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Rous Sarcoma Virus Mutant *dl*PA105 Induces Different Transformed Phenotypes in Quail Embryonic Fibroblasts and Neuroretina Cells

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***dl*PA105 is a spontaneous variant of Rous sarcoma virus, subgroup E, which carries a deletion in the N-terminal portion of the v-*src* gene coding sequence. This virus was isolated on the basis of its ability to induce proliferation of quiescent quail neuroretina cells. The altered v-*src* gene encodes a phosphoprotein of 45,000 daltons which possesses tyrosine kinase activity. DNA sequencing of the mutant v-*src* gene has shown that deletion extends from amino acid 33 to 126 of wild-type $p60^{v\text{-}src}$. We investigated the tumorigenic and transforming properties of this mutant virus. *dl*PA105 induced fibrosarcomas in quails with an incidence identical to that induced by wild-type virus. Quail neuroretina cells infected with the mutant virus were morphologically transformed and formed colonies in soft agar. In contrast, *dl*PA105 induced only limited morphological alterations in quail fibroblasts and was defective in promoting anchorage-independent growth of these cells. Synthesis and tyrosine kinase activity of the mutant $p45^{v\text{-}src}$ were similar in both cell types. These data indicate that the portion of the v-*src* protein deleted in $p45^{v\text{-}src}$ is dispensable for the mitogenic and tumorigenic properties of wild-type $p60^{v\text{-}src}$, whereas it is required for in vitro transformation of fibroblasts. The ability of *dl*PA105 to induce different transformation phenotypes in quail fibroblasts and quail neuroretina cells is a property unique to this Rous sarcoma virus mutant and provides evidence for the existence of cell-type-specific response to v-*src* proteins.**

Rous sarcoma virus (RSV) induces fibrosarcomas in birds and in vitro transforms avian fibroblasts, the natural target cells for this virus. Both cell transformation and tumor formation depend upon the expression of the viral *src* gene, v-*src* (24). The product of the v-*src* gene is a phosphoprotein ($p60^{v\text{-}src}$) with tyrosine kinase activity bound to the plasma membrane (5, 12, 13, 15, 26, 33, 39, 40).

Changes in cell morphology, anchorage-independent growth, and stimulation of cell proliferation are characteristic properties shared by transformed cells and tumor cells (24). Stimulation of cell proliferation is conveniently studied by using neuroretina (NR) cells from chicken or quail embryos as a host system. As a result of $p60^{v\text{-}src}$ expression, these differentiated cells are induced to proliferate upon infection with RSV, whereas uninfected NR cells rapidly cease to divide and cannot be propagated in vitro (8, 46). Proliferating NR cells also undergo morphological transformation, form colonies in soft agar, and become tumorigenic (8, 10, 48). Moreover, RSV mutants that are either partially or conditionally defective for transformation induce similar phenotypic changes in NR cells and fibroblasts (8–10, 27). These results led to the assumption that the transformed phenotype, induced by the v-*src* gene, is mediated by the same mechanisms in both cell types.

*dl*PA105 is a spontaneous variant of the Schmidt-Ruppin strain of RSV, subgroup E (SRE), that contains a partial deletion in the v-*src* gene but that has retained the ability to induce the growth of quail NR (QNR) cells. The deletion has been mapped to the N-terminal portion of the v-*src* coding sequence, between amino acids 32 and 127 (37). Genetic analysis of other RSV mutants has shown that a domain of $p60^{v\text{-}src}$ between amino acids 15 and 169 is required for a full morphological transformation of fibroblasts and that it may thus play a role in regulating substrate specificity of the v-*src* protein (16, 21, 32, 43, 55).

In this study, we investigated the transforming and tumorigenic properties of *dl*PA105. We report that, as expected from the location of the deletion, *dl*PA105 induces only limited morphological changes in quail embryo fibroblasts (QEF) and is defective in promoting anchorage-independent growth of these cells. However, QNR cells infected with the same virus are morphologically transformed and grow in soft agar. In spite of being defective for transformation of QEF in vitro, *dl*PA105 is as efficient as SRE in causing fibrosarcomas in quails.

The ability to induce distinct transformed phenotypes in QNR cells and QEF is a property of *dl*PA105 unique among RSV mutants and suggests that different cell types can respond differently to the transformation functions of v-*src* proteins.

MATERIALS AND METHODS

Cells and viruses. QEF and QNR cells were prepared, maintained, and infected as previously described (37). All infected-cell cultures were transferred twice to allow spreading of the virus before use.

SRE, the Rous-associated virus 60 (RAV-60) lymphomatosis virus, subgroup E, and the mutant *dl*PA105 are described in an accompanying paper (37).

Measurement of fibroblast growth. QEF were infected with undiluted virus and subcultured twice. When cultures infected with SRE became fully transformed, all virus-infected cells were seeded at low density in 60-mm dishes and counted at various intervals.

Anchorage-independent growth. QEF and QNR infected with virus and subcultured twice were suspended in agar at concentrations ranging between 1×10^5 and 3×10^5 cells per 60-mm dish. Solid medium was minimal essential medium

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containing 0.72% agar, 10% tryptose phosphate broth, 2% chicken serum, and either 8% newborn calf serum (for QEF) or 8% fetal calf serum (for QNR cells). Single-cell suspensions were prepared in the same medium containing 0.35% agar.

Plasminogen activator production. Plasminogen activator production (56, 57) in serum-free culture media was assayed by the caseinolysis of defatted dry milk in a gelled medium containing chicken serum as a source of plasmin (23). The cells seeded in 60-mm dishes are washed three times with serum-free medium and incubated for 24 h at 37°C in 2 ml of serum-free medium. The cells were harvested, and the number of cells per plate was determined. Plasminogen activator activity was measured as previously described (48).

Fluorescence labeling of cells. Cells that had been grown for at least 2 days on glass cover slips were fixed for 20 min with 3% formaldehyde in phosphate-buffered saline (pH 7.6) containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. The cells were then permeabilized by treatment with 0.1% Triton X-100 for 4 min at room temperature and stained for F-actin by using 1 U of 7-nitrosobenzo-2-oxo-1,3-diazole (NBD)-phallacidin (Molecular Probes Inc.) in phosphate-buffered saline (1). The fluorescence-labeled cytoskeleton was observed with a Leitz microscope equipped with a filter setting for fluorescein. The observations were made through a ×63 oil objective, and the photographs were taken on Ilford HP5 film.

