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Oncogenicity by Adenovirus Is Not Determined by the Transforming Region Only

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We have constructed a nondefective recombinant virus between the nononcogenic adenovirus 5 (Ad5) and the highly oncogenic Ad12. The recombinant genome consists essentially of Ad5 sequences, with the exception of the transforming early region 1 (E1) which is derived from Ad12. HeLa cells infected with the recombinant virus were shown to contain the Ad12-specific E1 proteins of 41 kilodaltons (E1a) and 19 and 54 kilodaltons (both encoded by E1b). The recombinant virus replicated efficiently in human embryonic kidney cells and HeLa cells, showing that the transforming regions of Ad5 and Ad12 had similar functions in productive infection. After the recombinant virus was injected into newborn hamsters, no tumors were produced during an observation period of 200 days. Thus, despite the fact that all products required for oncogenic transformation in vitro were derived from the highly oncogenic Ad12, the recombinant virus did not produce tumors in vivo. These data show that tumor induction by adenovirus virions is not determined only by the gene products of the transforming region.

Human adenovirus 12 (Ad12) efficiently induces tumors in newborn hamsters after a short latency period, whereas Ad5 completely fails to do so (11). Likewise, rat cells transformed in vitro by Ad12 are highly oncogenic in immunocompetent syngeneic rats and immunodeficient nude mice (1, 14, 16, 21, 24, 28, 34), whereas cells transformed by nononcogenic adenoviruses are, in general, nononcogenic in immunocompetent rats and only moderately oncogenic in nude mice (1, 15, 21, 29).

The region responsible for cell transformation in vitro coincides with the first region expressed early in productive infection, indicated as early region 1 (E1) (13). The E1 region of both oncogenic and nononcogenic adenoviruses is located at the left end of the viral genome and consists of two independent transcription units, E1a and E1b (13). Both regions E1a and E1b are required for cell transformation in vitro and contribute to the oncogenic potential of the transformed cells. Rat cells expressing only region E1a of Ad5 exhibit a semi-transformed phenotype and are nononcogenic in nude mice (23; P. J. van den Elson, Ph.D. thesis, State University of Leiden, Leiden, The Netherlands, 1982), and similar properties have been described for cells that express a normal E1b region but carry a mutation in region E1a (5, 18, 32).

Studies with rat cells transformed by Ad5/Ad12 hybrid E1 plasmids (1) have shown that two genes are responsible for the observed difference in oncogenicity between Ad5- and Ad12-transformed cells; one was localized in E1a and one was localized in E1b. The protein translated from the 13S E1a mRNA of Ad12, but not that of Ad5, has been shown to suppress the expression of class I transplantation antigens in transformed cells and thereby allows transformed cells to evade elimination by host cytotoxic T cells (3, 33). Region E1b further contributes to the difference in oncogenic potential between Ad5- and Ad12-transformed cells in the sense that it determines the efficiency with which the transformed cells induce tumors: if region E1b is derived from Ad5, tumor induction is inefficient, whereas if it is derived from Ad12, the tumors are readily induced (1, 3). This different behavior of the Ad5 and Ad12 E1b regions has been attributed to differences in the primary structure of the 54 to 58-kilodalton (kD) polypeptide (2).

An essential question is whether oncogenicity of adenovirus virions in vivo is solely dependent on the identity of the E1 products, as is the case for oncogenic transformation in vitro, or whether genes located in the remaining 90% of the viral genome also contribute to the oncogenic properties of the virus. To answer this question, we have constructed an Ad5/Ad12 recombinant virus by replacing the E1 region of Ad5 with the corresponding region of Ad12. This recombinant was nondefective in HeLa cells but did not induce tumors in newborn hamsters. This finding shows that tumor induction in vivo is also dependent on sequences located outside the transforming region.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Eagle minimal essential medium supplemented with 8% heat-inactivated newborn calf serum. The 293 cells were grown in minimal essential medium–10% fetal calf serum, and for growth of human embryonic kidney (HEK) cells, this medium was further supplemented with extra vitamins and amino acids (F15 medium). Infected cells were incubated in minimal essential medium–2% fetal calf serum. Plaque assays were performed by infecting confluent dishes of 293 cells or HEK cells with serial dilutions of virus phosphate-buffered saline (PBS)–2% fetal calf serum. After 1 h of absorption at 37°C, the virus was removed and cells were overlaid with 8 ml of F15 medium–2% fetal calf serum containing 0.7% agar. After 4 to 5 days, another 4 ml of overlay medium was added. On day 8, the cells were stained with neutral red, and plaques were counted on days 8 to 10. Replication of virus was also monitored by infecting two replicate subconfluent HeLa or BHK-21 cell cultures (in 6-cm dishes) with 10 PFU per cell.

