Adenovirus Type 9 E4 Open Reading Frame 1 Encodes a Transforming Protein Required for the Production of Mammary Tumors in Rats

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The E4 region of human adenovirus type 9 (Ad9) transforms established rat embryo fibroblasts and encodes an essential determinant for the production of estrogen-dependent mammary tumors in rats. Testing of the seven Ad9 E4 open reading frames (ORFs) individually for transformation of the established rat embryo fibroblast cell line CREF indicated that only Ad9 E4 ORF1 possessed a significant ability to generate transformed foci on these cells. In contrast, the E4 ORF1 sequences from human Ad5 and Ad12 lacked the transforming potential exhibited by Ad9 E4 ORF1. Cell lines derived from Ad9 E4 ORF1-transformed foci expressed the 14-kDa Ad9 E4 ORF1 protein and formed colonies in soft agar. In addition, the Ad9 E4 ORF1 protein was required for initiation of mammary oncogenesis *in vivo*, as E4 ORF1 mutant viruses failed while E4 ORF2 and ORF3 mutant viruses succeeded in eliciting mammary tumors in animals. A role for Ad9 E4 ORF1 in tumor maintenance was suggested by the fact that 100% of virus-induced mammary tumors expressed the E4 ORF1 protein. Taken together, the facts that the Ad9 E4 ORF1 protein exhibits transforming potential in culture and is required by Ad9 to produce mammary tumors in animals suggest that Ad9 E4 ORF1 is a new viral oncoprotein.

Human adenovirus type 9 (Ad9), a subgroup D adenovirus, is unique among DNA tumor viruses in eliciting exclusively estrogen-dependent mammary tumors in female rats (1, 2, 22), and mammary fibroadenoma is the predominant tumor found in these animals (2, 22). Because breast fibroadenoma is also the most prevalent type of breast neoplasm encountered by women under 35 years of age (34), determination of the molecular basis for Ad9 mammary oncogenicity may identify general mechanisms involved in the development of a common breast tumor of women.

The viral functions responsible for the oncogenic properties of adenoviruses are located within the E1 region (13). Within this region are encoded two well-known oncogenes, E1A (10) and E1B (38), and in the context of virus, this oncogene combination elicits mostly undifferentiated sarcomas at the site of virus inoculation (40). In contrast, the Ad9 E4 region is required for the specific hormone-dependent mammary oncogenicity of Ad9 and alone can produce transformed foci on established rat embryo fibroblasts in culture (23). Together, these findings suggest that the Ad9 E4 region encodes a novel oncoprotein involved in the ability of Ad9 to elicit mammary tumors in rats.

The adenovirus E4 region is a complex viral transcription unit which contains six different open reading frames (ORFs) (17, 19). These E4 ORFs express their corresponding proteins from alternatively spliced mRNAs initiated from a single promoter and terminated at a common polyadenylation site (3, 6, 26). For subgroup C adenoviruses (e.g., Ad2 and Ad5), in which the E4 region has been studied in detail, proteins corresponding to four of the E4 ORFs (ORF3, ORF4, ORF6, and ORF6/7) have been detected in cells (5, 8, 35). In general,

E4 ORFs encode small nuclear proteins ranging in size from 11 to 34 kDa and serve to regulate viral DNA synthesis and viral gene expression (15, 21, 27, 30–33, 41, 44).

It has previously been established that the Ad9 E4 function critical for mammary oncogenesis maps genetically to a region coding for ORF1, ORF2, and part of ORF3 (23). For subgroup C adenoviruses, these three E4 ORFs are dispensable for viral replication in cell culture (15). Protein products for E4 ORF1 and ORF2 have yet to be detected in cells, and the functions of these ORFs are not known. Interestingly, unlike other E4 mRNAs, the Ad5 E4 ORF1 mRNA behaves like a late viral gene requiring viral DNA synthesis, as well as E1B 55 kDa and E4 ORF6 functions, to accumulate to high levels late in infection (9). E4 ORF3 encodes a nuclear matrix-associated protein (35) which has partial functional redundancy with E4 ORF6 (4, 20).

The goal of the present study was to establish which Ad9 E4 ORF is responsible for the transforming potential of the Ad9 E4 region in culture and which Ad9 E4 ORF (ORF1, ORF2, or ORF3) is required for mammary tumor production in animals. The results presented here indicate that Ad9 E4 ORF1 encodes both the *in vitro* transforming function and the critical E4 determinant required for production of rat mammary tumors.

