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Isolation of Transformation-Defective, Replication-Nondefective Early Region 1B Mutants of Adenovirus 12

YASUHISA FUKUI,* IZUMU SAITO, KAZUKO SHIROKI, AND HIROTO SHIMOJO

Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108, Japan

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We isolated three adenovirus 12 early region 1B mutants (*in*205B, *in*205C, and *dl*205) by ligation of the cleaved DNA-protein complex and transfection of human embryo kidney cells with the ligation products. These mutants could replicate efficiently in human embryo kidney or KB cells but showed markedly reduced transforming capacities both in vitro and in vivo. In cells infected with the mutants, the early region 1B gene was transcribed efficiently. In cells infected with *in*205B, the products corresponding to the early region 1B-coded 19,000-molecular-weight polypeptide was detected by in vitro translation but not immunoprecipitated extract of labeled cells. In cells infected with *in*205C or *dl*205, the products corresponding to the same polypeptide were not detected by either in vitro translation or immunoprecipitation of labeled cell extracts. The results suggest that the 19,000-molecular-weight polypeptide encoded by early region 1B is required for cell transformation but not for viral propagation.

There have been many studies on early region 1B (E1B) gene functions of human adenoviruses. Some of the adenovirus 2 or 5 (Ad2 or Ad5) mutants have been isolated as host range mutants with mutations on the E1B gene (11, 13, 26). Most of these mutants can grow in 293 cells, Ad5-transformed human embryo kidney (HEK) cells (9), but fail to grow in HeLa, HEK, or KB cells. Although some host range mutants can grow in HEK cells (11), the results indicate that E1B gene products are required for the growth of viruses. The mutants also show much reduced capacities for transforming rodent cells (13, 23).

When rodent cells are transfected with DNA fragments encompassing both E1A and E1B genes, the cells are transformed completely (8, 27, 28). However, DNA fragments encompassing only the E1A gene transform cells only incompletely or partially (12, 24). It is deduced from these observations that E1B gene products play an important role in the transformation of cells. DNA sequence data of the E1B gene reveal that there are at least two polypeptides encoded by E1B (19,000-molecular-weight [19K] and 54K polypeptides in Ad12) (3, 4, 16, 25, 29). The smallest Ad12 DNA fragment capable of transforming rodent cells completely is the *Hin*dIII-G (0 to 6.8 map units) (22). This fragment encompasses the whole E1A, the whole coding region for the 19K polypeptide, and the left-hand part of the coding region for the 54K polypeptide (3, 16, 25). This observation suggests that the 19K polypeptide is required in the complete transformation of cells.

In this paper, we describe the isolation of Ad12 mutants with insertions or a deletion in the E1B-19K polypeptide-coding sequence of Ad12 and properties of the mutants for productive infection and cell transformation. The results show that these mutants have much reduced transforming capacities but are not defective in productive infection and suggest that the 19K polypeptide is required for cell transformation and not productive infection.

MATERIALS AND METHODS

Cells and viruses. KB and HEK cells were used for the propagation of viruses. A rat cell line 3Y1, established from a Fischer rat embryo (15), was used for the transformation of cells. Cells were cultured in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum. The prototype strain of Ad12 was used. As a source of the Ad12 E1B gene, plasmid pASC, which contains the Ad12 E1A and E1B sequence (0 to 10.2 map units), was used (6). Plasmid pASC was propagated in *Escherichia coli* HB101.

Preparation of Ad12 DNA-protein complex. Ad12 DNA-protein complex was prepared as described by Dunsworth-Browne et al. (5).

Enzymes and reaction conditions. Restriction endonucleases were purchased from Boehringer Mannheim Corp., Bethesda Research Laboratories, New England Biolabs, and Takara Shuzo. The conditions for DNA digestion were as specified by the suppliers. The conversion of cohesive ends of DNA fragments to flush ends was carried out with the large fragment of *E. coli* DNA polymerase I (Klenow's enzyme). For reactions, DNA fragments were dissolved in 10 μl of buffer containing 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 150 μM nucleotide triphosphate. To this solution, 1 U of enzyme was added. After incubation for 15 min at room temperature, the reaction was terminated by heating at 70°C for 10 min. For ligation, DNA fragments were dissolved in buffer containing 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM ATP. Then T4 DNA ligase was added (final solution, 2 to 3 U/ml) to the mixture, which was incubated at 4 or 12°C for 3 h.

DNA sequence analysis. The mutated parts of the virus genomes (1.7 to 6.8 map units) were cloned in pBR322. The 5′-end-labeled DNA fragments were obtained by using the *Hae*III sites (1,707-base-pair [bp] position, *in*205B and *in*205C) or *Pvu*II site (1,904-bp position; *dl*205) located near the mutations, and the DNA sequences were analyzed as described by Maxam and Gilbert (18), except that 4% formic acid was used in place of 4% pyridinium formate for the adenine plus guanine reaction.

Transfection of cells with DNA. This was carried out by the calcium phosphate technique as described by Graham and van der Eb (10).

Nuclease S1 mapping of mRNA. The procedures were as described previously (1, 21). Briefly, cytoplasmic RNA was extracted from cells and hybridized with an excess amount

* Corresponding author.

