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Human Adenovirus Type 9-Induced Rat Mammary Tumors

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Following subcutaneous inoculation of newborn Wistar-Furth rats with human adenovirus type 9 (Ad9), 16 of 16 female and 0 of 11 male rats developed mammary tumors. Tumor-positive animals usually developed tumors in multiple glands. Histopathological analyses indicated that three general categories of tumor could be identified. Mammary fibroadenomas were the most common tumor type encountered, but phyllodeslike tumors and solid sarcomas were also frequently found. In situ hybridization and immunohistochemical techniques established that benign fibroadenomas were derived from mammary fibroblasts (collagen type I- and vimentin-positive cells) and that malignant tumors were derived from myoepithelial cells (collagen type IV-, vimentin-, and muscle-specific actin-positive cells). The fact that mammary tumors were limited to female rats suggested that female hormones are essential for tumor growth and development. In this regard, ovariectomy of Ad9-infected female rats prevented tumor development, while subsequent diethylstilbestrol (DES) treatment elicited tumor formation. In addition, Ad9-infected and castrated male rats which received DES also developed mammary tumors. Established male mammary tumors regressed when DES treatment was stopped and reappeared after DES treatment was resumed. Together, these results indicate that estrogen is required for both initiation and maintenance of Ad9-induced mammary tumors. Southern blot analysis of high-molecular-weight tumor DNA showed that mammary tumor cells contained single or multiple integrated copies of the entire Ad9 genome. RNase protection experiments established that estrogen receptor as well as Ad9 E1a and E4 mRNAs were expressed in mammary tumors, but Ad9 E3 and, surprisingly, E1b mRNAs were not expressed at detectable levels.

Human adenovirus type 9 (Ad9) is a member of the subgroup D adenoviruses, which includes 20 different serotypes (48). In humans, some subgroup D viruses, including viruses closely related to Ad9, cause epidemic eye infections (1, 29, 37, 53), but in general, very little is known about these viruses.

Subgroup D viruses are classified as nononcogenic because they do not induce tumors following injection into newborn hamsters (50). Their ability to morphologically transform primary rodent cells in culture remains controversial. It was originally reported that several members of the subgroup D family, including Ad9, can morphologically transform primary Fisher rat embryo fibroblasts (REF) (36). In contrast, more recent studies suggest that transfection of Ad9 viral DNA into primary Fisher baby rat kidney (BRK) cells does not result in morphologically transformed foci (27). Ad9, however, is clearly able to transform established rat fibroblast cell lines. Rat 3Y1 cells, an established Fisher REF cell line, are efficiently transformed by Ad9 (12), and these Ad9-transformed cells typically contain multiple, tandemly integrated copies of the entire virus genome. As would be expected for an adenovirus, the ability of Ad9 to transform established rat fibroblasts maps to the left end of the viral DNA, since CREF cells (another established Fisher REF cell line) can be transformed by a cloned DNA fragment representing the left-most 20% of the virus genome (27).

Although the subgroup D adenoviruses are classified as nononcogenic, in 1974 Ankerst and colleagues found that following subcutaneous Ad9 inoculation of newborn Wistar-Furth (W/Fu) rats, 100% of the female rats developed benign mammary tumors (4). The male rats from the same litter remained tumor free. Histologically, the tumors have the appearance of mammary fibroadenomas, composed predominantly of stromal fibroblastic connective tissue interspersed with various amounts of alveolar and ductal epithelium. Later studies indicated that Ad9-induced mammary tumors express Ad9-specific T antigen (5); in a few instances, the tumor stroma appeared to progress to malignancy, converting to a fibrosarcoma, round cell liposarcoma, osteosarcoma, or malignant mesenchymoma (28).

Since male rats did not develop tumors following injection with Ad9, it was postulated that development of these mammary tumors might be dependent on estrogen. To this end, Macdonald et al. (33a) and Ankerst and Jonsson (3) demonstrated that Ad9-infected and castrated male rats which are treated with high doses of estrogen develop mammary tumors identical to those of their female counterparts.

Ad9 possesses several interesting traits which set it apart from other oncogenic adenoviruses. First, a rodent infected with an oncogenic human adenovirus, such as adenovirus type 12, generally develops a sarcomatous tumor at the site of virus injection (51). Ad9 deviates from this principle by targeting exclusively mammary tissue for tumorigenesis. Second, the mammary tumors elicited by Ad9 require estrogen for development. Finally, Ad9 does not appear to be able to transform primary rodent cells in culture (27, 27a). The underlying cause for these unusual distinctions and the fact that, in an animal model system, Ad9 causes a mammary tumor (fibroadenoma) that is common in women make it an attractive virus for tumor studies.

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In the present study, we confirm previous work regarding Ad9-induced tumorigenesis in W/Fu rats and make several new observations. First, Ad9-induced mammary tumors fall into three general histological categories—fibroadenoma, phyllodeslike tumor, and solid sarcoma. Second, they are derived from mammary fibroblasts (benign tumors) or myoepithelial cells (malignant tumors). Third, the histologic characteristics of the tumors (benign versus malignant) do not change with time. Fourth, the tumors require estrogen for both initiation and maintenance. Fifth, tumor cells contain single or multiple integrated copies of the entire Ad9 virus genome. Sixth, the tumor cells express detectable estrogen receptor and Ad9 E1a and E4 but not E1b or E3 mRNAs.

MATERIALS AND METHODS

Viruses and cells. A549 cells were maintained as monolayers in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and supplemented with penicillin and streptomycin. A seed stock of human Ad9 (Hicks strain) was obtained from the American Type Culture Collection (Rockville, Md.) and plaque-purified by limiting dilution on A549 cells. High-titer Ad9 stocks were obtained by multiple serial undiluted passages of the virus on A549 cells. When the infected cells showed maximal cytopathic effect (usually 48 to 72 h postinfection), virus was released from the cells by a single freeze and thaw cycle followed by 1 min of sonication at 0°C. Virus numbers in infected-cell lysates were counted on A549 cells, and plaques were counted 15 to 21 days postinfection. Virus-containing infected-cell lysates were stored at −80°C for later use.

Plasmids. For the Ad9 E1a plasmid, the Ad9 *Spe*I-*Nco*I fragment (1 to 936 nucleotides [nt] from the left end) was cloned into pGEM5Zf(+). The *Spe*I site was created by ligating *Spe*I linkers to the end of virus DNA as described previously (23). For production of Ad9 E1a-specific antisense RNA, the plasmid was digested with *Ssp*I (site at 763 nt). For the Ad9 E1b plasmid, the Ad9 *Nae*I-*Hin*cII fragment (nt 1628 to 1885 from left end) was cloned into the *Nae*I and *Eco*RI (blunt-ended with Klenow) sites of pGEM-3. For production of Ad9 E1b-specific antisense RNA, the plasmid was digested with *Nae*I. For the Ad9 E4 plasmid, the Ad9 *Kpn*I-*Eco*RI fragment (nt 789 to 1734 from the right end) was cloned into pGEM-3. For production of Ad9 E4-specific antisense RNA, the plasmid was digested with *Rsa*I (nt 1479). For the Ad9 E3 plasmid, the Ad9 *Pst*I-*Pst*I fragment (approximate map units 74 to 77) was cloned into pGEM3Zf(+). For production of Ad9 E3-specific antisense RNA, the plasmid was digested with *Nco*I (76 map units). All Ad9 clones were confirmed to be genuine by complete or partial sequence analysis (not shown).