Tumorigenicity in quails. The wing webs of 1-day-old Japanese quails (*Coturnix coturnix* Japonica) were subcutaneously injected with 0.1 ml of virus. Virus stocks were adjusted to 10^6 focus-forming or mitogenic units per ml. The mitogenic titer was defined as the reciprocal of the highest dilution of virus inducing proliferation of QNR cells after one subcultivation. In control experiments, quails were injected with 0.1 ml of medium. The birds were examined for tumor development every fourth day over a period of 9 weeks. Autopsies were performed on dead birds and at the end of the experiment. Tumors were fixed immediately in Hidenhain's fluid (4.5 g of mercury chloride, 0.5 g of sodium chloride in 20 ml of formalin and 80 ml of distilled water), embedded in paraffin, and sectioned. Sections 5 μm thick were stained with hematoxylin and eosin for histological examination.

Culture of tumor cells. Tumors occurring at the site of inoculation were excised, dissected into small pieces, and dissociated first with collagenase and then with trypsin. The dispersed cells were seeded into culture flasks.

Protein biochemistry. Labeling of cultures with [^{35}S]methionine and $^{32}P_i$ and preparation of cell extracts were done essentially as described previously (47). v-*src* proteins were immunoprecipitated with serum from tumor-bearing rabbits by a published procedure (5). Anti-p34 serum was kindly provided by R. Erikson. The immunoglobulin G (IgG) phosphorylation assay for determination of tyrosine kinase activity was performed as previously described (12).

Measurement of the amounts of acid-stable phosphoamino acids in total cellular proteins. Cells seeded in 35-mm dishes were labeled with 0.5 ml of medium containing 1 mCi of [^{32}P]P_i per ml (Commissariat à l'Energie Atomique, Saclay, France). The medium was removed, and the cultures were washed twice with phosphate-buffered saline and then fixed in 1 ml of 10% trichloroacetic acid at 70°C for 2 h. The cells were scraped from the plates and transferred to an Eppendorf tube. After three washes with 1 ml of 10% trichloroacetic acid at room temperature, the pellet was rinsed three times with ethanol. The pellet was hydrolyzed in 200 μl of 6 N HCl for 2 h at 110°C. Details of the analysis of the products of hydrolysis are described in an accompanying paper (37).

Cell fractionation. The method used for cell fractionation is similar to that described by Garber et al. (22). Cells were collected in phosphate-buffered saline, pelleted, swollen in 10 mM Tris hydrochloride (pH 7.4)-10 mM KCl-1 mM dithiothreitol-1 mM EDTA-1 mM phenylmethylsulfonyl fluoride–100 kallikrein-inactivating units of Trasylol per ml, and then homogenized in a tight-fitting Dounce homogenizer. Nuclei were pelleted by low-speed centrifugation, and the NaCl concentration of the postnuclear supernatant was adjusted (see Table 3). Membranes were pelleted by high-speed centrifugation at 100,000 × *g* for 30 min. The membrane pellet (P100) was suspended in buffer B-RIPA (22). RIPA detergents were added to the supernatant fraction (S100). Both P100 and S100 fractions were assayed for immune complex kinase activity.

RESULTS

Effects of *dl*PA105 on morphology of QEF and QNR cells. QNR cells infected with *dl*PA105 underwent extensive morphological transformation (Fig. 1c). They became rounded and refringent and were quite similar to SRE-infected cells (Fig. 1b). However, some mutant-virus-infected cells extend long processes which are seldom observed in QNR cell cultures transformed by wild-type virus.

In contrast, *dl*PA105 induced only limited changes in the morphology of QEF as compared with SRE. Fibroblasts chronically infected with the mutant virus remained flat, and virtually no rounded refractile cells were observed (Fig. 1f). When these cultures reached confluency, occasional areas of piled-up fusiform cells became visible under agar overlay (data not shown). The presence of cells with altered morphology depends on culture conditions, since they are observed only in dense cultures. Their number did not increase upon subsequent passages over a period of 2 months.

Cytoskeleton changes induced by *dl*PA105. The arrangement of actin cables within cells is greatly altered upon transformation by RSV (18). We analyzed the cytoskeletons of QEF and QNR cells infected with RAV-60, SRE, or *dl*PA105 by staining with the fluorescent probe NBD-phallacidin, which specifically binds to polymerized actin (1). RAV-60-infected QEF and QNR cells exhibited an overall polygonal shape, with numerous and relatively large actin cables (Fig. 2a and d). In contrast, both types of SRE-transformed cells were less spread, had fewer and thinner actin bundles, and exhibited, in general, a number of abnormal patches of actin (Fig. 2b and e).

As shown by phase-contrast microscopy, (Fig. 2f) the majority of *dl*PA105-infected QEF retained a polygonal shape and possessed relatively well-developed actin skeletons. However, they were, on average, less well spread than RAV-60-infected cells, and their actin cables were thinner. One feature clearly differentiated them from the latter cells: about 50% of the population of mutant-virus-infected fibroblasts exhibited some abnormal patches of actin which were almost exclusively concentrated at the cell periphery, near the ends of the actin bundles (Fig. 2c). In addition, a few cells (2%) displayed the characteristics of wild-type-virus-transformed cells.

Mutant-virus-infected QNR cells were clearly distinguishable from both RAV-60- and SRE-infected cells. They were distributed among two subpopulations, one (80%) composed of very elongated cells with few and thin actin cables and the

