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Mutations in the Gene Encoding the Adenovirus Early Region 1B 19,000-Molecular-Weight Tumor Antigen Cause the Degradation of Chromosomal DNA

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The adenovirus mutant Ad2*ts*111 has been previously shown to contain a mutation in the early region 2A gene encoding the single-stranded-DNA-binding protein that results in thermolabile replication of virus DNA and a mutation in early region 1 that causes degradation of intracellular DNA. A recombinant virus, Ad2*cyt*106, has been constructed which contains the Ad2*ts*111 early region 1 mutation and the wild-type early region 2A gene from adenovirus 5. This virus, like its parent Ad2*ts*111, has two temperature-independent phenotypes; first, it has the ability to cause an enhanced and unusual cytopathic effect on the host cell (cytocidal [*cyt*] phenotype) and second, it induces degradation of cell DNA (DNA degradation [*deg*] phenotype). The mutation responsible for these phenotypes is a single point mutation in the gene encoding the adenovirus early region 1B (E1B) 19,000-molecular-weight (19K) tumor antigen. This mutation causes a change from a serine to an asparagine in the 20th amino acid from the amino terminus of the protein. Three other mutants that affect the E1B 19K protein function have been examined. The mutants Ad2*lp*5 and Ad5*dl*337 have both the cytocidal and DNA degradation phenotypes (*cyt deg*), whereas Ad2*lp*3 has only the cytocidal phenotype and does not induce degradation of cell DNA (*cyt deg*$^+$). Thus, the DNA degradation is not caused by the altered cell morphology. Furthermore, the mutant Ad5*dl*337 does not make any detectable E1B 19K protein product, suggesting that the absence of E1B 19K protein function is responsible for the mutant phenotypes. A fully functional E1B 19K protein is not absolutely required for lytic growth of adenovirus 2 in HeLa cells, and its involvement in transformation of nonpermissive cells to morphological variants is discussed.

The early transcription region 1 (E1) of adenovirus, which occupies map units (m.u.) 0 to 11.2 on the viral genome, consists of two distinct transcription units, E1A and E1B, both of which are required for lytic growth of adenovirus and for complete morphological transformation of primary cells. Three lines of evidence indicate that the continued presence and expression of E1 is sufficient to cause oncogenic transformation. First, analysis of viral sequences has demonstrated that E1 is present and expressed in adenovirus-transformed cell lines (12, 14, 19, 21); second, restriction enzyme fragments of the adenovirus genome containing only E1A and most of E1B can cause successful and complete oncogenic transformation of cells (49); and third, adenovirus mutants with lesions in E1 are defective for transformation (2, 6, 8, 16, 41).

E1A (m.u. 1.3 to 4.4) directs the synthesis of 12S, 13S, and 9S mRNA species, which code for a series of proteins ranging from 30,000 to 60,000 molecular weight (30K to 60K) (42). The gene products of E1A regulate transcription of other early viral genes and of some cellular genes during lytic infection and during the transformation process (4, 24, 25, 40). In addition to regulating transcription, the role of E1A in transformation of primary cells is thought to include immortalization of primary cells and cooperation with other viral and cellular oncogenes to yield a complete transformation phenotype (38, 48).

E1B (m.u. 4.5 to 11.2) directs the synthesis of 13S and 22S mRNA species, which code for two major adenovirus tumor antigens of 19K and 57K (10, 20, 31). These E1B gene products are commonly called the adenovirus tumor antigens because animals with adenovirus-induced tumors have circulating antibodies directed against them. The 19K tumor antigen is translated from both mRNA species, whereas the 57K tumor antigen is synthesized only from the 22S mRNA, initiating at an AUG codon in a different open reading frame that is partially overlapping with the coding region for the 19K protein (7, 10). Recently, identification of an 18K protein related to the 57K protein has been reported (1, 30). The functions of the E1B proteins required for lytic infection and for cell transformation are not known, although mutations affecting the synthesis of the E1B 57K protein cause a delay in the onset of viral DNA synthesis (43) and reduced late gene expression and also fail to shut off host protein synthesis (S. Pilder, J. Logan, and T. Shenk, personal communication) (3).

A better understanding of the role of E1 proteins in lytic growth of the virus and in cell transformation should be obtained through the characterization of adenovirus mutants. Ad2*ts*111 is an adenovirus mutant that was found to be temperature sensitive for viral DNA replication (33), which also induced the degradation of chromosomal DNA during lytic infection (9). Biochemical and genetic analysis has revealed that the complex phenotype of Ad2*ts*111 is the result of two independent mutations, the replication defect being caused by an altered DNA-binding protein (DBP) that is encoded by early region 2A (E2A) and the DNA degradation by a mutation that has been mapped to E1 of the adenovirus genome (44). In addition to Ad2*ts*111, a number of other adenovirus mutants have been reported to cause the degradation of chromosomal DNA after infection. They are the Ad12*cyt* mutants (11, 45) and Ad5*dl*313 (28); some of

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these mutants are also defective for cell transformation (25, 32, 41, 45). Available evidence suggests that the Ad12*cyt* mutations map within E1B, but unfortunately the precise location within this region, and therefore the altered gene product, is not known (28). Ad5*dl*313 is a mutant of adenovirus 5 (Ad5) which has all of E1B and part of E1A deleted (m.u. 3.8 to 11), and hence E1B gene products cannot be made from this mutant. We report here that the DNA degradation phenotype of Ad2*ts*111 is caused by a mutation in the E1B 19K tumor antigen that is independent of the mutation present in the Ad2*ts*111 E2A gene and that these two mutations can be genetically separated. Furthermore, other E1B 19K deletion (*dl*) and large plaque (*lp*) mutants also induce degradation of chromosomal DNA during lytic infection. Preliminary analysis of the E1B 19K mutants is also presented.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were propagated in either monolayer or suspension cultures, and 293 cells, which are adenovirus-transformed human embryo kidney cells that contain and express E1 of Ad5 (17), were grown in monolayer cultures. Stocks of wild-type Ad2, Ad5, and Ad2*ts*111 (obtained from J. Sussenbach, State University of Utrecht, The Netherlands) were grown in suspension cultures of HeLa cells. Viruses were purified by methods described previously (29). Ad2*lp*3 and Ad2*lp*5 were obtained from G. Chinnadurai (St. Louis University Medical Center, St. Louis, Mo.) and Ad5*dl*337 was obtained from S. Pilder, J. Logan, and T. Shenk (State University of New York, Stony Brook, New York). Ad2*cyt*106 was constructed from Ad2*ts*111 and Ad5 viruses (see below). These mutant viruses were grown in monolayer cultures of HeLa or 293 cells, and stocks were maintained as crude lysates. Titers were determined by plaque formation on monolayer cultures of HeLa or 293 cells. All virus stocks were grown at 37°C except for Ad2*ts*111, which was grown at 32.5°C.

