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An Ovine Adenovirus Vector Lacks Transforming Ability in Cells That Are Transformed by AD5 E1A/B Sequences

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Adenoviruses of the Mastadenovirus and Aviadenovirus genera are able to transform certain cell types and induce tumor formation in susceptible animals. For the mastadenoviruses the E1A/B sequences are largely responsible for these properties but E4 sequences may also be involved. The transforming sequences of the aviadenoviruses, which lack E1A/B and E4 homologues, have not yet been fully identified. The recent proposal for a third genus of adenoviruses, which apparently lack an E1A homologue and have weak E1B homology, prompted an examination of the transforming properties of ovine adenovirus OAV287 (OAV), the prototype member of the new group. When OAV and human adenovirus type 5 (Ad5) were used to infect primary rat embryo cells, transformed foci developed in Ad5- but not in OAV-infected cultures. Similarly, after plasmid transfection, baby rat kidney cells were transformed by Ad5 E1A/B but not by OAV sequences. When CSL503 cells, an ovine cell line that is permissive for OAV, were transfected with Ad5 E1A/B sequences, transformed foci again appeared. However, plasmids or fragments containing complete or partial OAV genome sequences did not detectably transform CSL503 cells under the same conditions. When Ad5 E1A/B sequences were incorporated into the complete OAV genome and transfected, transformed clones were again obtained, showing that the gene dosage and transfection conditions were not limiting for transformation. The provision of Ad5 E1A and OAV sequences in combination marginally increased the number of morphologically altered foci in baby rat kidney cells but failed to induce multilayered focus formation. The data suggest that OAV lacks transforming functions in the cell types examined. Additional information suggesting that OAV may have a fundamentally distinct strategy for replication compared with other Ads is discussed.

INTRODUCTION

Many adenoviruses (Ads) are known to carry oncogenes. Members of the mastadenoviruses, including human and animal Ads, as well as CELO, a member of the aviadenoviruses, can readily transform cells in culture (Anderson *et al.,* 1969a,b; Darbyshire, 1966; Kinjo *et al.,* 1969; McAllister *et al.,* 1969; Trentin *et al.,* 1962). However, these viruses differ in their ability to induce tumor formation in animals. CELO virus rapidly induces tumors in newborn rodents (Sarma *et al.,* 1965). Among human Ads, the group A viruses such as Ad12 are highly oncogenic, while the group C (including Ad5) and E viruses are not known to be tumorigenic (reviewed in Flint, 1980; Horwitz, 1990; Shenk, 1996).

The transforming properties of the mastadenoviruses reside primarily in the E1A and E1B genes at the left end of the genome. These sequences are always present and expressed in transformed cells; transfection of these genes is sufficient to transform cells, and mutations in these genes abolish their transforming ability (reviewed in Flint, 1980; Horwitz, 1990; Shenk, 1996). The Ad2/5 E1A products bind to proteins of the cellular retinoblastoma (pRb) protein family (Whyte *et al.,* 1988), thereby releasing E2F transcription factors that regulate cell cycle progression into S phase (Nevins, 1992). The E1B 55-kDa protein binds to the tumor suppressor protein p53 and blocks p53-mediated apoptosis (White, 1998). The E1B 19-kDa protein is also anti-apoptotic (Rao *et al.,* 1992). Within the E4 region the ORF1 product of human Ad9 (group D) virus has oncogenic properties (Javier *et al.,* 1992; Thomas *et al.,* 1999). The Ad5 E4 ORF3 and ORF6 products also augment transforming activity of the E1A and E1B genes (Moore *et al.,* 1996; Nevels *et al.,* 1997, 1999a,b). The identity of the oncogenic genes in CELO virus is less clear. There are no identifiable E1A/B or E4 regions in the genome (Chiocca *et al.,* 1996), but recently two proteins, GAM-1 and ORF22, that interact with pRb were identified (Lehrmann and Cotten, 1999). GAM-1 may also functionally substitute for the E1B 19-kDa protein (Chiocca *et al.,* 1997).

Recently, based on phylogenetic analyses (Dan *et al.,* 1998; Harrach *et al.,* 1997) and the distinctive genome arrangement of newly characterized viruses (Hess *et al.,* 1997; Vrati *et al.,* 1996a), the existence of a third genus, Atadenovirus, has been proposed (Benko and Harrach, 1998). Members of this group include ovine adenovirus OAV287 (OAV) and egg drop syndrome virus-76 (both fully

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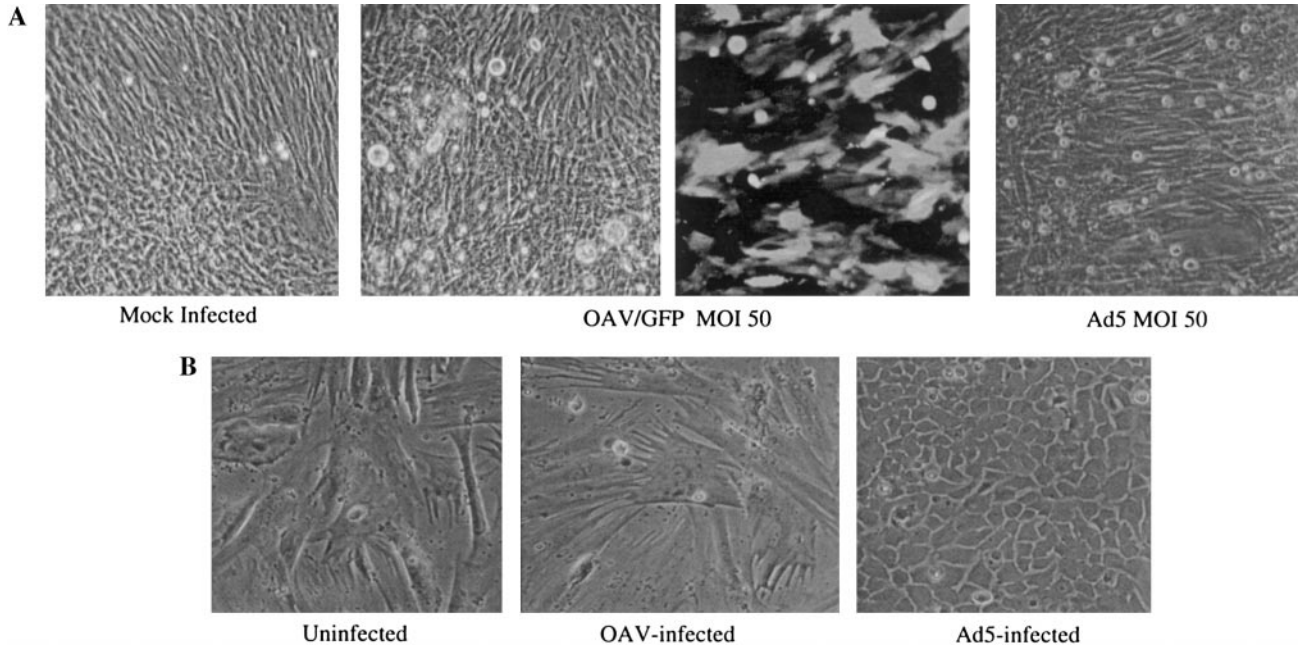