MATERIALS AND METHODS

Cell lines. A549 (human lung carcinoma cell line) and CREF (Fisher cloned rat embryo fibroblast cell line; 11) cells were maintained in culture medium (Dulbecco's modified eagle medium containing 20 μ g of gentamicin per ml and supplemented with 6 or 10% fetal calf serum [FCS]) at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

Plasmids. The seven Ad9 E4 ORFs were cloned into expression plasmid pCMV-Neo-Bam₃ (18) such that the first 5' ATG of the E4 ORF DNA insert was the predicted initiation codon. An Ad9 *Xho*I-*Spe*I (87 to 100 map units) pUC19

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plasmid was used as a template to amplify Ad9 E4 ORF1, ORF4, ORF6, and ORF6A by PCR with the following oligonucleotide pairs: ORF1, 5'-CTCGGATCCATGGCTGAATC TCTGTAT3' and 5'-CTCGAATTCACAGAAAGCATGCA CTAAG3'; ORF4, 5'-CTCGGATCCAAAATGGTTCTTCCAA TC3' and 5'-CTCGGATCCCTCAGATCGTGGTAAACGG3'; ORF6, 5'-CTCGGATCCACAATGCAAACCGAGATT3' and 5'-CTCGGATCCCTCAGTCTGGTGAATG3'; ORF6A, 5'-CTCGGATCCACCATGTGCTGCTGGCTT3' and 5'-CTCG GATCCTTATTCAGCAGCCCATAA3'. Ad9 E4 ORF6/7 was amplified by PCR from random hexamer-primed and reverse-transcribed (avian myeloblastosis virus reverse transcriptase) Ad9-infected cell total RNA with the oligonucleotide pair 5'-CTCGGATCCACAATGCAAACCGAGATT3' and 5'-CTC GGATCCTCATGTATCTTTATTGAT3'. Ad5 and Ad12 E4 ORF1 sequences were amplified by PCR from template Ad5 *w*300 or Ad12 strain Huie virion DNA by using the following oligonucleotide pairs: Ad5, 5'-CTCGGATCCATGGCTGC CGCTGTGGAA3' and 5'-CTCGAATTCCTTAAACATTAG AAGCCTG3'; Ad12, 5'-CTCGGATCCATGGCTGCTTTTG AGACT3' and 5'-CTCGAATTCCTTAAATTAAAGATGCC TG3'. The underlined nucleotides are the initiation and stop codons for each of the ORFs. E4 ORF6, ORF6A, *Bam*HI-digested E4 ORF4 and ORF6/7, *Bam*HI-*Eco*RI-digested Ad9, Ad5, and Ad12 ORF1 PCR products were blunt ended and cloned into the blunt-ended pCMV-Neo-Bam₃ plasmid *Bam*HI site. Ad9 E4 ORF2 and ORF3 were excised from the Ad9 *Bam*HI-*Spe*I (94 to 100 map units) pUC19 plasmid by restriction enzyme double digestion with *Sph*I-*Bsp*1286 and *Pst*I-*Eco*RI, respectively, which flanked these ORFs. These excised fragments were blunt ended and cloned into the blunt-ended pCMV-Neo-Bam₃ plasmid *Bam*HI site. All plasmid constructs were verified by sequence analysis of the entire insert.

Transfections. Calcium phosphate transfections were performed by standard methods (25). Briefly, 20 µg of plasmid DNA in 0.5 ml of a 250 mM CaCl₂ solution was mixed dropwise with 0.5 ml of 2× *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline solution (50 mM HEPES, 0.28 M NaCl, 1.5 mM Na₂HPO₄, pH 7.05) in a 4-ml polystyrene culture tube while being vortexed. After 30 min of incubation, the solution containing the calcium phosphate-DNA precipitate was mixed directly with 9 ml of culture medium (6% FCS) covering 50% confluent cells on a 100-mm-diameter culture dish. The cells were incubated with the precipitate for 16 h, washed three times with fresh culture medium, and then fed fresh culture medium.

Establishment of Ad9 E4 ORF1-transformed CREF cell lines. Ad9 E4 ORF1-transformed cell lines were generated from foci transformed by the cytomegalovirus (CMV)-Ad9 E4 ORF1 plasmid. Transformed foci were lifted from the monolayer with suction applied to a 200-µl micropipet tip. Isolated foci were dispersed with a trypsin-EDTA solution (0.05% [wt/vol] trypsin, 0.53 mM EDTA; Gibco), placed in culture medium (6% FCS), and plated on dishes for growth as monolayers. Four such transformed cell lines (9C-1, 9C-2, 9C-3, and 9C-4) were established.