The rat estrogen receptor (rER) cDNA was cloned by reverse transcription of Ad9-induced mammary tumor RNA followed by polymerase chain reaction (PCR) amplification with rER-specific (30) oligonucleotide primers as follows. Ad9-induced mammary tumor RNA (20 μg in 4.4 μl of water) was heat denatured at 95°C for 10 min and then placed on ice. To the denatured RNA were added 40 U of placental RNase inhibitor, 5 μl of a 4 mM deoxynucleoside triphosphate (dNTP) solution (4 mM each dATP, dCTP, dGTP, and dTTP), 2 μl of 10× PCR buffer (500 mM KCl, 200 mM Tris-HCl [pH 8.4], 25 mM $MgCl_2$, 1 mg of bovine serum albumin per ml), 10 pmol of RER2 oligonucleotide (5'AG GAGCAAACAGGAGCTT3'), and 500 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in a total volume of 20.5 μl. This solution was incubated at 25°C for 10 min, 43°C for 3 h, and 95°C for 10 min. Two microliters of this solution was added to 10 μl of 10× PCR buffer, 10 μl of a 2 mM dNTP solution, 10 pmol of the oligonucleotide RER1 (5'ACCATGACCAT GACCCTT3'), 10 pmol of the oligonucleotide RER2, and 2.5 U of *Taq* polymerase (Amplitaq) in a total volume of 100 μl. The following cycles were run for PCR amplification: 93°C for 80 s, 55°C for 100 s, and 72°C for 5 min for 35 cycles. The expected 1-kb fragment was gel purified with NA45 paper (Schleicher & Schuell BA85), digested with *Nco*I, and cloned into the *Nco*I and *Eco*RV sites of pGEM5Zf(+). Partial sequence analysis established the clone as genuine rER cDNA. For production of rER-specific antisense RNA, the plasmid was digested with *Xba*I, and as for all plasmid templates described above, T7 RNA polymerase was used to generate antisense RNA.

Animals. Near-term pregnant female W/Fu rats were obtained from Harlan Sprague Dawley, Indianapolis, Ind. Within 24 h of birth, newborn rats were inoculated subcutaneously (with a 26-gauge needle) on the dorsal side with 0.4 ml (0.2 ml on the right side and 0.2 ml on the left side) of a 10^8-PFU/ml stock of Ad9. Four weeks after birth, weanling rats were segregated according to sex (three animals per cage). Animals were given water and Rat Checkers (Purina 5001) ad libitum and observed by palpation for 1 year for tumor formation. Some males were castrated and some females were ovariectomized at 4 weeks of age. Selected gonadectomized rats were treated with diethylstilbestrol (DES) by subcutaneous implantation of DES-packed silastic tubing (Corning Glass Works, Corning, N.Y.). Silastic capsules were replaced every 2 weeks because a constant delivery rate may be impeded by connective tissue encapsulation.

Immunohistochemistry. Immunohistochemistry of tumor sections was performed as described by Wordinger et al. (56). Frozen sections of rat mammary tumors were mounted on chrome alum-treated slides and air dried for a minimum of 4 h in a room temperature desiccator. The sections were blocked with normal rabbit serum (1:100 dilution) for 30 min, drained, and incubated for 1 h with the primary antibody (diluted according to specifications with phosphate-buffered saline [PBS]). Following two washes with PBS, the sections were incubated with a biotinylated secondary antibody, washed twice with PBS, and incubated with the streptavidin-peroxidase complex. The reaction mixes were visualized with diaminobenzidine (10 μg/ml) for 5 min, washed with PBS, and counterstained with Harris's hematoxylin.

In situ hybridization. Fresh or frozen (−70°C) tissue was fixed in a PBS-buffered 4% formaldehyde solution, rinsed in PBS, dehydrated in ethanol, and embedded in paraffin. Tumor sections (5 μm) were floated on Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% Ficoll)-treated slides, incubated with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C, rinsed with distilled water, and fixed with an ethanol-acetic acid (3:1) solution (11). Following paraffin removal with xylene and ethanol, the sections were fixed in 4% formaldehyde buffered with PBS, digested with pronase (24), delipidated with 100 mM triethanolamine, and acetylated in 0.25% acetic anhydride (2). Prehybridization solution (10 μM Tris [pH 7.0], 150 μM NaCl, 1 μM EDTA, 10 μM dithiothreitol, 1 mg of salmon sperm DNA per ml, 1× Denhardt's solution, 50% [vol/vol] formamide) was placed on the tissue, and the slides were incubated for 16 h in a moist chamber at 42°C. The tumor sections were hybridized with fresh prehybridization

solution containing 10^5 cpm of α-^{35}S-labeled Ad9 E1a or E4 DNA probe (10^8 cpm/μg) at 42°C for 16 h (32) and washed three times with 2× SSC at room temperature, once with 1× SSC at room temperature, and five times in 0.1× SSC at 50°C. The tissue sections were then dehydrated with ethanol, air dried, and autoradiographed with Kodak NT-B emulsion. After exposure for 3 to 5 days, the slides were developed and counterstained with hematoxylin and eosin (H&E).

Isolation of high-molecular-weight DNA and total RNA. High-molecular-weight DNA was isolated from mammary tumors as follows. Rats were killed with CO_2, and well-isolated tumors were quickly removed and snap-frozen in liquid nitrogen. Frozen tumors were crushed to a fine powder with a liquid nitrogen-chilled mortar and pestle. The resulting frozen tumor powder was digested at 37°C for 12 h with DNA lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, 10 mM NaCl, 0.5% sodium dodecyl sulfate [SDS], 0.5 mg of proteinase K per ml), extracted with an equal volume of Tris-buffered phenol, and dialyzed overnight at 4°C against two changes of TNE (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 10 mM NaCl). The dialysate was treated with 20 μg of DNase-free RNase A per ml for 3 h at 37°C and extracted with an equal volume of Tris-buffered phenol and chloroform, and the DNA was precipitated with 2 volumes of ethanol. Precipitated DNA was lifted out of the ethanol with a Pasteur pipette and placed into a microfuge tube. Excess ethanol was aspirated, and the DNA pellet was dissolved in water.

