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Different Activities of the Adenovirus Types 5 and 12 E1A Regions in Transformation with the EJ Ha-*ras* Oncogene

A. G. JOCHEMSEN,* R. BERNARDS,† H. J. VAN KRANEN, A. HOUWELING, J. L. BOS, AND A. J. VAN DER EB

Department of Medical Biochemistry, Sylvius Laboratories, 2300 RA Leiden, The Netherlands

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We have compared the capacities of the E1A regions of nononcogenic adenovirus type 5 (Ad5) and highly oncogenic Ad12 to cooperate with the EJ bladder carcinoma Ha-*ras-1* oncogene in the transformation of primary baby rat kidney cells. Both E1A regions, when cotransfected with the Ha-*ras* oncogene, transformed the primary cells with a low frequency. Ad5 E1A plus Ha-*ras*-transformed cells differed in phenotype from cells transformed by Ad12 E1A plus Ha-*ras*. The cells expressing Ad5 E1A appeared highly transformed and practically failed to adhere to plastic. This phenotype may be due to the virtually complete absence of fibronectin gene expression in these cells. In contrast, the cells expressing Ad12 E1A were flatter and adhered to plastic, whereas fibronectin gene expression was reduced but not absent. The oncogenic potential of the two types of E1A plus *ras*-transformed cells was tested by their injection into both athymic nude mice and weanling syngeneic rats. The Ad5 E1A plus *ras*-transformed cells were found to be highly oncogenic in both animal species, whereas the Ad12 E1A plus *ras*-transformed cells were only weakly oncogenic in both syngeneic rats and nude mice. The difference in oncogenic potential of the Ad5 E1A plus *ras*- and the Ad12 E1A plus *ras*-transformed cells is discussed in terms of the different capacities of the Ad5 and Ad12 E1A-encoded proteins to modulate cellular gene expression.

Oncogenic transformation of primary (or secondary) cultures of rodent cells by nononcogenic adenoviruses (e.g., adenovirus type 5 [Ad5]) or highly oncogenic adenoviruses (e.g., Ad12) requires the continued expression of the left terminal 11% of the adenovirus genome (12, 14). This part of the viral genome harbors early region 1 (E1), which consists of two transcriptional units, E1A and E1B (39).

Considerable evidence now exists suggesting that the induction of oncogenic transformation of primary cells in vitro requires the concerted action of multiple E1-encoded genes. Cells transformed by region E1A are only semitransformed in phenotype, grow to low-saturation density, and have a very limited capacity, if at all, to cause tumors when injected into experimental animals (10, 17). Region E1B by itself has no detectable transforming activity in primary cells (35) but can cooperate with region E1A to yield cells that are fully transformed in phenotype, grow to high saturation density, and are oncogenic in immunodeficient nude mice (transformed by Ad5 and Ad12) (11, 21) and immunocompetent syngeneic rodents (transformed by Ad12 only) (8, 24).

The concept that multiple genes are involved in oncogenic transformation has also been shown to be valid for nonviral cancers, as indicated by the finding that the activated human c-*myc* and c-Ha-*ras* oncogenes can cooperate in oncogenic transformation of primary cells (22). Furthermore, it has been shown that oncogenic transformation can also be obtained with combinations of a viral (e.g., Ad2 E1A) and a nonviral (activated c-Ha-*ras*) transforming gene (28).

In the present study we have compared the abilities of the E1A regions of nononcogenic Ad5 and highly oncogenic Ad12 to cooperate with the Ha-*ras* bladder carcinoma oncogene in the oncogenic transformation of primary baby rat kidney (BRK) cells. These experiments appeared interesting because the E1A regions of these two adenoviruses have been shown to differ considerably in their activity to modulate viral and cellular gene expression (5, 29), properties which might well have profound effects on the transformed phenotype. We report that the adenovirus E1A plus *ras*-transformed cells differ considerably in morphology and in tumorigenicity, the Ad5 E1A plus *ras*-transformed cells being highly oncogenic and the Ad12 E1A plus *ras*-transformed cells being weakly oncogenic.

MATERIALS AND METHODS

Transformation of primary cells. Primary cultures of BRK cells were prepared from the kidneys of 1-week-old WAG-RIJ rats. Transformation of primary cells with plasmid DNA has been described in detail elsewhere (37). In our experiments, 10 replicate 6-cm dishes of primary cells were transfected with 10 μg of genome equivalent (1.6 μg of plasmid per dish for an E1A clone) of adenovirus DNA per dish (Table 1). In cotransfections with the EJ bladder carcinoma oncogene, 2 μg of pEJ6.6 per dish was added. To enhance transformation, the primary cell cultures were treated 4 h posttransfection for 90 s with a solution consisting of 10% dimethyl sulfoxide in phosphate-buffered saline. The E1A clones used were pAd5E1A, containing the 0-1572 *Hpa*I E fragment of Ad5, and pAd12E1A, containing the 0-1594 *Acc*I H fragment of Ad12 (3).

The procedures of Northern blotting, S1 nuclease RNA mapping, and testing of oncogenicity have been previously described (3, 29).

