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Adenovirus E1B 19-Kilodalton Protein Overcomes the Cytotoxicity of E1A Proteins

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Infection with adenovirus mutants carrying either point mutations or deletions in the coding region for the 19-kDa E1B gene product (19K protein) causes degradation of host cell and viral DNAs (*deg* phenotype) and enhanced cytopathic effect (*cyt* phenotype). Therefore, one function of the E1B 19K protein is to protect nuclear DNA integrity and preserve cytoplasmic architecture during productive adenovirus infection. When placed in the background of a virus incapable of expressing a functional E1A gene product, however, E1B 19K gene mutations do not result in the appearance of the *cyt* and *deg* phenotypes. This demonstrated that expression of the E1A proteins was responsible for inducing the appearance of the *cyt* and *deg* phenotypes. By constructing a panel of viruses possessing E1A mutations spanning each of the three E1A conserved regions in conjunction with E1B 19K gene mutations, we mapped the induction of the *cyt* and *deg* phenotypes to the amino-terminal region of E1A. Viruses that fail to express conserved region 3 (amino acids 140 to 185) and/or 2, (amino acids 121 to 185) or nonconserved sequences between conserved regions 2 and 1 of E1A (amino acids 86 to 120) were still capable of inducing *cyt* and *deg*. This indicated that activities associated with these regions, such as transactivation and binding to the product of the retinoblastoma susceptibility gene, were dispensable for induction of E1A-dependent cytotoxic effects. In contrast, deletion of sequences in the amino terminus of E1A (amino acids 22 to 107) resulted in extragenic suppression of the *cyt* and *deg* phenotypes. Therefore, a function affected by deletion of amino acids 22 to 86 of E1A is responsible for exerting cytotoxic effects in virally infected cells. Furthermore, transient high-level expression of the E1A region using a cytomegalovirus promoter plasmid expression vector was sufficient to induce the *cyt* and *deg* phenotypes, demonstrating that E1A expression alone is sufficient to exert these cytotoxic effects and that other viral gene products are not involved. Finally, placing E1A expression under the control of a strong promoter did not alter the requirement for E1B in the transformation of primary cells. One possibility is that the E1B 19K protein is required to overcome the cytotoxic effects of E1A protein expression and thereby enable primary cells to become transformed.

Early region one (E1) at the left end of the viral genome encodes two distinct transcription units, designated E1A and E1B, which function in concert to regulate expression of viral genes in productively infected cells (see reference 64 and references therein) and in the transformation of primary rodent cells (reviewed in references 6 and 17). The E1B gene encodes two distinct tumor antigens of adenovirus, the 19- and 55-kDa proteins (19K and 55K proteins, respectively), both of which are independently capable of cooperating with the E1A gene products in the transformation of primary rodent cells (63). Expression of the E1B 19K protein, however, is specifically associated with anchorage-independent growth (63). The E1B 19K protein has many additional features unique to the repertoire of transforming proteins. No obvious sequence homologies between the 19K protein and other transforming proteins have been reported. Furthermore, it possesses a dual subcellular localization pattern in that it is found associated with both cytoplasmic and nuclear envelope membranes, as well as the intermediate filament cytoskeleton (61, 62). Expression of the 19K protein profoundly alters the organization of two distinct classes of intermediate filament networks, i.e., vimentin-type filaments in the cytoplasm and the lamin filament network in the nucleus (62). Destruction of the integrity of the intermediate filament cytoskeleton occurs under conditions in which the 19K protein is transiently expressed via plasmid expression vectors (62), in virally infected cells (63a), and in stably transformed cell lines derived from oncogenic transformation of primary rodent cells with E1A and E1B DNAs (63). As intermediate filaments and the nuclear envelope membranes are subject to widespread structural modification by the E1B 19K protein and proper localization of the 19K protein to these cellular compartments is required for its biological activity, these cellular structures currently represent possible mediators of E1B 19K protein function (61–63).

Adenovirus E1B 19K gene mutants have been used to establish the role of the 19K protein in the productive phase of infection. Although by no means defective for virus replication, these E1B 19K mutant viruses possess numerous pleiotropic phenotypes which include degradation of host cell and viral DNAs (*deg* phenotype), enhanced cytopathic effect or the cytocidal (*cyt*) phenotype (46, 56, 66), the large-plaque (*lp*) phenotype (8), and a host range (*hr*) phenotype in which the mutant viruses display a growth advantage over the wild-type virus in human WI38 cells (65). Therefore, the E1B 19K protein functions in infected cells to (i) maintain the integrity of DNA within the nucleus, (ii) modify the morphology of the infected cell, and (iii) negatively regulate viral gene expression and replication (64). The mechanism by which the E1B 19K protein performed these apparently diverse functions was unclear; however, a functional interaction between the 19K protein and the E1A gene products was apparent.

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Previous work had identified two parameters which influenced the appearance of the DNA degradation (*deg*) phenotype: the host cell type and expression of the E1A proteins (67). The *deg* phenotype, while quite pronounced in HeLa and KB cells, is absent from human WI38 cells infected with E1B 19K mutant viruses (67). This may be due to many intrinsic differences between these different cell types. For example, while HeLa and KB cells are rapidly dividing transformed cells, WI38 cells are not transformed and strictly growth controlled (25, 26). The biochemical basis for these host cell effects on the integrity of cellular DNA is still not known.

The requirement of E1A gene expression for induction of the *cyt* and *deg* phenotypes of the E1B 19K gene mutant viruses was more tractable and specific. This finding was derived from the observation that the *cyt* and *deg* phenotypes were observed only in E1B 19K mutant viruses in which the E1A proteins were faithfully expressed and were lost upon substitution of a nonfunctional 9S E1A gene (67). The requirement for E1A expression was not limited to initiation of a productive viral infection, since the *deg* and *cyt* phenotypes were also not observed at high multiplicities of infection when efficient virus replication occurred, despite the absence of the E1A proteins (67). Therefore, expression of the E1A proteins induced DNA degradation and enhanced cytopathic effect, the appearance of which was normally prevented by expression of the E1B 19K protein in cells infected with the wild-type adenovirus.

