Altered Expression of Cellular Genes in Adenovirus-transformed Cells

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Human adenoviruses provide unique model systems for studies on basic mechanisms of carcinogenesis. An important advantage of this group of viruses is that both oncogenic and nononcogenic species are found within the same virus group. Although only the members of the subgenera A and B (e.g., Ad12 and Ad7, respectively) can cause tumors in newborn hamsters and some other rodents (Huebner 1967), all human adenoviruses studied so far are capable of transforming cells in vitro. In this article, we summarize a number of experiments designed to obtain some understanding of the role of the transforming genes of oncogenic Ad12 and nononcogenic Ad5 in oncogenesis and transformation.

The transforming activity of all human adenoviruses studied so far is associated with early region 1 (E1), which consists of two transcriptional units, E1A and E1B. In transformed cells, region E1A codes for two coterminal RNAs (12S and 13S) that differ only in the amount of RNA removed internally by splicing. The RNAs code for two polypeptides (of about 26 kD and 32 kD) that are structurally related. Region E1B codes for one major RNA species (in transformed cells) of 22S, which specifies two polypeptides of about 20 kD and 55 kD. These two proteins are translated in different reading frames and, hence, are not related (references in Pettersson and Akusjärvi 1983; van der Eb and Bernards 1983).

Rat cells transformed by region E1 of Ad5 or Ad12 are indistinguishable from cells transformed by intact DNA or virions, indicating that all transforming functions are located in region E1. DNA fragments comprising certain parts of region E1 may still contain transforming activity, although the resulting transformed cells show abnormal phenotypes. The smallest segment containing transforming activity is region E1A (0–4.5%). Cells transformed by E1A only exhibit a semitransformed phenotype and grow to low saturation densities. Ad5 E1A-transformed baby rat kidney (BRK) cells mainly differ from the untransformed primary cells in that they are immortalized (Houweling et al. 1980). Cells transformed by region E1A plus the adjoining 50% of region E1B appear almost completely transformed, but they differ from E1-transformed cells in that they have lost their oncogenicity in nude mice (Jochemsen et al. 1982). Region E1B alone has no detectable transforming activity, even under conditions where it is fully expressed (van den Elsen et al. 1983). These results indicate that region E1A has an initiating (and perhaps major) role in transformation (cf. van der Eb and Bernards 1984).

Several lines of evidence suggest that region E1B must have an important function in oncogenesis. First, introduction of a mutation in one of the two Ad12 E1B proteins results in a loss of oncogenicity, even in nude mice (Jochemsen et al. 1982; Bernards et al. 1983a). Second, transplantation studies using cells transformed by Ad5/Ad12 hybrid E1 regions, consisting of region E1A of nononcogenic Ad5 and E1B of oncogenic Ad12, and vice versa, have shown that the high oncogenic potential of Ad12-transformed cells in nude mice is determined by region E1B (Bernards et al. 1982), and specifically by the 54-kD E1B protein (Bernards et al. 1983a).

In the present study we report a major contribution of Ad12 region E1A in oncogenicity. We have found that expression of this region in transformed rat cells causes suppression of the production of class-I transplantation antigens and of a 32-kD cellular protein, resulting in a considerable reduction in susceptibility of the transformed cells to the cellular immune defense. This resistence to the host immune defense presumably causes the cells to be tumorigenic, even in immunocompetent animals.

Methods of Experimental Procedures

Transformed cells

All adenovirus-transformed cells discussed in this paper were obtained by transforming primary cultures of BRK cells (from 6-day-old Wag-Rij rats) with recombinant plasmids containing the viral DNA inserts. The transformations were carried out by use of the calcium technique (Graham and van der Eb 1973; van der Eb and Graham 1980). BRK cells transformed by the following recombinant plasmids were used:

pAd5XhoC (Ad5 region E1 of Ad5; Bernards et al. 1982)
pAd5HindIIIG (Ad5 region E1A + 19-kD E1B gene; Bernards et al. 1983a)
pAd5d1Sac (Ad5 region E1A + 55-kD E1B gene; Bernards et al. 1983a)
pAd5HpaIE (Ad5 region E1A; Bernards et al. 1982)
pAd12RIC (Ad12 region E1; Bos et al. 1981)
pAd12HindIIIG (Ad12 region E1A + 19-kD E1B gene; Jochemsen et al. 1982)

pAd12d1Acc (Ad12 region E1A + 54-kD E1B gene; Bernards et al. 1983a)
pAd512 (Ad5 E1A + Ad12 E1B; Bernards et al. 1982)
pAd125 (Ad12 E1A + Ad5 E1B; Bernards et al. 1982)
pAd512pm975 (Ad12 region E1 + Ad5 region E1A 13S product; Bernards et al. 1983b)
pAd512HL1007 (Ad12 region E1 + Ad5 E1A 12S product; Bernards et al. 1983b)
pAd51212 (Ad12 region E1 + Ad5 E1A; Bernards et al. 1983b)
pSVR7 (Ad12 region E1, but lacking 13S E1A product + SV40 enhancer; Bos et al. 1983)
pSVR11 (AD12 region E1, lacking 12S and 13S E1A products + SV40 enhancer; Bos et al. 1983)
pST12 (Ad5 + Ad12 region E1A + 19-kD Ad12 and 58-kD Ad5; Bernards et al. 1983a)
pLT12 (Ad5 + Ad12 region E1A + 19-kD Ad5 and 54-kD Ad12; Bernards et al. 1983a)

Assay for oncogenicity

Transformed cells (10^7) were injected subcutaneously into 4-day-old Wag-Rij rats or into adult athymic nude mice or nude Wag-Rij rats.

CTL reactions

Anti-RT1^u allogeneic cytotoxic T lymphocytes (CTL) were generated in a 6-day primary mixed lymphocyte culture: 10^7 spleen cells from an ACI rat were cultured with 3×10^6 x-irradiated spleen cells derived from Wag-Rij rats. Target cells were labeled in 100 μl of PBS containing 100 μCi of [^{51}Cr]sodium chromate for 30 minutes at 37°C. Lysis was measured in microtiter dishes during 4 hours at 37°C using 10^4 target cells per well. The specific lysis was calculated according to $(E-C)/(M-C) \times 100\%$, where E = counts released from target in the presence of CTL; C = counts released in the absence of CTL; and M = counts released from targets in the presence of 5% Triton X-100.