For the isolation of total RNA, snap-frozen tumors were crushed into a fine powder as described above, and total RNA was prepared. Briefly, the tumor powder was dissolved in tissue guanidinium solution (5 M guanidinium isothiocyanate, 50 mM Tris-HCl [pH 7.5], 10 mM EDTA, 5% β-mercaptoethanol), vortexed vigorously, and cleared by centrifugation at 12,500 × *g* for 10 min at 12°C. The resulting supernatant was drawn rapidly three times through a 20-gauge needle to shear the DNA, made to a final concentration of 2% *N*-lauryl sarcosine, and heated at 65°C for 2 min. Following the addition of 0.1 g of CsCl per ml of solution, the RNA was pelleted through a 1.5-ml 5.7 M CsCl cushion by centrifugation at 120,000 × *g* for 14 h at 15°C. The resulting RNA pellet was dissolved in 0.2 ml of tissue resuspension buffer (5 mM EDTA, 0.5% *N*-lauryl sarcosine, 5% β-mercaptoethanol), extracted with Tris-buffered phenol and chloroform, and ethanol precipitated.

Isolation of Ad9 virion DNA. Twelve 100-mm tissue culture dishes of A549 cells were infected with Ad9, and when extensive cytopathic effect was evident, the cells were harvested and pelleted (1,500 × *g* for 10 min at 4°C). The infected-cell pellets were pooled, frozen and thawed once, and sonicated for 3 min at 0°C. This infected-cell lysate was cleared of debris by centrifugation (1,500 × *g* for 10 min at 4°C), layered over a CsCl step gradient (3 ml of 1.25-g/ml CsCl over 3 ml of 1.4-g/ml CsCl), and centrifuged at 160,000 × *g* for 1 h at 14°C. The virion band was collected with a syringe needle and purified a second time by mixing with 11 ml of 1.34-g/ml CsCl and centrifuging at 160,000 × *g* for 16 h at 14°C. These twice-banded Ad9 virions were collected, 2 volumes of TNE (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, 1 mM EDTA) were added, and the virions were precipitated with ethanol. The virion pellet was resuspended in 2.2 ml of TNE containing 0.55% SDS, 20 mM EDTA, and 0.4 mg of proteinase K, incubated for 1 h at 37°C, and extracted with Tris-buffered phenol and chloroform, and the viral DNA was precipitated with ethanol and resuspended in water.

Southern analysis. High-molecular-weight tumor DNA (20 μg) was digested with 50 U of the restriction enzyme *Bgl*II (New England BioLabs) for 3 h, sequentially extracted with equal volumes of Tris-buffered phenol and chloroform, and then precipitated with ethanol. The DNA was resuspended in 25 μl of water and 1/10 volume of electrophoresis loading buffer (15% Ficoll [type 400], 0.25% bromophenol blue) and subjected to electrophoresis through a horizontal 0.8% agarose gel in 1× TBE (50 mM Tris-HCl, 50 mM boric acid, 0.5 mM EDTA) at 75 V for 12 h. The DNA was nicked in the agarose gel with 0.25 M HCl for 15 min, denatured with 0.5 M NaOH–1.5 M NaCl for 1 h, and neutralized with 1 M Tris-HCl (pH 8.0)–1.5 M NaCl for 1 h. The DNA was then transferred to a nitrocellulose membrane (Schleicher & Schuell BA85) by capillary action in 10× SSC for 12 to 24 h. The membrane was rinsed briefly with 6× SSC, baked for 2 h at 80°C, enclosed in a heat-sealable bag with prehybridization solution (25 mM KPO_4 [pH 7.4], 5× SSC, 5× Denhardt's solution, 50% deionized formamide, 200 μg of salmon sperm DNA per ml), and incubated with agitation at 42°C for 6 h. The prehybridization solution was replaced with hybridization solution (the same as prehybridization solution but including 10% dextran sulfate). The complete Ad9 genome was ^{32}P-labeled by the random primer method (18) with two radioactive nucleotide triphosphates. The resulting probe was denatured by boiling for 10 min, and then 5 × 10^5 cpm of probe per ml was added to the hybridization solution. The bag was agitated during hybridization at 42°C for 24 h. At the completion of the hybridization, filters were washed twice with 2× SSC–0.1% SDS at room temperature and three times with 0.25× SSC–0.1% SDS at 65°C and exposed to Kodak X-AR film at −80°C with an intensifying screen.

RNase protection analysis. Specific tumor RNAs were detected by the RNase protection assay (19). ^{32}P-labeled RNA was produced as follows: 4 μl of 5× transcription buffer (200 mM Tris-HCl [pH 8.0], 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl), 1 μl of 200 mM dithiothreitol, 2 μl of 4 mM dNTPs (4 mM each ATP, TTP, and GTP), 10 μl of [α-^{32}P]CTP (10 mCi/ml; 800 Ci/mmol), 1 μl of placental RNase inhibitor (40 U), and 1 μl of T7 RNA polymerase (5 U) were mixed in a microfuge tube and incubated at 37°C for 1 h. Three units of RNase-free DNase I was added, and incubation was continued at 37°C for 15 min. Following Tris-buffered phenol extraction, 10 μg of tRNA was added, and the ^{32}P-labeled RNA transcript was purified over a 5-ml Sephadex G50-150 (diethylpyrocarbonate-treated) column in water. Peak radioactive fractions were pooled, ethanol precipitated, and resuspended in hybridization buffer (40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4], 0.4 mM NaCl, 1 mM EDTA, 80% deionized formamide) at a concentration of 5 × 10^5 cpm/30 μl. For hybridizations, a 20-μg pellet of ethanol-precipitated tumor RNA was dissolved in 30 μl of probe in hybridization buffer, denatured at 85°C for 5 min, and hybridized at 58°C for 12 h. Then 350 μl of RNase digestion buffer (10 mM Tris-HCl [pH 7.5], 300 mM NaCl, 5 mM EDTA) containing 40 μg of RNase A and 2 μg of RNase T_1 per ml was added, and the tube was incubated at 30°C for 60 min. Next, 10 μl of 20% SDS and 2.5 μl of 20-mg/ml proteinase K was added, and after incubation at 37°C for 15 min, the solution was extracted once with an equal volume of phenol-chloroform and ethanol precipitated. The pellet was dissolved in 5 μl of RNA loading buffer (80% formamide, 1 mM EDTA [pH 8.0], 0.1% bromophenol blue, 0.1% xylene cyanol), heated to 85°C for 5 min, and loaded on a denaturing 6% polyacrylamide–urea gel. The gels were

TABLE 1. Induction of mammary tumors by Ad9 in W/Fu rats and their estrogen dependence[a]

Group[b]	Sex of animals	Gonadectomy at 4 wk	DES treatment[c]	No. with tumors/ no. in group
1	F	No	No	16/16
2A	F	Yes	No	0/7
2B	F	Yes	Yes	5/7
3	M	No	No	0/11
4	M	Yes	No	0/3
5A	M	Yes	Yes	7/7
5B	M	Yes	No	0/6
5C	M	Yes	Yes	6/6

[a] Approximately 24-h-old newborn rats were inoculated subcutaneously on the dorsal side with 0.4 ml (0.2 ml on right side and 0.2 ml on left side) of a 10^8-PFU/ml stock of Ad9.

