Human Adenovirus Type 9 E4 Open Reading Frame 1 Encodes a Cytoplasmic Transforming Protein Capable of Increasing the Oncogenicity of CREF Cells

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The induction of estrogen-dependent rat mammary tumors by human adenovirus type 9 (Ad9) requires the Ad9 E4 open reading frame 1 (9ORF1) protein, which alone can transform the rat embryo fibroblast cell line CREF in vitro. In the present study, independent pools of both 9ORF1-expressing and control CREF cells were generated by selection with G418 and compared with respect to transformed properties. Indirect immunofluorescence analyses revealed that more than 99% of the cells that made up the 9ORF1-transfected pools expressed 9ORF1 protein and, together with confocal laser scanning microscopy, indicated that this E4 protein was located predominantly within the cytoplasm of cells. With regard to transformation, cells of the 9ORF1-expressing pools differed from those of control pools by forming foci, displaying morphological alterations, growing more efficiently in soft agar, and reaching higher saturation densities. Following injection into immunocompetent syngeneic rats, the 9ORF1-expressing pool cells exhibited greatly enhanced oncogenicity compared with control pool cells. These results show that 9ORF1 protein (i) localizes predominantly within the cytoplasm, (ii) confers multiple general transformed characteristics to CREF cells in vitro, and (iii) increases the tumorigenic properties of these cells in vivo.

Human adenoviruses are classified as DNA tumor viruses by virtue of their ability to cause tumors when inoculated into rodents. Unlike other oncogenic adenoviruses, the subgroup D human adenovirus type 9 (Ad9) causes only estrogen-dependent mammary tumors in female rats (1, 2, 23). The prevailing mammary tumor produced in Ad9-infected rats is fibroadenoma, which is also the most common type of breast tumor encountered in young women (36). Thus, besides potentially yielding new insights into the regulation of cellular proliferation, the study of mammary tumor induction by Ad9 may reveal mechanisms involved in generating an important breast tumor of women.

The transforming and oncogenic activities of human adenoviruses have traditionally been ascribed to the viral E1 region, which consists of the E1A and E1B transcription units (13). Surprisingly, an Ad9-encoded function essential for mammary tumor induction genetically maps outside of E1, within the E4 region (24). The human adenovirus E4 transcription unit, encoded within the rightmost 3 kb of the viral genome, yields multiple mRNAs, which produce six different proteins (6–9, 17, 20, 25, 28, 37, 38). One of these proteins, Ad9 E4 open reading frame 1 (9ORF1), is required for mammary tumor formation by Ad9, because replication-competent mutant viruses unable to express it are nontumorigenic in rats (25). By itself, 9ORF1 expression induces focus formation in CREF cells, and cell lines derived from these transformed foci grow in soft agar (25). Therefore, it is likely that 9ORF1 functions as an oncoprotein in the production of rat mammary tumors.

The goal of the current work was to identify general transformed properties in vitro and the oncogenic potential in vivo of 9ORF1-expressing CREF cells. This was accomplished by

generating and examining pools of 9ORF1-expressing CREF cells, an approach which circumvents the inherent problems associated with the study of clonal cell populations. In addition to extending previous observations regarding the in vitro transforming potential of 9ORF1 (25), the results presented here indicate that 9ORF1 is a cytoplasmic protein whose expression significantly increases the tumorigenicity of CREF cells.

MATERIALS AND METHODS

Cell lines and plasmids. CREF cells (12) were maintained in culture medium (Dulbecco's modified Eagle's medium containing 20 µg of gentamicin per ml) supplemented with 6% fetal bovine serum (FBS) and grown at 37°C in a humidified incubator under a 5% CO₂ atmosphere.

The 9ORF1-coding sequence was PCR amplified from an Ad9 *XhoI-SpeI* (87 to 100 map units) pUC19 plasmid with the following oligonucleotide primer pair flanked by *Bam*HI and *Eco*RI sites (restriction enzyme sites underlined): 5'-CTCGGATCCATGGCTGAATCTGTAT3' and 5'-CTCGAATTCACAGAAAGCATGCACTAA3'. The resulting PCR product, digested with *Bam*HI and *Eco*RI, was inserted into the corresponding sites of the pJ4Ω expression plasmid (43), generating pJ4Ω-9ORF1. The 9ORF1 insert was sequenced to verify that it was correct.

