JOURNAL OF VIROLOGY, June 1997, p. 4385–4394 Vol. 71, No. 6
0022-538X/97/$04.00+0

Mutant Adenovirus Type 9 E4 ORF1 Genes Define Three Protein Regions Required for Transformation of CREF Cells

ROBERT S. WEISS,[1] MOSES O. GOLD,[1] HANNES VOGEL,[2] AND RONALD T. JAVIER[1]*

Division of Molecular Virology[1] and Department of Pathology,[2] Baylor College of Medicine, Houston, Texas 77030

Received 3 January 1997/Accepted 27 February 1997

Human adenovirus type 9 (Ad9) elicits exclusively estrogen-dependent mammary tumors in rats, and an essential oncogenic determinant for this virus is Ad9 E4 open reading frame 1 (9ORF1), which encodes a 125-residue cytoplasmic protein with cellular growth-transforming activity in vitro. In this study, we engineered 48 different mutant 9ORF1 genes in an attempt to identify regions of this viral protein essential for transformation of the established rat embryo fibroblast cell line CREF. In initial assays with CREF cells, 17 of the 48 mutant 9ORF1 genes proved to be severely defective for generating transformed foci but only 7 of these defective genes expressed detectable amounts of protein. To further examine the defects of the seven mutant proteins, we selected individual cell pools of stable CREF transformants for the wild-type and mutant 9ORF1 genes. Compared to cell pools expressing the wild-type 9ORF1 protein, most cell pools expressing mutant proteins displayed decreased growth in soft agar, and all generated significantly smaller tumors in syngeneic animals. The altered amino acid residues of the seven mutant 9ORF1 polypeptides clustered within three separate regions referred to as region I (residues 34 to 41), region II (residues 89 to 91), and C-terminal region III (residues 122 to 125). By using indirect immunofluorescence, we also assessed whether the mutant proteins localized properly to the cytoplasm of cells. The region I and region II mutants displayed approximately wild-type subcellular localizations, whereas most region III mutants aberrantly accumulated within the nucleus of cells. In summary, we have identified three 9ORF1 protein regions necessary for cellular transformation and have demonstrated that C-terminal region III sequences significantly influence the proper localization of the 9ORF1 polypeptide in cells.

Unlike other adenoviruses, subgroup D human adenovirus type 9 (Ad9) elicits only estrogen-dependent mammary tumors in female Wistar-Furth rats and is nontumorigenic in male rats (1, 2, 13, 15). While both benign and malignant mammary neoplasms arise in the Ad9-infected animals, benign fibroadenomas arise most frequently. Significantly, fibroadenoma also represents the most common benign breast disease of women (5). Therefore, studying Ad9-induced mammary tumors may help to elucidate the molecular mechanisms underlying the development of a common breast tumor of women and, in addition, may reveal new principles for viral oncogenesis.

Besides its unique ability to generate mammary tumors in animals, Ad9 is unusual among adenoviruses in requiring a viral function located outside of the E1 region for tumorigenicity (10, 14). Specifically, studies with recombinant and mutant viruses indicate that Ad9 E4 open reading frame 1 (9ORF1), encoding a 14-kDa polypeptide, is a key determinant for Ad9 oncogenicity in animals (16). Moreover, the fact that expression of the 9ORF1 protein alone in the established rat embryo fibroblast cell line CREF induces both morphological and growth transformation, including enhanced oncogenic potential in vivo (28), demonstrates that 9ORF1 is an oncogene. In these cells, the 9ORF1 polypeptide is distributed throughout the cytosol in a punctate staining pattern and is usually excluded from the nucleus (28). While the molecular mechanisms responsible for 9ORF1-induced transformation are not yet known, the predominantly cytoplasmic location of the 9ORF1 polypeptide may indicate that this viral oncoprotein functions by perturbing cellular signaling pathways.

* Corresponding author. Mailing address: Division of Molecular Virology, Rm. 928D, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3898. Fax: (713) 798-3586. E-mail: rjavier@bcm.tmc.edu.

Recent studies suggest that human adenovirus E4 ORF1 genes encode a family of functionally related polypeptides because, like subgroup D 9ORF1, the subgroup A, B, and C human adenovirus E4 ORF1s also possess cellular growth-transforming potential in vitro (27). The degree of amino acid sequence conservation among the different adenovirus E4 ORF1 polypeptides, approximately 45% identity and 65% similarity (27), may further indicate that these viral proteins utilize similar mechanisms for cellular transformation. Interestingly, the human adenovirus E4 ORF1 proteins may be distantly related to dUTP pyrophosphatases (dUTPases), essential cellular and viral enzymes involved in dTTP biosynthesis and DNA replication (27). The relatedness between E4 ORF1 and dUTPase polypeptides is evidenced by these proteins having both sequence and predicted structural similarity, as well as by the fact that avian adenovirus CELO codes for a bona fide dUTPase at a genomic location analogous to that of the human adenovirus E4 ORF1 genes. Nevertheless, because E4 ORF1 proteins do not exhibit demonstrable dUTPase activity and, conversely, transforming activity has not been detected for dUTPases, it is not yet clear whether these related proteins have a common function.

An important step toward understanding the unique mammary oncogenicity of Ad9 would be to determine the molecular mechanisms of the 9ORF1 transforming protein. Clearly, well-characterized 9ORF1 mutant proteins are essential to accomplishing this goal. In this study, we report the first mutational analysis of the 9ORF1 oncoprotein. Our results with mutant 9ORF1 polypeptides in CREF cells indicate that at least three different protein regions (regions I, II, and III) are important for transforming activity and, moreover, that C-terminal region III maintains the proper cytoplasmic localization of the 9ORF1 protein.

MATERIALS AND METHODS

Cells. CREF cells (8) were maintained as described previously (28) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% fetal bovine serum (FBS) and 20 μg of gentamicin per ml.

Mutagenesis. The gene constructs coding for truncated 9ORF1 proteins (*dl*1 to *dl*4) were obtained by several different approaches: mutant *dl*1 (residues 1 to 42) resulted from the formation of a stop codon at residue 43 during the random PCR mutagenesis procedure described below; mutant *dl*2 (residues 1 to 55) was described previously (16); mutant *dl*3 (residues 1 to 106) was engineered by digesting the 9ORF1 gene with *Kpn*I, removing overhanging ends with T4 DNA polymerase, and religating; and mutant *dl*4 (residues 61 to 125) was made by PCR amplification of wild-type 9ORF1 template sequences with the 5′ oligonucleotide 5′CTC GGA TCC ATG GCC TTG AAT GAC TAC3′ and a 3′ 9ORF1 oligonucleotide described previously (16).

The remaining 9ORF1 mutant gene constructs (mut1 to mut37, mutI-A, mutII-A, mutII-B, and mutIII-A to mutIII-D) were generated by random mutagenesis by PCR (20, 30), a method based on the ability of Mn^{2+} to reduce the fidelity of *Taq* polymerase. In four independent reactions with $MgCl_2$ and $MnCl_2$ concentrations ranging from 1.5 to 1.85 mM and 0.125 to 0.5 mM, respectively, 7.5 ng of template DNA was subjected to 30 cycles (the cycle parameters were 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) of PCR amplification in 1× *Taq* PCR buffer (Promega Corp.) containing 1 μM each 5′ and 3′ 9ORF1 oligonucleotide primer; 0.2 mM each dATP, dTTP, dCTP, and dGTP; and 5 U of *Taq* DNA polymerase (Promega Corp.). The template DNA plasmid, Ad9 *Xho*I-*Spe*I (87 to 100 map units) in pUC19, and the 5′ and 3′ 9ORF1 oligonucleotide primers were described previously (16). After digestion with *Bam*HI and *Eco*RI, the resulting PCR products were cloned into plasmid pGEX-2TK (Pharmacia Biotech, Inc.) for sequencing. Mutant 9ORF1 genes were subsequently directionally subcloned into the *Bam*HI and *Eco*RI sites of expression plasmid pJ4Ω (29) or blunt-end subcloned into the *Bam*HI site of expression plasmid pCMV-Neo-Bam_3 (cytomegalovirus [CMV] plasmid) (12). Mutants mut6 and mut8 were derived from mut5 and mut7, respectively, by subcloning the relevant C-terminal mutation(s) into a wild-type 9ORF1 background.