Sera and immunoprecipitation technique

Antisera against primary BRK cells were prepared by immunizing 10-week-old BALB/c mice with 10^7 BRK cells (derived from 6-day-old Wag-Rij rats, a highly inbred rat strain). Three intraperitoneal injections were given, with 10-day intervals, the first with complete Freund's adjuvant. Serum was collected 2 weeks after the last injection. Immunoprecipitations were carried out as described (Bernards et al. 1982). ^{125}I-labeling was carried out according to Markwell and Fox (1978).

Results

Region E1A has a major role in oncogenicity

In previous experiments, we had established a series of BRK lines transformed by Ad5 region E1, Ad12 region E1, Ad5 E1A + Ad12 E1B, and Ad12 E1A + Ad5 E1B. All four types of transformed cells were found to be oncogenic in athymic nude mice (Bernards et al. 1982). However, when the oncogenicity of the transformed cells was tested in immunocompetent syngeneic rats, a surprising result was obtained.

BRK cells transformed by intact region E1 of Ad5 were weakly oncogenic in nude mice and nononcogenic in immunocompetent rats, whereas cells transformed by region E1 of Ad12 were highly oncogenic in both types of animals. Cells transformed by Ad5 E1A and Ad12 E1B were highly oncogenic in nude mice but, surprisingly, completely nononcogenic in normal rats. Cells transformed by Ad12 E1A plus Ad5 E1B were weakly oncogenic, not only in nude mice but, to the same extent, also in normal rats (Table 1). These results indicated that region E1A determines whether or not a transformed cell is rejected in an immunocompetent animal: when E1A is derived from Ad12, the transformed cells apparently are sufficiently resistant to the host immune defense to be able to form a tumor, whereas when E1A is derived from Ad5, the transformed cells are always rejected, even when they are intrinsically highly tumorigenic in nude mice. This suggested that the oncogenic potential of transformed cells in immunocompetent animals is dependent on whether or not the cells can escape the immune surveillance of the host. Two alternative explanations might account for the observed differences in oncogenicity: (1) transformed cells that are oncogenic in immunocompetent rats are less immunogenic to the host than nononcogenic transformed cells or (2) oncogenic cells are resistant to the immune defense whereas nononcogenic cells are susceptible.

Table 1 Oncogenicity of Adenovirus-transformed Cells in Nude Mice, Syngeneic Rats, and Nude Rats

Plasmids used for transformation	Expression in transformed cell		Oncogenicity of transformed cells in:		
	E1A	E1B	nude mice	syngeneic rats	nude rats
pAd5XhoC	5	5	50% (15/31)	0% (0/51)	n.d.[c]
pAd12RIC	12	12	100% (23/23)	100% (18/18)[a]	n.d.
pAd512	5	12	100% (18/18)	0% (0/26)	100% (6/6)[d]
pAd125	12	5	10% (2/19)	10% (6/60)[b]	n.d.
p51212	5+12	12	100% (12/12)	0% (0/18)	n.d.

For each type of of transformed cells, at least three independently isolated cell lines were used. The numbers between brackets indicate the total number of animals with tumors/total number of animals injected.
[a]Average latent period, 6 weeks.
[b]Average latent period, 4 months.
[c]n.d., not done.
[d]Average latent period, 3 months.

The interactions between transformed cells and the immune system of the host will be either mediated by viral T antigens, by cellular proteins which are modulated by the viral transforming genes, or by both. To investigate whether specific cellular proteins play a role in determining whether or not a transformed rat cell is capable of inducing a tumor in immunocompetent oncogenic rats, we have prepared antisera against untransformed BRK cells in BALB/c mice. To screen for differences in cellular gene expression, the sera were used to analyze a panel of transformed cells, using the immunoprecipitation procedure.

Ad12 region E1A suppresses the synthesis of a cellular 32-kD protein

BALB/c mice were immunized with untransformed primary BRK cells derived from 6-day-old Wag-Rij rats. Preliminary experiments showed that the sera precipitated a rather limited set of proteins from the rat cells and that differences in protein content could be detected between untransformed and Ad12-transformed cells. The clearest differences were found for 38-kD and 32-kD proteins, which were both present in untransformed BRK cells but absent in Ad12-transformed cells. Surprisingly, the 32-kD protein was present in normal amounts in Ad5-transformed cells but the 38-kD protein was not detectable in this line. To study the occurrence of these proteins in more detail, a pool of several mouse sera was used to immunoprecipitate a panel of Ad-transformed cells including cell lines transformed by hybrid plasmids consisting of Ad5 E1A and Ad12 E1B (pAd512), or Ad12 E1A and Ad5 E1B (pAd125).

The results presented in Figure 1 show that the 32-kD protein is present in primary BRK cells and in all Ad5-transformed cell lines that contain Ad5 region E1A, irrespective of whether one of the E1B proteins is mutated, or even both are absent. The protein is absent in all cells expressing Ad12 E1A, again independent of the presence of mutations in one of the E1B proteins. The 38-kD protein is absent in all cell lines harboring Ad12 E1A and is present in some, but not all, lines containing Ad5 E1A. When the immunoprecipitation results are considered in relation to the oncogenicity data, it is obvious that a strict correlation exists between presence of region E1A of Ad12, the lack of the 32-kD protein, and oncogenicity in immunocompetent syngeneic rats. It appeared to be of interest, therefore, to investigate whether the 32-kD protein is a membrane protein ex-