Extraction of DNA. Low-molecular-weight DNA was isolated from HeLa cells by a modification of the method described by Hirt (23). The cells were lysed in neutral lysing solution (0.01 M Tris, pH 7.9, 0.005 M EDTA, 0.1 M NaCl, 0.5% sodium dodecyl sulfate) containing 1 mg of pronase (Sigma Chemical Co., St. Louis, Mo.) per ml (1 ml per 2×10^6 cells) for 2 h at 37°C, and then high-molecular-weight DNA was precipitated by addition of NaCl to 1 M and incubation at 4°C for 16 h. The high-molecular-weight DNA was removed by centrifugation for 20 min at 57,000 × *g* and 4°C. The DNA in the Hirt supernatant was extracted with phenol and chloroform:isoamyl alcohol (24:1) and precipitated with ethanol. RNase digestion (20 μg of pancreatic RNase A per ml) preceded agarose gel electrophoresis of the DNA samples. For complementation analysis of the DNA degradation phenotype, a more rapid method for isolating the degraded DNA was employed. Infected HeLa cells were harvested from plates and suspended in ice-cold 150 mM NaCl–10 mM Tris (pH 7.2)–2 mM $MgCl_2$–1 mM dithiothreitol and then Nonidet P-40 was added to 0.5%. After incubation on ice for 45 min, the nuclei were harvested by centrifugation and suspended in the same buffer without Nonidet P-40 but with 0.35 M NaCl and incubated for 30 min on ice. The nuclei were again harvested by centrifugation, and the supernatant containing the degraded DNA was treated with RNase and protease before electrophoresis on agarose gels.

Construction of recombinant plasmids and DNA sequencing. The following recombinant plasmid DNAs were constructed for the purpose of mapping and sequencing the mutation in the Ad2*ts*111 E1B gene region. The Ad2*ts*111 *Xma*I E fragment (m.u. 2.8 to 10.8) was cloned into the *Xma*I site of plasmid pUC8 such that the *Bam*HI site in pUC8 was located adjacent to the *Xam*I site at m.u. 2.8. The resulting plasmid, p111X2, was digested with *Bam*HI and *Hpa*I (which cuts the adenovirus insert at m.u. 4.3), and the large fragment was purified. A *Bam*HI-*Hpa*I fragment (m.u. 0 to 4.3) was excised from plasmid pLBH1 and ligated to the p111X2 *Bam*HI-*Hpa*I fragment, resulting in a plasmid, p111R1, containing adenovirus sequences from wild-type Ad5 (m.u. 0 to 4.3) and Ad2*ts*111 (m.u. 4.3 to 10.8). The plasmid pLBH1 contains Ad5 DNA sequences (m.u. 0 to 15.5) cloned into pACYC177 but contains a deletion in the E1B gene encoding the E1B 57K protein. This plasmid was a generous gift of L. Babiss (Columbia University, New York, N.Y.).

For sequencing of the *Hpa*I (m.u. 4.3) to *Xmn*I (m.u. 6.3) region of mutant and wild-type DNAs, the *Hpa*I-*Xmn*I fragment was gel purified from digests of p111RI or wild-type Ad2 DNA, ligated to *Eco*RI linkers (Collaborative Research Inc., Waltham, Mass.), and, after digestion with *Eco*RI, cloned into the *Eco*RI site of pBR322. The resulting plasmid DNAs, p111HX1 (from p111RI) and pAd2HX1 (from Ad2 DNA), were used as the source of DNA for cloning into bacteriophage M13mp18 and M13mp19 replicative-form DNA (35). The *Eco*RI to *Kpn*I (m.u. 5.6) fragments from p111HX1 and pAd2HX1 were individually cloned into both phage vectors, and the DNA sequences of these were determined by the chain-terminator method of Sanger et al. (39), using [^{35}S]dATP as radioactive label and buffer gradient gels as described by Biggin et al. (5). Plasmid DNAs were isolated by a modification of the procedure described by Mukhopadhyay and Mandal (34), and restriction enzymes were from New England Biolabs, Beverly, Mass.

DNA transfections. DNA fragments from recombinant plasmids and virus DNA were transfected into 293 cells by the calcium phosphate technique as described by Graham and van der Eb (18), followed by a boost with glycerol treatment 4 h after transfection.

Immunoprecipitations. HeLa monolayer cultures were labeled with [^{35}S]methionine (50 μCi/ml) at 17 to 22 h postinfection, harvested, washed in cold phosphate-buffered saline PBS (150 mM NaCl, 20 mM sodium phosphate-potassium phosphate, pH 7.4), and suspended in cold lysis buffer (1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline). The cell extract was disrupted by 20 passages through a 25-gauge needle, and the insoluble material was removed by centrifugation for 10 min in an Eppendorf microcentrifuge at 4°C. The supernatant was incubated with either anti-E1B 19K carboxy-terminal or amino-terminal antipeptide antibodies (20) (obtained from M. Green, St. Louis University Medical Center, St. Louis, Mo.) or anti-72K DNA-binding protein antibodies (obtained from C. Anderson, Brookhaven National Laboratory, Upton, N.Y.) for 2 h at 4°C on a rotator, and the antigen-antibody complexes were collected after binding to protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N.J.). The Sepharose was washed three times in lysis buffer and once with PBS before boiling sodium dodecyl sulfate-sample buffer. Immunoprecipitates were analyzed by electrophoresis through 15% sodium dodecyl sulfate–polyacrylamide gels (27) followed by autoradiography.

Transformation assays. Primary baby rat kidney (BRK) cells were prepared from 6-day-old Fisher rats, and 3×10^5 to 5×10^5 cells per 60-mm dish were plated and grown to

about 95% confluency for 2 days before infection with various viruses. Cultures were maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and antibiotics. Three weeks after infection, the plates were fixed and stained with Giemsa and examined for foci or morphologically transformed cells.

RESULTS

Isolation of temperature-independent derivatives of Ad2*ts*111 that degrade cellular DNA. We have previously demonstrated that the mutation in Ad2*ts*111 that caused degradation of cell DNA in infected cells was the result of a mutation that mapped in E1 (44). During the course of these studies, we isolated a number of recombinant viruses which physically separated the temperature-sensitive replication defect (in the gene encoding the DBP) from the E1 mutation. We infected 293 cells with a mixture of Ad2*ts*111 and wild-type Ad5, and temperature-insensitive recombinants were constructed in vivo at 32.5°C (permissive temperature for Ad2*ts*111). The yield of this coinfection was reinfected into HeLa cells and grown at 38.5°C (nonpermissive temperature for Ad2*ts*111), and the resulting viruses were plaque purified and tested for the degradation phenotype. One such recombinant contained, by restriction site analysis, Ad2*ts*111 DNA derived from m.u. 0 to 29 and m.u. 66 to 100 and Ad5 DNA derived from m.u. 29 to 66. This recombinant, which contains most of the gene for the DBP from the Ad5 wild-type virus, was not temperature sensitive for growth in HeLa cells but retained the ability to degrade cellular DNA. The recombinant virus also produced similar yields of virus after infection of either HeLa or 293 cells, and these yields were similar to the yields of wild-type Ad2 in these cells (data not shown). However, this recombinant virus showed an enhanced cytopathic effect on the infected HeLa or 293 cells, which was similar to the cytocidal effects observed in Ad12 *cyt* mutants (45). Thus, by analogy with the Ad12 *cyt* mutants, we have named the Ad2*ts*111/Ad5 recombinant virus Ad2*cyt*106.

