v-*src* Transformation of Rat Embryo Fibroblasts. Inefficient Conversion to Anchorage-independent Growth Involves Heterogeneity of Primary Cultures

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Abstract. To clarify whether a single oncogene can transform primary cells in culture, we compared the transforming effect of a recombinant retrovirus (ZSV) containing the v-*src* gene in rat embryo fibroblasts (REFs) to that in the rat cell line 3Y1. In the focus assay, REFs exhibited resistance to transformation as only six foci were observed in the primary cultures as opposed to 98 in 3Y1 cells. After G418 selection, efficiency of transformation was again somewhat lower with REFs compared to that with 3Y1 cells, but the number of G418-resistant REF colonies was much greater than the number of foci in REF cultures. Furthermore, while 98% of G418-resistant colonies of ZSV-infected REFs were morphologically transformed, only 25% were converted to anchorage-independent growth, as opposed to 100% conversion seen in ZSV-infected 3Y1 cells. The poor susceptibility of REFs to anchorage-independent transformation did not involve differences in expression and subcellular distribution of $p60^{v\text{-}src}$, or its kinase activity in vitro and in vivo. It rather reflected a property of the primary cultures, as cloning of REFs before ZSV infection demonstrated that only 2 out of 6 REF clones tested were permissive for anchorage-independent growth. The nonpermissive phenotype was dominant over the permissive one in somatic hybrid cells, and associated with organized actin filament bundles and a lower growth rate, both before and after ZSV infection. These results indicate that the poor susceptibility of REFs to anchorage-independent transformation by $p60^{v\text{-}src}$ reflects the heterogeneity of the primary cultures. REFs can be morphologically transformed by $p60^{v\text{-}src}$ with high efficiency but only a small fraction is convertible to anchorage-independent growth. REF resistance seems to involve the presence of a suppressor factor which may emerge from REF differentiation during embryonic development.

DURING the last 60 years, epidemiological studies (3, 9, 14) and experimental models of oncogenesis in vivo (4, 15, 16, 22) have provided evidence for the notion that carcinogenesis is a multistage process. Recent work with transforming proteins in vitro and in vivo is also consistent with this view. Thus, it is well known that the introduction of a single oncogene into primary rodent cells in culture is, in general, not sufficient to induce oncogenic transformation (33–35, 40). Only when additional genetic changes take place, e.g., introduction of an additional oncogene (34, 40) or long-term passage of the cells in culture (35), does full tumorigenic conversion occur. Similarly, transgenic mice harboring a single oncogene develop overt cancer only after a relatively long latency period, whereas the tissue-specific expression of two oncogenes greatly enhances tumor incidence and shortens the time for the emergence of malignancy (for reviews see 21, 25).

Nevertheless, there are limitations to this view. Spandidos and Wilkie (45) have shown that a *ras* oncogene alone can transform early passage rat embryo fibroblasts (REFs)[1], and it has been reported that the SV40 large T induces full tumorigenic conversion of REFs (10). Furthermore, both the SV40 large T and the *ras* oncogene produce liver tumors in transgenic animals (42), the emergence of which need not necessarily be construed as the result of multiple oncogenic events. However, it has been argued that in vitro transformation by the *ras* oncogene alone may require an exceptionally high level of $p21^{ras}$ expression (45), and the large T of the SV40 is a multifunctional protein (36), hence transformation by this protein may actually result from multiple tumorigenic events. The concept of oncogene cooperation in tumorigene-

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1. *Abbreviations used in this paper*: ffu, focus forming units; REF, rat embryo fibroblast; TBS-T, Tris-buffered saline with Tween-20; ZSV, Moloney leukemia retrovirus containing v-*src* and *neo* genes.

sis, therefore, is still widely accepted and the multistage nature of cancer remains to be challenged.

$p60^{v\text{-}src}$, the protein encoded by the v-*src* gene of the Rous sarcoma virus, transforms primary chicken fibroblasts in culture and induces tumors in animals (28). $p60^{v\text{-}src}$ also transforms rodent cell lines (5, 24, 27), yet it is not clear whether it can efficiently convert primary cultures of mammalian cells. Gilmer et al. (17) reported that v-*src* transforms Syrian hamster embryo cells, but considerable suppression was apparent when compared to the transforming effect in cells immortalized by carcinogens. MacAuley and Pawson (35) have also demonstrated that v-*src* poorly converts early passage rat adrenocortical epithelial cells to anchorage-independent growth. Higher transformation efficiency was observed only when v-*src* was introduced in conjunction with v-*myc*, a finding consistent with previous observations demonstrating synergism between v-*src* and v-*myc* in transforming avian primary cultures (1, 2). On the other hand, Hjelle et al. (23) found that v-*src* transfected into REFs induced full conversion to the tumorigenic phenotype, even though both growth in soft agar and tumor emerging in *nu/nu* mice were somewhat suppressed when compared to primary cells in which either the *ras* or *myc* oncogene was cotransfected with v-*src*.