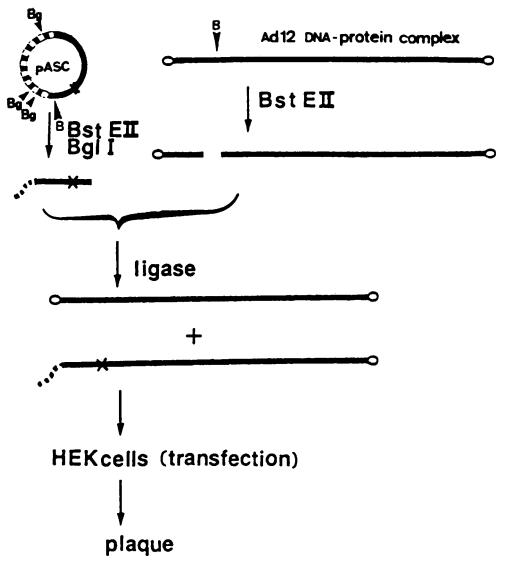

FIG. 1. An outline for the isolation of Ad12 mutants. The Ad12 DNA-protein complex was cut with *Bst*EII at a single site (10.2 map units) and mixed with *Bst*EII-*Bgl*-digested DNA fragments from pASC containing insertions or a deletion in the E1B-coding sequence for the 19K polypeptide. After ligation, HEK cells were transfected with the ligated complex. Mutants were isolated from plaques. Symbols: ▬, sequences of Ad12 genome; ····, sequences of pBR322; ▬×▬, sites for insertion or deletion; B, cutting site with *Bst*EII; Bg, cutting site with *Bgl*I.

of ^{32}P-labeled Ad12 DNA in hybridization buffer containing 80% formamide, 0.4 M NaCl, 0.04 M PIPES (piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (pH6.4), and 1 mM EDTA at 52°C for 3 h. The mixture was treated with single-strand-specific nuclease S1, and the S1-resistant DNA-RNA hybrids were analyzed by electrophoresis in a 1.4% agarose gel with alkaline buffer.

In vitro translation of mRNA. mRNAs complementary to E1A and E1B genes were selected by hybridization with DNA probes as described by Ricciardi et al. (20). Briefly, 1 μl of Ad12 *Hin*dIII-G was immobilized on a nitrocellulose filter, and 250 μg of cytoplasmic RNA extracted from Ad12-infected KB cells at 14 h postinfection (p.i.) was hybridized to the DNA probe in the hybridization buffer at 45°C for 2 h. The filter was washed 10 times with SSC (0.15 M NaCl plus 0.015 M sodium citrate) and 0.5% sodium dodecyl sulfate at 55°C and twice with buffer containing 2 mM EDTA (pH 7.9) at 55°C. Then the filter was soaked in distilled water, heated at 100°C for 1 min, and cooled quickly. The filter was removed, and the eluted RNA was precipitated with ethanol after the addition of 20 μg of *E. coli* tRNA as a carrier. Under this condition, mRNAs complementary to E1A and E1B genes are selected efficiently. These mRNAs were translated in nuclease-treated reticulocyte lysate containing [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.). The conditions were as specified by the supplier. The products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17) or by two-dimensional gel electrophoresis (19), and bands or spots of the products were detected by fluorography (2).

Immunoprecipitation. KB cells were infected with Ad12 at 10 PFU per cell. After the medium was replaced with methionine-free Eagle MEM at 12 h p.i., the cells were labeled with [^{35}S]methionine (200 μCi/ml) from 14 to 16 h p.i. Cells were harvested and suspended in 100 μl of RIPA buffer (10 mM Tris-hydrochloride [pH 7.5], 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 150 μM phenylmethylsulfonyl fluoride, 150 mM NaCl). The cell suspension was sonicated, kept at 4°C for 10 h, and centrifuged to remove the cell debris. A sample of anti-19K sera (sera from rats bearing Ad12 tumors) was added to the cell extract, and the mixture was incubated at 4°C for 4 h. A sample of the 20% suspension of formaldehyde-fixed *Staphylococcus* spp. (protein A [14]) was added to the mixture, which was incubated at 4°C for 30 min. The staphylococcal cells were collected by centrifugation and washed three times with RIPA buffer. The lysis buffer (9.5 M urea, 2% Nonidet P-40, 2% Ampholine) (19) was added to the cells and heated at 68°C for 10 min. The cells were removed by centrifugation, and the supernatant was analyzed by two-dimensional gel electrophoresis (19).

Transformation assay. The transformation assay was performed as described by Yano et al. (30). Monolayer cultures of rat 3Y1 cells were infected with viruses at 10 PFU per cell and subcultured at 10^5 cells per 5-cm dish. After 3 days, the medium was replaced with low calcium (0.1 mM Ca^{2+}) medium. Medium was changed every 3 days thereafter. Cells were fixed with a 10% Formalin solution and were stained with Giemsa 4 weeks after infection.

Colony formation in soft agar. Cells in a small dish were dispersed in phosphate-buffered saline containing 0.02% EDTA and collected by centrifugation. Then 10^5 cells were suspended in 4 ml of 0.4% agar medium (Eagle MEM, 0.1 mM Ca^{2+}, 10% fetal calf serum) and layered on the base layer of 0.8% agar with the same composition in a 5-cm dish. They were cultured for 3 weeks at 37°C.

Induction of tumors in hamsters. The virus suspension was injected subcutaneously into baby hamsters within 24 h after birth. The hamsters were observed for 90 days.

RESULTS

Isolation of Ad12 E1B mutants. The strategy for the isolation of Ad12 E1B mutants is outlined in Fig. 1. Since restriction enzyme *Bst*EII cleaves Ad12 DNA at a single site (10.2 map units), the Ad12 DNA-protein complex (DNA with terminal protein) was cleaved with *Bst*EII to produce small left-terminal and large right-terminal portions. Plasmid pASC, containing Ad12 E1A and a portion of E1B (0 to 10.2 map units), was mutagenized by insertion or deletion in the E1B sequence as described below. Cleaved DNA-protein complex and mutagenized plasmids were mixed and ligated. HEK cells were transfected with the ligated DNA-protein complex. After forming plaques in HEK cells, clones showing restriction cleavage patterns for insertion or deletion were selected.