FIG. 1. Infection of primary rat embryo cells with Ad5 or OAV217A. (A) Cells were mock-infected or infected with Ad5 or OAV217A viruses at an m.o.i. of 50 PFU/cell. The phase or fluorescence (third from left) images of the cells are shown at day 4 postinfection. No fluorescence was detected for uninfected cells. (B) Cells infected at m.o.i. 10 with OAV wild-type or Ad5 were cultured for up to 80 days. The cells in the right-hand panel were derived from an Ad5 focus picked at 20 days and amplified.

sequenced) and bovine Ads type 4–8. OAV and EDS-76 lack an identifiable E1A region but at the left end of the genome two reading frames that show a low level of homology with the Mastadenovirus E1B proteins are present (Hess *et al.,* 1997; Vrati *et al.,* 1996a). The right-hand regions of these genomes contain open reading frames (ORFs) that show strong homology with a motif that is found in E4 ORF6 of mastadenoviruses (Vrati *et al.,* 1996a). There are also additional ORFs whose function is unknown. The ability of bovine Ad types from this group to cause tumors *in vivo* has been examined. In one study tumor formation was reported in hamsters inoculated with Ad type 8 (Rondhuis, 1973). In the other study, none of Ads types 4–10 induced tumor formation (Mohanty, 1971). Thus, the oncogenic potential of individual members of the atadenoviruses is unclear. However, as current OAV vectors carry gene insertions and retain their full complement of viral genes, their transforming ability is of interest from both practical and fundamental viewpoints. Our intention is to use OAV vectors to deliver enzyme/prodrug gene therapy to cancer cells, in the first instance. In this regime, OAV-infected cells are killed when a prodrug is converted to the active form by an enzyme that is encoded by the therapeutic gene. This strategy provides a further safeguard against an undesirable outcome related to expression of viral genes.

In this work, we have investigated the ability of OAV sequences to transform primary rat cells and the sheep lung cell line CSL503 *in vitro.* The data indicate that neither cell type is transformed by OAV sequences under conditions where Ad5 E1A/B sequences induce multilayered, transformed focus formation.

RESULTS

Infection and transfection of primary rat cells by Ad5 and OAV

Primary rat embryo cells, in which Ad replication is semipermissive, have been used as one benchmark assay for transforming ability. To investigate whether OAV could transform such cells, 10^5 cells seeded at near confluence in 24-well plates were infected at a multiplicity of infection (m.o.i.) of 50 PFU/cell with OAV217A, a recombinant that expressed the GFP protein from the HCMV promoter. Cells were similarly infected with Ad5 as a positive control and initially observed for 4–6 days. During this time most cells in the OAV-infected monolayers produced GFP, showing that they were infected. For both the OAV and Ad5 infections, there were only a small number of rounded cells by day 4 (Fig. 1A) and this limited cytopathic effect (cpe) did not significantly progress by day 6 (data not shown). Moreover, when 5% of day 7 supernatant from these OAV217A-infected cultures was passaged on fresh primary rat embryo cells, few green cells were seen and no cpe was observed by day 14. Day 14 supernatant (5%) produced no detectable fluorescence after an additional passage. Thus, OAV did not replicate in these cells. Cells were also infected at an m.o.i. of 20 PFU/cell with Ad5 or OAV wild-type and Hirt DNA was prepared at 4 days postinfection (p.i.) (White *et*

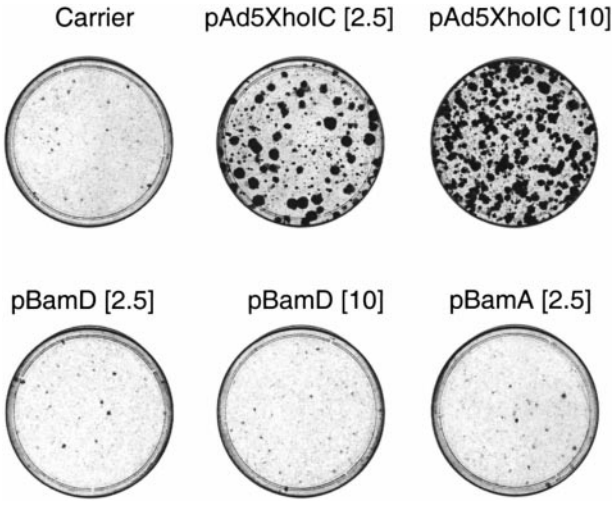

FIG. 2. Transformation of baby rat kidney cells with Ad5 E1A/B or OAV sequences. Cells were transfected with the indicated plasmids or salmon sperm carrier DNA. The numbers in brackets correspond to the microgram amounts of transfected plasmid DNA. Cells were stained with crystal violet 21 days posttransfection, and one representative plate for each transfection from one of several independent experiments (Table 1) is shown.

al., 1984). As shown by analysis of *Xba*I-digested DNA, and consistent with earlier observations (Gallimore, 1974), there was limited replication of Ad5 DNA during this time. Similar analysis of *Nhe*I-digested DNA showed that the OAV genome was not significantly amplified (data not shown). Other cells that survived infection with Ad5 or OAV wild-type at an m.o.i. of 10 were cultured. By ~40 days several morphologically distinct, flat epithelioid colonies grew in the Ad5-infected cultures (Gallimore, 1974) (Fig. 1B), but not in the OAV-infected cultures. These colonies were picked and amplified and DNA was extracted and tested for the presence of E1A and E1B sequences by PCR analyses. Whereas control reactions were negative, PCR products of 2.1 and 2.4 kb were amplified for E1A and E1B sequences, respectively, as expected (data not shown), consistent with the apparent transformed phenotype of the colonies. In contrast, in three similar experiments, no transformed foci were observed among the cells that survived infection with OAV, although these were maintained in parallel with the Ad5-infected cultures for up to 80 days (Fig. 1B).

Similarly, when baby rat kidney cells were transfected with pAd5XhoIC expressing E1A/B sequences, multilayered, transformed foci readily appeared (Fig. 2 and Table 1, lines 2 and 3). However, cells transfected alone or in combination with OAV plasmids pBamD and pBamA (containing ORFs for all nonstructural proteins except IVa_2) did not produce such foci (Fig. 2 and Table 1, lines 4–8). Collectively, these observations suggested that OAV sequences were not capable of transforming rat cells, or at least, transformed them with a much lower efficiency than Ad5 oncogenes.

