Adenovirus Type 12 E1B 19-Kilodalton Protein Is Not Required for Oncogenic Transformation in Rats

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The adenovirus type 12 mutants *in700* and *pm700* carry site-specific mutations within the reading frame encoding the E1B 19-kilodalton protein (19K protein) which prevent the production of the intact 19K protein. In cultures of human A549 cells, these mutants grow just as well as the wild-type virus does, but they display a large-plaque (*lp*), cytotoxic (*cyt*) phenotype. DNA in these infected cells is not degraded, but at late times in human KB cells infected by the mutants, the mutants display a DNA degradation (*deg*) phenotype. The transformation phenotype of these mutants is also host range. Although the mutants are defective for transformation of the 3Y1 rat cell line, they transform rat and mouse primary kidney cells in vitro at wild-type efficiency and are capable of inducing tumors in rats. These results support the view that the type 12 E1B 19K protein is not obligatory for oncogenic transformation.

The first evidence that a virus of human origin possessed oncogenic capacity came from experiments carried out in the early 1960s, in which rodents inoculated with adenovirus type 12 (Ad12) developed tumors (25, 40, 47, 58). Later in the 1960s, the first human adenovirus mutants of any kind to be isolated were the cytotoxic (*cyt*) mutants of Ad12 (46). Typically, these mutants produced large plaques on human embryonic kidney cultures and were found to possess reduced oncogenicity in newborn hamsters and defective transforming capacity in hamster kidney cells in vitro. It was proposed that the *cyt* gene is responsible for both adenoviral cytopathic effect and oncogenicity (46), although neither the chromosomal location nor the nature of the gene product in question was identified at that time.

As interest in the human adenoviruses grew throughout the 1970s, it was shown by a number of research groups, using a variety of molecular and genetic methods, that early region 1 (E1) gene products are necessary and sufficient for cellular transformation (reviewed in references 16 and 54). This is a complex region of the genome, composed of two discrete transcription units, E1A (map units [m.u.] 1.3 to 4.5) and E1B (m.u. 4.6 to 11.2). The organization of the Ad12 E1B region is depicted in Fig. 1. Early in the productive infection of human cells, precursor RNAs are generated by rightward transcription from the E1B promoter region located around position 4.6. Differential splicing of these precursors gives rise to two major mRNA species (2.22 and 0.97 kilobases [kb]; 22S and 13S) and two minor species (1.10 and 1.03 kb; 14.5S and 14S) with identical 5' and 3' termini (42, 48). The 2.22-kb mRNA encodes two proteins from overlapping reading frames (5), the first of which commences at the 5'-proximal AUG and generates a 19-kilodalton (163R) protein (19K protein), whereas the second initiates at the second AUG and gives rise to a 54K (482R) protein. The 0.97-kb mRNA also encodes the 19K protein, and this mRNA and the other two (1.10 and 1.03 kb) possess open reading frames encoding proteins, 8.4K and 11.6K respectively (48). The 54K and 19K proteins have been

detected by immunoprecipitation of infected and transformed cell extracts (7a, 20). At later times in infection, a small, 0.47-kb (9S), mRNA is generated from a separate transcriptional unit mapping between 9.7 and 11.2 m.u. This unspliced mRNA encodes the virion structural polypeptide IX (38).

Phenotypic analysis of group C mutants with alterations in the gene encoding the E1B 55K protein (the 55K gene) has revealed that the 55K protein is required for complete transformation of rat cells in vitro (1, 2, 17, 24, 31), although as yet little is known about the functional role(s) of this protein in transformation. We have also used mutational analysis to show that the Ad12 54K protein is required for full transformation of primary rat and mouse cells in vitro and for induction of tumor in rats (7a, 14).

The Ad12 E1B 19K (163R) protein is nonglycosylated and acylated (21), and this latter property may explain why the protein (in group C serotypes also) shows a strong preference for association with nuclear, cytoplasmic, and plasma membranes in both infected and transformed cells (9, 20, 21, 37, 49). A number of group C adenovirus mutants with lesions mapping to this gene have been isolated, and phenotypic characterization of these variants has shown that this protein (21K protein, 176R in Ad5) influences viral cytopathogenicity (the mutants are *cyt*) and prevents degradation of DNA by a nuclease activity present in infected cells (mutants with this phenotype are *deg*) (1, 2, 8, 39, 44, 45). These mutants have all been shown to be defective for transformation of rat cells in vitro, and, although the group C viruses are nononcogenic, it has been proposed that the E1B 21K protein is required for oncogenic transformation (1, 2, 8, 39, 44, 45). Recently Ad12 19K mutants have also been described, and these were found to be defective for transformation of rat 3Y1 cells and tumor induction in hamsters (12). In addition, some of the original Ad12 *cyt* mutants (46) have recently been characterized in more detail (30, 32). As with the group C *cyt* mutants, some of the Ad12 variants were found to be *deg*, and their mutational lesions were mapped to the E1B region by complementation analysis with Ad5 E1 mutants. Some of these *cyt* mutants were found to be defective for transformation of rat kidney cells, but one, H12 *cyt*61, transformed these cells at wild-type efficiency (32). None of these Ad12 *cyt* mutations were mapped physically