Cell labeling with [^{35}S]methionine and subsequent immunoprecipitation was performed essentially as described previously (30). An anti-Ad12 tumor serum, an anti-Ad5 E1A monoclonal serum (M73) (16), and an anti-*ras* monoclonal serum (Y13-259) (9) were used.

Construction of pMMTV-*ras*. The long-terminal repeat (LTR)-*tk* chimeric gene of plasmid 2.6 (15) was digested with *Bst*EII, which cuts in the U5 region of the mouse mammary tumor virus (MMTV-LTR) 98 nucleotides downstream from the LTR cap site. The ends were filled in with the Klenow

* Corresponding author.
† Present address: Whitehead Institute for Biomedical Research, Cambridge, MA 02142.

TABLE 1. Transforming activity of Ad5 and Ad12 E1A regions used singly and in cotransfection

Plasmid	Avg no. of foci/μg genome equivalent for expt(s):		
	1	2	1 and 2 (avg)
PAd5E1A	0.47	0.26	0.36
pEJ6.6	0	0	0
pAd5E1A + pEJ6.6	0.26	0.43	0.34
pAd5XhoC[a]	20	13	16.5
pAd12E1A	0	0	0
pAd12E1A + pEJ6.6	0.04	0	0.02
pAd12RIC[b]	ND[c]	ND	0.55[d]

[a] Contains Ad5 E1.
[b] Contains Ad12 E1.
[c] ND, Not done.
[d] From reference 3.

fragment of polymerase I, and subsequently the DNA was cut by *Pvu*I. A 4.6-kilobase (kb) fragment containing the MMTV-LTR region and part of pBR322 was isolated. pSVET24, containing the EJ *ras* gene under control of the simian virus 40 early promoter (6), was digested with *Eco*RI, the ends were blunted with the Klenow fragment of polymerase I, and the DNA was cut by *Pvu*I. A 8-kb fragment containing both the EJ *ras* gene and the larger part of the vector pBR322 was isolated and ligated to the 4.6-kb DNA fragment isolated from p2.6. The resulting chimeric MMTV-LTR-*ras* plasmid was called pMMTV-*ras*.

Adhesion to fibronectin-coated dishes. Dishes (6 cm) were coated with 10 μg of plasma fibronectin (5,000 molecules per μm^2).

RESULTS

Transformation of primary BRK cells. The ability of Ad5 and Ad12 E1A regions to cooperate with the c-Ha-*ras*-1 gene isolated from the EJ bladder carcinoma cell line (pEJ6.6) (33) in the transformation of primary BRK cells was tested after calcium phosphate-mediated gene transfer. Colonies of morphologically transformed cells appeared 2 to 3 weeks after transfection and were counted after 4 weeks. The efficiencies of focus formation of the various plasmids, used both singly and in combination, are shown in Table 1. In agreement with our previous results, it was found that focus formation by Ad5 E1A alone is a very inefficient process which is enhanced some 50-fold by the presence of region E1B (17, 34). No increase of focus formation was observed when Ad5 E1A was used in combination with the EJ *ras* oncogene, which indicates that immortalization by E1A and oncogenic transformation by the *ras* oncogene are independent events in the process of cell transformation. Immortalization by Ad12 E1A alone was not observed, but a small number of foci were found when Ad12 E1A was cotransfected with pEJ6.6. This result suggests that the *ras* oncogene can stimulate immortalization by Ad12 E1A. However, immortalization by Ad12 E1A alone with an efficiency similar to that by Ad12 E1A plus pEJ6.6 has been reported by others (10). Immortalization by Ad12 E1A may therefore be a rare event, and foci may easily be missed owing to their slow growth rate and untransformed phenotype. The actual efficiency of transformation by Ad12 E1A alone may not be significantly different from that obtained with the combination of Ad12 E1A plus pEJ6.6.

The primary foci of cells transformed by Ad5 E1A plus *ras* differed considerably in morphology from that of cells transformed by Ad12 E1A plus *ras* in that Ad5 E1A plus *ras*-transformed cells showed a marked decrease in their ability to attach to the plastic surface of culture dishes (Fig. 1A). When Ad5 E1A plus *ras*-transformed foci were isolated and established as cell lines, a further decrease in affinity to the substrate was usually observed, and eventually these cells became unable to adhere to plastic. In contrast, Ad12 E1A plus *ras*-transformed cells retained the capacity to grow in monolayers after prolonged growth in tissue cultures (Fig. 1B).

Expression of transforming genes. Individual foci of E1A plus *ras*-transformed BRK cells were isolated and established as cell lines. To determine whether and to what extent region E1A was expressed in the transformants, total cyto-