The cytocidal effect produced by Ad2*cyt*106 in lytically infected cells is characterized by rounding up of infected cells and their detachment from the plastic dish and other cells, which is far more severe and occurs more rapidly than the cytopathic effect caused by an infection with the wild-type adenovirus (Fig. 1). Reexamination of Ad2*ts*111 revealed that it also has the cytocidal phenotype at both the permissive and nonpermissive temperatures. The effect is slightly less severe than that seen with Ad2*cyt*106 at the nonpermissive temperature, presumably because the E1 mutation is in a genetic background containing a temperature-sensitive mutation. Ad2*cyt*106, which does not contain the temperature-sensitive mutation in the DNA-binding protein gene, was then used to reexamine the effect of the E1 mutation on lytic growth of the virus.

Since the E1 mutation caused both enhanced cytopathic effect on infected cells and DNA degradation, we have abbreviated these genotypes and defined the phenotypes as follows: *cyt*, the production of enhanced and abnormal cytopathic effect on infected cells (the wild-type is cyt^+); *deg*, the induction of DNA breaks in chromosomal DNA in mutant infected cells (the wild type is deg^+). This nomenclature is similar to that used for defining the Ad12 *cyt* mutants (45).

Replication of Ad2*cyt*106 virus DNA. Previous experiments with Ad2*ts*111 demonstrated that DNA degradation increased with the multiplicity of infection and also the time postinfection (44). Because Ad2*cyt*106 contained the E1 mutation that was not in a temperature-sensitive background, it was examined in the same way. HeLa cells were infected with either Ad2 or Ad2*cyt*106, and at various times postinfection Hirt supernatants were isolated and the DNA was analyzed by electrophoresis on agarose gels. At low multiplicities of infection (10 PFU per cell), the degraded DNA in mutant-infected cells appeared as a continuous smear of DNA in an agarose gel and was first detected at 24 to 36 h postinfection (Fig. 2a). DNA degradation was readily

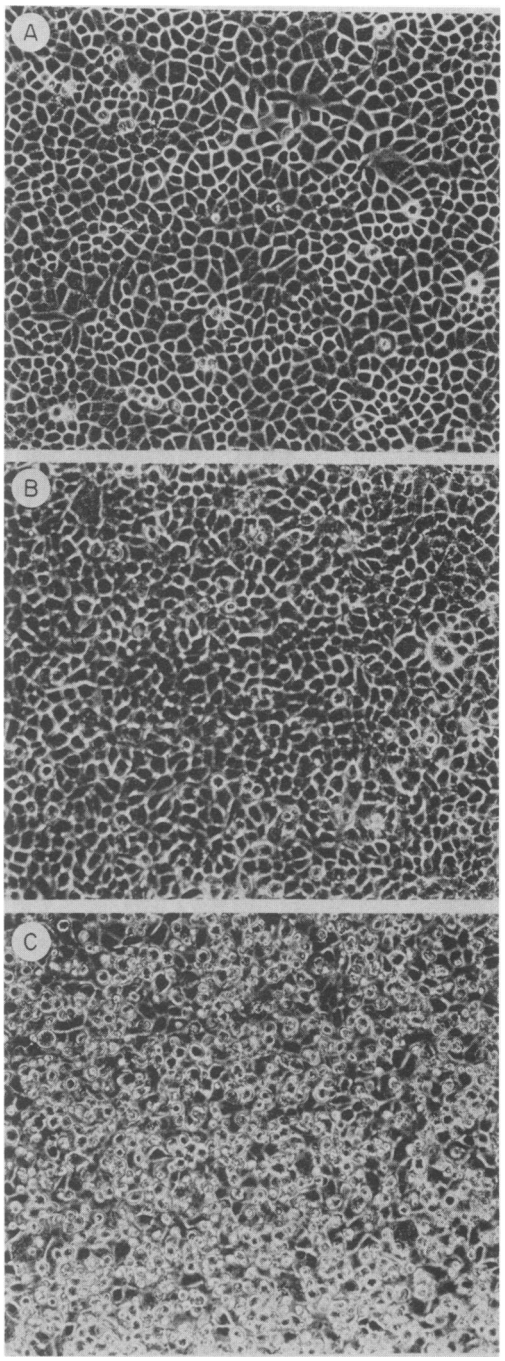

FIG. 1. Enhanced cytopathic effect of Ad2*cyt*106 in HeLa cells. HeLa cells were (A) mock infected or (B) infected with wild-type Ad2 or (C) Ad2*cyt*106 at a multiplicity of infection of 10 PFU per cell, and at 40 h postinfection the cells were photographed.

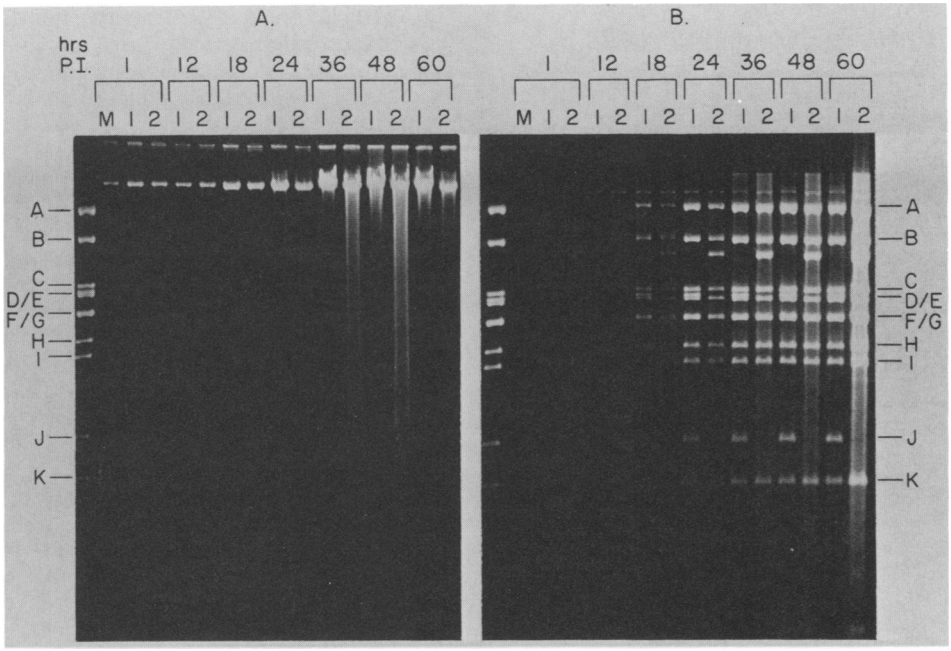

FIG. 2. Time course of DNA degradation and DNA synthesis in Ad2*cyt*106-infected HeLa cells. Cells were infected with either wild-type Ad2 or Ad2*cyt*106 at a multiplicity of infection of 10 PFU per cell, and at the indicated time postinfection, medium was removed and the cells were lysed directly in the plate. The Hirt supernatants were prepared and subjected to agarose gel electrophoresis either (A) undigested or (B) digested with *Hin*dIII. Lanes: M, mock-infected cells; 1, wild-type Ad2-infected cells; 2, Ad2*cyt*106-infected cells. The recombinant, Ad2*cyt*106, contains an altered *Hin*dIII restriction enzyme pattern. The marker lane at the left of each panel is Ad2 DNA digested with *Hin*dIII.

observable by 36 h and increased with time thereafter. At 60 h, many of the cells were floating in the medium and in this experiment were not included in the Hirt supernatant, contributing to the apparent by erroneous decrease in the amount of degraded DNA. Degradation of DNA was not apparent in either mock- or wild-type-infected cells at any time postinfection. Finally, like Ad2*ts*111, DNA degradation induced by Ad2*cyt*106 could be increased by increasing the multiplicity of infection (see Fig. 6); at high multiplicities of infection, the degraded DNA can be observed in ethidium bromide-stained gels as early at 18 h postinfection (data not shown).