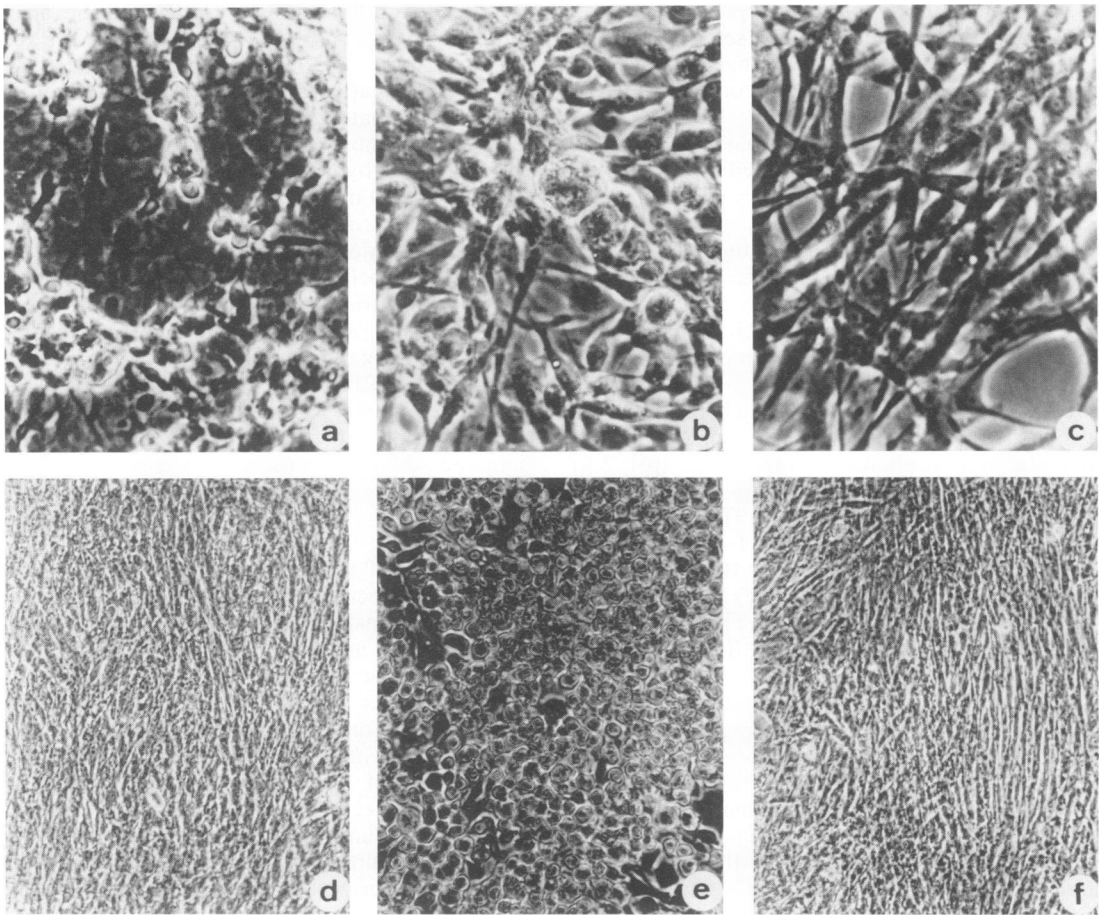

FIG. 1. Morphology of virus-infected cells. QNR cells were infected with RAV-60 (a), SRE (b), and *dl*PA105 (c). QEF were infected with RAV-60 (d), SRE (e), and *dl*PA105 (f). All cultures were passaged twice before microphotographs were taken. Magnifications are ×400 for QNR cells and ×250 for QEF.

other (20%) composed of polygonal cells similar to *dl*PA105-infected QEF. Like mutant-virus-infected fibroblasts, about one-half of the infected QNR cells had a few abnormal patches of actin concentrated at the cell periphery (Fig. 2f).

In summary, *dl*PA105 induces characteristic changes in the cytoskeletons of both cell types. Expression of these alterations appears to be more pronounced in QNR cells than in QEF.

Anchorage-independent growth. *dl*PA105 induced QNR cells to form colonies in soft-agar-containing medium with an efficiency that was consistently greater than that of SRE-infected cells (Fig. 3b and c). In contrast, QEF infected with the mutant virus gave rise to few colonies. Their efficiency of colony formation was estimated to be 1% that of *dl*PA105-infected QNR cells (Fig. 3f). QEF transformed by SRE formed colonies in soft agar with high efficiency (Fig. 3e).

Growth properties of monolayer cultures. *dl*PA105, like SRE, induces sustained growth on nondividing QNR cells (37). We investigated the effects of this mutant on the growth properties of QEF in monolayer cultures. Over a period of 10 days, the growth rate of QEF transformed by SRE was exponential, with an average doubling time of 36 h. In contrast, the growth characteristics of *dl*PA105-infected fibroblasts were closer to those of QEF infected with RAV-60. Compared with wild-type-virus-transformed cells, their growth rate was twice as slow, and they reached a density only 30% as high (Fig. 4).

Plasminogen activator production. RSV-transformed fibroblasts and NR cells produce increased levels of plasminogen activator (48, 56, 57). We compared the amounts of enzyme in culture media from both cell types infected with RAV-60, SRE, and *dl*PA105 (Table 1). In contrast to the other parameters of transformation tested above, the levels of plasminogen activator were comparable in mutant-virus-transformed QEF and QNR cells and were slightly lower than those of SRE-transformed cells.

Tyrosine kinase activity in mutant-virus-infected cells. The *src* gene of *dl*PA105 encodes a protein of 45,000 daltons ($p45^{v\text{-}src}$) which is phosphorylated on both serine and tyrosine residues and which possesses a tyrosine kinase activity when immunoprecipitated from QNR cell extracts (37). We considered the possibility that the differences in phenotypes between *dl*PA105-infected QNR cells and QEF might be due to a differential expression of the kinase activity.

Equal numbers of QEF and QNR cells chronically infected with RAV-60, SRE, or *dl*PA105 were labelled with $^{32}P_i$. Cell lysates were immunoprecipitated with tumor-bearing rabbit serum, and the v-*src* gene products were analyzed by established procedures. The amounts of phosphorylated $p45^{v\text{-}src}$ synthesized in QEF and QNR cells infected by the mutant virus were similar and were comparable to those of $p60^{v\text{-}src}$ in wild-type-virus-transformed cells. $p45^{v\text{-}src}$ proteins from either cell type were indistinguishable by V8 protease peptide mapping analysis (data not