Ad9 E4 ORF1-specific polyclonal antiserum. Ad9 E4 ORF1 was amplified by PCR from template Ad9 *Bam*HI-*Spe*I (94 to 100 map units) plasmid pUC19 by using the oligonucleotide pair 5'-CTCGGATCCATGGCTGAATCTCTGTAT3' and 5'-CTCAAGCTTACAGAAAGCATGCACTAA3'. The underlined nucleotides are the initiation and stop codons for the ORF. The resulting PCR product was digested with *Bam*HI and *Hind*III and cloned into the corresponding sites of plasmid pQE-9 (Qiagen). Plasmid pQE-9 introduces a six-histidine tag

at the amino-terminal end of the Ad9 E4 ORF1 protein which was expressed in bacteria and purified on nitrilo-tri-acetic acid (Ni-NTA)-agarose as recommended by the manufacturer (Qiagen). After an initial bleeding to obtain preimmune serum, a New Zealand White rabbit was injected intradermally with 200 µg of the purified Ad9 E4 ORF1 fusion protein as a 1:1 mixture with complete Freund's adjuvant. Four weeks later, the rabbit received an intradermal booster injection of 100 µg of the purified Ad9 E4 ORF1 fusion protein as a 1:1 mixture with incomplete Freund's adjuvant. A total of five such intradermal booster injections were administered at 4-week intervals, and 4 weeks after the final booster injection, the rabbit was exsanguinated for isolation of antiserum. The specificity of the antiserum was verified by immunoblot analysis of Ad9 E4 ORF1 fusion protein-expressing and -nonexpressing bacteria with preimmune and immune sera (24). Animal handling and care were done in accordance with institutional guidelines.

Immunoblot analysis. Immunoblot analysis was performed by standard methods (12). Briefly, proteins were diluted 1:1 in 2× sample buffer (0.13 M Tris [pH 6.8], 4% [wt/vol] sodium dodecyl sulfate, 20% [vol/vol] glycerol, 2% [vol/vol] β-mercaptoethanol, 0.003% [wt/vol] bromophenol blue), run on an either 12 or 13% Laemmli gel, and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon). The membrane was incubated for 1 h in blocking buffer containing 5% nonfat dry milk in TBST (50 mM Tris [pH 7.5], 200 mM NaCl, 0.1% [vol/vol] Tween 20), washed with TBST, incubated for 1 h with Ad9 E4 ORF1 rabbit antiserum (1:1,000 in TBST), washed three times with TBST, incubated for 1 h with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:1,000 in TBST; Fisher), and washed twice with TBST and twice with substrate buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 50 mM MgCl₂). The membrane was developed with a substrate solution containing 0.17 mg of nitroblue tetrazolium per ml and 0.17 mg of 5-bromo-4-chloro-3-indolylphosphate per ml.

Growth of cells in soft agar. Transformed cell lines and parental CREF cells were trypsinized to a single-cell suspension, suspended in culture medium (10% FCS), and counted with a hemacytometer. One hundred thousand viable cells were diluted in 3 ml of culture medium containing 0.4% Noble agar (Difco) and plated on top of 4 ml of a 0.6% Noble agar-culture medium underlay in 60-mm-diameter dishes. The plates were overlaid with culture medium containing 0.4% Noble agar every 3 days. After 10 days of incubation at 37°C in a humidified CO₂ incubator, the dishes were photographed with a camera-mounted Nikon inverted microscope.

Construction of E4 region mutant viruses. Restriction enzyme sites *Mlu*I, *Nhe*I, *Rsr*II, and *Eco*RV (see Fig. 3) were used to engineer E4 region mutations as follows. (i) The 9SS *Xho*I-*Spe*I (87 to 100 map units) pUC19 plasmid was digested with *Rsr*II-*Eco*RV (construct Δ*Rsr*II-*Eco*RV), *Nhe*I (construct in*Nhe*I), *Mlu*I (construct in*Mlu*I), *Nhe*I-*Eco*RV (construct Δ*Nhe*I-*Eco*RV), or *Mlu*I-*Nhe*I (construct Δ*Mlu*I-*Nhe*I). (ii) Constructs Δ*Rsr*II-*Eco*RV, in*Nhe*I, in*Mlu*I, and Δ*Mlu*I-*Nhe*I were blunt ended with Klenow and ligated, while construct Δ*Nhe*I-*Eco*RV was blunt ended with exonuclease VII and ligated. (iii) Mutations were verified by sequence analysis. (iv) The E4 region mutations were reconstructed into the larger 9SS *Xba*I-*Spe*I (65 to 100 map units) pUC19 plasmid, which was used to generate infectious virus by overlap recombination as described previously (23). Viral DNAs were examined by restriction enzyme analysis to ensure that the viruses were correct.

