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A Novel General Approach to Eucaryotic Mutagenesis Functionally Identifies Conserved Regions within the Adenovirus 13S E1A Polypeptide

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A new approach to the isolation of mutations in mammalian genes was developed which permits both the selection of infrequently occurring mutants that alter the cellular morphology of recipient cells and the rapid reisolation of the mutant gene. The adenovirus type 5 13S early region 1a (E1a) gene was mutagenized in vitro with sodium bisulfite and then efficiently transferred into cells with a retrovirus shuttle vector. Three classes of mutants of the 13S E1a gene product were isolated, each of which induced a distinct morphological alteration. The mutant E1a gene was reisolated from each cell line, and the precise nucleotide changes were determined. The E1a-induced morphological alterations were further examined by the construction of single and double point mutations within different regions of the polypeptides by utilizing the amino acid substitutions obtained from the original mutants. The results suggest that each of the three regions of highly conserved amino acids within the E1a 13S polypeptide has a distinct role in the alteration of cellular morphology and the activation of gene expression.

Adenovirus-mediated oncogenic transformation is a multistep event resulting from the constitutive expression of viral gene products which normally function transiently during a lytic infection. The region of adenoviral DNA involved in this process is composed of two subregions, early regions 1a (E1a) and 1b (E1b), both of which are necessary to establish the fully transformed state. The two E1a gene products involved in cellular transformation are encoded by two transcripts produced at early times of infection which share the same transcriptional initiation and termination sites but differ by the size of the intervening sequence that is removed (1, 4, 13, 19; Fig. 1A). The two proteins encoded by these transcripts are both capable of initiating cell transformation in the presence of the E1b gene products, although the effect of each on the transformation process is distinct (5, 6, 10). During a lytic infection, the larger protein is responsible for the activation of viral gene expression, whereas the smaller protein facilitates the growth of the virus in quiescent cells (2, 10, 11, 14, 17). Comparison of the amino acid sequences of the E1a gene from the human and simian adenoviruses reveals that the gene is highly conserved within three distinct regions, separated by a variable number of amino acids in the different viruses (8). Two of these regions are contained within both E1a proteins, whereas the third conserved region is composed of the amino acids unique to the larger protein (Fig. 1A).

With the use of cDNA copies of the E1a transcripts and site-directed mutagens, it should be possible to isolate mutations in each of the E1a proteins within specific regions of the polypeptide. However, for studies on mammalian cell transformation, the limitation of these methods has been the need to separately isolate each mutant DNA molecule and then individually test for alterations in function. In contrast, in bacterial and yeast systems one can mutagenize a DNA fragment and then introduce the total population of mutagenized DNA into recipient cells. Rare mutants can be identified by screening or selection, and the DNA can be rescued from these cells to discover the precise lesion. In principle this method is possible with mammalian systems, although the frequency of colony formation is often too low to provide an adequate selection, and the process of recovering the mutagenized DNA from the cells has required the difficult and time-consuming process of constructing and screening recombinant phage libraries.

Recent work with retrovirus vectors has demonstrated that this system might provide an easier approach to the isolation of mutants that alter mammalian genes (15). DNA can be incorporated into cells with high efficiency, allowing many mutants to be screened. When only one DNA molecule is integrated into the chromatin of a cell, it is possible to correlate phenotypic alterations with a specific lesion. Recovery of the mutant DNA sequences from isolated cells is easily achieved, allowing precise analysis of the lesions involved in each mutant.

I report here a method for producing mutations at high frequency within specific regions of the adenovirus type 5 13S E1a gene. By using retrovirus vectors, specific mutants were selected based on the E1a-induced morphological alterations of recipient cells, and these were characterized by morphology and the inability to activate viral gene expression. The results presented here suggest that each of the three regions of highly conserved amino acids in the E1a polypeptide might have a different role within the E1a protein in the alteration of cellular morphology and the induction of gene expression.

MATERIALS AND METHODS

Cells, bacterial strains, and viruses. NIH-3T3 and ψ2 cells were grown in Dulbecco modified medium supplemented with 10% calf serum, as were all the cell lines derived by the integration of the retrovirus. G418 was added to 1 mg/ml during selection. The bacterial strain BD1528 (*thyA met nadBF ung-1 gal supE supF hsdR hsdM*$^+$) was kindly provided by Bernard Weiss. The E1a deletion mutant of

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adenovirus type 5 (7) was propagated, and the titer was determined on 293 cells. The retrovirus vector DoL was a generous gift of Richard Mulligan.

Annealing. Heteroduplex molecules were formed by the procedure of Peden and Nathans (12). The annealing reactions were performed with 1 μg of gel-purified linear DNA from each of the two plasmids to be annealed. DNA from the annealed heteroduplex or from self-annealed control samples was used to transform HB101 or BD1528 cells to test the annealing reaction.

Bisulfite mutagenesis. The mutagenesis was carried out by the method of Shortle and Nathans (16) with final sodium bisulfite concentrations of 2.0, 1.0, and 0.5 M. The samples were overlayed with paraffin oil and incubated at 37°C for 1 h in the dark. After the final dialysis step, the samples were concentrated by lyophilization and used to transform BD1528 cells.

RNA dot blots. RNA was prepared from 10-cm-diameter dishes of cells at 60 to 80% confluence by the use of guanadinium isothiocyanate. The RNA samples were dried, suspended in 15× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–15% formaldehyde, and denatured at 60°C for 15 min. The RNA was spotted directly onto nitrocellulose that was equilibrated in 15× SSC by using a vacuum manifold device (Schleicher & Schuell, Inc., Keene, N.H.) under vacuum. The filter was dried for 1 h at room temperature and then baked for 2 h at 80°C under vacuum. The prehybridization and hybridization conditions were as described previously (18). The filters were washed in 2× SSC–0.1% sodium dodecyl sulfate at 55°C for 1 h with one change and then in 0.3× SSC–0.1% sodium dodecyl sulfate at 55°C for 1 h with one change and exposed to film. The film was quantitated with a microdensitometer (Beckman Instruments, Inc., Fullerton, Calif.).

DNA sequencing. All nucleotide sequences were determined by the partial chemical degradation method, as described by Maxam and Gilbert (9), with slight modifications. In the guanine-adenosine reaction, 10 μl of DNA was added to 25 μl of 88% formic acid and incubated at room temperature for 4 min. Hydrazine stop buffer (200 μl) was added, and the described procedure for the hydrazine reactions was followed.