G418 selection and generation of cell pools. A 20-µg portion of pJ4Ω-9ORF1 or empty pJ4Ω was cotransfected with 4 µg of pCMV-NEO-BAM₃ (18) onto subconfluent CREF cells by calcium phosphate precipitation with a glycerol shock (27). At 72 h posttransfection, cells were passaged at low density (approximately 5 × 10⁵ cells per 100-mm-diameter dish) into culture medium supplemented with 6% FBS and G418 (380 µg/ml in 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.3] [Gibco BRL Life Technologies, Inc.]). G418-supplemented growth medium was replaced at 4-day intervals for 10 days. After this time, each cell pool was prepared by collecting all of the G418-resistant colonies (approximately 200 to 300 [see Fig. 1A]) appearing on a single dish. Two independent experimental (pJ4Ω-9ORF1-transfected) cell pools gave similar results for all analyses described in this study, as did two control (pJ4Ω-transfected) cell pools.

Immunoprecipitation and immunoblotting. Cultured cell and tumor extracts were prepared in RIPA buffer (150 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium desoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate, 50 mM Tris-HCl [pH 8.0]) containing protease inhibitors (300 µg of phenylmethylsulfonyl fluoride per ml and 6 µg each of aprotinin and leupeptin per ml) as described previously (25). Immunoprecipitation and immunoblotting for 9ORF1 protein were performed as described elsewhere (25), except that a horseradish peroxidase-conjugated secondary antibody (Southern Biotechnology Associates,

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FIG. 1. (A) G418-selected CREF cell colonies on culture dishes. CREF cells, transfected with either pJ4 Ω -9ORF1 or empty pJ4 Ω and pCMV-NEO-BAM₃ as the G418-selectable marker, were cultured in G418-supplemented growth medium for 13 days. Colonies were fixed in methanol and stained with crystal violet. Focus formation is apparent for CREF cells transfected with pJ4 Ω -9ORF1. (B) A 9ORF1-induced transformed focus arising within a G418-selected colony (magnification, $\times 100$; phase-contrast). (C) 9ORF1 protein expression in G418-selected CREF pool cells. Cells were lysed in RIPA buffer containing protease inhibitors. A 200- μ g portion of total protein was resolved on a 12% protein gel and subjected to immunoblot analysis with 9ORF1-immune serum as described in Materials and Methods. The parental CREF cell extract was included as a negative control.

Inc.) was used for immunoblot analyses and membranes were developed by enhanced chemiluminescence (Kirkegaard and Perry Laboratories, Inc.).

Indirect immunofluorescence and confocal laser scanning microscopy. Indirect immunofluorescence was performed by standard methods (16). Briefly, cells grown on microscope coverslips were fixed in -20°C methanol, blocked for 30 min in 10% goat serum in TBS (200 mM NaCl, 50 mM Tris-HCl [pH 7.5]), and reacted for 1.5 h at 37°C with either preimmune or 9ORF1-immune polyclonal serum (25). After several washes with TBS, cells were again blocked in goat serum, incubated for 45 min at 37°C with a goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Gibco BRL Life Technologies, Inc.), and washed several times with TBS. The cells were rinsed briefly with a 0.5- μ g/ml 4',6-diamidino-2-phenylindole (DAPI) solution, and coverslips were affixed to microscope slides with mounting medium (25 mg of 1,4-diazabicyclo-[2.2.2]-octane per ml in glycerol). Cells were photographed on Kodak TMAX 400 black-and-white film with an Olympus Vanox-S microscope.