Transformation of CSL503 cells

The ability of OAV sequences to transform CSL503 cells was examined next because such cells are permissive for OAV replication and the viral promoters must therefore function. It was also necessary to determine whether Ad5 sequences could transform these cells and thus serve as a positive control.

With Ad5 E1A/B sequences. CSL503 cells were transfected with linear Ad5 sequences that included the E1A and E1B regions together (pE1-FR) or E1A sequences alone (pE1AdeIB). Cell monolayers were transfected with 4.5, 5.5, 6.5, or 7.5 μg of DNA and split 10 days later. Foci were first visible at ~day 14. Cells that were mock-transfected did not produce foci. A total of 75, 165, 170, and 182 foci of transformed cells developed, respectively, by 28 days posttransfection. In contrast, cells transfected with the same amounts of pE1AdeIB produced 1, 0, 9, and 1 clones, respectively, that were morphologically distinct from surrounding cells but flat, rather than multilayered. These grew poorly when subcloned for expansion. Several of the E1A/B-transformed clones (Fig. 3A)

TABLE 1

Multilayered Focus Formation after Transfection of Primary, Baby Rat Kidney Cells with Ad5- or OAV-Derived Plasmids

Line	Plasmids transfected	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 6	Expt. 7	Expt. 8
1	Carrier	0[a]	0	0	0	0	0	0
2	pAd5XhoIC (2.5)[b]	5	41	37	60	7	22	11
3	pAd5XhoIC (10)	nd	nd	42	155	35	nd	nd
4	pBamD (2.5)	nd	nd	0	0	0	0	nd
5	pBamD (10)	nd	nd	0	0	0	nd	nd
6	pBamA (2.5)	nd	nd	nd	nd	0	nd	0
7	pBamD (2.5)/pBamA (1)	nd	nd	nd	nd	0	nd	0
8	pBamD (2.5)/pBamA (2.5)	nd	nd	nd	nd	0	nd	0

Note. Expt., experiment. nd, not determined.

[a] Average number of transformed foci per 100-mm dish.

[b] Number in parentheses indicates the amount of transfected plasmid in micrograms.

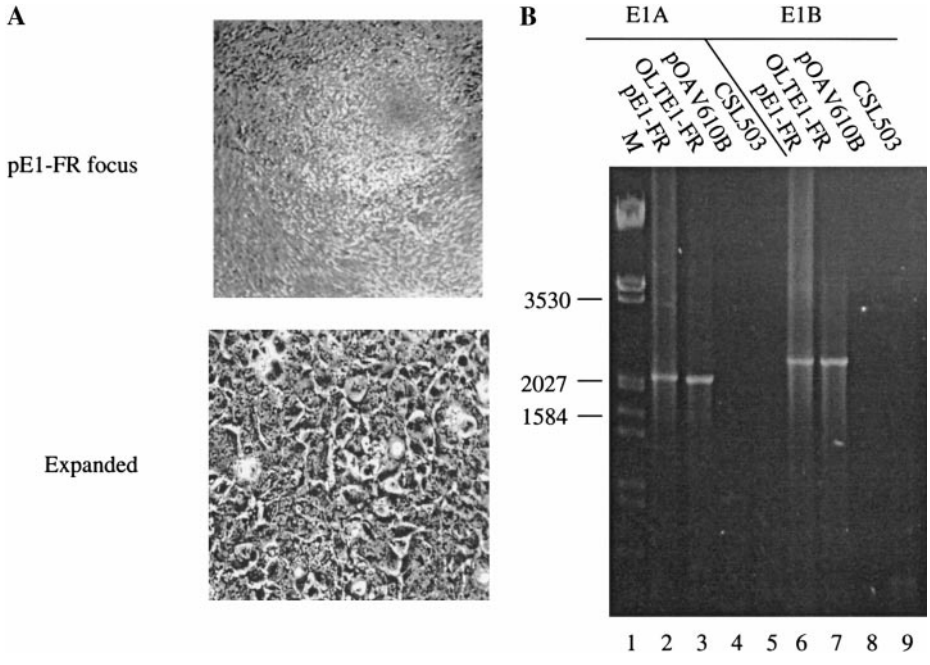

FIG. 3. Analysis of CSL503 cell clones transfected with Ad5 E1/AB sequences. (A) Morphology of transformed foci before and after expansion. (B) PCR analysis of DNA recovered from CSL503 cells transformed with pE1-FR. E1A and E1B sequences were amplified using primer pairs a–b (lanes 2–5) and c–d (lanes 6–9) (see Fig. 5A), respectively. pOAV610B and DNA from nontransfected CSL503 cells were used as controls.

were picked and expanded. Their morphology was distinct from the parental CSL503 cells (compare Fig. 3A, lower panel with Fig. 4A). DNA extracted from these cells was analyzed by PCR for the presence of Ad5 E1A/B sequences. Products of ~2.1 and ~2.4 kb were expected and observed for the E1A and E1B regions, respectively (Fig. 3B). Thus, the production of these clones indicated that there was no barrier to transformation of CSL503 cells.

With the left end of OAV. OAV pBamD encodes ORFs LH1, LH2, and LH3 on the *r*-strand and the structural protein p32k on the *l*-strand of the genome (Khatri and Both, 1998) (Fig. 5B). LH2 and LH3 may be distant homologues of the Ad5 E1B proteins, but LH1 is unique to OAV (Khatri and Both, 1998; Vrati *et al.,* 1996a). In an attempt to produce cells that were similar in principle to Ad5-transformed 293 cells (Graham *et al.,* 1977), a variety of transfection conditions were used to introduce pBamD sequences into CSL503 cells together with the neomycin-resistance gene cassette. However, very few G418-resistant colonies grew well. From four experiments a total of six colonies were transferred to 96-well plates but of these, only two (clones 1 and 2) grew well enough to allow stocks to be frozen down. The morphology of these clones was similar to that of CSL503 cells (Fig. 4A). These lines were thawed and reestablished in culture. DNA and RNA were then extracted from each line and analyzed by PCR. Primer pairs P1/P2 and P3/P4 (Fig. 5B) produced the expected products of 2147 and 1262 bp, respectively, from pBamD (Fig. 4B, lanes 4 and 8) and clone 1 and clone 2 DNA (Fig. 4B, lanes 2 and 6 and lanes 3 and 7, respectively), but not from control CSL503 DNA (Fig. 4B, lanes 1 and 5). Analysis of RNA extracted from OAV-infected CSL503 cells using RT-PCR and primer pairs AK3/AK7 and AK3/AK10 (Fig. 4C) produced the expected products of ~370 and ~435 bp, respectively (Fig. 4C, lanes 3 and 7), that were not seen in uninfected cells (Fig. 4C, lanes 2 and 6). These transcripts appear to code for products of ORFs LH1 and LH2. The same products were derived from clone 1 and clone 2 RNA (Fig. 4C, lanes 4 and 5 and lanes 8 and 9). The unspliced transcript that codes for LH3 (Fig. 5B) was also detected in clone 2 RNA (data not shown). Thus, these two clones contained integrated sequences that represented the complete left end of OAV and expressed transcripts for at least two of the three nonstructural ORFs. However, they did not grow well or have a transformed appearance (Fig. 4A).

