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# Expression of Adenovirus E1B Mutant Phenotypes Is Dependent on the Host Cell and on Synthesis of E1A Proteins

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**Adenovirus mutants containing genetic alterations in the gene encoding the E1B 19,000-molecular-weight (19K) tumor antigen induce the degradation of host cell chromosomal DNA (*deg* phenotype) and enhanced cytopathic effect (*cyt* phenotype) after infection of HeLa and KB cells. The *deg* and *cyt* phenotypes are a consequence of viral early gene expression in the absence of the E1B 19K protein. The role of the E1A proteins in induction of the *cyt* and *deg* phenotypes was investigated by constructing E1A-E1B double mutant viruses. Viruses were constructed to express the individual E1A 13S, 12S, or 9S cDNA genes in the presence of a mutation in the gene encoding the E1B 19K tumor antigen. Expression of either the 13S or 12S E1A proteins in the absence of functional E1B 19K protein produced the *deg* and *cyt* phenotypes. In contrast, a virus which expressed exclusively the 9S E1A gene product in the absence of the E1B 19K gene product did not induce the *deg* and *cyt* phenotypes, even at high multiplicities of infection. Therefore, both the 13S and 12S E1A gene products could directly or indirectly cause the *deg* and *cyt* phenotypes during infection of HeLa cells with an E1B 19K gene mutant virus. Furthermore, the *deg* phenotype was found to be host cell type specific, occurring in HeLa and KB cells but not in growth-arrested human WI38 cells. These results indicate that expression of the E1A *trans*-activating and transforming proteins is necessary for the induction of the *cyt* and *deg* phenotypes and that host cell factors also play a role.**

Expression of early region 1 (E1) of the adenovirus genome is sufficient to cause cellular transformation. E1 is divided into two distinct transcription units, E1A and E1B, which are expressed prior to viral DNA replication in infected cells. The E1A gene encodes two mRNAs expressed early in infection (see Fig. 1A), the 13S and 12S products (8, 17, 46, 53). The 13S mRNA encodes a 289-amino-acid polypeptide, whereas the 12S mRNA encodes a 243-amino-acid polypeptide. The 12S product differs from the 13S product only by the absence of 46 internal amino acids due to the use of an alternative splice site. The E1A region is responsible for *trans*-activating transcription of the adenovirus early transcription units (7, 13, 23, 26, 35, 42, 52, 56) and are also known to regulate the expression of some endogenous cellular genes (40, 44, 65).

In addition to their role in transcriptional regulation, the E1A gene products have the ability to immortalize rodent cells in culture (32, 38, 39, 49, 51, 59, 75, 77). However, complete transformation by adenovirus requires expression of the E1B gene (9, 14, 24, 27, 28, 38, 43, 59). Other functions of the E1A gene products include efficient replication of adenovirus DNA in quiescent cells (40, 49, 63), stimulation of cell cycle progression and induction of cellular DNA synthesis (6, 45, 63), and repression of some enhancer-dependent promoters (10, 58, 60, 70). The E1A region encodes a third 9S mRNA produced late in infection (see Fig. 1A) (17, 62, 71), which has no known functions (77).

Viruses constructed by various genetic manipulations that separately express each of the E1A gene products have been useful in assigning functions to the individual E1A proteins (28, 39, 49–51, 75, 77). Viruses capable of expressing solely the 13S gene product replicate normally, are capable of *trans*-activating viral gene transcription, and can immortalize rodent cells (39, 49–51, 75). Without expression of the 12S E1A gene product, however, the 13S-producing viruses are deficient for replication in growth-arrested WI38 cells (49, 63). Viruses expressing the 12S gene product exclusively do not replicate in HeLa cells at low multiplicities of infection (28, 37, 49–51, 56), which has been attributed to an inability to activate transcription of the viral early genes. Immortalization functions of viruses expressing the 12S E1A gene alone are, however, unimpaired (5, 28, 49, 51). A virus which is capable of expressing only the late E1A 9S mRNA does not function in *trans* activation, nor does it immortalize rodent cells (51). Phenotypically, this 9S E1A virus resembles the E1A deletion virus Ad5*dl*312 (43).

The E1B region produces three transcripts early in infection. These mRNAs encode the 19,000-molecular-weight (19K) and 55K tumor antigens (11, 20, 31, 48) and a 17K polypeptide related to the 55K tumor antigen (1) (see Fig. 1A). Through characterization of adenovirus mutants, the 55K tumor antigen has been assigned a role in the transport or processing of late mRNA (4, 55; J. Williams, B. D. Karger, Y. S. Ho, C. L. Castiglia, T. Mann, and S. J. Flint, Cancer Cells, in press) and the shutoff of host cell protein synthesis (3; Williams et al., in press).

The precise role of the 19K tumor antigen in infection or cellular transformation is less well defined. Adenovirus mutants which carry either point mutations or deletions within the coding region for the E1B 19K tumor antigen do have, however, a number of interesting phenotypes. First, they cause enhanced and unusual cytopathic effect, commonly called the cytocidal (*cyt*) phenotype, in infected HeLa and KB cells (54, 67–69, 74). The *cyt* phenotype is also associated with the formation of large plaques (*lp*); consequently, some 19K gene mutants are designated *lp* mutants (14, 67). Second, infection of HeLa or KB cells with these mutant viruses results in degradation of the host cell and viral DNA (*deg* phenotype) (18, 21, 47, 54, 66, 74). Third, mutations in the E1B 19K gene result in an inability of adenovirus to transform rodent cells (2, 9, 14, 25, 67–69, 74). Fourth, the E1B 19K gene alterations produce a host range

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phenotype for virus replication in infected human cells (73). Mutant virus replication in HeLa cells is unaffected compared with the wild-type virus, while in KB cells it is partially defective (down 10- to 100-fold) and in WI38 cells it is enhanced (up 10- to 500-fold). The increased replication of mutant viruses in infected WI38 cells is due to higher levels of early gene expression in these growth-arrested cells. These data implicated a role for the E1B 19K protein in negatively regulating early-gene expression (73). We have proposed that one function of the E1B 19K tumor antigen during infection is to regulate the expression of the E1A proteins and that the *cyt* and *deg* phenotypes are the pleiotropic result of this altered regulation (73).