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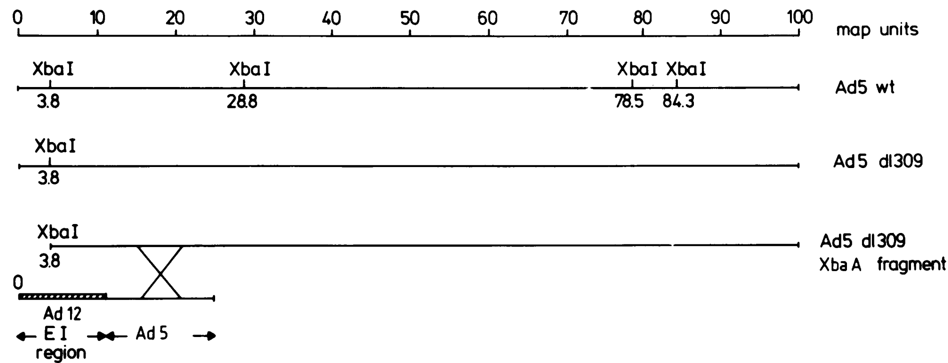

FIG. 1. Construction of Ad5/Ad12 recombinant viruses. The *Xba*I cleavage sites of wild-type Ad5 and of the Ad5 mutant dl309 are indicated. Recombinant viruses arise by homologous recombination between the Ad5 dl309 *Xba* A fragment and an Ad5/Ad12 hybrid plasmid after cotransfection onto permissive human cells.

After absorption of the virus for 1 h at 37°C, the cells were washed three times with PBS to remove unabsorbed virus and fed 5 ml of minimal essential medium–2% fetal calf serum. After 1 h, cells and medium were collected from one of the dishes (t = 0, control); the other dish was incubated for 4 days at 37°C before cells and medium were collected. After 6 cycles of freezing and thawing, cell debris was removed by centrifugation, and serial dilutions of the supernatant were made in PBS–2% fetal calf serum and used to infect 293 cells for a plaque assay. Procedures for ^{35}S labeling and immunoprecipitation have been described previously (1).

Recombinant DNA techniques. Plasmid p5125E1 was constructed from a plasmid containing the Ad12 *Eco*RI C fragment (pAd12RIC) (6). In the first step, the left terminal *Pvu*II fragment (nucleotides 0 to 398) of Ad12 in this plasmid was replaced by the left terminal *Pvu*II fragment of Ad5 (nucleotides 79 to 451, isolated from pAd5 *Xho*C [1]). In the second step, this plasmid was further modified by removing the Ad12 sequences downstream from the E1b polyadenylation signal. Finally, to obtain homologous recombination with the dl309 *Xba* A fragment, the Ad5 *Bgl*II B fragment was ligated downstream from Ad12 E1b, yielding p5125E1. Methods used for these constructions have been described previously (1, 2, 6). Before transfection into 293 cells, the plasmid was digested with *Eco*RI. This enzyme cleaves the plasmid once at 29 base pairs left of the Ad5 inverted terminal repetition (ITR).