With the other OAV sequences. Sequences in addition to the left end of the OAV genome may be required for transformation. Although it was not originally intended, a *de facto* assessment of the transforming ability of OAV in CSL503 cells was provided by the procedure used to generate recombinant viruses. CSL503 cells were transfected with linear OAV DNA using lipofectamine and incubated for up to 4 weeks. The development of cpe indicated virus rescue (Vrati *et al.,* 1996b; Xu *et al.,* 1997). Although many OAV recombinants have now been rescued in our laboratory, over 100 dishes of cells (60 mm) that were transfected never developed cpe and no multilayered, transformed foci were observed. Additional ex-

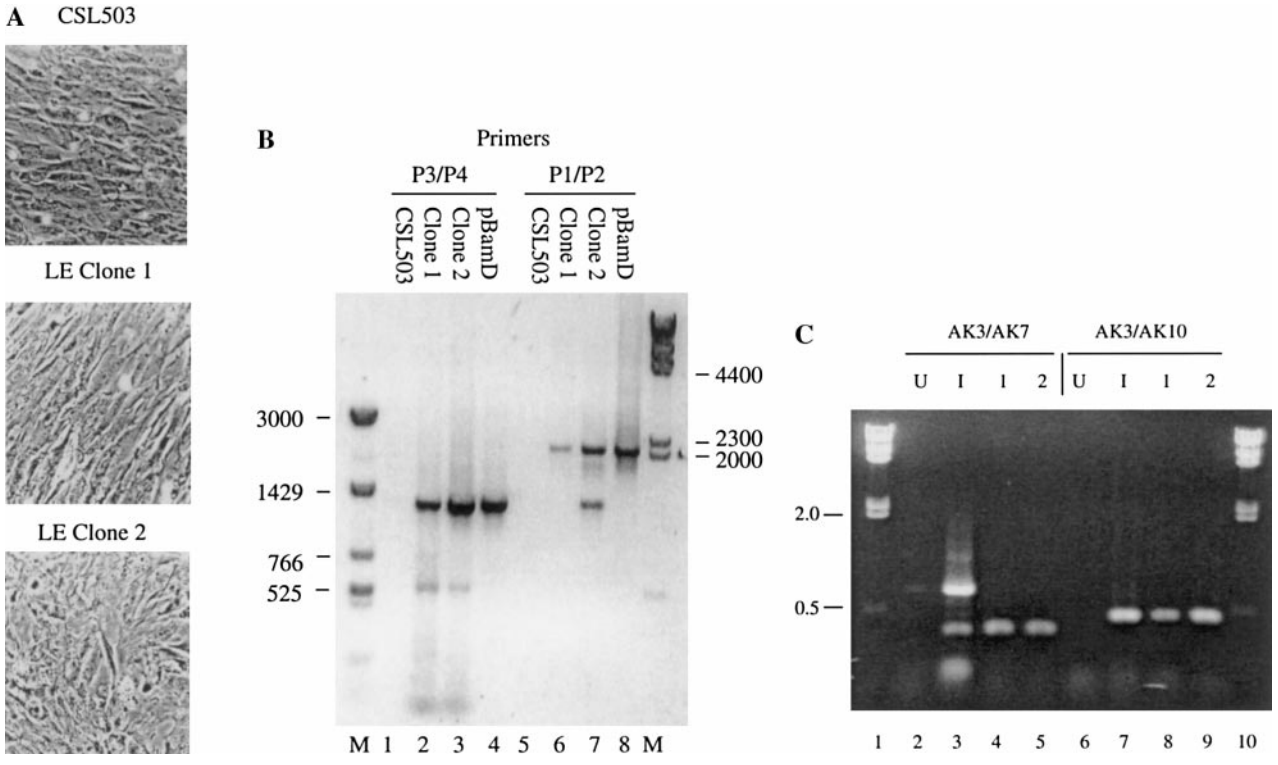

FIG. 4. Analysis of CSL503 cell clones transfected with OAV left end sequences. (A) Morphology of CSL503, clone 1, and clone 2 cells. (B) PCR analysis of CSL503, clone 1, clone 2, and pBamD DNA using primer pairs P3/P4 (Fig. 5B) (lanes 1–4) and P1/P2 (lanes 5–8). (C) RT-PCR analysis of transcripts from uninfected (U), OAV-infected (I) CSL503 and clone 1 and clone 2 cells using primer pairs AK3/AK7 (lanes 2–5) and AK3/AK10 (lanes 6–9), as indicated. Lanes 1 and 10 contained markers of the sizes indicated.

periments to assess whether this was due to inefficient transfection are described below.

In case the integration efficiency of the intact OAV genome into the chromosomes was too low to induce transformation, the viral genome was digested with *Bam*HI/*Kpn*I to generate genome fragments of 4–8 kb. *Kpn*I released the intact, linear genome while *Bam*HI cut internally five times such that the nonstructural regions (except for IVa_2) remained intact (Vrati *et al.,* 1996a,b). *Kpn*I/*Bam*HI fragments were transfected into CSL503 cells (4 × 60-mm dishes; 3.4 μg DNA/5 × 10^5 cells). Alternatively, in case potentially cooperative OAV sequences were separated by *Bam*HI digestion, pOAV169 (~15 kb) (lacking ORFs for structural proteins; Fig. 5) was digested with *Kpn*I only and transfected under the same gene dosage conditions. The cells were observed and the medium was changed weekly. However, no multilayered, transformed foci had appeared by 56 days although occasional flat, morphologically distinct foci, reminiscent of those seen with pE1AdelB, were seen.