The objective of the present studies was to clarify whether nonestablished cell cultures can efficiently be transformed by a single oncogene. Particularly, if only a fraction of the primary cells can be transformed, is this due to higher levels of the transforming protein, special culture conditions, and/or inherent heterogeneity in cellular susceptibility? To accomplish these goals, we have studied the ability of a recombinant retrovirus carrying the v-*src* and *neo* genes to transform REFs and compared the results to those obtained with the rat cell line 3Y1.

Materials and Methods

Antibodies and Reagents

Monoclonal antibody 327 against $p60^{src}$ was kindly provided by J. Brugge and monoclonal antibody against phosphotyrosine (4G10) was purchased from UBI (Lake Placid, NY). FITC-conjugated anti-mouse IgG and peroxidase-conjugated anti-mouse IgG were obtained from Amersham (Arlington Heights, IL). Unless otherwise indicated, all cell culture products and chemical reagents were from GIBCO BRL (Gaithersburg, MD) and Sigma Chem. Co. (St Louis, MO), respectively.

Retrovirus Construction, Cell Culture, and Infection Protocol

A replication-defective retrovirus (ZSV) was constructed by transfecting the pZIPNeoSrc plasmid, which contains the lung terminal repeats of Moloney murine leukemia virus, the neomycin resistance gene, and the v-*src* gene, into the Ψ_2 packaging cell line as previously described (26). REFs were prepared from Fisher rat embryos at their 16th d of gestation, grown in culture for 48 h, and stored in liquid nitrogen. Unless otherwise indicated, cells were cultured at 37°C in DMEM containing 10% FCS. Rat 3Y1 cells and REFs were infected with ZSV as follows. In the focus assay, cells were inoculated at 2.5×10^5/6-cm dish, and, after an overnight culture, infected with 100 focus forming units (ffu) of ZSV/dish. Thereafter, cells were cultured in normal growth medium containing no selection drug, and foci were scored 7–12 d after ZSV infection. When selection was desired, cells were inoculated and infected as above, and cultured in the presence of 0.2 (REFs) or 0.4 (3Y1 cells) mg/ml G418. Resistant colonies[2] were scored 8–12 d after G418 addition. During this time, G418 resistant colonies which were thoroughly separated from each other were picked with cloning cylinders, grown in appropriate selection medium in a 24-well plate (Becton and Dickinson, Lincoln Park, NJ), and, if morphologically homogeneous, further expanded in 6-cm dishes and stored. Clones that appeared morphologically heterogeneous were either discarded or subcloned. Both in the focus assay and G418 selection protocol, three infection experiments were carried out, in each of which duplicate dishes were used. In all instances, growth medium was changed every 3 d of culture.

Cell Fusion Procedure

Two clones of ZSV-infected REFs were fused using the polyethylene glycol method. Parental clones were first transfected with a pBABE (37) vector containing either the puromycin or hygromycin resistant gene using the calcium phosphate technique (47). Thereafter, 2.5×10^5 cells of each clone were inoculated into a 6-cm dish, cultured overnight, and treated with a 50% solution of polyethylene glycol for 60 min at 37°C. Cells were then washed, and incubated overnight with 10% FCS-DMEM. After trypsinization, 2×10^5 cells were inoculated into 6-cm dishes containing 10% FCS-DMEM and 0.3 mg/ml hygromycin and 3 μg/ml puromycin. After 10–12 d in culture, some of the surviving colonies were picked, expanded, and stored until used for further studies. As controls, cells from either clone were fused to each other and cultured in selection medium as above. Under these conditions, all cells died during a 10-d culture period.

Determination of Growth Rates

Cells were inoculated at $2–4 \times 10^4$/6-cm dish. After attachment (~12 h), the medium was discarded and cultures were washed three times with serum-free medium. Fresh DMEM containing 0.1, 1.0, or 10.0% FCS was then added. For each FCS concentration, six duplicate dishes were prepared and used for daily cell counting from day 0 (just after fresh medium was added) through day 5. Cells were counted in a hemocytometer after washing and trypsinization. Doubling time was determined during exponential growth.