Much is known about the function of the E1A proteins and the cellular proteins with which they interact. The E1A gene encodes a series of related polypeptides translated from 13S, 12S, 11S, 10S, and 9S mRNA species generated by alternative splicing (4, 9, 34, 45, 53, 57, 60). All biological activities of E1A have thus far been attributed to the 13S 289-amino-acid and 12S 243-amino-acid products (reviewed in reference 42). Functions of the E1A proteins include transactivation and repression of transcription (reviewed in reference 3), stimulation of cellular DNA synthesis (33, 51), and immortalization of primary cells (28). Extensive mutational analysis of the E1A gene products has resulted in identification of three discrete, autonomously functional regions, conserved regions 1, 2, and 3 (see Fig. 1) (reviewed in reference 42). This has enabled assignment of functions to specific domains of the E1A protein. For example, conserved region 3, which is unique to the E1A 13S mRNA product, encodes the transcriptional transactivation function. This region is required for transactivation of transcription of the viral early promoters in the productive phase of infection (reviewed in reference 3) but is, for the most part, dispensable for transformation (22, 39, 74).

Conserved region 2 is functionally homologous to similar regions shared by other DNA tumor virus transforming proteins (40) and is required but not sufficient for transformation (30, 35, 37, 44, 49, 50, 69, 75). This conserved region serves as binding sites for specific cellular proteins (24, 72), including the product of the retinoblastoma susceptibility gene (68) and cyclin A (19, 47). The direct correlation between binding of host cell proteins to E1A and the transforming ability of the E1A proteins has suggested that these proteins represent the means by which this region contributes to the transforming activity of the E1A proteins (70).

Conserved region 1 resides in the amino-terminal portion of E1A and, like conserved region 2, is required but not sufficient for E1A transforming functions (30, 43, 50, 55, 69). It is interesting that conserved regions 1 and 2 can function in *trans* in transformation assays (43), reinforcing the notion that E1A protein functions are discrete and separable and that regions of the E1A proteins function autonomously. This amino-terminal region of E1A also serves as a binding site for host cell proteins, some of which are different from those associated with conserved region 2 (70). Binding to these cellular proteins correlates with the enhancer repression activity of E1A and the ability of E1A to induce cellular DNA synthesis (29, 30, 52).

How these functions associated with the E1A proteins are related to the cytotoxic effects of E1A displayed in E1B 19K viral mutant-infected cells was unclear. Identification of the subregion of E1A responsible for induction of the *cyt* and *deg* phenotypes may provide insight into the mechanism of E1A cytotoxicity, as well as how the E1B 19K protein overcomes the effects of E1A expression. We therefore designed genetic experiments whereby a series of E1A gene mutations affecting specific functional domains of the protein were introduced into the background of E1B 19K mutant virus to map the functional subregion within the E1A proteins responsible for induction of DNA degradation in E1B 19K viral mutant-infected cells.

MATERIALS AND METHODS

Cells and viruses. Human HeLa cells were grown in monolayer culture in Dulbecco modified Eagle medium with 10% fetal bovine serum. Human 293 cells (21), which express adenovirus E1A and E1B gene products, were maintained in Dulbecco modified Eagle medium with 10% calf

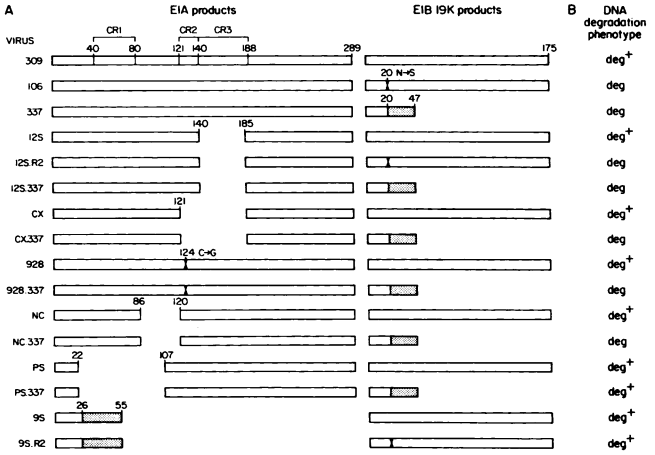

FIG. 1. Schematic representation of E1A and E1B 19K protein products from wild-type *dl*309 virus and the panel of mutant adenoviruses. The *cyt*106 (66), *dl*337 (46), 12S, 9S (41), 12S.R2, 9S.R2 (67), CX, 928, NC, and PS (41, 43, 44, 74) viruses have been previously described. (A) Conserved regions (CR) 1, 2, and 3 of E1A (42) are indicated schematically, with numbers representing amino acids from the amino terminus of the wild-type 289-amino-acid (13S) protein product. Discontinuous regions represent amino acids not present in the mutant proteins as a consequence of mutations. The positions of amino acid substitutions are indicated (×), and shaded areas represent regions shifted into another reading frame. The 175-amino-acid 19K E1B protein and mutant proteins are similarly indicated. (B) The presence of degraded DNA (*deg* phenotype) was determined as described in Materials and Methods, and the results were tabulated as either wild type (deg^+), when no degraded DNA was apparent, or mutant (deg), when DNA degradation was apparent.