Combined immunoprecipitation and immunoblot analysis. Immunoprecipitations were performed by standard methods

TABLE 1. Transformation of CREF cells by plasmids expressing each of the Ad9 E4 ORFs^a

Plasmid	No. of transformed foci	
	Expt 1	Expt 2
Group A		
CMV vector	0	0
CMV-Ad9 E4 ORF1	191	226
CMV-Ad9 E4 ORF2	0	0
CMV-Ad9 E4 ORF3	0	0
CMV-Ad9 E4 ORF4	0	0
CMV-Ad9 E4 ORF6	0	0
CMV-Ad9 E4 ORF6A	0	0
CMV-Ad9 E4 ORF6/7	0	1
Group B		
CMV-Ad9 E4 ORF1(antisense)	0	0
CMV-Ad9 E4 ORF1(Δ RsrII-EcoRV)	0	0

^a Fifty percent confluent CREF cells in 100-mm-diameter dishes were transfected with 20 μ g of each CsCl-purified supercoiled expression plasmid by the calcium phosphate precipitation method (14). The expression plasmid without an insert (CMV vector) was included as a control. After approximately 16 h, the cells were washed three times with 6% FCS-supplemented culture medium and then fed fresh 6% FCS-supplemented culture medium with feedings twice a week thereafter. After 4 weeks, the cells were fixed in methanol for 1 h, stained with Giemsa (diluted 1:10 in methanol) overnight and rinsed with water and transformed foci were counted.

(16). Briefly, cells were lysed in ice-cold RIPA buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% [wt/vol] sodium dodecyl sulfate, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] deoxycholate) containing the protease inhibitors aprotinin (2 μ g/ml), leupeptin (2 μ g/ml), and phenylmethylsulfonyl fluoride (100 μ g/ml) and incubated for 20 min on ice. Debris was cleared from the lysate by centrifugation (16,000 $\times$ g for 10 min). The cleared lysates were subjected to immunoprecipitation by incubation with 0.5 μ l of Ad9 E4 ORF1 antiserum for 1 h on ice and then shaking with 20 μ l of protein A-Sepharose beads for 1 h at 4°C. The beads were washed three times with 0.7 ml of ice-cold RIPA buffer, pelleted, and boiled in 30 μ l of 2 $\times$ sample buffer, and the eluted proteins were run on a 13% Laemmli gel. Immunoblot analysis was carried out as described above, except that the secondary antibody used was alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (γ heavy chain specific; Fisher).

Viral mammary oncogenicity in rats. Virus tumorigenicity was determined as described previously (22,23). Briefly, viruses were amplified in A549 cells to a titer of approximately 10^8 PFU/ml. Near-term pregnant Wistar-Furth rats were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.) and monitored for delivery of rat pups. At 24 to 48 h after birth, male and female newborn rat pups were inoculated subcutaneously with a 26-gauge needle over each shoulder with 0.2 ml of undiluted cell lysate containing virus (approximately 5×10^7 PFU). The rats were observed for tumor development during a 7-month period following virus infection. At the end of this observation period, animals were sacrificed and tumors were removed and either fixed in 10% formalin for histological examination or snap frozen in liquid nitrogen for subsequent protein analysis. Animal handling and care were done in accordance with institutional guidelines.

RESULTS

Ad9 E4 ORF1 sequences exhibit transforming potential in the continuous rat embryo fibroblast cell line CREF. The first

FIG. 1. (A) Alignment of the predicted Ad9, Ad5, and Ad12 E4 ORF1 protein sequences. The sequences were optimally aligned by using the PileUp program of the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package. The lengths of the Ad9, Ad5, and Ad12 E4 ORF1 proteins are 125, 128, and 127 amino acids, respectively. The asterisks denote positions of amino acid identity, and the numbers above the protein sequences correspond to Ad9 E4 ORF1 amino acid positions. (B) Comparison of Ad5, Ad9, and Ad12 E4 ORF1 expression plasmids for production of transformed foci on the established rat embryo fibroblast cell line CREF. The expression plasmid without an insert (CMV vector) was included as a control. Transformation assays with 20 μ g of plasmid were performed as described in the footnote to Table 1. Cells were stained 4 weeks after transfection.

experiments focused on determining whether a single E4 ORF was responsible for the transforming properties previously observed with the entire Ad9 E4 region. This was accomplished by introducing each of the seven Ad9 E4 ORFs (ORF1, ORF2, ORF3, ORF4, ORF6, ORF6A, and ORF6/7) into a eukaryotic expression plasmid (pCMV-Neo-Bam₃; 18) under the transcriptional control of the CMV immediate-early promoter. E4 ORF6A has been found only in subgroup D

adenoviruses, such as Ad9 (23); the significance of ORF6A has not been established. The expression plasmid constructs then were individually transfected into the established rat embryo fibroblast cell line CREF (11) to test their ability to generate transformed foci. As shown for group A in Table 1, only Ad9 E4 ORF1 possessed significant transforming potential in this assay, indicating that ORF1 is responsible for the *in vitro* transforming behavior of the Ad9 E4 region. Interestingly, the Ad9 E4 ORF1 expression plasmid generated considerably more transformed foci in this assay than when the entire Ad9 E4 region under the control of its own promoter was used (23). It is not clear whether this difference is due to a higher level of ORF1 expression by the CMV-Ad9 E4 ORF1 expression plasmid or interference with ORF1-induced transformation by other E4 ORF products when the entire E4 region plasmid is used.