Cloning of REFs

REFs which were grown for ~48 h (2–3 divisions) and stored, were reinoculated and cultured for ~24 h. Cells were then trypsinized, plated at a density of ~200 cells/25-cm dish, and cultured as above. After attachment the medium was changed, and individual cells well separated from each other were identified and numbered under the plate with an ink marker. Cells in each clone were then counted over 12-h periods for up to 3 d and photomicrographed. At this point, colonies (including 20–50 cells) were transferred to a 24-well plate with cloning cylinders, and grown to semiconfluence. Each clone was then infected with ZSV ($\sim 10^4$ ffu) and selection was achieved with 0.2 mg/ml G418 in growth medium. After 7–10 d, resistant cells were inoculated into soft agar as described below.

Anchorage-independent Growth

Soft agar assays were carried out using 6-cm dishes prepared with a lower layer of 0.5% Bacto-Agar (Difco Laboratories, Detroit, MI) in DMEM containing 10% bovine calf serum. 5×10^3 cells were plated in upper layer containing 0.4% Noble Agar (Difco) in 10% FCS-DMEM. For each experiment, duplicate dishes were prepared and a positive (v-*src* transformed 3Y1 clone) and negative (3Y1 cells) control were invariably included. Colonies were scored after a 21-d incubation period at 37°C.

Immunofluorescence Experiments

Immunofluorescence staining was performed to examine the cellular localization of $p60^{v\text{-}src}$ and the organization of actin cables. Cells ($2-4 \times 10^4$) were inoculated into wells of Lab-Tek chamber slides (Nunc, Naperville, IL) and cultured for ~24 h. For actin cable visualization, cells were then washed with PBS and fixed in 4% formaldehyde in serum-free DMEM. After washing and permeabilization with a 0.5% Triton X-100 solution, cells were incubated for 30 min with 250 μl of a phalloidin-FITC solution (Molecular Probes Inc., Eugene, OR) as previously described (49). Cultures

2. Throughout the manuscript, the term "colony" is used to indicate a well defined group of cells growing under G418 selection conditions after infection with ZSV. After isolation and expansion in normal growth medium, a colony is then referred to as "clone".

were then thoroughly washed in PBS, and, after plastic well moldings were removed, slides were mounted with a 50% glycerol/0.1% *p*-phenylendiamine solution in PBS and inspected under a fluorescent microscope. For $p60^{v\text{-}src}$ staining, cells were prepared, washed, fixed, and permeabilized as above. Anti $p60^{v\text{-}src}$ antibodies were then added at 1:25–250 dilutions and cultures incubated at 37°C for 60 min. After washing, cells were incubated at room temperature for 30 min with FITC-conjugated anti-mouse IgG (1:100), washed, and slides were mounted and visualized as above.

$p60^{v\text{-}src}$ Kinase Activity

The procedure for determining $p60^{v\text{-}src}$ kinase activity in vitro was essentially that described by Sabe et al. (41). Cells (~10^6/6-cm dish) were lysed with RIPA buffer [1% Triton X-100; 1% Na-deoxycholate; 0.1% SDS; 20 mM Tris-HCl, pH 7.4; 100 mM NaCl; 5 mM EDTA; 50 mM NaF; 10 μM Na_2MoO_4; 1 mM PMSF; 100 Kallikrein inactivator units of aprotinin/ml (1%); 20 μg/ml leupeptin; 1 mM Na_3VO_4; and 5 mM benzamidine], and lysate containing 20–50 μg of total protein was immunoprecipitated in 0.5 ml of RIPA buffer with ~1 μg of monoclonal antibody against $p60^{v\text{-}src}$. Kinase activity was then determined in a reaction mixture (50 μl) containing: 0.1% Triton X-100; 20 mM Tris-HCl, pH 7.2; 0.2 mM Na_3VO_4; 5 mM $MgCl_2$; 5 μg of acid-denatured enolase; and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham). Kinase reaction was carried out at 30°C for 15 min and stopped by the addition of twofold concentrated Laemmli sample buffer (125 mM Tris-HCl, pH 6.8; 10% mercaptoethanol; 4% SDS; 20% glycerol; 10 mM EDTA; 2 mM Na_3VO_4; and 0.02% bromophenol blue). Samples were then boiled for 5 min and applied to a 10% SDS-PAGE gel. After electrophoresis, the gel was fixed, dried, and exposed to film.