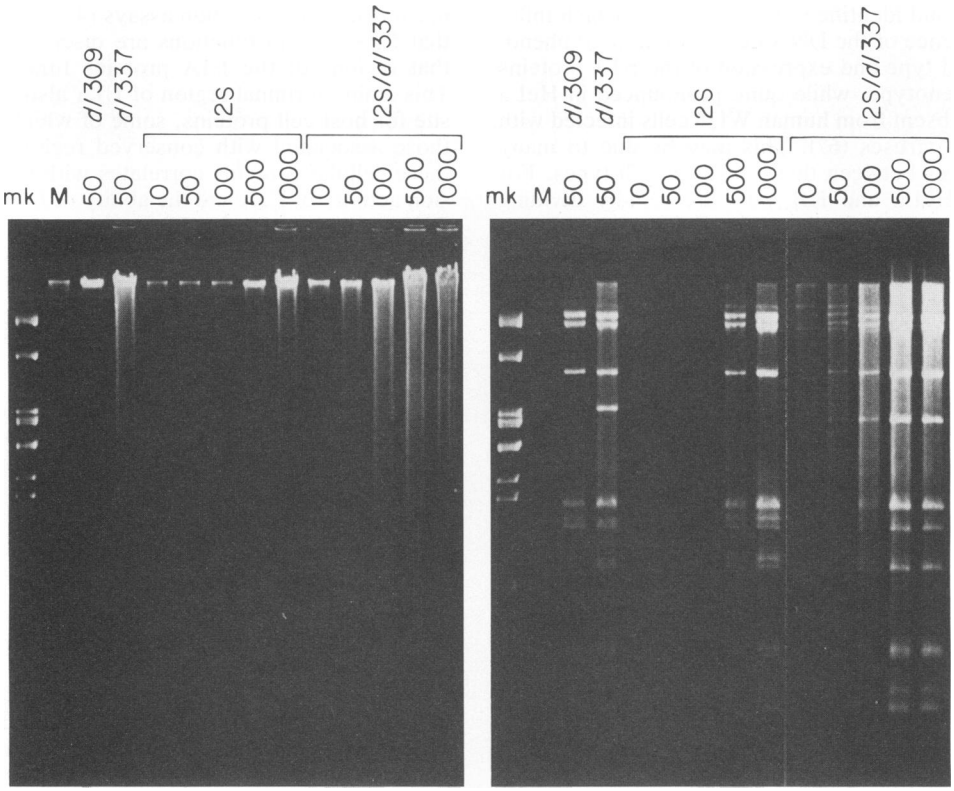

FIG. 2. Induction of DNA degradation with E1B 19K and E1A conserved region 3 double-mutant virus. HeLa cells were infected at multiplicities of infection of 10, 50, 100, 500, and 1,000 PFU per cell with either the 12S E1A virus or a 12S E1A virus bearing an E1B 19K gene deletion mutation (12S/*dl*337). DNA was isolated at 48 h postinfection by the method of Hirt (27), and the low-molecular-weight DNA supernatant fraction was separated by electrophoresis in an agarose gel. Lanes: M, mock infected; *dl*309, wild-type virus infected; *dl*337, E1B 19K gene deletion mutant virus infected. mk, adenovirus type 2 DNA digested with *Hin*dIII as size markers. Panels: left, undigested DNA; right, *Sac*I-digested DNA.

serum. Primary Fisher baby rat kidney (BRK) cells were prepared as previously described (63). Transfection of BRK cells was performed by either calcium phosphate-DNA precipitation (63) or electroporation (10).

All viruses used in this investigation are described in Fig. 1. Ad5*cyt*106 contains an amino acid substitution (asparagine to serine) and has been characterized previously (66). Ad5*dl*337 (46) possesses a deletion encompassing most of the E1B 19K-coding region and was a generous gift of T. Shenk (Department of Molecular Biology, Princeton University, Princeton, N.J.). Viral DNA from Ad5*dl*337 was isolated from $CsCl_2$ band-purified virions by methods described previously (38). The E1A 12S, 9S, CXd1, pm928, NCd1, and PSd1 viruses (41, 43, 44, 74) were generously provided by Betty Moran (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). The 12S.R2 and 9S.R2 viruses expressing the E1A12S and 9S gene products, respectively, in conjunction with an E1B 19K gene point mutation, were constructed and described previously (67). All viruses were grown and their titers were determined on 293 cells, and stocks were maintained as crude lysates. All of the viruses described above, as well as the viruses constructed below, were derived from Ad5*dl*309 (32), and this virus was therefore used as the wild-type strain.

Plasmids. Constructs containing alterations within the E1A coding region (12S, CXdl, pm928, NCdl, and PSdl; 43, 44, 75) were a gift of Betty Moran. E1B plasmid expression vectors pCMV19K, pCMV55K, and pCMVE1B, in which production of the E1B 19K or 55K protein or all of E1B is under the direction of the cytomegalovirus (CMV) early promoter and enhancer, have been previously described (62, 63). Construction of the pCMVE1A expression vector, which was generously provided by Gilbert Morris (Cold Spring Harbor Laboratory), was accomplished by substituting the E1A coding region (nucleotides 493 to 1830) for the E1B 19K-coding region in pCMV19K. Expression of the E1A proteins was detected by indirect immunofluorescence (62) with E1A-specific monoclonal antibody M73 (23) in transient expression assays (62) following transfection of HeLa cells with 10 μg of plasmid pCMVE1A.

Construction of E1A-E1B double mutant viruses. Viruses containing a series of E1A mutations in combination with an E1B 19K gene deletion mutation were constructed to map the subregion of E1A responsible for inducing the *deg* phenotype. Plasmids bearing E1A deletion and point mutations (12S, CXd1, pm928, NCdl, and PSdl) were cleaved at 0 and 3.8 map units (*Eco*RI and *Xba*I sites, respectively) and ligated in excess in vitro to *Xba*I-digested Ad5*dl*337 viral DNA. This resulted in generation of intact viral genomes containing 0 to 3.8 map units derived from E1A mutant plasmid DNAs and 3.8 to 100 map units, including the E1B 19K gene deletion mutation, derived from Ad5*dl*337. Human 293 cells were transfected with ligated DNAs by calcium phosphate precipitation (67). Plaques arising from transfec-

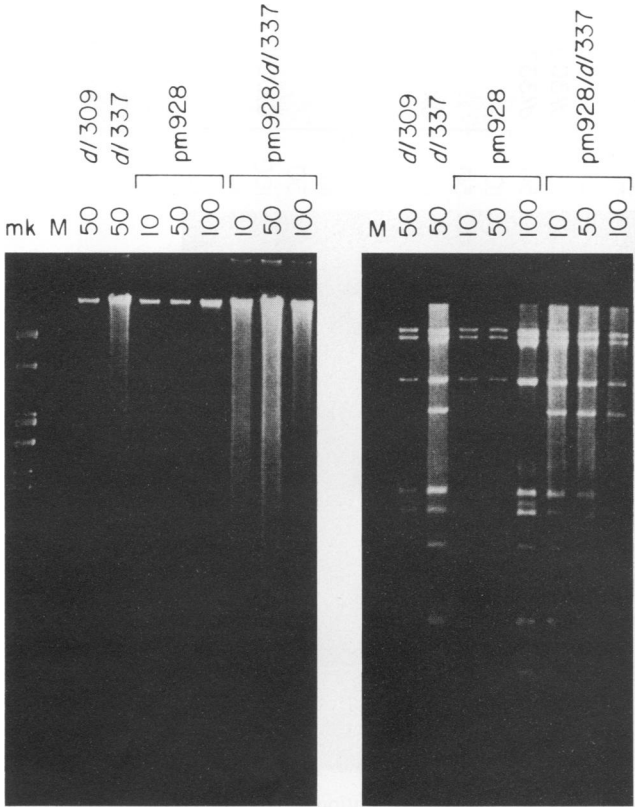

FIG. 3. Induction of DNA degradation with E1B 19K and E1A conserved region 2 (point mutation) mutant virus. Cells were infected as described in the legend to Fig. 2, except that the 928 region 2 E1A point mutation viruses containing either a wild-type E1B gene (pm928) or an E1B 19K gene deletion mutation (pm928/*dl*337) were used. Multiplicities of infection were 10, 50, and 100 PFU per cell, as indicated.

