digested with S1 nuclease and denatured, and the products were analyzed by polyacrylamide gel electrophoresis (Fig. 4). This protocol allowed us to specifically measure the alternative 5' exons that can be produced from the E1a gene. The major E1a mRNAs of 13S and 12S, which code for the 289R and 243R proteins, respectively, generate protected fragments of 615 and 475 nucleotides, respectively, and the 9S species that is observed in infected cells at late times gives a 120-nucleotide protected fragment. We detected the major 13S and 12S mRNAs, but not the 9S mRNA, in all of our cell populations that contain the E1a gene. However, the levels of the E1a mRNA varied markedly; the pLeHpa (Ad E1a) cells had low levels of E1a mRNA, while pLeBcl (Ad E1ab), pMTE1a, and pMTE1ab cells had progressively higher levels. Even the highest levels, however, were much lower than those found in Ad2-infected HeLa cells (track f had 15-fold less RNA). As expected, the MT promoter was significantly stronger than the viral E1a promoter, inducing about 10- to 15-fold-higher levels of E1a gene expression. The formal equivalence in focus formation of the presence of the MT promoter to the presence of region E1b was also reflected in this assay, since the presence of the E1b gene results in four- to fivefold-higher levels of E1a mRNA. The effects of the MT promoter and the E1b gene on E1a expression appear to be independent and additive.

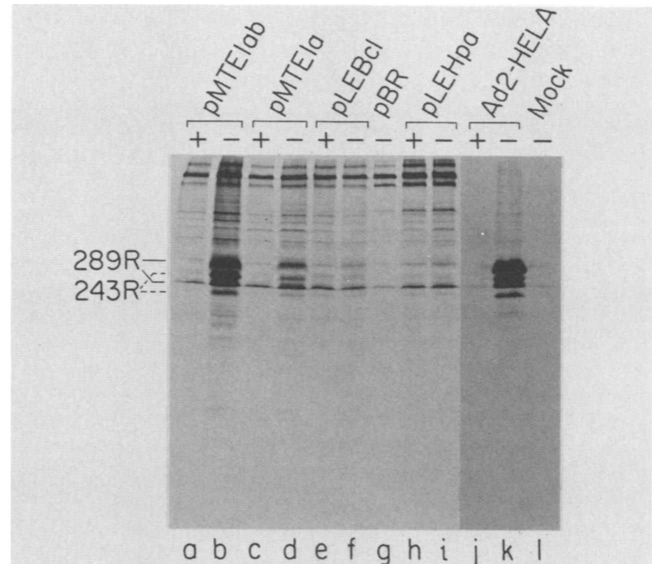

FIG. 5. Analysis of proteins from transfected cells. Transfected cells (lanes a through i), Ad2-infected HeLa cells (lanes j and k), or mock-infected HeLa cells (lane l) were labeled by incubation with 250 μCi of [^{35}S]methionine in 3 ml of methionine-free medium containing 5% fetal bovine serum per 100-mm dish for 3 h. To control for nonspecific precipitation of cellular proteins, the antibodies used in the lanes marked (+) were first incubated with an excess (20 μg) of the peptide used for immunization. Fluorography of the sodium dodecyl sulfate-17.5% acrylamide electropherogram was for 7 days (lanes a through i) or 1 day (lanes j through l).

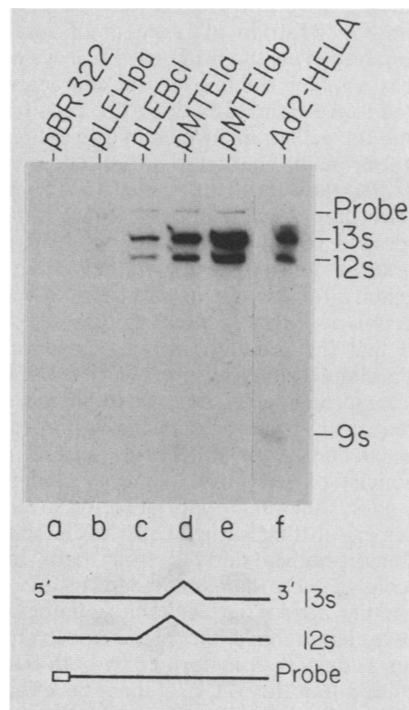

FIG. 4. Analysis of RNA from transfected cells. RNA extracted from populations of NIH 3T3 cells transfected with pko.neo plus plasmids containing Ad2 E1 genes was hybridized to a 3' end-labeled complentary strand DNA probe (structures of the two major E1a RNA species and of the probe are shown above); hybrids were digested with S1 nuclease and analyzed by polyacrylamide gel electrophoresis.

