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Role of Adenovirus Types 5 and 12 Early Region 1b Tumor Antigens in Oncogenic Transformation

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Recently we have reported that the difference in oncogenic potential between adenovirus type 5 (Ad5)- and Ad12-transformed cells in athymic nude mice is specified by early region 1b. In order to determine which of the two early region 1b (E1b) tumor antigens is responsible for the observed difference in oncogenicity we have constructed two Ad5/Ad12 hybrid plasmids: one allowing expression of the Ad5 19kD and Ad12 54kD E1b proteins, the other of the Ad5 58kD plus Ad12 19kD E1b polypeptides. Both hybrid plasmids contain the intact E1a regions of both serotypes. The chimeric plasmids were used to transform primary cultures of baby rat kidney cells and the resulting transformed cells were tested for oncogenicity in athymic nude mice. It was found that the degree of oncogenicity is determined by the identity of the large E1b tumor antigen. Studies with cells transformed by an Ad12 region E1 plasmid in which the gene coding for the 19kD tumor antigen was mutated showed that expression of this protein is nevertheless required for manifestations of the oncogenic phenotype of the transformed cell.

INTRODUCTION

Human adenoviruses (Ad) can be classified into two groups according to their oncogenic potential in newborn hamsters: the oncogenic viruses (e.g., Ad12) and the nononcogenic viruses (e.g., Ad5). All human adenoviruses, however, have the capacity to transform rodent cells *in vitro* (Flint, 1980). Rat cells transformed by oncogenic adenoviruses are tumorigenic when injected into syngeneic rats (Freeman *et al.*, 1967; Shiroki *et al.*, 1977; Mak *et al.*, 1979; Harwood and Gallimore, 1975) and in athymic nude mice (Gallimore and Paraskeva, 1980; Bernards *et al.*, 1982; Jochemsen *et al.*, 1982), while cells transformed by nononcogenic adenoviruses are nononcogenic in syngeneic rats (McAllister *et al.*, 1969; Harwood and Gallimore, 1975) and only weakly oncogenic in nude mice (Gallimore *et al.*, 1977; Gallimore and Paraskeva, 1980; Bernards *et al.*, 1982). It is not known which gene(s) are responsible for viral oncogenicity or for modulating the tumorigenic potential of the transformed cell.

Since the region required for cell transformation *in vitro* comprises no more than the leftmost 11% of the viral genome which corresponds to early region 1 (Flint, 1980) it is to be expected that the difference in oncogenic potential between cells transformed by Ad5 and Ad12 is determined by one or more of the genes encoded by this DNA segment. Recently, we have investigated this matter in more detail by use of Ad5/Ad12 hybrid early region 1 plasmids in which region E1a of Ad5 was combined with region E1b of Ad12 and vice versa. We found that baby rat kidney cells transformed by Ad5 E1a plus Ad12 E1b are highly oncogenic in nude mice (Bernards *et al.*, 1982; Van den Elsen *et al.*, 1982), while cells transformed by Ad12 E1a plus Ad5 E1b proved only weakly oncogenic (Bernards *et al.*, 1982), thus suggesting that the difference in oncogenicity in nude mice between Ad5- and Ad12-transformed cells

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is due to differences in the gene products specified by the E1b region.

Since the E1b region of adenoviruses codes for two major tumor antigens (T-antigens) of 19kD and of 54-58kD, it is not clear which of these proteins, if not both, is responsible for the observed difference in oncogenicity of the transformed cells. Recently, it was shown that the two E1b proteins are translated from different reading frames which are only partially overlapping (Bos *et al.*, 1981). This arrangement makes it possible to mutate one of the two E1b proteins without affecting the other, so that the contribution in oncogenicity of each of the E1b products can be measured separately.

In order to investigate which of the two E1b proteins determines the degree of oncogenicity we have constructed plasmids containing essentially Ad5/Ad12 hybrid E1b regions, i.e., expressing the small E1b tumor antigen of Ad5 and the large tumor antigen of Ad12, and vice versa. Analysis of the oncogenicity in nude mice of cells transformed by these hybrid plasmids revealed that the difference in oncogenic potential between Ad5- and Ad12-transformed cells is determined by the large E1b T-antigen. The small E1b T-antigen is also required for the expression of the oncogenic phenotype but its contribution to oncogenicity does not appear to be serotype specific.