Inversion of the ORF1 sequence in the antisense orientation [CMV-Ad9 E4 ORF1(antisense)] or truncation of the 125-residue ORF1 coding region to the N-terminal 55 residues [CMV-Ad9 E4 ORF1(Δ RsrII-EcoRV)] abolished the transforming potential of the plasmid (Table 1, group B). This experiment extended the results summarized for group A in Table 1 by suggesting that expression of the intact ORF1 protein is necessary for transformation by the Ad9 E4 ORF1 expression plasmid. The fact that the Ad9 E4 ORF1 transforming sequences lie within the portion of the E4 region previously shown to be essential for Ad9-induced mammary oncogenesis lent further support to the idea that E4 ORF1 and the Ad9 E4 function required for mammary oncogenesis are the same.

E4 ORF1 sequences derived from Ad5 and Ad12 do not transform CREF cells. Other human adenoviruses, which do not elicit mammary tumors, possess E4 ORF1 sequences capable of encoding proteins similar to that of Ad9 (Fig. 1A). Therefore, it was of interest to determine whether the ability to transform cells in culture is a property common to all adenovirus E4 ORF1 sequences. This question was answered by introducing the E4 ORF1 sequences from nononcogenic Ad5 and oncogenic Ad12 into the CMV expression plasmid used in the experiments whose results are shown in Table 1. The predicted ORF1 amino acid sequences of Ad5 and Ad12 exhibited approximately 50% identity and 70% similarity to that of Ad9 E4 ORF1 (Fig. 1A); however, unlike the Ad9 E4 ORF1 expression plasmid, transfection of CREF cells with the Ad5 or Ad12 E4 ORF1 expression plasmid did not produce transformed foci (Fig. 1B). This suggested that among human adenoviruses, Ad9 E4 ORF1 possesses an unusual transforming property, which is consistent with the notion that Ad9 E4 ORF1 is an important factor contributing to the unique mammary oncogenicity of Ad9.

Ad9 E4 ORF1-transformed CREF cells express the predicted 14-kDa ORF1 protein and grow in soft agar. The results obtained with the transformation-defective Ad9 E4 ORF1 expression plasmids that make up group B in Table 1 suggested that ORF1 protein expression is required to transform CREF cells. This argument was strengthened by direct examination of ORF1 protein expression in transformed cells with a polyclonal antiserum generated against an Ad9 E4 ORF1 fusion protein purified from bacteria. Transformed CREF cell foci induced by the Ad9 E4 ORF1 expression plasmid were isolated from a culture dish, and four independent transformed cell lines (9C-1, 9C-2, 9C-3, and 9C-4) were established. Two passages after establishment of these lines, cell lysates were prepared and examined for Ad9 E4 ORF1 protein expression by immunoblot analysis with Ad9 E4 ORF1-specific antiserum. In contrast to control CREF cells, all four of the Ad9 E4 ORF1-transformed cell lines expressed the 14-kDa ORF1

FIG. 2. (A) Immunoblot analysis for expression of the Ad9 E4 ORF1 protein in four different CREF cell lines (9C-1, 9C-2, 9C-3, and 9C-4) transformed by the CMV-Ad9 E4 ORF1 plasmid. At 2 passages postestablishment, 10^7 transformed cells from each cell line were lysed in 0.5 ml of $2\times$ sample buffer and boiled for 5 min. A control CREF cell lysate was produced in the same way. Immunoblot analysis with 50 μ l of each cell lysate was performed as described in Materials and Methods. Proteins were separated on a 12% Laemmli gel. (B) Analysis of Ad9 E4 ORF1-transformed cell lines for growth in soft agar. One hundred thousand normal or Ad9 E4 ORF1-transformed CREF cells were suspended in culture medium supplemented with 10% FCS and 0.4% Noble agar. The photographs were taken 10 days postplating (magnification, $\times 20$).

FIG. 3. The E4 region genomic structures of five E4 region mutant viruses derived from mammary oncogenic virus 9SS (23). The Ad9 E4 region with its seven ORFs is shown above the virus structures (black bars). Gaps in the virus structures represent deletions (viruses Δ RsrII-EcoRV, Δ NheI-EcoRV, and Δ MluI-NheI), and the inverted triangles (viruses inNheI and inMluI) represent small insertions. For virus 9SS and the E4 mutant viruses, Ad9 DNA is present to the right of the *Sma*I site and Ad26 DNA is present to the left of this site. The arrow indicates the start of transcription, and ITR is the inverted terminal repeat.

protein (Fig. 2A). This demonstrated that Ad9 E4 ORF1 does indeed encode a protein and, more importantly, provided additional evidence correlating Ad9 E4 ORF1 protein expression with the transformation of CREF cells.