tions were screened by restriction endonuclease mapping to identify E1A and E1B 19K double gene deletion mutations in 12.337, CX.337, NC.337, and PS.337 viruses and plaque purified twice on 293 cells. The E1A 928 point mutation in the 928.337 virus was identified by differential hybridization of the 928 mutagenic oligonucleotide. E1A-E1B 19K double mutant viruses were checked by immunoprecipitation and Western blotting (immunoblotting) for the size and stability of the mutant E1A products, and all were found to make stable protein of the anticipated molecular weight (data not shown).

Assay for DNA degradation. HeLa cells were infected with increasing multiplicities of infection of mutant viruses, and low-molecular-weight DNA was isolated by the method of Hirt (27) with minor modifications (66). DNA from the supernatant fraction was analyzed by electrophoresis through 1% agarose gels and visualized by ethidium bromide staining. Appearance of low-molecular-weight DNA in the undigested Hirt DNA supernatant fractions was used as an indication of the *deg* phenotype. All experiments included both positive (*dl*337 infection) and negative (*dl*309 infection) controls for appearance of the *deg* phenotype. Digestions of Hirt DNA samples with restriction enzymes were also included as a means of demonstrating the level of viral DNA replication in the various viral mutant-infected cells.

RESULTS

Mapping of DNA degradation induction to the amino terminus of E1A. A panel of E1A mutant plasmids containing either point or deletion mutations within E1A conserved and nonconserved regions (12S, CX, 928, NC, and PS; 43, 44, 75) were used to construct E1A-E1B 19K gene double mutant adenovirus. The mapping strategy was directed at exon 1 of E1A, since previous work had indicated that exon 2 of E1A was probably not involved. This derived from the observation that Ad5*dl*313, a virus with a substantial part of exon 2 deleted along with E1B (32), was fully capable of inducing DNA degradation (36).

Restricted DNAs encoding various mutations within the E1A region from 0 to 3.8 map units were ligated in vitro to 3.8 to 100 map units derived from Ad5*dl*337, which contains a deletion mutation within the E1B 19K gene, to reconstruct intact E1A-E1B 19K double mutant viral genomes. After recovery of virus following transfection of 293 cells, the ability of the double mutant viruses, designated 12S.337, CX.337, 928.337, NC.337, and PS.337 (Fig. 1), to induce degradation of cellular DNA was determined.

The point mutation producing an amino acid substitution at position 20 in the 19K protein Ad5*cyt*106 virus and the 19K gene deletion mutation contained within Ad5*dl*337 both effectively induce the *deg* phenotype in infected HeLa cells (Fig. 1; 46, 66). We have previously shown that introduction of the Ad5*cyt*106 19K gene point mutation into the background of an E1A 9S virus results in no *deg* phenotype, demonstrating the requirement for functional E1A proteins to induce DNA degradation (Fig. 1; 67). In contrast, a virus bearing the same 19K gene point mutation in the background of an E1A gene capable of expressing only the 13S 289-amino-acid E1A protein but not the 12S 243-amino-acid E1A protein (13S.R2) did so effectively (67), as did a similar virus expressing only the 12S 243-amino-acid protein but not the 13S 289-amino-acid product (12S.R2) (Fig. 1; 67). These results established that induction of the *cyt* and *deg* phenotypes was an activity common to the 289- and 243-amino-acid E1A proteins. These results were further substantiated by analysis of an E1A 12S virus with the E1B 19K gene deletion of Ad5*dl*337 (12S.337; Fig. 1), which also possessed the *deg* phenotype (Fig. 2). Therefore, the region of E1A unique to the 13S mRNA, which encodes the transactivation function, is completely dispensable for induction of DNA degradation.

A point mutation which results in an amino acid substitution within conserved region 2 (pm928) results in complete loss of known functions attributable to domain 2 (52, 75), except that pm928 binds the 60K protein while domain 2 deletions do not (18). When pm928 was placed in the background of a virus possessing an E1B 19K gene deletion, the *deg* phenotype was readily apparent (Fig. 3). The CXd1 mutation takes out conserved region 2, including the 12S splice donor site, resulting in an E1A product missing conserved regions 1 and 2 (Fig. 1; 43, 75). The CX mutation in the background of the E1B 19K deletion was still capable of inducing the *deg* phenotype, although the virus replicated somewhat less efficiently than the CX virus with a wild-type E1B gene (Fig. 4). Deletion of the nonconserved region (NC mutation) separating regions 1 and 2 similarly did not affect DNA degradation (Fig. 5). As the induction of the *deg* phenotype mapped outside of these regions, the E1A functions associated with conserved region 2, or both regions 2 and 3 must not be required for DNA degradation.