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FIG. 1. Physical map of Ad12 E1B mRNAs, coding regions, and 19K mutations (adapted from references 42 and 48).

to the 19K gene, and the possibility that they also bore lesions in other E1 genes was not excluded, but by analogy of their phenotypic properties with those of the group C *cyt* mutants, it may be concluded that they do alter that gene.

To more definitively address the role of the Ad12 19K protein in oncogenic transformation, we have constructed two mutants which carry mutations specifically within the E1B 19K reading frame. These site-specific mutations prevent the production of intact 19K protein, resulting in a *cyt* phenotype in human A549 cells. The DNA in these infected cells is not degraded, but the mutants do possess a *deg* phenotype in human KB cells. The mutants are not defective for the transformation of rat or mouse kidney cells in vitro or tumor induction in rats, although they are unable to transform cells of the rat 3Y1 line. Thus, while the transformation phenotype is host range, the results are consistent with the view that the 19K protein is not required for oncogenic transformation.

MATERIALS AND METHODS

Cells and viruses. The parental virus used was type 12 strain Huie, originally obtained from the American Type Culture Collection and plaque purified on cultures of the A549 lung carcinoma cell line (15) obtained from C. Tibbetts, Vanderbilt University. High-titer stocks were grown on these cells or on cells of the Ad12-transformed human embryo retinal (HER) line HER3 (6, 14) obtained from P. Gallimore, Birmingham University, England. The latter cells were derived following transfection of HER cells by the Ad12 plasmid pAsc2 (m.u. 0 to 16.5 of Ad12 DNA [7], and they contain and express only three or four copies of the Ad12 E1 sequences (P. J. Byrd, Ph.D. thesis, University of Birmingham, Birmingham, England, 1983). HER3 cells support efficient growth and plaque production of Ad12, and we have previously used them for isolation, growth and assay of

Ad12 host range (*hr*) mutants (5a, 14). Mutants *hr*703 and *in*702, which carry mutations (a C-to-T transition at nucleotide [nt] 2245 and termination codon at that point, and a 4-base-pair insert at nt 2318, respectively) within the 54K reading frame, were grown on these cells and have been described elsewhere (5a). The Ad5 mutant *d*/313 (all of E1B deleted [26]) was originally obtained from T. Shenk, Princeton University; stocks of this mutant were grown on 293 cells (18) used previously for isolation of Ad5 *hr* mutants (22). The HeLa cells used in some tests were derived from stocks used previously in studies with Ad5 temperature-sensitive (*ts*) and *hr* mutants (22, 55, 57). Human KB cells were originally obtained from H. Ginsberg, Columbia University. Cells were maintained in Dulbecco modified Eagle medium supplemented with either 7% calf serum (A549, HeLa, and KB cells) or 7% fetal calf serum (HER3 and 293 cells). The infectivities of wild-type and mutant viruses were measured by plaque assay on the appropriate cells (53).

Mutagenesis and mutant isolation. This E1B 19K mutants *in*700 and *pm*700 were generated site specifically by insertion and oligonucleotide-directed mutagenesis, respectively. The *in*700 mutation was constructed in plasmid pAsc6.8, which contains the Ad12 E1 sequence from 0 to 6.8 m.u. This and other Ad12 E1 plasmids (see pAsc10.3 below) were obtained from P. Gallimore (7). The Ad12 sequence in pAsc6.8 contains a single *Acc*I restriction site at nt 1595 in E1B, and to make the mutation the plasmid was opened up by digestion with *Acc*I and the single-stranded ends were filled in with Klenow fragment (33) and subsequently ligated. Prior to oligonucleotide mutagenesis the E1 sequence in pAsc6.8 was cloned into the M13 vector mp19 (36). The 12-base oligomer carrying a single substitution of the G at nt 1543 by a C (a *Taq*I site is created at that point) was obtained from the DNA Synthesis Service of the University of Pennsylvania, Philadelphia. The mutagenesis procedures were carried out by previously described methods (34, 60). Following mutagenesis, the mutated fragments were first transferred into the pAsc10.3 plasmid (0 to 10.3 m.u.). The E1 10.3-m.u. fragment from this plasmid was then released by double digestion with *Eco*RI and *Sal*II and rescued by overlap recombination (24) with the *Bst*EII A fragment (10.2 to 100 m.u.) of Ad12 viral DNA complex. *Bst*EII cuts the Ad12 genome only once, at 10.2 m.u. (12, 13). To effect rescue, we transfected mixtures containing these two fragments onto either HER3 or A549 cells by means of the calcium phosphate-glycerol boost procedure (11, 19). The transfection efficiency of Ad12 viral DNA complex on HER3 cells is quite good, and we generally obtain between 5.0×10^4 and 1.5×10^5 PFU/ μ g of complex, whereas the efficiency on A549 cells is about 50 to 100 times lower (D. Breiding, C. Lamberti, and J. Williams, unpublished results). The *in*700 mutation was rescued by transfection onto HER3 monolayers. These were harvested 1 week later, and lysates were titrated by plaque assay upon HER3 and A549 cells; large-plaque variants formed on the latter and were isolated. Most of the plaques on these cultures were, however, of the small-plaque type and presumably arose from wild-type complex which escaped digestion by *Bst*EII. Because of this, and because an indirect mode of isolation was used here, the rescue efficiency could not be accurately determined. However, in the rescue of an Ad12 E1A *hr* mutation by using identical overlapping fragments and direct plaque isolation, some 5% of the plaques were found to be *hr* (C. Lamberti and J. Williams, unpublished results). The *pm*700 mutation was rescued in the same way as the *in*700 mutation; it was also successfully rescued by transfection directly onto A549