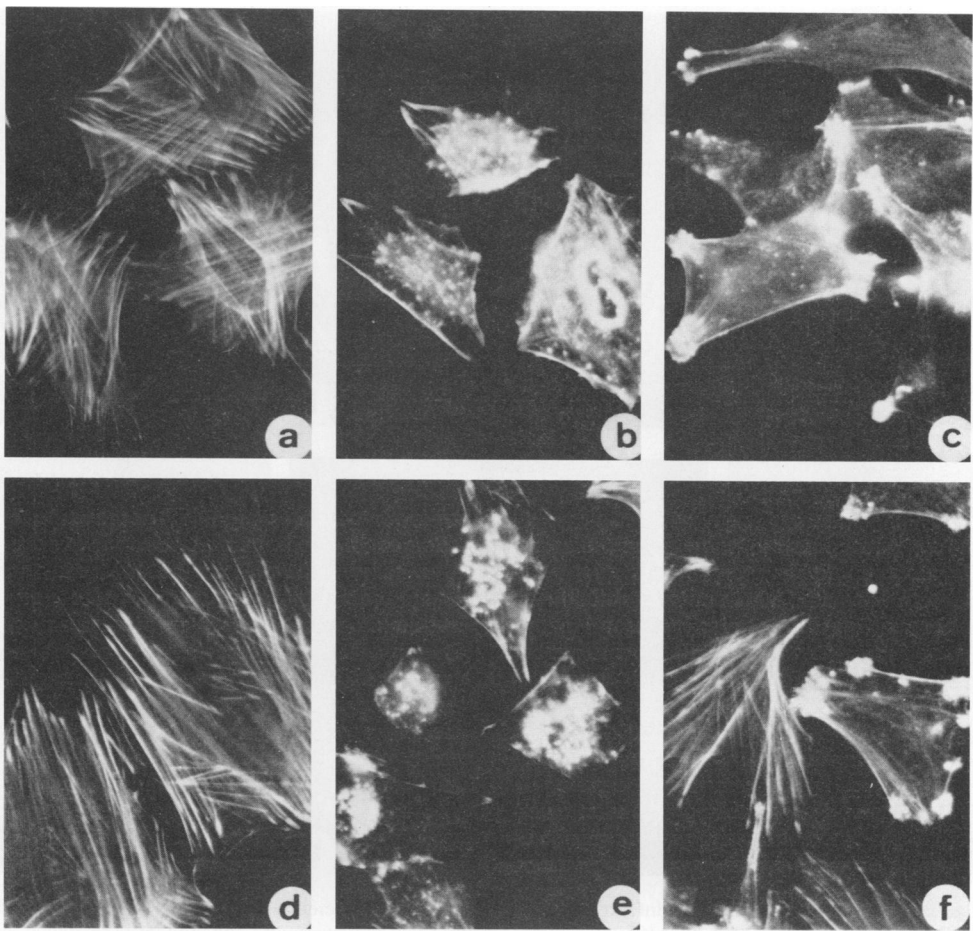

FIG. 2. Organization of polymerized actin in virus-infected cells. QNR cells were infected with RAV-60 (a), SRE (b), and *dl*PA105 (c). QEF were infected with RAV-60 (d), SRE (e), and *dl*PA105 (f). Chronically infected cells were seeded in 35-mm dishes containing glass cover slips (3×10^4 cells per dish). Two days later, cells were fixed and incubated with NBD-phallacidin.

shown). The 50,000- and 90,000-molecular-weight cellular phosphoproteins which coprecipitate with $p60^{v\text{-}src}$ (2–4, 14, 42) were also detected in comparable abundance in immunoprecipitates from both cell types (Fig. 5A).

Tyrosine kinase activity of $p45^{v\text{-}src}$ was first measured on the basis of its ability to phosphorylate IgG in immune complexes (12, 39). In this in vitro assay, comparable levels of $p45^{v\text{-}src}$ kinase activity were detected in immunoprecipitates from both QNR cells and QEF (Fig. 5B).

RSV-transformation of fibroblasts and NR cells results in a dramatic increase in total cellular phosphotyrosine (26, 47, 53), presumably a direct consequence of the catalytic activity of $p60^{v\text{-}src}$. We measured the levels of tyrosine phosphorylation of cellular proteins in virus-infected QEF and QNR cells (Table 2). The phosphotyrosine content of both QEF and QNR cells infected with *dl*PA105 was about 10 times higher than that of cells infected with RAV-60. The relative abundance of phosphotyrosine in mutant-virus-infected cells was comparable to that observed in SRE-transformed cells.

A cellular protein, p34, is phosphorylated on both serine and tyrosine residues in transformed cells and is considered a potential substrate of $p60^{v\text{-}src}$ kinase activity in vivo (19, 20, 50, 51). QEF and QNR cells infected with either SRE or *dl*PA105 were labeled with [^{35}S]methionine or $^{32}P_i$, and cell extracts were immunoprecipitated with anti-p34 serum. We found that p34 is phosphorylated equally in wild-type- and mutant-virus-infected cells (Fig. 6B).

Subcellular localization of $p45^{v\text{-}src}$ in QEF. Wild-type $p60^{v\text{-}src}$ is associated with the inner surface of the plasma membrane, within focal adhesion plaques and at points of junction with adjacent cells (15, 35, 36, 41, 52, 58). The N-terminal 13 kilodaltons of $p60^{v\text{-}src}$ has been implicated in this association (38). Amino acids 1 to 14 are required for N myristylation of $p60^{v\text{-}src}$ which, in turn, is required for plasma membrane association and cell transformation (6, 16, 17, 29, 44, 45).

To investigate whether the low transforming potential of $p45^{v\text{-}src}$ in fibroblasts correlated with an abnormal subcellular localization of this protein, we compared the distribution of $p45^{v\text{-}src}$ kinase activity in membrane and cytoplasmic subcellular fractions of QEF infected with mutant and wild-type virus. No major differences in the relative amounts of membrane-associated immunoprecipitable kinase activity were observed; in QEF infected with either virus, about two-thirds of the phosphorylating activity was detected in the membrane fraction (Table 3). The results are in agreement with the finding that $p45^{v\text{-}src}$ is labeled with myristic acid (37).

The binding of $p60^{v\text{-}src}$ to the plasma membrane is normally not affected by treatment with high concentrations of salt or EDTA or with 4 M urea (33). The v-*src* proteins of