RESULTS

General approach. The overall approach that was developed to mutagenize subregions of the E1a gene is illustrated in Fig. 1B. Full-length cDNA clones of the individual E1a transcripts (15) were first mutated within a defined region of the gene with a highly efficient mutagen, in this case bisulfite. The total population of mutagenized E1a DNA was inserted into a retrovirus vector which was then propagated in bacteria and transfected onto the ψ2 cell line by the procedure of Cepko et al. (3). As this cell line constitutively produces the retrovirus structural proteins, full-length vector RNA is packaged into infectious virus. However, as the packaged RNA does not encode the information for the retroviral structural proteins, infection of this virus onto NIH 3T3 cells results only in the integration of the viral DNA into the cellular genome without the production of progeny virus. Because the multiplicity of infection is typically one virus per 1,000 cells, only one copy of the viral DNA is integrated into the cellular genome, resulting in the expression of an individual E1a gene copy in each infected cell. Two days after the infection the cells were subcultured into medium containing G418. Only cells which contained the vector and express the gene for drug resistance to G418 survived, resulting in individual colonies which were visible to the naked eye 7 days later.

In the case of the E1a gene, the presence of a mutated gene in the resulting cell lines can be determined by visual inspection of the cellular morphology and by functional assays of the viral gene product with mutant adenoviruses. Furthermore, the mutant DNA can be readily recovered from each of the cell lines and subjected to nucleotide sequence analysis to determine the precise lesion. The reisolation of the DNA allows for further experimentation with each mutant.

Mutagenesis of the E1a gene. To mutagenize subregions of the E1a gene efficiently, bisulfite mutagenesis of single-stranded gaps was utilized, which changes deoxycytosine to deoxyuracil (12, 16). The E1a gene was first constructed to contain a *Bam*HI site at the 5′ end and a *Sal*I site at the 3′ end, to facilitate unidirectional insertion of the gene into the retrovirus vector (Fig. 1A). The E1a gene was also inserted into the pUC13 vector, and large quantities of this DNA were prepared. The pUC-E1a plasmid was cleaved at the unique *Sca*I site within the pUC13 vector, or at the *Cla*I and *Xba*I sites which deletes 306 base pairs in the central portion of the E1a gene. These latter two sites were chosen because they were each unique within the plasmid and created a gap that included two of the three highly conserved regions within the E1a gene (8; Fig. 1a). The large fragments of DNA were then purified by agarose gel electrophoresis and annealed together, creating either linear molecules or a circular molecule with a single-stranded gap. When this mixture was introduced into bacteria, the gapped circular molecule produced 100-fold more transformants than did the linear molecules (unpublished data). This then created a suitable substrate for the bisulfite mutagenesis.

The annealed molecule was incubated in 2, 1, or 0.5 M bisulfite and transformed into a strain of *Escherichia coli* deficient in the enzyme uracil *N*-glycosylase (*ung*) to prevent loss of the deoxyuracil residues created by the mutagen (12). Plasmid DNA was prepared from the total population of *ung* transformants, and the E1a gene fragment was isolated and ligated into a retrovirus vector. The ligation mixture was transformed into an *E. coli* strain deficient in the *recA* protein (HB101) to prevent recombination through the duplicated retrovirus long terminal repeats. DNA prepared from the resulting pool of transformants was transfected onto the ψ2 cell line, and infectious virus recovered 22 h later was infected onto NIH 3T3 cells. After 3 days the cells were subcultured into media containing G418, and individual colonies were observed after an additional 7 days.

Selection of mutant cell lines. NIH-3T3 cell infected with retroviruses containing each of the E1a transcripts produced G418-resistant colonies with markedly different morphologies (15; unpublished data). Colonies of cells containing the retrovirus vector expressing the 13S transcript appeared small and dense to the naked eye, whereas larger, diffuse colonies were obtained with the vector expressing the 12S transcript. With the vector alone, the colonies were very large and readily distinguishable from colonies containing either of the E1a gene products. Under microscopic examination, the 13S cells appeared small and square, with virtually no cell processes, and often appeared to be tightly associated at their edges (Fig. 2B). In contrast, the 12S cells were larger and well spread out, with obvious cellular processes (Fig. 2D). These cells were distinctive for the absence of nucleoli and cytoplasmic granules. Cells containing the vector alone were indistinguishable from the parental NIH 3T3 cells and were characterized by the overlapping