The mutant plasmid DNAs were constructed as shown in Fig. 2. Since the *Acc*I site at 4.5 map units is located on the coding sequence for the E1B-19K polypeptide, we introduced mutations at this site. Plasmid pASC was modified to produce pIN5B (insertion of 12 bp), pIN5C (insertion of 16 bp), and pDL5-6 (deletion of 175 bp).

These mutated plasmids (pIN5B, pIN5C, and pDL5-6) were purified and amplified in *E. coli*. After cleavage with *Bgl*I and *Bst*EII (Fig. 1), mutated DNA fragments were isolated by agarose gel electrophoresis and electroelution. Each of the mutated fragments was mixed with the Ad12 DNA-protein complex digested with *Bst*EII at 37°C, and the mixtures were treated with T4 DNA ligase. HEK cells were transfected with each of the ligation products and kept under agar overlay. After incubation for 3 weeks, plaques became visible. Virus clones were isolated from plaques and propagated in KB cells. Restriction enzyme cleavage patterns of

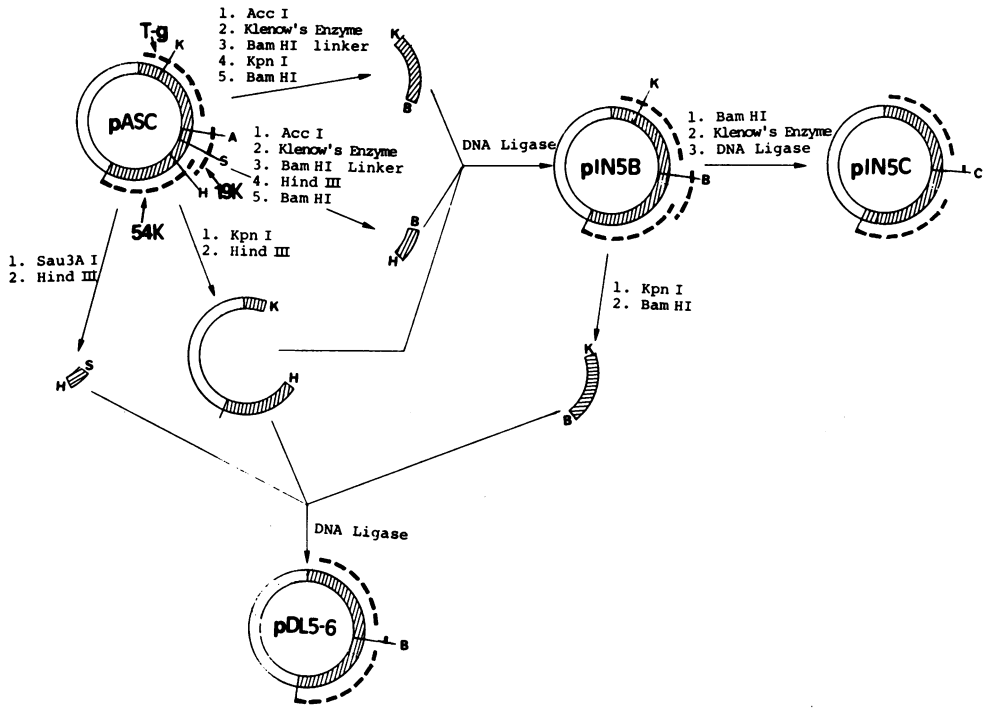

FIG. 2. Introduction of the insertions and the deletion in the E1B-coding sequence for the 19K polypeptide. Plasmid pASC was cleaved with *Acc*I. The ends were converted to flush ends, resulting in the addition of 2 bp, and *Bam*HI linkers (10 bp; CCGGATCCGG) were ligated to the ends. The products were cleaved with *Kpn*I, *Hin*dIII, and *Bam*HI, and the two fragments (K-B and B-H) were isolated. Plasmid pASC was also cleaved with *Kpn*I and *Hin*dIII, and the large fragment (K-H) was isolated. The ligation of these three fragments resulted in the formation of pIN5B, in which 12 bp was inserted into pASC. Next, pIN5B was cleaved with *Bam*HI, and the cohesive ends were converted to flush ends. The product was circularized to produce pIN5C, in which the *Bam*HI site was converted to the *Cla*I site, resulting in the insertion of 16 bp into pASC. As for the deletion mutant, pIN5B DNA was digested with *Kpn*I and *Bam*HI, and the smaller fragment (K-B) was isolated. The *Sau*3AI-*Hin*dIII fragment of pASC (S-H) was also isolated. These two fragments were inserted between the *Kpn*I and *Hin*dIII site of pASC, resulting in the formation of pDL5-6, in which the sequence between the *Bam*HI and *Sau*3AI sites of pIN5B was deleted (deletion of 175 bp compared with pASC). This could be done because the cohesive ends of DNAs cut with *Bam*HI and *Sau*3AI were the same. Single capital letters indicate cleavage sites of restriction endonucleases: A, *Acc*I; B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; K, *Kpn*I; and S, *Sau*3AI. Symbols: – – – –, coding regions for the proteins: T-g, T antigen g encoded by E1A; 19K, the 19K polypeptide encoded by E1B; 54K, the 54K polypeptide encoded by E1B. Symbols for origins of sequences: ▨, Ad12 *Sal*I-C; ▭, pBR322.

viral DNA from each clone were examined by electrophoresis. The virus clones which contained expected insertions or deletions were selected and established as mutants after plaque purification in HEK cells (*in*205B from pIN5B, *in*205C from pIN5C, and *dl*205 from pDL5-6). For instance, transfection of HEK cells with ca. 50 μg of pIN5C DNA ligated with ca. 50 ng of Ad12 DNA-protein complex resulted in the formation of six plaques. Of these plaques, one consisted of a mutant, one of a wild-type (WT) virus, and the remaining four of the mixture. Mutants were named as by the method of Ginsberg et al. (7).