In vitro transformation assays and generation of stable CREF cell pools. Focus assays with CREF cells were performed as described previously (16). Initial focus assays assessing the transforming proficiencies of the 48 mutant 9ORF1 genes were performed with CMV plasmid constructs. Cell pools of stable CREF transformants were established by selection with G418 (18, 28), using 4 μg of CMV plasmid as a selectable marker and 20 μg of either empty pJ4Ω (vector), pJ4Ω encoding wild-type 9ORF1, or pJ4Ω encoding mutant 9ORF1 genes. The CREF cell pool expressing lower levels of 9ORF1 protein (9ORF1-low) was generated as described above, except that 2.5 μg of plasmid pJ4Ω encoding wild-type 9ORF1 and 0.5 μg of CMV plasmid were used in the transfection. The anchorage-independent growth of CREF cells was assessed as previously described (16), with cells suspended in DMEM containing 3% FBS and 0.4% Noble agar.

Immunoblot analyses. Cells lysates, prepared in RIPA buffer (150 mM NaCl, 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate, 50 mM Tris-HCl [pH 8.0]) and containing protease inhibitors (300 μg of phenylmethylsulfonyl fluoride per ml and 6 μg each of aprotinin and leupeptin per ml), were subjected to immunoblot analyses with a 9ORF1 polyclonal antiserum as described previously (16).

Tumor and histological analyses. Following trypsinization and suspension in 0.2 ml of DMEM, 10^5 viable CREF cells were injected via a 1-ml syringe and a 21-gauge needle subcutaneously above the right shoulder of 2-day old Fischer 344 rats (Harlan Sprague-Dawley). Animals were monitored for tumor formation by palpation every other day. At 34 days postinjection, the animals were sacrificed and the tumor tissues were removed and weighed. The two-sample *t* test for independent samples with unequal variances (Satterthwaite's method) was used for statistical analyses, and *P* values were determined with statistical software (Minitab, Inc.). The results were considered statistically significant when the *P* values were less than 0.05. For histological examination, all or a portion of each tumor specimen was fixed in 10% neutral-buffered formalin. Tumor sections mounted on slides were stained with hematoxylin and eosin. Animal handling and care were performed in accordance with institutional guidelines.

Indirect immunofluorescence assays. Cells were grown on glass coverslips, fixed in −20°C methanol, blocked with 10% goat serum in TBS (200 mM NaCl, 50 mM Tris-HCl [pH 7.5]), and reacted with either pre-immune or 9ORF1-immune polyclonal rabbit serum. After several TBS washes, the cells were reblocked with 10% goat serum, incubated with a goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Gibco BRL Life Technologies, Inc.), and washed extensively with TBS. The coverslips were briefly soaked in a 0.5-mg/ml 4′,6-diamidino-2-phenylindole (DAPI) solution and affixed to slides with mounting medium (28). The cells were photographed on Kodak TMAX 400 black-and-white film with an Olympus Vanox-S microscope.

RESULTS

Generation of a large collection of 9ORF1 mutants. Mutant 9ORF1 proteins would greatly facilitate studies aimed at deciphering the mechanisms for 9ORF1-induced cellular transformation and oncogenicity. In hopes of obtaining multiple transformation-defective proteins, we mutagenized 9ORF1 by various approaches (see Materials and Methods) and isolated 48 different mutant genes. Based on the types of mutations, the mutant 9ORF1 proteins were categorized into either a large group of 44 polypeptides with primarily missense mutations (Fig. 1A and B, and 2A) or a small group of 4 polypeptides with extensive N- or C-terminal deletions (Fig. 1C). Collectively, the former group of missense mutants had sustained 85 amino acid substitutions distributed over the entire length of the 9ORF1 polypeptide, with individual proteins possessing one to seven mutations. In addition, 59 of the 125 9ORF1 amino acid residues had been altered within this group of mutant proteins, with changes observed at 13 of the 34 residues conserved in adenovirus E4 ORF1 proteins (27).

Seven stably expressed 9ORF1 genes show defective focus-forming potentials in CREF cells and define three protein regions involved in cellular transformation. Following transfection into CREF cells, an expression plasmid encoding a wild-type 9ORF1 gene induces the formation of large numbers of transformed foci on cell monolayers (16). In an attempt to isolate transformation-defective 9ORF1 mutants with functional rather than protein stability deficiencies, we used this convenient and sensitive transformation assay to identify mutant 9ORF1 genes unable to form foci on CREF cell monolayers and subsequently examined such transformation-defective mutants for their ability to express 9ORF1 protein in these same cells.

Upon completion of this screening procedure for the 48 mutant 9ORF1 genes, it was found that 31 of the missense mutants (mut1 to mut31 [Fig. 1A]) generated transformed foci on CREF cells similar to wild-type 9ORF1 (data not shown). Six other missense mutants (mut32 to mut37; [Fig. 1B]) and all four deletion mutants (*dl*1 to *dl*4 [Fig. 1C]) failed to show detectable focus-forming potential in CREF cells, but we were unable to demonstrate protein expression for any of these mutant 9ORF1 genes after transient transfection of expression plasmids into CREF cells and immunoblot analysis with 9ORF1 antiserum (data not shown). This assay is capable of detecting 9ORF1 protein expression to approximately 1/10 that of the wild-type protein. Our failure to detect these mutant proteins was probably the result of unstable protein expression in these cells because, upon expression in bacteria, these 10 mutant polypeptides did react with the 9ORF1 antiserum in immunoblot assays (data not shown). Therefore, due to either proficient transforming activity (mut1 to mut31) or lack of stable protein expression (mut32 to mut37 and *dl*1 to *dl*4), these 9ORF1 mutant genes were not further examined.

Similar to the 10 transformation-defective mutants (mut32 to mut37 and *dl*1 to *dl*4) described above, the remaining 7 mutant 9ORF1 genes, containing mostly missense mutations (Fig. 2A), also exhibited greatly reduced capacities for generating transformed foci on CREF cells (Fig. 2B). In such focus assays, five of the mutants (mutI-A, mutII-A, mutII-B, mutIII-A, and mutIII-B) showed no detectable transforming potentials whereas mutIII-C and, to a lesser degree, mutIII-D (data not shown) displayed weak transforming activities by producing fewer and smaller foci than did wild-type 9ORF1. Significantly, all seven of these transformation-defective genes expressed detectable amounts of protein in CREF cells (data not shown; see below), suggesting that these mutant polypeptides possessed specific functional deficiencies.

Inspection of sequences for the seven stably expressed 9ORF1 transformation-defective polypeptides revealed that mutations clustered within three separate regions, designated