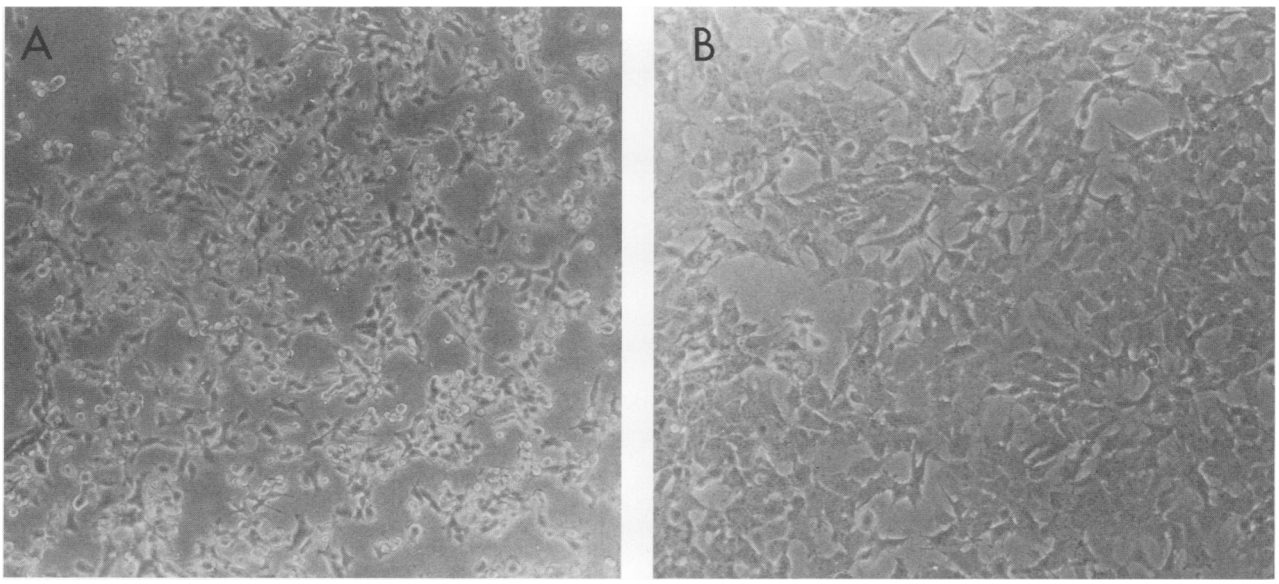

FIG. 1. Phase-contrast photomicrographs of primary BRK cells transformed by Ad5 E1A plus pEJ6.6 (A) and Ad12 E1A plus pEJ6.6 (B).

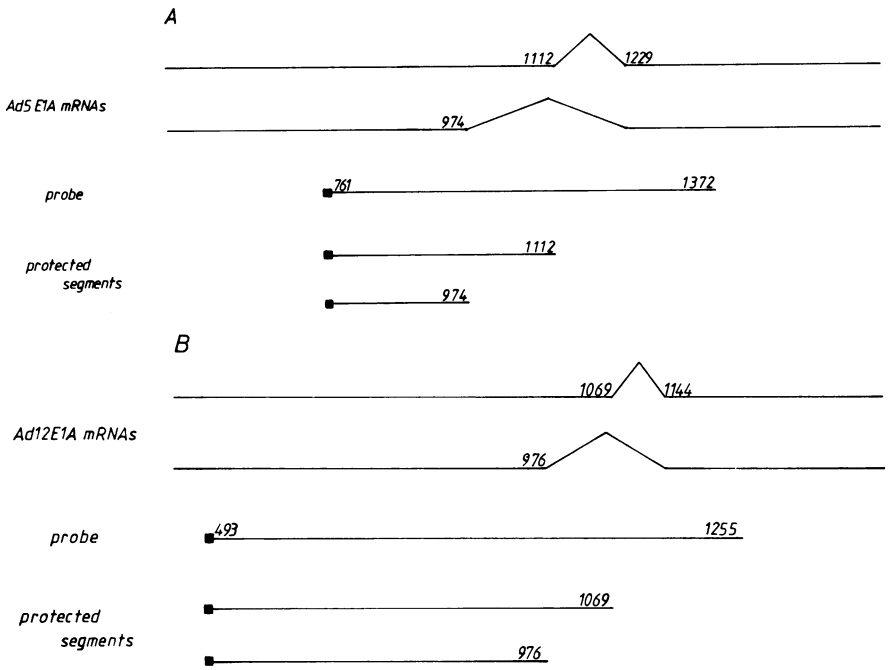

FIG. 2. Diagram of the end-labeled probes used to detect Ad5 (A) and Ad12 (B) E1A mRNAs by S1 nuclease analysis. Included are the lengths of the protected segments expected. The numbers indicate the endpoints of the DNA fragments.