monolayers. In this case the background level of Ad12 plaques (from wild-type complex) was zero, and all of the plaques arising on the rescue plates were large. Compared with the efficiency of Ad12 DNA complex on A549 cells, the transfection efficiency of the digested-fragment mixture was about 1%. Large-plaque mutant isolates were plaque-purified on A549 again and then grown on A549 cells to provide high-titer stocks. Restriction enzymes were obtained from Boehringer Mannheim Biochemicals.

Verifying the presence of the mutations. The presence of the mutations in both the plasmid and the viral stages during construction of *in700* and *pm700* was checked by digestion with restriction endonucleases *AccI* and *TaqI*, respectively, since the respective mutations should result in the loss of an *AccI* site and the acquisition of a *TaqI* site. The presence of the mutation at each stage was also checked by nucleotide sequence analysis with the dideoxy chain termination method (43).

Polypeptide analysis in infected cells. A549 cells were infected at an input multiplicity of 30 PFU per cell. At various times after infection (up to 36 h), cells were labeled for 2 h with [³⁵S]methionine (30 μ Ci/ml) in DMEM lacking methionine. In some cases, when labeling was directed toward E1 proteins, cytosine arabinoside (100 mg per plate) was added to prevent DNA synthesis and expression of viral late genes. Methods for extraction of cells and for immunoprecipitation of E1 proteins have been previously described (41). Two of the Ad12 tumor antisera used (one against rat cells transformed by pAsc2 and the other against rat cells transformed by pAsc6.8) were given to us by Phillip Gallimore. Others, made by ourselves, were directed against Ad12 virus-transformed rat cells. For measurement of viral late protein synthesis, cultures were pulse-labeled with [³⁵S]methionine (10 μ Ci/ml) for 2 h, at 24, 36, 48 h postinfection. In all cases the labeled samples were analyzed by electrophoresis on sodium dodecyl sulfate SDS-12.5% polyacrylamide gels (29). Gels were subsequently fixed, fluorographed (4), and exposed to X-ray film. ¹⁴C-labeled molecular weight standards were obtained from Bethesda Research Laboratories. In most cases, purified Ad12 virion proteins ([³⁵S]methionine labeled) were used as size markers.

Analysis of DNA in infected cells. Confluent monolayers of A549 (or, in some cases, KB) cells were infected with mutant or wild-type virus at an input multiplicity of 30 PFU per cell and incubated for various lengths of time, usually until 35 h postinfection. Infected cells were collected by scraping into the medium, and after low-speed centrifugation the total cellular and viral DNA was extracted from pelleted cells as described previously (52). In some cases, low-molecular-weight DNA was isolated by a modification of the Hirt procedure (23). Cells were lysed in extraction buffer (10 mM Tris pH 7.9), 5 mM EDTA, 100 ml of NaCl, 0.5% sodium dodecyl sulfate), and the extract was incubated for 2 h in the presence of RNase A (20 μ g/ml) and proteinase K (200 μ g/ml). Samples were taken to 1 M NaCl and incubated for 16 h at 4°C to allow precipitation of high-molecular-weight DNA, which was removed by centrifugation at high speed, and the DNA in the supernatant was extracted with phenol and with chloroform-isoamyl alcohol (24:1), precipitated with ethanol, and suspended in sample buffer prior to agarose gel electrophoresis. To monitor for viral DNA in the samples, the DNA (total or low-molecular-weight material) was first cut with *EcoRI* prior to electrophoretic analysis. In all cases DNA was visualized by staining the gels with ethidium bromide.