A

mut1 MAESLYAFIDSPGGIAPV^{L}EGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut2 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLER^{M}IFPSVKIATLV

mut3 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALND^{C}HARDILTQSDVIF^{T}GRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut4 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVI^{L}PSVKIATLV

mut5 MAESLYAFIDSPGGIAPV^{R}EGTSNRY^{N}FFCP^{G}SFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHA^{G}DILTQSDVIFAGRRQELTVLLFNHTDRFL^{F}VRKGHPVGTLLLERV^{V}FPSVK^{V}ATLV

mut6 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERV^{V}FPSVK^{V}ATLV

mut7 MAESLYAFIDSPGGIAPVQEGTSNRYTF^{L}CPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHA^{S}DILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVK^{T}ATLV

mut8 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVK^{T}ATLV

mut9 MAESLYAFIDSPGGIAPVQ^{G}GTSNRYTFFCPES^{I}HIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut10 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGR^{L}MALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut11 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHP^{A}GTLLLERVIFPSVKIATLV

mut12 MAESLYAFIDSPGGIAPVQEGTSNRY^{A}FFCPESFHIPPHGVVLLH^{P}KVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut13 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHAR^{G}ILTQSDVIFAGRRQELTVLLFNHTDRFLY^{A}RKGHPVGTLLLERVIFPSVKIATLV

mut14 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVR^{M}GHPVGTLLLERVIFPSVKIATLV

mut15 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTD^{Q}FLYVRKGHPVGTLLLERVIFP^{P}VKIATLV

mut16 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVG^{S}LLLERVIFPSVKIATLV

mut17 MAESLYAF^{T}DSPGGIAPV^{R}EGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut18 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTG^{N}QGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut19 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVS^{A}LVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLER^{A}IFPSVKIATLV

mut20 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQS^{E}VIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut21 MAESLYA^{L}IDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLH^{I}KVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut22 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLK^{A}SVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut23 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLL^{R}LKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut24 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESF^{A}IPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut25 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVL^{V}FNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut26 MAESLYAFIDSPGGIAPVQ^{G}GTS^{S}RYTFFCPESF^{R}IPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut27 MAESLYAFIDSPGGIAPVQEG^{A}SNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMAL^{S}DYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut28 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMAL^{S}DYHA^{W}DILTQSDVI^{L}AGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLFRVIFPSVKIATLV

mut29 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRF^{I}ALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut30 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRR^{R}ELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut31 M^{G}ESLYAFIDSPGGIAPVQEGTSNRYTFFCPES^{L}HIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

B

mut32 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLF^{I}HTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut33 MAESLYA^{L}ID^{G}PGGIAP^{A}QEGTSNRYT^{I}FCPESFHIPPHGVVLLHLKV^{G}VLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYV^{C}KGHPVGTLLLERVIFPSVKIATLV

mut34 MAESLYAFIDSPGGIAP^{I}QEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRF^{I}ALNDYHAR^{V}ILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut35 MAESL^{C}AFI^{V}SPGGIAPVQEGTSNRYTFFCPESFH^{L}PPHGVVLLHLKVSVLV^{H}TGYQGR^{S}MALND^{C}HARDIL^{A}QSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut36 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGY^{R}GRFMAL^{K}D^{C}HARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

mut37 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQE^{F}TV^{P}LFNHTDRF^{S}YVRKGHPVGTLLLERVIFPSVKIATLV

C

*dl*1 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVV

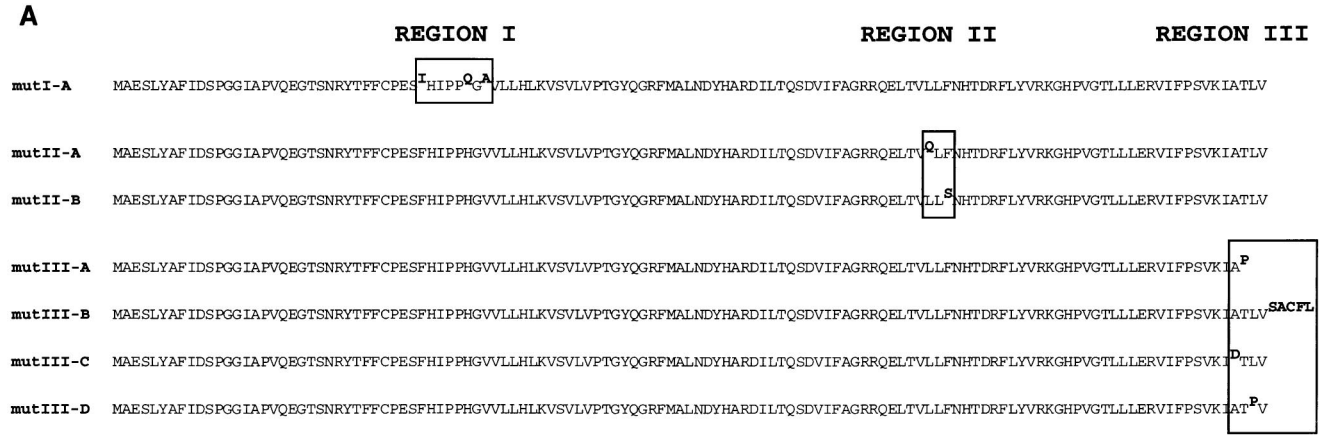
*dl*2 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTG

*dl*3 MAESLYAFIDSPGGIAPVQEGTSNRYTFFCPESFHIPPHGVVLLHLKVSVLVPTGYQGRFMALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPV

*dl*4 MALNDYHARDILTQSDVIFAGRRQELTVLLFNHTDRFLYVRKGHPVGTLLLERVIFPSVKIATLV

FIG. 1. Amino acid sequences of 9ORF1 mutant proteins. (A) Transformation-proficient 9ORF1 missense mutants, mut1 to mut31. (B and C) Transformation-defective 9ORF1 missense mutants, mut32 to mut37 (B), and deletion mutants, *dl*1 to *dl*4 (C), neither groups of which expressed detectable amounts of 9ORF1 protein by immunoblot analysis with 9ORF1 antiserum after transient transfection of expression plasmids into CREF cells (data not shown). The transforming capacities of 9ORF1 mutants were determined by transfecting subconfluent CREF cells with 20 μg of each CMV plasmid encoding a mutant 9ORF1 gene and, after 4 weeks, quantitating the number and size of transformed foci (data not shown).

region I, region II, and region III (Fig. 2A). Region I was the portion of 9ORF1 containing the three missense mutations at residues 34, 39, and 41 of mutI-A. Because transformation-proficient mut9 possessed an identical mutation at residue 34 (Fig. 1A), region I may actually be limited to a smaller region bounded by residues 39 and 41. Region II was the section of 9ORF1 including mutated residues 89 and 91 of mutII-A and mutII-B, respectively. The facts that region II overlapped with



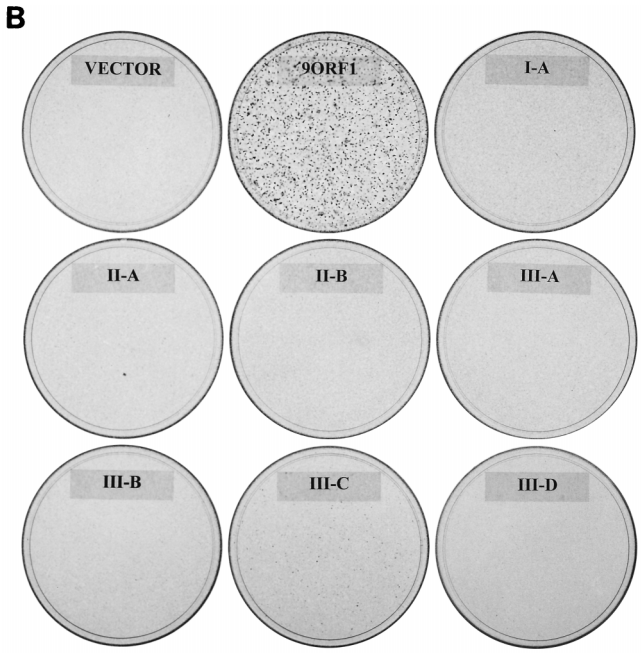


FIG. 2. (A) Amino acid sequences of the seven transformation-defective 9ORF1 mutants that expressed detectable amounts of protein. 9ORF1 protein expression was assessed by immunoblot analysis with 9ORF1 antiserum following transient transfection of expression plasmids into CREF cells (data not shown). Boxes indicate 9ORF1 protein regions required for transformation (region I, residues 34 to 41; region II, residues 89 to 91; region III, residues 122 to 125). (B) Focus transformation assays in CREF cells for wild-type and mutant (mutI-A, mutII-A, mutII-B, mutIII-A, mutIII-B, mutIII-C, and mutIII-D) 9ORF1 proteins shown above in panel A. Focus assays were performed as described in the legend to Fig. 1, except that pJ4Ω expression plasmids were used. The dish labelled VECTOR was transfected with empty pJ4Ω plasmid. Note that mutIII-C showed weak focus-forming activity but the weaker transforming activity of mutIII-D was not apparent in this experiment.

a highly conserved region (9ORF1 residues 88 to 93) of the adenovirus E4 ORF1 proteins (27) and that mut32, having a single missense mutation at residue 92 (Fig. 1B), was not stably expressed may indicate that region II plays important roles in both the functional activity and structural integrity of the 9ORF1 polypeptide. The remaining four transformation-defective proteins (mutIII-A to mutIII-D) had limited deletion, addition, or missense mutations at amino acid residues 122 to 125, thereby defining this C-terminal region of 9ORF1 as region III.