MATERIALS AND METHODS

1. Construction of an Ad5 plasmid defective in expression of the 19kD E1b polypeptide. In order to construct the 19kD defective plasmid the Ad5 *Hin*dIII G fragment was first subcloned from pAd5XhoC (Bernards *et al.*, 1982). This plasmid, pAd5HindIIIG, was cleaved at the single *Sac*I site at position 1770 of the Ad5 sequence, mapping between the first and second ATG-triplet of the E1b region. After phenol extraction and ethanol precipitation 10 μg of the linearized plasmid was incubated for 2 hr at 11° with 1 unit/μl of S1 nuclease in 50 μl final volume. After another cycle of phenol extraction and ethanol precipitation the plasmid was recircularized using T4 DNA ligase. The plasmid was then once more cleaved with *Sac*I to remove wild-type plasmid molecules and was subsequently transfected into *E. coli* HB101. From ampicillin-resistant colonies a plasmid was selected lacking 11 base pairs at the *Sac*I site, as established by DNA sequence analysis (Maxam and Gilbert, 1980). This mutant *Hin*dIII G fragment was then reintroduced into the Ad5*Xho*C plasmid using standard recombinant DNA procedures, yielding p5dlSac.

2. Construction of an Ad5 plasmid defective in expression of the 58kD E1b polypeptide. pAd5XhoC has an unique *Hin*dIII site in the coding sequence of the 58kD E1b T-antigen. To construct a 58kD defective plasmid, 10 μg of pAd5XhoC was cleaved with *Hin*dIII. After phenol extraction and ethanol precipitation the 5'-protruding ends of the linearized plasmid were repaired by incubation with DNA polymerase I (Klenow fragment) and 50 μM of each of the four dNTPs for 1 hr at 37°. After recircularization of the plasmid with T4 DNA ligase and cleavage of the remaining wild-type molecules with *Hin*dIII, this plasmid was used to transfect *E. coli* HB101. From ampicillin-resistant colonies a plasmid was isolated which had lost the *Hin*dIII site at position 2805 of the Ad5 sequence. DNA sequence analysis showed that this plasmid, p5dlHind, has the expected insertion of four base pairs at the *Hin*dIII site.

3. Construction of an Ad12 plasmid defective in expression of the 19kD E1b polypeptide. To construct an Ad12 E1 plasmid with a mutation at the *Acc*I site at position 1594 of the Ad12 sequence (mapping between the first and second ATG of Ad12 E1b), 20 μg of pAd12RIC (Bos *et al.*, 1981) was partially digested with 3 units of *Acc*I for 30 min at 37°. The 5'-protruding *Acc*I ends were repaired with Klenow enzyme as described above. After phenol extraction and ethanol precipitation the DNA was cleaved with *Rru*I and *Bam*HI. After agarose gel electrophoresis the 4.3-kb *Acc*I–*Bam*HI fragment extending from nt 1594 of the Ad12 sequence via the *Eco*RI site at 16 map units to the *Bam*HI site of the plasmid, was isolated (Vogelstein and Gilles-

pie, 1979). Likewise, 20 μg of pAd12RIC was digested to completion with *Acc*I and the ends were repaired with Klenow enzyme. After phenol extraction and ethanol precipitation the plasmid was digested with *Eco*RI and the 0-1594 *Eco*RI-*Acc*I fragment was recovered from an agarose gel. This fragment, together with the 4.3-kb *Acc*I-*Bam*HI fragment (see above) was added to the *Bam*HI + *Eco*RI double-digested vector pAT153 (Twigg and Sherrat, 1980). The mixture was first ligated with T4 DNA ligase and 100 μm ATP for 16 hr at 14° to join cohesive *Bam*HI and *Eco*RI ends, then more ligase was added, ATP to 1 m*M*, and the repaired *Acc*I ends were joined by incubating for another 6 hr at 37°. After transfection into *E. coli* HB101, tetracycline-resistant colonies were screened for the loss of the *Acc*I site at position 1594 (Birnboim and Doly, 1980). DNA sequence analysis and restriction enzyme fine mapping showed that one of these clones, p12dlAcc, has no other alteration than an insertion of two base pairs at the *Acc*I site at nt 1594.