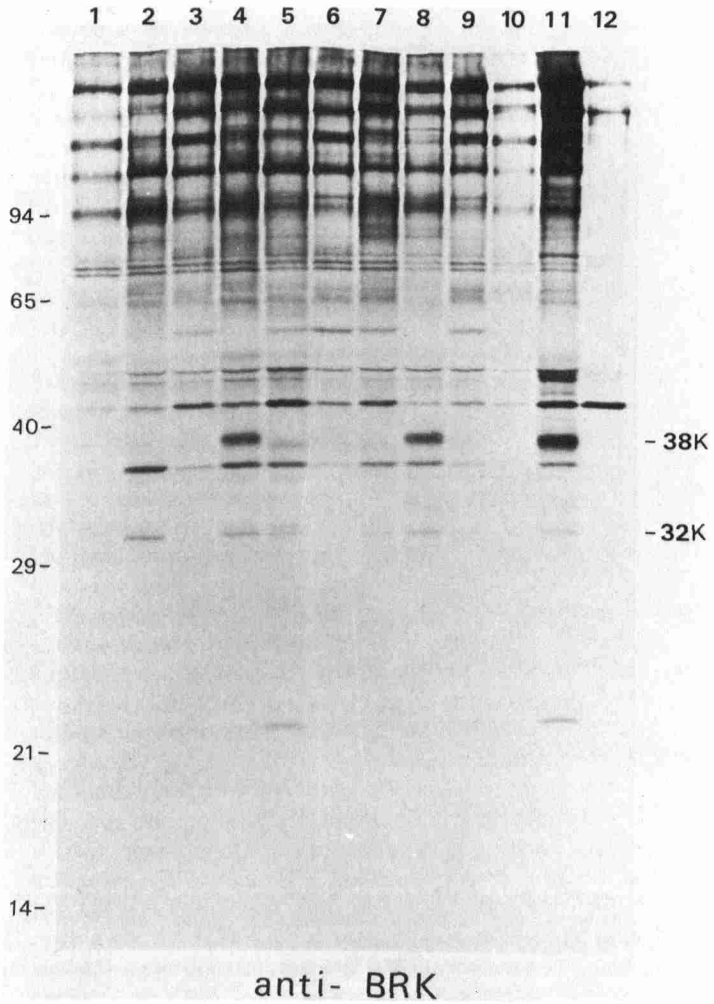

Figure 1 Proteins immunoprecipitated from extracts of [^{35}S]methionine-labeled adenovirus-transformed rat cells with pooled serum from BALB/c mice immunized with primary baby rat kidney (BRK) cells. The rat cells were transformed by the following plasmids: (lane *1*) pAd12RIC (containing Ad12 region E1); (lane *2*) pAd5XhoC (containing Ad5 region E1); (lane *3*) pAd12HindIIIG (containing Ad12 region E1A + part of E1B; lacks the 54-kD protein); (lane *4*) pAd5HindIIIG (containing Ad5 region E1A + part of E1B; lacks the 58-kD protein); (lane 5) pAd5HpaIE (containing Ad5 region E1A); (lane *6*) pAd12d1Acc (Ad12 region E1, lacking the 19-kD E1B protein); (lane *7*) pAd5dlSac (Ad5 region E1, lacking the 19-kD E1B protein); (lane *8*) pAd512 (Ad5 E1A + Ad12E1B); (lane *9*) pAd125 (Ad12 E1A + Ad5 E1B); (lane *10*) cell line derived from a tumor induced by pAd125-transformed cells; (lane *11*) primary BRK cells; (lane *12*) primary BRK cells precipitated with normal mouse serum.

posed on the outside of the cell membrane, where immune recognition is expected to occur.

To this end, primary BRK cells and Ad12-transformed BRK cells were labeled with ^{125}I and immunoprecipitated with the pooled BALB/c anti-BRK serum. No radioactive 32-kD protein could be precipitated, suggesting that it is not a membrane component, or at least is not exposed on the outer surface. However, prominent 38-kD and 12-kD proteins were precipitated from the primary cells which were absent in the Ad12-transformed cells (Fig. 2, lanes 1b and 2b). The 38-kD protein comigrated with the [^{35}S]methionine-labeled 38-kD protein precipitated by the same serum.

The coprecipitation of the 38-kD protein with a 12-kD component suggested that the two polypeptides might belong to the class-I major histocompatibility complex (MHC), which is known to consist of a heavy chain in the 40–45-kD molecular-weight range, associated with β_2-microglobulin (β_2-m; 12-kD molecular weight). Therefore, the ^{125}I-labeled extracts were immunoprecipitated with an antiserum directed against human β_2-m, which cross-reacts with rat β_2-m. A considerable amount of 12-kD β_2-m was precipitated from untransformed BRK cells, but only very small amounts of this protein were found in the extract of Ad12-transformed cells (Fig.2, lanes 3d and 5d). No heavy-chain molecules were co-