The *cyt* and *deg* phenotypes which occur after infection of HeLa and KB cells with the E1B 19K gene mutant viruses are known to be the result of adenovirus early-gene functions, since they can be observed prior to and in the absence of viral DNA replication (54, 74). A deletion mutant, Ad5*dl*337 (54), which does not express the E1B 19K gene product also induces the *cyt* and *deg* phenotypes. This led to the proposal that another adenovirus early-gene product causes these phenotypes in the absence of the 19K protein (74). We have sought to identify the early gene product responsible for this phenomenon, since this protein(s) would be a candidate for functional interaction with the E1B 19K tumor antigen.

To define functional interactions between the E1B 19K gene product and other early gene products, a series of double mutant viruses have been constructed. Toward this end, viruses which express 13S, 12S, or 9S E1A cDNA genes in conjunction with an E1B 19K gene mutation have been used to assess the involvement of the E1A gene products. These E1A-E1B double mutant viruses were assayed for *cyt* and *deg* phenotypes. It was anticipated that eliminating expression of the factor causing the *deg* and *cyt* phenotypes would result in a wild-type phenotype ($deg^+$ $cyt^+$) even in the absence of the E1B 19K protein. We found that expression of either 13S or 12S E1A gene products in a $19K^-$ viral background was sufficient to induce *cyt* and *deg*. Eliminating expression of both the 13S and 12S gene products by substituting an E1A 9S gene, however, resulted in reversion to the wild-type $cyt^+$ and $deg^+$ phenotypes in a $19K^-$ viral background. Finally, in addition to the E1A proteins, host cell factors are important for induction of the *deg* phenotype, since $19K^-$ viral mutants do not cause DNA degradation on growth-arrested WI38 cells.

## MATERIALS AND METHODS

**Cells and viruses.** Mammalian cells were grown in monolayer culture in Dulbecco modified Eagle medium with 10% fetal bovine serum. Human diploid embryonic lung cells (WI38) were purchased from Whittaker M.A. Bioproducts, Walkersville, Md. Viruses were grown in, and titers were determined by plaque assay on, 293 cells (29). Virus stocks were maintained as crude lysates. Adenovirus mutants which contain E1A 13S, 12S, or 9S cDNA genes in place of wild-type E1A sequences were obtained from E. Moran and B. Zerler, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., as were the E1 plasmids pLE13S, pLE12S, and pLE9S that were used to construct them (51, 77). The Ad2*cyt*106 virus, which contains a point mutation (G-to-A transition at nucleotide 1769) and results in an amino acid substitution (Ser to Asn) in residue number 20 of the E1B 19K protein, was isolated and described previously (74). Ad5*dl*337, which contains a deletion in the E1B 19K coding region (54), was obtained from T. Shenk, Princeton University, Princeton, N.J. Ad5*dl*337 and the viruses described below were derived from the Ad5*dl*309 virus (42); therefore, Ad5*dl*309 was used as the wild-type virus in experiments with these viruses. Ad2*lp*3 and Ad2*lp*5 contain point mutations in the E1B 19K coding region (14) and were obtained from G. Chinnadurai, St. Louis University Medical School, St. Louis, Mo. These viruses are isogenic with the wild-type adenovirus type 2 (Ad2). Confluent monolayers of HeLa cells were used for virus infections, whereas WI38 cells monolayers were used 3 days after reaching confluence and were growth arrested.

**Plasmid and recombinant virus construction.** For the purpose of obtaining E1A-E1B double mutant viruses, a series of plasmids was constructed which contain DNA from the left-end 15.5 map units (m.u.) of the adenovirus genome. E1 sequences containing the point mutation in the E1B 19K gene from Ad2*cyt*106 were isolated from virus DNA (74) and cloned so that the resulting plasmid (p111R2) had 0 to 15.5 m.u. of the adenovirus genome with a *Bam*HI site at 0 m.u. and the 19K gene mutation at 5.9 m.u. The *Cla*I-*Hpa*I fragments from plasmids pLE13S and pLE12S, containing E1A 13S and 12S cDNA sequences, respectively, were substituted for wild-type E1A sequences in p111R2. The final constructs contained 0 to 15.5 m.u. of the left end of the viral genome with E1A 13S or 12S genes in conjunction with the E1B 19K gene point mutation (plasmids p13S.R2 and p12S.R2, respectively). The engineered E1 sequences were rebuilt into intact viral chromosomes by overlap recombination (15). DNA from the p13S.R2 and p12S.R2 plasmids was digested with *Bam*HI and *Bgl*II, liberating adenovirus sequences from 0 to 9 m.u., and used in cotransfections with the *Xba*I large fragment (3.8 to 100 m.u.) from Ad5*dl*309 (42). DNA fragments were transfected into 293 cells by the calcium phosphate technique (30) followed by a glycerol boost at 4 h after transfection.

Recombinant viruses arising from transfections were plaque purified on 293 cells and screened by restriction endonuclease mapping for the appropriate E1A cDNA sequences and the E1B 19K gene point mutation (see below). The resulting 13S.R2 and 12S.R2 viruses contain left-end sequences donated by the p13S.R2 and p12S.R2 plasmids, and the majority of the right-hand end of the viral chromosome was contributed by Ad5*dl*309 DNA (see Fig. 1B).

Construction of the E1A 9S cDNA-E1B double mutant virus used plasmid pLE9S, containing E1 sequences with an *Eco*RI site at 0 m.u. through to the *Bgl*II site at 9 m.u. and the E1A 9S cDNA gene in place of the wild-type E1A gene (51, 77). The *Xba*I-*Hin*dIII fragment (3.8 to 7.9 m.u.) from p111R2, encompassing the E1B 19K gene point mutation at 5.9 m.u., was substituted for the corresponding wild-type E1B sequences in the pLE9S plasmid. The resulting p9S.R2 construction contained the E1A 9S cDNA gene in conjunction with the E1B 19K gene mutation. E1 sequences from 0 to 7.9 m.u. were excised from the p9S.R2 plasmid by *Eco*RI-*Hin*dIII digestion and used in cotransfections with the *Xba*I large fragment from Ad5*dl*309 as described above. Recombinant viruses were plaque purified on 293 cells and screened by restriction endonuclease mapping. The resulting 9S.R2 virus contained the E1A 9S cDNA gene and the E1B 19K gene mutation at the left end of the viral chromosome in an Ad5*dl*309 wild-type virus background (see Fig. 1B).