Transformation procedures. The transformation assays used are basically the same as those we have used previously (17, 56, 57). Primary rat and mouse kidney cell cultures were prepared by Dispase (Boehringer Mannheim) digestion of kidneys removed from 10-day-old inbred hooded Lister rats and BALB/c mice, respectively, and grown in 60-mm dishes in DMEM containing 7% fetal calf serum. When confluent (usually after 3 to 4 days), these cultures were infected at a defined input multiplicity, and following infection the dishes were washed with medium to remove unabsorbed virus. For rat kidney cells, DMEM plus 7% fetal calf serum was added to these cultures at this point, and they were then incubated at 37°C. Mouse kidney cultures, on the other hand, were split immediately after infection by trypsinization, and replicate dishes were seeded with 5×10^5 cells in DMEM plus 7% fetal calf serum. In some cases, primary rat embryo cultures were used. These were prepared by trypsinization of 16- to 18-day-old embryos from pregnant Hooded Lister rats and grown in 60-mm dishes in DMEM plus 7% calf serum. Following infection of these cells, the cultures were split by trypsinization, and replicate dishes were seeded with 10^6 cells each. Transformation experiments were also carried out with cells of the continuous rat cell line 3Y1-2. This line is a flat, low-density subline, which we derived by cloning from the original 3Y1 line (27). These cells grow to a very low final density of around 10^6 cells per 60-mm dish (400 cells per mm²). In all cases, at 4 days after infection the original medium was removed and replaced with calcium-free DMEM plus 7% calf serum (10), and for the rest of the experiment (usually up to 4 to 5 weeks) this medium was renewed every 3 to 4 days. At 37°C Ad12 foci first appeared at around 18 to 20 days on primary cultures, and final counts were made at 28 to 30 days postinfection. On 3Y1-2 cells these times were around 25 and 35 to 40 days, respectively.

RESULTS

The mutants make no detectable 19K product in A549 cells. Two mutations were created site-specifically within the reading frame of the E1B 19K (163R) protein. The mutant *in700* has lost the *AccI* site and acquired the insertion of an AT base pair at nt 1595 (Fig. 1). This 2-base-pair insert introduces a frame shift and a stop signal at codon 38 of the 19K reading frame and should result in truncation of the 163R product to 37R (17 missense residues). In mutant *pm700* the ATG of the 163R reading frame was changed to an ATC codon (specifying isoleucine) by substitution of the G at nt 1543 by a C (Fig. 1). Because the AUG is removed and the replacement AUG codon lies within a sequence of only moderate context (28), it is most unlikely that intact protein will be made. The next methionine in the 19K reading frame is at position 114 and could theoretically be used to make a 50R C-terminal fragment. It is, however, in very poor context and is preceded by the AUG of the E1B 54K frame, which is in moderate context. Theoretically, reinitiation could also occur to give a 50R C-terminal fragment in cells infected by *in700*.

To test for the presence of 19K protein or related N- or C-terminal fragments in A549 cells infected with these mutants, we carried out immunoprecipitation tests with a variety of tumor antisera which contain antibodies directed against the Ad12 E1 proteins. An immunoprecipitation test with a serum which precipitates 19K (E1B) and 40K and 45K (E1A) products from wild-type-infected lysates is shown in Fig. 2. Clearly, the 19K product is missing from cells infected with either *in700* or *pm700*. This result has also been

FIG. 2. Immunoprecipitation of A549-infected cell lysates ($[^{35}\text{S}]$ methionine labeled for 2 h at 36 h after infection in the presence of ara-C[1- β -D-arabinofuranosylcytosine]) with Ad12 tumor antiserum. Lanes: A, Size standards (purified Ad12 virion proteins); B, wild-type lysate; C, *pm700* lysate; D, *in700* lysate; E, noninfected cell lysate.

observed with a number of other antisera, some of which precipitate the E1B 54K protein (in 19K mutant-infected cells, too) in addition to the others. The small putative 37R N-terminal (*in700*) and 50R C-terminal (*pm700*) fragments (which would in theory run close to the bottom of the gels) were not observed on the autoradiograph depicted in Fig. 2 or in a number of other cases in which other antisera were used. Most probably they either are not made in these mutant-infected cells or are unstable. It cannot be ruled out, however, that the antisera used were not capable of reacting with the fragments in question. Unfortunately, antisera directed against N- and C-terminal peptides of this Ad12 protein, which could perhaps be useful in resolving this problem, are not yet available. It should be noted that although immunoprecipitation tests did not reveal the 19K protein or related products in A549 cells infected by the mutants, Northern (RNA) blot analysis revealed wild-type levels of the E1B 2.2- and 0.97-kb mRNAs in these cells (J. Tong, unpublished observations).