Isolation of recombinant viruses. The isolation of the Ad5 dl309 *Xba* A fragment-terminal protein complex has been described previously (4). To obtain Ad5/Ad12 recombinants, human 293 cells in 6 cm dishes were cotransfected with 1 μg of the *Xba* A fragment and 5 μg of *Eco*RI-linearized plasmid p5125E1 by the calcium phosphate coprecipitation technique (39). At 4 h after transfection, the medium was removed. The cells were washed twice with PBS to remove the precipitate, and the cells were overlaid with agar as described above. After 9 to 10 days, individual plaques were picked and, after

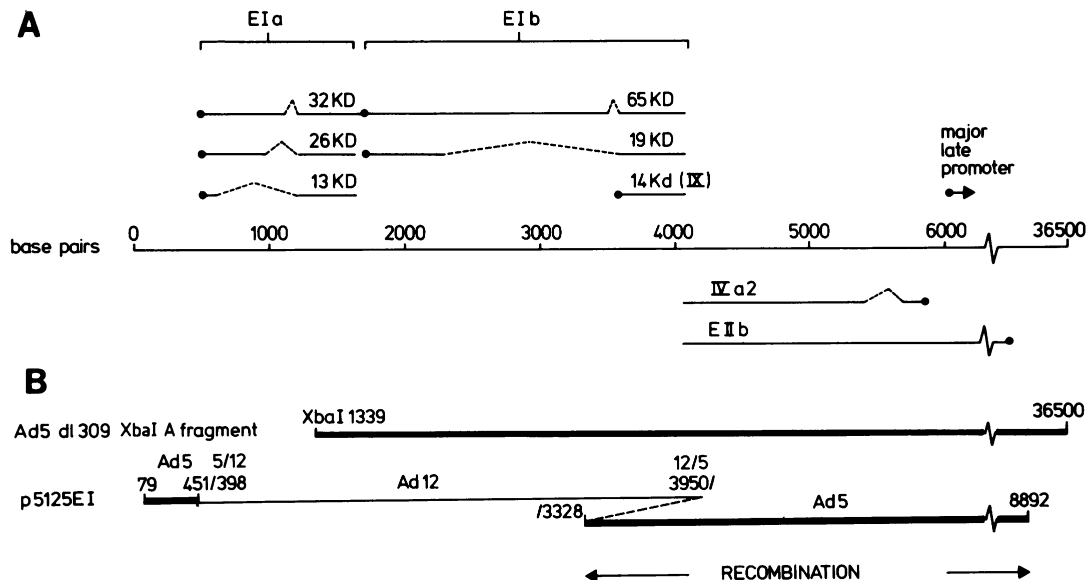

FIG. 2. Schematic representation of the major RNAs transcribed from the left end of the adenovirus genome (A). Dotted lines represent introns, solid lines represent exons, and black dots represent ATG start codons. (B) Structure of the Ad5 dl309 *Xba* A fragment and of the plasmid p5125E1. Thick lines represent Ad5-derived sequences; thin lines represent Ad12-derived sequences. The numbers at the Ad5/Ad12 junctions indicate the exact positions of the crossover points (see text for further explanation).