**Analysis of DNA.** Low-molecular-weight DNA was isolated from infected cells by the method of Hirt (36) with minor modifications (74). DNA from the supernatant fraction

was analyzed by electrophoresis through 1% agarose gels, followed by ethidium bromide staining.

## RESULTS

**Construction and isolation of viral mutants.** The involvement of the individual E1A gene products in induction of the *cyt* and *deg* phenotypes was tested via the construction of E1A-E1B double mutant viruses. The desired mutations were constructed in plasmids containing the entire E1 region, and the mutations were then rebuilt back into the virus by in vivo overlap recombination with Ad5*dl*309 DNA. Viruses were constructed so that the wild-type E1A gene was replaced with cDNA sequences for the 13S, 12S, or 9S E1A genes in the background of an E1B 19K gene mutation (Fig. 1). Viruses containing the 13S cDNA gene (13S and 13S.R2 viruses) expressed only the 289-amino-acid E1A protein, and viruses containing the 12S cDNA gene (12S and 12S.R2 viruses) expressed only the 243-amino-acid protein (51, 77; data not shown). The 9S cDNA viruses (9S and 9S.R2 viruses) were capable of expressing only the 9S E1A product, but a corresponding gene product has not been detected in infected HeLa cells (77).

In the context of a wild-type E1A gene, a virus possessing a point mutation in the E1B 19K coding region (Ad2*cyt*106) induces the *cyt* and *deg* phenotypes after infection of HeLa cells (72, 74). The point mutation at nucleotide 1769 results in an amino acid substitution which renders the 19K protein unstable and affects its localization within the infected HeLa cells (72, 74). The E1A cDNA genes were separately combined with this E1B 19K point mutation from Ad2*cyt*106 producing the E1A-E1B double mutant viruses (Fig. 1), and the viruses were then assayed for induction of the *cyt* and *deg* phenotypes.

**Analysis of DNA degradation phenotype.** The effect of production of specific E1A 13S, 12S, and 9S gene products on expression of the *cyt* and *deg* phenotypes was assayed in infected HeLa cells. HeLa cells were infected with the 13S and 13S.R2 viruses at increasing multiplicities of infection, and at 48 h postinfection, Hirt supernatant DNA was isolated and subjected to agarose gel electrophoresis. Under these conditions, the degraded DNA appeared as a smear and was clearly evident in 13S.R2- but not 13S-infected cells (Fig. 2A). When the Hirt supernatant DNA was digested with restriction enzymes to reveal the viral DNA fragments, the 13S and 13S.R2 viruses replicated viral DNA to similar extents (Fig. 2B) and to levels comparable to the wild-type virus (see Fig. 6). Therefore, introducing an E1B 19K gene mutation into an E1A 13S gene background had no detectable effect on viral DNA replication in HeLa cells. The difference in the pattern of DNA fragments in *Sac*I digests between the two viruses was the result of the 19K gene mutation, which alters the *Sac*I site at 5.9 m.u. (74). An F-G fusion fragment was produced (3,632 base pairs) from the left end of the virus genome with a wild-type E1A gene, which was reduced in size by 111, 252, or 589 base pairs by insertion of E1A 13S, 12S, or 9S cDNA sequences, respectively (Fig. 1; see Fig. 6 for comparison).

The 12S and 12S.R2 viruses were assayed for the *deg* phenotype by the same methods used for the 13S viruses. Since the 243-amino-acid protein was the only E1A gene product synthesized by these two viruses, transcription of the adenovirus early genes was not *trans*-activated efficiently during infection (35, 49, 51). The multiplicity-dependent leaky phenotype of E1A mutant viruses (42), however, permitted early viral gene expression at high multiplicities of infection in HeLa cells. At 72 h postinfection, the *deg* phenotype was severe in 12S.R2-infected HeLa cells but was most prominent in cells infected with 100 and 500 PFU/ml (Fig. 3A). Surprisingly, DNA degradation was also observed at low multiplicities of infection with the 12S.R2 virus. Little DNA degradation was evident in 12S virus-infected cells at any multiplicity of infection (Fig. 3A). As expected, the 12S and 12S.R2 viruses synthesized significant levels of virus DNA only at high multiplicities of infection, while the amounts of viral DNA synthesized were similar (Fig. 3B).

The effect of synthesis of only the 9S E1A gene product on induction of the *deg* phenotype was assayed by infection of HeLa cells with the 9S and 9S.R2 viruses. Like the 12S viruses, the 9S viruses did not synthesize the E1A 13S (289-amino-acid) gene product to *trans* activate transcription; therefore, this effect was overcome by infection at high multiplicities. At all multiplicities of infection tested (10 to 500 PFU/cell), the 9S.R2 virus did not induce host cell DNA degradation (Fig. 4A). The small amounts of degraded DNA that appeared in this gel of the 9S.R2 Hirt DNA were no greater than appeared in the mock-infected control plate (Fig. 4A). We found that this small amount of endogenous degraded DNA in either uninfected or defective-virus-infected HeLa cells could be surpressed by expression of the E1B 19K protein. This was clearly demonstrated in 12S virus-infected HeLa cells (Fig. 3A). There was a considerable reduction in the amount of degraded DNA when HeLa cells were productively infected with the 12S virus (multiplicity of infection of 500) compared with infections at low multiplicities, in which early gene expression was compromised. It was clear, however, that the DNA degradation phenotype could easily be distinguished from endogenous DNA degradation in side-by-side comparisons of *deg* and *deg*$^+$ mutant-virus-infected cells in Fig. 6 (see below).

In addition, the lack of DNA degradation in 9S.R2-infected HeLa cells even at high multiplicities of infection was not due to the lack of early-gene transcription. Viral DNA was synthesized in 9S- and 9S.R2-infected HeLa cells nearly to levels found in infected 293 cells (Fig. 4B). In addition, both 9S viruses synthesized large amounts of the 72K DNA-binding protein in HeLa cells at high multiplicities of infection (Fig. 5).