Growth of and plaque production by the mutants in human cells. As mentioned above, both mutants produce large plaques on A549 monolayers. The production of Ad12 wild-type plaques on these cells is highly dependent on the presence of MgCl_2 in the overlay medium (J. Williams, unpublished results), an effect first observed for Ad5 on HeLa cells (53). In the absence of MgCl_2 , Ad12 plaques appear only after 15 to 16 days of incubation on A549 cells and remain very small, whereas they appear after 9 to 10 days in the presence of 25 mM MgCl_2 (12 mM MgCl_2 results in an intermediate effect). With the 19K mutants, plaques are first visible around 7 days after seeding, and MgCl_2 has no effect upon the time at which they appear or upon the size they attain. These differences between Ad12 and *in700* are clearly shown in Fig. 3, which depicts plaque size at 12 days postinfection. It is worth pointing out that the large-plaque phenotype is cell dependent. On HER3 cells, which contain and express Ad12 E1 sequences, the large plaque size is suppressed, and both the rate of production and the size of

FIG. 3. *cyt* mutant plaque morphology on A549 cells. The upper dishes were incubated in the presence of 12 mM MgCl_2 , while the lower two received 25 mM MgCl_2 . These cultures were stained with neutral red at 12 days postinfection to make the plaques more visible and photographed 1 day later.

Ad12 and 19K mutant plaques are identical, although the efficiency of plaque formation of both wild-type and mutant viruses is slightly higher on HER3 cells (Table 1). In contrast, the E1B 54K mutant *in702* is strongly host range and shows greatly reduced plaque-forming efficiency upon A549 cells (Table 1). On HeLa cells Ad12 makes very small plaques at reduced efficiency by comparison with its behavior on A549 cells (Williams, unpublished results); the 19K mutations do not alter the plaque-forming phenotype of Ad12 on these cells, and mutant plaques remain small.

We have examined the growth rates of the E1B 19K mutants and Ad12 on A549 cells; this comparison is shown for the *in700* mutant and the wild type in Fig. 4. On measuring cell-associated virus, we found that growth rate and final viral yields were very similar for the two viruses. Further, these growth rates were very similar to those obtained with HER3 cells (data not shown). Measurement of extracellular virus levels in A549 cells, however, revealed a striking difference between the mutant and the wild type (Fig. 4). Clearly, the rate of release of the mutant virus was considerably higher than that of Ad12, and this would certainly explain why large plaques are produced on these cells. The mechanism by which virus is more rapidly re-

TABLE 1. Infectivity of Ad12 E1B mutants plated on A549 and HER3 cells^a

Virus	Infectivity (PFU/ml)		HER3/A549 ratio
	HER3 cells	A549 cells	
Wild type	5.8×10^9	4.8×10^9	1.2
<i>in700</i>	1.2×10^{10}	4.0×10^9	3.0
<i>pm700</i>	1.7×10^{10}	1.0×10^{10}	1.7
<i>in702</i> ^b	1.6×10^{10}	1.5×10^5	1.1×10^5

^a Final plaque counts were made at 18 days postinfection.

^b An E1B 54K *hr* mutant, with a 4-base-pair insert at nt 2318.

FIG. 4. Growth of wild-type Ad12 and *in700* on A549 cells. Samples harvested at various times were frozen and thawed three times to release the virus and assayed for infectivity on HER3 monolayers. Extracellular virus represents the virus present in the fluid medium from which cells and debris were removed by centrifugation at 1,200 rpm for 10 min. Symbols: ○, cell-associated virus; ●, extracellular virus.

leased is not yet known, and of course it may simple involve more-rapid or more-profound lysis of the cells by the mutant. Microscopical examination of the cells infected by Ad12 and *in700* at 36 to 60 h postinfection showed them both to be rounded but not markedly lysed or broken up. The *in700* cultures did contain a much higher proportion of rounded cells at those times, however, with many of these floating off into the medium.

The fate of DNA in mutant-infected cells. A number of Ad12 Ad2, and Ad5 *cyt* mutants have been shown to induce degradation of both viral and cellular DNA during infection of HeLa or KB cells (2, 30, 39, 44). We have examined this phenotypic property in A549 cells infected at an input multiplicity of 30 PFU per cell with *in700* and *pm700*. First, to examine viral DNA replication, the total, high-molecular-weight DNAs (or in some cases the modified Hirt supernatant DNAs) collected at various times after infection were digested with *Eco*RI, and the resultant components were separated by electrophoresis on agarose gels. Ethidium bromide staining revealed the discrete bands diagnostic of viral DNA. One such gel, loaded with DNA from wild-type- and *in700*-infected cells, is shown in Fig. 5. Viral DNA in both cases was first detected at 20 h postinfection and reached identical steady-state levels at around 30 h after infection. There is no evidence of smearing, indicative of degradation, on these gels. The normal levels of viral DNA made by the mutant are consistent with the normal yields of virus observed (Fig. 4). It is also worth noting that we observed normal levels of all major late proteins of the virus in these cells (polyacrylamide gel electrophoresis of [³⁵S]methionine-labeled cell extracts; data not shown), which is consistent with both results.