Most CREF cell pools expressing mutant 9ORF1 proteins display reduced growth in soft agar. To further assess the transforming deficiencies of the seven 9ORF1 mutants, we established pools of stable CREF cell transformants with either the wild-type 9ORF1 gene or each mutant gene by selection with G418. In agreement with the results shown in Fig. 2B, transformed foci formed within the G418-resistant cell colonies selected for wild-type 9ORF1 and to a lesser degree for mutIII-C and mutIII-D but not for the other 9ORF1 mutants (data not shown). By immunoblot analysis with 9ORF1 antiserum, it was determined that four of the mutant proteins (mutI-A, mutII-A, mutIII-A, and mutIII-C) were expressed at relatively high levels in the cell pools whereas two others (mutII-B and mutIII-D) accumulated to slightly lower levels (Fig. 3). Because of this variation in protein expression, two different wild-type 9ORF1 cell pools, expressing higher or lower levels of 9ORF1 protein (9ORF1-high and 9ORF1-low) (Fig. 3), were generated to provide suitable protein expression-matched controls for the mutant cell pools in subsequent experiments. The mutIII-B cell pool, however, expressed less 9ORF1 protein than did the 9ORF1-low cell pool.

We have previously shown that wild-type 9ORF1 increases the ability of CREF cells to grow in soft agar (16, 28). Thus, using the CREF cell pools described above, we wanted to determine whether the transformation defects of the 9ORF1 mutants included reduced capacities for promoting anchorage-independent growth in these cells. As expected, both of the wild-type 9ORF1 cell pools (9ORF1-high [Fig. 4B] and 9ORF1-low [Fig. 4C]) showed significantly higher growth activity in soft agar than did the vector cell pool generated with an empty expression plasmid (Fig. 4A). In contrast to the wild-type 9ORF1 cell pools, the mutII-B (Fig. 4F), mutIII-A (Fig. 4G), and mutIII-B (Fig. 4H) cell pools displayed little detectable anchorage-independent growth, similar to that of the vector cell pool. The mutI-A (Fig. 4D), mutII-A (Fig. 4E), and mutIII-C (Fig. 4I) cell pools, on the other hand, showed some weak activities in the soft agar assays by growing more efficiently than the vector cell pool yet significantly less well than the wild-type 9ORF1 cell pools. Interestingly, even though mutIII-D displayed barely perceptible focus-forming activity (Fig. 2B and data not shown), the CREF cell pool expressing this mutant protein grew nearly as well as the 9ORF1-low cell pool in soft agar (Fig. 4J), suggesting that focus formation and anchorage-independent growth may be separable properties of the 9ORF1 protein. Nevertheless, taken together, the results of these soft agar transformation assays indicated that in addition to reduced focus-forming po-

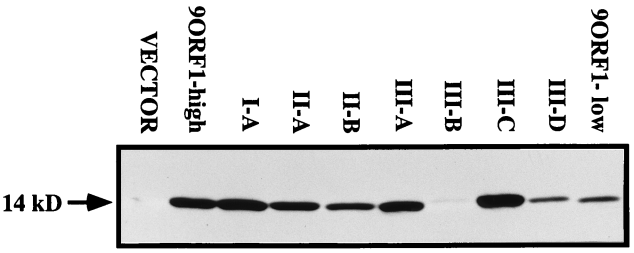


FIG. 3. 9ORF1 protein expression in the stable CREF cell pools. Stable CREF cell pools containing either empty pJ4Ω plasmid (vector), pJ4Ω encoding wild-type 9ORF1 (9ORF1-high and 9ORF1-low), or pJ4Ω encoding mutant 9ORF1 genes (mutI-A, mutII-A, mutII-B, mutIII-A, mutIII-B, mutIII-C, and mutIII-D) were generated by G418 selection. Cells were lysed in RIPA buffer containing protease inhibitors, and 150 μg of total cellular protein was subjected to immunoblot analysis with 9ORF1 antiserum. Protein bands were visualized by chemiluminescence detection (Pierce).

tential, most of the 9ORF1 mutants were also significantly impaired for inducing anchorage-independent growth in the CREF cells.

CREF cell pools expressing the mutant 9ORF1 proteins exhibit decreased tumorigenic growth in animals. It has been previously established that CREF cells expressing wild-type 9ORF1 elicit significantly larger tumors in animals than do control CREF cells (28). Therefore, because the most pertinent measure of cellular transformation is oncogenicity, we next sought to determine whether the seven mutant 9ORF1 proteins were restricted in the ability to increase the tumorigenic growth of CREF cells. For this purpose, each cell pool was injected subcutaneously into a small group of newborn syngeneic rats and, at 34 days postinjection, the tumor masses produced by the cell pools were quantitated by weight (Table 1). Consistent with previous results (28), the 9ORF1-low and 9ORF1-high cell pools formed large tumors with masses 190-fold and 360-fold greater, respectively, than the small tumors induced by the vector cell pool ($P < 0.02$). More important, all the tumors generated by cell pools expressing any of the seven mutant 9ORF1 proteins were significantly smaller ($P < 0.03$) than those produced by either of the wild-type 9ORF1 cell pools (Table 1). In fact, although decreased tumor latency periods were frequently observed, most tumors produced by the mutant protein-expressing cell pools were comparable in mass to those generated by the vector cell pool. While the mutIII-C and mutIII-D cell pools formed tumors slightly larger than those made by the vector cell pool, these differences were not statistically significant ($P = 0.052$ and 0.056, respectively). These results clearly demonstrated that all of the mutant 9ORF1 proteins exhibited significantly reduced capacities for increasing the oncogenic growth of CREF cells in animals.

Besides their significantly larger sizes, tumors formed by wild-type 9ORF1-expressing CREF cells also show more malignant histologies than do those produced by control CREF cells (28). Therefore, tumors generated by the different CREF cell pools were subjected to histological examination (Table 1). From such analyses, we determined that tumors derived from the vector cell pool were exclusively benign fibromas (Fig. 5A) whereas tumors arising from the wild-type 9ORF1 cell pools were entirely malignant high-grade fibrosarcomas exhibiting nuclear pleomorphism, necrosis, large numbers of atypical mitotic figures, and high mitotic indices and nuclear-to-cytoplasmic ratios (Fig. 5C). As a group, the mutant protein-expressing cell pools behaved heterogeneously in the animals by producing both benign fibromas and high-grade fibrosarcomas, as well as low-grade fibrosarcomas with an intermediate tumor grade (Fig. 5B). Nevertheless, mutant 9ORF1 proteins could be segregated into two groups with respect to the histological grade of the tumors generated. Specifically, five of the mutants (mutI-A, mutII-A, mutII-B, mutIII-A, and mutIII-B) elicited predominantly benign fibromas or low-grade fibrosarcomas while the remaining two mutants (mutIII-C and mutIII-D) generated exclusively high-grade fibrosarcomas (Table 1). Thus, even though all the mutant proteins induced significantly smaller CREF cell tumors than did wild-type 9ORF1 in animals, some mutants still produced malignant tumor histologies, indicating the retention of limited but measurable transforming potential for these proteins.