plasmic RNA was isolated and analyzed by nuclease S1 protection experiments with probes identifying the Ad5- or Ad12-specific E1A transcripts. A schematic diagram of the analysis is shown in Fig. 2. The Ad5 E1A plus *ras*-transformed cell lines expressed both the 12S and 13S E1A mRNA in about equal quantities (Fig. 3A, lanes 3, 4, and 5). This level is considerably lower than that in Ad5 E1A plus E1B-transformed cells (Fig. 3A, lane 2) but is comparable to that in Ad5 E1A-immortalized cells (not shown). Similarly, the Ad12 E1A plus *ras*-transformed cells expressed the Ad12 E1A region at a considerably lower level than did the Ad12 E1A plus E1B-transformed cells (Fig. 3B, lanes 2, 3, 4, and 5). The E1A expression was also investigated by immunoprecipitation (Fig. 4B). The anti-Ad12 tumor serum recognized both the E1A products (41 kilodalton) and the E1B large T antigen (55 kilodalton). The results showed that the level of E1A protein expression in the Ad12 E1A plus *ras*-transformed cells (Fig. 4B, lanes 1 and 2) is significantly lower than in the Ad12 E1-transformed cells (Fig. 4B, lane 3), although the difference does not appear to be as spectacular as that seen at the RNA level. Essentially the same result was found with an Ad5 E1A monoclonal antibody. Although the Ad5 E1A protein bands (which appear as a smear between 36 and 40 kilodaltons) are not very clear in Fig. 4B, lane 6 (Ad5 E1-transformed cells), they are hardly visible in lanes 4 and 5 (Ad5 E1A plus *ras*-transformed cells). This indicates that at the protein level as well, the Ad5 E1A expression is much lower in the Ad5 E1A plus *ras*-transformed cells than in Ad5 E1-transformed cells. These results show that the expression of region E1A is very low in cells immortalized by region E1A alone (36) and in E1A plus *ras*-transformed cells and is at least 10-fold higher in cells transformed by E1A plus E1B. Apparently, the *ras* oncogene is unable to enhance E1A expression, as opposed to that of region E1B.

The expression of the Ha-*ras* oncogene in the two types of E1A plus *ras*-transformed cells was measured by immunoprecipitation with the anti-*ras* monoclonal antibody Y13-259 (9). The level of expression of the *ras* oncogene in various Ad12 E1A plus *ras*- and Ad5 E1A plus *ras*-transformed cell lines was basically the same (Fig. 4A). The expression of the Ha-*ras* oncogene appeared to be similar to the *ras* expression in the T24 bladder carcinoma cell line (data not shown).

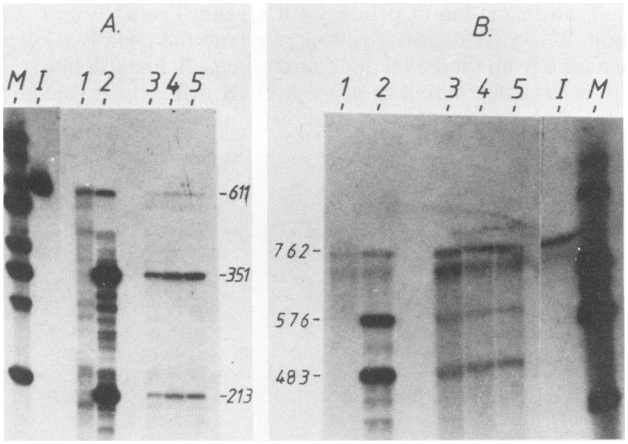

FIG. 3. S1 nuclease analysis of Ad5 and Ad12 E1A mRNA. 20 μg of the total cytoplasmic RNA was hybridized to the 3'-end-labeled DNA probes and treated with S1 nuclease, and the samples were separated on a 5% acrylamide 7 M urea gel. (A) Analysis of the Ad5 E1A expression. Shown are protected segments after hybridization to *Escherichia coli* tRNA (lane 1), to RNA from an Ad5 E1-transformed cell line (lane 2), and to RNA from three Ad5 E1A plus *ras*-transformed cell lines (lanes 3, 4, and 5). I, Untreated DNA probes; M, marker digest. (B) Analysis of the Ad12 E1A expression. Shown are protected segments after hybridization to *E. coli* tRNA (lane 1), to RNA from an Ad12 E1-transformed cell line (lane 2), and to RNA from three Ad12 E1A plus *ras*-transformed cell lines (lanes 3, 4, and 5).