Cells transformed in culture may exhibit different degrees of the transformed state, so it was of interest to examine the extent to which Ad9 E4 ORF1 had transformed the CREF cells. This was determined by assaying the Ad9 E4 ORF1-transformed CREF lines for the ability to form colonies when suspended in soft agar. Anchorage-independent growth of cells is associated with a highly transformed state, as it most commonly correlates with the ability to produce tumors in animals (39). All four of the Ad9 E4 ORF1-transformed CREF lines formed colonies in soft agar after 10 days, while the parental CREF cells did not (Fig. 2B). The results were the same when the experiment was extended to 21 days (24). This approach suggested that Ad9 E4 ORF1 alone can confer a highly transformed phenotype on CREF cells.

Construction of Ad9 E4 ORF1 mutant viruses. The properties of Ad9 E4 ORF1 in cultured cells suggested that this protein functions as an oncoprotein in vivo, so it was important to establish a specific requirement for Ad9 E4 ORF1 in the production of mammary tumors in animals. This was done by constructing viruses having mutations within E4 ORF1, as well as the two adjacent E4 ORFs, ORF2 and ORF3. In bacteria, the Ad9 plasmid necessary for construction of Ad9-derived viruses generated only rearranged constructs upon multiple attempts to introduce the E4 region mutations. In contrast, an equivalent plasmid derived from mammary oncogenic Ad9-Ad26 recombinant virus 9SS was stable upon subcloning, and consequently, E4 region mutations were introduced and tested within the 9SS viral genetic background (Fig. 3). For these experiments, viruses 9SS and Ad9 are equivalent because virus 9SS possesses the relevant Ad9 E4 region sequences which make it oncogenically identical to Ad9 (23).

Single and double E4 ORF mutant viruses with mutations in Ad9 E4 ORF1, ORF2, and ORF3 were constructed (Fig. 3). The ORF1 mutant virus (Δ RsrII-EcoRV) was engineered with a small deletion identical to that found within the CMV-Ad9 E4 ORF1(Δ RsrII-EcoRV) expression plasmid which was defective for transformation of CREF cells (Table 1, group B). Besides serving as controls, the ORF2 and ORF3 mutant

FIG. 4. Combined immunoprecipitation and immunoblot analysis for expression of the Ad9 E4 ORF1 protein by virus 9SS and the five E4 mutant viruses in A549 cells (a human lung carcinoma cell line permissive for viral replication). Ten million A549 cells were mock infected (MOCK) or infected with 5 PFU of each virus per cell, and at 20 h postinfection, the cells were harvested and lysed in 1 ml of ice-cold RIPA buffer containing protease inhibitors. A 0.25-ml volume of each cleared lysate was subjected to combined immunoprecipitation and immunoblot analysis as described in Materials and Methods. Proteins were separated on a 13% Laemmli gel.

viruses (inNheI and inMluI) were generated because ORF2 and ORF3 are contained, along with ORF1, within the portion of the Ad9 E4 region previously defined as being required for mammary oncogenesis (23). The three different mutations of the single E4 ORF mutant viruses were all predicted to truncate their respective proteins by approximately half. The two double E4 ORF mutant viruses, Δ NheI-EcoRV and Δ MluI-NheI, each had a large deletion affecting two adjacent E4 ORFs, ORF1 and ORF2 or ORF2 and ORF3, respectively. Each deletion would theoretically truncate the predicted protein of the upstream ORF by approximately half while eliminating protein expression from the downstream ORF.

Upon amplification, the mutant viruses were found to attain viral titers comparable to that of parental virus 9SS (approximately 10^8 PFU/ml), indicating that gross replication defects due to the introduction of these mutations were not acquired (24). Consistent with this observation, Ad2 and Ad5 with mutations disrupting E4 ORF1, ORF2, and ORF3 replicate similarly to their wild-type counterparts (15). As E4 ORF1 was the focus of these experiments, each of the E4 mutant viruses was tested for the ability to express this particular protein. Analysis of infected cell lysates indicated that viruses Δ RsrII-EcoRV and Δ NheI-EcoRV, which have mutations affecting E4 ORF1, did not express detectable amounts of the Ad9 E4 ORF1 protein (Fig. 4). Moreover, the 6-kDa truncated ORF1 protein expected to be made by these two mutant viruses was not found. This may be due to the fact that the truncated protein either is not recognized by the antiserum or is unstable in cells. As expected, the remaining viruses did express the Ad9 E4 ORF1 protein, which under these electrophoretic conditions, was resolved into a protein doublet band (Fig. 4). The doublet band may indicate that Ad9 E4 ORF1 is posttranslationally modified in cells.