The C-terminal region III of the 9ORF1 protein affects its proper subcellular localization. Consistent with our previous findings (28), the wild-type 9ORF1 protein expressed in both 9ORF1-high (Fig. 6B) and 9ORF1-low (Fig. 6C) cell pools exhibited punctate cytoplasmic staining and exclusion from the nucleus by indirect immunofluorescence whereas the negative-control vector cell pool showed only low background staining (Fig. 6A). By using this assay, we also examined the 9ORF1 mutant proteins to determine whether they were aberrantly localized within cells. In these analyses, the subcellular locations of region I and II mutant proteins (mutI-A [Fig. 6D], mutII-A [Fig. 6E], and mutII-B [Fig. 6F]) resembled that of the wild-type 9ORF1 protein. In contrast, striking differences were evident for most of the region III mutant proteins. Whereas mutIII-C (Fig. 6I) showed an approximately wild-type staining pattern in cells, the remaining region III mutant proteins (mutIII-A [Fig. 6G], mutIII-B [Fig. 6H], and mutIII-D [Fig. 6J]) aberrantly localized to the nucleus of cells. The nuclear staining of mutIII-A was granular rather than diffuse like that of mutIII-B and mutIII-D, but for all three of these mutant proteins, both nuclear and cytoplasmic staining coexisted in the majority of cells. Some cells, though, appeared to have larger amounts of these 9ORF1 mutant proteins in the nucleus than in the cytoplasm. These results suggested that C-terminal region III sequences influenced the proper localization of 9ORF1 protein within CREF cells.

DISCUSSION

In this study, we generated 48 mutant 9ORF1 genes and, from this collection of mutants, identified seven transformation-defective mutant proteins, whose properties are summarized in Table 2. In general, most of these mutant proteins were expressed at approximately wild-type levels in CREF cells but proved to be defective for inducing focus formation and growth in soft agar, as well as for increasing the tumorigenic growth of these cells. Further work, however, is necessary to determine whether these mutant 9ORF1 genes, reconstructed into Ad9 viruses, will also fail to promote mammary tumors in animals. With regard to other DNA tumor virus transforming proteins, the study of transformation-defective mutant proteins has played a central role in revealing mechanisms involved with their oncogenicity (references 6, 17, 21, and 23 to 25 and references therein). Because the seven 9ORF1 mutants are incompetent for functions essential to transformation, identifying their altered activities is expected to aid in revealing the molecular mechanisms responsible for the tumorigenic activity of 9ORF1.

Most DNA tumor virus oncoproteins are assemblages of discrete functional domains acting cooperatively to mediate transformation (6, 21, 24). From the locations of amino acid changes within the seven mutant polypeptides, we discovered that three separate protein regions (regions I, II, and III) were essential for the growth-transforming activity of 9ORF1. Be-

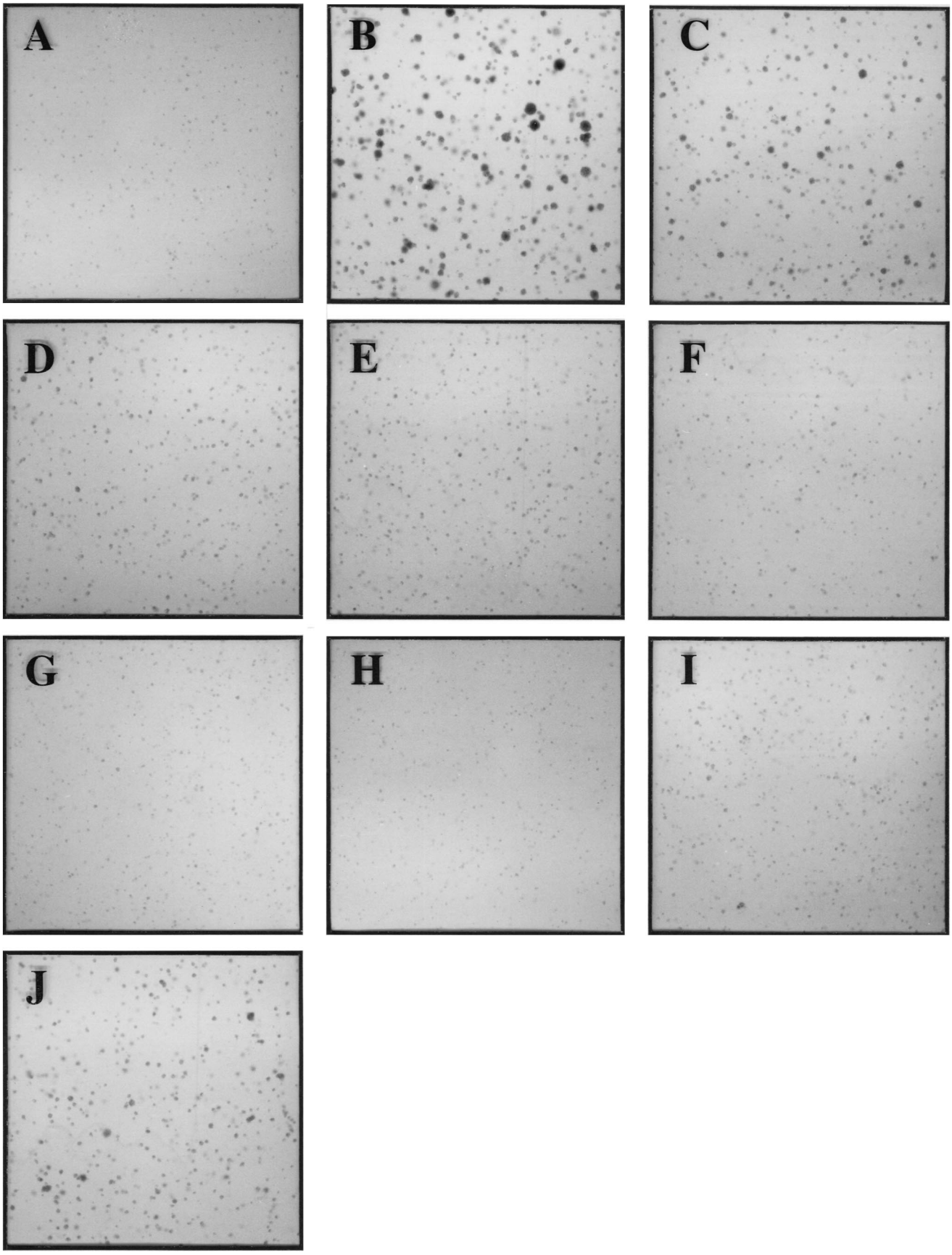


FIG. 4. Soft agar transformation assays with the stable CREF cell pools. The CREF cell pools tested were vector (empty pJ4Ω) (A), 9ORF1-high (B), 9ORF1-low (C), mutI-A (D), mutII-A (E), mutII-B (F), mutIII-A (G), mutIII-B (H), mutIII-C (I), and mutIII-D (J). Growth in soft agar was determined by suspending 10^5 viable cells in growth medium containing 3% FBS and 0.4% Noble agar. Cells were photographed 13 days after plating in soft agar. Magnification, ×18.8.

TABLE 1. Tumorigenicity of wild-type and mutant 9ORF1 protein-expressing CREF cell pools[a]

CREF cell pool	No. of rats with palpable tumors/total no. of rats	Mean tumor latency (days) ± SD[b]	Tumor mass (mg)	Tumor histological grades[c]
Vector	3/4	20 ± 4	8	Fibroma
			10	Fibroma
			20	Fibroma
9ORF1-high	4/4	12 ± 2	1,980	High-grade fibrosarcoma
			2,070	High-grade fibrosarcoma
			3,038	High-grade fibrosarcoma
			11,516[d]	High-grade fibrosarcoma
9ORF1-low	3/3	20 ± 4	2,020	High-grade fibrosarcoma
			2,690	High-grade fibrosarcoma
			2,770	High-grade fibrosarcoma
mutI-A	3/3	15 ± 1	82	Low-grade fibrosarcoma
			92	Low-grade fibrosarcoma
			284	High-grade fibrosarcoma
mutII-A	4/4	16 ± 3	27	Fibroma
			42	Fibroma
			73	Fibroma
			336	Low-grade fibrosarcoma
mutII-B	3/4	21 ± 0	15	Fibroma
			20	Fibroma
			76	Fibroma
mutIII-A	4/4	20 ± 2	17	Low-grade fibrosarcoma
			18	Fibroma
			49	Fibroma
			88	Low-grade fibrosarcoma
mutIII-B	3/3	18 ± 3	11	Fibroma
			13	Fibroma
			18	Fibroma
mutIII-C	3/3	12 ± 2	210	High-grade fibrosarcoma
			389	High-grade fibrosarcoma
			497	High-grade fibrosarcoma
mutIII-D	3/3	14 ± 0	230	High-grade fibrosarcoma
			242	High-grade fibrosarcoma
			454	High-grade fibrosarcoma

[a] Two-day-old Fischer 344 rats were given subcutaneous injections on the right flank with 10^5 viable cells and were monitored for tumor formation for 34 days, after which time the animals were sacrificed and the tumors were removed.