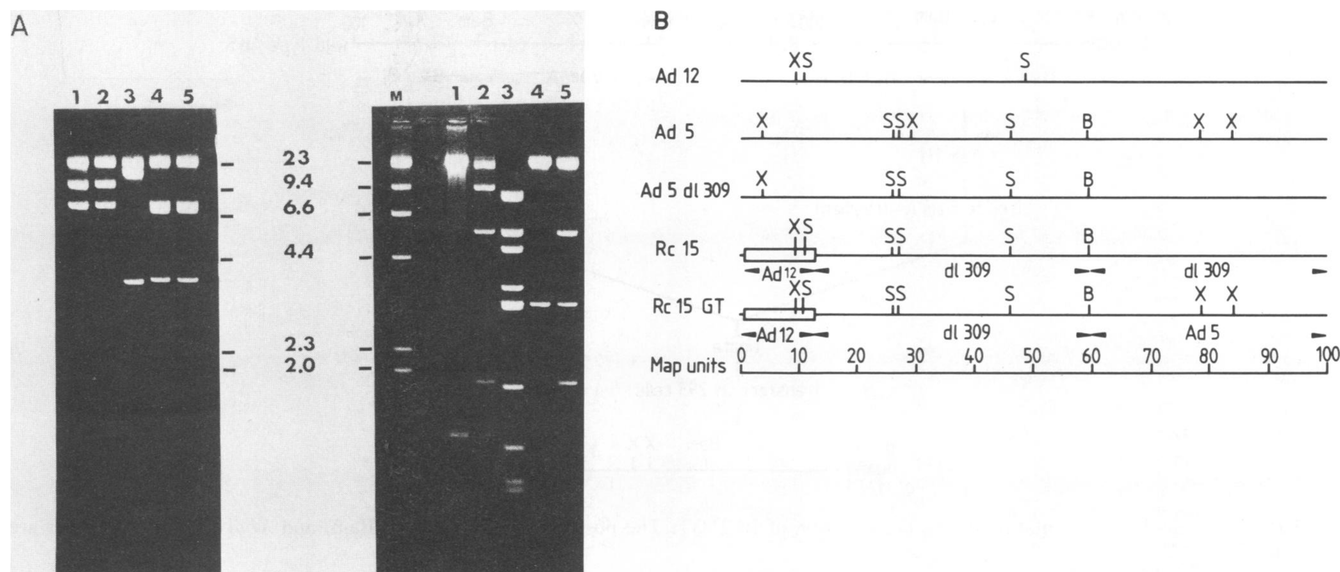

FIG. 3. (A) Agarose gel electrophoresis of *Sal*I digests (left panel) and *Xba*I digests (right panel) of the viruses Ad5 dl309 (lane 1), Ad5 (lane 2), Ad12 (lane 3), RC15 (lane 4), and RC15GT (lane 5). (B) Cleavage maps of the viruses Ad5, Ad5 dl309, Ad12, RC15, and RC15GT for the restriction enzymes *Sal*I (S), *Xba*I (X), and *Bam*HI (B).

two cycles of freeze-thawing, used to infect human 293 cells in 6-cm dishes. After the development of cytopathic effect, usually after 3 to 4 days, viral DNA was isolated as described previously (4) and analyzed by restriction endonuclease digestion. Recombinants were plaque purified two more times before use in further analyses.

RESULTS

Construction of the recombinant virus. The method used to replace E1 of Ad5 with the corresponding region of Ad12 was based on the high frequency of homologous recombination of adenoviruses and is schematically shown in Fig. 1. In short, this method involves the cotransfection, onto permissive human cells, of a plasmid containing Ad12 E1 and a subgenomic fragment of Ad5 which lacks a part of E1. Infectious virus can arise only when the two DNA fragments recombine to yield a complete Ad5/Ad12 recombinant genome. Two modifications of the Ad12 E1 plasmid (5) were required before it could be used in this experiment. The first modification was required to allow the Ad5/Ad12 recombinant to replicate. Replication of the linear genome of adenoviruses requires base-pairing of the two inverted terminal repetitions (ITRs) present at the left- and right-end of the genome (11, 13). Thus, since the ITRs of Ad5 and Ad12 are not identical, replication of an Ad5 genome, containing Ad12 E1 in the proper position (i.e., at the left end), is possible only when the Ad12 ITR in such a construction is replaced by the Ad5 ITR. Therefore, the Ad12 ITR in the Ad12 E1 plasmid was replaced by the Ad5 ITR (see above). The use of an incomplete Ad5 ITR in this experiment (nucleotides 0 to 78 were deleted from our Ad5 left-end clone) was not problematic since Stow (36) has shown that such a defect is repaired in vivo by using the right-end ITR of the virus as a template. The second modification of the Ad12 E1 plasmid was required to facilitate homologous recombination between the plasmid and the Ad5 genome. To this end, the Ad12 E1 plasmid was further modified by inserting Ad5 sequences, derived from the region downstream from the Ad12 E1 region, to the right of the Ad12 E1 region, thus yielding the plasmid p5125E1 (see Fig. 2 for details). Reconstruction of an intact Ad/Ad12 recombinant genome was performed by cotransfection of the plasmid p5125E1 and the *Xba*I A fragment (3.8 to 100 map units) of the Ad5 mutant dl309 into human 293 cells (19) in a plaque assay (see above). If the *Xba*I A fragment is sufficiently pure, plaques can be formed only when the plasmid recombines with the *Xba*I A fragment. Because the plasmid and the dl309 *Xba* A fragment shared the Ad5 sequences between nucleotides 3328 and 8892, homologous recombination was most likely to occur in this region. Such a recombinational event would result in the replacement of region E1 of Ad5 by the corresponding region of Ad12 in the Ad5 genome.