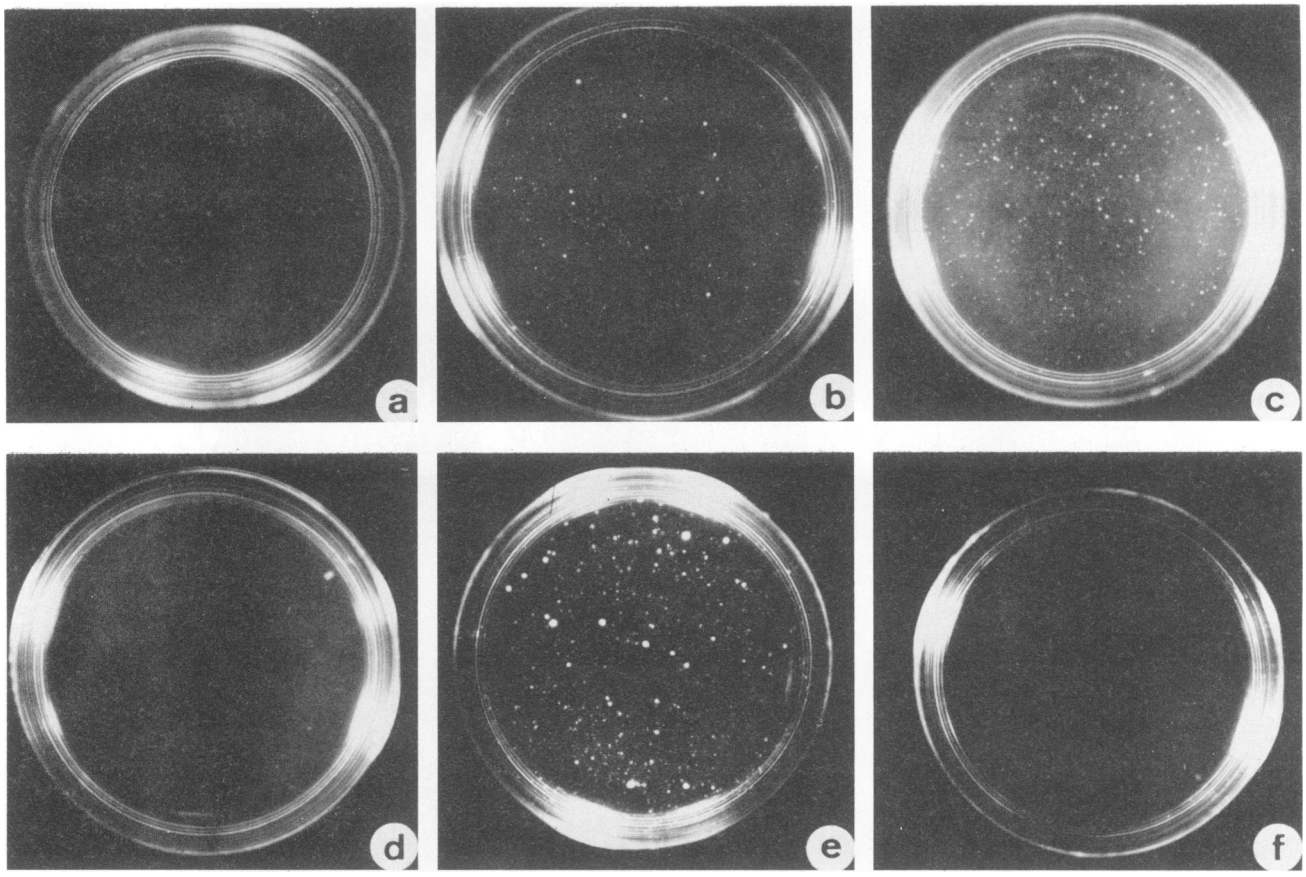

FIG. 3. Anchorage-independent growth of virus-infected cells. QNR cells were infected with RAV-60 (a), SRE (b), and *dl*PA105 (c). QEF were infected with RAV-60 (d), SRE (e), and *dl*PA105 (f). Cultures were passaged twice and subsequently seeded in soft-agar-containing medium. Colonies were observed 10 to 14 days later.

certain RSV mutants were shown to be normally attached to the plasma membrane, although the nature of this association was weaker than that of wild-type $p60^{v\text{-}src}$, as indicated by a greater salt sensitivity of the interaction (34). Both mutant $p45^{v\text{-}src}$ and wild-type $p60^{v\text{-}src}$ kinase activities displayed similar salt-resistant membrane association (Table 3).

Tumorigenicity of *dl*PA105 in quails. One-day-old quails were injected subcutaneously with SRE or *dl*PA105. Both mutant and wild-type virus induced malignant tumors that grew rapidly, extended massively beyond the wing web, and caused the death of all birds within 3 weeks (Table 4). Autopsy revealed tumors in both groups of birds at the site of inoculation and on the lungs and liver. Histological examination showed that the tumors induced by both viruses exhibited the microscopic features of fibrosarcomas. They were composed of areas of spindle-shaped fibroblasts mixed with pleiomorphic, irregular cells containing numerous mitotic figures and large hyperchromic nuclei, as described by Campbell (11) and Purchase and Payne (49). The bundles of fibroblasts were growing aggressively and infiltrating the neighboring tissue. In tumors growing at the site of inoculation which were at later stages of tumor growth, cells with polymorphic transitional forms and irregular sizes were observed together with various inflammatory and necrotic alterations. In primary tumors taken at early stages of growth and in secondary foci, typical waves of fusiform fibroblasts peculiar to fibrosarcomas were predominant with both viruses (Fig. 7).

In vitro cultures were generated from tumor explants obtained from each group of quails. Tumors derived from wild-type-virus injection gave rise to cultures of typical rounded and refringent cells (Fig. 8A, panel 1). In contrast, cells from *dl*PA105-induced tumors had an almost normal, fibroblastlike morphology (Fig. 8A, panel 2). Moreover, the efficiency of soft-agar colony formation of *dl*PA105 tumor cells was greatly reduced in comparison with that of wild-type-virus-induced tumor cells (Fig. 8B).

Development of fibrosarcomas induced by *dl*PA105 was not due to the generation of wild-type recombinant virus, as reported with other RSV mutants carrying partial deletions in the v-*src* gene (25, 30, 31). Instead, the biological properties of virus released by *dl*PA105-induced tumor cells were not distinguishable from those of the original mutant virus; (i) its v-*src* gene encoded a $p45^{v\text{-}src}$ protein, (ii) it promoted multiplication and morphological transformation of QNR cells, and (ii) it was able to induce QNR cells but not QEF to grow in soft agar (data not shown).