[b] Groups 2A and 2B represent the same animals. Group 2A are Ad9-infected and ovariectomized females which were monitored for 5 months for tumor formation. Group 2B are the animals of group 2A subsequently treated with DES. Groups 5A, 5B, and 5C also represent the same animals. Group 5A are Ad9-infected and castrated male rats treated with DES. Group 5B are six of the seven animals of group 5A following withdrawal of DES treatment, and group 5C are the animals of group 5B following resumption of DES treatment.

[c] Rats were treated with DES by repeated subcutaneous implantation of DES-packed silastic tubing.

dried down on Whatman 3MM paper at 80°C and exposed to Kodak X-AR film at −80°C with an intensifying screen.

RESULTS

Ad9-induced tumorigenesis. To confirm the reported tumorigenicity of Ad9 in W/Fu rats, we obtained the Ad9 Hicks strain from the American Type Culture Collection, plaque-purified it, and produced high-titer stocks (approximately 10^8 PFU/ml). Newborn W/Fu rats (within 24 h of birth) were inoculated subcutaneously with approximately 5×10^7 PFU of Ad9 and observed for the formation of tumors. Over a 1-year period, 16 of 16 females and 0 of 11 males developed tumors (Table 1, groups 1 and 3). Tumors were first detected when the animals were about 3 months old, and all females had palpable tumors by 5 months of age. Tumors were subcutaneous on the ventral body surface near one of the 12 mammary glands. Most rats developed multiple mammary tumors, some of which grew to huge dimensions (>5 cm in diameter) after several months. As has been reported previously (4, 28), only mammary tumors were observed, and the tumors were neither metastatic nor life-threatening.

Three histological classes of mammary tumors are induced by Ad9. H&E-stained mammary tumor tissue sections were examined histologically (Fig. 1). The most frequently encountered tumors were benign mammary fibroadenomas, as reported earlier (4). These tumors were contained within a well-defined capsule and moved freely beneath the skin. Histologically, they showed a sparse cellular stroma embedded within a dense extracellular matrix which was interspersed with various amounts of ductal and alveolar epithelium (Fig. 1A).

The second histological tumor type, not previously reported for Ad9, resembled a phyllodeslike tumor (cystosarcoma phyllodes), which is a rare breast tumor found in women (39). Two variants of this tumor were identified. In the first type, the overall cellular architecture was similar to that of the fibroadenoma but showed increased stromal cellularity (Fig. 1B). The second variant exhibited a highly atypical stroma with mitoses and was identified as a malignant phyllodeslike tumor (Fig. 1C). Both the benign and malignant forms of the phyllodeslike tumor contained benign ductal and alveolar elements.

The third tumor type elicited in Ad9-infected rats was a solid sarcoma (Fig. 1D). These tumors were composed exclusively of highly atypical stromal cells with mitotic activity; there were no glandular components. Some tumors were seen with a solid stromal sarcoma bordering on a malignant phyllodeslike tumor, suggesting that the solid sarcomas may originate from phyllodeslike tumors by loss of glandular elements.

Additionally, we did not observe conversion of fibroadenomas to malignant tumors when 10 different fibroadenomas were rebiopsied multiple times during a 1-year period. Thus, it appears that benign fibroadenomas do not progress to malignant tumors.

Ad9-induced mammary tumors are estrogen dependent. The fact that normal male rats are resistant to Ad9 tumorigenesis suggested that the mammary tumors in females might be dependent on estrogen for development. Since estrogen synthesis occurs in the ovaries, we initially tested our hypothesis by castrating Ad9-infected females prior to tumor formation at 4 weeks of age. As shown in Table 1, ovariectomized female rats were completely resistant to Ad9-induced mammary tumor development (group 2A), behaving identically to male rats (group 3). Treatment of these Ad9-infected ovariectomized females with an estrogen analog, DES, subsequently elicited the formation of mammary tumors (group 2B). These results indicate that estrogen is absolutely required for the initiation of Ad9-induced mammary tumor growth.

Since male rats were completely resistant to Ad9-induced tumor formation, we wanted to determine whether they could be made susceptible to Ad9 by castration and treatment with DES. The results of such experiments are presented in Table 1. Ad9-infected castrated male rats which received DES treatment developed mammary tumors (group 5A). Similar results have been reported previously (3, 33a). With two controls, we were able to show that uninfected and DES-treated castrated male rats (not shown) as well as Ad9-infected castrated male rats (group 4) remained tumor free. Male mammary tumors regressed when the DES treatment was withdrawn (group 5B) and reappeared upon resumption of the DES treatment (group 5C). These results establish for the first time that estrogen is required for both initiation and maintenance of Ad9-induced mammary tumors.

Ad9 DNA is present in mammary tumor cells. Tumors elicited by oncogenic adenoviruses in rodents invariably contain integrated within the cellular DNA at least a portion of and more often an entire copy of the virus genome (21, 26, 33). To determine the state of the Ad9 virus genome in mammary tumors, we performed Southern blot analysis on high-molecular-weight tumor DNA. Figure 2 shows the results of such an analysis, in which tumor DNA was digested with *Bgl*II and probed with the entire Ad9 genome. Several conclusions may be drawn from this experiment. First, the entire virus genome is present in the tumor cells, since all internal *Bgl*II fragments were detected. Second, the viral DNA is probably integrated, because virus terminal fragments show unique altered mobilities (Fig. 2 and data not shown). Third, the copy number varies from less than one to several Ad9 genomes per tumor cell. Fourth, the tumors are monoclonal, since unique virus terminal fragments were detected for each tumor DNA.