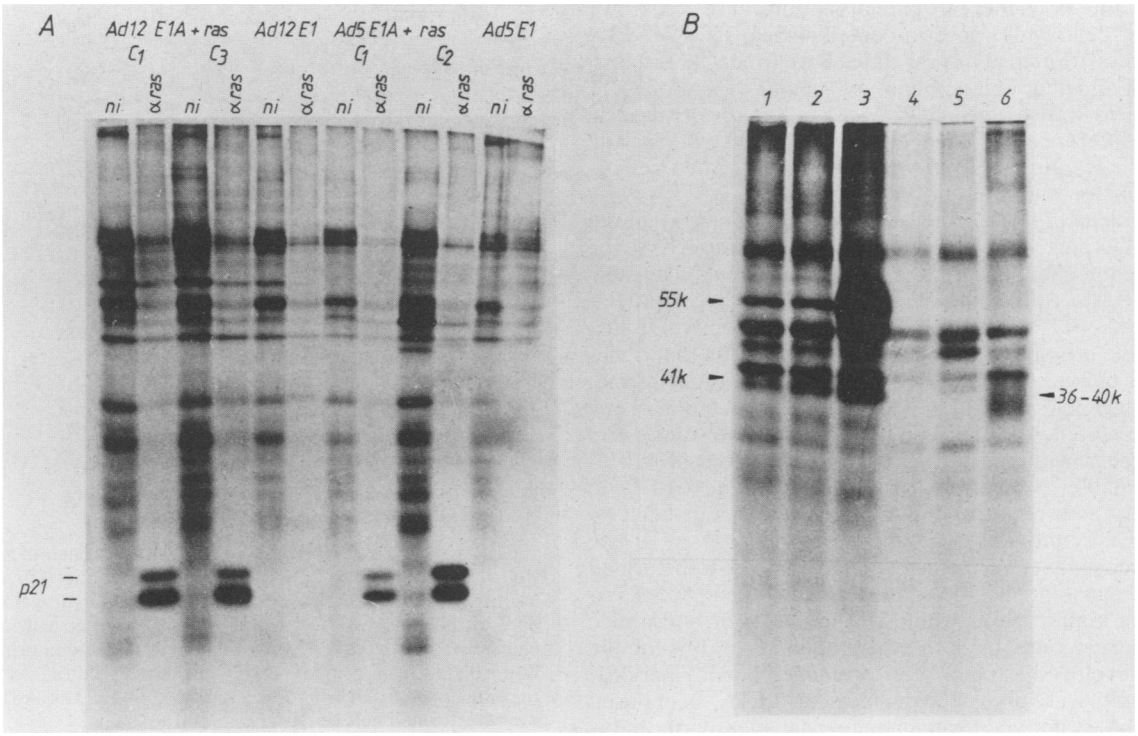

FIG. 4. Analysis of the expression level of the transforming proteins in transformed BRK cells. (A) Polyacrylamide gel electrophoresis of the p21 *ras* molecules precipitated from BRK cells transformed by Ad12 E1A plus *ras*, Ad12 E1, Ad5 E1A plus *ras*, and Ad5 E1. The labeled extracts were immunoprecipitated with normal rat serum (ni) or an anti-*ras* (α*ras*) monoclonal antibody. (B) Detection of the adenovirus E1A proteins in BRK cells transformed by Ad12 E1A plus *ras* C1 and C3 (lanes 1 and 3, respectively), Ad12 E1 (lane 3), Ad5 E1A plus *ras* C1 and C2 (lanes 4 and 5, respectively), and Ad5 E1 (lane 6). Immunoprecipitation was performed with an anti-Ad12 tumor serum and an anti-Ad5 E1A monoclonal antibody.

Similar results were obtained from Northern blotting analysis of the c-Ha-*ras* mRNA expression in the various cell lines.

To demonstrate unequivocally that p21 *ras* expression does not influence the adenovirus E1A expression, as is found for E1B (36), we have also transformed BRK cells by Ad5 E1A in combination with the T24 *ras* gene under control of the MMTV-LTR. It has been shown that genes located 3′ of the MMTV-LTR can be induced by glucocorticoid hormones (15, 18, 23). To activate transcription, we added 1 μM dexamethasone to the medium. We found that this addition to the medium was effective for the activation of transcription. To prevent transcription of the *ras* gene constructed by glucocorticoids in the serum, we treated the serum with active coal and sterilized it by filtration. Transfections were performed both in the presence and absence of dexamethasone. The transformation frequency was similar in both conditions and comparable to that with Ad5E1A plus pEJ6.6. In the absence of dexamethasone, the colonies were very flat and looked semitransformed, like Ad5 E1A-immortalized cells. In the presence of the hormone, however, the cells exhibited a morphology similar to Ad5 E1A plus *ras*-transformed cells. To determine whether p21 *ras* influences the Ad5 E1A expression in the transformed cells, we grew cell lines, each isolated from a single colony, in the absence or presence of dexamethasone in the medium and investigated the *ras* expression by immunoprecipitation and the Ad5 E1A expression by S1 nuclease analysis. The results obtained with one cell line are presented in Fig. 5. It was found that the amount of p21 was highly elevated in the presence of dexamethasone (Fig. 5A), whereas the Ad5 E1A expression was very similar in both growth conditions (5B). The same results were obtained with other cell lines (not shown). These results demonstrate that p21 *ras* is unable to enhance the Ad5 E1A expression, as has been described for Ad5 E1B. More characteristics of the Ad5 E1A plus MMTV-*ras*-transformed cells will be described elsewhere. It is interesting that *ras* expression in the majority of the isolated cell lines could be regulated effectively by dexamethasone in early passages. However, after prolonged passaging in tissue cultures, many lines lost this ability and did not respond any more to dexamethasone, i.e., the cells kept their typical rounded, nonadherent phenotype, even in the absence of the hormone. The reason for this constitutive expression of MMTV-*ras* in the absence of dexamethasone is unclear.

Synthesis of fibronectin in transformed cells. Since a decrease in the amount of matrix fibronectin often appears to be the cause of decreased adhesion of transformed cells to substrate (1, 19, 40), we investigated whether the different capacities of the two types of E1A plus *ras*-transformed cells to adhere to plastic was caused by differences in fibronectin gene expression. Fibronectin-specific mRNA was detected by Northern blot hybridization with a rat fibronectin cDNA probe (31). This probe detects a major transcript of 8.8 kb in RNA isolated from untransformed BRK cells (Fig. 6). In Ad12 E1A plus *ras*-transformed cells, a substantial reduction in the amount of fibronectin-specific mRNA was observed (Fig. 6, lanes 5 and 6), whereas Ad5 E1A plus *ras*-transformed cells were found to be almost completely devoid of the 8.8-kb fibronectin transcript (Fig. 6, lanes 2, 3, and 4). To investigate whether the virtual absence of fibronectin expression in Ad5 E1A plus *ras*-transformed cells is the only

cause of the nonadherent phenotype of these cells, we seeded the cells on fibronectin-coated dishes. The Ad5 E1A plus *ras*-transformed cells now did adhere to the culture dish within 3 h and acquired a phenotype similar to that of Ad12 E1A plus *ras*-transformed cells (Fig. 7). This result suggests that the difference in fibronectin expression between Ad5 E1A plus *ras*- and Ad12 E1A plus *ras*-transformed cells is responsible for the difference in morphology.