4. Construction of an Ad12 plasmid defective in expression of the 54kD E1b polypeptide. To introduce an early stopcodon in the reading frame for the Ad12 54kD E1b T-antigen, pAd12RIC was cleaved with *Aos*I. This enzyme has a single cleavage site in the Ad12 *Eco*RI C fragment at position 2089. Subsequently, the *Aos*I-digested plasmid was incubated with Klenow enzyme in the presence of 40 μ*M* dATP, 40 μ*M* dGTP, and 40 μ*M* dTTP, for 1 hr at 37°. After phenol extraction and ethanol precipitation the protruding 5′ ends of the *Aos*I site were made blunt by incubating with S1 nuclease as described in section 1. After cleavage with *Eco*RI the digest was subjected to agarose gel electrophoresis and the 0-2089 *Eco*RI-*Aos*I plus the 2089-5500 *Aos*I-*Eco*RI fragments were isolated. These fragments were then inserted into the *Eco*RI-cleaved vector pAT153. Ligation was as described in section 3. Three clones could be isolated lacking 1, 4, and 5 nucleotides, respectively, at the *Aos*I site. In the work presented here the plasmid lacking five nucleotides was used: p12dlAos5.

Procedures for transformation of primary rat kidney cells, cell labeling with [^{35}S]methionine and subsequent immunoprecipitation, and S1 nuclease analysis have been described previously (Bernards *et al.*, 1982).

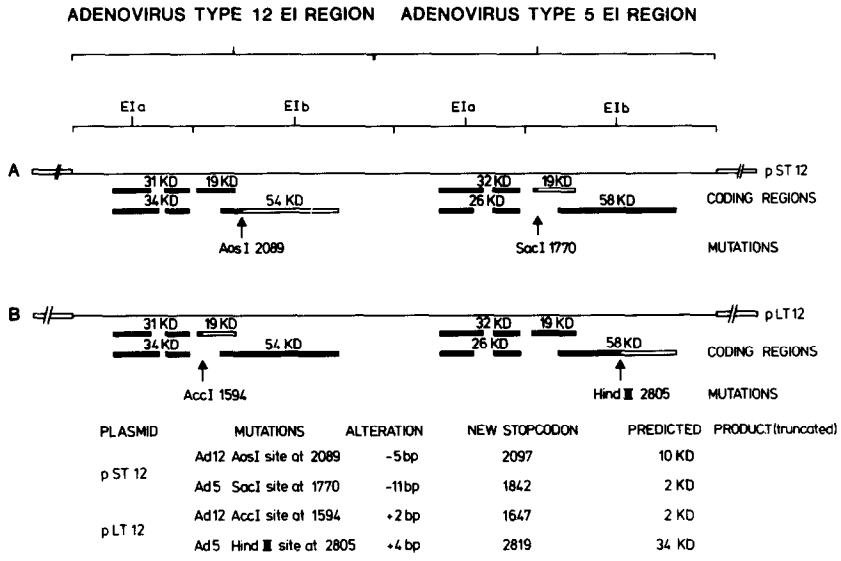
RESULTS

Construction of Ad5/Ad12 Chimeric E1 Plasmids

To investigate the role of the two major E1b T-antigens of Ad5 and Ad12 in oncogenic transformation, we have constructed two plasmids carrying hybrid E1b regions, i.e., one plasmid expressing the Ad12 19kD and the Ad5 58kD proteins and another expressing the Ad5 19kD plus the Ad12 54kD E1b T-antigens. These two plasmids were derived from four adenovirus region E1 plasmids containing specific mutations in the E1b region: p5dlSac, defective in the expression of the Ad5 19kD E1b T-antigen; p5dlHind, defective in expression of the Ad5 58kD E1b T-antigens; p12dlAcc, defective in expression of the Ad12 19kD E1b T-antigen, and p12dlAos5, defective in the expression of the Ad12 54kD E1b T-antigen. The exact lesions present in each plasmid and the predicted effects on the E1b genes are given in Fig. 1. These four plasmids were used to construct the two Ad5/Ad12 chimeric E1 plasmids: p5dlSac and p5dlHind were linearized with *Eco*RI and used as vectors to clone the mutated *Eco*RI inserts of p12dlAos5 and p12dlAcc, respectively, yielding pST12 and pLT12 (ST12 for Small T-antigen of Ad12, LT12 for Large T-antigens of Ad12, see Fig. 1). pST12 contains the complete coding information for the Ad5 58kD and the Ad12 19kD E1b T-antigens, pLT12 for the Ad5 19kD and the Ad12 54kD T-antigens. Both plasmids contain the intact E1a regions of both serotypes.