It is important to note that many mammary tumors exhibit

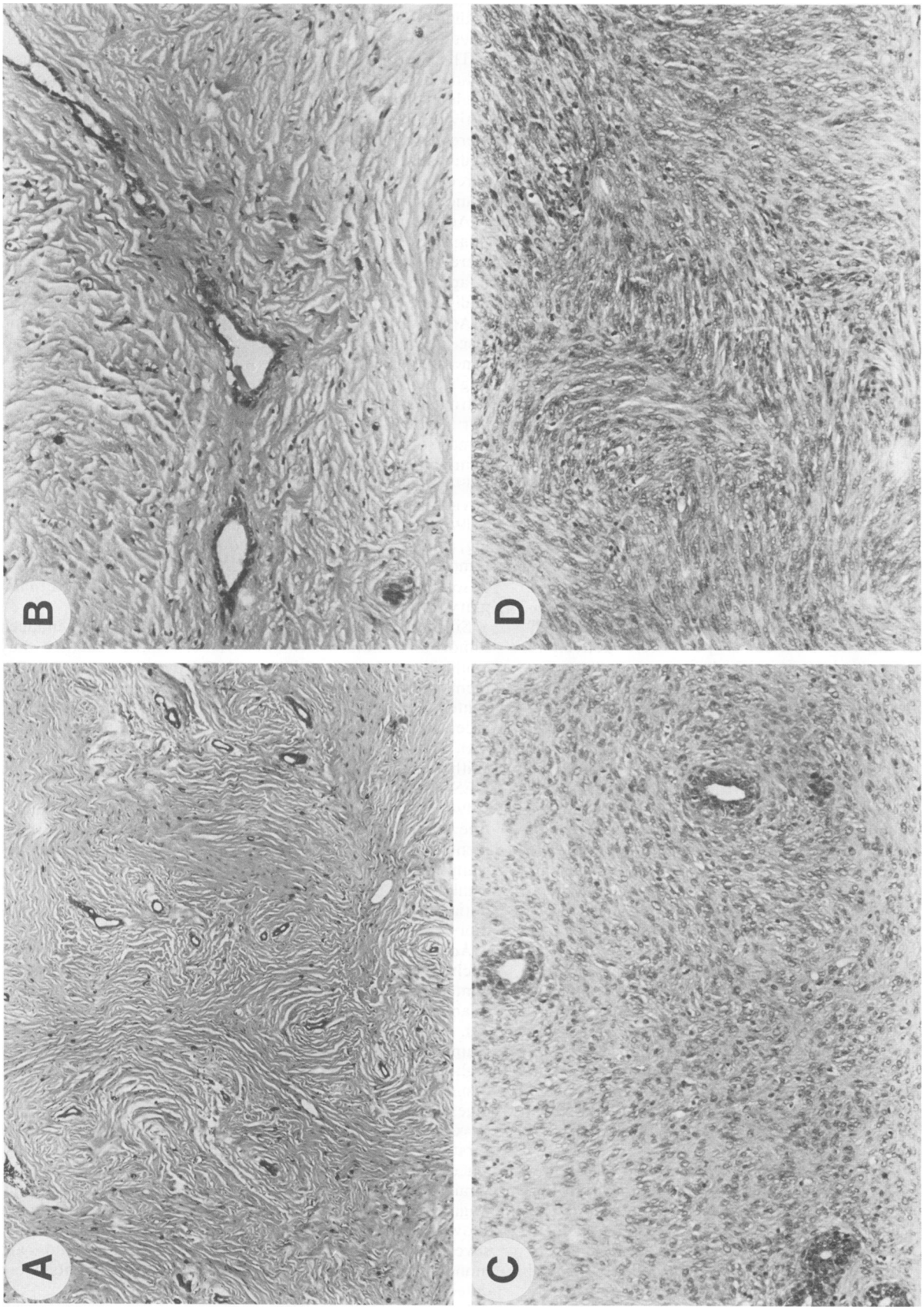

FIG. 1. Histological appearance of Ad9-induced mammary tumors in female W/Fu rats. (A) Fibroadenoma (H&E). Glands are surrounded by a dense stroma consisting of sparse stromal cells embedded in extracellular matrix. (B) Phyllodeslike tumor with benign stroma (H&E). The overall architecture is that of a fibroadenoma, but there is marked stromal cellularity. (C) Phyllodeslike tumor with malignant stroma (H&E). The glands are surrounded by a highly cellular, atypical stroma displaying mitotic figures. (D) Solid sarcoma (H&E). Highly atypical stroma is devoid of glandular components, and there are numerous mitoses. Original magnification: (A) ×100; (B, C, and D) ×400.

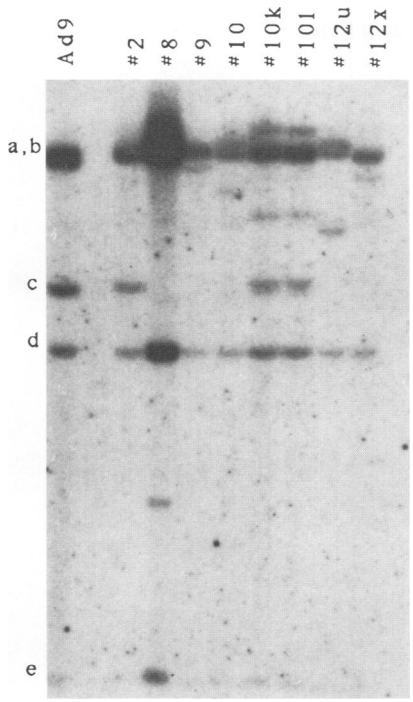

FIG. 2. Southern analysis of Ad9-induced rat mammary tumor high-molecular-weight DNAs (20 μg per lane) digested with *Bgl*II and probed with the entire Ad9 virus genome. Lanes: Ad9, Ad9 DNA (1 virus genome per cell equivalent) added to 20 μg of W/Fu rat cellular DNA; tumor 2, fibroadenoma; tumor 8, malignant solid sarcoma; tumor 9, fibroadenoma; tumor 10, fibroadenoma; tumor 10k, benign phyllodeslike tumor; tumor 101, same as 10k but different region of tumor; tumor 12u, fibroadenoma; tumor 12x, malignant phyllodeslike tumor. The *Bgl*II DNA fragments a and c represent the right and left termini of the Ad9 virus genome, respectively.

less than the reconstruction value of one Ad9 genome per cell (Fig. 2, tumors 2, 9, 10, 12u, and 12x). We believe that these tumors consist of a mixture of tumor and nontumor cells. By performing reconstructions with known amounts of Ad9 virus DNA, we estimate that 30 to 50% of the cells from these tumors contain a single entire integrated copy of Ad9 virus DNA (not shown).

As shown in lane 4, the solid sarcoma (tumor 8) contained high levels of Ad9 DNA. Multiple integrated virus genomes are present within each cell of this tumor, since we can detect a virus DNA fragment with the predicted size of a head-to-tail linkage of the right and left ends of the virus genome as well as two independent left-end integration junction fragments (data not shown). High Ad9 DNA copy number does not seem, however, to be a requirement for malignant tumors in general, since two malignant phyllodeslike tumors studied contained only a single integrated copy of the Ad9 genome in the tumor cells (Fig. 2, tumor 12x, and not shown). To date, all benign fibroadenomas analyzed contain a single integrated copy (Fig. 2, and not shown), but malignant tumors have one or multiple copies of the entire Ad9 genome in each tumor cell. The significance of DNA copy number to the tumor type, if any, is not clear.