To identify recombinant viruses, individual plaques from the cotransfection assay were picked and used to infect 293 cells in 6-cm dishes. After 3 to 4 days, viral DNA was purified from the infected cells and analyzed by digestion with restriction endonuclease *Sal*I. If homologous recombination had occurred between the plasmid and the dl309 *Xba*I A fragment, the resulting recombinant genome would have an additional *Sal*I site located at 10 map units when compared with Ad5 dl309 (Fig. 3B). One of the plaques, RC15, had a novel *Sal*I digestion pattern (Fig. 3A). The disappearance of the left-terminal *Sal*IB band and the appearance of two novel bands of 10 and 16 map units (Fig. 3A, left panel, lane 4) was consistent with the insertion of the Ad12 E1 region at the left end of the Ad5 genome. Additional fine mapping of the recombinant genome further confirmed the organization of RC15 as presented in Fig. 3B.

Transfer of an intact region E3 into the recombinant virus. The Ad5 mutant dl309 used to construct RC15 lacked three of the four *Xba*I cleavage sites present in Ad5 (26). Although dl309 is nondefective in HeLa cells, at least one of the *Xba*I sites was lost as a result of a deletion (83 to 85 map units) mapping in E3 (26). One of the products of this region is a 19-kD glycoprotein which has been shown to be associated with the class I transplantation antigens in transformed cells (35). Since these class I antigens play a crucial part in the process of tumor rejection (3), the E3 deletion of dl309, and thus of

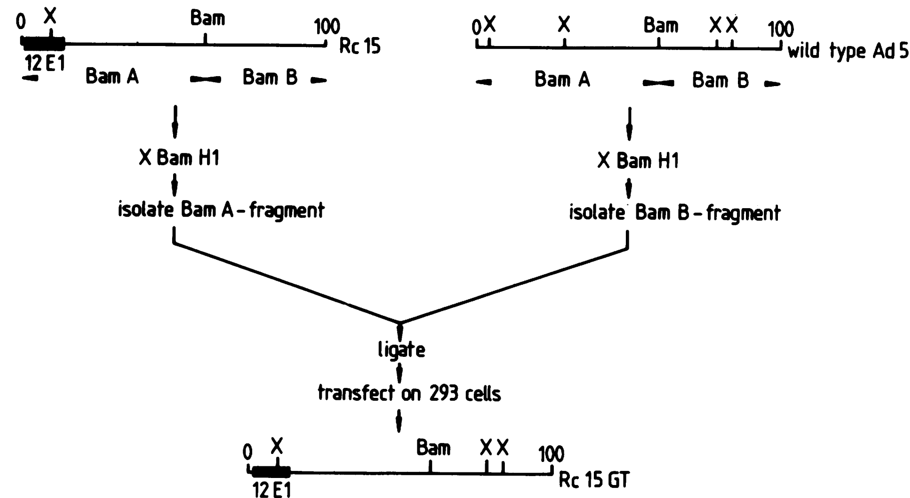

FIG. 4. Schematic representation of the construction of RC15GT. The positions of the *Bam*HI (Bam) and *Xba*I (X) cleavage sites are indicated.