Nature of mutants. The restriction enzyme cleavage patterns of DNAs from mutants are shown in Fig. 3. In Ad12 WT DNA, *Bam*HI-A (8,020 bp; 0 to 23.4 map units) was located at the left end of the genome. Cleavage with *Bam*HI showed that new *Bam*HI sites were introduced in *in*205B and *dl*205 DNAs (Fig. 3, lanes b and d). Since *in*205B DNA was formed after the addition of *Bam*HI linkers, the presence of the *Bam*HI site was rational. As for *dl*205 DNA, since the ligation of *Bam*HI site and *Sau*3AI site in *in*205B regenerated the *Bam*HI site as deduced from the sequence date (3, 25), the presence of a new *Bam*HI site was also rational. Cleavage with *Cla*I showed that a new *Cla*I site was introduced instead of the *Bam*HI site in *in*205C DNA (Fig. 3, lane g). In *dl*205 DNA, the *Hin*dIII-G (2,320 bp; 0 to 6.8 map units) is a little shorter than that of WT DNA because of the deletion of 175 bp (Fig. 3, lane 1). Next, we cloned mutated parts of the virus genomes and analyzed the DNA sequences. The results are shown in Fig. 4. They were as expected by the construction procedures of the plasmids. These three mutants (*in*205B, *in*205C, and *dl*205) have mutations on the coding sequence for the E1B-19K polypeptide and not on the coding sequence for the 54K polypeptide. These mutants could grow as efficiently as the WT virus in HEK (Table 1) or KB (data not shown) cells, unlike most of the host range mutants of Ad5.

Transcription and translation of mutants. To confirm the presence of the mutations, we examined the transcription and translation of the E1B mutant. mRNA in cells infected with mutants and WT virus at 14 h p.i. was analyzed with nuclease S1 mapping by using the ^{32}P-labeled E1B probe (4.1 to 11.2 map units) (Fig. 5A). Since a great, excess amount of the DNA probe was used in RNA-DNA hybridization, the intensity of the bands was proportional to the amounts of the RNAs. Since the intensities of the bands were almost equal among cells infected with mutants and WT viruses, the levels of E1B mRNAs were estimated to be nearly equal among these cells. We next selected mRNAs complementary to E1A and E1B by hybridization with the *Hin*dIII-G of Ad12 DNA and translated the mRNAs eluted from the

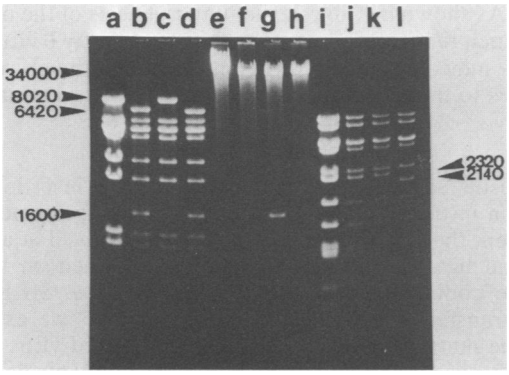

FIG. 3. Restriction endonuclease cleavage patterns of the viral DNAs from mutants. Viral DNAs from WT virus (lanes a, e, and i), *in*205B (lanes b, f, and j), *in*205C (lanes c, g, and k), and *dl*205 (lanes d, h, and l) were cleaved with restriction endonucleases and analyzed by electrophoresis in a 1% agarose gel with Tris-acetate-EDTA buffer. The bands were visualized by staining with ethidium bromide. When DNAs were cleaved with *Bam*HI (lanes a to d), WT virus (lane a), and *in*205C (lane c) DNAs showed similar patterns. Two extra bands were detected in *in*205B DNA (the 6,420- and 1,600-bp bands; lane b) and *dl*205 DNA (the band which is a little shorter than 6,420 bp and the 1,600-bp band; lane d) with the disappearance of the *Bam*HI-A band (8,020 bp) in WT DNA (lane a), indicating the introduction of new *Bam*HI sites in *in*205B and *dl*205 DNAs. When DNAs were cleaved with *Cla*I (lanes e to h), the new band (1,600 bp) was detected only in *in*205C DNA (lane g), whereas in other DNAs (lane e for WT DNA, lane f for *in*205B DNA, and lane h for *dl*205 DNA) only the bands of the full genome length (34,000 bp) were detected, indicating the presence of the new *Cla*I site in *in*205C DNA and the absence of the site in WT, *in*205B, and *dl*205 DNAs. When DNAs were cleaved with *Hin*dIII (lanes i to l), the cleavage patterns of *in*205B DNA (lane j) and *in*205C DNA (lane k) were the same as that of WT DNA (lane i). In *dl*205 DNA (lane l), the *Hin*dIII-G band (2,320 bp in WT DNA) is a little short to be 2,140 bp because of the deletion of 175 bp.

hybrids by using a reticulocyte lysate system (Fig. 5). The E1A 38 to 40K polypeptides and E1B-19K and -54K polypeptides, identified in previous reports (21a), could be discerned, although the pictures contain some backgrounds present in mock-infected cells (Fig. 5B, panel e). The E1B-19K polypeptide synthesized by *in*205B was a little larger in size with a more basic pI value (Fig. 5B, panels a and b) than that synthesized by WT virus. Since the mutation in *in*205B is expected to result in the insertion of four amino acids (Pro-Asp-Pro-Asp), this result is rational. As for the other two mutants, *in*205C and *dl*205, we could not detect any polypeptide corresponding to the 19K polypeptide. Since the reading frames of the 19K polypeptide were shifted by insertion or deletion in these mutants, the 19K polypeptide was expected to be a 3K polypeptide, which was too small to be detected in the gel. Since the products of the E1A and 54K polypeptide encoded by E1B were detected in the same cells, the results may not be due to unfavorable conditions for translation in our system. The results are explained by the expected sequences in mutant DNA, and they show that the expected sequences are transcribed in cells infected with the mutants.