To examine DNA degradation in these cells, the Hirt supernatants isolated from cells at 24 and 48 h postinfection were analyzed by electrophoresis on agarose gels. In some cases also, total DNA (including the higher-molecular-weight component) was also examined. As a positive control, extracts of A549 cells infected with the Ad5 E1B mutant *dl313* (26) were also included, since this virus had previously been shown to induce DNA degradation in KB cells (30). Neither the Ad12 E1B mutant *in700* nor Ad5 *dl313* induced

FIG. 5. Viral DNA synthesis in A549 cells infected with Ad12 and *in700*. DNA samples were prepared as described in Materials and Methods and digested with *Eco*RI prior to electrophoretic separation on an agarose gel. Lanes: A, noninfected A549 cells; B, Ad12 after 20 h; C, Ad12 after 25 h; D, Ad12 after 30 h; E, Ad12 after 35 h; F, *in700* after 20 h; G, *in700* after 25 h; H, *in700* after 30 h; I, *in700* after 35 h.

DNA degradation in A549 cells (Fig. 6, lanes A to G). Ad12 mutant *pm700* behaves in a similar fashion (data not shown). In contrast, both mutants induce some degradation in KB cells (lanes K and M). In the *dl313* KB cell lysate, the DNA is presumably cellular, since the mutant fails to make viral DNA in restrictive cells (30). We did not determine in these experiments whether viral or cellular DNA or both are degraded. Two important conclusions can, however, be drawn from these tests. First, the *deg* phenotype of the Ad12

FIG. 6. DNA degradation in A549 and KB cells infected with Ad12, *in700*, and Ad5 *dl313*. DNA in the low-molecular-weight fraction was separated by electrophoresis in an agarose gel. Lanes A to G were loaded with A549 cell lysates. Lanes H to N were loaded with KB cell lysates. Lanes: A, noninfected A549 cells; B and H, Ad12 after 27 h; C and I, Ad12 after 48 h; D and J, *in700* after 24 h; E and K, *in700* after 48 h; F and L, Ad5 *dl313* after 24 h; G and M, Ad5 *dl313* after 48 h; N, noninfected KB cells.

FIG. 7. Relationship between input multiplicity of infection and transformation frequency in primary baby hooded rat kidney cells (BHRK; upper panels) and rat 3Y1-2 cells (lower panels) infected by wild-type Ad12, and *in700*, *pm700*, and *in702* mutants. The transformation assay was as described in Materials and Methods. The cultures were incubated at 37°C. For BHRK cells the final counts were made at 28 days postinfection, whereas for 3Y1-2 cells the final counts were made at 38 days after infection.

E1B mutants is cell dependent. Second, since neither viral nor cellular DNA is degraded in A549 cells infected by the 19K mutants, it follows that the *cyt* phenotype observed in these cells is independent of DNA degradation.

Transformation of primary rodent cells by the 19K mutants is not defective. Ad2, Ad5, and Ad12 *cyt* mutants have been shown to be defective for transformation of rodent cells in vitro, and it has been proposed that the 19K protein is required for oncogenic transformation (1-3, 8, 12, 32, 39, 44, 45). To test this hypothesis in our system, we first measured the ability of *in700* and *pm700* to transform primary rat kidney cells. In view of the previous results from other groups, we were surprised to find that both mutants transform these cells at greater-than-wild-type frequencies over a wide range of input multiplicities. The complete results of one such experiment are shown in the upper panels of Fig. 7. An E1B 54K mutant, *in702* (which has a 4-base-pair insert at nt 2318 in the 54K gene) was included as a negative control, since we had previously demonstrated that this gene product is required for the oncogenic transformation of rat cells (7a). As expected, this mutant was totally defective for transformation of these cells. Foci arising on *in700*- and *pm700*-infected cultures appear to be fully morphologically transformed, and remain thus and grow vigorously upon isolation and repeated passage. Similar results were obtained with primary rat embryo cells, and we have found that the mutants are not *ts* for transformation (data not shown). In a parallel set of experiments we obtained similar results for transformation of primary kidney cells from BALB/c mice (Table 2). In this case, *hr703*, a 54K mutant which has a very

similar overall phenotype to *in702*, was used. Clearly, the Ad12 19K protein is not required for the transformation of primary rat or mouse cells.