RC15, may drastically influence the oncogenic potential of the recombinant virus. We therefore repaired the E3 deletion in RC15 according to the scheme outlined in Fig. 4. RC15 and wild-type Ad5 were propagated on a large scale in human KB cells, and viral DNA was isolated. The viral DNAs were digested with *Bam*HI, which has a single cleavage site at 59.5 map units in both viruses. After preparative gel electrophoresis, the *Bam*HI A fragment (0 to 59.5 map units) of RC15 and the *Bam*HI B fragment (59.5 to 100 map units) of wild-type Ad5 were isolated, ligated, and transfected onto 293 cells in a plaque assay. After 6 days, individual plaques were picked and analyzed by digestion with the restriction enzyme *Xba*I. One of the isolates of this experiment, RC15GT, had the expected two extra *Xba*I cleavage sites when compared with RC15 (Fig. 3A, right panel, lanes 4 and 5). Furthermore, the *Sal*I pattern of RC15GT showed that the Ad12 E1 region was still present at the left terminus of the recombinant (Fig. 3A, left panel, lane 5). These data indicate that in RC15GT all sequences between 59.5 and 100 map units, including region E3, are derived from wild-type Ad5.

Expression of E1 genes in infected HeLa cells. To test expression of the Ad12 E1 genes in the recombinants RC15 and RC15GT, HeLa cells were infected with these viruses at a multiplicity of 100 PFU per cell and labeled 19 to 22 h postinfection with [^{35}S]methionine. Ad5- and Ad12-specific proteins were immunoprecipitated from infected cells with sera from animals immunized with Ad5- and Ad12-transformed cells, respectively. HeLa cells infected with either RC15 or RC15GT produced Ad12-specific proteins of 19, 41, and 54 kD (Fig. 5). Proteins of the same molecular weights were detected in Ad12-infected cells, although the expression of the 54-kD protein seemed somewhat reduced in the recombinant-virus-infected cells. The 41-kD protein is the major product of Ad12 E1a, and the 19-kD and 54-kD proteins have been shown to be encoded by E1b (25). The only protein that could be precipitated with the Ad5 antiserum from RC15- and RC15GT-infected cells, but not from Ad12-infected cells, was 14 kD and is probably encoded by Ad5 E4 (10). When HeLa cells were infected with any of the three viruses and labeled 5 to 9 h postinfection, only the 41-kD Ad12 E1a protein could be precipitated (data not shown). We conclude from these experiments that the E1 region of Ad12 in the recombinant genome is, both qualitatively and temporally, faithfully expressed.

Ad5/Ad12 recombinant virus found to be nondefective. It is well documented that adenoviruses with mutations in either region E1a or region E1b are defective for growth in HeLa cells (17, 20, 22, 26). It appeared to be of interest, therefore, to determine whether the recombinant virus RC15GT had the capacity to replicate in these cells. This experiment was complicated by the finding that RC15GT, like Ad12, failed to form plaques when titrated directly on our HeLa cells. As we could harvest large amounts of infectious recombinant virus from infected HeLa cells, the failure of the recombinant to form plaques on HeLa cells appeared to be due not to an inability of the recombinant to replicate in HeLa cells, but rather to the inability of the virus to lyse infected cells. Growth of the recombinant virus in HeLa cells was therefore monitored indirectly. Replicate dishes of HeLa cells were infected at a multiplicity of 10 PFU per cell. After infection, cells were washed three times with PBS and fresh medium was added. Cells were harvested at either 1 h postinfection or 4 days postinfection, and virus yield was determined in a plaque assay on 293 cells. No differences in growth potential in HeLa cells between Ad5 or Ad12 and the recombinant RC15GT could be detected in this assay (Table 1). This finding shows that the recombinant virus was nondefective, which was also demonstrated by the fact that the recombinant efficiently formed plaques on HEK cells (Table 1).

Oncogenicity of the recombinant virus. The in vivo oncogenic potential of the recombinant viruses RC15 and RC15GT was tested by injecting 10^8 PFU of virus subcutaneously into 1-day-old Syrian hamsters. None of the 20 animals injected with either RC15 or RC15GT developed tumors during a period of 200 days, whereas in a control experiment, Ad12 induced tumors in 9 of 10 animals within 2 months. These data show that the Ad12 transforming region, placed in a genomic background of a nononcogenic virus, was not capable of converting the recombinant into a virus with detectable oncogenic properties, at least not within the period of observation.