For confocal laser scanning microscopy, indirect immunofluorescence was performed on cells as described above except that after reaction with the secondary antibody, coverslips were incubated with a 5- μ g/ml propidium iodide solution instead of a DAPI solution. A MultiProbe 2001 confocal laser scanning microscope (Molecular Dynamics, Inc.) equipped with a 100 $\times$ objective (1.4 numerical aperture; Nikon Inc.) and an argon/krypton laser ($\lambda = 488/565$ nm) was used for optical sectioning. Multiple optical sections through samples were collected and pseudocolored with ImageSpace software (Molecular Dynamics, Inc.). Images were recorded on Kodak EP2-100 color slide film.

Monolayer and anchorage-independent growth. For monolayer cell growth in 10 or 1% FBS, 10^5 cells were seeded onto 60-mm dishes in culture medium supplemented with the same FBS concentration used for the growth analysis. For growth studies in 0.1% FBS, cells were initially plated for 16 h in culture medium containing 10% FBS and, after three washes with Dulbecco's modified Eagle's medium were incubated with culture medium containing 0.1% FBS. Growth medium was replaced every 48 h, and cells from duplicate dishes were counted at each time point with an automated Coulter cell counter. Cell viability was determined by trypan blue dye exclusion.

Anchorage-independent growth in 0.4% soft agar was assessed as previously described (25). At 18 days postplating, the cells were photographed with a camera-mounted Nikon inverted microscope and Kodak TMAX 100 black-and-white film. The efficiency of soft agar growth was assessed after 19 days by determining the percentage of colonies larger than 0.2 mm in diameter, a size significantly larger than that observed for control CREF pools.

Tumorigenicity in rats. The indicated number of viable cells, suspended in 0.2 ml of Dulbecco's modified Eagle's medium, was injected subcutaneously at a single site on the right flank of suckling (3- to 4-day-old) or juvenile (19-day-old) Fischer 344 rats (Harlan Sprague Dawley) with a 1-ml syringe and a 21-gauge needle. Animals were monitored for tumor formation by palpation every other day. At the end of each experiment, the rats were sacrificed and tumor tissues were removed and frozen in liquid nitrogen for subsequent extract preparation. For statistical analyses, the two-sample *t* test for independent samples with unequal variances was used and *P* values were determined with Minitab statis-

FIG. 2. Indirect immunofluorescence analyses of 9ORF1-expressing and control CREF pools. (A and B, C and D, E and F, G and H) Paired identical fields examining either fluorescein fluorescence (A, C, E, and G) or DAPI fluorescence (B, D, F, and H) to show nuclei. 9ORF1-expressing (A, B, E, and F) or control (C, D, G, and H) pool cells were incubated with either 9ORF1-immune (A to D) or preimmune (E to H) serum. Magnification, $\times 111.25$.

FIG. 3. Confocal laser scanning microscopic analysis of 9ORF1 immunofluorescence in CREF cells. All three panels display the same representative section through the middle of two 9ORF1-expressing cells. (A) Combined propidium iodide and fluorescein fluorescence. (B) Propidium iodide fluorescence, indicating the location of nuclei. (C) Fluorescein fluorescence, showing the position of 9ORF1-containing punctate bodies. Scale bar, 20 μ m.

tical software (Minitab, Inc.). A result was considered statistically significant when $P < 0.05$. Animal handling and care were performed in accordance with institutional guidelines.

For histological analyses, tumors were fixed in buffered formalin (4% formaldehyde, 46 mM sodium phosphate dibasic, 29 mM sodium phosphate monobasic [pH 7.0]), embedded in paraffin, sectioned at 3 to 5 μ m, and stained with hematoxylin and eosin. Tumor sections were examined and photographed with an Olympus BH-2 photomicroscope equipped with an Olympus PM-10ADS automatic photomicrographic system.

RESULTS

Establishment of 9ORF1-expressing CREF cell pools. Pools of 9ORF1-expressing CREF cells were generated to examine the general transformed properties of nonclonal cell populations. This was accomplished by transfecting CREF cells with a G418-selectable marker and either a 9ORF1 expression plasmid (pJ4 Ω -9ORF1) or the empty expression plasmid (pJ4 Ω) as a control. The pJ4 Ω -9ORF1 expression plasmid, which utilizes the Moloney leukemia virus promoter (43), was chosen for these studies because, in preliminary experiments, it was found to potently induce focus formation on CREF cells.