Immunoprecipitation of the 19K polypeptide synthesized in cells infected with mutants. KB cells were infected with mutants and WT virus labeled with [^{35}S]methionine and harvested at 16 h p.i. From each of the cell extracts, gene products were immunoprecipitated with anti-19K sera (Fig. 6). We could detect the 19K polypeptide distinctly in the extract from the cells infected with WT virus (Fig. 6, panel a) (21a). However, we failed to detect polypeptides encoded by the E1B-19K polypeptide-coding sequences in extracts from all the cells infected with the mutants. In extracts from cells infected with *in*205C or *dl*205, the results can be explained by the fact that their sizes were too small to be detected (Fig. 6, panels c and d). In the extract from cells infected with *in*205B, the failure to detect the 19K polypeptide was unexpected (Fig. 6, panel b). There may be two possibilities to explain these results. One possibility is that the product is very unstable in KB cells, and the other is that the property of the product is changed so as not to be solubilized under the present condition. Further investigation will be required to solve the question.

S1 mapping of mRNAs. It is shown in the preceding section that all three mutants are not defective in replication. To confirm whether the above notion is correct, we carried out S1 mapping of viral mRNAs in permissive cells infected with the mutants (Fig. 7). The results indicate that mRNA patterns at early and late stages in cells infected with the mutants were the same as those in cells infected with WT virus. Thus, transcriptional control is not affected by the mutation.

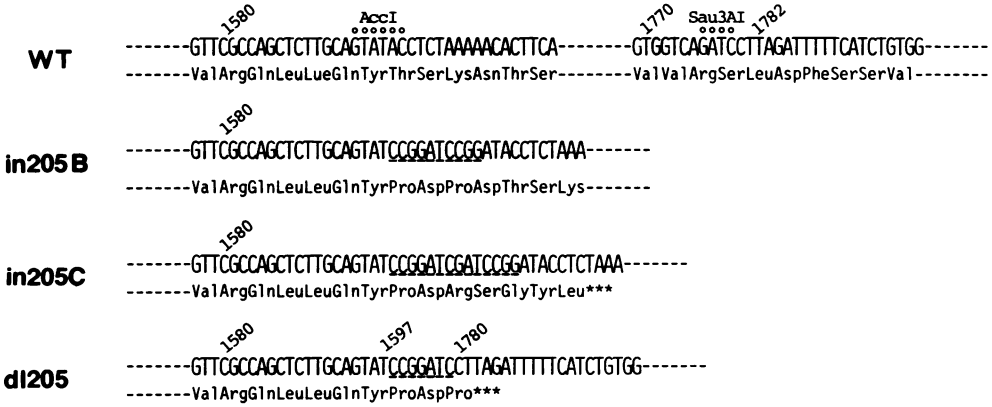

FIG. 4. DNA and the corresponding amino acid sequences of the mutated viruses. The mutated parts of the virus genomes were cloned in pBR322, and the nucleotide sequences were analyzed. Underlines show the DNA sequences derived from linker sequences. The numbers indicate bp positions counted from the left end of the virus genome. Symbols: ° ° ° , cutting sites of restriction enzymes; * * *, termination codons; – – – , the DNA or amino acid sequence continues.

TABLE 1. Growth of WT virus and mutants in HEK cells[a]

Virus	Virus yield (PFU/ml)	
	Expt I	Expt II
WT	1.0×10^8	1.7×10^7
*in*205B	2.6×10^8	6.5×10^7
*in*205C	2.6×10^8	4.5×10^7
*dl*205	2.2×10^8	8.0×10^7

[a] HEK cells were infected at ca. 5 to 10 PFU per cell, and virus yields in cell-medium lysates at 72 h p.i. were measured by plaque assay in HEK (experiment I) or 293 (experiment II) cells.

Transformation of cells with mutants. The abilities of the mutants to transform rat 3Y1 cells were examined. As shown in Fig. 8, the mutants could induce much reduced numbers of foci compared with those induced by WT virus.

We then subcultured the transformed cells from the foci and established transformed cell clones. The ability of cells in each clone to grow in soft agar medium was tested. As shown in Table 2, most of the transformed cell clones induced by the mutants failed to form colonies in soft agar medium, whereas most of those induced by WT virus could grow to form colonies of distinct sizes in soft agar medium. These results indicate that both the frequency of cell transformation and the phenotype of transformed cells were different between the mutants and the WT virus.

Next, tumor formation in vivo was assayed. The virus suspensions were injected subcutaneously into baby hamsters. As shown in Table 3, the tumorigenesis of the mutants was much reduced compared with that of the WT virus. The results indicate that the three mutants have much reduced abilities to transform rodent cells both in vitro and in vivo.

DISCUSSION

We isolated three Ad12 mutants with either insertions or a deletion in the E1B-19K polypeptide-coding sequence. For isolation, the DNA-protein complex was cleaved at a single site and ligated with DNA fragments mutated on the sequence coding for the E1B-19K polypeptide. HEK cells were transfected to isolate mutants. At first, we expected that the mutant would be obtained as a mixed virus with a WT, because it was known that most Ad5 mutants defective in E1B grow in 293 cells (8) but not in HEK or HeLa cells (11, 13). Unexpectedly, we found that Ad12 mutants defective in the E1B-19K polypeptide could grow efficiently in HEK cells. This finding enabled us to isolate mutants with the use of only HEK cells and without the use of 293 cells. Mutants were selected by restriction endonuclease cleavage patterns of their DNAs and established after plaque purification in HEK cells. The reason for the difference between Ad5 and Ad12 E1B mutants is not known. However, careful comparison of mutants suggests that the larger polypeptide (54K) encoded by E1B is required and that the smaller polypeptide (15K or 19K) encoded by E1B is not required for virus growth. Ad5 *dl*313 is defective in virus growth and in the synthesis of the larger polypeptide (13), whereas Ad12 E1B mutants discussed above are nondefective in virus