With Ad5 E1A/B sequences in an OAV plasmid. To distinguish between transformation-deficient OAV sequences and factors relating to gene dosage and/or transfection efficiency, hybrid plasmids in which Ad5 E1A/B sequences were incorporated into the OAV genome were used to transfect CSL503 cells under conditions used for OAV rescue. Ad5 E1A/B sequences were inserted into the OAV genome between the pVIII and the fiber genes (pOAV219) (Fig. 5) or into a site ~1 kb from the right end of the genome (pOAV619). A plasmid (pOAV610B) carrying unrelated sequences was used as a control. These plasmids were partially digested with *Kpn*I to release at least one end of the linear genome without cutting the internal site within the E1B gene. DNAs were transfected with lipofectamine using standard conditions. Although this could have resulted in rescue of recombinant OAV/Ad5 hybrid viruses, in two experiments no cytopathic effect developed in any dish of transfected cells. However, transformed foci with morphology similar to that of pE1-FR-transformed cells developed in dishes transfected with pOAV219 and pOAV619 (Table 2) but not with the control plasmid pOAV610B. More foci were observed with pOAV619 where the Ad5 sequences were incorporated near one end of the OAV genome. Cells transfected with pOAV619B, in which only the Ad5 E1A sequences were intact, produced a few morphologically distinct foci but these were not multilayered.

Ovine lung transformed OLT219 and OLT619 colonies were picked and amplified. Their morphology was clearly distinct from CSL503 cells (Fig. 6A) and similar to that of the cells transformed by E1A/B sequences (Fig. 3A). DNA from the expanded cells was analyzed by PCR. E1A and E1B sequences were detected in OLT619 DNA (Fig. 6B,

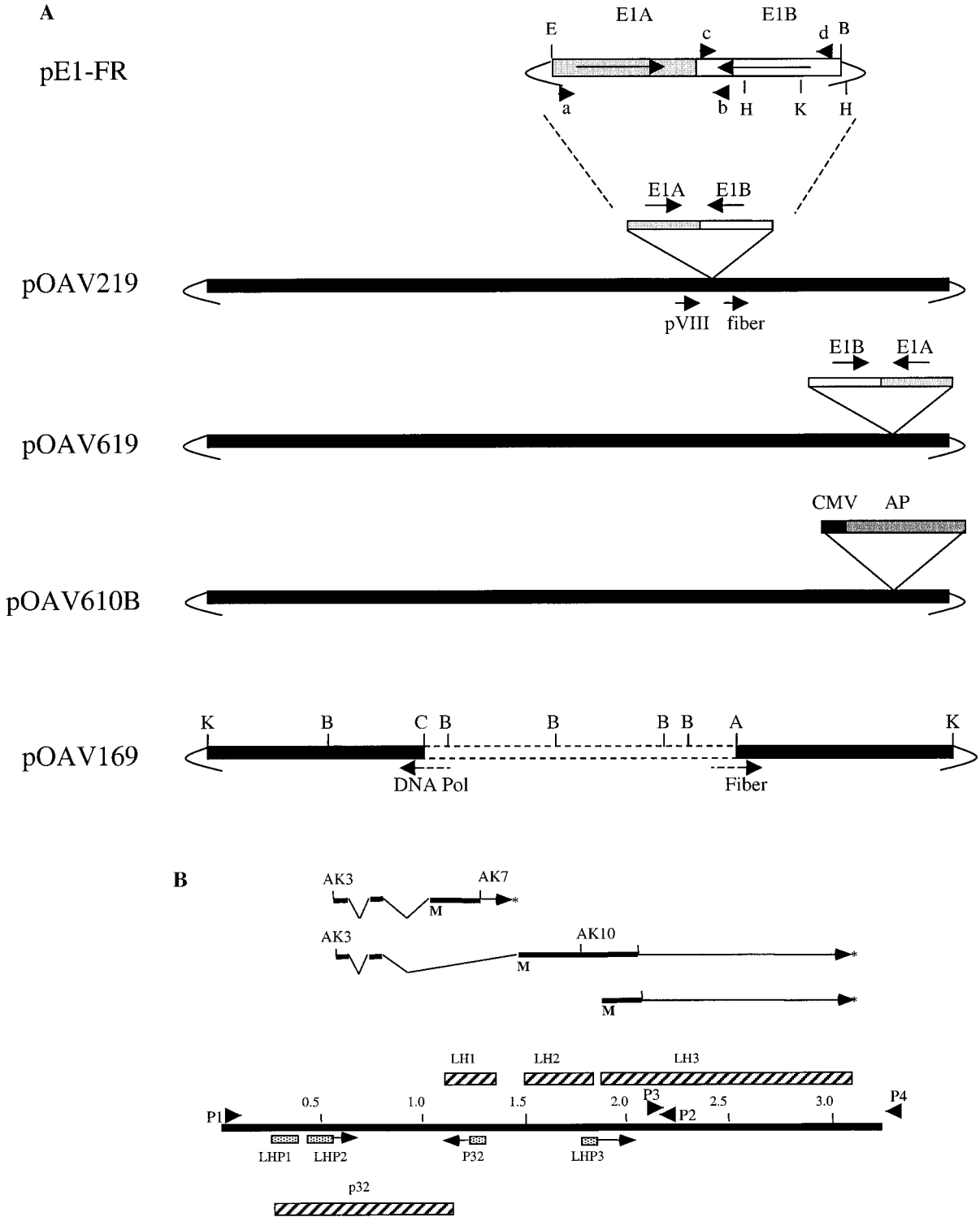

FIG. 5. Structure of plasmids and transcripts. (A) pE1-FR contains the Ad5 E1A/B regions in an inverted orientation. pE1AdelB was derived by *Hin*dIII digestion and religation. pAd5E1A is a similar plasmid (not shown) derived from pXhoIC. The orientation of PCR primers a–d is indicated. Restriction enzymes sites for *Bam*HI (B), *Hin*dIII (H), *Kpn*I (K), *Cla*I (C), and *Age*I (A) are indicated. Solid bar indicates OAV genome sequences. Dashed lines in pOAV169 indicate the deleted region. (B) Structure of the left end of the OAV genome. The open reading frames (striped bars), promoter regions (stippled boxes), key transcripts (top), and relevant RT-PCR primers (AK3, AK7, and AK10) (Khatri and Both, 1998) are shown. OAV genome sequences (black bar, scale in kilobases) were analyzed by PCR primers P1–P4.

TABLE 2

Appearance of Transformed Foci after Transfection of CSL503 Cells with Hybrid OAV Plasmids

DNA	5[a]	6	7	8
Plasmid				
Experiment 1				
pOAV219	0[b]	0	2	3
pOAV619	0	1	5	6
pOAV610B	0	0	0	0
Experiment 2				
pOAV219			2	3
pOAV619	0	2	4	4
pOAV619B	0	0	1[c]	1[c]

[a] μg DNA used for transfection.

[b] Total number of transformed foci in two 60-mm dishes of cells after 23 days.