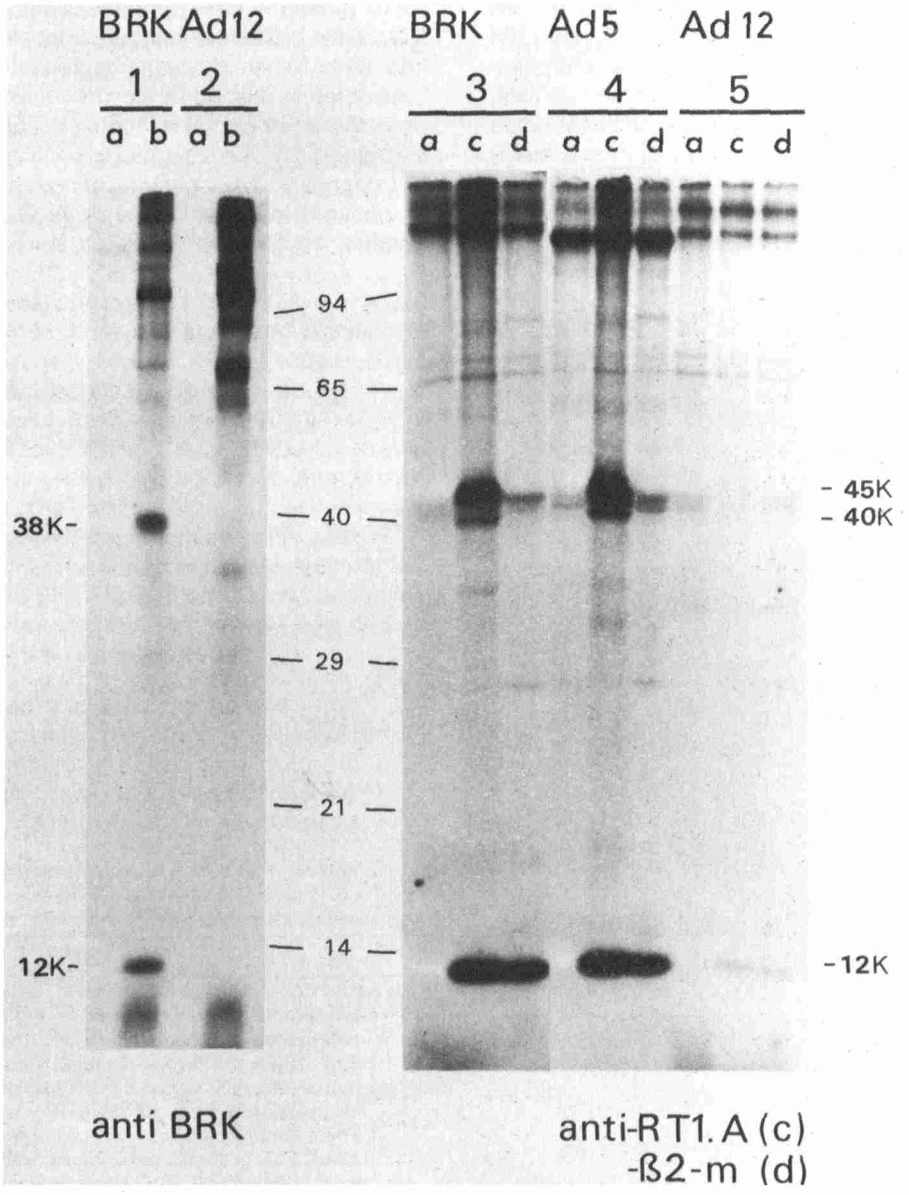

Figure 2 Proteins immunoprecipitated from extracts of ^{125}I-labeled rat cells with anti-BRK mouse serum, anti-Wag-Rij alloantiserum, and an antiserum against human β_2-m. Extracts of the following cells were used for immunoprecipitation: (lanes *1* and *3*) primary BRK cells; (lanes *2* and *5*) pAd12RIC-transformed BRK cells; (lane *4*) pAd5XhoC-transformed BRK cells. The following sera were used: normal mouse serum (lanes *a*); mouse anti-BRK serum (lanes *b*); Lewis anti-Wag-Rij hyperimmune alloantiserum (lanes *c*); and anti-human β_2-m serum (lanes *d*).

precipitated, probably due to the fact that the heterologous antiserum does not precipitate β_2-m when it is complexed to the heavy chain. In later experiments, an antiserum against rat β_2-m was used that did coprecipitate 45-kD heavy-chain molecules. The finding that Ad12-transformed cells contain minimal amounts of β_2-m molecules at the cell surface directly suggested to us that the amount of class-I heavy-chain molecules, to which the β_2-m molecules are normally complexed, would also be reduced.

Expression of RT1.A class-I genes is greatly reduced in cells harboring Ad12 region E1

The expression of class-I MHC antigens in adenovirus-transformed cells was further investigated with specific rat alloantisera. The rat cells used for the transformation assays originated from Wag-Rij rats, a highly inbred strain carrying the $RT1^u$ haplotype. (The RT1 complex of the rat comprises five loci, two of which, RT1.A and RT1.E, code for the serologically detectable class-I antigens.) Figure 2, lane 3c, shows that these alloantisera precipitated predominantly a 45-kD and a 12-kD protein from ^{125}I-labeled untransformed BRK cells, representing the class-I heavy-chain molecules and β_2-m, respectively. These two proteins were also precipitated from cells expressing Ad5 E1 (Fig. 2, lane 4c), but they were absent, or present in greatly reduced amounts, in the lines expressing Ad12 E1 (Fig.2, lane 5c). Basically similar results were obtained with ^{125}I-labeled and ^{35}S metabolically labeled extracts. Further experiments showed that expression of the class-I heavy-chain molecules exactly paralleled that of the 32-kD protein detected by the BALB/c sera: Expression of 32-kD protein is greatly reduced in the presence of Ad12 E1A but normal with Ad5 E1A (Table 2). This suggests that expression of class-I protein and of 32-kD protein is controlled in a similar way by Ad12 E1A.

Expression of class-I genes is suppressed at the level of mRNA accumulation

The almost complete absence of class-I molecules on the surface of Ad12-transformed BRK cells can have several causes, for example, inhibition of transcription of class-I genes, reduced stability of class-I mRNAs, or absence of β_2-m. To test the latter possibility, transformed cells were metabolically labeled with [^{35}S]methionine and subsequently precipitated with antiserum against human β_2-m. It was found that Ad12-transformed cells, which lack 45-kD heavy chains, contain normal amounts of ^{35}S-labeled β_2-m as compared with Ad5-transformed cells. To investigate whether reduced expression of heavy-chain molecules is caused by the absence of mRNA transcripts, a number of transformed cell lines were studied by Northern blot analysis, using a DNA probe containing conserved sequences of the human HLA B7 gene. The probe was found to hybridize to a 1.7-kb RNA, which most likely represents the mRNA coding for the RT1.A heavy chain. This RNA band was present in untransformed BRK cells and in cells containing Ad5 E1A, but it was almost completely lacking in cells expressing Ad12 E1A (Fig. 3). This demonstrates that the absence of heavy-chain molecules is caused by a greatly reduced concentration of mRNA transcripts. Since the HLA B7 probe will react with all RT1.A gene transcripts, this result also shows that no other (alien) class-I alloantigens are expressed instead of the $RT1.A^u$ heavy chains.