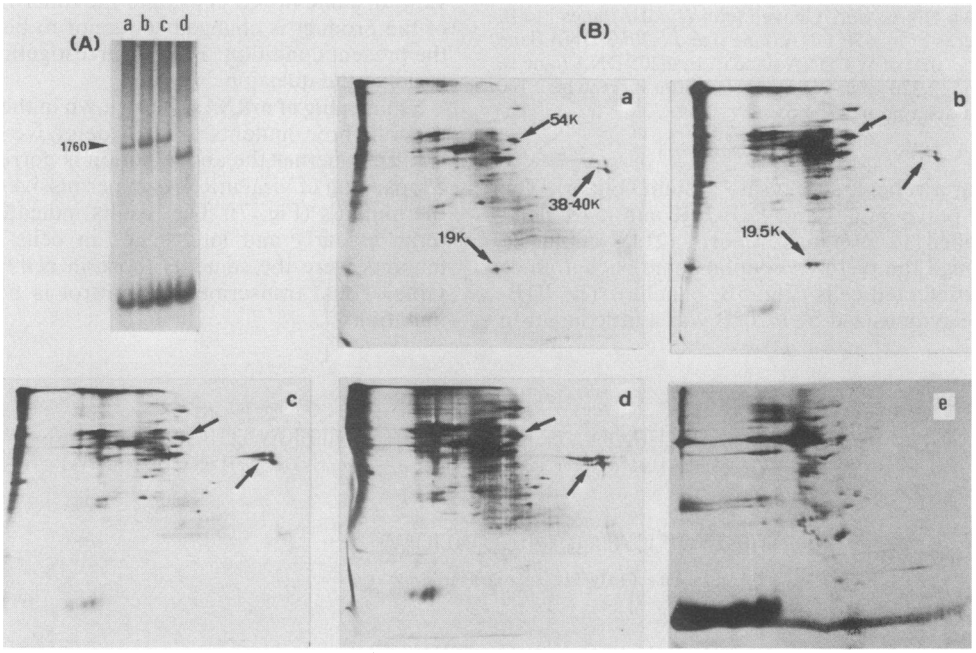

FIG. 5. Transcription and translation of the E1 gene in cells infected with the mutants. (A) S1 gel analysis of E1B mRNAs. The cytoplasmic RNAs were extracted from KB cells infected with WT virus and mutants at 14 h p.i. and hybridized with ^{32}P-labeled E1B probe (4.1 to 11.2 map units). The S1-resistant DNA-RNA hybrids were analyzed by electrophoresis in a 1.4% agarose gel with alkaline buffer. The bands of 1,760 nucleotides are E1B mRNAs in cells infected with WT virus (lane a), *in*205B (lane b), and *in*205C (lane c). The band a little shorter than 1,760 nucleotides is E1B mRNA in cells infected with *dl*205 (lane d). The lower bands are mRNAs for polypeptide IX. (B) In vitro translation of mRNAs complementary to E1. The mRNAs complementary to E1 were selected by hybridization with *Hin*dIII-G of Ad12, eluted and translated in vitro. The products were analyzed by two-dimensional gel electrophoresis. The polypeptides were separated by pH gradients from the left (pH 7.5) to the right (pH 4.0) and by molecular weight from top to bottom. Arrows indicate the products of E1: 38 to 40K, the polypeptide encoded by E1A (T antigen g); 19K, 19.5K, and 54K, the polypeptides encoded by E1B. (a) WT; (b) *in*205B; (c) *in*205C; (d) *dl*205; (e) mock infected.

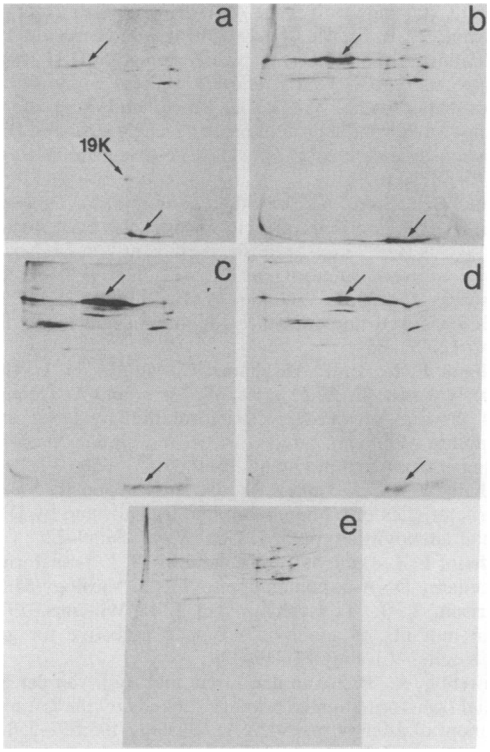

FIG. 6. Two-dimensional gel electrophoresis of immunoprecipitates of extracts from cells infected with the mutants with anti-19K sera. KB cells were infected with WT virus or mutants and labeled with [^{35}S]methionine from 14 to 16 h p.i. The cell extracts were prepared and the gene products were immunoprecipitated with anti-19K sera. The conditions for gel electrophoresis are the same as in Fig. 5B. When cells were infected with WT virus or mutants, the spots indicated by the arrows were detected as virus-specific antigens. The spot for the 19K polypeptide was detected in cells infected with WT virus (a), but the spots corresponding to the 19K polypeptide could not be detected in cells infected with *in*205B (b), *in*205C (c), and *dl*205 (d). Panel e shows the mock-infected cell extract. Arrows show Ad12 gene products. The upper arrow may be an E2-coded DNA-binding protein, and the lower arrow may be an E4 gene product.