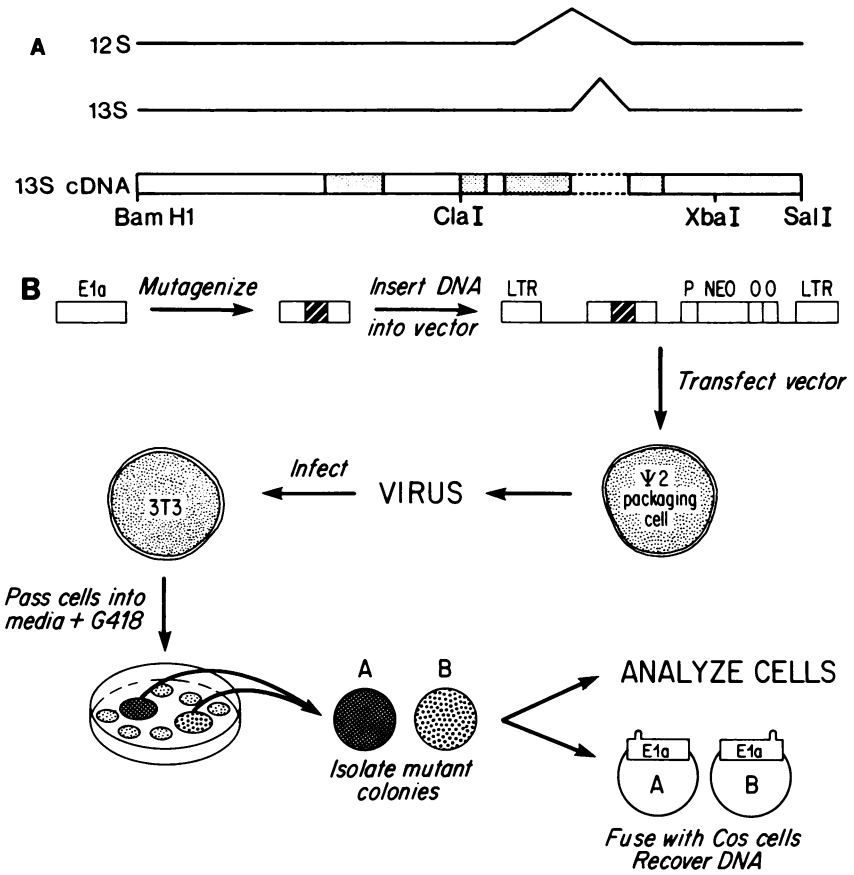

FIG. 1. Procedure used in this study. (A) The two early E1a transcripts are shown with a schematic diagram of the 13S cDNA clone used in these experiments. The shaded areas indicate the three highly conserved regions. (B) The E1a gene was mutated within a specific region, and then the total population of mutagenized DNA was inserted into the retrovirus vector at unique *Bam*HI and *Sal*I sites. The vector-E1a DNA was transfected into ψ2 cells, and the infectious virus recovered 22 h later was used to infect NIH 3T3 cells by the procedure of Cepko et al. (3). Colonies were selected in G418-containing medium added at 3 days postinfection, and morphologically altered cells were isolated and propagated as separate cell lines. The cell lines were analyzed by visual inspection of the cellular morphology and by a complementation assay with a mutant adenovirus. The cells were also fused with Cos cells to recover the mutant E1a gene. Abbreviations: LTR, long terminal repeat; P, the simian virus 40 promoter; NEO, the gene encoding drug resistance to G418; O, either the pBR or simian virus 40 origins of replication (R. Mulligan, personal communication). Not shown are the DNA sequences from polyomavirus that were attached at both ends of the vector, forming a circular molecule which could be propagated in bacteria.

and abundant cellular processes, the readily apparent nucleoli and cytoplasmic granules, and the size and shape of the cells.

Colonies of cells produced by retroviruses containing the E1a 13S gene mutagenized by 2 or 1 M bisulfite were examined, and mutant colonies were identified both by the size and appearance of the colony and by microscopic inspection. In the absence of mutagen, very few mutant colonies were observed. Twenty-five colonies were isolated and propagated as separate cell lines. Although many colonies appeared to be morphologically similar, at least three distinct cell types were readily observed. For example, the 5-3 cell line represented a frequently occurring morphological type. These cells were larger than the 13S cells or the cells with the vector alone, and the nucleoli and cytoplasmic granules appeared less distinct under phase-contrast microscopy (Fig. 2C; compare Fig. 2A and B), similar to cells containing the 12S gene product (Fig. 2D). A morphological type not previously observed is represented by the 3-1 cells. These cells were as large as the 5-3 and 12S cells (Fig. 2E; compare Fig. 2C and D), although they were frequently observed to be severalfold larger (Fig. 2F). The nucleoli were readily apparent in these cells, unlike the 5-3 or 12S cells, with a distinct ring of cytoplasmic granules surrounding the nucleus (Fig. 2E and F). Finally, one isolate was obtained that appeared to be identical to the DoL cells (1-12; data not shown). These results suggest that the individual cell lines might contain different E1a mutants, resulting in the variety of morphologies that was observed.

Activation of viral transcription. The 13S gene product functions during the lytic growth cycle to activate early viral gene transcription. Viruses which are mutant in the E1a gene are unable to synthesize early mRNA and are completely defective for growth at a low multiplicity of infection. However, infection of these mutant viruses onto cell lines containing the 13S gene product results in the transcription of all the viral early regions. This complementation can be utilized as an assay for the presence of the wild-type 13S function in different cell lines expressing E1a gene products (15).

The E1a mutant adenovirus d1312, which contains a deletion of the entire E1a region, was used to infect the various cell lines; and the levels of the early viral transcripts were assayed directly by RNA dot hybridization. The 13S cell line (2G) efficiently induced the accumulation of early region 3 mRNA, whereas infection of the control cells (DoL)