The Hirt supernatant DNAs from cells infected with either wild-type Ad2 or Ad2*cyt*106 were also digested with *Hin*dIII and subjected to agarose gel electrophoresis to follow the replication of virus DNA, indicated by the characteristic restriction pattern (Fig. 2b). When compared with wild-type Ad2, Ad2*cyt*106 viral DNA replication was not significantly affected by the cytopathic effect and DNA degradation (Fig. 2b). Furthermore, DNA replication was not delayed in this mutant, as it is after infection with some host-range mutants with defects in E1B (43). Southern blots of the degraded DNA with adenovirus DNA as probe revealed that a small proportion of adenovirus DNA is degraded in cells infected with Ad2*cyt*106 (data not shown); thus, the bulk of the degraded DNA must be of host cell chromosomal origin. This is consistent with the fact that viral DNA is replicated and that the yield of mutant virus is not significantly different from the yield of wild-type virus, which could not occur if the viral DNA was being extensively degraded.

Expression of the *deg* phenotype before replication of virus DNA. When viral DNA replication, and therefore late protein synthesis (47), was blocked by the addition of hydroxyurea at the time of infection, DNA degradation was still observed in Ad2*cyt*106-infected cells (Fig. 3). At this multiplicity of infection and in the absence of hydoxyurea, DNA degradation was just detectable by ethidium bromide staining at 24 h postinfection; however, in the drug-treated cultures, the DNA degradation was more pronounced (Fig. 3). DNA degradation must therefore be the result of a protein produced early in infection. In addition, the majority of the degraded DNA in hydoxyurea-treated cells must have been of host chromomsomal origin since virus DNA was not synthesized under these conditions. The reason why DNA degradation is enhanced in the presence of hydroxyurea is not yet clear, but it may be related to the overproduction of one or more adenovirus early proteins in the absence of DNA replication and late transcription.

Identification of E1 mutation that causes DNA degradation. Previous results demonstrated that the mutation in Ad2*ts*111 that caused DNA degradation and enhanced cytopathic effect mapped between m.u. 3.8 and 9.1 on the adenovirus genome (44). To determine the location of the mutation more precisely, a plasmid DNA containing an insert of m.u. 0 to 4.3 from wild-type Ad5 and m.u. 4.3 to 10.8 from Ad2*ts*111 was constructed (see above). This plasmid, p111R1, contained wild-type E1A sequences from Ad5 and E1B sequences from Ad2*ts*111 cloned into pUC8. The adenovirus DNA insert was digested with *Hin*dIII and the resulting m.u. 0 to 8 fragment was cotransfected into 293 cells with the *Xba*I A fragment (m.u. 3.8 to 100) from *dl*309, which is a nondefective virus derived from Ad5 and containing a single *Xba* site at m.u. 3.8 (26). Recombinants were isolated and screened for the *deg* phenotype by isolation of Hirt supernatant DNA from infected cells and for the *cyt* phenotype by visual inspection of the infected cells at 40 to 46 h postinfec-

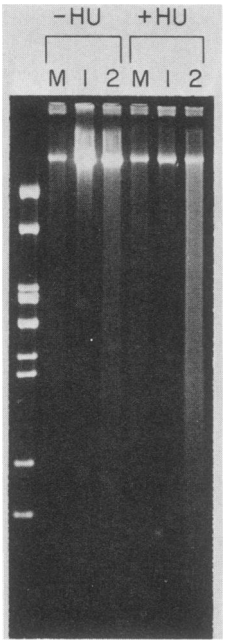

FIG. 3. Enhanced degradation of cell DNA by inhibition of DNA synthesis. HeLa cells were mock infected (lanes M) or infected with either wild-type Ad2 (lanes 1) or Ad2*cyt*106 (lanes 2) at a multiplicity of infection of 100 PFU per cell, and at 1 h postinfection, hydroxyurea was added to a final concentration of 10 mM where indicated. At 24 h postinfection, cells were harvested, and Hirt supernatants were prepared and subjected to agarose gel electrophoresis. The marker lane at left is Ad2 DNA digested with *Hin*dIII.

tion. Furthermore, the recombinant virus DNAs were screened for the presence of an *Xmn*I site at m.u. 6.3, which lies just outside the coding region for the 19K protein and is present Ad2 and Ad2*ts*111 but absent from Ad5 and Ad5*dl*309. In addition, they were also screened for the presence of the *Sac*I site at m.u. 4.9, which is absent in Ad2*ts*111 and Ad2*cyt*106 (see below) but present in the wild-type Ad2 and Ad5. Of 11 recombinants examined, 5 had the *cyt* and *deg* phenotypes, and only these recombinants were missing the *Sac*I site. Four *cyt deg* recombinants also had the *Xmn*I site derived from p111R1, but one *cyt deg* recombinant did not contain this *Xmn*I site. These results suggest that the mutation causing DNA degradation maps to the left of the *Xmn*I site at m.u. 6.3. The six cyt^+ deg^+ recombinants analyzed had the *Sac*I site, but did not contain the m.u. 6.3 *Xmn*I site. From these results, it can be concluded that the DNA degradation phenotype of Ad2*ts*111 and Ad2*cyt*106 maps between m.u. 4.3 and 6.3 in E1B. This region contains all of the E1B 19K protein coding region and the amino-terminal coding region of the E1B 57K protein (Fig. 4A).

To confirm the location of the mutation in the m.u. 4.3 to 6.1 region, the entire region from either Ad2*ts*111 or Ad2 wild-type DNA was cloned into bacteriophage M13 vectors, and the DNA sequence was determined. When the mutant and wild-type sequences were compared with each other and to the known sequence of Ad2 (15), a single G-to-A transition was found at nucleotide position 1769, destroying the *Sac*I site that is normally found in wild-type adenovirus DNA (Fig. 4B). This nucleotide change destroys the *Sac*I restriction site normally present in wild-type Ad2 DNA and explains why this site is missing in both Ad2*ts*111 and Ad2*cyt*106 and indeed in all *cyt deg* recombinant derivatives of Ad2*ts*111 (see above). The predicted amino acid alteration is a change from a serine to an asparagine residue in the E1B 19K protein. The mutation lies within the region which does not overlap with the coding sequence for the E1B 57K protein (Fig. 4A and B).