The 19K mutants are defective for transformation of 3Y1 rat cells. The above results seem at first sight to be at odds with the conclusions drawn by others (1-3, 8, 12, 32, 39, 44, 45). Since many of the experiments of others were carried out with cell lines, we repeated the transformation tests on cells of the flat 3Y1 derivative 3Y1-2 (see Materials and Methods for details of this cell line). The transformation phenotypes of the E1B mutants on these cells are the exact reverse for the phenotypes displayed on primary cells.

TABLE 2. Transformation of BALB/c baby mouse kidney cells by E1B mutants

Input multiplicity (PFU/cell)	No. of foci/dish (5×10^5 cells seeded) ^a		
	Wild type	<i>pm700</i>	<i>hr703</i> ^b
100	17, 20, 21 ^c	23, 21, 20	0, 0, 0
30	19, 23, 20	26, 23, 25	0, 0, 0
16	11, 12, 14	14, 16, 17	0, 0, 0
3	3, 4, 5	6, 10, 8	0, 0, 0
1	1, 1, 0	2, 3, 4	0, 0, 0
0.3	0, 0, 0	2, 1, 2	0, 0, 0
0.1	0, 0, 0	0, 1, 0	0, 0, 0

^a Values are for triplicate determinations.

^b Ad12 E1B 54K mutant with termination codon at R134 in E1B 54K reading frame.

^c Final counts at 26 days postinfection.

FIG. 8. Transformation of baby hooded rat kidney cells (BHRK) and 3Y1-2 cells by wild-type and *in700*, *pm700*, and *in702* mutants. Cells were infected at an input multiplicity of 30 PFU per cell and incubated at 37°C as described in Materials and Methods. At 28 days (BHRK) and 38 days (3Y1-2) postinfection the cultures were fixed with 10% formaldehyde and stained with Giemsa stain.

Mutants *in700* and *pm700* are totally defective, whereas the 54K mutant transforms at wild-type frequencies (Fig. 7, lower panels). The clear-cut, all or none transformation phenotype in BRK and 3Y1 cells is illustrated in Fig. 8. Two trivial explanations for the inability of the 19K mutants to transform 3Y1 cells can be discounted. First, we have found that the survival (by colony-forming efficiency) of 3Y1 cells infected by the 19K mutants is identical to that of wild-type-, 54K mutant-, and mock-infected cells, showing that the reduction in transformation does not result from enhanced cell killing by the mutants. Second, by measuring the ability of each virus to induce Ad12 T antigen (determined by an immunofluorescence assay with Ad12 tumor antisera), we have shown that the mutant viruses are taken up and expressed at early times by 3Y1 cells with the same efficiency as the wild type (and similar to uptake efficiencies measured the same way in BRK cells). Thus, both the E1B 19K and the 54K mutants display a strong cell-dependent transformation phenotype.

The E1B 19K mutants are still capable of tumor induction in rats. The ultimate test of the oncogenic transforming capacity of a virus is its ability to induce tumor formation in animals. Accordingly, we have inoculated newborn rats of the hooded Lister strain with each of the 19K mutants, in parallel with Ad12 wild type. These tests are not extensive (for example, we have not yet carried out dose-response experiments with these mutants), but they serve to show that both mutants induce tumor formation within the same time frame (latency period) and with almost wild-type frequency in these animals (Table 3). In addition, we have shown that *in700* and *pm700* transformed cell lines are highly tumorigenic in rats. As expected, these cells contain no 19K antigen (as detected by immunoprecipitation), and the tumor antiserum induced in these animals did not precipitate the 19K

protein from Ad12-infected cell extracts (J. Tong and J. Williams, unpublished observations). Collectively, these various results lead us to conclude that the Ad12 E1B 19K protein is not required for oncogenic transformation of rat cells.

DISCUSSION

The Ad12 mutants *in700* and *pm700* fail to produce the intact E1B 19K protein, yet they display wild-type efficiency for growth in human A549 cells and transformation of rodent primary kidney and embryonic cells. Although viral growth per se is not altered in A549 cells, the viruses do display the cytocidal (or *cyt*) phenotype and produce large plaques on monolayers of these cells. These, of course, are properties typical of the many Ad2, Ad5, and Ad12 mutants with lesions in this viral E1 gene (2, 8, 12, 30, 39, 44, 45). The molecular basis for the enhanced cytopathogenicity of these mutants remains to be established, but recent analysis of E1A-E1B double mutants has led to the view that the *cyt* phenotype depends upon expression of the E1A 13S and/or 12S gene (51). On the basis of these results and results from the analysis of viral early gene expression in mutant- and wild-type-infected cells, White et al. concluded that the Ad5 E1B 21K protein negatively regulates the expression of the E1A genes and that the mutant phenotype stems from a change in this regulation (50, 51). Others, however, using various cotransfection and transient-expression assays have found evidence that this E1B protein activates transcription from other viral early promoters, including that of E1A (35, 59; M. Mathews, personal communication; C. Lamberti, unpublished observations). Furthermore, we have recently used Northern analysis to show that the steady-state levels of E1A mRNAs in A549 cells infected with Ad12 19K mutants are about twofold lower than those in wild-type-infected cells (Tong, unpublished results). This does not suggest negative regulation of E1A by Ad12 E1B 19K protein, but the apparent differences between our results and those of others could be due in part to the fact that we used different cellular and viral systems.