Ad9 and estrogen receptor mRNAs are expressed in mammary tumor cells. Since the entire Ad9 genome is contained within mammary tumor cells, we wished to examine mRNA expression from the adenovirus transforming genes E1a and E1b, as well as some of the other early viral genes. In order to accomplish this, we cloned gene-specific fragments of the Ad9 genome into plasmids from which radiolabeled antisense RNAs could be generated for RNase protection analysis. As shown in Fig. 3, we were able to detect both E1a and E4 Ad9 mRNAs in all mammary tumors examined. Similarly, rER mRNA was expressed in all tumors. This raises the possibility that the mammary tumor cells are directly responsive to estrogen. We were not able, however, to detect Ad9 E1b or E3 mRNA in the mammary tumors. Lack of detectable E1b mRNA expression is unprecedented for adenovirus-induced tumors (20). Also worth noting is that expression of both E1a and E4 was elevated in the malignant solid sarcoma (tumor 8) compared with the benign fibroadenomas and phyllodeslike tumors (benign and malignant) examined here. Elevated virus gene expression has also been found for one malignant phyllodeslike tumor (not shown). Thus, some but not all malignant tumors express higher levels of E1a and E4 mRNAs, but the importance of this observation, if any, has not been established.

Mammary tumors arise from both mammary fibroblast and myoepithelial cells. Since mammary tissue is composed of several distinct cell types (epithelial, myoepithelial, and fibroblast), it was of interest to find which of these cells is the target of Ad9. It seems unlikely that epithelial cells are targeted by Ad9, since we and others (4, 28) have not detected Ad9-induced adenomas or carcinomas. These two tumor types are, by definition, derived from epithelial cells. In fact, the types of tumors that Ad9 elicits are classified as mesenchymal tumors (fibroadenomas, phyllodeslike tumors, and solid sarcomas), which strongly suggests at the outset that mammary stromal fibroblasts or myoepithelial cells are targeted. To formally exclude epithelial cells from consideration, we performed in situ hybridization with Ad9 E1a and E4 as probes to determine the location of viral mRNA-expressing cells in fibroadenomas and phyllodeslike tumors, which contain both stromal and epithelial cells. As shown in Fig. 4A to D, only stromal cells expressed E1a and E4 mRNAs in these tumors, and the expressing cells represented only a fraction of the total stromal cells. After examining many tumor sections, we have not been able to detect Ad9 gene expression in epithelial cells.

It is interesting that the in situ hybridization experiments indicate that only a small fraction of the tumor stromal cells accumulate detectable Ad9 mRNA at any given moment. On the other hand, the Southern analysis suggests that a much greater fraction of tumor cells than are seen expressing viral mRNA by in situ hybridization contain Ad9 DNA (Fig. 2). Perhaps viral mRNA expression within tumor cells is dependent on a specific stage of the cell cycle.

Expression of Ad9 E1a and E4 mRNAs was also examined in solid sarcomas (Fig. 4E and 4F). As with fibroadenomas and phyllodeslike tumors, only a small fraction of cells from the solid sarcoma expressed viral mRNAs at levels that could be detected by in situ hybridization. We did not perform an RNase protection assay on this particular tumor's RNA to determine whether its Ad9 E1a and E4 mRNA levels were elevated. Thus, presently we do not know whether elevated viral RNA expression exhibited by some malignant tumors is the result of higher expression per cell or a larger proportion of expressing cells.

As established above, stromal cells give rise to the mammary tumor cells. Unfortunately, in situ hybridization cannot distinguish between mammary fibroblasts and myoepithelial cells. To do this, it is necessary to examine cell type-specific markers. As expected, all tumor stromal cells

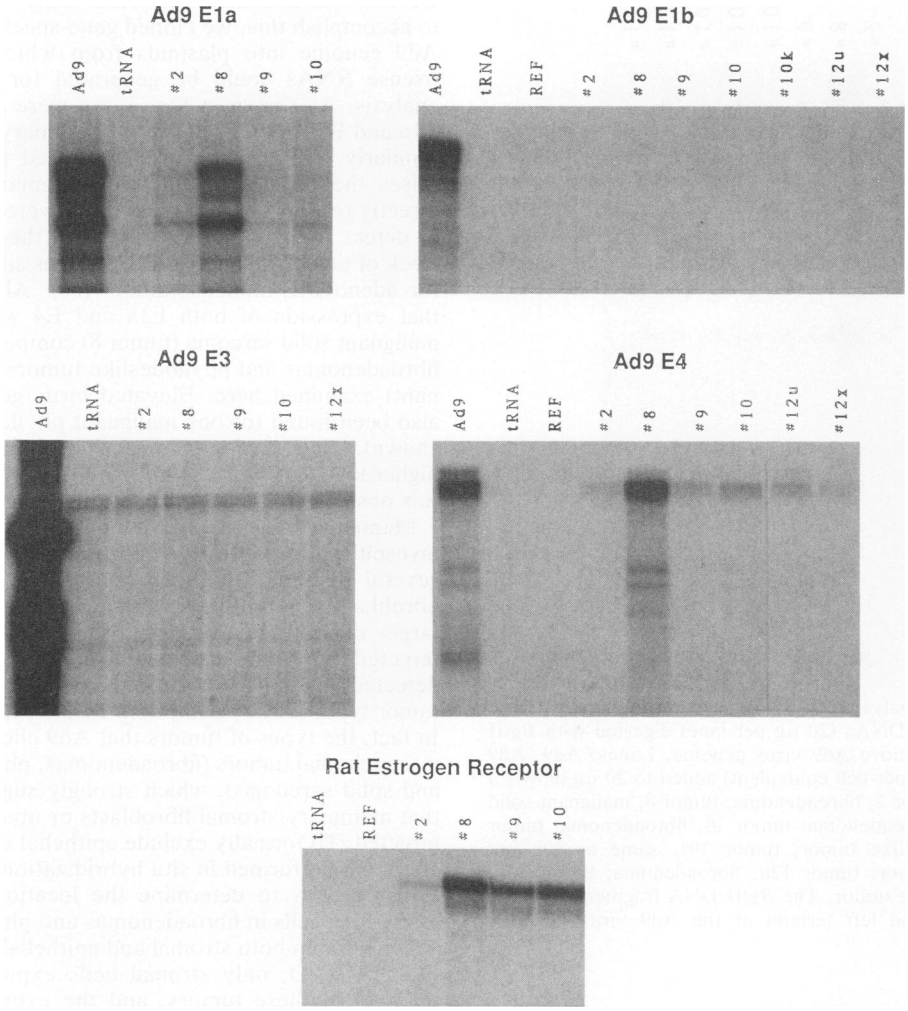

FIG. 3. RNase protection analysis of Ad9-induced rat mammary tumor total RNAs. Examination of Ad9 E1a, E1b, E3, and E4 and rat estrogen receptor mRNAs (20 μg per lane). Lanes: Ad9, 3 ng of total RNA isolated from Ad9-infected A549 cells (9 h postinfection). Numbered tumors are explained in the legend to Fig. 2.

expressed vimentin, an antigen specific for stromal cells in general, but did not express detectable levels of cytokeratins, which are found predominantly in epithelial cells (Table 2). It is known that mammary fibroblasts specifically express collagen type I and that mammary myoepithelial cells specifically express collagen type IV as well as muscle-specific actin (14, 52). The stromal cells of five of five benign fibroadenomas expressed collagen type I but did not express collagen type IV. In contrast, three of three malignant phyllodeslike tumors were found to express collagen type IV and muscle-specific actin (Table 2). The one solid sarcoma tested was positive for collagen type IV, while one of the three malignant phyllodeslike tumors also expressed epithelial membrane antigen (54). The heterogeneous antigen profiles of the malignant myoepithelial cells may result from the fact that intermediate variants between epithelial and myoepithelial cells exist in vivo and in vitro (41).