Six out of nine revertants of Ad2*ts*111, selected for growth at the nonpermissive temperature (38.5°C) by passaging virus three times at 38.5°C followed by plaque purification, also reverted from *cyt deg* to cyt^+ deg^+. When the DNA from two such revertants, Ad2*ts*111R:7 and Ad2*ts*111R:9 was digested with *Sac*I enzyme, the restriction site at m.u. 4.9 was present (data not shown). This result demonstrates that reversion to cyt^+ and deg^+ is accompanied by a reversion to produce the wild-type *Sac*I restriction site in the DNA, and this unambigously identifies the G-to-A transition in Ad2*cyt*106 and Ad2*ts*111 DNA at nucleotide 1769 from the left end as being responsible for the mutant phenotypes. Three other Ad2*ts*111 temperature-sensitive revertants were *cyt deg* and did not contain the *Sac*I site at m.u. 4.9 in their genome (data not shown).

Other adenovirus E1B mutants cause DNA degradation. A number of mutants of Ad12, and Ad2, and Ad5 have been isolated which have a cytocidal phenotype similar to that of Ad2*cyt*106. The Ad12*cyt* mutants (45) also cause DNA degradation (11) and map in the E1B region. A number of *lp* mutants of Ad2, which produce large clear plaques on human KB cells because of their cytocidal effects, were sequenced and found to have mutations in the E1B 19K protein (8); however, it was not determined whether these mutants induced degradation of cell DNA. Ad2*lp*3 contains a single point mutation at nucleotide 1718 which results in an amino acid substitution (alanine to valine) near the amino terminus of the 19K protein (Fig. 4A). Ad2*lp*5 has two point mutations in the gene for the E1B 19K protein, one at nucleotide 1954 and the other at 2237 (8). The first mutation results in an amino acid substitution (asparagine to tyrosine) and the second changes the termination codon to a leucine residue, and as a result additional amino acids are added to the carboxy terminus of the mutant protein. Depending on whether the 13S or 22S message is utilized for translation of the Ad2*lp*5 E1B 19K protein, either 11 or 14 amino acids are added to the carboxy terminus. This second mutation in Ad2*lp*5 is also in the region of overlap with the E1B 57K protein, causing an amino acid substitution there as well. A deletion mutant, Ad5*dl*337, which contains a small out-of-frame deletion in the E1B 19K protein (Fig. 4A), also caused cytocidal effects (S. Pilder, J. Logan and T. Shenk, personal communication). Because the Ad2*lp*3, Ad2*lp*5, and Ad5*dl*337 mutations were known to alter the E1B 19K protein and these mutants produced a cytocidal phenotype similar to that of Ad2*cyt*106, they were examined for the DNA degradation phenotype.

When Hirt supernatants of mutant-infected HeLa cells were examined for the presence of DNA degradation, both Ad2*lp*5 and Ad5*dl*337 induced DNA degradation as did Ad2*cyt*106; however, Ad2*lp*3 did not (Fig. 5A). From this result it is clear that lytic infection by E1B 19K mutants other than Ad2*ts*111 and Ad2*cyt*106 could induce DNA degradation; however, in the case of Ad2*lp*3, the cytocidal effects were independent of DNA degradation. This indicates that the *deg* phenotype is not a consequence of the massive changes in cell morphology induced by these mutants, since a similar *cyt* phenotype in Ad2*lp*3-infected cells does not result in DNA degradation. Again, like Ad2*cyt*106, the other E1B 19K mutants replicate their DNA, some more efficiently than others, despite their aberrant phenotype

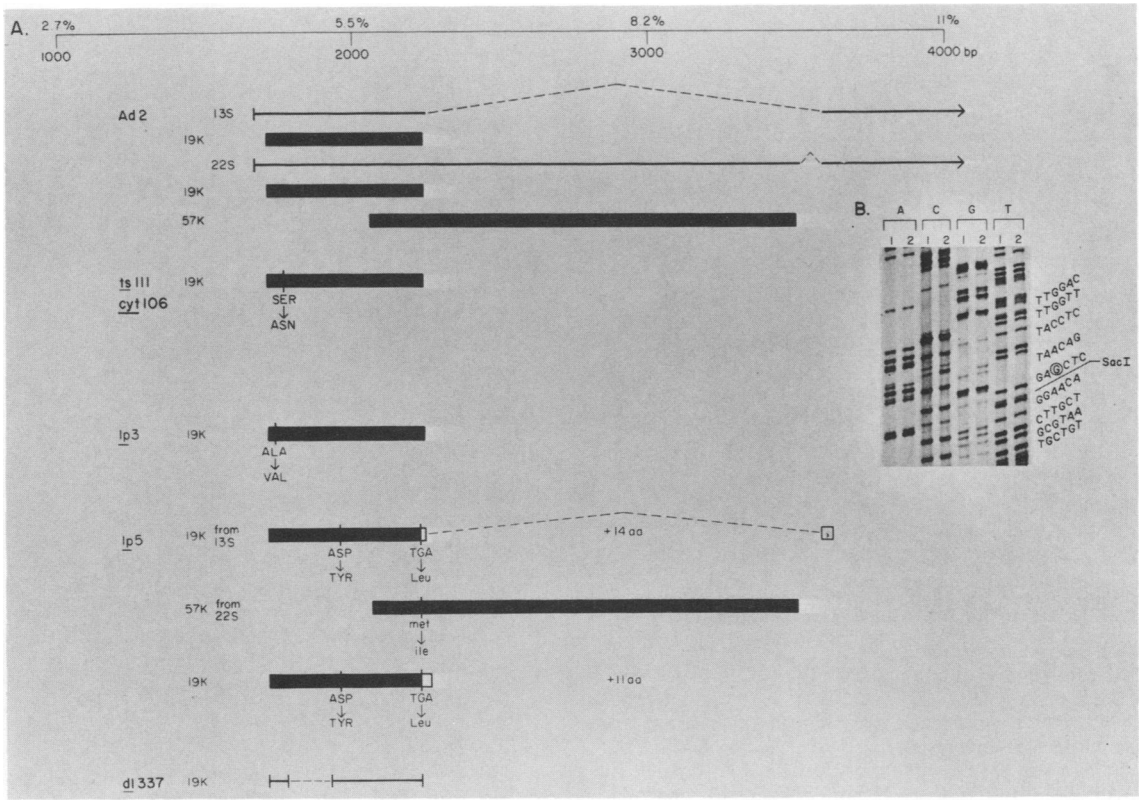

FIG. 4. Structure of the adenovirus E1B gene region and location of the *cyt* mutations. (A) Top line represents nucleotide numbers and m.u. from the left end of adenovirus, and the thin lines below represent the structure of the spliced 22S and 13S mRNA species. The thick bars represent open reading frames in the DNA sequence which encode the 19K and 57K proteins. Also shown are the location and amino acid alterations in the proteins produced by Ad2*ts*111 and Ad2*cyt*106, Ad2*lp*3, Ad2*lp*5, and Ad5*dl*337 (this deletion should be out of frame; S. Pilder, J. Logan, and T. Shenk, personal communication). Two altered 19K proteins are made from Ad2*lp*5, depending on the mRNA species used to synthesize the protein (8). Ad2*ts*111, Ad2*cyt*106 Ad2*lp*3, and Ad5*dl*337 should produce wild-type 57K protein. (B) Region from a DNA sequencing gel showing the DNA sequence alteration in the gene encoding E1B 19K protein in Ad2*ts*111 and Ad2*cyt*106 DNA. Lanes 1 contain mutant sequence and lanes 2 contain wild-type sequence. The circled wild-type guanine residue is changed to an adenine residue in the mutant, and this alters the *Sac*I site, which is underlined.