We analyzed [^{35}S]methionine-pulse-labeled proteins from these cells by immunoprecipitation with an antibody raised against a synthetic peptide homologous to the C terminus of the E1a 289R and 243R proteins (Fig. 5). This antiserum specifically immunoprecipitates four polypeptides from Ad2-infected HeLa cells that migrate with the four polypeptides synthesized in vitro from purified E1a RNA (data not shown). Each of the two major E1a mRNAs gives rise to two polypeptide forms, presumably owing to posttranslational modification (27). In addition to nonspecific precipitation of several cellular polypeptides, the anti-E1a serum specifically (the reaction is blocked by preincubation of the antiserum with the synthetic peptide) precipitates the four expected polypeptides from the E1a-transfected cells. Although the presence of host cell polypeptides in the E1a region of the gel made quantitation difficult, the results were qualitatively similar to those found with mRNA. Cells transfected by

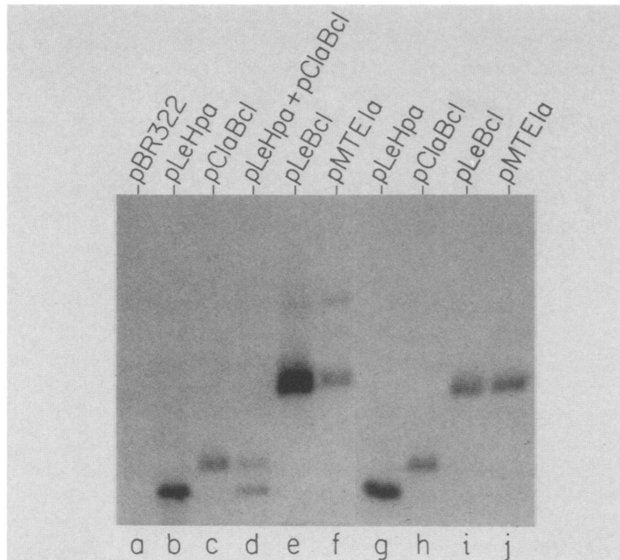

FIG. 6. Analysis of DNA from transfected cells. DNA from transfected cell populations (lanes a through f) or plasmid DNAs (lanes g through j) were digested with *Eco*RI and *Hin*dIII, analyzed by agarose gel electrophoresis, transfered to nitrocellulose, and hybridized to a nick-translated probe containing the entire E1a coding region (N494 to N1569). Autoradiography of cellular DNA samples (lanes a through f) was for 22 h; autoradiography of plasmid DNA samples (lanes g through i), which contained approximately 10 molecules of plasmid DNA per cell DNA equivalent, was for 110 min.

pMTE1ab synthesized the highest level of E1a protein, with less in cells transfected with pMTE1a and still lower (not measurable owing to the background of nonspecifically precipitated cellular proteins) amounts in cells transfected by pLeBcl (Ad E1ab) and pLeHpa (Ad E1a).

The higher levels of E1a RNA and protein seen in transformed cells that contain either the MT promoter or region E1b are most probably due to higher levels of expression of the cotransfected E1a gene(s) induced by either of these two elements. To eliminate an alternative possibility, that the transfection frequency of E1a genes is higher in the presence of E1b or the MT elements, Southern blot analysis for E1a and E1b sequences was performed on several populations of transfected cells (Fig. 6). An average of one to two copies of E1 sequences per cell were contained in each population, with no more E1a DNA in MT E1a or Ad E1ab than in Ad E1a cell populations. Thus, increased gene dosage cannot account for increased levels of E1a gene products in the first two cases compared with the last.