The effect of synthesis of the individual E1A proteins on occurrence of the *deg* phenotype was analyzed by comparing all the viruses in the same experiment, as shown in Fig. 6. Expression of all E1A gene products (Ad5*dl*337 virus) or only the 13S (13S.R2 virus) or 12S (12S.R2 virus) gene products caused degradation of host cell chromosomal DNA in the absence of a functional E1B 19K gene product. Eliminating expression of both the 13S and 12S gene products (9S.R2 virus) alleviated the requirement for the E1B 19K protein in preventing DNA degradation during infection. No DNA degradation occurred with the wild-type virus (Ad5*dl*309) or the 13S, 12S, and 9S viruses expressing wild-type E1B proteins.

**Analysis of *cyt* phenotype.** The role of the individual E1A gene products in inducing the *cyt* phenotypes was examined in infected HeLa cells. An infection with the wild-type virus (Ad5*dl*309) induced rounding of the cells that was only prominent at late times postinfection. In contrast, infection with an E1B 19K gene mutant virus (Ad5*dl*337) produced an enhanced and more dramatic cytopathic effect earlier during infection (Fig. 7). This *cyt* phenotype also caused the cells to become detached from the dish. Expression of either the 13S or 12S E1A gene product in conjunction with an E1B 19K

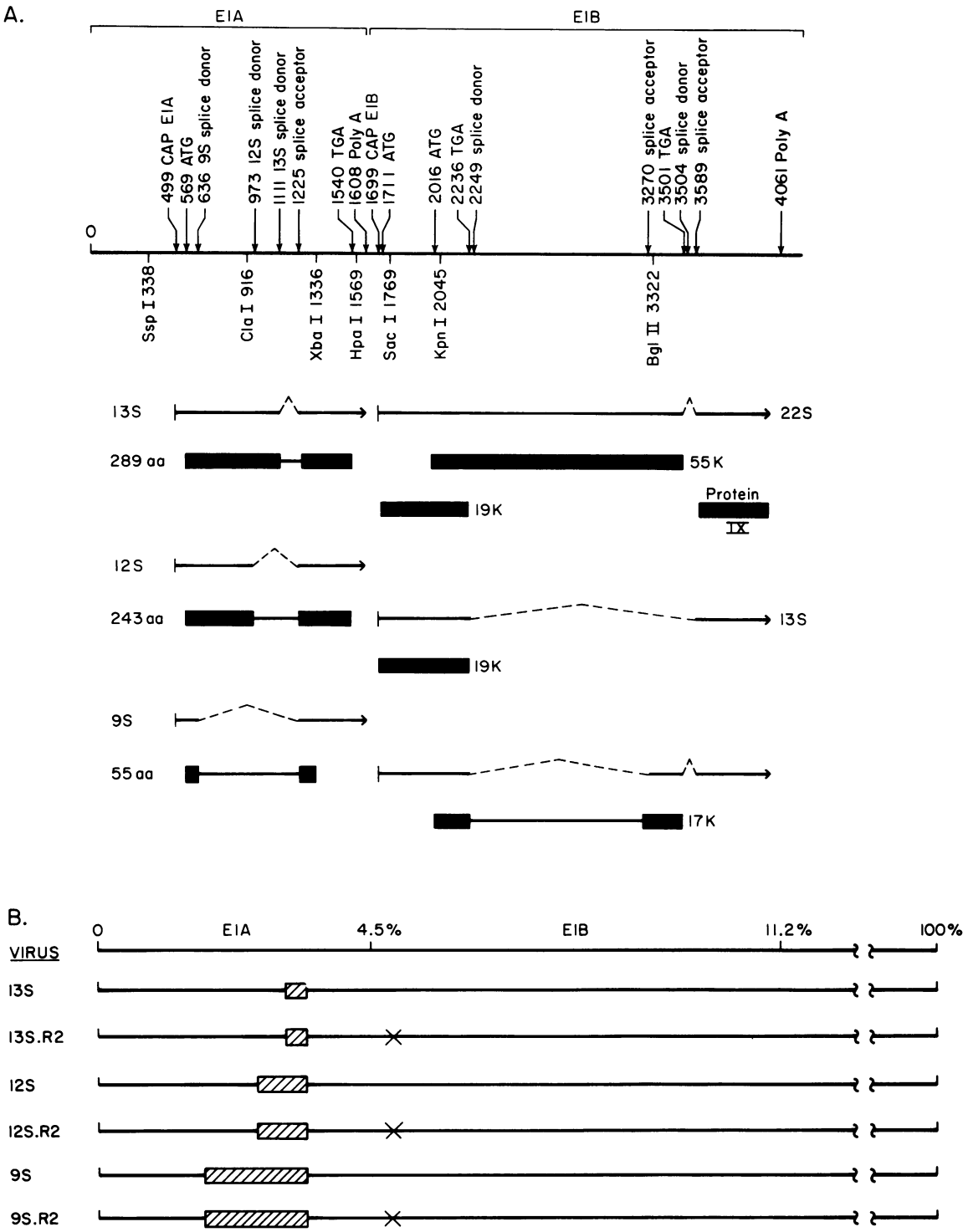


FIG. 1. Schematic representation of E1 and adenovirus mutants. (A) Map of E1, delineating positions of differentially spliced mRNAs (thin arrows; carets indicate introns removed by splicing), corresponding translation products (solid boxes), and relevant restriction sites. Numbers indicate base pairs, beginning from the left end of the genome. Poly A, Polyadenylation site; aa, amino acids. (B) Map of mutant virus genomes, with numbers indicating map units (percent genome length) from the left end of the genome. Hatched boxes indicate deletions of intron sequences due to the insertion of cDNA genes. X indicates the position of the point mutation and amino acid substitution in the E1B 19K gene and protein, respectively.