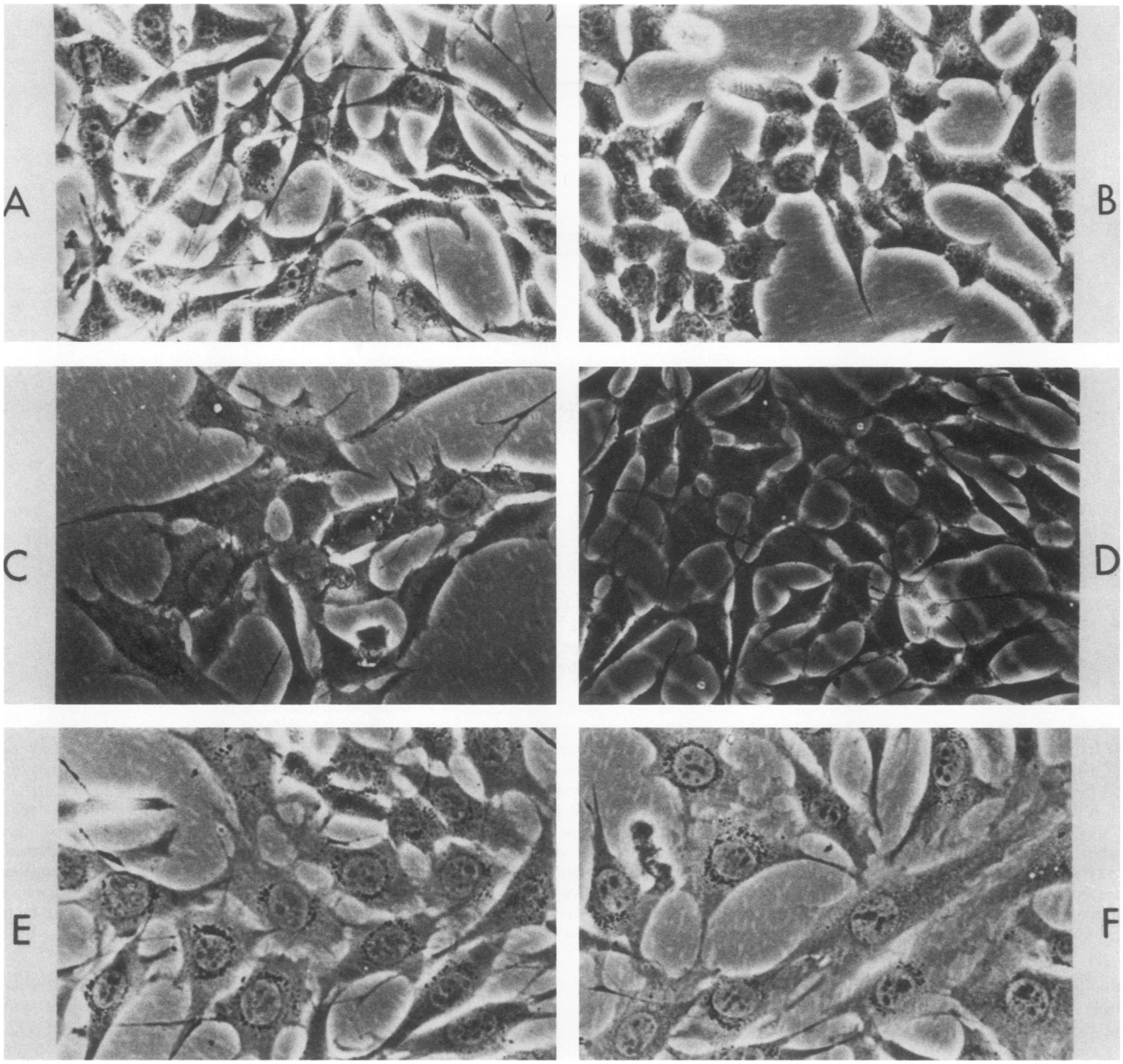

FIG. 2. Morphology of the mutant and wild-type cell lines. Each of the cell lines was photographed at the same magnification and then enlarged to the same extent. The cell lines shown are as follows: DoL (A); 2G (13S wild-type) (B); mutant 5-3 (C); 12-3 (12S wild-type) (D); mutant 3-1 (E and F). Note the very large cells in the lower right of panel F. Magnification, ×200.

did not result in detectable viral early gene expression (Fig. 3). Two independently isolated 12S cells lines (Fig. 3, 12-2 and 12-3) appeared to stimulate weakly early viral transcription, in accordance with previous results (15). Examination of the mutant cell lines revealed a wide range in the ability to activate viral transcription. Cell line 1-12, for example, did not induce detectable accumulation of viral mRNA, whereas the other cell lines activated transcription at the level of the 12S gene product or a few-fold higher. Infection of cell lines 2-1 and 3-11, which contain a mixed population of mutant and wild-type cells (unpublished data), resulted in wild-type levels of early viral mRNA. Except for cell lines 2-1 and 3-11, these results demonstrate that each of the cell lines contains a mutant E1a gene product.

Nucleotide sequence analysis of the E1a mutants. To determine the precise lesion that results in the mutant phenotype, the proviral sequences from each of the cell lines were rescued as bacterial plasmid recombinants (3). The E1a gene in the plasmids was first analyzed by digestion with the restriction enzymes *Bam*HI and *Sal*I, which cleave at both ends of the gene. In 24 of 25 cases, the E1a gene was found to be intact (data not shown). Cell line 1-12, however, contains a spontaneously arising deletion beginning at approximately 180 base pairs after the translation initiation site to a site beyond the 3′ end of the gene. The other plasmids were digested with either *Cla*I or *Xba*I and labeled at their 5′ termini with ^{32}P by using polynucleotide kinase. As these were the two enzymes utilized to create the single-stranded gap, sequence analysis of the 306 base pairs between these two sites should contain all of the nucleotide changes. The

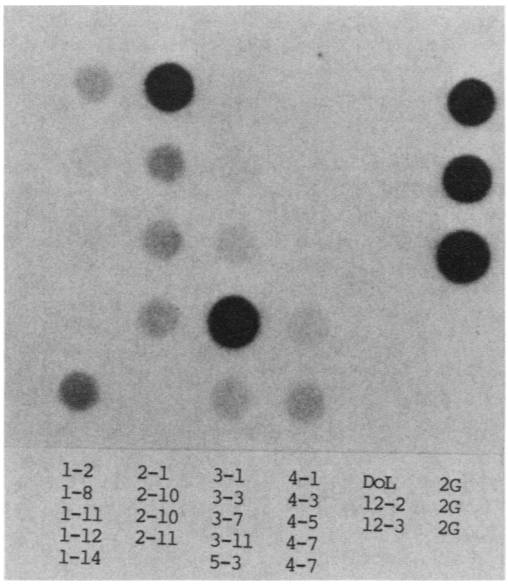

FIG. 3. Induction of E3 transcription. Each of the cell lines was infected with adenovirus deletion mutant d1312 at a multiplicity of 5 PFU per cell. RNA was isolated 23 h later, bound to a nitrocellulose filter, and hybridized with a ^{32}P-labeled fragment from the adenovirus type 5 E3 region. Each column of dots is labeled below the column, beginning with the dot in the first row. Duplicate samples of 2G, 2-10, and 4-7 are from separate infections of the same cell line.

precise changes were located by the method of Maxam and Gilbert (9), and observed mutations were confirmed by sequence analysis of both strands. In all cases in which multiple mutations occurred, the changes were all on the same DNA strand and were all changes from cytosine to thymine, indicating that the mutations arose as the result of bisulfite mutagenesis of a single strand. The predicted amino acid changes are illustrated in Fig. 4.

To determine whether these mutations were responsible for the observed phenotypes, the 306-base-pair fragment from the *Cla*I to *Xba*I sites were isolated from each of the mutants listed in Fig. 4 and ligated into a wild-type gene from which this fragment was removed. This was then inserted into a retrovirus vector, and G418-resistant colonies were produced. In all cases colonies of mutant morphology were observed. For example, vectors containing DNA from cells lines 2-7 and 4-7 produced colonies that grew in tightly packed clusters (Fig. 5A and B). Microscopic inspection of these cells revealed that they had the morphology characteristic of cells containing the 12S gene. Colonies produced with DNA from the 3-1 mutant were much less packed and contained larger cells with a ring of granules surrounding the nucleus (Fig. 5C). In contrast, the 13S wild-type gene produced very small colonies that were characteristically refractile at their edges (Fig. 5D). These results demonstrate that the morphological alterations are a property of the mutated E1a gene and that the nucleotide changes within the mutated region are responsible for the observed morphologies.