Strikingly different results were obtained when a more

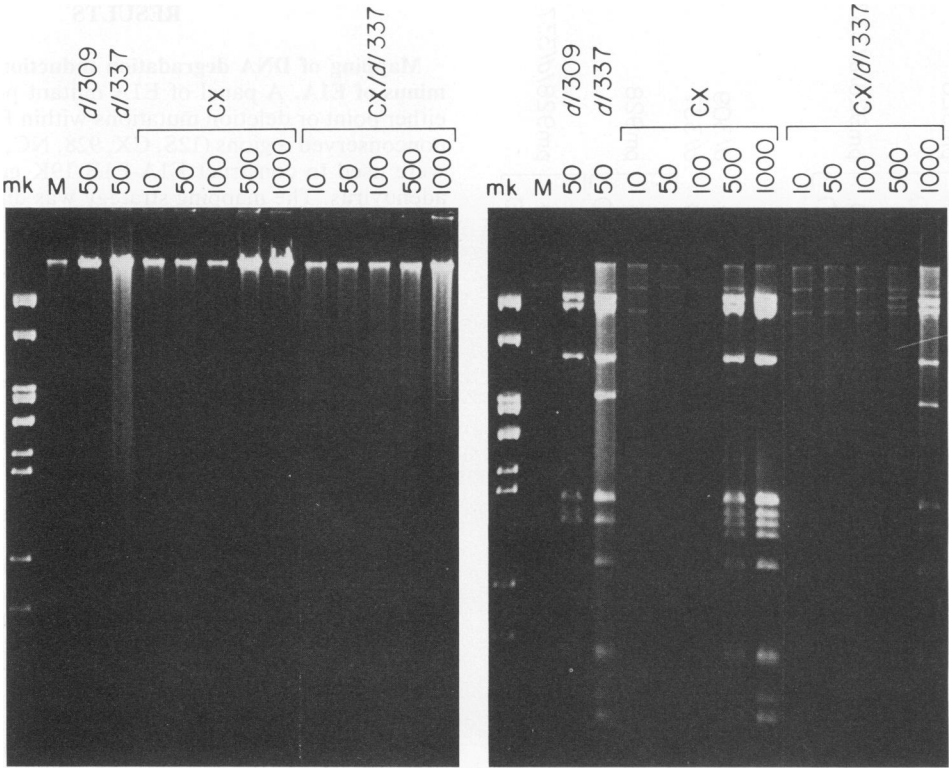

FIG. 4. Induction of DNA degradation with E1B 19K and E1A conserved region 2 (deletion mutation) double-mutant virus. Cells were infected as described in the legend to Fig. 2, except that the CX region 2 E1A deletion mutant viruses containing either a wild-type E1B 19K gene (CX) or an E1B 19K gene deletion mutation (CX/*dl*337) were used.

amino-terminal E1A mutation was examined. The PS deletion removes E1A coding sequences between residues 22 and 107, the E1A product of which is stable, although reduced in molecular weight (43). Although the PS mutation renders the E1A proteins defective for transformation, the CX and PS mutations complement each other in transformation assays, indicating that region 1 is functional in region 2 mutants and vice versa (43). The E1A PS mutation in the background of a 19K deletion mutant virus (PS/*dl*337) was incapable of inducing degradation of host cell DNA (Fig. 6). Therefore, the *deg* function lies within amino acids 22 to 107 defined by the PS deletion. However, as the NC deletion (amino acids 86 to 120) still encodes the *deg* function, this implicated the region of E1A inclusive of residues 22 to 86 as responsible for exerting the cytotoxic effects observed during infection in the absence of a functional E1B 19K protein.

Although the results obtained with the panel of E1A mutants clearly implicated the involvement of the amino terminus of E1A in the induction of DNA degradation, evaluation of the *cyt* phenotype was less decisive. This may be due in part to the lack of a quantitative assay (visual inspection), compounded by the various abilities of the different mutant viruses to replicate. It appeared that amino-terminal E1A mutant PS/dl337 did not cause occurrence of the *cyt* phenotype, whereas the other E1A-E1B double mutants did (data not shown). This suggested that the sequences required for induction of *cyt* and *deg* map within the same region of E1A.

Sufficiency of E1A expression alone to induce enhanced cytopathic effect and DNA degradation. The cytotoxicity of the E1A proteins could be a direct consequence of E1A expression or an indirect effect of E1A on another viral gene product. To distinguish between these possibilities, a plasmid expression vector (pCMVE1A) was constructed to enable transient, high-level expression of the E1A proteins in cells independently of adenovirus infection (Fig. 7). Calcium phosphate precipitation was used to introduce the pCMVE1A plasmid into HeLa cells. Indirect immunofluorescence with an anti-E1A monoclonal antibody demonstrated bright nuclear fluorescence typical of E1A in infected and transformed cells (Fig. 7). As expected from the analysis of other CMV expression vectors (62, 63), the level of E1A expression was at least five times greater than that observed when E1A expression was driven from the E1A promoter (data not shown). This transient expression of the E1A proteins produced drastic morphological changes strikingly similar to those observed in cells infected with the 19K mutant adenoviruses (Fig. 8). In experiments in which 50% of the cells were positive for E1A expression by indirect immunofluorescence, a cytopathic effect became apparent 24 h posttransfection and increased steadily until 72 h, when a significant proportion of the cells had rounded up and detached from the surface of the dish (Fig. 8). Electron microscopic examination of the nuclei and cytoplasm of E1A-expressing cells indicated that the cells underwent pronounced necrosis (data not shown). This cytopathic effect induced by transient E1A expression was mitigated by cotransfection of pCMVE1A with an E1B 19K gene expression vector (pCMV19K; 59) (Fig. 8), whereas cotransfection of pCMVE1A with another CMV expression vector, pCMV55K, expressing a different, unrelated E1B protein (63) did not (data not shown).

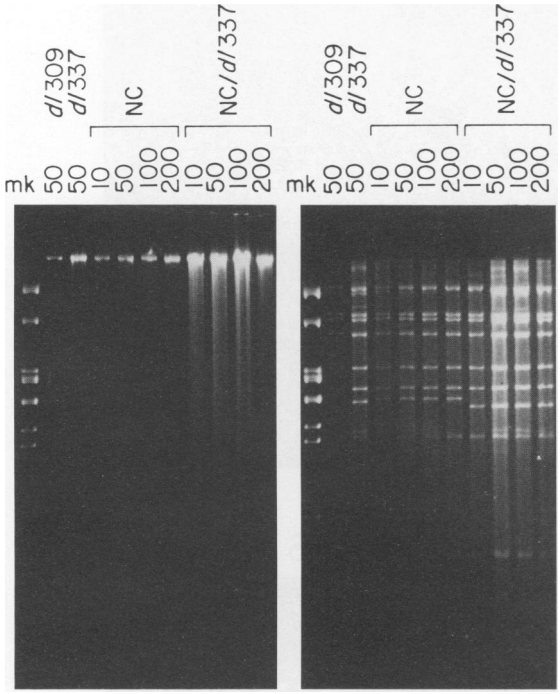

FIG. 5. Induction of DNA degradation with E1B 19K and E1A nonconserved region double-mutant virus. Cells were infected as described in the legend to Fig. 2, except that the nonconserved region E1A deletion mutation viruses containing a wild-type E1B gene (NC) or an E1B 19K gene deletion mutation (NC/*dl*337) were used. *Hin*dIII was used to digest the Hirt supernatant DNA in the right panel, and the multiplicities of infection were 10, 50, 100, and 200 PFU per cell, as indicated.