DISCUSSION

Efficiency of transformation by adenoviral oncogenes. We have examined the two-step model of adenoviral transformation by asking which Ad2 E1 genes are sufficient to produce foci of cells altered in morphology and growth properties when transfected into established rodent cells. In some experiments the appearance of foci that escaped the growth restrictions imposed by the monolayer was assayed. In others, cells were cotransfected with E1 plasmids and a plasmid containing a selectable marker, and the properties of those G418-resistant or TK^+ colonies that also appeared morphologically transformed were assayed. Although transformation following infection by Ad or transfection of Ad DNA is inefficient, the high percentage (30 to 90%) or morphologically transformed colonies seen with appropriate E1 constructions in the experiments involving cotransfection with a dominant selectable marker demonstrates that Ad E1 genes are capable of efficient transformation when taken up by cells capable of expressing foreign genes and are thus dominant transforming oncogenes. Consequently, the limiting factor in transformation by Ad is likely to be viral functions involved in inserting viral DNA into the cell in a way in which it can be efficiently expressed, rather than the functioning of the E1 oncogenes once expressed. Evidence for the role of the former functions in viral transformation is provided by group II host range mutants, which are defective for transformation by virus but wild type for transformation via transfection (18). The polyoma middle T antigen has similarly been shown to be a much more efficient oncogene when introduced via means other than polyoma virus infection (5).

Increased transforming capacity of the E1a gene expressed at high levels. The limited transformation capability previously ascribed to E1a (10) on the basis of its inability to fully transform primary rodent cells is reflected in these results by the inability of the same E1a sequences to efficiently transform established rodent cell lines. A comparison of the capacity to transform cells of plasmids in which Ad E1a genes are controlled by homologous (Ad) upstream sequences and by plasmids controlled by heterologous (MT) upstream sequences demonstrates that this limited capability originates from the regulation of E1a expression, not from intrinsic limitations of E1a gene products. Thus the Ad E1a construction exhibited very limited capacity for morphological transformation, while the MT E1a construction was essentially as capable as either the Ad E1ab or MT E1ab constructions. We have not proved that the MT E1a gene will substitute for E1ab in all aspects of morphological transformation; however, the properties that we have investigated address a major fraction of the characteristics that were attributed to Ad transformation. The cells transformed by MT E1a and the cells transformed by the entire E1 region share at least four major characteristics of adenovirus transformed cells: (i) they produce cells that overgrow the monolayer, (ii) they have similarly altered morphology (much smaller and rounded), (iii) they have a greatly increased saturation density, and (iv) they have a greatly increased capacity for growth in soft agar. None of these genes appeared to appreciably affect the growth rate of NIH 3T3 cells, so that the production of foci cannot be easily attributed to a simple increase in growth rate. Characteristics of Ad-transformed cells that we have not examined include altered serum requirements, growth at low calcium levels, changes in fibronectin or other cell surface molecules, and tumorigenicity of cells in vivo. Many of these characteristics, however, including tumorigenicity, are not universally attributes of Ad-transformed cells, even including lines of epitheloid morphology derived from virus infection of primary rat cells (7). Further studies are clearly needed to determine whether appropriate cells morphologically transformed by E1a alone have the same spectrum of these characteristics as do cells transformed by both E1a and E1b.

Our supposition that the MT E1a chimeras exhibit greater capability for transformation because of higher levels of E1a expression was confirmed by S1 nuclease analysis of E1a mRNA and by radioimmune precipitation of E1a proteins in populations of transformed cells. Although the immunoprecipitation experiments are not as sensitive as the RNA determinations, both approaches demonstrate that transformation efficiency correlates with increased levels of expres-

sion up to the level seen with either MT E1a or Ad E1ab. The further increase in expression seen with MT E1ab is not correlated with a further increase in the efficiency of transformation, presumably reflecting a plateau of maximum effect of E1a gene products upon the process of transformation. These experiments also indicate that at least one effect of the presence of E1b genes is to increase the level of E1a gene expression, so that both increased transformation and increased E1a expression can be achieved either by the presence of E1b or by regulation by MT sequences. The model of transformation favored by our results is that although E1a may be capable of immortalizing primary cells at low levels of expression, it is also capable of morphologically transforming established cell lines at a higher level of expression. We have not yet done experiments to measure the levels of E1a required for immortalization, but van den Elsen et al. (24) have reported that some cell lines partially transformed (immortalized) by E1a alone have unusually low levels of E1a RNA.

Montell et al. (16) recently demonstrated that both of the E1a proteins (289R and 243R) are involved in the process of full transformation by Ad. Although expression of both types of E1a protein is increased by the MT promoter, we do not yet know whether increased expression of only one would be sufficient.