[c] Cells morphologically different from E1A/B transformed foci.

lanes 3 and 7) but not in the control pOAV610B or DNA from CSL503 cells or (Fig. 6B, lanes 4 and 5). Identical results were obtained on analysis of OLT219 cell DNA (Fig. 6C, lanes 3 and 7). Clearly therefore, CSL503 cells were transformed by Ad5 E1A/B sequences, whereas, under the same conditions of gene dosage and transfection, OAV sequences were never observed to achieve this result.

Cotransfection of Ad5/E1A and OAV sequences

As OAV lacks an identifiable E1A function and apparently cannot transform cells, we tested whether the combinations of Ad5 E1A and OAV sequences could cooperate to induce the appearance of transformed foci. CSL503 cells were transfected with pE1-FR or pE1AdelB plus various OAV plasmids and the appearance of multilayered foci was monitored. pBamC was used to adjust DNA levels between experiments. However, foci were observed only when pE1-FR, the Ad5 E1A/B control plasmid, was used. Combinations of Ad5 E1A sequences with OAV pBamD (left end) or pOAV169 (all the nonstructural coding sequences) failed to induce multilayered colony formation (Fig. 7). When baby rat kidney cells were transfected with combinations of Ad5 E1A and the left end clone (pBamD) of OAV a small increase in the number of transformed colonies was observed compared to transfections with E1A alone (Fig. 8). However, this increase may not be significant and these colonies did not exhibit the multilayered morphology that was typical of foci transformed by E1A/B sequences.

DISCUSSION

A large body of work conducted over many years has investigated the oncogenic properties of adenoviruses. It is now clear that the mastadenoviruses, which include all known human Ads, contain several groups of viruses whose tumor-inducing properties differ, although they all share an ability to transform susceptible cells during nonlytic infection (reviewed in Flint, 1980; Horwitz, 1990). In addition, mastadenoviruses of animal origin can also transform certain cell types or cause tumors (Darbyshire, 1966; Mittal *et al.,* 1995; Panigrahy *et al.,* 1976; Tsukiyama

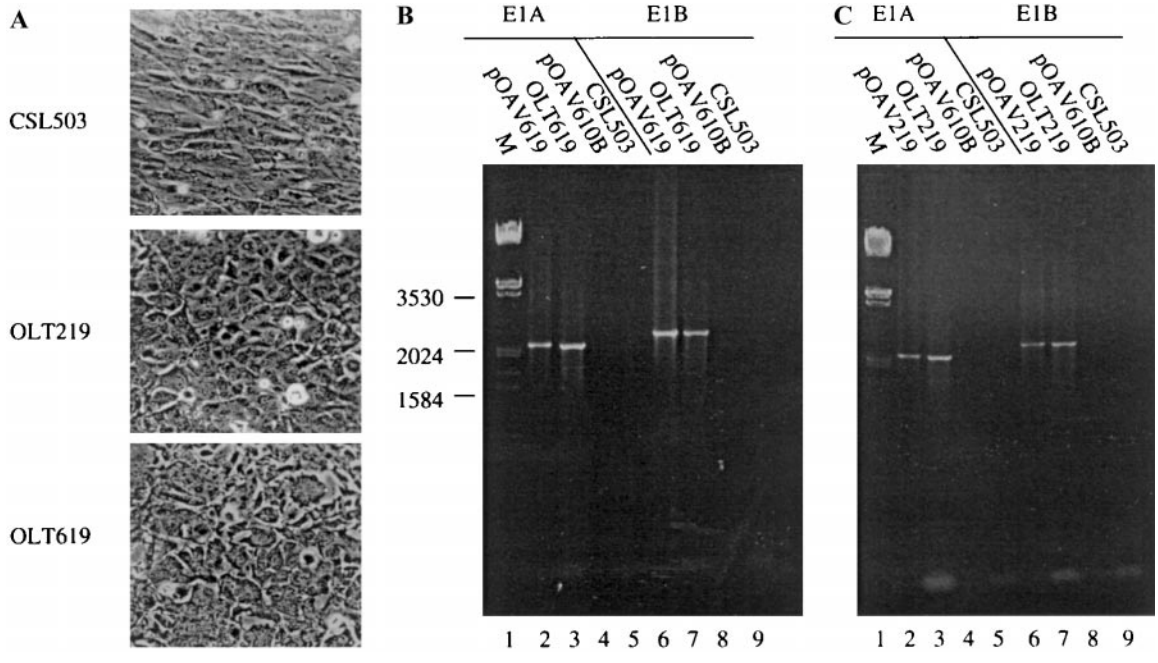

FIG. 6. Analysis of OLT619 and OLT219 clones. (A) Morphology of clones transformed with pOAV219 and pOAV619 plasmids. Primers pairs a–b and c–d (Fig. 5) representing E1A and E1B sequences, respectively, were used for amplification of OLT619 (B) and OLT219 (C) DNAs, respectively. pOAV610B and DNA from nontransfected CSL503 cells were used as controls. Lanes M contain markers of the sizes indicated.

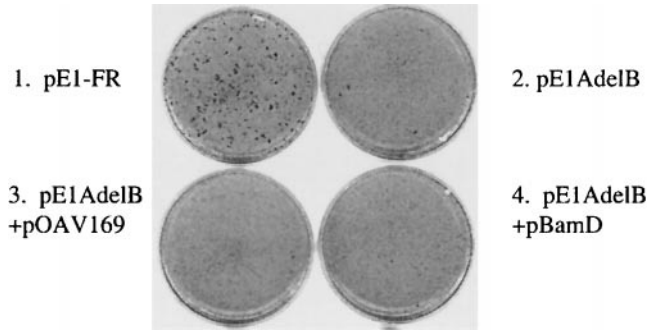

FIG. 7. Transformation of CSL503 cells with Ad5 E1A plus OAV sequences. Cells were transfected with (1) pE1-FR (5 μg) + pBamC (5 μg), (2) pE1AdelB (5 μg) + pBamC (5 μg), (3) pE1AdelB (6 μg) + pBamD (6 μg), or (4) pE1AdelB (5 μg) + pOAV169 (5 μg). Cells were split 1:2 at day 13 and stained with crystal violet at day 35 posttransfection.

et al., 1988). Similarly, CELO virus, a member of the aviadenoviruses, can induce tumors in rodents (Sarma *et al.,* 1965) and transform heterologous cells (Anderson *et al.,* 1969a,b; Kinjo *et al.,* 1969; McAllister *et al.,* 1969; Trentin *et al.,* 1962). There is only one unconfirmed report of tumor induction in hamsters by bovine Ad8 (Rondhuis, 1973), a member of the proposed atadenovirus group (Benko and Harrach, 1998). However, in an earlier study using a related Ad8 isolate, tumor formation in hamsters was not observed (Mohanty, 1971). The difference may be due to the smaller amount of virus that was used for inoculation in the first study (Mohanty, 1971).