(Fig. 5B). Among the mutants there are some slight differences in the extent of the cytopathic effect and degradation of host cell and viral DNA.

Complementation and dominance analysis of E1B 19K mutants. Wild-type Ad2 and Ad2*cyt*106 were coinfected into HeLa cells at various multiplicities of infection to determine whether the wild type was dominant or recessive over the E1B 19K mutants for both *cyt* and *deg* phenotypes. At 40 h postinfection, the *cyt* phenotype was determined by visual inspection of the infected cells, and the *deg* phenotype was determined by extracting low-molecular-weight DNA from isolated nuclei with high concentrations of NaCl followed by agarose gel electrophoresis (Fig. 6; data not shown). The *cyt deg* mutant viruses are recessive to the cyt^+ deg^+ wild-type virus as long as the amount of wild-type virus is equal to or exceeds the amount of mutant virus in the infection. DNA degradation only occurs when the mutant virus is in excess. Complementation analysis among the E1B 19K *cyt deg* mutants (Ad2*cyt*106, Ad2*lp*5, and Ad5*dl*337) revealed that they cannot complement one another for both *cyt* and *deg*. Furthermore, Ad2*lp*3, which is *cyt* deg^+, is dominant over Ad2*cyt*106, Ad2*lp*5, and Ad5*dl*337 for the *deg* phenotype, given the same qualification regarding the ratio of each virus present, but Ad2*lp*3 cannot complement the other mutants for the *cyt* phenotypes. In summary, the *deg* and *cyt* mutants are recessive to deg^+ and cyt^+ viruses only when the complementing E1B 19K protein is present in equivalent amounts or in excess (see below).

The mutant Ad2*cyt*106 also induced enhanced cytopathic effect and degradation of cell DNA after infection of 293 cells, even at multiplicities of infection as low as 1 PFU per cell, whereas wild-type virus did not show these abnormal phenotypes (data not shown). In addition, the amount of degraded DNA in mutant-infected 293 cells was more than that observed after infection of HeLa cells at the same multiplicity of infection. Although these cells contain E1B 19K protein, it is either not functional or is not in sufficient amounts to complement the mutant viruses.

Analysis of proteins produced by E1B 19K mutants. HeLa cells were infected with either E1B 19K mutants or wild-type virus, labeled with [^{35}S]methionine in vivo, and examined by immune precipitation with 19K carboxy-terminal antipeptide antisera (20) for production of the 19K protein and with anti-72K DNA-binding protein antisera for production of the DBP. The amount of 19K detected in a 5-h label at 17 h postinfection in Ad2*ts*111 and Ad2*cyt*106 was much less than the level found in wild-type-infected cells, although the protein has the correct molecular weight (Fig. 7B). This is probably due to a decrease in the stability of the protein (data not shown). Ad2*lp*3 and Ad2*lp*5 make the E1B protein

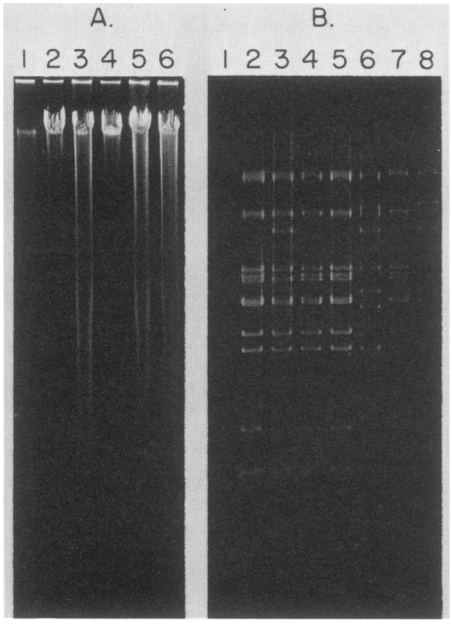

FIG. 5. DNA degradation induced by E1B 19K mutants. HeLa cells were infected with viruses at a multiplicity of 10 PFU per cell, and at 40 h postinfection Hirt supernatants were prepared. The DNA present in the Hirt supernatant was subjected to agarose gel electrophoresis either (A) undigested or (B) digested with *Hin*dIII. Lanes: 1, mock-infected; 2, Ad2 wild type; 3, Ad2*cyt*106; 4, Ad2*lp*3; 5, Ad2*lp*5; 6, Ad5*dl*337. In (B), lanes 7 and 8 contain Ad2 and Ad5 virion DNA, respectively.

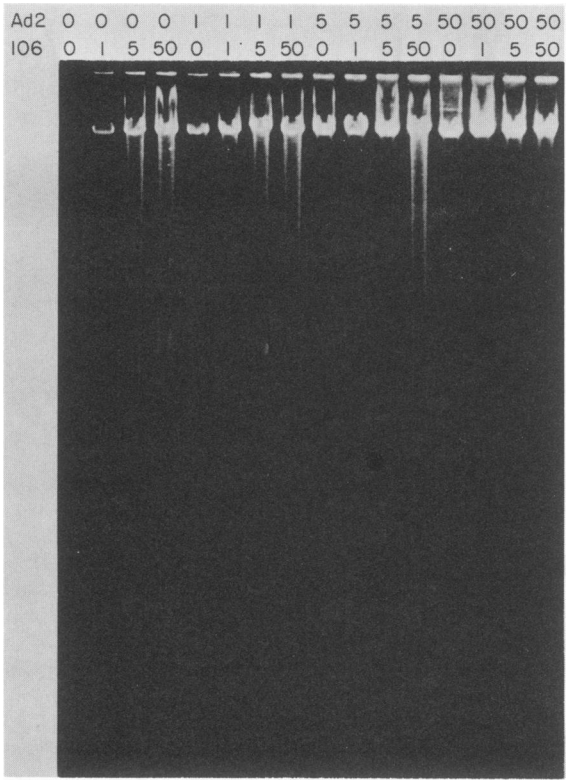

FIG. 6. Complementation between wild-type Ad2 and Ad2*cyt*106. HeLa cells were infected with both Ad2 and Ad2*cyt*106 at the indicated multiplicities of infection and at 42 h postinfection, and the degraded DNA was extracted from isolated nuclei (see the text). The DNA was subjected to agarose gel electrophoresis without digestion in vitro.

in amounts comparable to, but slightly less than, that of the wild-type virus, but in Ad2*lp*5, the protein migrates at 21K due to the mutation in the termination codon (8). Ad5*dl*337 does not make a 19K protein which is detectable by immune precipitation with either carboxy- or amino-terminal antipeptide antisera (Fig. 7B). If Ad5*dl*337 makes a truncated gene product it must be less than 10K and not be resolved on 15% gels. There is a 55K band precipitated by the carboxy-terminal antisera which may represent an abnormal splicing product or cross-reaction of the antipeptide antisera with another Ad5 viral protein, but this protein is not recognized by the amino-terminal antisera. This protein only appears when the small E1B protein is absent. All E1B 19K mutants make late proteins (Fig. 7A) and the DBP (Fig. 7C) in levels comparable to that of wild-type, which further emphasizes that the mutations in E1B 19K protein do not render the virus defective for lytic growth in HeLa cells.