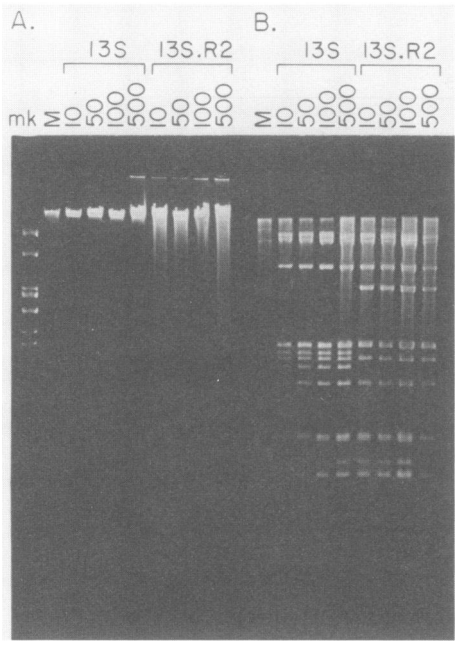


FIG. 2. Analysis of DNA degradation phenotype in 13S and 13S.R2 mutant-virus-infected HeLa cells. Cells were infected at multiplicities of infection of 10, 50, 100, and 500 PFU per cell. DNA was isolated at 48 h postinfection by the method of Hirt (36), and the DNA in the supernatant fraction was separated by electrophoresis in an agarose gel. Lanes M, mock-infected cells. (A) Undigested DNA. (B) *Sac*I digest of Hirt supernatant DNA. Lane mk, Ad2 DNA digested with *Hin*dIII as size markers.

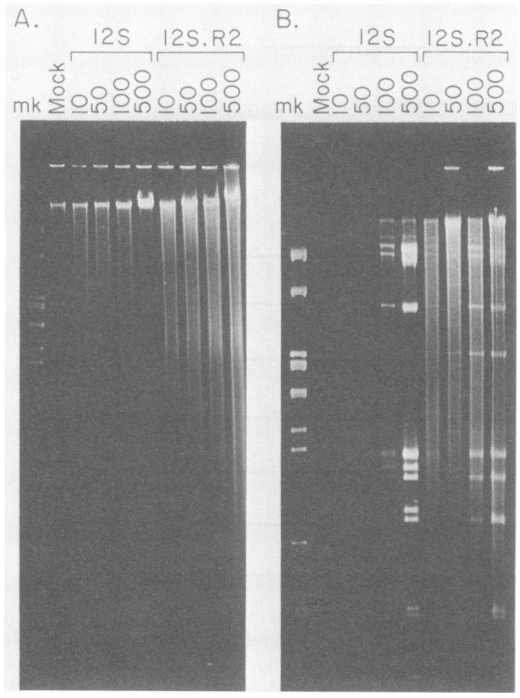


FIG. 3. Analysis of DNA degradation phenotype in 12S and 12S.R2 mutant-virus-infected HeLa cells. Cells were infected as described in the legend to Fig. 2, except that DNA was isolated at 72 h postinfection. (A) Undigested DNA. (B) *Sac*I-digested DNA.

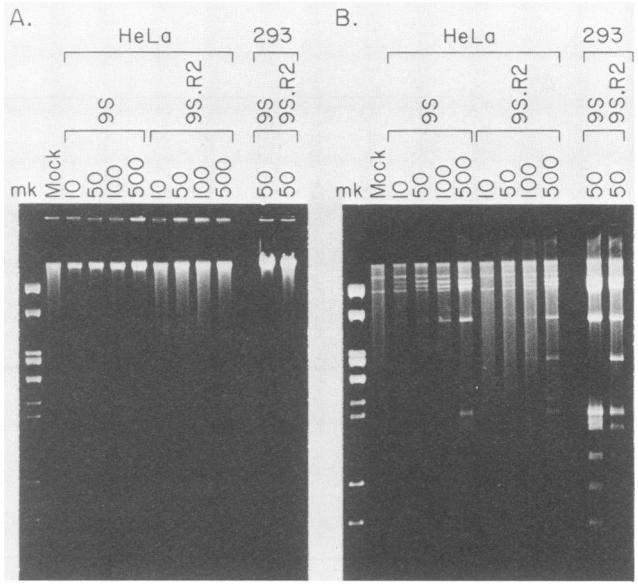


FIG. 4. Analysis of DNA degradation phenotype in 9S and 9S.R2 mutant-virus-infected HeLa cells. HeLa and 293 cells were infected as described in the legend to Fig. 3. (A) Undigested DNA. (B) *Sac*I-digested DNA.

gene mutation was sufficient to induce the *cyt* phenotype, although in the 13S.R2-infected cells the phenotype was less marked (Fig. 7). In contrast, infection with the 9S.R2 virus did not induce the *cyt* phenotype and infected-cell morphology was indistinguishable from that in either wild-type Ad5*dl*309 or 9S virus infections. Therefore, eliminating expression of either the 13S or 12S gene product was insufficient to prevent the *cyt* and *deg* phenotypes in an E1B 19K$^-$ virus infection. By eliminating expression of both 13S and 12S E1A gene products, the E1B 19K protein was not required to prevent the *cyt* and *deg* phenotypes in infected HeLa cells.

**Absence of the DNA degradation phenotype in WI38 cells.** In addition to the *cyt* and *deg* phenotypes, the E1B 19K gene mutant viruses also possess a host range phenotype for virus replication in human WI38 cells (73). These cells are nontransformed secondary lung fibroblasts (33, 34) which can be easily growth arrested. In comparable infections, the E1B mutant viruses have a substantial growth advantage

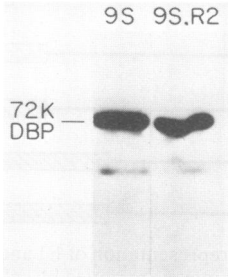


FIG. 5. Expression of the 72K DNA-binding protein (DBP) in 9S virus-infected HeLa cells. Cells were infected with either the 9S or 9S.R2 virus at a multiplicity of 500 PFU/cell and harvested at 48 h postinfection. Extracts were prepared and analyzed in a Western blot with antibodies directed against the E2A 72K DNA-binding protein (73).