OAV is the prototype member of the proposed third genus of adenoviruses (Benko and Harrach, 1998; Harrach and Benko, 1999; Harrach *et al.,* 1997) and is being developed as a potential gene delivery vector. Current vectors carry an expression cassette at a nonessential site in the genome while retaining the full complement of viral genes (Khatri *et al.,* 1997; Vrati *et al.,* 1996b; Xu *et al.,* 1997). Thus, the issue of whether this virus has transforming ability is of interest from a fundamental viewpoint and from a safety perspective. Many ORFs and transcripts of the OAV genome have been defined (Khatri and Both, 1998; Vrati *et al.,* 1996a) but the proteins have not been identified. As many of the ORFs encoding nonstructural proteins have no obvious homology with other Ad sequences, their functions are unknown. OAV may have homologues of the Mastadenovirus E1B and E4 coding sequences but apparently it has no E1A gene. However, this is also true for CELO (Chiocca *et al.,* 1996), a virus that has both transforming and tumorigenic activities (Anderson *et al.,* 1969a,b; Darbyshire, 1966; Kinjo *et al.,* 1969; McAllister *et al.,* 1969; Sarma *et al.,* 1965; Trentin *et al.,* 1962). The situation for bovine Ad8 is unknown because its sequence has not been determined.

To ascertain whether OAV has oncogenic properties, we investigated its ability to transform cells that have been used in similar studies with other viruses. Primary rat embryo cells were infected in parallel with OAV or Ad5, a virus that is known to carry transforming sequences (reviewed in Flint, 1980; Horwitz, 1990). Both Ad5 and OAV apparently infected primary rat embryo cells but only after Ad5 infection were clones of distinct morphology observed to grow out from surviving cells. PCR analysis showed that these carried Ad5 E1A/B sequences, as expected. Similarly, Ad5 E1A/B sequences transformed baby rat kidney cells following transfection while sequences from the left end of the OAV genome did not. The trivial explanation, that OAV promoters did not function in rat cells, seems unlikely as they do function in other animal and human cell types (Khatri *et al.,* 1997).

To rule out the trivial explanation, OAV sequences were transfected into the sheep fetal lung cell line, CSL503 (Pye, 1989), in which OAV is known to replicate (Boyle *et al.,* 1994). This situation mimicked the transfection of human embryonic kidney cells with sheared Ad5, a strategy that produced the transformed 293 cell line (Graham *et al.,* 1977). Several approaches to transform CSL503 cells with OAV sequences were tried. Cell clones that were stably transfected with sequences from the left end of the genome were selected using neomycin, expanded, and frozen down. Upon revival two clones that carried the appropriate OAV genome sequences were identified and shown by RT-PCR to produce transcripts for at least two of the ORFs. Clone 2 also expressed the unspliced LH3 transcript. However, despite this stable expression, both clones grew poorly and were morphologically similar to the parental CSL503 cell line.

In case transformation required cooperation between sequences that were located in distant parts of the genome, CSL503 cells were transfected with a mixture of restriction fragments representing the entire genome or a plasmid in which the nonstructural genes were intact.

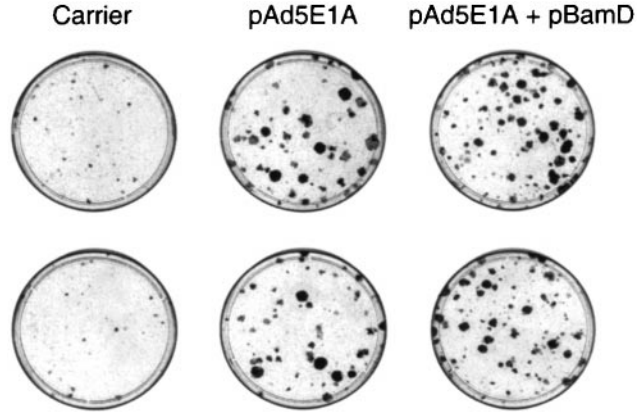

FIG. 8. Transformation of baby rat kidney cells with Ad5 E1A plus OAV sequences. Cells were transfected with the indicated plasmids (5 μg) or salmon sperm carrier DNA and stained with crystal violet 21 days posttransfection. Two representative plates for each transfection from one of two independent experiments are shown.

In addition, transfection of the entire OAV genome into CSL503 cells during many attempts at virus rescue provided a *de facto* assessment of the transforming ability of OAV sequences. With none of the above approaches did multilayered transformed foci appear in CSL503 cell monolayers. In contrast, transfection of CSL503 cells with Ad5 E1A/B sequences resulted in the appearance of many transformed foci that, upon expansion, were shown to carry Ad5 E1A/B sequences. Thus, there was no impediment to transformation of CSL503 cells. Importantly, when Ad5 E1A/B sequences were incorporated into OAV plasmids and introduced into CSL503 cells under transfection and gene dosage conditions identical to those used for virus rescue, transformed foci again appeared. The conclusion therefore is that OAV lacks sequences that are capable of transformation in the cell types that were tested. OAV sequences also failed to suppress transformation by Ad5 E1A/B sequences.

As OAV lacks identifiable E1A sequences, attempts were also made to provide this function using plasmids carrying the Ad5 E1A region. However, combinations of Ad5 E1A with the left end or the left plus right ends of the OAV genome (including all of the nonstructural ORFs) again failed to induce multilayered focus formation. Baby rat kidney cells transformed with Ad5 E1A plus OAV pBamD sequences did not display the multilayered morphology that is typical for E1A/E1B transformed foci. These results suggest that at best, sequences from the left end of OAV may cooperate with Ad5 E1A to enhance the efficiency with which morphologically altered cells arise without creating a fully transformed phenotype.

The apparent inability of OAV to transform cells in the way that Ad5 E1A/B sequences do is consistent with the absence of an E1A homologue or a substituting function in the OAV genome. The E1A protein binds the pRb family of proteins (Whyte *et al.,* 1988), thereby releasing E2F transcription factors. Promoters that control cell cycle progression into S phase as well as the Ad E1A and E2 promoters are activated (Kovesdi *et al.,* 1987; Nevins, 1992). Although CELO virus lacks E1A it also codes for proteins that bind pRb, thus modulating the E2F protein family (Lehrmann and Cotten, 1999). It is noteworthy therefore that the OAV E2 promoter region, whose sequence and approximate location have been determined (Khatri and Both, 1998; Vrati *et al.,* 1996a), lacks an E2F binding site of the type TTTc/gGCGCc/g (Zheng *et al.,* 1999). Nevertheless, the promoter is active in numerous cell lines although OAV replication is abortive (Khatri *et al.,* 1997). Thus, OAV interacts with the cellular machinery in a way that differs fundamentally from the mastadenoviruses and aviadenoviruses. Although many different cell lines are infected by OAV (Khatri *et al.,* 1997), the only known cell line in which OAV replicates to high titer is the fetal lung cell line CSL503 (Pye, 1989). Similarly, bovine Ad members of the proposed new genus show a strong preference for replication in primary cultures (Mohanty, 1971). Viruses in this group therefore seem to have a narrower host range for replication compared with other Ads, perhaps because they cannot induce the cell to enter S phase (bovine Ad8 is a possible exception). This warrants further investigation.