Transformation of primary rat cells by Ad2*ts*111. Since the mutation in Ad2*ts*111 and Ad2*cyt*106 that causes DNA degradation is located in the gene encoding the E1B 19K tumor antigen, we examined the ability of these mutant viruses to induce foci of morphologically transformed cells after infection of primary baby rat kidney BRK cells. BRK cells were infected with various multiplicities of either Ad2*ts*111, Ad2*cyt*106, or 1×51i (an Ad2 and Ad5 wild-type recombinant virus) or were mock infected, and foci were scored after growth for 3 weeks. At the nonpermissive temperature for viral DNA synthesis (38.5°C), Ad2*ts*111 formed foci at levels equivalent to that of wild-type (Table 1), whereas Ad2*cyt*106-infected cells did not form foci at any of the multiplicities that were tested because of massive cell death which resulted from the infection with this virus (data not shown). The ability of Ad2*ts*111-infected cells to become morphologically transformed was probably due to the reduction in virus replication and hence cell death at the nonpermissive temperature. The E1B 19K mutants Ad2*lp*3, Ad2*lp*5, and Ad5*dl*337 also caused cell death after infection of primary BRK cells, so that the effects of these E1B 19K mutations on foci formation could not be assessed. In fact, there were not any cells left on the plate after 3 weeks of the assay when primary BRK cells were infected with Ad2*cyt*106, Ad2*lp*5, and Ad5*dl*337 (data not shown).

DISCUSSION

The adenovirus 2 mutant Ad2*ts*111 was originally described as a mutant with a temperature-sensitive defect in viral DNA synthesis (33) and was subsequently shown to induce degradation of intracellular DNA (9). The temperature-sensitive defect in virus DNA replication was shown to be due to an alteration in the adenovirus single-stranded DNA-binding protein, and the mutation causing DNA degradation was previously localized to E1 (44). We have shown that this latter mutation is a single base change at nucleotide 1769 from the left end of the genome. This mutation results in an amino acid substitution in the E1B 19K tumor antigen and does not affect the E1B 57K tumor antigen, nor does it alter the putative proteins that may be encoded from the virus "l" strand in this region (15). A recombinant virus, Ad2*cyt*106, has been isolated, which contains this E1 19K mutation and a wild-type DNA-binding protein gene. The E1B 19K mutation causes two pronounced phenotypes in Ad2*ts*111- and Ad2*cyt*106-infected cells: enhanced cytopath-

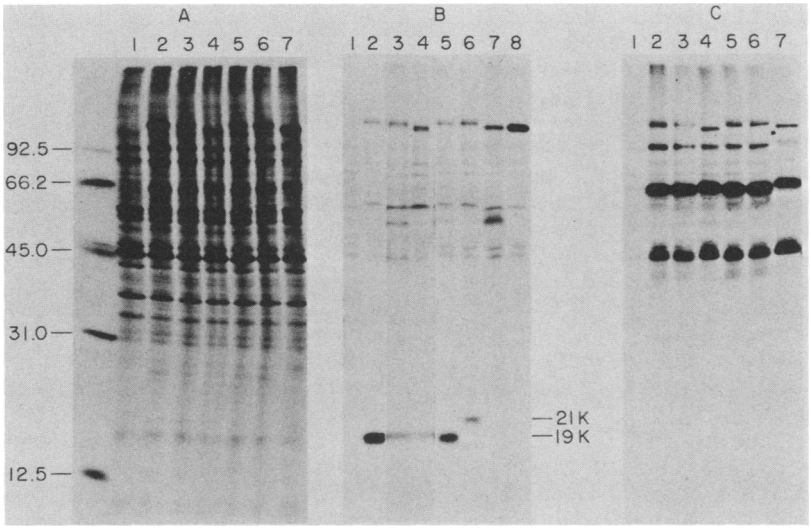

FIG. 7. Analysis of proteins produced in HeLa cells infected with various E1B 19K mutants. HeLa cells were infected with virus at a multiplicity of infection of 50 PFU per cell and then labeled with [^{35}S]methionine from 17 to 22 h postinfection. Protein extracts were prepared and subjected to electrophoresis through 15% sodium dodecyl sulfate–polyacrylamide gels. (A) Total protein extract. (B) Immunoprecipitates with anti E1B 19K carboxy-terminal peptide antiserum (lanes 1 to 7) and amino-terminal peptide antiserum (lane 8). (C) Immunoprecipitates with anti-Ad2 DBP antiserum, which recognizes the 72K DBP and a 44K proteolytic cleavage product. Lanes: 1, mock infected; 2, wild-type Ad2; 3, Ad2*ts*111; 4, Ad2*cyt*106; 5, Ad2*lp*3; 6, Ad2*lp*5; 7 and 8, Ad5*dl*337. In (B), lanes 3 and 4 show a fivefold longer exposure of the gel to film. The positions of protein markers and the 21K and 19K proteins are indicated. Note that the virion hexon protein, which often contaminates immunoprecipitations, has a different molecular size in Ad2 and Ad5.

ic effect of the virus on the host cell (*cyt*) and the induction of breaks in the host cell chromosomal DNA (*deg*). Other E1B 19K mutants (Ad2*lp*5 and Ad5*dl*337) have the *cyt* and *deg* phenotypes similar to the Ad2*cyt*106 mutant, but another E1B 19K mutant Ad2*lp*3, only displays the cytocidal phenotype and does not induce degradation of host cell DNA. This result is of some importance since it demonstrates that the significant alterations in cell morphology do not induce degradation of cell DNA but that a specific and partially independent set of events leads to induction of the DNA damage. The *lp* mutants were originally isolated because of their ability to generate abnormally large plaques on human KB cells (8), but we have observed that the large plaque phenotype is not apparent in human 293 or HeLa cells. Rather, plaques of all the E1B 19K mutants described here appeared more rapidly in HeLa cells (3 to 4 days) when compared with the rate of appearance of wild-type Ad2 plaques in HeLa cells (unpublished data).

TABLE 1. Transformation of BRK cells by Ad2*ts*111

Virus	Multiplicity of infection	No. of colonies (PFU per cell)[a]	No. of plates
Ad2*ts*111	1	0	5
	0.5	0	5
	0.1	7 (0,2,2,1,2)	5
	0.05	8 (4,1,1,2)	4
	0.01	3 (0,1,1,1,0)	5
	0.005	3 (0,0,1,1,1)	5
Wild-type (1×51i)	1	0	2
	0.5	0	2
	0.1	3 (1,2)	2
	0.05	5 (5,0,0)	3
	0.01	2 (1,1,0,0,0)	5
Mock infected		0	4

[a] Numbers in parentheses are the number of foci observed in each 6-cm-diameter plate.