Following selection with G418, hundreds of colonies arose on each of the pJ4 Ω -9ORF1-derived experimental and pJ4 Ω -derived control dishes (Fig. 1A). Since 9ORF1 expression induces focus formation on CREF cells (25), it was not surprising that transformed foci arose within the drug-resistant colonies formed by cells transfected with the pJ4 Ω -9ORF1 plasmid (Fig. 1A). The foci appeared as clumps of aggregated cells visibly raised above the surrounding monolayer and, microscopically, were found to be composed of small refractile cells (Fig. 1B). In contrast, each control colony resulting from drug selection with the empty pJ4 Ω plasmid formed a normal monolayer similar in appearance to that of parental CREF cells.

Two independent sets (pool 1 and pool 2) of matched experimental and control CREF pools were generated and analyzed for 9ORF1 protein expression by immunoblotting. The experimental pools but not the parental CREF cells or control pools produced the 14-kDa 9ORF1 protein (Fig. 1C).

9ORF1 protein localizes within the cell cytoplasm. The 9ORF1-expressing CREF pools were analyzed by indirect im-

muno fluorescence to ascertain the subcellular location of 9ORF1 protein and assess the proportion of cells expressing 9ORF1 protein. Cells of the 9ORF1-expressing pools incubated with 9ORF1-immune serum exhibited punctate and predominantly extranuclear fluorescence (Fig. 2A and B) which was specific, since this staining was absent from control pool cells incubated with either the 9ORF1-immune (Fig. 2C) or preimmune serum (Fig. 2G) as well as from 9ORF1-expressing pool cells treated with preimmune serum (Fig. 2E). These findings were verified by using an independent 9ORF1-immune polyclonal serum, a monoclonal antibody directed against epitope-tagged 9ORF1 protein, and alternative fixation methods (40).

By conventional fluorescence microscopy, the 9ORF1-containing punctate structures appeared to be present within the cytoplasm of the CREF cells. This was corroborated by confocal laser scanning microscopy, which allows for the visualization of thin cross sections through cells. A representative confocal microscopy image through the midsection of two 9ORF1-expressing cells is shown in Fig. 3. The analysis of many such sections, both horizontally and vertically with respect to the plane of the substratum, confirmed that the majority of 9ORF1 protein was indeed distributed throughout the cytoplasm. In addition to its predominantly cytoplasmic localization, 9ORF1 protein was occasionally observed in small quantities within the nuclei of CREF cells and, for other 9ORF1-expressing cell lines, along the cell periphery and at cell-cell junctions (41). The significance of these observations is not known, but they may hint at the possibility that 9ORF1 protein is functionally targeted to several locations within the cell.

Since relatively homogeneous populations of 9ORF1-expressing cells were needed to accurately measure the growth properties of the cell pools, it was important to know whether the 9ORF1-expressing pools contained a significant number of nonexpressing cells. By indirect immunofluorescence, no non-expressing cells were observed within the experimental cell pools during the examination of more than 10^3 cells (e.g., Fig. 3A). Therefore, greater than 99.9% of the cells making up the 9ORF1-expressing pools stably expressed 9ORF1 protein.

FIG. 4. Morphology of control (A and B) and 9ORF1-expressing (C and D) CREF pool cells that were subconfluent (A and C) or confluent (B and D). Arrows indicate examples of either elongated projections, more commonly seen in control pool cells, or lamellipodia, more frequently observed in the 9ORF1-expressing pool cells. Magnification, $\times 200$ (subconfluent cells) or $\times 100$ (confluent cells) (phase-contrast microscopy used in all panels).

FIG. 5. Soft agar growth comparison for control (A) and 9ORF1-expressing (B) CREF pools. A total of 10^5 viable cells were suspended in growth medium containing 10% FBS and 0.4% Noble agar. Photographs were taken 18 days postplating. Magnification, $\times 20$.