Growth of the recombinant virus in hamster cells. It is well established that hamster cells are semipermissive for Ad5

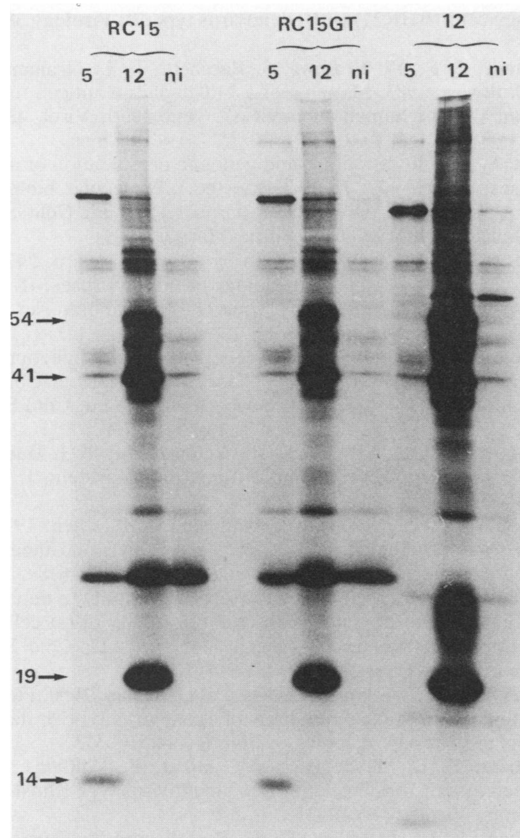

FIG. 5. Polyacrylamide gel electrophoresis of tumor antigens precipitated from HeLa cells infected with the virus RC15, RC15GT, or Ad12 and labeled 19 to 22 h postinfection with [^{35}S]methionine. Viral proteins were precipitated from infected cells with preimmune serum (lanes n.i.), Ad5 antitumor serum (lanes 5) or Ad12 antitumor serum (lanes 12).

but nonpermissive for Ad12 (9, 38), and it has been suggested that replication of Ad5 in hamster cells prevents oncogenesis (30). Because the factors that influence this host range phenotype are largely unknown, we measured the growth of RC15GT in BHK-21 cells by plaque titration on 293 cells. RC15GT did indeed have the capacity to replicate in hamster cells, with an efficiency which was clearly lower than that of Ad5 but higher than that of Ad12, for which these cells are strictly nonpermissive (Table 1). Thus, the differences in growth potential in hamster cells between Ad5 and Ad12 are to a large extent, but not completely, determined by the E1 products. Whether the limited replication of RC15GT in hamster cells caused this virus to be nononcogenic remains uncertain as yet.

DISCUSSION

We constructed a recombinant between the nononcogenic Ad5 and the highly oncogenic Ad12 by transferring the Ad12 transforming region into an Ad5 genome. HeLa cells productively infected with this recombinant virus produced the expected E1a and E1b proteins of Ad12 but not those of Ad5. When compared with that of Ad12, the level of expression of the 54-kD E1b polypeptide in cells infected with the recombinant virus seemed somewhat reduced. This difference may have been caused by a slight shift in the temporal control of the ratio of 13S over 22S E1b mRNAs (42). However, since adenovirus mutants which lack either the 13S E1a product or the 54-kD E1b polypeptide are defective for growth in HeLa cells (17, 20, 22, 26), we believe that the finding that the recombinant virus grew to wild-type levels in HeLa cells shows that the level of expression of the Ad12 E1 region in the recombinant was within the normal range. These results further show that Ad12 E1 could fully replace Ad5 E1 in productive infection, a finding which is in good agreement with the results of others, who have shown complementation in productive infection of Ad5 mutants with wild-type Ad12 in both region E1a and region E1b (7, 31, 40).