Examination of the phenotype produced after coinfection of wild-type virus and the E1B 19K mutants (Ad2*cyt*106, Ad2*lp*3, Ad2*lp*5, and Ad5*dl*337) revealed a complex pattern of dominance. The wild-type was only dominant when present in amounts equal to or greater than those of the mutant. The *cyt* phenotype could be observed in cells coinfected with *cyt* mutants and wild-type virus, and its extent was proportional to the amount of mutant virus in excess over the amount of the wild-type. The same pattern was observed for the *deg* phenotype. Moreover Ad2*lp*3, which is *cyt deg*$^+$, was dominant over Ad2*cyt*106, Ad2*lp*5, and Ad5*dl*337 (which are *cyt deg*) for the DNA degradation phenotype, but only when Ad2*lp*3 was present in excess. Similarly, wild-type virus is not dominant over Ad5*dl*337 when the mutant is present in excess. Our inability to detect any E1B 19K protein in Ad5*dl*337 mutant-infected cells (only a small amino-terminal peptide would be expected) supports the hypothesis that the *cyt* and *deg* phenotypes of the E1B 19K mutants are not due to an altered E1B 19K enzymatic function. Rather, the phenotypes are most likely a result of the complete absence of the E1B 19K protein (as in Ad5*dl*337) or an inactive protein product (as in Ad2*cyt*106 and Ad2*lp*5). Consistent with this suggestion is the finding that Ad5*dl*313, which contains a deletion of the entire E1B region (25), also induces degradation of intracellular DNA (28). Thus, another virus protein or proteins or a cellular protein must be directly responsible for the occurrence of breaks in the chromosomal DNA. The E1B 19K protein might be present in a fixed ratio to another virus-induced protein, and an alteration in this ratio might induce degradation of intracellular DNA. We are currently searching for such a protein. This protein must be produced in the early stages of virus infection since DNA degradation is enhanced in the presence of hydroxyurea (9), which inhibits virus DNA replication and thus late gene expression.

D'Halluin et al. (9) originally reported that the DNA degradation phenotype was temperature sensitive and that upon a shift to 39.5°C, all of the virus DNA is degraded. We have reported that the DNA degradation phenotype is not temperature sensitive (44) that the two mutations present in Ad2*ts*111 can be genetically separated. It is possible that a combination of the temperature-sensitive DBP and the altered E1B 19K protein in Ad2*ts*111 leads to enhanced degradation of viral DNA at 39.5°C compared to 32.5°C, as was observed by D'Halluin et al. (9), but we have not observed any difference between Ad2*ts*111 and Ad2*cyt*106 in the extent of cell DNA degradation at 39.5 or 37°C. Furthermore, we have shown that the majority, but not all, of the viral DNA is stable and not degraded in Ad2*cyt*106, which is consistent with the normal yields of viral DNA (Fig. 2), late proteins (Fig. 7A), and virus.

A number of Ad12 *cyt* mutants (Ad12*cyt*) have been isolated and partially characterized (11, 32, 45, 46). The *cyt* mutation has been localized to early region E1B on the Ad12 genome (11), but the effected E1B product has not been determined. The Ad12*cyt* mutants cause enhanced cytopathic effect in human KB cells and also induce degradation of cell DNA in these cells (11). One class of the Ad12 *cyt* mutants is also defective for morphological transformation of cells, but another class is not (32, 46). Since the organization of the Ad12 E1B region is very similar to that of Ad2 and Ad5 (50), it is most likely that the Ad12 *cyt* mutants contain a mutation in the gene encoding the Ad12 E1B 19K protein equivalent.

The E1B 19K mutant Ad2*cyt*106 is not defective for virus production in HeLa cells, and similar results have been obtained for the Ad5*dl*337 mutant (S. Pilder, J. Logan, and T. Shenk, personal communication). Thus, a fully functional E1B 19K protein is not required for lytic growth or adenovirus in HeLa cells. Recently, Fukui et al. (13) have demonstrated that the Ad12 E1B 19K protein is also not required for lytic growth of Ad12 in human embryo kidney cells, although these mutants were not examined for any possible cytocidal or DNA degradation phenotypes. It is likely, however, that the Ad2*cyt* mutants suffer a slight growth disadvantage when compared with wild-type virus (unpublished data). Relative to wild-type Ad12, the Ad12*cyt* mutants synthesize reduced amounts of viral DNA, and the virus yield is low in some lines of KB cells but not in other KB cell lines or human embryo kidney cells (11, 46). If the Ad12*cyt* mutations map in the E1B 19K gene, this result suggests that the Ad12 E1B 19K protein may be necessary for efficient growth of the virus but that some human cell lines contain a protein which complements the Ad12 defect. A role for both E1B gene products in determining the host range of the virus is suggested by the ability of mutants to grow in some human cell lines but not in others (22, 46).

E1B gene products are clearly required for morphological and oncogenic transformation of both continuous cell lines and primary cells. However, the identification of which E1B protein or proteins are required, their role in the transformation process, and their requirement for maintenance of the transformed phenotype are not known, and existing evidence is for the most part confusing and in many cases contradictory. Many investigators use different primary cells and cell lines, making comparison of results difficult. Different results with the same virus mutant can also be obtained depending on whether virus infection or plasmid DNAs are used to introduce the E1A and E1B gene regions into cells (37). A further complication in interpreting transformation results must now be considered since we have shown that E1B 19K *cyt* mutations cause enhanced cytopathic effect and that viruses carrying these mutations caused cell death in the primary BRK cells. Thus, a negative result for transformation may not necessarily imply that the E1B 19K protein is required for morphological transformation. Negative transformation results were obtained when Ad*cyt*106, Ad2*lp*3, Ad2*lp*5, and Ad5*dl*337 were used in transformation assays with primary BRK cells (see above). However, when the Ad2*cyt*106 mutation was present in the defective virus Ad2*ts*111, transformed foci were observed at the nonpermissive temperature of 38.5°C. This is presumably due to the inability of this virus to replicate extensively at the restrictive temperature, thus limiting cell death. This result suggests that, at least for the Ad2*cyt*106 mutation, the alteration in the E1B 19K protein does not affect a possible role of the protein in morphological transformation. Chinnadurai (8) reported that Ad2*lp*3 and Ad2*lp*5 showed either reduced levels or no focus formation after infection of the continuous cell line 3Y1 and stated that these viruses did not cause excessive cell death in these cells. Thus, the E1B 19K protein is required for morphological transformation of that continuous rat cell line. We do not wish to speculate on the role of the E1B 19K protein in cell transformation but note that the protein plays an important role in determining cell morphology of lytically infected human cells (this report) and nonpermissive cells (unpublished data).

The function of the E1B 19K tumor antigen in infected cells is still not resolved. Persson et al. (36) have shown that approximately 50% of E1B 19K protein labeled with [^{35}S]methionine at intermediate times postinfection localizes in a nuclear fraction, whereas the remainder fractionates with cellular membranes. Whether this reflects two functional subpopulations of the protein is not known, but it is most likely that the nuclear subpopulation plays a role in preventing extensive DNA damage during an adenovirus infection. We are currently investigating the intracellular localization of the E1B 19K protein and its function as well as examining the nature of the degraded DNA produced after infection of cells with the *cyt* mutants.

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