MATERIALS AND METHODS

Cells and viruses

Embryos were removed from a Copenhagen rat at ~10 days of gestation (Loo and Cotman, 1994), the heads were removed, and primary cells were prepared and maintained in DMEM plus 10% FCS. Baby rat kidney cells were prepared as previously described (Nevels *et al.,* 1997) and maintained in the same medium. The sheep fetal lung cell line CSL503 (Pye, 1989) was grown in EMEM plus 10% FCS. Human adenovirus type 5 was obtained from the ATCC. The source of the OAV isolate OAV287 has been previously described (Boyle *et al.,* 1994; Peet *et al.,* 1983). A recombinant, OAV217A, that expressed the GFP protein was also constructed. A humanized GFP gene (provided by Dr. Shinichi Aota, Biomolecular Engineering Research Institute, Japan) was blunt-cloned into the *Xho*I/*Sma*I sites of plasmid pCI (Promega Corp., Madison, WI). The cassette was subcloned as a *Bam*HI/*Bgl*II blunt-ended fragment into the *Xba*I site of pGem11zf (Promega). The intron in the cassette was removed by fusing the two *Afl*II sites. The cassette was then subcloned as an *Apa*I/*Not*I fragment into site I of plasmid OAV200 and the virus was rescued as described previously (Vrati *et al.,* 1996b). Ad5 and OAV were titrated by plaque assays on 293 or CSL503 cells, respectively (Graham and Prevec, 1991).

Plasmids

Plasmid pAd5XhoIC contains the left end of the Ad5 genome, including the E1A/B regions (Logan *et al.,* 1984). Plasmid pAd5E1A was derived from pAd5XhoIC by restriction digestion with *Bst*EII and religation, eliminating most of the E1B coding sequences. Plasmid pE1-FR (provided by Dr. R. I. Garver, Gene Therapy Program, University Alabama at Birmingham) also contains the Ad5 E1A and E1B sequences but in an inverted juxtaposed configuration (Fig. 5A). This plasmid was previously characterized and used to complement deleted E1A/B functions in replication-deficient Ad5 viruses (Dion *et al.,* 1996). Digestion with *Hin*dIII, followed by ligation, eliminated the E1B promoter and much of the E1B coding sequence, producing pE1AdeIB. Plasmids pBamD and pBamA (Boyle *et al.,* 1994) carry the left-hand ~4.25-kb and right hand ~8.7-kb *Bam*HI fragments of the OAV genome, respectively, in the *Bam*HI/*Hin*cII sites of Bluescribe M13+ (Stratagene, San Diego, CA). *Kpn*I sites were subsequently added at the ends of the genome in pBamA and pBamD (Vrati *et al.,* 1996b). pOAV169

was prepared from pOAV100, a plasmid containing the entire OAV genome in a modified Bluescribe plasmid (Vrati *et al.,* 1996b), by digestion with *Cla*I and *Age*I, followed by blunt-end repair and ligation (Sambrook *et al.,* 1989). These enzymes cut within ORFs for DNA polymerase and fiber, respectively, leaving nonstructural sequences intact while deleting all structural proteins (except for p32k). To construct pOAV219, Ad5 E1A/B sequences were excised from pE1-FR as an *Eco*RI/*Bam*HI fragment, blunt-end repaired, and subcloned into the blunted *Xba*I site of pGem11zf (Promega). The flanking *Apa*I/*Not*I sites were used to subclone the Ad5 sequences into the *Apa*I/*Not*I sites of pOAV200, located between the pVIII and the fiber genes (Vrati *et al.,* 1996b) (Fig. 5A). Similarly, the *Sal*I site of pOAV287Cm (~1 kb from the right end of the genome) was modified with a synthetic oligonucleotide containing *Sal*I/*Apa*I/*Not*I sites. The *Apa*I/*Not*I-flanked Ad5 sequences were also cloned into that plasmid to construct pOAV619 (Fig. 5A). pOAV619B contains only the pE1AdelB sequences in the same insertion site (not shown). pOAV610B is a control plasmid in which the HCMV/alkaline phosphatase cassette was inserted at the same site (Khatri *et al.,* 1997) (Fig. 5A).

Transfection of cells and transformation assays

Primary baby rat kidney cells were transfected with plasmids and assayed for focus formation as previously described (Nevels *et al.,* 1997, 1999b). OAV plasmids (~33 kb) were cut with *Kpn*I to release the linear genome and DNA (5–8 μg per 5×10^5 cells) was transfected into CSL503 cells using lipofectamine (Gibco BRL) (12–16 μl) (Vrati *et al.,* 1996b). Plasmids pE1-FR and pE1AdelB were made linear by *Sca*I digestion prior to transfection under similar conditions. To produce cell lines stably transfected with the OAV left end, pBamD was made linear with *Bam*HI and transfected into CSL503 cells using electroporation or $CaPO_4$ precipitation together with a plasmid (ratio 10:1) carrying the neomycin-resistance gene expressed from the thymidine kinase or phosphoglycerate kinase promoter. Cells were maintained in medium containing 200–300 μg/ml of G418. Neomycin-resistant clones that appeared were lifted into 96-well plates and expanded for further analysis.

PCR analysis

DNA was extracted from transfected cells by SDS/pronase treatment (White *et al.,* 1984). Primers for PCR amplification of DNA from the left end of OAV (Fig. 5B) corresponding to bases 72–93 (P1, +sense), 2198–2219 (P2, −sense), 2137–2159 (P3, +), and 3379–3400 (P4, −) were designed from the known sequence of OAV (GenBank Accession No. U40839). Transcripts from OAV promoters LH1 and LH2 were amplified by RT-PCR using primer pairs AK3/AK7 and AK3/AK10 (Khatri and Both, 1998) (primer details available on request). Primer pairs used to amplify Ad5 E1A sequences in pE1-FR corresponded to bases 30–53 (primer "a") and 3644–3666 (primer "b") of the Ad5 genome. Ad5 E1B sequences were amplified using primers corresponding to bases 1677–1691 (primer "d") and 4067–4090 (primer "c") (Fig. 5A).

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