Surprisingly, the recombinants did not induce tumors when injected into newborn hamsters. A positive result was anticipated because hamster cells transformed in vitro by Ad12 are highly oncogenic when transplanted into hamsters (27), and in the recombinant virus, all sequences required for oncogenic transformation in vitro were derived from Ad12. There could be several reasons for this negative result. First, the Ad5 mutant dl309, which was used to construct the first Ad5-Ad12 recombinant (RC15), lacks three *Xba*I cleavage sites when compared with Ad5 (26). It could be argued that the loss of these sites introduces mutations in the genome of dl309 (and thus of RC15) in regions required for manifestation of the oncogenic phenotype. Especially the loss of the *Xba*I site in E3 was initially thought to be of importance in this respect, since E3 encodes a glycoprotein which is associated with the major histocompatibility class I antigens in transformed cells (35). However, the recombinant RC15GT, which has an intact E3 region, was similarly nononcogenic. We therefore do not consider this a very likely explanation. Second, one of the early regions of Ad5 could encode a strong transplantation antigen, whereas Ad12 might lack such a product. If this were true, then cells transformed by either RC15 of RC15GT would be more susceptible to the cellular immune defense of hamsters than would cells transformed by Ad12. Again, we do not favor this hypothesis, because Ad12 E1a suppresses in hamster cells (as in rat cells [33]) the expression of class I major histocompatibility antigens and thereby bypasses the T cell defense (R. T. M. J. Vaessen, unpublished data). The failure of the recombinant viruses to induce tumors in newborn

TABLE 1. Host range phenotype of the recombinant virus

Virus	Virus yield (PFU/ml) on following cells[a]:					
	293	HEK	HeLa		BHK-21	
			$t = 0$	$t = 4$	$t = 0$	$t = 4$
Ad5 dl309	1×10^9	6×10^8	8×10^2	2.8×10^9	2.2×10^3	2.4×10^7
Ad12	3.2×10^7	8.4×10^7	1.6×10^3	1.4×10^8	5×10^3	4×10^3
RC15GT	1.4×10^9	1×10^8	3×10^3	8×10^8	3×10^3	1.4×10^5

[a] Virus yield on 293 and HEK cells was determined in a direct plaque assay. Values for HeLa and BHK-21 cells were determined in an indirect assay (see text). $t = 0$, Virus yield at 1 h postinfection; $t = 4$, virus yield at 4 days postinfection.

hamsters might therefore be due not to an inherent inability of in vivo transformed cells to grow out and form a tumor but rather to an inability to cause transformation in vivo. Failure to transform in vivo could be related to permissivity, as it has been shown that the ability of adenoviruses to transform cells in vitro is inversely correlated with permissivity (12). For example, human cells are fully permissive for Ad5 and Ad12, and transformation of human cells with virus has never been reported, although transformation with subgenomic DNA fragments is possible (8, 19) and hamster cells, for which Ad5 is semipermissive, can be transformed efficiently only when the replicating capacity of the virus has been abolished by using nonpermissive mutants (30, 37, 41). The simplest explanation for these observations is that cells in which replication takes place are killed before transformation can occur. Thus, the lack of oncogenicity of Ad5 in hamsters could also be due to the fact that Ad5, but not Ad12, can replicate in hamster cells (9, 38). This view is further supported by the demonstration that hamster cells transformed in vitro by Ad5 are oncogenic in newborn hamsters (41). Our present results indicate that the Ad5/Ad12 recombinant virus, when compared with Ad5, has a limited capacity to replicate in hamster cells. However, BHK-21 cells are clearly more permissive for the recombinant virus than for Ad12, for which these cells are strictly nonpermissive (Table 1). At this stage, we cannot estimate the extent to which the limited replication of the recombinant virus in hamster cells contributes to the nononcogenic character of the virus. Definite proof for this relation has to await the construction of an Ad5/Ad12 recombinant virus that is strictly nonpermissive for growth in hamster cells.

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