Construction of single and double point mutants in the E1a gene. The morphology of the 3-1 cell line is readily distinguished by the large size of these cells and by the ring of the granules surrounding the nucleus (Fig. 2E and F). Two other mutants with this morphology, 1-11 and 4-5, were isolated and characterized to help to understand the basis for this phenotype. Although all three of these mutants contained multiple amino acid substitutions, they did not all contain an alteration in the same amino acid (Fig. 4). However, all three

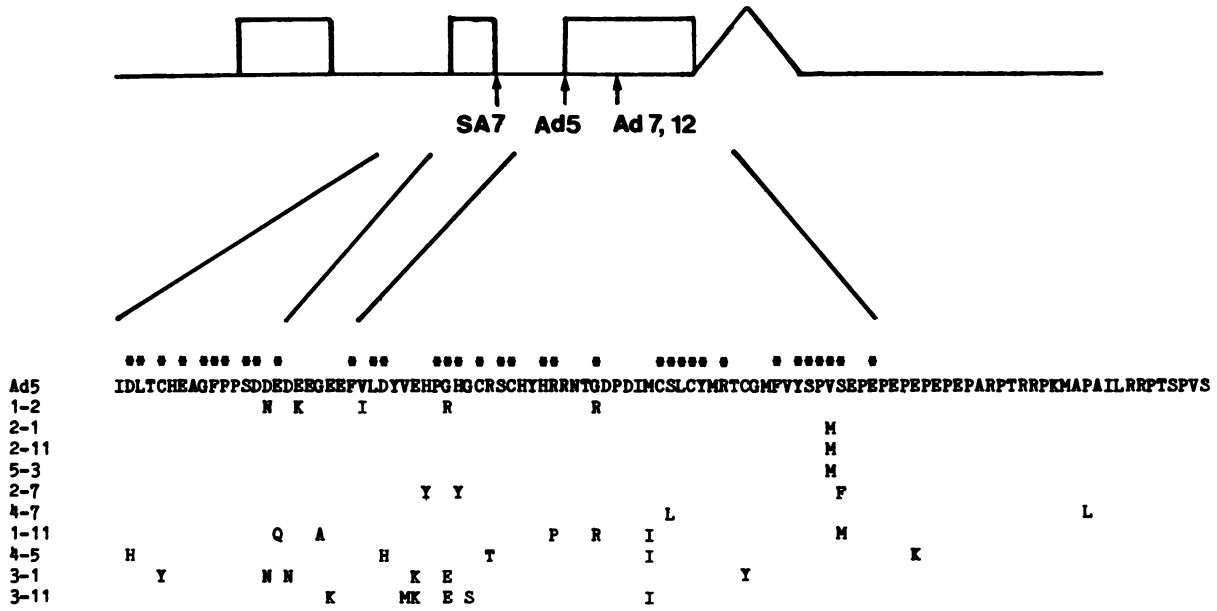

FIG. 4. Nucleotide sequence of the E1a mutants. The three regions of highly conserved amino acids contained within the 13S transcript are shown, with the 5′ splice junction of the 12S E1a transcript of each of the adenoviruses (Ad) indicated by the arrows. The 3′ splice junction of the 12S transcript is the same as that for the 13S transcript. The mutated region, which is bounded by a *Cla*I site at the 5′ end and a *Xba*I site at the 3′ end, contains the second and third conserved regions, as indicated by the oblique lines. The amino acid alterations in 10 of these mutants are listed below the wild-type amino acid sequence of the mutated region. The asterisks indicate the highly conserved amino acids within this region. For the 2-1 and 3-11 cell lines, which contained a mixed population of mutant and wild-type cells, only the alteration in the mutant E1a molecule is shown.