FIG. 6. Growth curves for 9ORF1-expressing (●) and control (□) CREF pool cells. A total of 10^5 cells from pool 1 (A, C, and E) or pool 2 (B, D, and F) were plated on 60-mm dishes in culture medium containing 10% (A and B), 1% (C and D), or 0.1% (E and F) FBS as described in Materials and Methods. The $\log_{10}$ mean viable cell number for duplicate dishes was plotted. Error bars represent the spread between two data points and are not visible when data points are very close to their mean.

9ORF1 induces morphological alterations and anchorage-independent growth in CREF cells. Transformed fibroblasts can exhibit a variety of morphological alterations as a result of cytoskeletal rearrangements (4). When they were subconfluent, 9ORF1-expressing pool cells displayed elongated projections less frequently than did control pool cells but exhibited

more numerous lamellipodia (sheet-like membrane extensions) (22). Representative examples of these morphological differences in subconfluent cells are presented in Fig. 4. These changes in cellular morphology suggested that 9ORF1 expression may directly or indirectly affect the organization of polymerized actin within cells, a process involving the rho sub-

TABLE 1. Saturation densities of 9ORF1-expressing and control CREF pool cells in 10 and 1% FBS

CREF pool	Growth medium conditions (% FBS)	Final time point of growth analysis (h) ^a	No. of cells/dish at final time point (10 ⁵) ^b	% Increase in cell number above control pool
Pool 1				
Control	10	168	93.0	
9ORF1-expressing	10	168	189	103
Control	1	264	85.8	
9ORF1-expressing	1	264	157	83
Pool 2				
Control	10	170	130	
9ORF1-expressing	10	170	172	32
Control	1	262	54.6	
9ORF1-expressing	1	262	102	87

^a The final time points are taken from the 10% and 1% growth curves shown in Fig. 6.^b These values represent the means of duplicate samples.

family of low-molecular-weight GTPases (31, 34, 35). Upon reaching confluency, the appearance of the 9ORF1-expressing pool monolayers became indistinguishable from that of the control pools (Fig. 4). With prolonged incubation at confluency, however, 9ORF1-expressing pool cells but not control pool cells formed numerous foci, acidified the culture medium, and, as a result of cell death, detached from the culture dishes. These properties are commonly associated with highly transformed cells.

When suspended in soft agar, the 9ORF1-expressing pool cells also exhibited significantly increased anchorage-independent growth compared with control pools (Fig. 5). This result was consistent with the previous observation that clonal CREF cell lines derived from 9ORF1-transformed foci also grow in soft agar (25). The mean cloning efficiencies for the 9ORF1-expressing pools, determined by counting more than 200 colonies, were $33 \pm 2\%$ (pool 1) and $31 \pm 5\%$ (pool 2). These findings, together with those presented above, demonstrated that some general effects of 9ORF1 expression in CREF cells include morphological alterations and focus formation, as well as increased anchorage-independent growth.

9ORF1 increases the saturation density of CREF cells. Since transformed cells may demonstrate increased growth rates or saturation densities (4), the ability of 9ORF1 to affect the monolayer growth properties of CREF cells was also examined. This was accomplished by comparing the growth of the cell pools on culture dishes under several growth medium conditions (10, 1, or 0.1% FBS).

In 10 and 1% FBS, 9ORF1-expressing and control pool cells showed nearly identical exponential growth rates at subconfluent densities (less than 2×10^6 cells per 60-mm dish) (Fig. 6). Several days to 1 week after reaching confluency under these two growth conditions, cells of the highly transformed 9ORF1-expressing pools began to die and detach from dishes. This complication for the growth analyses was circumvented by concluding each experiment at the time point prior to observing these changes. At these final time points, the 9ORF1-expressing pool cells achieved 32 to 103% higher saturation densities than did the control pool cells (Table 1; Fig. 6). Thus, the 9ORF1-expressing pools displayed decreased density regulation of growth.

In 0.1% FBS, the 9ORF1-expressing pool cells briefly divided at rates similar to those of control pool cells but at later times showed significantly reduced viability and declining cell numbers (Fig. 6). The cause of this lower cell viability remains to be determined. One intriguing possibility is that 9ORF1 overexpression, like *c-myc* overexpression (11), induces programmed cell death under serum-deficient conditions.