Expression of Ad9 E4 ORF1 protein is required for production of mammary tumors in rats. The mammary oncogenic potential of each E4 mutant virus was tested by subcutaneous inoculation of 5×10^7 virus PFU into newborn Wistar-Furth

TABLE 2. Mammary oncogenicities of E4 mutant adenoviruses in newborn Wistar-Furth rats^a

Virus	E4 genotype	No. of rats with mammary tumors	
		Females	Males
9SS	Wild type	3/3	0/2
Δ RsrII-EcoRV	Mutant ORF1	0/3	0/2
inNheI	Mutant ORF2	3/3	0/2
inMluI ^b	Mutant ORF3	3/3	0/2
Δ NheI-EcoRV	Mutant ORF1 and ORF2	0/3	0/2
Δ MluI-NheI ^b	Mutant ORF2 and ORF3	3/3	0/2

^a Newborn Wistar-Furth rat pups 24 to 48 h old were inoculated subcutaneously with approximately 5×10^7 PFU of virus. The rats were observed for tumor development during a 7-month period following virus infection.

^b Viruses inMluI and Δ MluI-NheI displayed greater oncogenicity than the other oncogenic viruses analyzed here (see text). The female rats infected with these viruses had developed numerous large mammary tumors by 5 months postinfection, at which time all animals infected with these two viruses were sacrificed.

rats. Ad9 and virus 9SS are oncogenic in female rats but nononcogenic in male rats (22), so both males and females were infected with the mutant viruses. Animals were observed for tumor development for 7 months postinfection. The two E4 mutant viruses (Δ RsrII-EcoRV and Δ NheI-EcoRV), which did not express detectable amounts of the Ad9 E4 ORF1 protein, failed to elicit tumors of any kind in the animals (Table 2). Parental virus 9SS and the remaining E4 mutant viruses, on the other hand, produced mammary tumors in all of the female rats and were nontumorigenic in male rats. Upon examination, the tumors were found to be similar both grossly and histologically to Ad9-induced mammary tumors (24). These results indicated that Ad9 E4 ORF1 protein expression is required for production of mammary tumors. This conclusion was further supported by the fact that eight independent mammary tumors generated by the oncogenic viruses (Table 2) expressed the Ad9 E4 ORF1 protein (Fig. 5). This particular result also suggested that continuous expression of Ad9 E4 ORF1 in the mammary tumors is necessary for tumor maintenance.

E4 ORF3 mutant viruses exhibit increased mammary oncogenicity. In the experiment summarized in Table 2, viruses inMluI (ORF3 mutant) and Δ MluI-NheI (ORF2 and ORF3 mutant) exhibited greater oncogenicity than the other viruses tested. Female rats infected with either of these two E4 mutant viruses exhibited palpable tumors a month earlier than did animals infected with the other oncogenic viruses (2 versus 3 months postinfection). In addition, female rats infected with the inMluI or Δ MluI-NheI virus had developed numerous large mammary tumors at 5 months postinfection, a time when animals infected with the other viruses had a few small tumors. The alteration shared by viruses inMluI and Δ MluI-NheI was a mutation of E4 ORF3 (Fig. 3). Thus, besides providing further evidence that the E4 ORF3 function had been altered in these particular mutant viruses, the results suggested that ORF3 protein expression reduces viral mammary oncogenicity. From these experiments, it was not clear whether ORF3 directly affected ORF1 protein expression, as the steady-state levels of ORF1 protein appeared not to be significantly altered in tumors induced by virus inMluI but were slightly elevated in those induced by virus Δ MluI-NheI (Fig. 5). The mechanism by which Ad9 E4 ORF3 negatively influences mammary oncogenicity remains to be determined.

FIG. 5. Combined immunoprecipitation and immunoblot analysis for expression of the Ad9 E4 ORF1 protein in virus-induced mammary tumors. Approximately 200 mg of snap-frozen tumor tissue was ground into a fine powder with a mortar and pestle chilled in liquid nitrogen. The tumor powder was vortex resuspended with 1 ml of ice-cold RIPA buffer containing protease inhibitors. A 0.25-ml volume of each cleared tumor lysate was subjected to immunoprecipitation and immunoblot analysis as described in Materials and Methods. As a control, one Ad9-induced mammary tumor was analyzed with both Ad9 E4 ORF1-specific antiserum and the corresponding preimmune serum. Proteins were separated on a 13% Laemmli gel. Each lane represents a single mammary tumor sample, and the virus used to produce the tumor and the animal number are indicated.