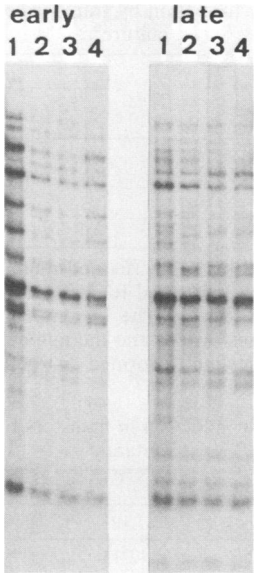

FIG. 7. S1 mapping of viral mRNAs. Cytoplasmic RNA was extracted from KB cells infected with the viruses at 14 h p.i. (early) or at 27 h p.i. (late) and was hybridized with an excess amount of ^{32}P-labeled Ad12 whole DNA. S1-resistant DNA-RNA hybrids were analyzed by electrophoresis in a 1.4% agarose gel with alkaline buffer. mRNA patterns in cells infected with the mutants (lanes 2 for *in*205B, lanes 3 for *in*205C, and lanes 4 for *dl*205) were almost the same as that in cells infected with WT virus (lanes l).

growth and in the synthesis of the larger polypeptide. To substantiate this suggestion, the isolation of Ad12 E1B mutants defective in the synthesis of 54K polypeptides is necessary.

The first mutant, *in*205B, has an insertion of 12 bp (4 amino acids, in frame) at the *Acc*I site (4.5 map units). The second mutant has an insertion of 16 bp (frame shift) at the same site. The third mutant, *dl*205, has a deletion of 175 bp (sequence between 4.5 and 5.1 map units; frame shift). From sequence data, it is expected that *in*205B will produce a longer (4 amino acids) polypeptide and that *in*205C and *dl*205 will produce polypeptides that are as small as 3K, instead of the E1B-coded 19K polypeptide detected in WT-infected cells (Fig. 4).

After infection of KB cells with mutants, transcription of the E1B gene was found to proceed efficiently in the same way as in WT infection. In vitro translation of E1B mRNA revealed that *in*205B produces a polypeptide that is a little longer and more basic than the 19K polypeptide and that *in*205C and *dl*205 produce polypeptides that are too small to be detected under the present condition. This observation agrees with the expectation. When the extract of cells infected with *in*205B and labeled with [^{35}S]methionine was immunoprecipitated and analyzed in gel electrophoresis, no specific band or spot was detected. This observation may be explained by the alteration in stability or solubility or both of the gene product.

Although all three mutants replicate in permissive cells as efficiently as in WT cells, the mutants show much reduced capacities for cell transformation. This fact was confirmed by the very low frequencies in the appearance of transformed cell foci in rat cells, no or poor formation of colonies in soft agar culture with cells transformed by the mutants,

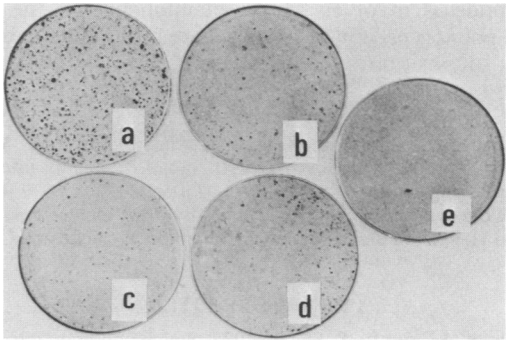

FIG. 8. Transformation of rat cells with Ad12 WT virus and mutants. Rat 3Y1 cells were infected with WT virus and mutants at 10 PFU per cell and maintained in low calcium Eagle MEM with 10% fetal calf serum. After incubation for 4 weeks, the cells were fixed with 10% Formalin and stained with Giemsa. A number of foci are seen in the dish infected with WT virus (a). Fewer numbers of foci are seen in the dishes infected with *in*205B (b), *in*205C (c), and *dl*205 (d). No focus is seen in the mock-infected dish (e).

TABLE 2. Colony formation by transformed cells in soft agar culture[a]

Cells transformed by:	Duration (wk)	
	2	3
WT	3/5	4/5
*in*205B	0/4	0/4
*in*205C	0/3	0/3
*dl*205	0/6	1/6

[a] Cells transformed by WT virus and mutants were subcultured from each of the foci and tested for colony formation in soft agar cultures for 2 and 3 weeks. The denominator is the number of transformed cell clones tested, and the numerator is the number of transformed cell clones which formed distinctly visible colonies.

TABLE 3. Tumor induction in hamsters by Ad12 WT virus mutants[a]

Virus	Dose (PFU)			
	10^4	10^5	10^6	10^7
WT	0/2	2/11	5/6	ND
*in*205B	ND	ND	0/11	0/9
*in*205C	ND	ND	0/11	0/12
*dl*205	ND	0/12	0/6	0/10

[a] The virus with titers shown was injected subcutaneously into baby hamsters and observed for tumor induction for 90 days. The denominator is the number of hamsters observed, and the numerator is the number of hamsters with tumors. ND, Not determined.

and very low frequencies of tumor induction in hamsters. The process of cell transformation by virus may be divided into the initiation of cell transformation and the maintenance of the transformed phenotype. Although inefficient, the mutants induce the foci of transformed cells (nondefective in initiation). Some of the foci induced by the mutants became flat again during the observation period, resulting in the disappearance of the foci (nondefective in initiation and defective in maintenance). The cells transformed with the mutants formed no or poor colonies in soft agar cultures, although they formed foci efficiently. This suggests the necessity of a phenotype for colony formation in addition to the phenotype for focus formation. The mutants formed tumors very inefficiently in hamsters. These observations can be explained by a supposition that the E1B-coded 19K polypeptide is involved in the maintenance of the transformed phenotype. Further studies are required to substantiate this supposition.