9ORF1 enhances the oncogenicity of CREF cells. CREF cells are reported to be nontumorigenic in syngeneic rats (3, 10); therefore, the oncogenicity of the 9ORF1-expressing pools was tested by subcutaneous injection into Fischer 344 rats. Because the 9ORF1-expressing pools appeared to be highly transformed in vitro, a relatively small number of cells was initially inoculated into juvenile rats. During a 41-day obser-

TABLE 2. Tumorigenicity of 9ORF1-expressing and control CREF pool cells in rats at 14 days postinoculation^a

CREF pool	No. of cells injected	No. of rats with palpable tumors/ total no. of animals	Latency ^b (days)	Mean tumor mass (mg) $\pm$ SEM
Group A				
Control pool 1	10 ⁶	0/3		
9ORF1-expressing pool 1	10 ⁶	3/3	4–6	283 $\pm$ 48
Group B				
Control pool 2	10 ⁶	0/3		
9ORF1-expressing pool 2	10 ⁶	3/3	4–6	179 $\pm$ 95
Group C				
Control pool 1	10 ⁵	0/3		
9ORF1-expressing pool 1	10 ⁵	3/3	6–10	99 $\pm$ 19

^a Four-day-old Fischer 344 rats were given subcutaneous injections on the right flank with the indicated number of cells and were monitored for tumor formation for 14 days. At this time, the animals were sacrificed and the tumors were removed.^b The time range postinjection when palpable tumors were first detected.

FIG. 7. 9ORF1 protein expression in tumors. Groups A, B, and C are the same as shown in Table 2. Total tumor protein (200 μ g) was subjected to combined immunoprecipitation and immunoblotting with 9ORF1-immune serum as described in Materials and Methods. As controls, an extract from cultured 9ORF1-expressing pool cells (pool 2) was immunoprecipitated with 9ORF1-immune or preimmune serum.

vation period, two of three animals receiving 10^3 9ORF1-expressing pool cells (pool 2) developed visually apparent tumors while none of three animals given the same number of matched control pool cells developed any detectable tumors. This preliminary result suggested that 10^3 cells approached the lower limit of cell numbers required for tumor formation by 9ORF1-expressing CREF pools in juvenile rats.

In subsequent experiments, more cells and younger rats were used. Under these conditions, suckling rats given injections of either 10^5 or 10^6 9ORF1-expressing pool cells developed palpable tumors as early as 4 days after injection, and all of these animals developed tumors within 10 days (Table 2). These tumors expressed 9ORF1 protein, verifying their derivation from the 9ORF1-expressing pools (Fig. 7); the slightly reduced levels of 9ORF1 protein in tumors (Fig. 7, group B) relative to the corresponding cells in vitro (Fig. 7, cultured 9ORF1-expressing pool 2) presumably occurred from dilution of injected cells by excess extracellular matrix and infiltrating host cells in vivo. The rats inoculated with control pool cells were free of palpable tumors when this experiment was ended 14 days postinjection (Table 2). As opposed to Ad9-induced rat mammary tumors (2, 23), these 9ORF1-expressing CREF cell-derived tumors grew equally well in both male and female animals. Clearly, the 9ORF1-expressing pool cells were more oncogenic in rats than were the control pool cells; however, because of the short observation periods for these animal experiments, it was premature to conclude that control pool cells were completely nontumorigenic in the rats.

During another experiment with an extended observation period, suckling rats inoculated with 2×10^6 control pool cells (pool 1) did eventually develop small tumors 21 days postinjection. In juvenile rats, control pool cells also produced tumors but with longer latency periods. The observation of anisotropic collagen fibrils under polarized light suggested a fibrocytic origin and thus a CREF cell derivation for these control pool-derived tumors. More important, these tumors lacked detectable 9ORF1 protein expression and consequently did not result from contamination of control pools by 9ORF1-expressing cells. In addition, the oncogenicity of the control pool cells could not be attributed to selection in G418, because parental CREF cells formed identical tumors (42). The disparity between these results and others (3, 10) regarding the tumorigenicity of CREF cells could be due to differences in the CREF cell passage history or in the ages of rats used for the studies.