[b] The number of days postinjection when tumors first became palpable.

[c] Histological grades were assigned as follows: fibroma (zero to one mitotic figure per ×40 field, no necrosis, rare dying cells), low-grade fibrosarcoma (zero to two mitotic figures per ×40 field, focal necrosis, scattered dying cells), high-grade fibrosarcoma (one to five mitotic figures per ×40 field, atypical mitotic figures, multifocal necrosis, numerous dying cells, increased nuclear-to-cytoplasmic ratio, and nuclear hyperchromasia).

[d] This unusually large tumor was excluded to calculate *P* values for pairwise comparisons of mean tumor masses.

cause these mutational analyses were not exhaustive, however, subsequent studies may reveal additional 9ORF1 protein regions involved in transformation. Furthermore, the results do not prove that the three identified 9ORF1 protein regions each represent functional domains because the mutations of the transformation-defective 9ORF1 mutants may be remote from such domains and disrupt activity through conformational changes in the polypeptide. The fact that the mutant 9ORF1 polypeptides were stably expressed in cells, however, favors the idea that functional rather than structural elements were affected. Even so, if the three identified protein regions were located within functional domains, the actual number of such domains within the 9ORF1 polypeptide may be less than three because a complex domain may be assembled from multiple protein regions distant from one another on the linear molecule. Therefore, ascertaining the precise effects of mutations within the transformation-defective 9ORF1 proteins and the actual number of different functional domains within the 9ORF1 polypeptide must await the completion of further studies.

Because other human adenovirus E4 ORF1 genes besides 9ORF1 also possess cellular growth-transforming potential (27), we were prompted to look for sequence conservation of regions I, II, and III within these related polypeptides. These analyses reveal that region II is highly conserved and therefore may form part of a functional domain, because such domains frequently exhibit high sequence conservation among related proteins. Regions I and III, on the other hand, lie within regions of lower sequence identity between the adenovirus E4 ORF1 proteins. This observation may indicate that these particular protein regions represent structural elements or parts of functional domains that tolerate some sequence variation. While it is also possible that regions I and III carry out distinct functions for each of the different E4 ORF1 proteins, this seems less likely, because all these polypeptides share the ability to transform cells. Mutational analyses of other adenovirus E4 ORF1 proteins may help to clarify which of these possibilities is correct.

In contrast to the acutely transforming retroviruses, DNA tumor viruses normally encode oncoproteins that display only limited regions of sequence similarity to cellular proteins (21, 22). Adenovirus E4 ORF1 transforming proteins, however, do show sequence and predicted structural similarity to cellular and viral dUTPase enzymes but possess neither dUTPase enzymatic activity nor any discernible cellular protein motifs (27). Nevertheless, in addition to their conservation within adenovirus E4 ORF1 transforming proteins, sequences equivalent to 9ORF1 region II are relatively well conserved within most eukaryotic dUTPases, hinting that E4 ORF1 proteins may share an undiscovered activity with these enzymes. Interestingly, in addition to its catalytic function, dUTPase has recently been found to complex with and inhibit the activity of certain cellular transcription factors (4). Perhaps adenovirus E4 ORF1 proteins mimic or perturb this novel activity of cellular dUTPases. In contrast, sequences equivalent to 9ORF1 regions I and III are not well conserved within dUTPases, possibly reflecting the acquisition of novel activities by the 9ORF1 transforming protein. Using the known crystal structure of the *E. coli* dUTPase monomer as a structural model (3, 19), we predict that in the native 9ORF1 molecule, regions I and II lie in close proximity to one another whereas region III resides in a separate part of the molecule. This finding may indicate that 9ORF1 possesses two different functional domains formed by regions I and II and by region III. Such an idea is supported by the results of indirect immunofluorescence experiments (Fig. 6), which showed that both region I and II 9ORF1 mutant pro-

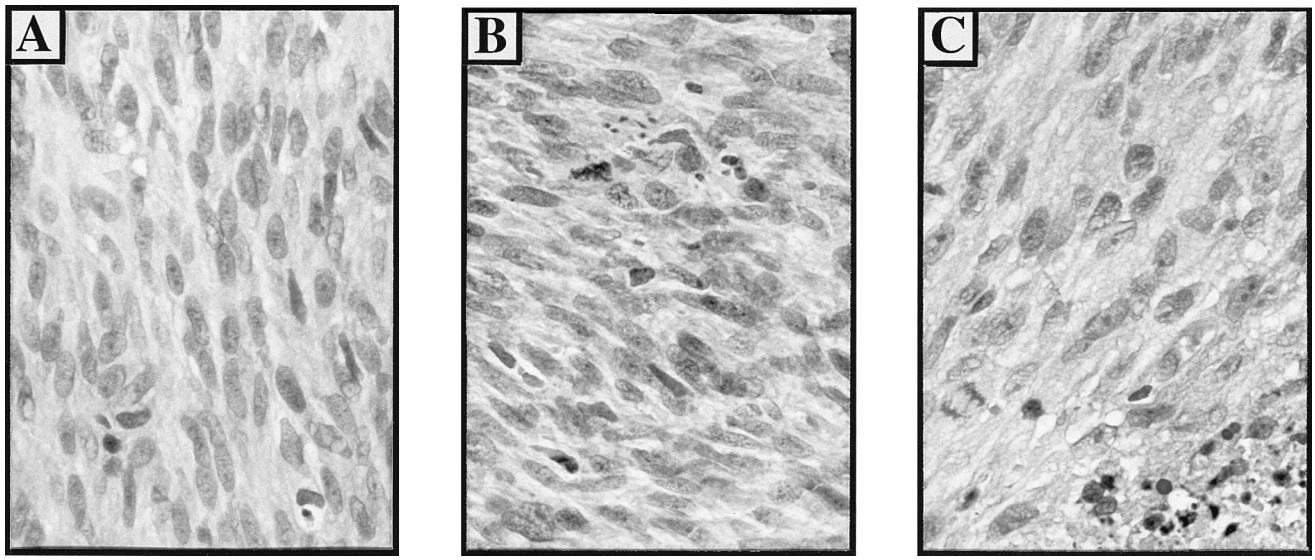


FIG. 5. Tumors generated by the stable CREF cell pools in syngeneic rats. Shown are representative hematoxylin-and-eosin-stained sections of a benign fibroma (A), malignant low-grade fibrosarcoma (B), or malignant high-grade fibrosarcoma (C). See Table 1 for tumor grading criteria. Magnification, ×48.6.

teins exhibited wild-type subcellular localizations whereas most region III mutant proteins behaved differently by localizing aberrantly within cells. Also, considering that dUTPases form homotrimers and that adenovirus E4 ORF1 proteins may also have this property, we may find that the functional defects for some 9ORF1 mutants result from failures to properly homo-oligomerize. While much remains to be learned about the E4 ORF1 transforming proteins, comparisons with dUTPase enzymes may help to provide important insights into the structure and function of these viral transforming proteins.