Oncogenicity of transformed cells. The oncogenic potential of the E1A plus *ras*-transformed cells was tested by the subcutaneous injection of 10^7 cells into weanling syngeneic WAG-RIJ rats ($RT1^u$ haplotype), weanling allogeneic BN and ACI rats ($RT1^n$ and $RT1^a$ haplotypes, respectively), and 12-week-old athymic nude mice (Table 2). It was found that Ad5 E1A plus *ras*-transformed cells are highly oncogenic, both in syngeneic rats and in nude mice, with tumors arising after short latency periods. When injected into allogeneic rats, the cells within 7 days even caused tumors of 0.5- to 1.0-cm diameter, which regressed again in the next 10 days, most likely as a result of the killing of the tumor cells by allo-specific cytotoxic T lymphocytes.

The oncogenicity of the Ad12 E1A plus *ras*-transformed cells was substantially lower than that of their Ad5 E1A-expressing counterparts when assayed in both nude mice and syngeneic rats. In both cases only 25 to 30% of the animals developed a tumor after prolonged latency periods. These results were unexpected, since Ad12 E1A, as opposed to Ad5 E1A, is derived from an oncogenic adenovirus and is known to suppress the activity of class I major histocompatibility (MHC) antigens in transformed cells, which should increase rather than decrease oncogenicity as compared with what Ad5 E1A plus *ras*-transformed cells should do (see Discussion).

Thus, our data indicate that E1A of nononcogenic Ad5 can efficiently cooperate with the EJ bladder carcinoma *ras* oncogene in oncogenic transformation, whereas the E1A region of highly oncogenic Ad12, when used in combination with the activated Ha-*ras* oncogene, is far less efficient in conferring an oncogenic phenotype to transformed cells.

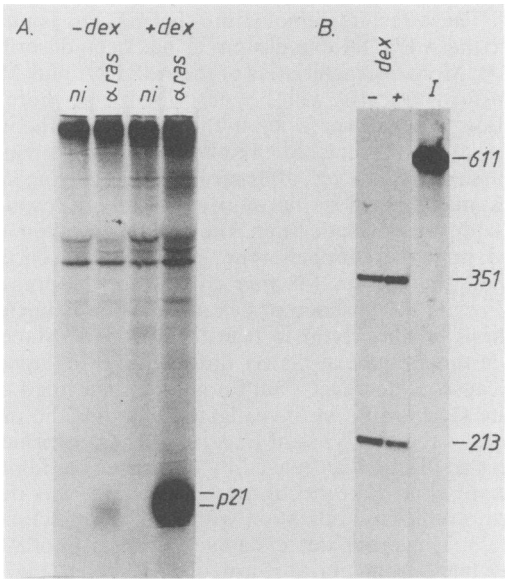

FIG. 5. Analysis of the expression level of the transforming genes in BRK cells transformed by Ad5 E1A plus MMTV *ras* with or without induction of the *ras* expression by dexamethasone. (A) Immunoprecipitation of the p21 *ras* proteins. The labeled extracts were either precipitated with normal rat serum (ni) or an anti-*ras* (α*ras*) monoclonal antibody. (B) S1 nuclease analysis of the Ad5 E1A mRNA expression. The 3'-end-labeled DNA probe and the expected protected segments are described in Fig. 2.

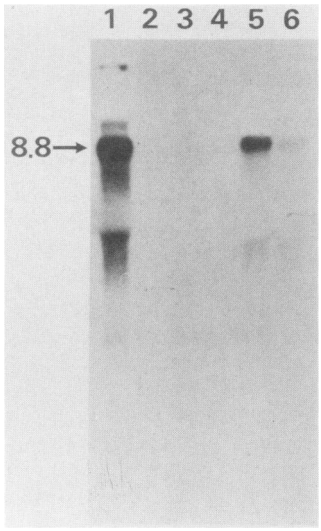

FIG. 6. Northern blotting analysis of the fibronectin gene expression. Twenty micrograms of total cytoplasmic RNA was size fractionated on a 1% agarose, 2.2 M formaldehyde gel and blotted onto nitrocellulose. Hybridization was performed with a 500-base-pair fibronectin cDNA fragment isolated from clone prlf (31). RNA was isolated from primary BRK cells (lane 1), three Ad5 E1A plus *ras*-transformed cell lines (lanes 2, 3, and 4), and two Ad12 E1A plus *ras*-transformed cell lines (lanes 5 and 6).