Reduced expression of 32-kD and 45-kD proteins is a function of the Ad12 13S mRNA product

In transformed cells, the E1A region of human adenoviruses is transcribed into two coterminal mRNAs of 12S and 13S, which only differ in the amount of RNA removed internally by splicing. To investigate which of these RNAs, if not both, is responsible for suppression of 32-kD protein production, we investigated the presence of this protein in rat cells transformed by an Ad12-region E1 plasmid carrying a mutation affecting the 13S mRNA product only (pSVR7; Bos et al. 1983). Figure 4, lane 8B, shows that cells transformed by pSVR7 have normal expression of the 32-kD protein. Similar results were obtained with cells transformed by pSV11, which carries a mutation affecting both E1A gene products (Fig. 4, lane 9B), indicating that the 13S mRNA product must be responsible for turning-off 32-kD gene expression. Similar results were obtained for expression of class-I genes (Bernards et al. 1983b; Table 2).

Ad5 E1A prevents Ad12 E1A from inhibiting expression of 32-kD and 45-kD proteins

Expression of the 32-kD protein was also studied in three cell lines containing the E1A regions of both Ad5 and Ad12. S1 nuclease analysis had shown that the two E1A regions were transcribed to the same extent (Bernards et al. 1983a,b). Analysis by immunoprecipitation with anti-BRK serum showed that all three cell lines expressed normal levels of 32-kD proteins, which indicates that the E1A region of Ad5 blocks the inactivating activity of Ad12 E1A (Fig.4, lanes 5, 6, and 7). To investigate which Ad5 E1A product is responsible for the dominant effect, 32-kD protein expression was determined in transformed cells carrying region E1 of Ad12 as well as a mutant E1A region of Ad5. Two plasmids were tested, one allowing expression of Ad12 E1 plus Ad5 13S E1A RNA (pAd512 pm975), the other of Ad12 E1 plus Ad5 12S E1A RNA (pAd512 HL1007) (Bernards et al. 1983b). It was found that coexpression of the Ad5 13S E1A mRNA product and Ad12 region E1 resulted in normal production of the 32-kD protein, whereas coexpression of the Ad5 12S E1A mRNA product and Ad12 region E1 gave rise to suppression of this protein (Fig. 4, lanes 3 and 4). Thus, the dominant effect exerted by Ad5 E1A on Ad12 E1A is also a function of the 13S mRNA. Again, the expression of class-I heavy-chain molecules paralleled that of the 32-kD protein in the above-mentioned cell lines. Thus, rat cells coexpressing all transforming genes of Ad12 as well as region E1A of Ad5 had normal levels of class-I genes (not shown). These cells were nononcogenic in immunocompetent rats, in spite of their high oncogenicity in nude mice (Table 1). This suggests

Table 2 Cellular Proteins Present in a Series of Adenovirus-transformed Cell Lines with Different Oncogenic Potential

	Expression of Ad5 or Ad12 region		Proteins detected in transformed cells[b]			Oncogenicity	
transformed by[a]	E1A	E1B	32 kD	(45 kD)	(12 kD)	mice	rats
BRK (untransformed)	—	—	+	+	+	−	−
pAd5XhoC	5	5	+	+	+	+/−	−
pAd5HindIIIG	5	5 (only 19 kD)[c]	+	+	+	−	n.d.
pAd5d1Sac	5	5 (only 58 kD)[c]	+	+	+	−	n.d.
pAd5HpaIE	5	—	+	+	+	−	n.d.
pAd12RIC	12	12	−	−	+	+	+
pAd12HindIIIG	12	12 (only 19 kD)[c]	−	−	+	−	−
pAd12dlAcc	12	12 (only 54 kD)[c]	−	−	+	−	−
pAd512	5	12	+	+	+	+	−
pAd125	12	5	−	−	+	+/−	+/−
pAd51212	5 + 12	12	+	+	+	+	−
pST12	5 + 12	Ad12 19 kD + Ad5 58 kD	+	+	+	+/−	−
pLT12	5 + 12	Ad5 19 kD + Ad12 54 kD	+	+	+	+	−
pAd512pm975	12 + 5 (only 13S product)[c]	12	+	+	+	n.d.	n.d.
pAd512HL1007	12 + 5 (only 12S product)[c]	12	−	−	+	n.d.	n.d.
pSVR7	12 (only 12S product)[c]	12	+	+	+	+	n.d.
pSVR11	12 (no 12S product)[c] (no 13S product)	12	+	+	+	+	n.d.

[a]The composition of the various recombinant plasmids is given in Methods of Experimental Procedures.
[b]+, Highly oncogenic; +/−, weakly oncogenic; −, nononcogenic; n.d., not done.
[c]Truncated products from mutated genes can still be present.

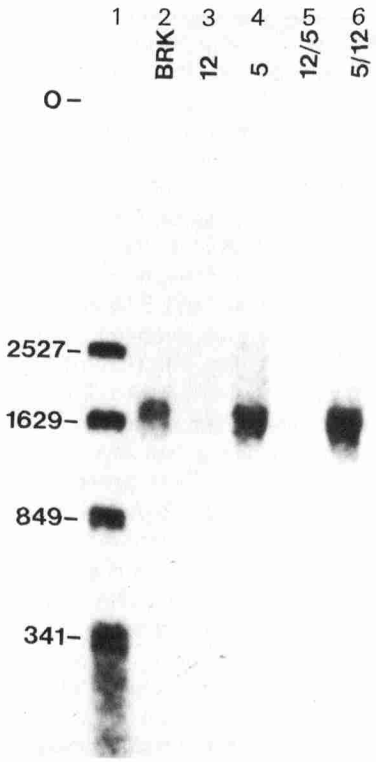

Figure 3 Northern blot analysis of RNA extracted from primary and adenovirus-transformed BRK cells, using a nick-translated human HLA B7 cDNA clone as described by Schrier et al. (1983). (Lane *1*) *Hae*III-digested M13mp8 marker DNA; (lane *2*) RNA isolated from primary BRK cells; (lane *3*) RNA from cells transformed by pAd12RIC; (lane *4*) DNA from cells transformed by pAd5XhoC; (lane *5*) RNA from cells transformed by pAd125; (lane *6*) RNA from cells transformed by pAd512.