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LITERATURE CITED

1. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.
3. **Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, P. J. van den Elsen, A. J. van der Eb, and H. van Ormondt.** 1981. The 2.2 kb Elb mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell **27**:121–131.
4. **Dijkema, R., B. M. M. Dekker, and H. van Ormondt.** 1980. The nucleotide sequence of the transforming *Bgl*II-H fragment of adenovirus type 7 DNA. Gene **9**:121–156.
5. **Dunsworth-Browne, M., R. E. Schell, and A. J. Berk.** 1980. Adenovirus terminal protein protects single stranded DNA from digestion by a cellular exonuclease. Nucleic Acids Res. **8**:544–554.
6. **Fukui, Y., I. Saito, K. Shiroki, H. Shimojo, Y. Takebe, and Y. Kaziro.** 1983. The 19K protein encoded by early region 1b of adenovirus type 12 is synthesized efficiently in *Escherichia coli* only as a fused protein. Gene **23**:1–13.
7. **Ginsberg, H. S., J. F. Williams, W. H. Doerfler, and H. Shimojo.** 1973. Proposed nomenclature for mutants of adenoviruses. J. Virol. **12**:653–654.
8. **Graham, F. L., P. J. Abrahams, C. Mulder, H. L. Heijneker, S. O. Warnaar, F. A. J. Vries, W. Fiers, and A. J. van der Eb.** 1974. Studies on *in vitro* transformation by DNA and DNA fragments of human adenoviruses and simian virus 40. Cold Spring Harbor Symp. Quant. Biol. **39**:637–650.
9. **Graham, F. L., J. Smiley, W. C. Russel, and R. Nairn.** 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. **36**:59–72.
10. **Graham, F. L., and A. J. van der Eb.** 1973. Transformation of rat cells by DNA of human adenovirus 5. Virology **54**:536–539.
11. **Harrison, T. J., F. Graham, and J. F. Williams.** 1977. Host range mutants of adenovirus type 5 defective for growth in HeLa cells. Virology **77**:319–329.
12. **Houweling, A., P. J. van den Elsen, and A. J. van der Eb.** 1980. Partial transformation of primary rat cells by the left most 4.5% fragment of adenovirus 5 DNA. Virology **105**:537–550.
13. **Jones, N., and T. Shenk.** 1979. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. Cell **17**:683–689.
14. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**:1617–1624.
15. **Kimura, G., A. Itagaki, and J. Summers.** 1975. Rat cell line 3Y1 and its virogenic polyoma- and SV40-transformed derivatives. Int. J. Cancer **15**:694–706.
16. **Kimura, T., Y. Sawada, M. Shinagawa, Y. Shimizu, K. Shiroki, H. Shimojo, H. Sugisaki, M. Takanami, Y. Uemizu, and K. Fujinaga.** 1981. Nucleotide sequence of the transforming early region E1b of adenovirus type 12 DNA: structure and gene organization, and comparison with those of adenovirus type 5 DNA. Nucleic Acids Res. **9**:6571–6589.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. **74**:560–564.
19. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
20. **Ricciardi, R. P., J. S. Miller, and B. E. Roberts.** 1979. Purification and mapping specific mRNAs by hybridization-selection and cell-free translation. Proc. Natl. Acad. Sci. U.S.A. **76**:4927–4931.
21. **Saito, I., J. Sato, H. Handa, K. Shiroki, and H. Shimojo.** 1981. Mapping of RNAs transcribed from adenovirus type 12 early and VA RNA region. Virology **114**:379–398.

21a. **Saito, I., K. Shiroki, and H. Shimojo.** 1983. mRNA species and proteins of adenovirus type 12 transforming regions: identification of proteins translated from multiple coding stretches in 2.2kb region 1B mRNA in vitro and in vivo. Virology **127**:272–289.

22. **Shiroki, K., H. Handa, H. Shimojo, S. Yano, S. Ojima, and K. Fujinaga.** 1977. Establishment and characterization of rat cell lines transformed by restriction endonuclease fragments of adenovirus 12 DNA. Virology **82**:462–471.
23. **Shiroki, K., K. Maruyama, I. Saito, Y. Fukui, and H. Shimojo.** 1981. Incomplete transformation of rat cell by a deletion mutants of adenovirus type 5. J. Virol. **38**:1048–1054.

24. **Shiroki, K., H. Shimojo, Y. Sawada, Y. Uemizu, and K. Fujinaga.** 1979. Incomplete transformation of rat cells by a small fragment of adenovirus 12 DNA. Virology **95**:127–136.
25. **Sugisaki, H., K. Sugimoto, M. Takanami, K. Shiroki, I. Saito, H. Shimojo, Y. Sawada, Y. Uemizu, S. Uesugi, and K. Fujinaga.** 1980. Structure and gene organization in the transforming *Hin*dIII-G fragment of Ad12. Cell **20**:777–786.
26. **Tooze, J.** 1980. DNA tumor viruses. Molecular biology of tumor viruses, part 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
27. **van der Eb, A. J., and A. Houweling.** 1977. Transformation with specific fragments of adenovirus DNAs. II. Analysis of the viral DNA sequences present in cells transformed with a 7% fragment of adenovirus 5 DNA. Gene **2**:133–146.
28. **van der Eb, A. J., C. Mulder, F. L. Graham, and A. Houweling.** 1977. Transformation with specific fragments of adenovirus DNAs. I. Isolation of specific fragments with transforming activity of adenovirus 2 and 5 DNA. Gene **2**:115–132.
29. **van Ormondt, H., J. Maat, and C. P. van Beveren.** 1980. The nucleotide sequence of the transforming early region E1 of adenovirus type 5 DNA. Gene **11**:299–309.
30. **Yano, S., S. Ojima, K. Fujinaga, K. Shiroki, and H. Shimojo.** 1977. Transformation of a rat cell line by an adenovirus type 12 DNA fragment. Virology **82**:214–220.