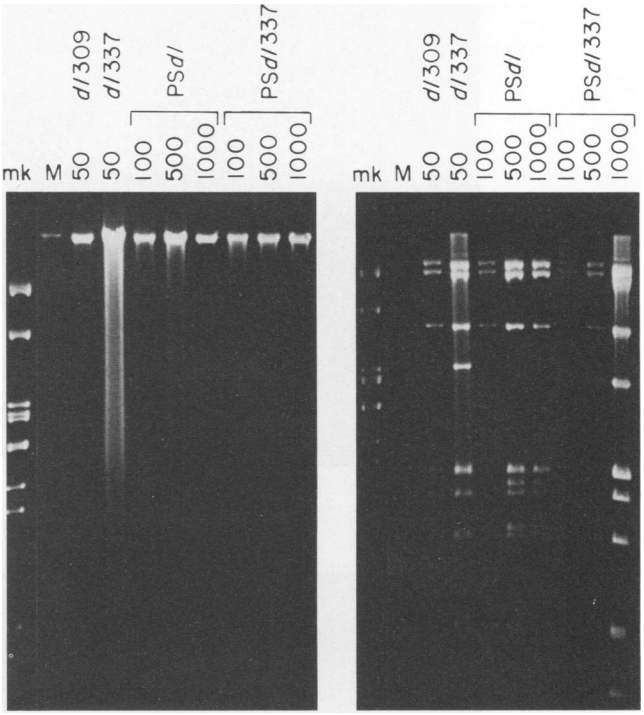

FIG. 6. Absence of DNA degradation induction with E1B 19K and E1A conserved region 1 double mutant. Cells were infected as described in the legend to Fig. 2, except that PS region 1 E1A deletion mutation viruses containing either a wild-type E1B gene (PS*dl*) or an E1B 19K gene deletion mutation (PS*dl*337) were used. Multiplicities of infection were 50, 100, 500, and 1,000 PFU per cell, as indicated.

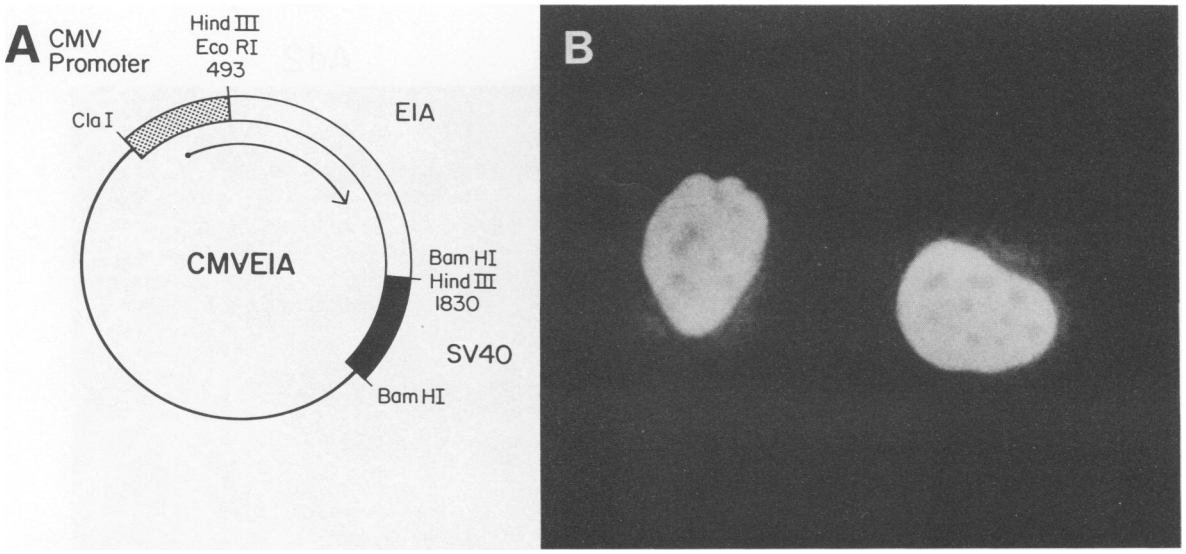

FIG. 7. E1A plasmid expression vector and transient expression of E1A proteins. (A) The E1A coding region (nucleotides 493 to 1830) was cloned into a CMV promoter expression vector (62). SV40, simian virus 40. (B) Expression of E1A proteins was detected in HeLa cells by indirect immunofluorescence with an E1A-specific monoclonal antibody 48 h following calcium phosphate-mediated DNA precipitation. Original magnification, ×1,000.