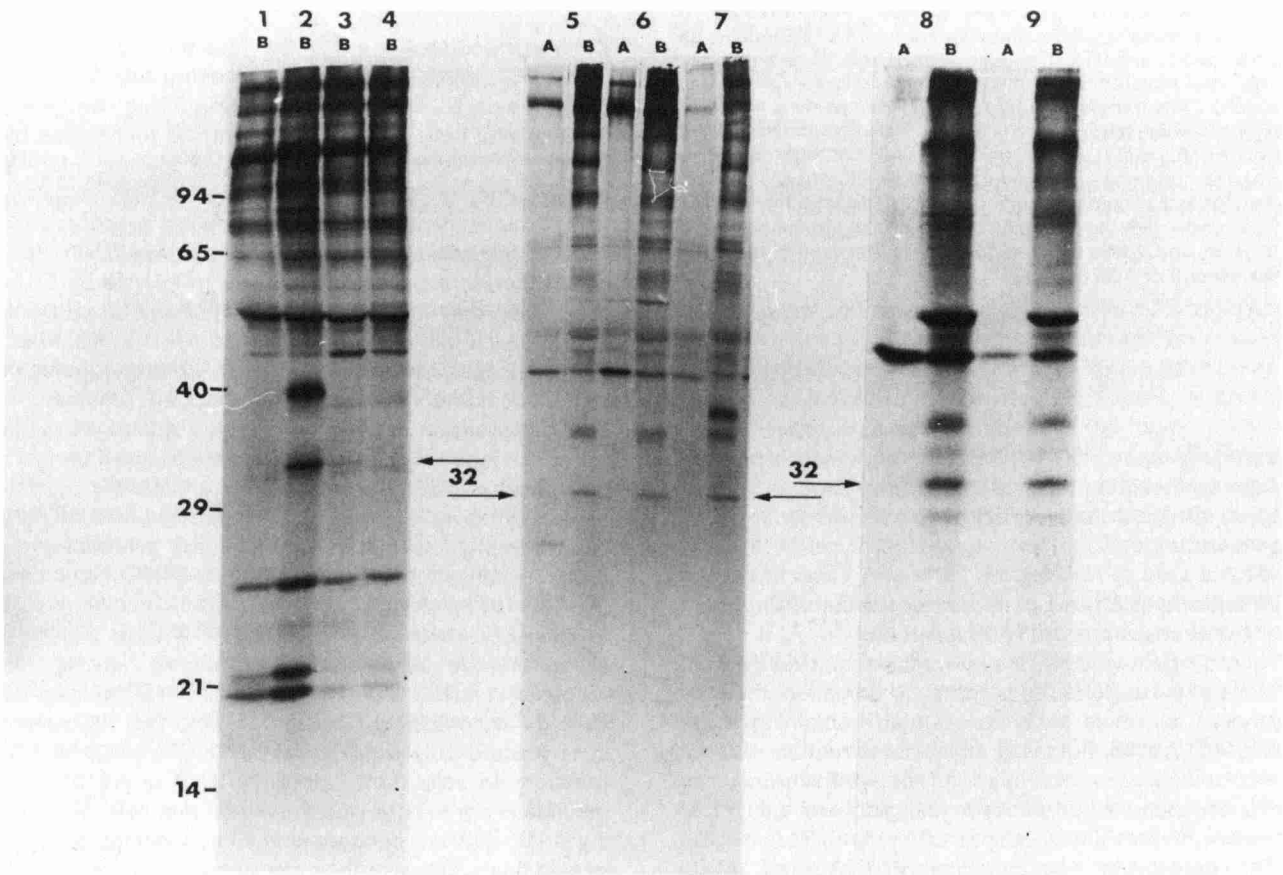

Figure 4 Proteins immunoprecipitated from extracts of [^{35}S]methionine-labeled transformed cells using an anti-BRK serum (lanes *B*) or nonimmune serum (lanes *A*). (Lane 1) pAd12RIC; (lane *2*) pAd5HindIIIG; (lane *3*) p512 pm975; (lane *4*) p512HL1007; (lane *5*) p51212; (lane *6*) pLT12; (lane *7*) pST12; (lane *8*) pSVR7; (lane *9*) pSVR11. For details on plasmids, see Methods section.

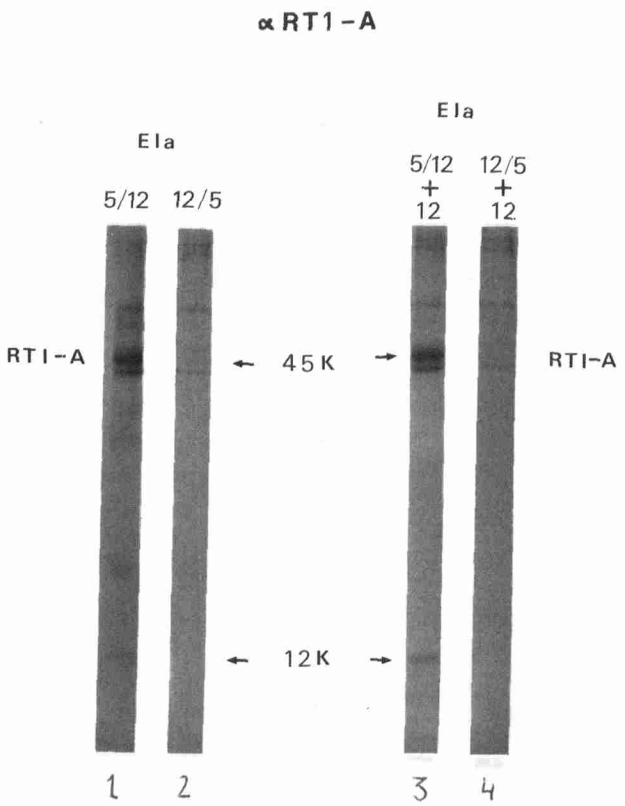

Figure 5 Proteins immunoprecipitated from extracts of [^{35}S]methionine-labeled adenovirus-transformed BRK cells, using a specific anti-RT1.A^{u} alloantiserum. Extracts were obtained from cells transformed by the following adenoviral DNAs. (*Left panels*) Cells transformed by Ad2 E1B plus either a hybrid E1 region in which the first exon is derived from Ad5 and the second from Ad12 (lane *1*), or a hybrid E1A region with the first exon of Ad12 and the second of Ad5 (lane *2*). (*Right panels*). Cells transformed by complete region E1 of Ad12 plus either a hybrid E1A region with the first exon of Ad5 and the second of Ad12 (lane *3*), or a hybrid E1A region with the first exon of Ad12 and the second of Ad5 (lane *4*).

that the property of Ad12 E1A to suppress class-I antigen synthesis is the basis of the highly oncogenic phenotype of Ad12-transformed cells in immunocompetent animals.