DISCUSSION

We studied the biological properties of *dl*PA105, a spontaneous variant of SRE with a deletion of 278 base pairs in the N-terminal portion of the v-*src* gene-coding sequence. This virus was selected on the basis of its ability to induce proliferation of quiescent QNR cells. We have shown (i) that the alteration of v-*src* results in a dissociated expression of

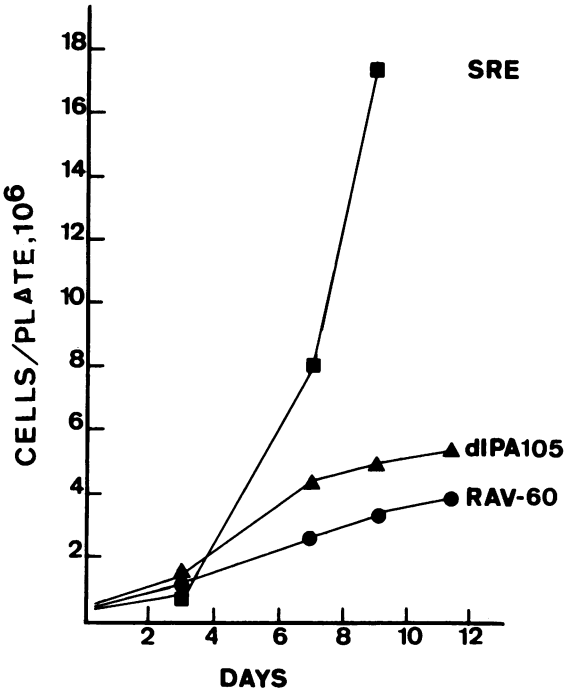

FIG. 4. Growth kinetics of virus-infected QEF. QEF were infected with the indicated viruses and subcultured twice. When transformation of QEF infected with SRE was complete, 2×10^5 cells from each culture were seeded in 60-mm dishes. Medium was renewed daily, and cells were counted on the days indicated. At day 12, dishes of SRE-transformed cells contained 7.2×10^5 cells per cm^2, whereas the saturation density of *dl*PA105-infected cells corresponded to 1.8×10^5 cells per cm^2.

the transformation functions of v-*src* and (ii) that this mutant induces distinct phenotypes in QEF and QNR cells.

Nucleotide sequence analysis of the mutant v-*src* gene has shown that the deletion is localized in the N-terminal one-third of the protein and that it extends from amino acid 33 to 126 of $p60^{v\text{-}src}$. The C-terminus of $p45^{v\text{-}src}$ appears to be intact, and its tyrosine kinase activity is similar to that of wild-type $p60^{v\text{-}src}$ (37).

TABLE 1. Plasminogen activator production in virus-infected cells[a]

Cell type	Virus	Caseinolysis dilution[b]
QEF	RAV-60	0[c]
	SRE	1/8
	*dl*PA105	1/4
QNR	RAV-60	0[c]
	SRE	1/8
	*dl*PA105	1/4

[a] Culture fluids were collected from confluent, virus-infected cells as described in the text. Cell numbers were determined, and the volumes of culture fluids were adjusted so that 1 ml corresponded to 10^6 cells. Serial dilutions (1/2) were prepared, and 0.1 ml of each dilution was assayed as indicated in the text.

[b] The results are expressed as the highest dilution inducing total caseinolysis in 48 h at 37°C.

[c] No caseinolysis was observed with supernatants of RAV-60-infected cells.

The mitogenic and tumorigenic properties of this truncated v-*src* protein resemble those of wild-type $p60^{v\text{-}src}$. *dl*PA105 stimulates the growth of QNR cells to an extent similar to that of SRE and induced fibrosarcomas in quails with an incidence comparable to that of wild-type virus. Yet $p45^{v\text{-}src}$ induces only limited changes in the morphology of fibroblasts; it does not release these cells from growth inhibition by saturation density, and it is largely defective in promoting anchorage-independent growth of QEF. These results demonstrate that the N-terminal portion of the v-*src* protein, which is deleted in $p45^{v\text{-}src}$, is dispensable for both the mitogenic and tumorigenic properties of v-*src*, whereas it is required for morphological transformation and alteration of growth properties of fibroblasts in vitro.

Both QNR cells and QEF infected with SRE undergo similar phenotypic and biochemical changes correlated with the expression of $p60^{v\text{-}src}$. Infected QNR cells become morphologically transformed and grow in soft-agar-containing medium. In addition, transformation of QNR cells by SRE leads to a 10- to 20-fold elevation of the relative amounts of

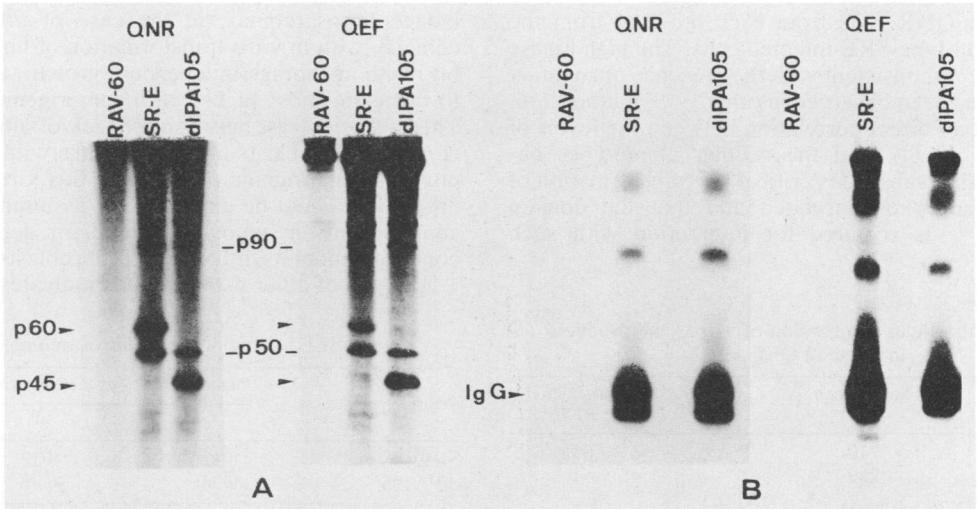

FIG. 5. Synthesis and in vitro protein kinase activity of v-*src* proteins (A) QNR cells and QEF were infected with the indicated viruses and subcultured twice. All cells were then seeded in 35-mm dishes (2×10^6 cells per dish) and labeled the next day with 0.5 mCi of $^{32}P_i$ for 5 h. Cell extracts were prepared and immunoprecipitated with tumor-bearing rabbit serum. The reaction products were separated on a sodium dodecyl sulfate-polyacrylamide gel (8.5% polyacrylamide). (B) Cell lysates were obtained from unlabeled parallel cultures, immunoprecipitated, and assayed for IgG phosphorylation as described in the text.

TABLE 2. Phosphoamino acid content of infected cells[a]

Cell type	Virus	% Phospho-serine	% Phospho-threonine	% Phospho-tyrosine
QEF	RAV-60	90	9.2	0.09[b]
	SRE	92	6.7	1.3
	*dl*PA105	91	7.0	1.4
QNR	RAV-60	92	7.8	0.06
	SRE	87	11	1.3
	*dl*PA105	88	9.8	1.9

[a] QNR cells (2×10^6) and QEF chronically infected with the indicated viruses were seeded in 35-mm dishes. Cells were labeled with 0.5 mCi of $^{32}P_i$ for 16 h. Phosphoproteins were extracted, hydrolyzed, and resolved in two dimensions as described in the text. The radioactivity associated with each phosphoamino acid is expressed as a percentage of the total radioactivity recovered in phosphoserine, phosphothreonine, and phosphotyrosine.