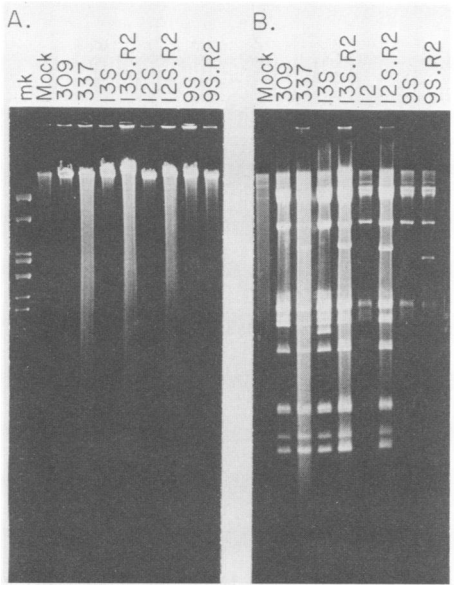


FIG. 6. Summary of DNA degradation phenotypes. HeLa cells were mock infected or infected with Ad5*dl*309, Ad5*dl*337, 13S, and 13S.R2 viruses at a multiplicity of 100 PFU/cell or the 12S, 12S.R2, 9S, and 9S.R2 viruses at a multiplicity of 500 PFU/cell. Hirt supernatant DNA was isolated 48 h postinfection and subjected to electrophoresis in an agarose gel. (A) Undigested. (B) *Sac*I digested. Lane mk, Ad2 DNA digested with *Hin*dIII as size markers.

over the wild-type virus in these growth-arrested cells (73). This effect occurs at the level of early-gene expression (73). While monitoring mutant and wild-type virus replication in WI38 cells, it was observed that the E1B 19K mutant viruses did not induce the *deg* phenotype in these cells. Infection of HeLa cells with Ad2*cyt*106 resulted in the degradation of host cell chromosomal DNA (Fig. 8) (74), whereas infection of WI38 cells at the same multiplicity did not (Fig. 8). Similarly, infection with Ad5*dl*337 and Ad2*lp*5, which carry different genetic alterations in the E1B 19K gene than Ad2*cyt*106, produced the *deg* phenotype in HeLa cells but not in WI38 cells (Fig. 8B). The E1B 19K gene mutant viruses still failed to induce the *deg* phenotype in WI38 cells at high multiplicities of infection (>200 PFU/cell; data not shown) or at prolonged times postinfection (Fig. 8B). The absence of the *deg* phenotype in mutant-infected cells was not a result of an unproductive infection, since there was efficient early and late gene expression and virus production in infected WI38 cells (73). It was difficult to assess the appearance of the *cyt* phenotype in WI38 cells because of differences in the growth rates of the wild-type and mutant viruses and the morphology of the cells.

**Viral DNA replication in mutant-infected WI38 cells.** The absence of the *deg* phenotype in growth-arrested WI38 cells indicated that in addition to the 13S and 12S E1A proteins, cell type or cell growth physiology may also play a role in inducing the degradation of host cell chromosomal DNA. The 12S E1A gene product was shown to be required for maximal virus replication in growth-arrested WI38 cells (49, 63). This is thought to occur through the 243-amino-acid E1A protein altering the growth physiology of resting cells and thereby producing a more favorable environment for viral replication (63). In contrast, the E1B 19K protein has a negative effect on adenovirus replication in growth-arrested WI38 cells (73). That is, infection with 19K$^-$ viral mutants

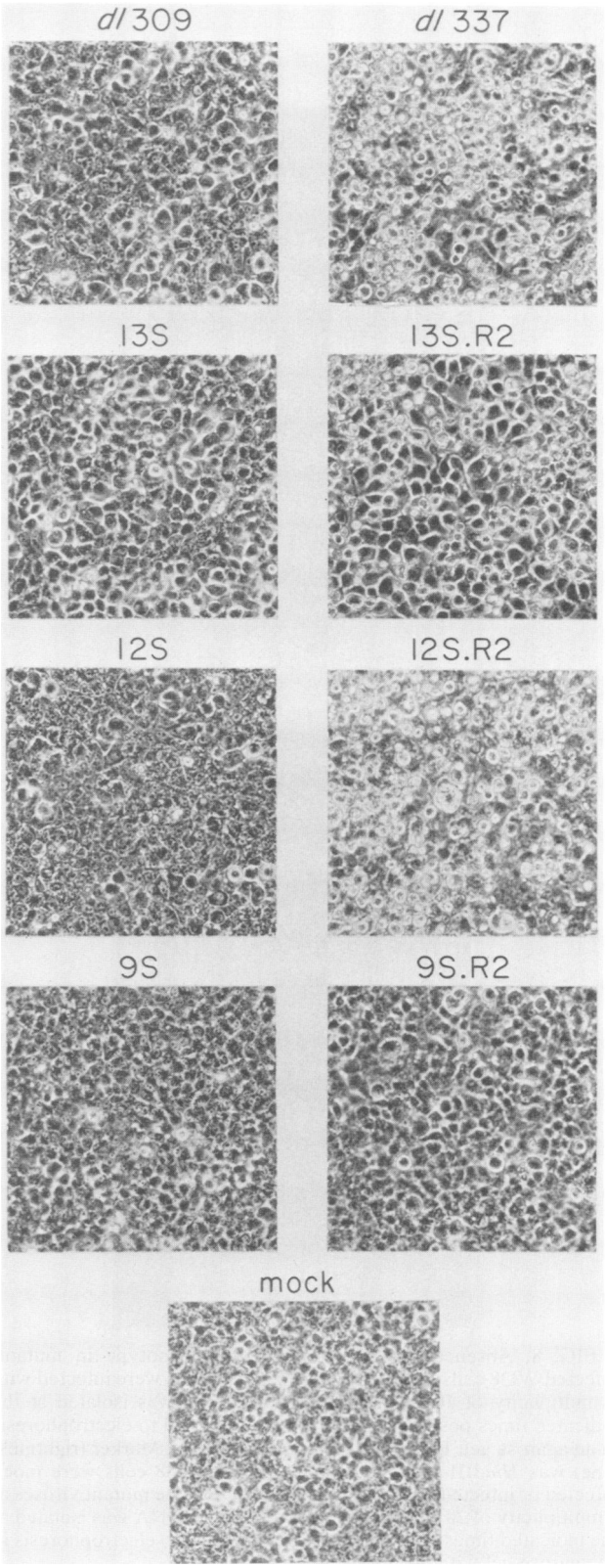


FIG. 7. Analysis of cytocidal phenotype (*cyt*) in mock-infected and mutant-virus-infected HeLa cells. Cells were infected as described in the legend to Fig. 6 and photographed at 48 h postinfection.