Western Blot Analysis

Western blotting was performed to determine the expression of phosphorylated proteins in vivo and $p60^{v\text{-}src}$. Cells lysates were prepared using RIPA buffer described above and SDS-PAGE was performed either after loading whole cell lysate (20–40 μg total protein) onto gel or after immunoprecipitating the desired protein from cell lysate (50–300 μg total protein) with the specific antibody. Separated proteins were then transferred onto a PVDF membrane (Millipore, Bedford, MA) which was blocked for 60 min with 3% bovine serum albumin in TBS-T (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, and 0.1% Tween-20), incubated with first antibody (1–3 h) in 1% bovine serum albumin in TBS-T, washed in TBS-T (6 × 5 min), reincubated with second antibody conjugated with HRP (1 h), washed again, and the immunoreactivity detected with the ECL system (Amersham).

Results

v-src Transformation of 3Y1 Cells and REFs

When cells were grown without selection, ZSV infection induced foci in both 3Y1 cells and REFs. However, whereas infection with 100 ffu of ZSV produced an average of 98 foci in 3Y1 dishes, only six foci were observed in REF plates (Table I, Exp. 1). Furthermore, foci in REF cultures were smaller than in 3Y1 cells at any time of the 14 d after infection period. Mock infections produced no foci in both cultures. In separate experiments, G418 was added to cultures infected with 100 ffu of ZSV and G418-resistant colonies in both 3Y1 cells and REFs were obtained (Table I, Exp. 2). With 3Y1 cells, the average number of G418-resistant colonies was similar to the number of foci formed in non-selected plates, and 98% of the G418-resistant colonies were morphologically transformed. With REFs, the average number of G418-resistant colonies was much greater than the number of foci in non-selected cultures, although somewhat less than those observed with 3Y1 cells. As with the latter, 98% of the REF colonies were morphologically transformed, although to varying degrees (see below). Thirty six of these colonies were isolated as "ZSV-REF clones."

To determine whether the morphologically transformed cells were anchorage independent, 12 and 36 G418-resistant colonies from ZSV-infected 3Y1 and REF cultures, respectively, were picked, expanded, and inoculated onto soft agar (Table I, Exp. 3). Of the 3Y1 colonies chosen, nine were morphologically transformed and three displayed a normal phenotype. We also attempted to grow flat REF colonies, but, like the uninfected REFs, these cells could not be propagated any longer after 15–20 d in culture. We assumed that such a growth crisis reflected the lack of an integrated (intact) v-*src* gene (as suggested by the results with the flat 3Y1 colonies, see below) and thus refrained from further studying these colonies. Fig. 1 depicts the morphology of representative 3Y1 and REF G418-resistant clones. All morphologically transformed 3Y1 clones showed a similar phenotype; most cells were round and refractile, and invariably piled up forming clusters. On the other hand, the morphology of ZSV-REF colonies was quite variable. Some clones (25%) were similar to transformed 3Y1 cells, while other clones were variable in shape (elongated or polygonal). These latter clones invariably failed to overgrow into clusters. As summarized in Table I, all morphologically transformed 3Y1 clones grew efficiently in soft agar, whereas the flat clones failed to form any colonies, as did the uninfected 3Y1 cells. On the other hand, only 12 out of the 36 morphologically transformed REF clones displayed anchorage-independent growth at high efficiencies.

Table I. Number of Foci and G418 Resistant Colonies after ZSV Infection of 3Y1 Cells and REFs

		3Y1 Cells	REFs
Experiment 1			
Foci		87;108;98 (98)	4;8;6 (6)
Experiment 2			
G418	MT	89;82;92 (88)	60;53;52 (55)
Colonies	Flat	2;2;4 (3)	1;0;2 (1)
	Total	91;84;96 (90)	61;53;54 (56)
Experiment 3			
Anchorage	MT	9/9 (100%)	12/36 (33%)
Independent growth	Flat	0/3 (0%)	ND

Number of foci/dish and G418 resistant colonies/dish were determined in three separate infection experiments using duplicate 6-cm dishes (average values are shown in parenthesis). In mock infections, no foci or G418 resistant colonies were detected. *MT*, Morphologically transformed; *Flat*, Normal phenotype. Anchorage-independent growth is expressed as the number of G418 resistant colonies able to grow in soft agar at >10% efficiency over number of tested clones (percentage shown in parenthesis). *ND*, Not determined since REF colonies with normal morphology were not immortalized. For more details see text and Table II.