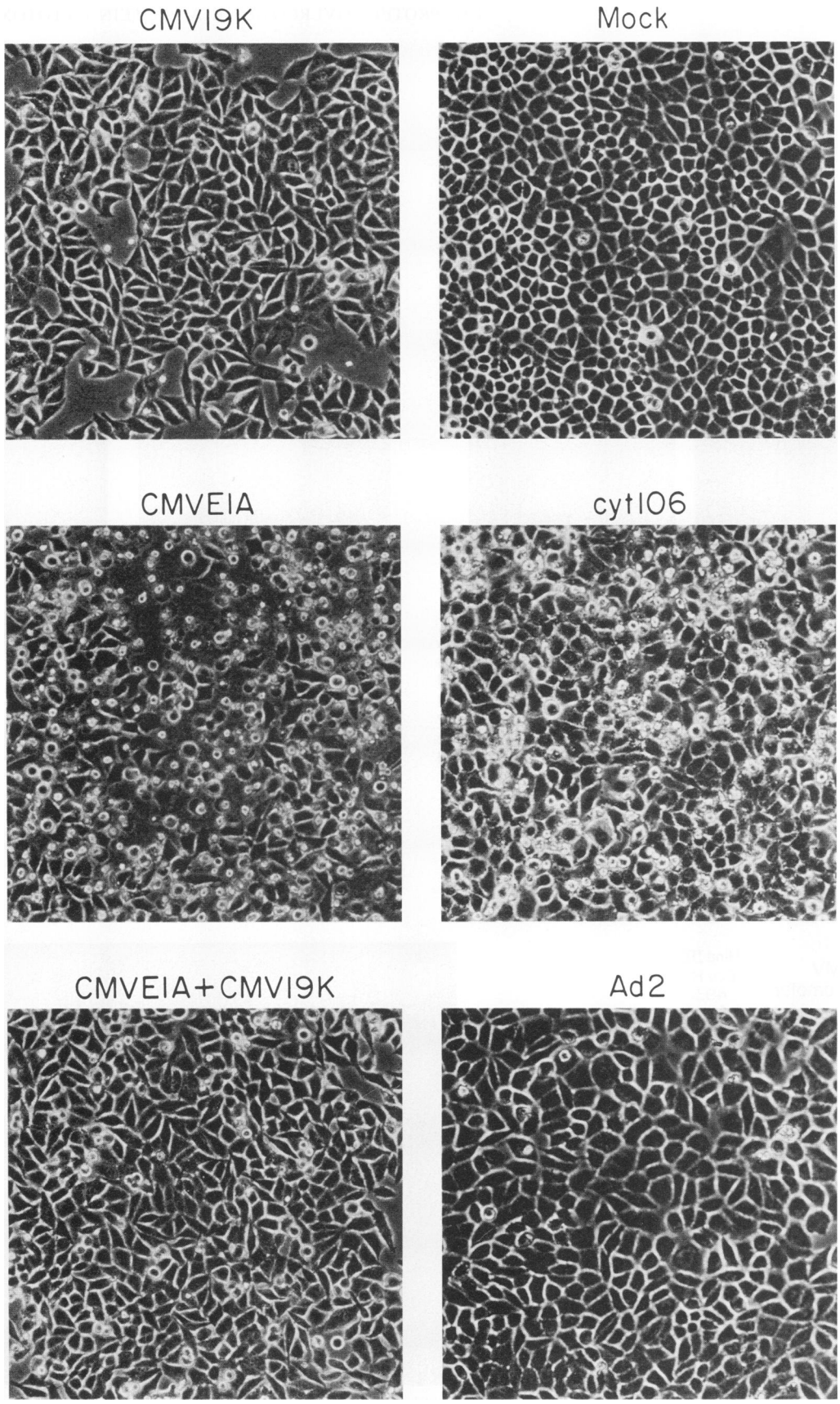

FIG. 8. Induction of an enhanced cytopathic effect by transient E1A expression. HeLa cells were transfected with 10 μg of CMV 19K, CMVE1A, or CMVE1A + CMV19K plasmid DNA. For comparison, cells were also mock infected or infected with cyt106 (Ad5*cyt*106 19K mutant) or wild-type adenovirus type 2 (Ad2) at a multiplicity of 50 PFU per cell. Magnification, ×25.

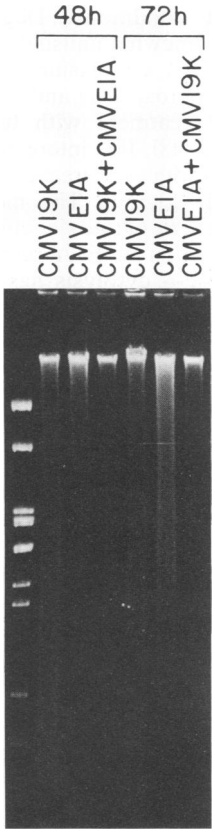

FIG. 9. Degradation of cellular DNA caused by transient E1A expression. HeLa cells were transfected with 10 μg of CMV19K, CMVE1A, or CMVE1A + CMV19K plasmid DNA, and Hirt DNA was isolated at 48 and 72 h posttransfection. Leftmost lane, adenovirus type 2 DNA digested with *Hin*dIII as size markers.

The resemblance of the cytopathic effect induced upon transient E1A expression to the *cyt* phenotype of E1B 19K mutant viruses prompted an examination of the integrity of the DNA within the cells. The presence of degraded DNA began to be apparent above the background in pCMVE1A-transfected cells at 48 h and was maximal at 72 h, when the cytopathic effect was most severe (Fig. 9). Although this DNA degradation was not as extensive as the *deg* phenotype in virus-infected cells, this would be expected since at best only half of the cells transfected with pCMVE1A DNA express the E1A proteins, whereas all cells do so during viral infection. As with the cytopathic effect, cotransfection of the E1B 19K expression vector (pCMV19K; 62) with pCMVE1A suppressed the appearance of degraded DNA (Fig. 9). Therefore, transient E1A expression induces a cytopathic effect similar to that observed in cells infected with E1B 19K gene mutant viruses, as well as degradation of cellular DNA. Furthermore, as in the case of viral infection, coexpression of the E1B 19K protein with E1A was specifically required to prevent the occurrence of the *cyt* and *deg* phenotypes.

Transformation of primary rodent cells is unaffected by overexpression of E1A proteins. The *cyt* and *deg* phenotypes observed upon overexpression of the E1A proteins in HeLa cells raised the possibility that these toxic effects affect transformation of primary BRK cells. pE1A, a construct in which E1A is expressed from its own promoter at much lower levels than with the CMV promoter (62), and pCMVE1A were used in BRK cell transformation assays. pCMVE1A and pE1A showed equally poor abilities to induce focus formation in primary BRK cells (Table 1). Transfection of either plasmid resulted in small numbers of abortive foci characteristic of E1A in the absence of a second, cooperating oncogene. This suggested that the level of E1A expression is not a significant factor regulating the frequency of focus formation or the characteristics of the resulting cells.

TABLE 1. Induction of focus formation in primary BRK cells

DNA(s)	No. of foci/no. of plates (no. of foci/plate) in:		
	Expt 1	Expt 2	Expt 3[a]
pE1A	[4][b]/12 (0.3)		
pCMVE1A	[5]/12 (0.4)	[2]/10 (0.2)	
pE1A + pE1B	13/8 (1.6)	12/3 (4.0)	78/4 (19.5)
pCMVE1A + pE1B	11/12 (0.9)		
pCMVE1A + pCMVE1B			85/4 (21.3)
pCMVE1A + pCMV19K		9/ 0(0.9)	
pCMVE1A + pCMV55K		17/10 (1.7)	
pCMVE1A + pCMV19K + pCMV55K		21/10 (2.1)	

[a] Transfection by electroporation.
[b] Brackets indicate abortive foci.