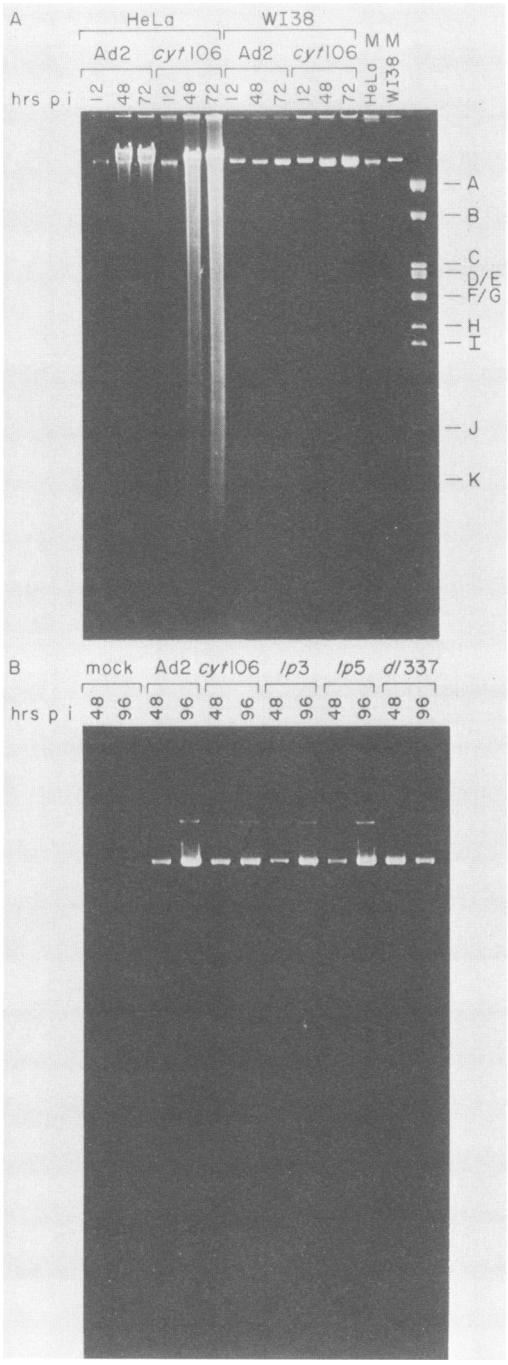


FIG. 8. Absence of DNA degradation phenotype in mutant-infected WI38 cells. (A) HeLa cells or WI38 cells were infected with a multiplicity of 10 PFU/cell, and Hirt DNA was isolated at the indicated times postinfection (p.i.) and subjected to electrophoresis in an agarose gel. Lanes M, Mock-infected cells. Marker (rightmost lane) was *Hin*dIII-digested Ad2 DNA. (B) WI38 cells were mock infected or infected with Ad2 or the E1B 19K gene mutant viruses at a multiplicity of 20 PFU/cell. Hirt supernatant DNA was isolated at the indicated times postinfection and subjected to electrophoresis in an agarose gel without prior digestion.

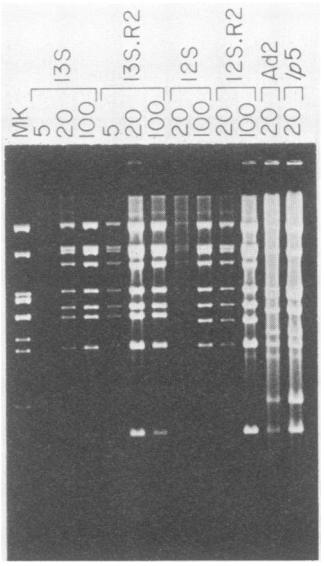


FIG. 9. Replication of virus DNAs in WI38 cells. WI38 cells were infected at the indicated multiplicities (PFU per cell) with wild-type or mutant viruses. Hirt supernatant DNA was isolated at 48 h postinfection, digested with *Hin*dIII, and subjected to electrophoresis in an agarose gel. Lane MK, Ad2 DNA digested with *Hin*dIII as size markers.

resulted in enhanced replication (73). The apparently opposing effects of the E1A 12S and E1B 19K gene products on virus replication in WI38 cells led us to investigate potential interactions between these E1A and E1B proteins. This was accomplished genetically by using the E1A-E1B double mutant viruses.

Infection of WI38 cells with the 13S.R2 virus produced a severalfold increase in the amount of virus DNA during infection relative to infection with the 13S virus (Fig. 9). This was consistent over a wide range of multiplicities of infection. Similar results were obtained with 12S.R2 virus-infected cells (Fig. 9). Thus, the absence of functional E1B 19K protein in the 12S.R2-infected cells resulted in elevated levels of viral DNA, comparable to the amount synthesized by the 13S virus at the same multiplicity of infection. For example, compare the amounts of DNA synthesized in 13S virus- and 12S.R2 virus-infected cells at a multiplicity of 20 PFU/cell (Fig. 9).

In the absence of the E1B 19K protein, the enhancement of viral DNA replication during infection of WI38 cells was found to be clearly E1A 13S and 12S gene product-dependent, since infection with the 9S or 9S.R2 virus did not cause this effect (Fig. 10). The 9S virus did not replicate efficiently in WI38 cells, presumably because of the lack of endogenous E1A-like activity in these growth-arrested cells (40). Even at high multiplicities of infection, at which viral DNA replication would normally occur with the 9S viruses in HeLa cells (Fig. 4), viral DNA replication was minimal in 9S- and 9S.R2-infected WI38 cells (Fig. 10). Therefore, the ability of the E1B 19K protein to repress early gene expression and virus replication in WI38 cells (73) is dependent on synthesis of either the 13S or 12S E1A gene product.