The higher level of E1a gene expression that occurred with the MT promoter could reflect either a higher intrinsic strength of the MT promoter than the E1a promoter or differences in the manner in which the two promoters respond to levels of the two E1a proteins, which have been shown to regulate transcription both positively and negatively from a variety of promoters, including the E1a promoter (3, 16). An issue that we have not yet addressed is whether the important factor in enhanced transformation is (i) the overall increase in the level of E1a gene expression, (ii) a change in the level of E1a expression when the cell is at a particular point of the cell cycle, (iii) changes in E1a expression when cell growth is inhibited by culture conditions.

Implications for the two-step model of transformation. The results described above are in conflict with the simplest form of the two-step model of transformation, which suggest that separate classes of genes are responsible for induction of immortalization and of morphological transformation. Although E1a gene products may be intrinsically more active as immortalizing agents, they are also active in morphological transformation. Similar crossover in activity between the two classes of oncogenes has been observed for other prototypical oncogenes. Spandidos and Wilkie (20) and Yoakum et al. (28) reported that appropriate *ras* gene containing plasmids can, in at least some cell types, produce both immortalization and malignant transformation. Similarly, Keath et al. (12) demonstrated that the introduction into NIH 3T3 or Rat2 cells of the c-*myc* oncogene linked to viral promoters could induce tumorigenic cell lines. These results, as well as ours and those of Land et al. (13) and Ruley (19), are not all necessarily directly comparable, since there may be important differences between different cell types with respect to gene functions necessary for morphological transformation. For example, it will be of especial interest to determine whether high levels of expression of E1a will also morphologically transform primary cells. One could imagine that NIH 3T3 cells are not only immortalized but also partially transformed, so that enhanced levels of E1a might provide the functions needed to morphologically transform these cells, but not primary cells. Taken together, these results suggest that the division of the transformation process into immortalization and morphological changes is artificial and at least partially misleading. Rather, these two types of phenotypic change may actually represent different partial disruptions of a single mechanism regulating cellular growth and structure.

Modulation of E1a expression by E1b. A major implication of the results described above is that the presence of the E1b gene significantly increases the level of expression of E1a gene products, the reverse of the well-established role of E1a in enhancing the expression of E1b (2, 11). This effect of E1b on E1a expression correlates with an enhancement of transforming activity and may represent a role of E1b in transformation, as has also been suggested by van den Elsen et al. (24). The presence of the E1b gene could increase E1a gene expression, either because of the presence of *cis*-acting regulatory signals contained within the E1b region, or because of a *trans*-acting interaction of an E1b protein with E1a regulatory signals, located either upstream of (9) or within the transcribed portion of the E1a gene (17). Preliminary results from transient expression experiments performed with both NIH 3T3 and HeLa cells demonstrate that at least a large fraction of the stimulation of E1a gene expression by cotransfection of E1b occurs even if E1a and E1b genes are on separate plasmids, and is thus due to a *trans*-acting E1b function (unpublished data). However, *cis*-acting sequences may also contribute to the effect of E1b upon E1a. We currently do not know whether this effect is at the level of transcription, RNA processing, or mRNA stabilization.

Role of E1b in transformation. Finally, we do not wish to imply that the E1b gene necessarily plays no role in transformation beyond regulating E1a expression. Results of preliminary experiments with plasmids in which either the 175 or the 495 codon reading frame of E1b has been interrupted suggest that different E1b gene products are required for the complementation of E1a in the transformation (focus formation) assay and for the stimulation of E1a expression in the transient expression assay (unpublished results). Furthermore, although the cells transformed by the cooperation of E1a and E1b genes and those transformed by the MT E1a gene are similar with respect to four major features of morphological transformation, more detailed studies may reveal some differences between the two cell types. Alternatively, the two types of cells may be transformed by very different molecular mechanisms even if their phenotypes are similar. The E1b gene may have activities (other than the regulation of E1a expression) that are normally important for transformation, but which become dispensable if E1a proteins are expressed at sufficient levels, much as *myc* and *ras* oncogenes can cooperate to induce full tranformation when expressed at low levels, but can apparently act independently when expressed at high levels. The relative importance of the two sets of genes may also depend on the cellular environment in which they function. E1a and E1b genes may well have evolved as a single functional unit and cooperate not only by regulating the expression of each other but also by working together in other ways.

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