Further evidence regarding the origin of the malignant tumor cells was provided by electron microscopy. Two malignant phyllodeslike tumors were examined and found to exhibit basal lamina, a characteristic of myoepithelial cells, which is not produced by mammary fibroblasts (data not shown). Interestingly, the one benign phyllodeslike tumor examined contained a mixture of collagen type I- and IV-expressing cells (not shown). Therefore, while the cell type origin leading to benign phyllodeslike tumors was not established, it appears that Ad9 targets mammary fibroblasts to elicit benign fibroadenomas and myoepithelial cells to elicit malignant tumors.

DISCUSSION

In this study, we have examined the histology, estrogen dependence, viral DNA and RNA, and cell types of Ad9-induced mammary tumors in W/Fu rats, and we reached several new conclusions. First, besides fibroadenomas and solid sarcomas, Ad9 also induces a phyllodeslike tumor (Fig. 1). Presently, Ad9 infection of W/Fu rats represents the only model system for the study of cystosarcoma phyllodes, a rare breast tumor of women (39). Second, the mammary tumors are estrogen dependent not only for initiation, but also for maintenance (Table 1). Third, the mammary tumors

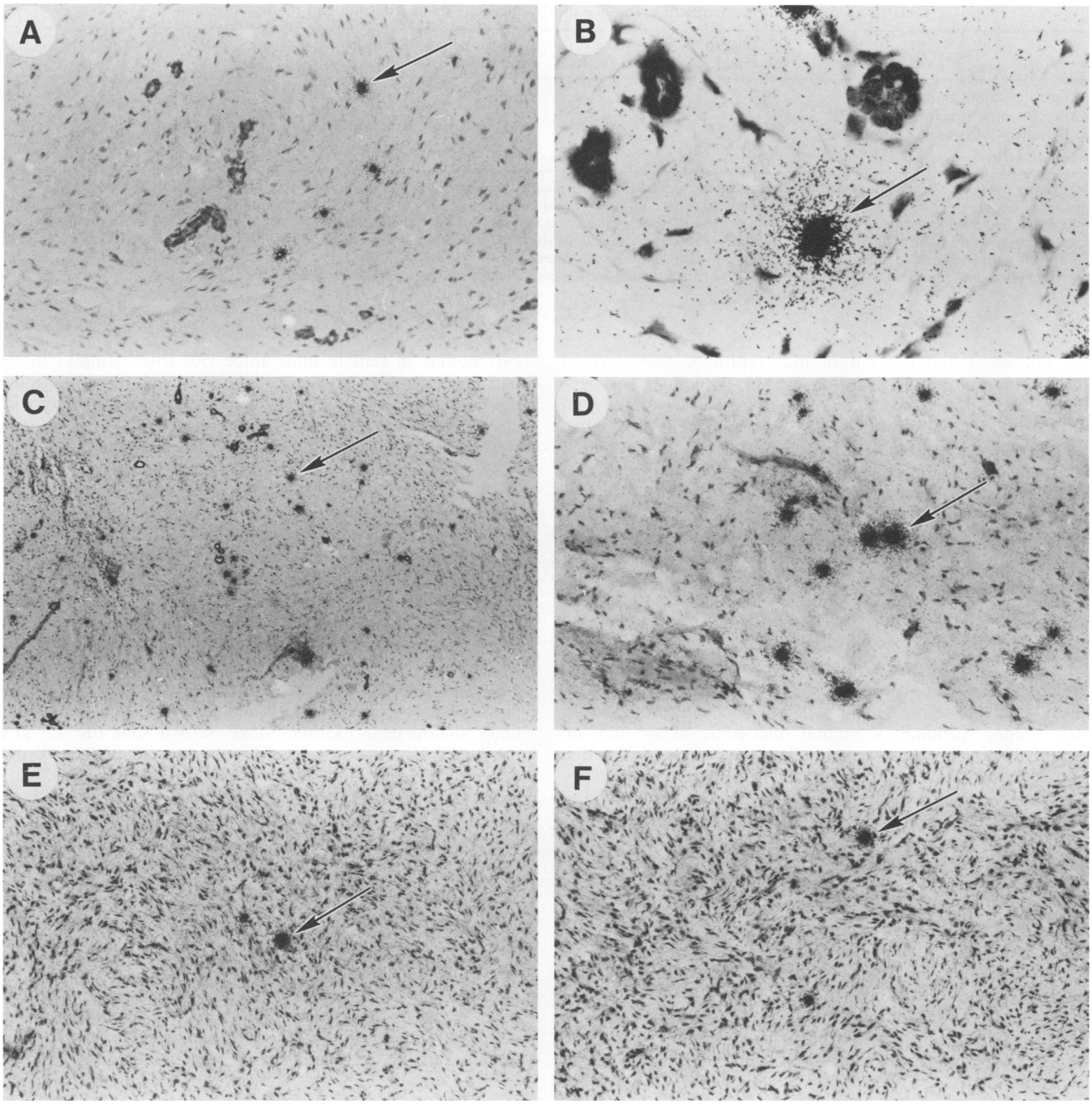

FIG. 4. In situ hybridization of Ad9-induced rat mammary tumors for expression of Ad9 E1a and E4 mRNAs. Arrows point to representative viral mRNA-expressing stromal cells. (A and B) Fibroadenoma hybridized with the Ad9 E1a probe (A) and Ad9 E4 probe (B). (C and D) Phyllodeslike tumor hybridized with the Ad9 E1a probe (C) and Ad9 E4 probe (D). (E and F) Solid sarcoma hybridized with the Ad9 E1a probe (E) and Ad9 E4 probe (F). Original magnification: (A, C, D, E, and F) ×100; (B) ×400.

contain one or multiple integrated copies of the entire Ad9 genome (Fig. 2). Fourth, the mammary tumors express detectable estrogen receptor and Ad9 E1a and E4, but not Ad9 E1b or E3 mRNA (Fig. 3 and 4). Fifth, both fibroblasts (fibroadenomas) and myoepithelial cells (malignant tumors) are the targeted mammary cells for Ad9 tumorigenesis (Fig. 4 and Table 2). We have also confirmed previous results concerning specific induction of mammary tumors in female W/Fu rats by Ad9 (4) and the estrogen dependence of these mammary tumors (3, 33a). We found that Ad9 elicits only mammary tumors, with approximately 100% efficiency in female W/Fu rats, and that Ad9-infected male rats develop mammary tumors following castration and treatment with DES (Table 1).