## DISCUSSION

Two independent factors, the E1A gene products and the host cell type, influence the ability of E1B 19K gene mutant viruses to induce the *deg* and *cyt* phenotypes after infection. Both the 289-amino-acid (13S) and the 243-amino-acid (12S) E1A proteins have the ability to induce these phenotypes. The requirement for the E1A proteins for manifestation of

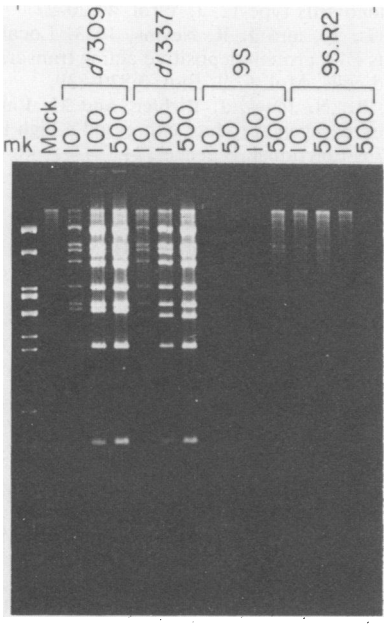


FIG. 10. Lack of viral DNA replication in 9S and 9S.R2 virus-infected WI38 cells. Cells were infected and processed as described in the legend to Fig. 9. Lane mk, Marker DNA. Numbers indicate multiplicity of infection (PFU per cell).

the *cyt* and *deg* phenotype is not just for *trans*-activating transcription of the other viral genes. At high multiplicities of infection, the 9S.R2 virus did not induce the *cyt* and *deg* phenotypes, even though viral genes were transcribed and the viral DNA replicated. Thus, the E1A 289-amino-acid and 243-amino-acid proteins must be providing an activity in addition to *trans* activation of early genes.

The ability of the host cell to influence the *deg* phenotype may be related to the growth state of the cells. Human WI38 cells and human embryonic kidney (HEK) cells are easily growth arrested, whereas HeLa, KB, and 293 cells are transformed, rapidly dividing cells that cannot be easily growth arrested. DNA degradation did not occur in growth-arrested WI38 cells (Fig. 8) or primary HEK cells (data not shown), but occurred readily in HeLa, KB, and 293 cells (54, 66, 74). Moreover, the host cell specificity of the DNA degradation phenotype may be related to the presence of E1A or E1A-like activities in HeLa, KB, and 293 cells but not WI38 or HEK cells (40).

The E1A proteins may induce the *cyt* and *deg* phenotypes by either direct or indirect mechanisms. A direct mechanism for induction of DNA degradation would require that the E1A proteins possess nuclease activity. There is no evidence that this is the case. Several indirect mechanisms are also possible. First, E1A could activate transcription of an endogenous cellular or viral nuclease. The E1A proteins are known to activate transcription of a number of cellular genes (40, 44, 65); whether one of these is a nuclease is not known. It has been reported, however, that levels of topoisomerase I increase after adenovirus infection of HeLa cells (16). It has also been reported that infection of KB cells with an E1B 19K gene mutant virus increases the level of cellular nuclease activity (19), but we have been unable to repeat this result (unpublished experiments). Therefore, the source and identity of the nuclease causing the DNA degradation are unclear. It is unlikely that other viral early genes directly cause the *deg* phenotype, since mutations in the E1B 55K, E2A, or E3 genes, in combination with an E1B 19K gene mutation, have no effect on induction of DNA degradation (unpublished experiments).

Second, E1A gene products have been shown to induce cellular DNA synthesis in growth-arrested rodent cells (12, 45, 61, 64, 76) and to a much lesser extent in growth-arrested human WI38 cells (63). Changes in the proliferative state of the cell may indirectly cause higher cellular nuclease activity. This possibility is raised by the fact that no DNA degradation occurred in mutant-virus-infected WI38 cells, which, unlike rapidly dividing HeLa and KB cells, are strictly growth controlled (33, 34). Expression of E1A in WI38 cells may be insufficient to induce the significant proliferative conditions needed for chromosomal DNA degradation.

Third, the E1A proteins may induce DNA degradation by affecting chromatin structure. The functions of the E1A proteins in regulating cellular gene transcription and transformation may be mediated through changes in the structure of cellular chromatin. Changes in the structure of chromatin in the nucleus during adenovirus infection might render cellular DNA more sensitive to endogenous nucleases in the absence of the E1B 19K protein. This is plausible, since the E1A proteins are associated with the nuclear matrix (22). Moreover, the E1B 19K protein is associated with the nuclear envelope, and this association appears to be important for preventing DNA degradation (72). Coordinated activity between the E1A and E1B 19K proteins may be required to preserve or maintain nuclear structure during adenovirus infection and transformation.

It is clear that the E1A proteins play a role in the induction of the *cyt* as well as the *deg* phenotype. The molecular mechanism by which this occurs is also unknown. Induction of the *cyt* phenotype by E1A may be related to the function of E1A in regulating cellular morphology and inducing the transformed phenotype (41, 57). Alternatively, the *cyt* phenotype could be an indirect result of uncontrolled gene expression brought about by the absence of the E1B 19K tumor antigen (73).

It is important to note that these experiments provide genetic evidence for functional interaction between the E1A and E1B 19K gene products. E1A can effect the *cyt* and *deg* phenotypes of E1B 19K$^-$ viruses, and in turn, the E1B 19K protein can effect E1A-dependent viral replication in growth-arrested cells. Available evidence indicates that one function of the E1B 19K tumor antigen is to negatively regulate early-gene expression (73). Until recently, the negative effect of the 19K protein on gene expression was thought to be restricted to growth-arrested W138 cells (73). We have since observed this effect in infected HeLa cells, in which 19K$^-$ viral infection causes enhanced early-gene expression (manuscript in preparation). This enhanced early-gene expression accounts for the *deg* phenotype in 12S.R2 virus-infected HeLa cells at a low multiplicity of infection (Fig. 3). The genetic evidence presented here demonstrates that this effect of the E1B 19K tumor antigen on gene expression is mediated through the E1A proteins. Defining the molecular basis of the E1A-E1B 19K gene product interactions should yield insights into the control of gene expression.

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