Many of the *cyt* mutants isolated by others have been found to induce degradation of DNA in infected cells, and it is believed that the protein prevents DNA degradation resulting from nuclease activity in these cells (the *deg* phenotype) (2, 30, 39, 44). Neither *in700* nor *pm700* displays this phenotype on A549 cells (although they do so on KB cells), and one must conclude that the cytopathic effect does not depend on or result from DNA degradation (or vice versa). Others have come to this conclusion and have also shown that the *deg* phenotype of group C *cyt* mutants is cell type dependent (39, 51). These investigators have also shown that the growth efficiency of Ad5 *cyt* mutants is cell dependent; in HeLa cells they behave as the wild type, in KB cells they grow poorly, whereas in W138 cells they give higher yields than the wild type does (51). We have not yet tested our Ad12 mutants in this way, but such studies may reveal information pertinent to the role of the 19K protein during infection of the natural host.

In virtually every case in which the property has been examined, it was found that the *cyt* mutants were defective for the transformation of rodent cells in vitro, and it has been proposed that the E1B protein is required for oncogenic transformation (1-3, 8, 12, 32, 39, 44, 45). It is here that our results appear to differ radically from those of others. Again, at least in part, this difference can perhaps be explained by differences in cell type and in virus serotype. Even within

TABLE 3. Induction of tumors in newborn hooded Lister rats by the E1B 19K mutants

Virus ^a	Tumor-positive rats/no. inoculated (%)	Latency period (days) ^b
Wild type	12/12 (100)	63
<i>in700</i>	10/11 (91)	63
<i>pm700</i>	7/9 (78)	67

^a 10⁸ PFU inoculated intraperitoneally.

^b Time at which palpable tumors first appeared in the animals.

our own system, we find that the transformation phenotype is markedly host range; *in700* and *pm700* transform primary cells with wild-type efficiency, whereas they are totally defective for the transformation of 3Y1 cells. In fact, the 3Y1 part of our results agrees with the findings of Fukui et al., who showed that Ad12 *cyt* mutants are defective for transformation of 3Y1 cells (12). Unfortunately, they did not test their mutants on primary rat or mouse cells. Many of the Ad2 and Ad5 *cyt* mutants were tested for transformation on continuous cell lines such as CREF, which could perhaps behave in the same way as 3Y1. Some, however, were tested on primary cells; for example, Ad5 *dl337* exhibits defective transformation on primary rat embryo cells (39) and Ad5 *dl19* shows reduced transforming capacity on baby rat kidney cells (3). In the latter work, however, an Ad5 E1 plasmid with a frameshift mutation (identical to that in *dl19*) close to the N-terminus of the E1B 21K reading frame showed only a two fold reduction in transformation frequency on these same cells. This may simply reflect a fundamental difference in transforming efficiency of plasmid and viral DNA. However, the difference between these Ad5 viral transformation results and our own could reflect a basic difference between the Ad5 protein and its Ad12 counterpart; the Ad5 product may well be required for transformation of primary rodent cells, whereas the Ad12 equivalent is not.

Of course, in the Ad12 system the ability of a mutant to induce tumors can be directly measured, and the Ad12 mutants *in700* and *pm700* are not defective in this respect. In contrast, although they do transform 3Y1 rat cells, the Ad12 54K mutants we isolated are totally defective for transformation of rat kidney and embryo cells in vitro and totally defective for tumor induction in rats (Fig. 7 and 8) (7a). There is therefore in our system, at least for the hooded Lister rats, a good correlation between the ability to transform primary cells in vitro and the capacity for tumor induction in vivo. However, it is worth pointing out that we have found that the oncogenicity of Ad12 is both strain and species specific; the virus transforms Fischer rat and BALB/c mouse cells efficiently in vitro, but fails to induce tumors in these animals (Williams, unpublished results). This strain and species dependency notwithstanding, the Ad12 serotype is clearly oncogenic, whereas the group C adenoviruses (Ad2 and Ad5) are not. When the viral gene function(s) which convey oncogenicity on one virus group and not the other are considered, our results suggest that those of the E1B 19K protein can be excluded.

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