Inhibitions of class-I gene expression is a function of the first exon of Ad12 E1A mRNA

To investigate whether the suppressing activity of Ad12 E1A can be assigned to a particular domain of the gene product, plasmids were constructed containing hybrid Ad5/Ad12 E1A regions. For the construction of these recombinants, use was made of an *Ava*II site occurring at corresponding positions in the Ad5 and Ad12 E1A regions, at the splice-acceptor site of both E1A mRNAs. Cells transformed by these hybrid E1A regions plus Ad12 E1B were tested for the presence of class-I antigens. It was found that class-I proteins were virtually absent in cells transformed by a hybrid E1A region in which the first exon was derived from Ad12 and the second from Ad5, while these proteins were normally present with an E1A region in which the first exon is derived from Ad5 and the second from Ad12 (Fig. 5). Thus, inhibition of class-I gene expression is an activity associated with the first "domain" of the 13S mRNA product of Ad12. Since the 12S mRNA product(s) apparently do not influence MHC gene expression, the segment that is unique to 13S mRNA must have an important role in this activity. Further studies showed that the dominant activity of Ad5 E1A over Ad12 E1A in suppressing class-I gene expression is also encoded by the first exon of the Ad5 13S mRNA (Fig. 5). This indicates that these opposite activities of Ad5 and Ad12 E1A are functions of the same protein domains, and hence might be based on similar pathways or mechanisms.

Evidence has recently been presented that the Ad5 DNA region in front of the E1A major cap site has an enhancer activity. To investigate the possibility that the effect of Ad12 E1A on class-I gene expression is caused by an activity of this upstream DNA sequence, we have studied cell lines transformed by an Ad12 E1 region in which this enhancer region is replaced by the corresponding segment of Ad5. It was found that in these lines class-I gene production was as strongly suppressed as in control Ad12-transformed cells, showing that this effect is not a function of the Ad12 DNA segment in front of the E1A cap site (results not shown).

Cells expressing Ad12 E1A are less susceptible to cytotoxic T cells than those expressing Ad5 E1A

Cells of a particular animal expressing foreign antigens, such as viral tumor antigens, will only be recognized by the animal's cytotoxic T cells (CTL) if the neoantigens are presented on the cell surface together with the class-I MHC molecules. Cells having reduced expression of class-I antigens, such as Ad12-transformed BRK cells, would therefore be able to escape from lysis by CTL, even when they express viral T antigens on the plasma membrane. If this assumption were correct, it would explain why cells expressing Ad12 E1A are oncogenic in immunocompetent animals. To investigate whether reduced expression of class-I molecules indeed results in lower susceptibility to CTL, we have measured the lysis of a panel of Ad5- and Ad12-transformed cells by allogeneic cytotoxic T lymphocytes generated in a primary mixed lymphocyte culture. Such CTLs are mainly directed against the allogeneic class-I MHC molecules (Bevan 1977). Absence of class-I molecules would therefore prevent killing by cytotoxic T cells. Figure 6 shows that the allogeneic CTLs exhibited a strong lytic response against Ad5 E1- and pAd512-transformed cells (both containing E1A of Ad5), but that the spleen cells reacted only weakly with Ad12 E1- and pAd125-transformed cells (both containing E1A of Ad12). This result is in agreement with the model that cells expressing Ad12 E1A are oncogenic in immunocompetent syngeneic hosts because they can escape from the T-cell-mediated immune response. That the phenomenon is not restricted to rat cells of a particular haplotype was shown by an experiment in which the CTLs were derived from