DISCUSSION

We have compared the properties of BRK cells transformed with Ad5 or Ad12 E1A in combination with an activated c-Ha-*ras* oncogene. Two major differences were observed between Ad5 E1A plus *ras*- and Ad12 E1A plus *ras*-transformed cells. First, the two types of transformed cells were different in phenotype in that the Ad5 E1A-expressing cells practically failed to adhere to the surface of the plastic culture dishes, whereas the Ad12 E1A-expressing cells were adherent and grew stably in a monolayer. This difference could be due to the much lower levels of fibronectin mRNA in the Ad5 E1A plus *ras* cells as compared with those in the Ad12 E1A plus *ras* cells. This explanation is supported by the finding that the nonadherent Ad5 E1A plus *ras* cells acquired an adherent Ad12 E1A plus *ras* phenotype when the cells were grown in fibronectin-coated dishes.

A second major difference between the two types of transformed cells became apparent when oncogenicity was tested in nude mice and syngeneic weanling rats. The Ad5 E1A plus *ras*-transformed cells were highly oncogenic in both animal species, with tumors appearing after short latency periods, whereas the Ad12 E1A plus *ras*-transformed cells produced tumors in only a fraction of the injected nude mice and syngeneic rats after prolonged latency periods. This result was unexpected, since Ad12 E1A is known to reduce class I MHC expression in transformed rat cells (29). This should have resulted in an increase of the oncogenic potential of the transformed cells rather than in a decrease, as compared with the potential of Ad5 E1A plus *ras*-transformed cells, which have normal levels of class I

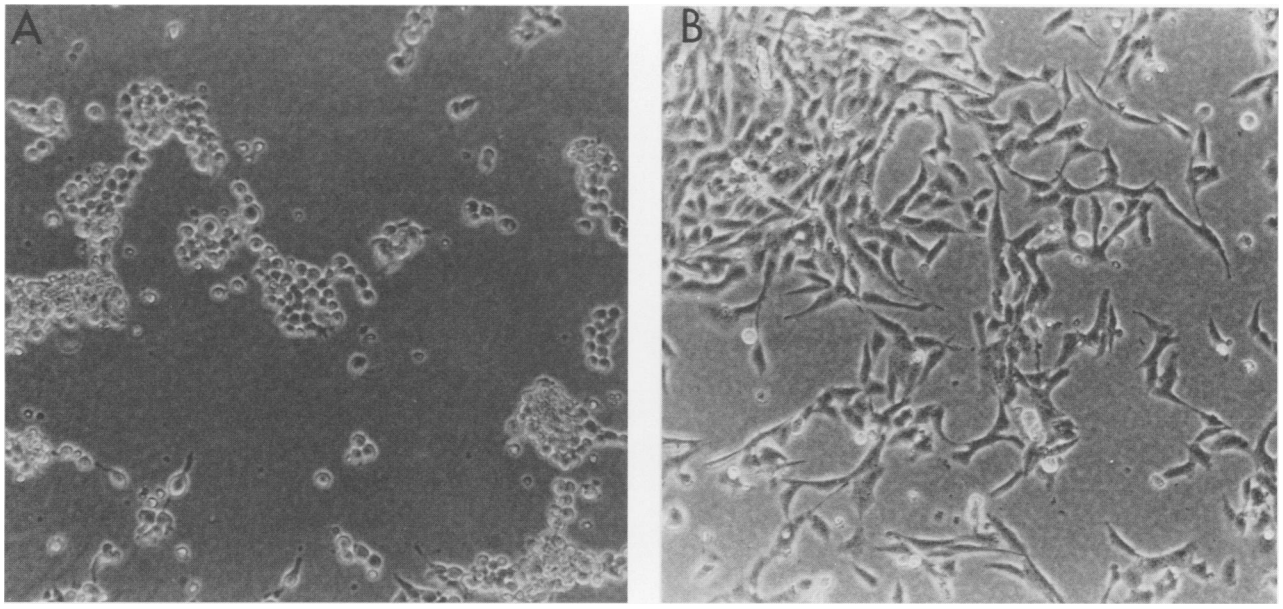

FIG. 7. Phase-contrast photomicrograms of Ad5 E1A plus *ras*-transformed cells 3 h after seeding on normal tissue culture dishes (A) or on fibronectin-coated dishes (B).

MHC antigens. However, measurements of the level of class I gene expression showed that the Ad12 E1A plus *ras*-transformed cells have little if any reduction in the level of class I MHC antigens (R. T. M. J. Vaessen, personal communication), probably as a result of the low E1A expression (see below). This suggests that the difference in oncogenic potential between the two types of E1A plus *ras*-transformed cells was not caused by a difference in immune response against the two types of cells. The fact that the E1A plus *ras*-transformed cells were oncogenic in immunocompetent hosts can be explained by the assumption that these cells have no viral transplantation antigens on their surface, which are presumably encoded by region E1B (7). This interpretation is supported by the finding that a similar difference in oncogenicity between Ad12 E1A plus *ras*- and Ad5 E1A plus *ras*-transformed cells was found in immunodeficient nude mice (Table 2).