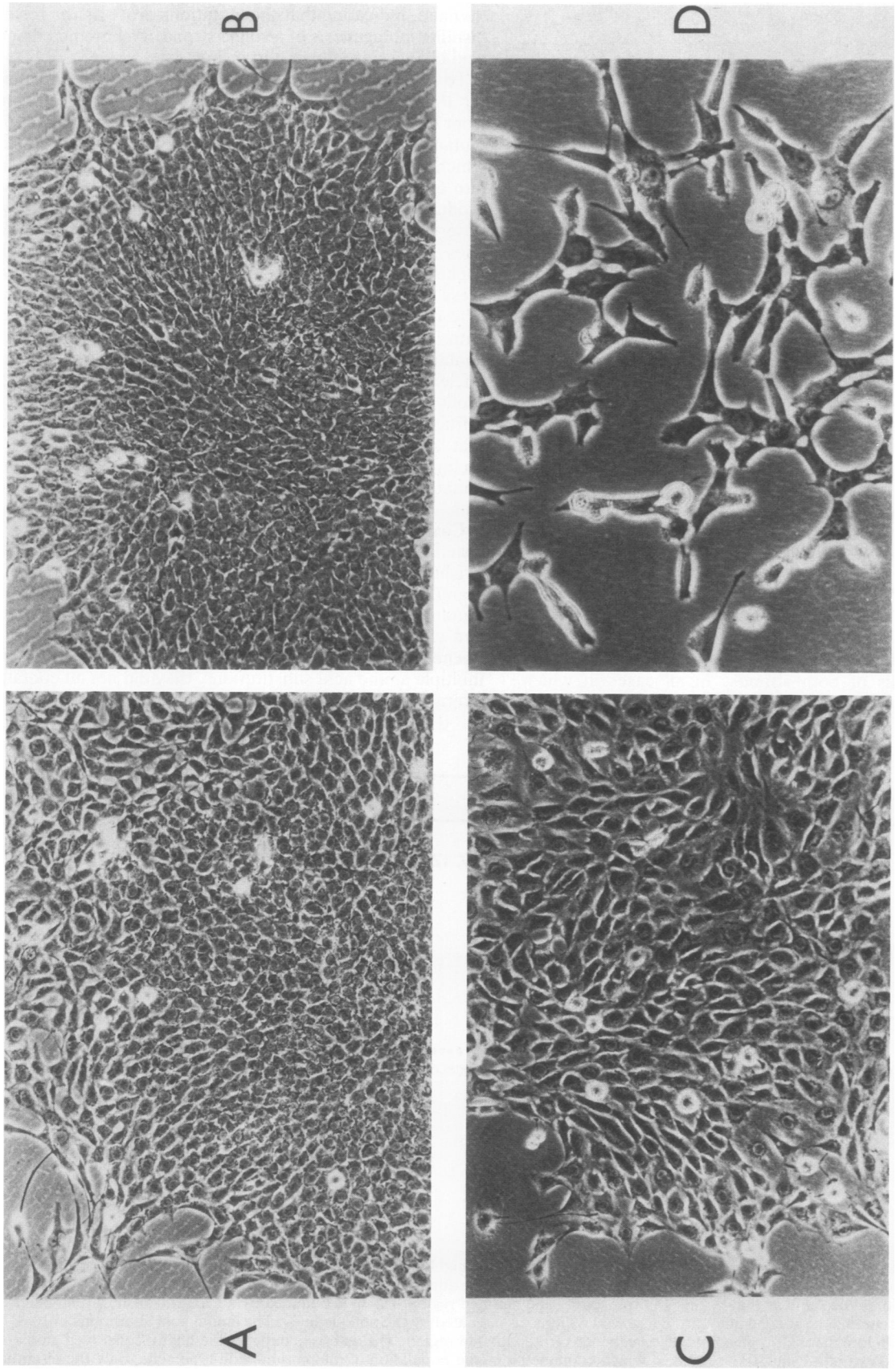

FIG. 5. Colonies produced by the reisolated E1a genes. The *Cla1-Xba1* fragment from mutants 2-7, 3-1, and 4-7 were inserted into the wild-type E1a gene, replacing the wild-type sequences; and then these molecules were inserted into the retrovirus vector. Colonies were produced with these vectors, as well as with the 13S wild-type vector, and photographed. The 13S wild-type cells were photographed at a twofold higher magnification to reveal greater detail. All of the pictures were enlarged to the same extent from the original exposures. The colonies shown are as follows: mutant 2-7 (A); mutant 4-7 (B); mutant 3-1 (C); 13S wild-type (D). Magnification: A, B, and C, ×50; D, ×100.

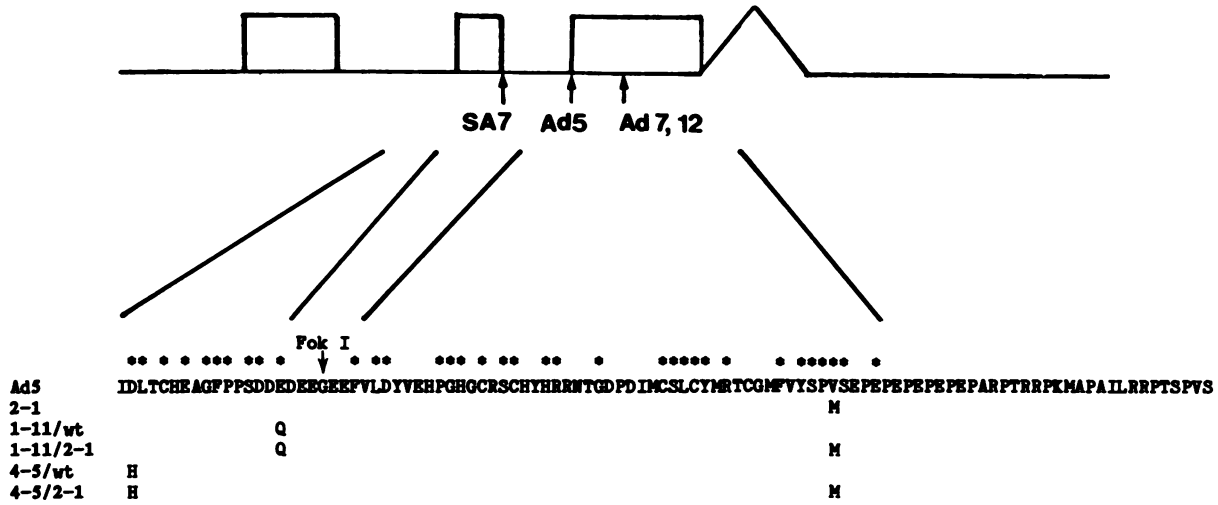

FIG. 6. Nucleotide sequence of the reconstructed E1a mutants. The *Bam*HI-*Fok*I fragment of mutants 1-11 and 4-5, containing the 5′ half of these genes, was combined with the *Fok*I-*Sal*I fragment of either the 13S wild-type or mutant 2-1 genes, producing the two single point mutants 1-11/wt and 4-5/wt or the two double point mutants 1-11/2-1 and 4-5/2-1. A schematic diagram (as in Fig. 4) is shown to indicate the position of each of the conserved regions relative to the region containing the mutations. The asterisks indicate highly conserved amino acids within this portion of the E1a polypeptide.

of these mutants contained an alteration in a conserved amino acid in both the highly conserved second and third regions of the E1a polypeptide, a feature not found in any of the other mutants. Precise analysis of these mutants was complicated by the multiple changes found in each of these three mutants. Multiple changes alone did not produce the 3-1 morphology, because 1-2 and 3-11 had numerous changes but retained the 12S morphology.

To simplify this analysis, DNA isolated from mutants 1-11 and 4-5 was digested with restriction enzymes *Bam*HI and *Fok*I, and the 5′ halves of these genes were isolated. As the *Fok*I recognition site is between the second and third conserved regions, only the single alteration in the second region was retained in this DNA fragment (Fig. 6). These fragments were then combined with the *Fok*I-*Sal*I 3′ fragment of the 13S wild-type (wt) gene, producing the two mutants with a single alteration in the second conserved region, 1-11/wt and 4-5/wt (Fig. 6). Alternatively, the 5′ *Bam*HI-*Fok*I fragment was combined with the *Fok*I-*Sal*I 3′ fragment of the mutant 2-1 gene, which contained a single alteration in the third conserved region. This then produced two double point mutants, 1-11/2-1 and 4-5/2-1, which had a single alteration in a conserved amino acid of both the second and third conserved regions (Fig. 6).