It should not come as a surprise that Ad9 targets stromal cells (fibroblasts and myoepithelial cells) of the mammary gland for tumorigenesis, since oncogenic subgroup A and B adenoviruses usually elicit mesenchymal tumors (sarcomas

TABLE 2. Immunocytochemical reactivity of stromal cells from Ad9-induced mammary tumors[a]

Tumor[b]	Collagen type I	Collagen type IV	Cytokeratins	Epithelial membrane antigen	Muscle-specific actin	Vimentin
Benign fibroadenomas (5)	+	−	−	−	−	+
Malignant phyllodeslike tumor (1)	−	+	−	+	+	+
Malignant phyllodeslike tumors (2)	−	+	−	−	+	+
Malignant solid sarcoma (1)	−	+	−	−	−	+

[a] Frozen tissue was sectioned and processed for immunocytochemical staining as described in Materials and Methods. Antibody to collagen type I was a product of Chemicon Inc., Temecula, Calif.; antibody to collagen type IV was a kind gift from P. Yurchenco, Robert Wood Johnson Medical School; antibodies to vimentin and muscle-specific actin were obtained from Biogenex Corp., San Ramon, Calif.; antibody to epithelial membrane antigen was a product of Dako Corp., Carpenteria, Calif. The cytokeratin antibody cocktail contained antibodies MNF 116 (Dako Corp.), MAK-6 (Triton, Alameda, Calif.), AE1/AE3 (Hybritech, San Diego, Calif.), and Cam 5.2 (Becton-Dickinson, Mountain View, Calif.).

[b] Numbers in parentheses are the numbers of the specified tumors having the indicated antigen expression profile.

and fibromas) and not carcinomas (epithelial cell-derived tumors) in animals (50, 51). Interestingly, mammary myoepithelial cells, which produce the malignant tumors analyzed here, appear to be derived from epithelial cells through a differentiation step (8, 14). The reason for the mesenchymal cell tropism of adenoviruses in rodents is not understood.

In contrast to the results of Jonsson and Ankerst (28), our results do not show that malignant conversion of fibroadenomas is the source of the malignant tumors observed. If this were the case, we would expect to find fibroadenomas in the process of progressing to malignancy; that is, tumors should be identified that contain regions composed of both benign fibroadenoma and malignant tumor tissues. Also, we would predict that significantly more malignant tumors would develop in older animals. To date, we have not obtained such results. Thus, it seems possible that the potential for a tumor to evolve as benign or malignant may be determined at an early stage. Although a second oncogenic event may be responsible for progression to malignancy, we favor the idea that fibroadenomas result from Ad9 infection of mammary fibroblasts while malignant tumors result from Ad9 infection of myoepithelial cells. The fact that benign fibroadenomas are derived from fibroblasts and malignant tumors are derived from myoepithelial cells (Table 2) supports this notion.

The malignant tumors that we have encountered are limited to malignant phyllodeslike tumors and solid sarcomas (Fig. 1). Jonsson and Ankerst (28), who originally characterized Ad9 tumorigenesis, reported finding many types of malignant tumors, including round cell liposarcomas, osteosarcomas, and malignant mesenchymomas. We can only speculate that factors such as the source of W/Fu rats, rat diet, type of cells used to propagate Ad9, and the fact that we used a plaque-purified derivative of Ad9 might contribute to these differences.

In some respects, nitrosomethylurea (NMU) carcinogenesis resembles Ad9 carcinogenesis in female rats. A single injection of NMU into female rats elicits mammary tumors with high incidence (22). Unlike Ad9 tumorigenesis, however, mostly adenocarcinomas are produced, but depending on the dose of NMU given, some fibroadenomas do arise (40). These tumors seem to arise predominantly as a result of a single point mutation within the *ras* oncogene (31, 49). In the NMU-induced mammary tumor system (6, 55), as with Ad9-induced mammary tumors (Table 1), estrogen is required for both tumor initiation and maintenance. We do not yet know whether the Ad9-induced mammary tumors contain *ras* mutations as well, but we think it unlikely since these tumors already express at least one viral oncogene, E1a. It is also worth mentioning that the A2 strain of polyomavirus induces estrogen-dependent mammary adenocarcinomas in female nude mice (9). Interestingly, in this particular system, estrogen is required only for initiation of the mammary carcinomas, since subsequent hormone withdrawal does not lead to tumor regression.

The molecular analyses of Ad9-induced mammary tumors have provided both familiar and unexpected results. As with most Ad12-induced tumors (20) and Ad9-transformed 3Y1 cells (12), the mammary tumors elicited by Ad9 contain the entire virus genome integrated into the cellular DNA. Equally familiar is the finding that Ad9 E1a mRNA is expressed, since this gene is invariably detected in adenovirus-induced tumors. Furthermore, in tumors containing the right end of the adenovirus genome, it is not unusual to find expression of E4 mRNA or an E4-derived T antigen (13, 16, 17, 35, 42). The fact that both Ad9 E1a and E4 mRNAs are expressed in all mammary tumors examined raises the possibility that, besides E1a, E4 also contributes to mammary oncogenesis. Others have shown that the E4 gene product can contribute to soft-agar growth of transformed cells in vitro (46), and studies with Ad12 (38) and Ad5-Ad12 recombinant viruses (10, 44) suggest that a gene outside the E1 region may contribute to oncogenicity. We are currently testing the possibility that the E4 gene may play a role in Ad9 tumorigenesis.

We have not been able to detect mRNA expression from the Ad9 E1b gene in rat mammary tumors. This result was unexpected because of the well-established role of both E1a and E1b gene products in adenovirus tumorigenesis (reviewed in reference 20). We cannot, however, rule out low, undetectable, but significant E1b expression in the mammary tumors. Therefore, Ad9 E1b mutant viruses must be tested for induction of mammary tumors before firm conclusions regarding the need for E1b expression can be made. If Ad9 is, indeed, capable of producing mammary tumors in rats without expression of the E1b gene, one must argue that susceptible rat cells can complement the lack of Ad9 E1b expression. Although it is well known that both E1a and E1b are needed to transform primary rodent cells in culture, there are several reports which support the idea that established rodent fibroblast cells do not absolutely require adenovirus E1b for transformation. Ad12 E1a alone can transform the established REF line 3Y1 (47), and Ad5 E1a alone can transform both the established REF line CREF (7) and the established mouse embryo fibroblast line 3T3 (45). Perhaps established rodent fibroblast cell lines and specialized stromal cells of the W/Fu rat mammary gland share a similar alteration(s) which allows transformation by Ad9.

E1b-independent oncogenesis could, in theory, occur if the target cells contain mutant p53 alleles (15, 25). This would bypass the need for the adenovirus E1b 55-kDa

protein which, like mutation of p53, stabilizes p53 protein (34, 43, 57) and might contribute to oncogenesis by interfering with the function of this cellular tumor suppressor gene. Work is in progress to test the possibility that a cellular oncogene contributes to Ad9 tumorigenesis.

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