Transformation of Baby Rat Kidney Cells with Chimeric E1 Plasmids

pST12 and pLT12 were used to transform subconfluent primary cultures of baby rat kidney cells by means of the calcium phosphate technique (Van der Eb and



PLASMID	MUTATIONS	ALTERATION	NEW STOPCODON	PREDICTED PRODUCT(truncated)
pST12	Ad12 AosI site at 2089	-5bp	2097	10 KD
	Ad5 SacI site at 1770	-11bp	1842	2 KD
pLT12	Ad12 AccI site at 1594	+2bp	1647	2 KD
	Ad5 Hind III site at 2805	+4bp	2819	34 KD

FIG. 1. Schematic representation of the two Ad5/Ad12 hybrid E1b plasmids. The construction of the plasmids is described under Materials and Methods. Black bars indicate protein coding regions. Arrows indicate the site of mutation in the protein coding segments.

Graham, 1980). Colonies of morphologically transformed cells were isolated 3–4 weeks after transfection and established as cell lines. Cells transformed by either of the two plasmids manifested the typical epithelial or polygonal morphology, characteristic for cells transformed by adenovirus.

Attempts were also made to transform BRK cells with p12dlAcc and p12dlAos5, defective in expression of the Ad12 19kD and 54kD proteins, respectively. Plasmid p12dlAcc transformed BRK cells nearly as efficiently as wild-type region E1 DNA of Ad12 (=pAd12RIC, Bernards *et al.*, 1982) and the transformed cells are morphologically indistinguishable from Ad12 E1-transformed cells (not shown). Surprisingly, p12dlAos5 did not show any transforming activity, in spite of extensive attempts.

Characterization of Cells Transformed by Chimeric E1 Plasmids

On the basis of the structural organization of the adenovirus E1b region (Bos *et al.*, 1981) it was expected that pST12-transformed cells expressed the 19kD E1b T-antigen of Ad12 and the 58kD E1b product of Ad5, while pLT12 should express the reverse combination of T-antigens, i.e., 19kD of Ad5 and 54kD of Ad12. In addition both types of transformed cells should express the E1a region of both serotypes.

Expression of the E1b genes in the transformed cells was verified by immunoprecipitation of [^{35}S]methionine-labeled extracts using highly specific Ad5 or Ad12 anti-tumor sera. The same sera were used to immunoprecipitate T-antigens from extracts of pAd5XhoC- or pAd12RIC-transformed cells as controls.

Figure 2 shows the autoradiogram of the 10–15% SDS-polyacrylamide gradient gel used to separate T-antigens precipitated from cells transformed by pST12, pLT12, pAd5XhoC, and pAd12RIC. It is clearly demonstrated that pST12-transformed cells express the Ad5 58kD plus the Ad12 19kD T-antigens, while pL12-transformed cells express the reverse combination of E1b proteins. Similar experiments were performed with p12dlAcc-transformed cells, demonstrating that in these cells only the Ad12 54kD E1b T-antigen is expressed (not shown). Expression of E1a genes cannot be assayed in this way since these proteins are only weakly precipitated with our sera, if at all. Therefore, we

used the S1 nuclease RNA mapping technique to test expression of the E1a regions. ^{32}P-3′-labeled DNA fragments derived from the Ad5 and Ad12 E1a regions (Fig. 3) were hybridized to total cytoplasmic RNA from pST12- and pLT12-transformed cells. S1 nuclease-resistant DNA fragments were separated on a denaturing polyacrylamide gel. Cytoplasmic RNA from pAd5XhoC- and pAd12RIC-transformed cells was hybridized to the same DNA probes as controls. Using these probes the 5′ splice sites of the E1a mRNAs can be detected.