Cotransfection of E1A with E1B not only dramatically increased the frequency of transformation (Table 1) but conferred an increased growth rate and morphological transformation and enabled growth to high density in an anchorage-independent fashion (63). The strength of the promoter driving expression of the E1A gene made no significant difference in cooperation assays with E1B (Table 1). This was true whether the E1B gene used its own promoter or the CMV promoter (Table 1). The transforming activities of both E1B proteins were clearly detectable in cotransfections with the pCMVE1A plasmid with either the pCMV19K or pCMV55K construct (Table 1). This suggested that up regulating the level of E1A expression does not affect the requirement for either E1B protein in transformation.

DISCUSSION

The E1B 19K protein has long been known to protect DNA from degradation and prevent the occurrence of abnormal and enhanced cytopathic effect (46, 56, 66). Genetic studies with mutant adenoviruses have demonstrated that expression of the E1A proteins is responsible for the appearance of the *cyt* and *deg* phenotypes, suggesting that expression of the E1A proteins themselves is cytotoxic in the absence of the E1B 19K protein (67). We sought to determine which functional domain of E1A was responsible for this cytotoxicity and how it may relate to other functions of E1A. The results demonstrate that despite deletions spanning most of exon 2 and amino acids 86 to 185 in exon 1, encompassing conserved regions 3 and 2 encoding the transactivation and retinoblastoma tumor susceptibility gene product-binding activities, the E1A proteins retain the ability to induce the *cyt* and *deg* phenotypes. A deletion encompassing conserved region 1 in the amino terminus of the protein, however, eliminated this cytotoxicity.

The amino terminus of E1A serves as a binding site for a discrete set of cellular proteins and is known to be essential for induction of cellular DNA synthesis and enhancer repres-

sion, activities thought to be linked to the transforming activity of E1A (16, 52, 70). Whether any or all of these activities are related to the cytotoxic effects of E1A remains to be determined. It is conceivable that aberrant or inappropriate induction of cellular DNA synthesis by E1A is cytotoxic to cells. Alternatively, E1A may repress the expression of certain cellular genes, resulting in cytotoxic effects. The E1B 19K protein may be required to counteract or modulate these phenomena. Conversely, E1A-dependent enhancer repression may be a consequence of E1A cytotoxicity. Evidence of this is supplied by the observation that the E1B 19K protein relieves enhancer repression by E1A (60a, 73). Further genetic analysis of E1A and E1B 19K protein function will help determine whether any or all of these possibilities are viable.

Alleviation of the cytotoxic effects of E1A expression may be all or part of the role of the E1B 19K protein in adenovirus transformation. The *cyt* and *deg* phenotypes are so pronounced in E1B 19K viral mutant-infected primary BRK cells that transformation assays using virus result in no focus formation because of extensive cell death (66). Therefore, cytotoxicity of E1A in BRK cells is a significant factor affecting the frequency of transformation by virus. Adami and Babiss (1) have described a mutant adenovirus that expresses 100-fold less E1A than the wild-type virus. This mutant possesses eightfold greater transforming ability, suggesting that higher levels of E1A expression actually can be detrimental to the frequency of transformation by virus.

With plasmid DNA, the cytotoxicity of E1A, under control of the E1A or CMV promoter, is not readily apparent, most likely because of the low efficiency of DNA transfection in BRK cells. E1A cytotoxicity may explain the failure to obtain E1A-transformed BRK cell lines that express high levels of E1A in the absence of E1B (31). Although transfection of DNA encoding E1A alone is not sufficient for significant focus formation, coexpression of either the E1B 19K or 55K protein will enable transformation of primary BRK cells to occur (63). The E1B 19K protein may permit E1A expression to be tolerated at higher levels and thereby enhance transformation. In support of this possibility, cell lines transformed by E1A and E1B have been reported to contain higher levels of E1A mRNA than do E1A-immortalized lines (58).

The mechanism by which the E1B 19K protein overcomes the cytotoxic effects of E1A expression is not known. Studies with E1B 19K mutant adenoviruses suggest that suppression of the *cyt* phenotype and suppression of the *deg* phenotype are genetically separable functions of the 19K protein (54, 61, 66). Furthermore, the relationship between the function of the 19K protein in productively infected cells and that in transformed cells has not been established. A complete genetic analysis of the E1B 19K protein and its functions in infected and transformed cells is required to address this issue. The 19K protein does, however, share one important feature in both infected and transformed cells, i.e., disruption of intermediate filaments. Expression of the 19K protein alters the organization of two distinct classes of intermediate filament proteins in the cell, i.e., the cytoplasmic vimentin and nuclear lamin filaments (62, 63). It is not difficult to imagine that this perturbation of the cytoskeletal system and nuclear architecture by the 19K protein is involved in preventing abnormal cytopathic effects and protecting DNA from degradation. These events may ultimately precipitate the alterations in cell growth attributable to the 19K protein in transformed cells.

Cytotoxicity is induced by a wide variety of agents under numerous different conditions. Degradation of cellular DNA, however, is somewhat unusual and occurs not only as a consequence of E1A expression but under the specific circumstances of apoptosis (71) and cell-mediated cytolysis (15) and following treatment with tumor necrosis factor alpha (TNF-α) (5, 12, 48). It is interesting that expression of the E1A proteins produces increased sensitivity to TNF-α treatment (2, 7, 11, 13, 59). The mechanisms by which TNF-α and E1A exert cytotoxic effects may in fact be related, since the region of E1A responsible for inducing susceptibility to TNF-α cytolysis has been mapped to the amino terminus within the region defined here for induction of DNA degradation and enhanced cytopathic effect (14). Since the E1B 19K protein can protect cells from the cytotoxic effects of E1A expression, it is interesting that this protective effect can be extended to TNF-α-mediated cytolysis of adenovirus-infected cells (20). Prevention of cytotoxicity may, therefore, be a universal function of the E1B 19K protein that may be required for oncogenic transformation by adenovirus.

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