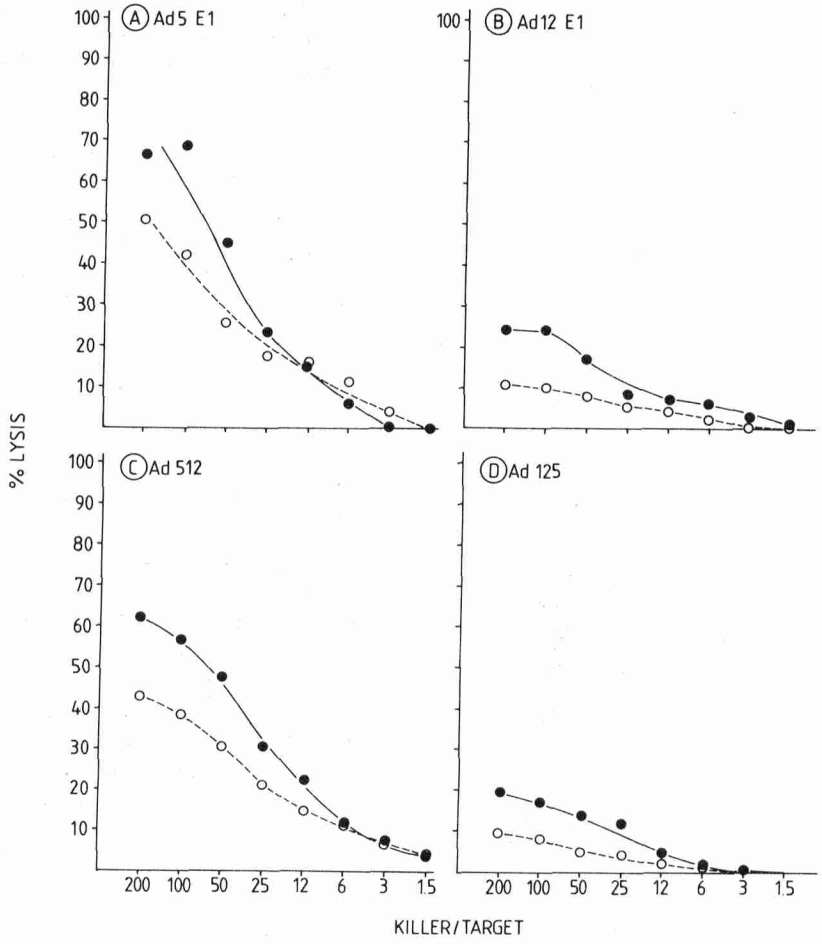

Figure 6 Susceptibility of adenovirus-transformed Wag-Rij cells to allogeneic CTL. In each of the four panels, percentage of cell lysis by allogeneic CTLs is shown for two independently derived transformed cell lines. Cell killing is plotted against different ratios of killer lymphocytes versus target adenovirus-transformed cells. (*A*) Cells transformed by Ad5 region E1 (pAd5XhoC); (*B*) cells transformed by Ad12 region E1 (pAd12RIC); (C) cells transformed by a plasmid containing Ad5 E1A plus Ad12 E1B (pAd512); (*D*) cells transformed by a plasmid containing Ad12 E1A plus Ad5 E1B (pAd125).

Wag-Rij rats and the stimulator lymphocytes and target cells from Brown Norway (BN) rats ($RT1^n$ haplotype). Again, a similar difference in susceptibility to allogeneic CTLs was found between Ad5- and Ad12-transformed BN cells.

To further confirm the model, the reactivity of virus-specific syngeneic T cells was measured against Ad-transformed cells of the same haplotype as the T cells, in a secondary syngeneic CTL reaction. The T killer cells were now specifically directed against the viral antigens. It was found that Ad5-transformed cells were efficiently killed by Ad5-stimulated CTLs whereas Ad12-transformed cells were only poorly lysed by Ad12-stimulated CTLs (results not shown). Thus, basically similar results were obtained with virus-specific syngeneic CTLs as with allogeneic primary CTLs.

Discussion

The study reported in this paper was undertaken to search for differences at the protein level between oncogenic and nononcogenic adenovirus-transformed rat cells. Two proteins were identified, of 32 kD and 45 kD, that were absent in Ad12-transformed cells (or present in greatly reduced amounts), but present in normal concentrations in Ad5-transformed and untransformed cells. The 45-kD protein was identified as the heavy chain of the class-I MHC antigens, RT1.A, of the rat. Suppression of heavy-chain production was shown to occur at the level of mRNA accumulation, whereas synthesis of the light chain, β_2-m was unaffected. The suppressing activity of Ad12 region E1 was shown to be a function of the 13S mRNA (probably its product) encoded by region E1A, and specifically of the first exon. We have recently shown that the same Ad12 13S mRNA product is also required for activation of the E1B promoter (Bos and Ten Wolde-Kraamwinkel 1983). A similar property has been described for the Ad5 13S E1A mRNA, not only for activation of region E1B but also of the other adenoviral early regions, as well as for activation of a cellular gene coding for a heat-shock protein (Nevins 1982). Thus, the E1A regions of both Ad5 and Ad12 are capable of activating their own early genes and cellular heat-shock genes (shown for Ad5 only), whereas Ad12 E1A, but not Ad5 E1A, can suppress the activity of at

least two different cellular genes, the 45-kD class-I MHC genes and a gene coding for a 32-kD protein. The suppressing activity of Ad12 E1A is counteracted by Ad5 E1A, if both regions are present within the same rat cell. Since the suppressing activity of Ad12 E1A on class-I gene expression and the Ad5 activity in preventing this suppression by Ad12 E1A are both associated with the first exon of the respective 13S mRNAs, a reasonable working hypothesis would be that the two viral polypeptides compete for the same cellular binding or effector site. The Ad5 product would then have a higher affinity than the Ad12 product. More difficult to explain is how the Ad12 product would cause inhibition of class-I gene expression while the Ad5 product does not affect expression of these genes, although the E1A products of the two viruses are otherwise homologous in function and can complement each other in transformation.

So far, we have assumed that the strongly reduced expression of the 45-kD and 32-kD proteins is caused by an inhibition of mRNA transcription or a reduction of mRNA stability. It cannot be excluded, however, that Ad12 region E1, and more specifically region E1A, can only transform cells that have no, or reduced, expression of these two proteins. This possibility is supported by the observation that Ad12 usually transforms with an at least 10-fold lower efficiency than Ad5. On the other hand, preliminary experiments have shown that the level of expression of Ad12 region E1A is inversely correlated with that of class-I genes. An Ad12-transformed hamster line, derived from an Ad12 DNA-induced tumor, and a subclone of this line, were found to differ in viral T antigen expression, one containing high levels of T antigens, the other low levels. Northern blotting analysis showed that the line with high T antigen concentration had a very low expression level of class-I antigens, and vice versa. This result suggests that the Ad12 products can indeed switch-off expression of class-I genes. Further experiments to distinguish between the alternative possibilities are in progress.

The results presented in the last paragraph show that transformed BRK cells containing Ad12 E1A are much less susceptible to allogeneic CTLs than cells containing Ad5 E1A. Whether this observation can be extrapolated to an in vivo situation is not certain, but is strongly suggested by the finding that cells expressing Ad12 E1A can form tumors in immunocompetent rats whereas cells expressing Ad5 E1A cannot. Thus, as a result of the decreased susceptibility to CTL, a sufficient number of Ad12-transformed cells will escape the immune surveillance so that a tumor can be formed, while transformed cells expressing Ad5 E1A will be eliminated and hence will only be able to form tumors when they are introduced into immunodeficient animals, such as nude mice.

Preliminary experiments with a viable Ad5 recombinant virus containing a genome in which the E1 region is replaced by the corresponding E1 region of Ad12, have shown that this virus may be nononcogenic in hamsters. If this is correct, it would indicate that other regions of the viral genome can also influence oncogenicity of intact virions.

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