Figure 3 shows the schematic representation and the results of the S1 mapping experiment. Using the Ad5 E1a probe RNA from pAd5XhoC-transformed cells protected the expected segments of 160 and 298 nucleotides, respectively, against S1 nuclease digestion. The same fragments could be protected by use of RNA from both pST12- and pLT12-transformed cells, indicating that the Ad5 E1a region is most likely correctly expressed in these transformed cells. Likewise, RNA from pAd12RIC-, pST12-, and pLT12-transformed cells protected the same segments of the Ad12 E1a probe of 339 and 432 nucleotides. These data indicate that both pST12- and in pLT12-transformed cells the E1a regions of the two serotypes are faithfully expressed. Since the same amount of RNA was used in each experiment it seems that there are no major differences in the level of expression of the E1a regions in the various cells. From these experiments we conclude that both pST12 and pLT12 direct the expression of the expected genes.

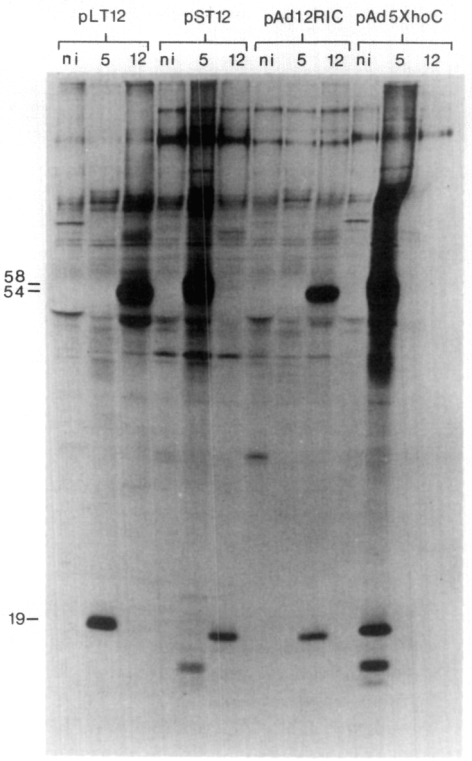


FIG. 2. Polyacrylamide gel electrophoresis of T-antigens precipitated from BRK cells transformed by pLT12 (lanes 1–3), pST12 (lanes 4–6), pAd12RIC (lanes 7–9), and pAd5XhoC (lanes 10–12). [^{35}S]Methionine-labeled extracts of transformed cells were immunoprecipitated with normal hamster serum (lanes indicated: ni), Ad5 anti-T serum (lanes indicated: 5), and Ad12 anti-T serum (lanes indicated: 12), and separated on a 10–15% SDS-polyacrylamide gradient gel. The positions of the E1b T-antigens are indicated by their apparent molecular weights.

Oncogenicity of Chimeric E1 Plasmid-Transformed Cells

Primary colonies of BRK cells transformed by pST12, pLT12, and p12dlAcc were isolated and established as cell lines. The oncogenic potential of these lines was tested by inoculating 10^7 or 10^6 cells subcutaneously into adult congenitally athymic nude mice. The animals were inspected weekly over a period of six months for development of palpable tumors. The results of these experiments are shown in Table 1. Data for oncogenicity of pAd12RIC- and pAd5XhoC-transformed cells are taken from Bernards *et al.* (1982).

Cells transformed by pLT12, and thus expressing Ad5 19kD and Ad12 54kD E1b T-antigens, proved highly oncogenic, while cells transformed by pST12, expressing Ad5 58kD plus Ad12 19kD E1b polypeptides, were only weakly oncogenic with tumors developing after a prolonged latency period. These results show that the difference in neoplastic growth potential between Ad5- and Ad12-transformed cells in nude mice is determined by the large (54–58kD) tumor antigen of the E1b region.