While the weak tumorigenicity of the CREF cells precluded conclusions regarding the ability of 9ORF1 to oncogenically transform cells, the results presented above did strongly suggest that 9ORF1 significantly enhanced the tumorigenic growth of these cells. This observation was further illustrated by the fact that rats inoculated with 9ORF1-expressing pool cells developed massive tumors 27 days postinjection whereas rats treated identically with control pool cells developed small tumors that were not visually apparent. Two representative tumor-bearing animals are shown in Fig. 8.

In another experiment, differences in tumor growth rates were quantitated by inoculating 10^5 experimental or control pool cells (pool 2) into juvenile rats and weighing the resulting tumors at 30 days postinjection. Three animals treated with the 9ORF1-expressing pool cells developed average tumor masses of $1,219 \pm 382$ mg, while only small tumors, weighing 22 ± 22 mg, were observed in three animals given the control pool cells. These differences in tumor sizes were statistically significant ($P = 0.032$). Therefore, under these conditions, 9ORF1-expressing CREF pool cells generated 55-fold-larger tumor masses than did control pool cells in animals.

In support of the results described above showing that experimental tumors grew more aggressively than those produced by control cells, 9ORF1-expressing pool-induced tumors also proved to be more malignant on the basis of histopathological criteria. Tumors induced by the 9ORF1-expressing pool cells presented as high-grade fibrosarcomas, demonstrating hypercellularity, central necrosis, hemorrhage, large nuclear size, pleomorphism, and a high mitotic index (five to six mitotic figures per field at $\times 165$ magnification) with occasional atypical mitotic figures (Fig. 9). In contrast, control pool cells formed low-grade fibrosarcomas, which grew in interwoven bundles and sheets with finger-like projections of local invasion but displayed less than one mitotic figure per field at $\times 165$ magnification. Taken together, these in vivo studies indicated that 9ORF1 expression greatly enhances the oncogenic potential of CREF cells.

DISCUSSION

This study was undertaken to identify general transformed properties of 9ORF1-expressing cells, and the results presented here indicate that 9ORF1 induces many of the hallmark features associated with highly transformed cells. Significantly, it is shown for the first time that 9ORF1-expressing CREF cells

FIG. 8. 9ORF1-expressing CREF pool cells generate larger tumors than control CREF pool cells in syngeneic rats. A total of 2×10^6 control (A) or 9ORF1-expressing (B) CREF pool cells (pool 1) were injected subcutaneously into the right flanks of 3-day-old Fischer 344 rats. The arrow points to a large tumor formed by the 9ORF1-expressing pool cells 27 days postinjection. The small tumor present in the control animal was not visually evident.

FIG. 9. Histology of tumors induced by control (A) or 9ORF1-expressing (B) CREF pool cells. Three-day-old Fischer 344 rats were given subcutaneous injections of 2×10^6 9ORF1-expressing or control CREF pool cells (pool 1). Tumors were removed 27 days postinjection and fixed in buffered formalin, and sections were stained with hematoxylin and eosin. The arrow indicates an aberrant mitotic figure within a tumor generated by the 9ORF1-expressing pool cells. Magnification, $\times 68.5$.

exhibit morphological changes and higher saturation densities, as well as enhanced oncogenicity in animals. Together with the requirement of 9ORF1 protein for Ad9-induced mammary oncogenesis (25), the elevated tumorigenicity of 9ORF1-expressing CREF cells provides strong support for the idea that 9ORF1 encodes an oncoprotein.

Also presented for the first time is the result that 9ORF1 protein exists primarily within the cytoplasm of cells. In theory, the 14-kDa 9ORF1 protein is sufficiently small to diffuse into the nucleus (32); consequently, retention in the cytoplasm may be due to sequestration within a membrane-enclosed organelle, to anchoring to a cytosolic protein or structure, or to existence as a high-molecular-weight multimer. Additional work is needed to determine which of these three possibilities is correct, because all are consistent with the punctate staining observed by immunofluorescence for 9ORF1 protein.