[b] Phosphotyrosine spots in RAV-60-infected QEF contained 556 cpm, whereas 176 cpm was detected in blanks.

cellular phosphotyrosine and to an increased phosphorylation of the cellular protein p34. These data are in agreement with our previous results obtained in chicken NR cells transformed by RSV (47). Furthermore, we have shown that conditionally and partially transformation-defective RSV mutants induced expression of the same subset of transformation parameters in both fibroblasts and NR cells (9, 10, 27). These results support the hypothesis that v-*src* proteins transform both cell types by similar mechanisms.

*dl*PA105 is unique among RSV mutants, since it induces distinct phenotypes in QNR cells and QEF. QNR cells induced to multiply by *dl*PA105 are morphologically transformed and form colonies in semisolid medium. In contrast, QEF infected with the mutant virus express only a partially transformed phenotype, as manifested by the limited changes in cell morphology and the lack of alteration of growth properties.

The v-*src* gene product of *dl*PA105 is myristylated, and the majority of $p45^{v\text{-}src}$ molecules are attached to the plasma membrane in QEF. Hence, the limited transformation of QEF cannot be explained by alterations in these properties, nor do the tyrosine kinase activities associated with $p45^{v\text{-}src}$ differ in QEF or QNR cells from each other or from the levels seen in wild-type-SRE-infected cells. The high kinase activity of $p45^{v\text{-}src}$ is consistent with the presence of an intact C terminus, as has been observed in other v-*src* mutants (16, 32). The absence of direct correlation between the levels of tyrosine kinase activity and the cellular phenotypes observed suggests that substrates critical for transformation of fibroblasts are not phosphorylated and that the domain deleted in $p45^{v\text{-}src}$ is required for interaction with such substrates.

TABLE 3. Subcellular localization of v-*src* gene products in infected QEF

Virus	NaCl concn (mM)	% Kinase activity in P100[a]
SRE	10	66
	200	64
*dl*PA105	10	78
	200	71

[a] Results are expressed as a percentage of the v-*src* gene product kinase activity recovered in the P100 fractions (see Materials and Methods). They represent the mean of two experiments.

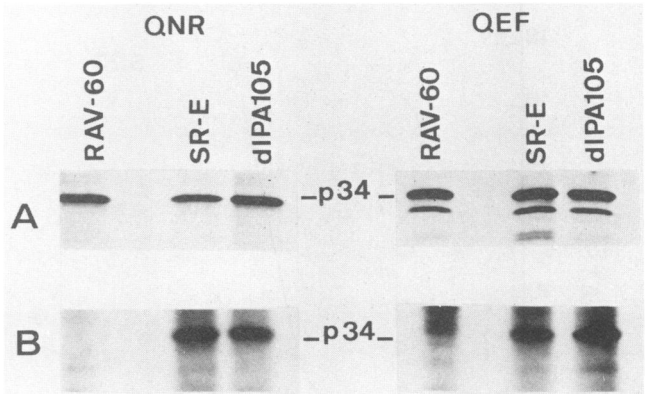

FIG. 6. Immunoprecipitation of radiolabeled p34 cellular protein in virus-infected cells. QNR cells and QEF were infected with the indicated viruses and subcultured twice. A total of 2×10^6 cells of each type were seeded in 35-mm dishes and metabolically labeled with either [^{35}S]methionine (A) or $^{32}P_i$ (B). Cell extracts were immunoprecipitated with anti-p34 serum, and the reaction products were analyzed on a sodium dodecyl sulfate-polyacrylamide gel (8.5% polyacrylamide). Only the p34 region of each gel is displayed.

How, therefore, does $p45^{v\text{-}src}$ succeed in transforming QNR cells? There are several possible explanations for the different phenotypic responses of QEF and QNR cells to infection with *dl*PA105. It is possible that the capacity of $p45^{v\text{-}src}$ to phosphorylate specific substrates for transformation is reduced in both cell types but that QNR cells have lower requirements for such substrates than have QEF. Alternatively, conformational changes in $p45^{v\text{-}src}$ could confer upon the mutant protein a broader specificity which allows its interaction with substrates present only in QNR cells. Finally, it is possible that activation of DNA replication may lead per se to activation of cellular pathways which would complement $p45^{v\text{-}src}$ for the transformation of QNR cells.

*dl*PA105 is highly tumorigenic in quails. Yet fibroblasts explanted in vitro from tumors induced by *dl*PA105 morphologically resembled QEF infected in vitro by this mutant and were not able to grow in soft agar. Hence, the ability to induce fibrosarcomas, in the case of *dl*PA105, does not coincide with in vitro transformation of fibroblasts or, notably, with anchorage-independent growth, a property shown to correlate most highly with tumorigenicity (28, 48, 54). There is a contrast between the lack of alteration of growth control of fibroblasts infected in vitro with *dl*PA105 and the powerful tumorigenic potential of this virus in vivo. These differences could be explained by assuming that a factor(s) supplied only in vivo is required for deregulating growth control of mutant-virus-infected fibroblasts.

Analysis of other v-*src* mutants indicates that amino acids

TABLE 4. Incidence of fibrosarcomas in quails[a]

Virus	Tumorigenicity at the following day postinfection[b]:		
	7	10	12
SRE	0/12	8/12	12/12
*dl*PA105	9/36	36/36	34/34[c]

[a] Groups of 1-day-old Japanese quails maintained in separate isolation rooms were inoculated in each wing web with 0.1 ml of undiluted virus adjusted to identical mitogenic titers (see text).

[b] Numbers represent sites with a palpable tumor out of the total amount of inoculation sites. These numbers are cumulative.

[c] All of the birds died from tumors.