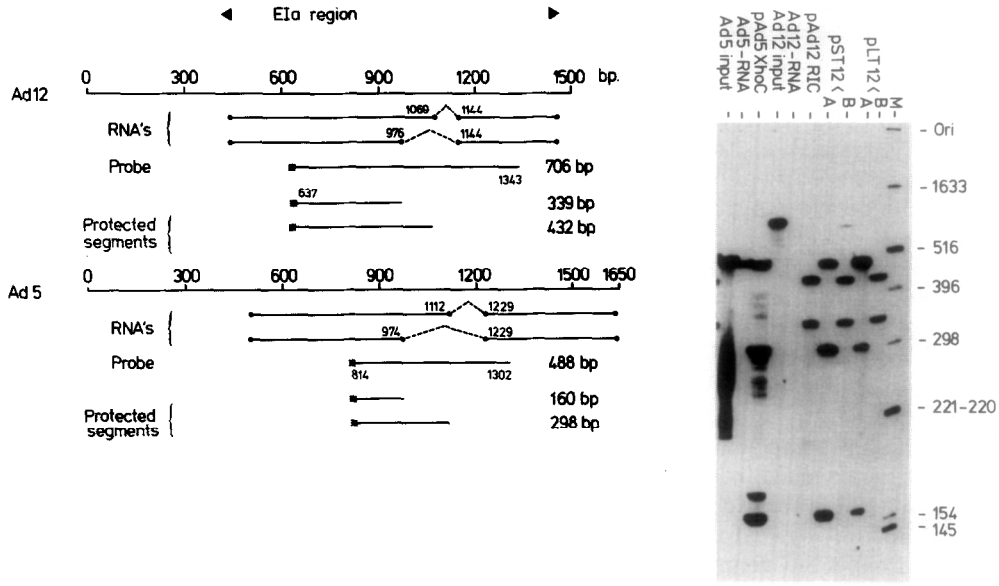


FIG. 3. Nuclease S1 mapping of E1a RNAs in transformed cells. 3′ end-labeled DNA fragments were hybridized to total cytoplasmic RNA from transformed cells, treated with S1 nuclease, and separated on a 5% polyacrylamide-7 *M* urea gel. Ad12 E1a transcripts were detected with a 706-bp *Mbo*I fragment (position 637-1343 of the Ad12 sequence). Ad5 E1a RNAs were detected with a 488-bp *Nar*I fragment (position 814-1302). The Ad12 E1a probe was hybridized to total cytoplasmic RNA from pAd12RIC-transformed cells (lane pAd12RIC), pST12-transformed cells (lane pST12 B), pLT12-transformed cells (lane pLT12 B), and in the absence of RNA (lane Ad12-RNA). The lane Ad12 input shows the untreated DNA probe. The Ad5 E1a probe was hybridized to RNA isolated from cells transformed by pAd5XhoC (lane pAd5XhoC), pST12 (lane pST12 A), and pLT12 (lane pLT12 A). Controls were: the E1a probe hybridized in the absence of RNA (lane Ad5-RNA) and the untreated Ad5 probe (lane Ad5 input). A ^{32}P-5′-end-labeled *Hin*fI digest of pAT153 was used as a size marker (lane M).

From the finding that p12dlAcc-transformed cells are completely nononcogenic in nude mice we conclude that expression of the 19kD gene is required for oncogenicity (compare p12dlAcc and pAd12RIC in Table 1). However, the contribution of the 19kD protein in oncogenicity is, in contrast to that of the 54-58kD E1b T antigen, not serotype specific (compare pLT12 and pAd12RIC in Table 1).

DISCUSSION

We have used site-directed mutagenesis in Ad5 and Ad12 early region 1 plasmids to investigate the role of the two major adenovirus E1b tumor antigens in oncogenic transformation. Two Ad12 mutant early region 1 plasmids, p12dlAcc and p12dlAos5, were constructed, carrying stopcodons in the genes for the 19kD and 54kD E1b polypeptides, respectively. Similarly, two Ad5 mutant plasmids were made with mutations in either the 19kD E1b gene (p5dlSac) or in the 58kD E1b gene (p5dlHind). These plasmids were then joined crosswise to form two new plasmids: pST12, allowing expression of Ad5 58kD plus Ad12 19kD E1b polypeptides, and pLT12 expressing Ad5 19kD plus Ad12 54kD polypeptides. Cells transformed by either of these latter two plasmids express the intact E1a regions of both serotypes and manifest the typical adenovirus-transformed phenotype, suggesting that the E1b proteins of both serotypes are compatible at the level of transformation. Plasmids p12dlAcc (19kD deficient) was

TABLE 1

ONCOGENICITY OF BABY RAT KIDNEY CELLS TRANSFORMED BY ADENOVIRUS MUTANT E1b PLASMIDS[a]