These mutants were inserted into retrovirus vectors, and cells were produced containing each of these genes. Cells containing the wild-type E1a gene were small and often tightly associated at their boundaries (Fig. 7A), whereas cells produced by the 2-1 mutant had the distinctive large size and opaque appearance of the 12S morphology (Fig. 7B). Cells produced by the single point mutants 1-11/wt and 4-5/wt appeared to be very similar to cells containing the 13S wild-type gene, suggesting that the failure to isolate any mutants that altered only the second conserved region in the original screen was due to the lack of any distinct morphological alterations caused by these mutants. However, the combination of the second conserved region change with a single alteration in the third region, as in 1-11/2-1 and 4-5/2-1, resulted in cells with the characteristic 3-1 morphology (Fig. 7E and F; compare Fig. 2E and F). Infection of these cell lines with d1312, the E1a deletion mutant of adenovirus, revealed that 1-11/wt and 4-5/wt activated E3 expression to the same level as did the 13S gene product, whereas 1-11/2-1 and 4-5/2-1 were as defective for the induction of E3 expression as was the 3-1 cell line (unpublished data). These results demonstrate that two substitutions in conserved amino acids, one within each of the two conserved regions, are sufficient to produce cells of the 3-1 phenotype.

DISCUSSION

To examine the transforming functions of the E1a gene, I isolated a series of mutants in the largest E1a gene product. This polypeptide, the product of the 13S E1a transcript, activates early viral gene expression during a lytic infection and encodes functions involved in cell transformation (2, 10, 11, 14, 17). Furthermore, this protein contains all three of the highly conserved amino acid sequences found in the E1a region of human and simian adenoviruses (8).

An efficient analysis of this gene requires the production of a large number of distinct mutations, together with a means to isolate frequently and rarely occurring alterations. A solution to this problem was provided by a combination of site-directed mutagenesis together with retrovirus shuttle vector technology. Bisulfite treatment of a single-stranded gap within the E1a gene produced highly efficient mutagenesis of any predefined region within the gene. Limiting the size of the gap to 300 base pairs directed the mutations to a specific site and facilitated the rapid nucleotide sequence analysis of resulting mutations. The mutagenized population of DNA was efficiently transferred into cells by use of retrovirus vectors, producing a large number of mutagenized colonies which could be screened for infrequently occurring phenotypes. In the case of the E1a region, the wild-type 13S gene caused drastic morphological alterations in recipient cells, and hence, mutant cells could be selected by visual examination. Previous examination of colonies produced with retrovirus vectors demonstrated that each of the colonies contained cells with only one copy of the E1a gene, such that any observed phenotypic alteration must be the result of the integrated DNA (15). If more than one gene was inte-

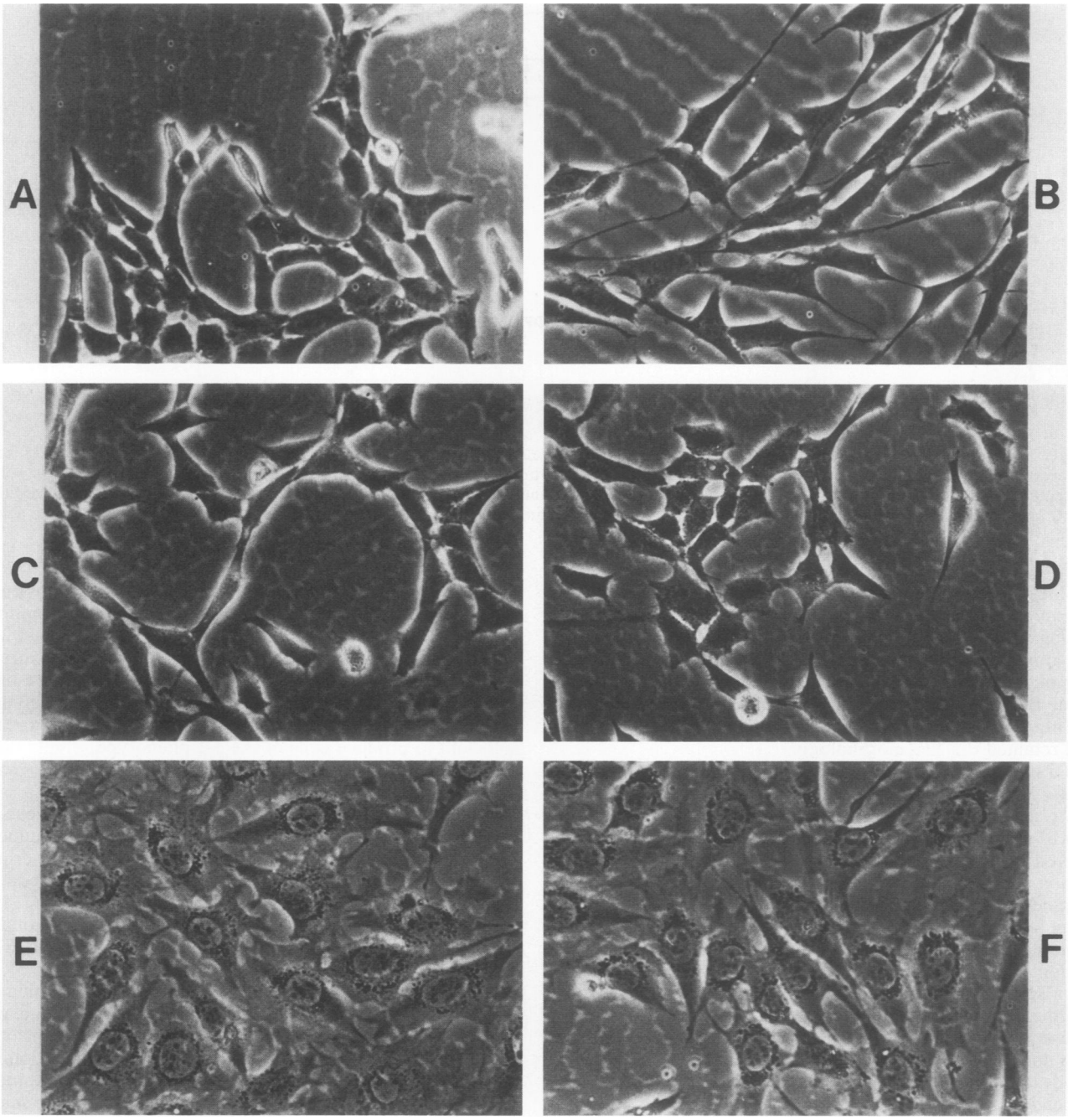

FIG. 7. Cells produced with vectors containing the reconstructed E1a mutants. Cells were produced with retrovirus vectors containing each of the mutants illustrated in Fig. 6, as well as from the vector containing the wild-type 13S gene, and photographed at the same magnification. The original exposures were then enlarged to the same extent. The cells shown are as follows: 13S wild-type (A); mutant 2-1 (B); mutant 1-11/wt (C); mutant 4-5/wt (D); mutant 1-11/2-1 (E); and mutant 4-5/2-1 (F). Magnification, ×200.