In general, DNA tumor virus oncoproteins operate by either inactivating tumor suppressor proteins or activating the products of proto-oncogenes (4). The mechanism by which 9ORF1 protein transforms cells has not been established but may be intimately linked to its subcellular location. Thus, it is possible that expression of 9ORF1 perturbs the activity of a cytoplasmic factor, particularly one with known oncogenic potential. Most candidates for such a cellular factor can be grouped according to their involvement in one of three processes: (i) translation (e.g., eukaryotic initiation factor 4E and the inhibitor of double-stranded-RNA-dependent protein kinase), (ii) transcription (e.g., c-Abl, c-Rel, and signal transducers and activators of transcription), and (iii) signal transduction (e.g., c-Raf, MEK, c-Mos, and Bcr). While all of the polypeptides listed above

must be considered possible targets of 9ORF1 protein, other DNA tumor virus gene products, such as bovine papillomavirus E5 (33), polyomavirus middle T antigen (26), and Epstein-Barr virus LMP-1 (30), function by activating components of signal transduction pathways. This common transforming mechanism for extranuclear viral oncoproteins suggests that cellular cytoplasmic factors involved with signal transduction deserve special attention with regard to the transforming activity of 9ORF1 protein.

The inability of normal cells to exhibit anchorage-independent growth results from a block in cyclin A expression, and ectopic cyclin A expression efficiently induces anchorage-independent growth yet fails to confer other transformed properties to cells (14). These findings suggest that the anchorage-independent growth commonly associated with oncogenic transformation is specifically mediated through cyclin A. In support of this idea, c-Myc and E2F-1 oncoprotein-transformed cells grow in soft agar and display elevated cyclin A levels (19, 44). Because 9ORF1-transformed cells also grow efficiently in soft agar, it follows that one consequence of 9ORF1 expression may be deregulated cyclin A expression. Interestingly, cyclin A is also implicated in apoptosis (19, 29), and thus, altered cyclin A expression could be responsible, in part, for the cell death exhibited by 9ORF1-expressing cell pools when cultured under serum-deficient conditions.

The role of E4 ORF1 proteins in the adenovirus life cycle is unknown, because these conserved proteins (25) are dispensable for viral replication in cultured permissive cells (5, 15, 21, 25). The ability of 9ORF1 to transform CREF cells, however, implies that E4 ORF1 proteins, in general, may serve to un-

dermine the regulation of cellular proliferation during a viral infection. This interpretation may seem unsatisfactory since Ad5 and Ad12 E4 ORF1s fail to induce focus formation in CREF cells (25). Nonetheless, because human epithelial cells represent the natural targets for adenovirus infections in vivo, rodent fibroblast cell lines such as CREF cells may be unsuitable for evaluating the transforming potential of some E4 ORF1 proteins. If this notion is correct, E4 ORF1 proteins from all adenovirus subgroups may be competent for transformation when assayed in relevant cell types.

In contrast to other adenoviruses, for which the E1 region is both necessary and sufficient for oncogenic transformation, E4 ORF1 may encode the major transforming protein of Ad9. Several observations support this hypothesis. 9ORF1 is required for Ad9-induced mammary tumors and is highly transforming in CREF cells (25; also see above). Moreover, unlike 9ORF1-expressing CREF pools, Ad9 E1 region-transformed CREF pools fail to demonstrate increased tumorigenicity in rats (39). Nevertheless, because the Ad9 E1 region can cooperate with the Ad9 E4 region (24) or 9ORF1 (25) to transform CREF cells, the mammary oncogenicity of Ad9 may require activities from both of these viral gene regions. Whereas the cellular targets and molecular mechanisms of the adenovirus E1 proteins are well characterized, little is yet known about these aspects of 9ORF1 protein. The identification of cellular factors associated with 9ORF1 protein will probably clarify pathways deregulated by this protein and, perhaps, reveal novel mechanisms involved in mammary gland oncogenesis.

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