DNA used for transformation	Expression in transformed cells		No. of cells/ animal	No. of cell lines tested	No. of animals with tumors / No. of animals injected	Average latent period (days)
	E1a	E1b				
pAd12RIC	Ad12	Ad12	10^7	6	21/21	40–50
			10^6	1	2/2	45–55
pAd5XhoC	Ad5	Ad5	10^7	9	15/31	60–80
pST12	Ad5 + Ad12	Ad5 58kD + Ad12 19kD	10^7	2	3/12	50–70
			10^6	2	1/12	100
pLT12	Ad5 + Ad12	Ad5 19kD + Ad12 54kD	10^7	3	15/15	20–30
			10^6	3	12/12	30/40
p12dlAcc	Ad12	Ad12 54kD	10^7	2	0/14	—
			10^6	1	0/6	—

[a] 10^6 or 10^7 cells in PBS were injected subcutaneously into adult homozygous athymic Balb/C nude mice.

found to transform BRK cells to a level comparable to that obtained with wild-type region E1 DNA of Ad12 (=pAd12RIC), while p12dlAos5 (54kD deficient) did not transform BRK cells at all. This latter finding is somewhat unexpected since we have previously shown (Jochemsen *et al.*, 1982) that the Ad12 *Hin*dIII G fragment (0–7.2%) does transform BRK cells, albeit at a reduced frequency. Possibly, this difference can be explained by the fact that the Ad12 *Hin*dIII G fragment codes for a larger truncated 54kD E1b polypeptide (16kD) than the p12dlAos5 plasmid (10kD). If this explanation is correct it would mean that normal transformation can only be obtained in the presence of 54kD E1b T-antigen or a sufficiently large N-terminal segment. Consistent with this view is the finding that the p12dlAcc-transformed cells are morphologically identical to wild-type E1-transformed cells, so that our previous conclusion that morphological transformation is a function of the 19kD E1b T-antigen (Schrier *et al.*, 1979) is presumably not correct.

Recently, we have shown that cells transformed by Ad5 E1a/Ad12 E1b hybrid plasmids are highly oncogenic in nude mice, while cells transformed by Ad12 E1a/Ad5 E1b plasmids proved only weakly oncogenic (Bernards *et al.*, 1982). This suggests that oncogenicity of adenovirus-transformed cells in nude mice is determined by one or more of the E1b genes. Our present results allow us to draw further conclusions on the function of the adenovirus E1b tumor antigens in oncogenic transformation: First, the finding that cells transformed by p12dlAcc, expressing only the 54kD E1b T-antigen, are completely nononcogenic in the nude mice system shows that expression of the 19kD E1b polypeptide is required for oncogenicity of the transformed cell. Since cells transformed by the Ad12 *Hin*dIII G fragment, which lack an intact 54kD E1b T-antigen, are also nononcogenic in nude mice (Jochemsen *et al.*, 1982) we conclude that both E1b tumor antigens play an important role in oncogenesis of the transformed cell. Second, the difference in oncogenic poten-

tial between Ad5- and Ad12-transformed cells is encoded by the large (54-58kD) E1b tumor antigen. This is demonstrated by the finding that pST12-transformed cells (expression of 19kD Ad12 plus 58kD Ad5 E1b genes) are very weakly oncogenic in nude mice, while cells transformed by pLT12, expressing the reverse set of E1b T-antigens, are highly oncogenic. Since cells transformed by pLT12 display the same high oncogenic potential as cells transformed by the Ad12 E1 region, we conclude that the origin of the 19kD protein has no serotype-dependent influence on oncogenicity in nude mice.

Comparison of the predicted primary structure of the Ad5 and Ad12 54-58kD E1b proteins (Bos *et al.*, 1981) reveals that these proteins are considerably homologous with identical amino acids in 48% of the positions. The region of highest sequence divergence is found in the N-terminal part (amino acid 24-120) of the proteins, where homology drops below 20%. Whether the difference in biological properties of the proteins is determined by this N-terminal region is not known. An interesting question is whether the difference in oncogenicity of the various adenovirus serotypes in newborn hamsters is solely dependent on the nature of their transforming region, and more in particular of the large E1b tumor antigen, or whether the remaining 90% of the genome also has an influence on oncogenicity. To answer this question we have recently constructed an Ad5 virion expressing Ad12 E1b instead of Ad5 E1b. Preliminary evidence suggests that, although this hybrid virus is fully viable, it is nononcogenic in newborn hamsters (Bernards *et al.*, manuscript in preparation), suggesting that oncogenicity of infectious virions is dependent on either other or additional factors compared to oncogenicity of transformed cells.

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