grated into each cell, as with typical $CaPO_4$ transfections, then only dominant mutants could be isolated. Most importantly, the retrovirus shuttle vectors permitted the DNA to be reisolated from cells, which is not possible with most other transfection schemes. Thus, the precise lesion in the gene that causes the mutant phenotype could be determined. In addition, the mutant DNA was available for further studies.

The 13S gene was mutated within the second and third conserved regions of the gene, which includes the region shared between the 12S and 13S gene products, and the region unique to the 13S gene. Over 500 individual G418-resistant colonies were examined, and 25 potential mutants were selected and propagated as separate cell lines. A summary of the results obtained with the 11 mutants examined in detail is presented in Table 1. The most frequently occurring morphological phenotype was represented by cell line 5-3 (Fig. 2C) and occurred in other mutants that con-

TABLE 1. Characteristics of mutants examined in detail

Mutant	Morphology[a]	*trans*-Activation[b]	Mutation in[c]:	
			Second domain	Third domain
1-2	12S	+ +	NC	C
1-11	3-1	+	C	C
1-12	DoL	0	Deleted	Deleted
2-1	12S	+ +		C
2-7	12S	+ +		C
2-11	12S	+ +		C
3-1	3-1	+	C	C
3-11	12S	+ +		C
4-5	3-1	+	C	C
4-7	12S	+ +		C
5-3	12S	+ +		C
1-11/wt	wt	wt	C	
1-11/2-1	3-1	+	C	C
4-5/wt	wt	wt	C	
4-5/2-1	3-1	+	C	C
DoL	DoL	0	Absent	Absent
12S	12S	+		Absent
13S	wt	wt		

[a] The morphologies of the cells are indicated as being similar to the wild-type 13S (wt), 12S, 3-1, or DoL cell lines.

[b] The extent of stimulation of the E3 gene of d1312 in the infection assay is listed. wt indicates the levels of the wild-type 13S gene.

[c] The mutations are listed as in conserved (C) or nonconserved (NC) residues. Where changes in both conserved and nonconserved amino acids occurred, only the conserved change is indicated.

tained single or multiple lesions within conserved amino acids of the region unique to the 13S gene. These cells were very similar morphologically to cells containing the 12S gene product (Fig. 2D), although they enhanced d1312 expression two- to threefold above the level of the 12S cell lines, suggesting that the unmutagenized amino acids in the third region could still contribute weakly to the induction of transcription.

An additional morphological type was found with cell lines 3-1 (Fig. 2E and F). Nucleotide sequence analysis of these mutants revealed several amino acid changes, although all of these mutants did not share an alteration in the same amino acid (Fig. 4). Unlike the other class of mutants, these E1a genes contained lesions in conserved amino acids of both the second and third regions, suggesting that the morphological phenotype is due to an alteration in both conserved regions.

This proposition was tested by constructing single and double point mutations in the second and third regions of the E1a gene, utilizing the amino acid substitutions found in the original mutants. These results demonstrated that a single point mutation in the third conserved region produces the 12S phenotype (Fig. 7B), whereas a single point mutation in the second region produces no morphological alteration (Fig. 7C and D). However the combination of an alteration in a conserved amino acid of the second region with an alteration in a conserved amino acid of the third region produced the 3-1 morphology (Fig. 7E and F). Hence, the 3-1 morphology might be due to a function encoded within the first conserved region operating together with the nonconserved amino acids of the polypeptide. This view is supported by two lines of evidence. First, single substitutions of different conserved amino acids within a conserved region are sufficient to cause the same morphological alteration, demonstrating that a single change will alter the entire region. Hence, the two amino acid substitutions in 1-11/2-1 and 4-5/2-1 appear to be sufficient to inactivate the entire activity of the second and third regions, although additional amino acid substitutions in these regions must be tested to establish this point. Second, the only mutant isolated that produced the same morphology as cells containing the vector alone was a spontaneously arising deletion mutant that truncated most of the 13S gene, most likely eliminating all activity of the 13S polypeptide. The failure to isolate point mutants with this phenotype might be due to the activity of the unmutagenized first region, conferring some morphological alteration to cells containing any of the point mutants. This is further supported by the *trans*-activation studies which show that only the deletion mutant 1-12 has no *trans*-activation activity, whereas the mutants 1-11, 3-1, 4-5, 1-11/2-1, and 4-5/2-1 can all activate d1312 expression to a measurable extent (Table 1). Hence, the first region not only appears to cause morphological alterations but is also able to weakly stimulate gene expression. However, until mutants within the first conserved region have been analyzed, it is not possible to rule out the possibility that the 3-1 morphology is due to contributions from amino acids outside the first region. In summary, it is suggested that an active first region produces the morphology characteristic of mutant 3-1, whereas an active first and second region produces the morphology induced by the 12S gene product. Finally, the active first and third regions, or all three of the conserved regions together, produce the morphology of the 13S wild-type gene and are fully able to induce the expression of other adenoviral early genes.

Thus, these results suggest that the three conserved regions of the polypeptide, which were identified from a comparison of the amino acid sequences, might encode different functions of the polypeptide. It will be of use to construct point mutants that alter the first region alone, and to combine these with the mutants that affect the second and third regions, to understand the interrelationship between each of the conserved regions. Finally, it will be necessary to combine these E1a mutants with the E1b region in cellular transformation studies to understand how each of these morphology-altering mutations affects the cellular-transforming property of the E1a gene.

The mutants that I isolated were all chosen on the basis of morphological alterations to recipient cells. This methodology should work well for any gene the product of which causes either an observable change in recipient cells or a change that is readily assayable in the initial colonies. Proteins involved in morphological alterations, secreted proteins, and various enzymes might be amenable to a similar approach.

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