DISCUSSION

The results presented here indicate that the Ad9 E4 ORF1 protein possesses transforming potential in continuous rat embryo fibroblasts and is required for virus-induced mammary oncogenicity in rats. The continued presence of the Ad9 E4 ORF1 protein in transformed CREC cells and mammary tumors further suggests that this protein is necessary for maintenance of both the transformed and neoplastic states. Taken together, these in vitro and in vivo results strongly suggest that Ad9 E4 ORF1 is an oncoprotein. Additionally, since Ad9 E4 ORF1 exhibits unique transforming properties compared with other adenovirus E4 ORF1 sequences, one intriguing possibility is that Ad9 E4 ORF1 is responsible for the unusual oncogenic tropism of Ad9 for the mammary gland. Further experiments are necessary to determine whether this notion is correct.

It should be stressed that this report does not indicate that Ad9 E4 ORF1 is the sole viral function required for mammary oncogenesis. The E1A and E1B genes of the adenovirus E1 region encode well-known viral oncoproteins that inactivate cellular tumor suppressor proteins Rb and p53, respectively (28, 29, 42). In support of a role for the Ad9 E1 region in mammary oncogenesis, this viral gene region has been shown to cooperate with the Ad9 E4 region (23) and Ad9 E4 ORF1 (24) to transform cells in culture. E1A mRNA, like E4 mRNA, has been detected in mammary tumors caused by Ad9, suggesting that E1A does indeed participate in mammary oncogenesis (22). In contrast, Ad9 E1B mRNA has not been detected in mammary tumors (22). Nevertheless, Ad9 E1B could be necessary for oncogenesis caused by Ad9, as this gene may be expressed at low yet biologically significant levels in tumors. Therefore, it seems possible that, besides E4 ORF1, E1A, and perhaps also E1B, contributes to Ad9-induced mammary oncogenesis.

Ad9 E4 ORF1 alone was found to generate transformed CREC cells that exhibited anchorage-independent growth

(Fig. 1B). In this regard, the Ad12 E4 region has also been shown to confer an increased potential for growth in soft agar on Ad12 E1 region-transformed cells (37). It is tempting to conclude that Ad12 E4 ORF1 is responsible for inducing anchorage-independent growth upon Ad12 E1 region-transformed cells. The fact that Ad12 E4 ORF1 alone did not transform CREF cells (Fig. 2B), however, is not inconsistent with a role in anchorage-independent growth, since the abilities to generate transformed foci and confer anchorage-independent growth may be separate characteristics of the E4 ORF1 protein. Consequently, while a requirement for Ad12 E4 ORF1 in oncogenesis has not been evaluated, E4 ORF1 may be found to be a common viral determinant required for the oncogenicity of adenoviruses.

The significance of the Ad9 E4 region for mammary oncogenesis was previously elucidated by using recombinant viruses made with oncogenic virus Ad9 and nononcogenic virus Ad26 (23). A region encompassing E4 ORF1, ORF2, and ORF3 was found to contain the relevant genetic difference between these viruses. Even though this relevant genetic difference has not been further defined, the results presented here indicate that it is localized to E4 ORF1. Surprisingly, however, both the Ad9 and Ad26 E4 regions (23) and their corresponding E4 ORF1 sequences (24) exhibit similar transforming potentials in CREF cells. Such results suggest that Ad26 E4 ORF1 possesses transforming potential *in vitro* yet lacks oncogenic potential *in vivo*. The Ad5 E1 region serves as an example of such a region encoding transforming but nononcogenic viral proteins (13). Alternatively, the genetic difference between Ad9 and Ad26 may be localized to E4 ORF2 or ORF3 rather than to ORF1. Such a result would suggest that the Ad9 and Ad26 E4 ORF1 proteins are identical with respect to transformation and oncogenicity and that either Ad26 E4 ORF2 or ORF3 dominantly represses mammary oncogenicity. The fact that Ad9 E4 ORF3 was found to influence mammary tumor production negatively (Table 2) supports the idea of such an inhibitor of oncogenesis. The analysis of more Ad9-Ad26 recombinant viruses should distinguish between these two possibilities.

Ad9 E4 ORF1 encodes the first known transforming protein located outside of the adenovirus E1 region. A search of sequence data bases with the Ad9 E4 ORF1 amino acid sequence has yielded strong similarities only to other human adenovirus E4 ORF1 sequences and not to known proteins which might suggest a function for this viral protein (23). Future studies must be directed at determining the mechanism by which Ad9 E4 ORF1 transforms cells. In this regard, viral oncoproteins almost always complex with critical cellular regulatory proteins (7, 28, 29, 36, 42, 43) and the identification of cellular proteins which bind to Ad9 E4 ORF1 may lead to a novel mechanism of tumorigenesis by a viral oncoprotein and perhaps also identify similar mechanisms leading to the production of some breast neoplasms in women.

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