Tumorigenicity of transformed cells most often correlates with an ability to grow in soft agar (9, 26). The cell pool expressing mutIII-D, however, exhibited anchorage-independent growth nearly identical to the cell pool expressing wild-type 9ORF1 (compare Fig. 4C and J) yet produced significantly smaller tumors in animals (Table 1). These results suggested that for 9ORF1 expression in CREF cells, inducing growth in soft agar per se is insufficient to promote wild-type tumorigenic growth in animals. Instead, the focus-forming potentials of the 9ORF1 proteins represented a better in vitro indicator for oncogenic growth, since all 9ORF1 mutants demonstrated severe deficiencies for both of these properties in CREF cells. This correlation was further strengthened by tumor histological analyses, which showed that mutant 9ORF1 proteins without perceptible focus-forming activities (mutI-A, mutII-A, mutII-B, mutIII-A, and mutIII-B) produced benign or low-grade malignant tumors whereas mutant 9ORF1 proteins with weak yet detectable focus forming potential (mutIII-C and mutIII-D) generated exclusively high-grade malignant tumors. In addition, the tumor growth of cell pools expressing the leaky mutIII-C and mutIII-D mutants may have been slightly elevated above that of the vector cell pool (Table 1), even though the small numbers of tumors analyzed did not allow definitive conclusions to be drawn from these results. Nevertheless, the results with these mutant proteins suggested that limited 9ORF1 focus-forming activity in CREF cells leads

TABLE 2. Summary of relative protein expression levels, transforming activities, and subcellular localizations of wild-type and mutant 9ORF1 proteins in CREF cells

CREF cell pool	9ORF1 protein steady-state levels[a]	Focus formation[b]	Growth in soft agar[c]	Tumorigenicity[d]	9ORF1 protein localization[e]
Vector	−	−	−	+	NA
9ORF1-high	++++	+++++	+++++	+++++	C
9ORF1-low	++	+++++	++++	+++++	C
mutI-A	++++	−	+	+	C
mutII-A	++++	−	−	+	C
mutII-B	++	−	+	+	C
mutIII-A	++++	−	−	+	C/N
mutIII-B	+	−	−	+	C/N
mutIII-C	++++	++	+	++	C
mutIII-D	++	+	++++	++	C/N

[a] Summary of results in Fig. 3. ++++, high 9ORF1 protein expression; ++, moderate 9ORF1 protein expression; +, low 9ORF1 protein expression; −, no 9ORF1 protein expression.
[b] Summary of results in Fig. 2B and data not shown. +++++, numerous large foci; ++, few small foci; +, rare small foci; −, no foci.
[c] Summary of results in Fig. 4. +++++, extensive growth; ++++, moderate growth; +, minimal growth; −, no growth.
[d] Summary of results in Table 1 and Fig. 5. +++++, large high-grade fibrosarcomas; ++, small high-grade fibrosarcomas; +, small fibromas or low-grade fibrosarcomas.
[e] Summary of results in Fig. 6. C, cytoplasmic localization; C/N, cytoplasmic and nuclear localization; NA, not applicable.

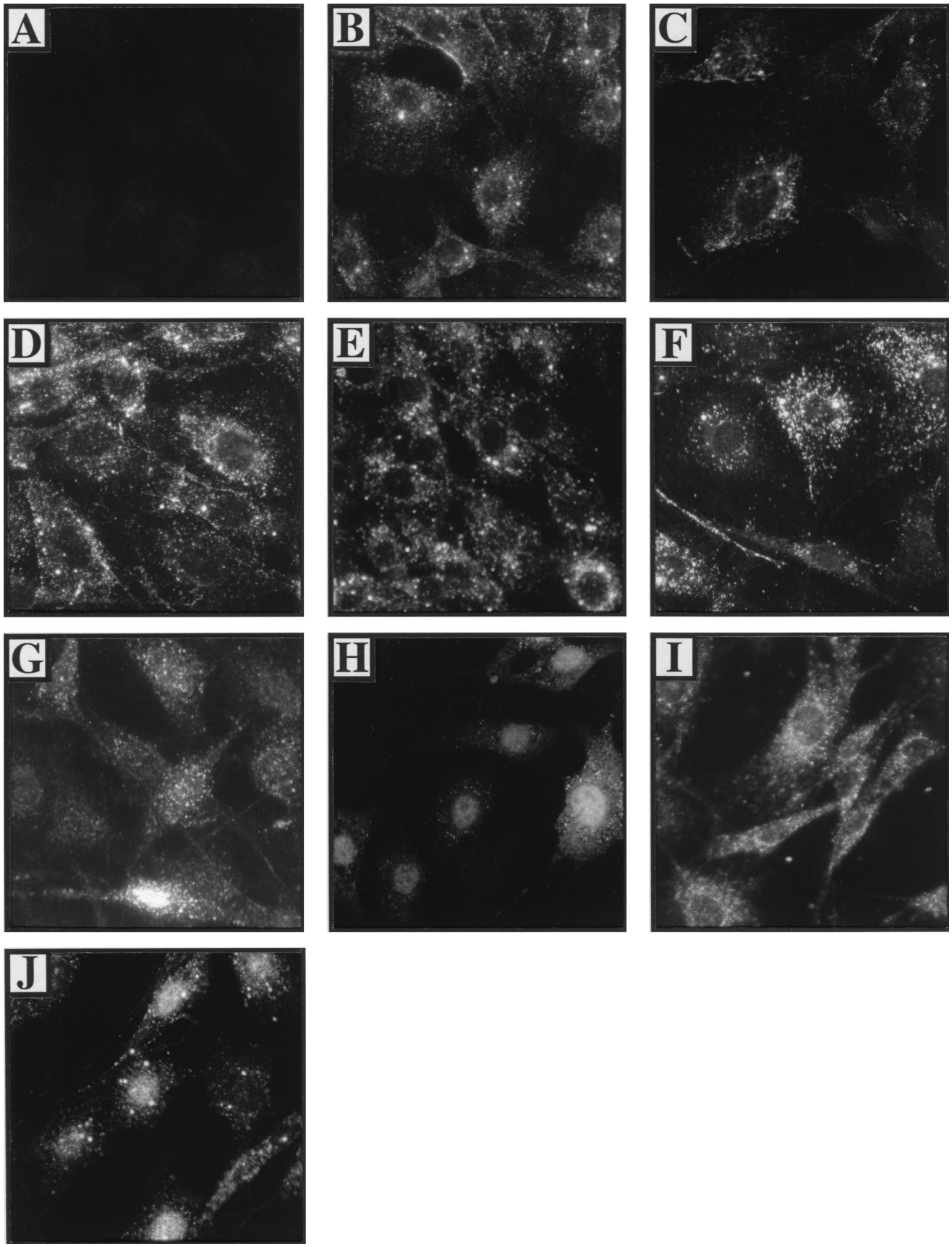


FIG. 6. Subcellular localizations of wild-type and mutant 9ORF1 proteins in the stable CREF cell pools. Indirect immunofluorescence assays with a 9ORF1-specific polyclonal antiserum were performed with the following CREF cell pools: vector (empty pJ4Ω) (A), 9ORF1-high (B), 9ORF1-low (C), mutI-A (D), mutII-A (E), mutII-B (F), mutIII-A (G), mutIII-B (H), mutIII-C (I), and mutIII-D (J). Magnification, ×131.6.

to small tumors with high-grade malignant histologies whereas increasing this activity serves solely to enhance tumor growth. Why tumors having identical malignant histologies showed such profound growth differences is not known (e.g., compare 9ORF1-low and mutIII-C cell pools [Table 1]) but could be due to different amounts of apoptosis, because this process may significantly affect the growth of tumors (7, 11).