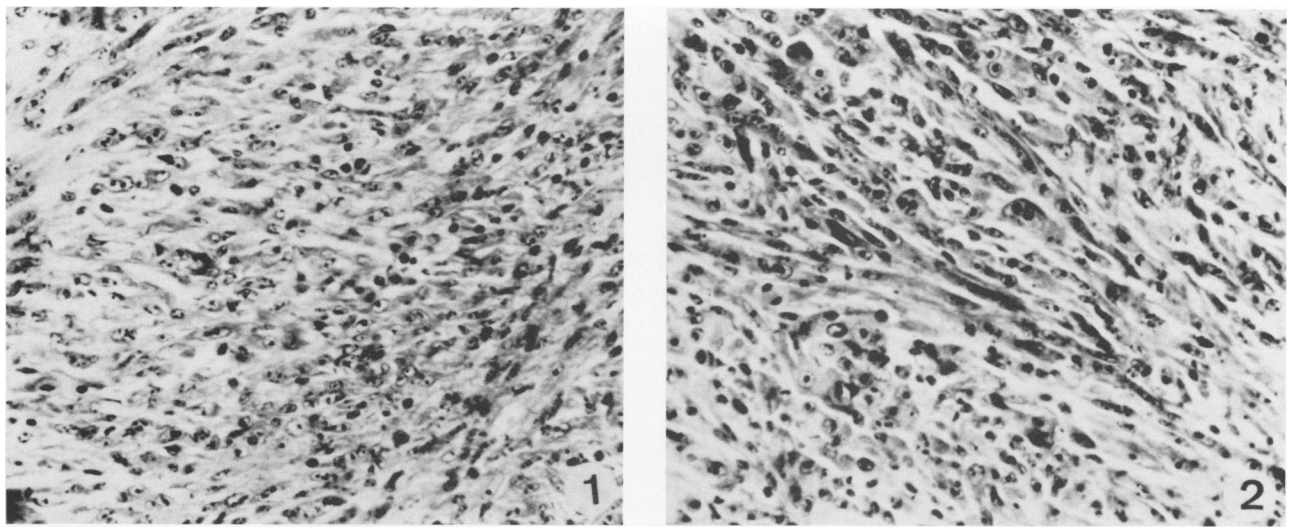

FIG. 7. Histological sections of secondary fibrosarcomas induced on liver by SRE (1) and *dl*PA105 (2) stained with hematoxylin and eosin. Magnification, ×80.

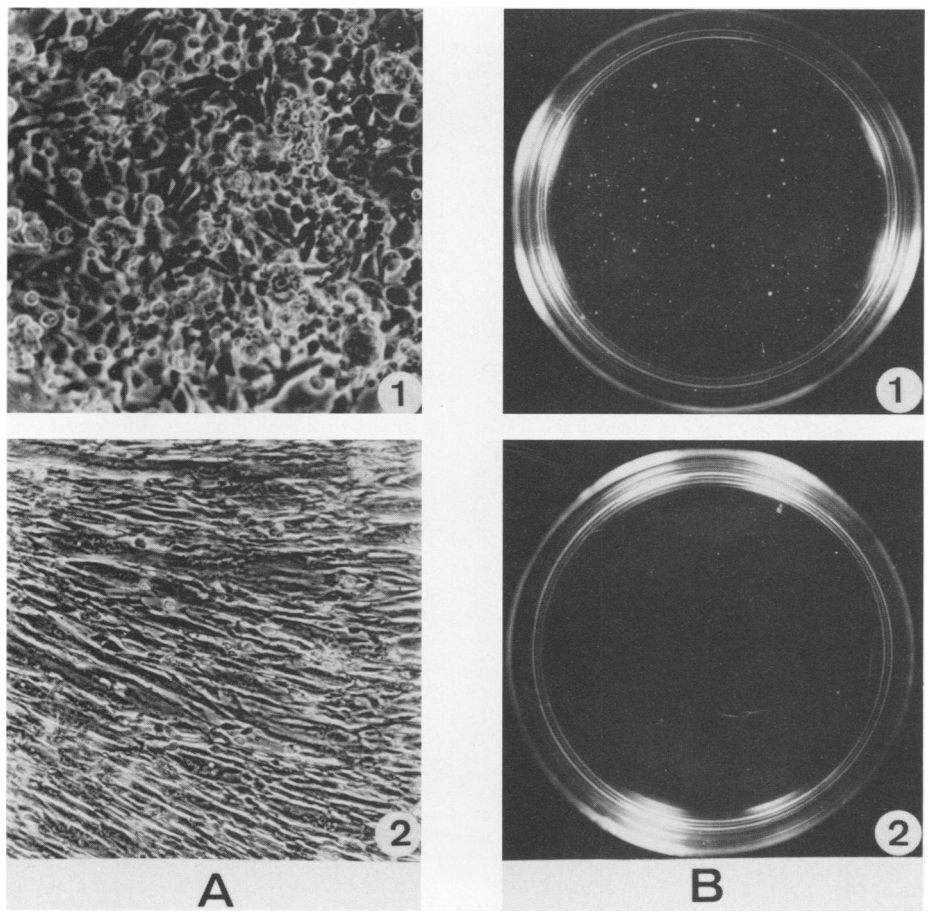

FIG. 8. Morphology and anchorage-independent growth of tumor-derived cells. Cells derived from tumors induced by SRE (1) and *dl*PA105 (2) were microphotographed (A) (magnification, × 250) or assayed for colony formation in soft-agar-containing medium (B) as described in the legend to Fig. 3.

15 to 169 of p60$^{v\text{-}src}$ are required for morphological transformation but not for anchorage-independent growth of fibroblasts (16, 21, 32, 43, 55). By using RSV mutants with in vitro-generated N-terminal deletions, we have previously shown that this domain is also dispensable for the induction of NR cell proliferation, although the rate of cell growth is reduced when the deletions extend beyond amino acid 81. In addition, these mutants induced similar phenotypic changes in both fibroblasts and NR cells (7).

The deletion in the v-*src* gene of *dl*PA105 is included within this modulatory region of v-*src*. In agreement with the proposed role of this domain, we have shown that fibroblasts infected with this mutant virus exhibited only limited changes in morphology. However, *dl*PA105 has also lost the capacity to induce growth of fibroblasts in soft agar. The other biological properties of *dl*PA105 differ from those of mutants with comparable N-terminal deletions in three aspects. First, the growth rate of QNR cells induced to proliferate by this mutant is comparable to that of wild-type-virus-infected cells, although the deletion extends well beyond amino acid 81 in the v-*src* protein. Second, *dl*PA105 has retained its full tumorigenic potential, whereas the tumorigenicity of other deletion mutants is reduced (17). Third, *dl*PA105 induces distinct phenotypes in QNR cells and QEF, whereas the other viruses induce a partially transformed phenotype in both cell types. Further molecular characterization of the v-*src* gene of *dl*PA105 should help explain these differences.

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