It seems more likely, therefore, that the difference in oncogenic potential between Ad12 E1A plus *ras*- and Ad5 E1A plus *ras*-transformed cells should be explained either by differences in the expression levels of the transforming genes in the two cell types or by differences in the biological activity of the proteins encoded by the E1A regions of Ad5 and Ad12. No differences were observed, however, in the levels of expression of the EJ *ras* oncogene in the two cell types (Fig. 4A) or in those of the E1A regions of Ad5 and Ad12. The fact that expression of the E1A regions was some 10-fold lower than that in cells transformed by an intact region E1 (Fig. 3, 4B) was most likely caused by the absence of region E1B, which appears to be required for efficient expression of the E1A region (36). Recent experiments in our lab and in others have indicated that E1B can indeed directly enhance the expression level of E1A (32; unpublished observations). The Ha-*ras* oncogene product is apparently unable to perform this activity. The only plausible alternative explanation is that the difference in oncogenicity between the two types of E1A plus *ras*-transformed cells is a consequence of differences in the functional activities of the Ad5 and Ad12 E1A proteins. One of the best-documented activities of region E1A is the capacity to influence the expression of both viral and cellular genes (2, 13, 25, 26).

TABLE 2. Oncogenicity of E1A plus EJ *ras*-transformed cells

Plasmids transforming cell lines	No. of cell lines tested	Oncogenicity[a] and tumor formation (days)[b] in:			
		Nude mice	Syngeneic rats	Allogeneic rats	
				BN	ACI
pAd5E1A + pEJ6.6	3	15/15 (15)	9/9 (10)	0/9[c]	0/9[c]
pAd12E1A + pEJ6.6	3	5/15 (45)	2/9 (90)	0/9	0/9

[a] Indicated as the ratio of the number of animals with tumors to the number of animals injected. Cells (10^7) in phosphate-buffered saline were injected subcutaneously.

[b] Average latency period.

[c] Tumors of 0.5 to 1 cm in diameter appeared within 1 week but regressed in the next 10 days.

Evidence has been presented suggesting that the E1A proteins of Ad5 and Ad12 differ in their capacity to modulate gene expression. This was reported by Bos and ten Wolde-Kraamwinkel (5), who found that Ad5 E1A is four to five times more potent in activating the Ad12 E1B promoter than Ad12 E1A. A further difference between these proteins is that the E1A product of Ad12, but not that of Ad5, has the capacity to reduce the expression of class I MHC antigens (29). These functional differences between Ad5 and Ad12 E1A regions may be relevant for the interpretations of our present results, since E1A not only has a role in the immortalization of primary cells but also plays a crucial part in conferring oncogenicity upon transformed cells. This latter activity of E1A was first demonstrated by the use of E1A mutants which retained the capacity to transform primary cells and to express region E1B but nevertheless were nononcogenic (4, 27).

The mechanism by which E1A brings about the changes in

primary cells that eventually lead to immortalization and transformation is unknown. As proposed previously, an attractive hypothesis is that these phenotypical changes are the result of E1A-induced alterations of the expression of one or more cellular genes (20, 38), and thus the difference in oncogenicity between the two types of E1A plus *ras*-transformed cells may be explained by differences in the capacity of the E1A proteins of Ad5 and Ad12 to modulate gene expression.

We have previously reported that the oncogenicity in nude mice of cells transformed by region E1A plus E1B is determined to a large extent by the origin of the E1B region. When E1B is derived from oncogenic Ad12, the transformed cells are highly oncogenic in nude mice, whereas when E1B is derived from Ad5, the cells are weakly oncogenic (3). The origin of the E1A region has only a minor influence on this property. This result appears to be in contradiction with our present observations showing that the degree of oncogenicity of E1A plus *ras*-transformed cells is determined by the nature of the E1A region, with Ad5 E1A conferring higher oncogenicity than Ad12 E1A. Since the expression levels of the E1A region and of *ras* are approximately the same in these transformed cells, the differences in oncogenicity must be ascribed to differences in the biological activities of the E1A regions, e.g., their capacities to modulate cellular gene expression. It is possible that these differences become apparent only in combination with *ras*, when the E1A regions are expressed at approximately 10-fold-lower levels than in the presence of an E1B region. If this interpretation is correct, it would predict that Ad12 E1A plus *ras*-transformed cells in which the E1A region is expressed at a high level will be highly oncogenic. Unfortunately, we have never succeeded in obtaining transformed primary cells with a high expression level of E1A in the absence of E1B, not even when the E1A coding region was regulated by a strong heterologous promoter. An alternative explanation is that the difference in oncogenicity of the cells is not a direct result of the different capacities of the E1A regions to modulate cellular gene expression but is determined by the ability of the E1A regions to cooperate with a second oncogene, in our case either E1B or *ras*. Cooperation could take place, e.g., at the level of protein-protein interactions. Ad5 E1A would then be more effective in collaborating with *ras* than would Ad12 E1A, which could explain why Ad5 E1A plus *ras* cells are more highly oncogenic than Ad12 E1A plus *ras* cells are.

A limited cooperation in oncogenic transformation was also reported for the activated human c-*myc* and Ha-*ras* oncogenes. Primary cells transformed by this combination of genes could only produce tumors that grew to a certain static size and did not kill the animals. Our present results are thus in agreement with the two-step transformation model proposed by Land et al. (22) and Ruley (28), although *myc* and Ad12 E1A do not seem to be such active members of the family of immortalizing genes as Ad5 E1A.

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