By indirect immunofluorescence, the subcellular localizations of region I and II 9ORF1 mutant proteins were similar to that of the wild-type protein whereas most region III mutant proteins exhibited aberrant staining patterns, most notably nuclear localization within some cells (Fig. 6G, H, and J). These results indicated that the C-terminal sequences of the 9ORF1 protein serve to actively retain this viral protein within the cytoplasm, perhaps through association with a cellular factor(s). Such a model, however, might predict that mutations within region III would lead to 9ORF1 protein evenly distributed in the cytoplasm and nucleus of all cells. The fact that region III mutant proteins were localized predominantly to the nucleus of some cells may indicate that altered C-terminal sequences unmask a latent capacity of the 9ORF1 polypeptide for transport to the nucleus. As the 9ORF1 protein does not possess an identifiable nuclear localization signal, such nuclear localization might be indirectly mediated by interactions with additional cellular factors that are themselves actively recruited to the nucleus. Nonetheless, because the region III mutant protein, mutIII-C, did localize properly within cells, it must also be concluded that besides maintaining proper 9ORF1 protein localization, region III performs another function essential for transformation. Further studies will be needed to identify this additional 9ORF1 C-terminal protein activity.

Viral oncoproteins utilize numerous molecular mechanisms to transform cells. In general, the transformation of cells by viral oncoproteins is accomplished through functional domains that perform a wide variety of activities, including intermolecular interactions and enzymatic reactions (22). DNA tumor virus oncoproteins, however, invariably physically associate with cellular growth regulatory proteins, suggesting that 9ORF1 may also hetero-oligomerize with such cellular factors, perhaps through 9ORF1 protein regions revealed in this study. For these protein-protein interactions and other possible 9ORF1 functions, use of the mutant 9ORF1 proteins described here should aid in identifying 9ORF1 protein activities that correlate with transforming activity. The detection of novel 9ORF1 activities and their assignment to specific 9ORF1 protein regions is likely to provide significant insights into how 9ORF1 functions in transformation.

ACKNOWLEDGMENTS

We thank Andy Rice for critically reviewing the manuscript.

R.S.W. was the recipient of predoctoral fellowships from the National Science Foundation and a U.S. Army Breast Cancer Training Grant (DAMD17-94-J4204). This work was supported by a grant from the National Cancer Institute (CA 58541) to R.T.J.

REFERENCES

1. **Ankerst, J., and N. Jonsson.** 1989. Adenovirus type 9-induced tumorigenesis in the rat mammary gland related to sex hormonal state. J. Natl. Cancer Inst. **81:**294–298.
2. **Ankerst, J., N. Jonsson, L. Kjellen, E. Norrby, and H. O. Sjogren.** 1974. Induction of mammary fibroadenomas in rats by adenovirus type 9. Int. J. Cancer **13:**286–290.
3. **Cedergren-Zeppezauer, E. S., G. Larsson, P. O. Nyman, Z. Dauter, and K. S. Wilson.** 1992. Crystal structure of a dUTPase. Nature **355:**740–743.
4. **Chu, R., Y. Lin, M. S. Rao, and J. K. Reddy.** 1996. Cloning and identification of rat deoxyuridine triphosphatase as an inhibitor of peroxisome proliferator-activated receptor α. J. Biol. Chem. **271:**27670–27676.
5. **Cotran, R. S., S. L. Robbins, and V. Kumar.** 1994. Robbins pathologic basis of disease, 5th ed. The W. B. Saunders Co., Philadelphia, Pa.
6. **Fanning, E.** 1992. Simian virus 40 large T antigen: the puzzle, the pieces, and the emerging picture. J. Virol. **66:**1289–1293.
7. **Fisher, D. E.** 1994. Apoptosis in cancer therapy: crossing the threshold. Cell **78:**539–542.
8. **Fisher, P. B., L. E. Babiss, I. B. Weinstein, and H. S. Ginsberg.** 1982. Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF). Proc. Natl. Acad. Sci. USA **79:**3527–3531.
9. **Freedman, V. H., and S. Shin.** 1974. Cellular tumorigenicity in *nude* mice: correlation with cell growth in semi-solid medium. Cell **3:**355–359.
10. **Graham, F. L.** 1984. Transformation by and oncogenicity of human adenoviruses, p. 339–398. *In* H. S. Ginsberg (ed.), The adenoviruses. Plenum Press, New York, N.Y.
11. **Green, D. R., and S. J. Martin.** 1995. The killer and the executioner: how apoptosis controls malignancy. Curr. Opin. Immunol. **7:**694–703.
12. **Hinds, P. W., C. A. Finlay, R. S. Quartin, S. J. Baker, E. R. Fearon, B. Vogelstein, and A. J. Levine.** 1990. Mutant p53 cDNAs from human colorectal carcinomas can cooperate with ras in transformation of rat cells. Cell Growth Differ. **1:**571–580.
13. **Javier, R., K. Raska, Jr., G. J. Macdonald, and T. Shenk.** 1991. Human adenovirus type 9-induced rat mammary tumors. J. Virol. **65:**3192–3202.
14. **Javier, R., K. Raska, Jr., and T. Shenk.** 1992. Requirement for the adenovirus type 9 E4 region in production of mammary tumors. Science **257:**1267–1271.
15. **Javier, R., and T. Shenk.** 1996. Mammary tumors induced by human adenovirus type 9: a role for the viral early region 4 gene. Breast Cancer Res. Treat. **39:**57–67.
16. **Javier, R. T.** 1994. Adenovirus type 9 E4 open reading frame 1 encodes a transforming protein required for the production of mammary tumors in rats. J. Virol. **68:**3917–3924.
17. **Kiefer, F., S. A. Courtneidge, and E. F. Wagner.** 1994. Oncogenic properties of the middle T antigens of polyomaviruses. Adv. Cancer Res. **64:**125–157.
18. **Kingston, R. E.** 1987. Stable transfer of genes into mammalian cells, p. 9.5.1–9.5.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York, N.Y.
19. **Larsson, G., L. A. Svensson, and P. O. Nyman.** 1996. Crystal structure of the *Escherichia coli* dUTPase in complex with a substrate analogue (dUDP). Nat. Struct. Biol. **3:**532–538.
20. **Leung, D. W., E. Chen, and D. V. Goeddel.** 1989. A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. Technique **1:**11–15.
21. **Moran, E., and M. B. Matthews.** 1987. Multiple functional domains in the adenovirus E1A gene. Cell **48:**177–178.
22. **Nevins, J. R., and P. K. Vogt.** 1996. Cell transformation by viruses, p. 301–343. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 1. Lippincott-Raven Publishers, Philadelphia, Pa.
23. **Nilson, L. A., R. L. Gottlieb, G. W. Polack, and D. DiMaio.** 1995. Mutational analysis of the interaction between the bovine papillomavirus E5 transforming protein and the endogenous β receptor for platelet-derived growth factor in mouse C127 cells. J. Virol. **69:**5869–5874.
24. **Pipas, J. M.** 1992. Common and unique features of T antigens encoded by the polyomavirus group. J. Virol. **66:**3979–3985.
25. **Shenk, T.** 1996. Adenoviridae: the viruses and their replication, p. 2111–2148. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
26. **Shin, S.-I., V. H. Freedman, R. Risser, and R. Pollack.** 1975. Tumorigenicity of virus-transformed cells in *nude* mice is correlated specifically with anchorage independent growth *in vitro*. Proc. Natl. Acad. Sci. USA **72:**4435–4439.
27. **Weiss, R. S., S. S. Lee, B. V. V. Prasad, and R. T. Javier.** 1997. Human adenovirus early region 4 open reading frame 1 genes encode growth transforming proteins that may be distantly related to dUTP pyrophosphatase enzymes. J. Virol. **71:**1857–1870.
28. **Weiss, R. S., M. J. McArthur, and R. T. Javier.** 1996. Human adenovirus type 9 E4 open reading frame 1 encodes a cytoplasmic transforming protein capable of increasing the oncogenicity of CREF cells. J. Virol. **70:**862–872.
29. **Wilkinson, D., R. R. P. de Vries, J. A. Madrigal, C. B. Lock, J. P. Morgenstern, J. Trowsdale, and D. M. Altmann.** 1988. Analysis of HLA-DR glycoproteins by DNA-mediated gene transfer. J. Exp. Med. **167:**1442–1458.
30. **Zhou, Y., X. Zhang, and R. H. Ebright.** 1991. Random mutagenesis of gene-sized DNA molecules by use of PCR with Taq DNA polymerase. Nucleic Acids Res. **19:**6052.