Growth Properties of v-src Transformed Cells

To establish a possible relationship between serum dependent and anchorage-independent growth, we measured the doubling time of some clones of 3Y1 cells and REFs transformed by v-*src* in the presence of 10%, 1%, and 0.1% FCS. The results are summarized in Table II. Compared to the uninfected cells, ZSV-transformed 3Y1 cells exhibited an increase in growth rate with higher FCS concentrations. On the other hand, only the ZSV-REF clones [RF-T(I)] which displayed anchorage-independent growth exhibited growth rates higher than that of the parental cells. In clones not capable of efficient growth in soft agar [RF-T(II)], the average growth rate was significantly decreased at each concentration of FCS. The low growth rates of the nonpermissive

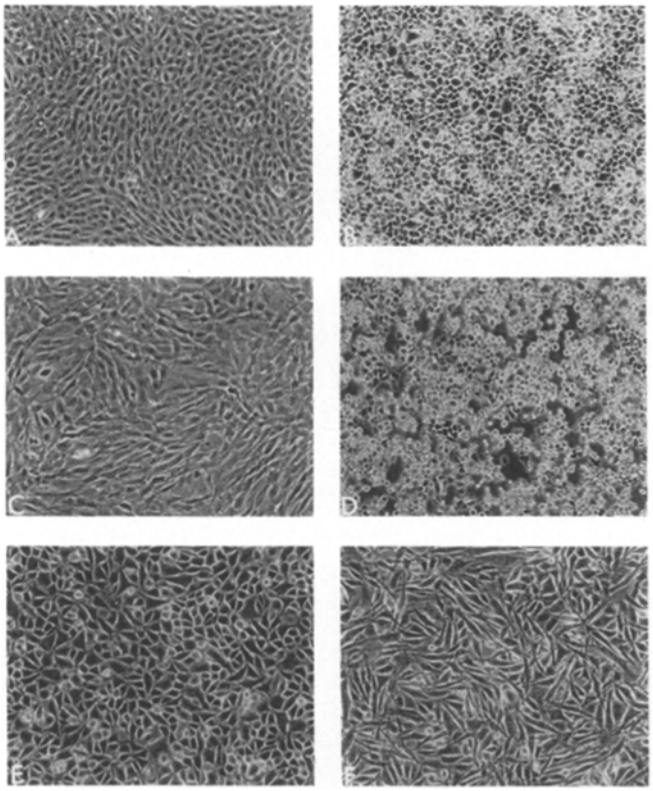

Figure 1. Morphologic appearance of 3Y1 cells (*A* and *B*) and REFs (*C, D, E,* and *F*) before (*A* and *C*) and after (*B, D, E,* and *F*) ZSV infection. Morphologically transformed 3Y1 cells (*B*) grew efficiently in soft agar and so did the REF clone shown in *D*. On the other hand, REF clones shown in *E* and *F* were not converted to anchorage-independent growth. Original magnification ×100.

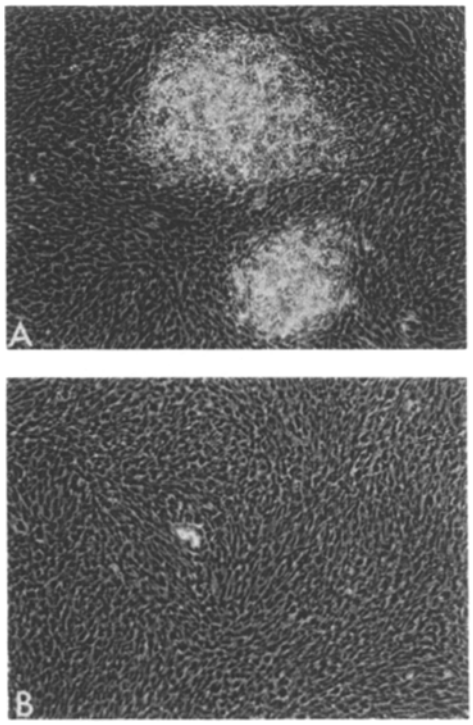

Figure 2. Focus formation by ZSV-infected REF clones cocultured with normal REFs. One hundred ZSV-infected, morphologically transformed REF cells either converted (*A*) or not converted (*B*) to anchorage-independent growth were simultaneously inoculated with uninfected REFs (5×10^5/6-cm dish) and cultured for 8 d in 10% FCS-DMEM. Note that only the fully converted clone produced large foci. For more details, see text and Table III. Original magnification ×100.

clones did not involve release of an inhibitory factor(s), for addition of the supernatant from these clones to culture which exhibited faster growth rates did not produce an inhibitory effect. Regardless of their growth differences in liquid medium and soft agar, all ZSV-REF clones listed in Table II ($n = 11$) were immortalized as they showed no sign of senescence after being passaged for more than 3 months. During such a time, both the morphology and growth rate of most ZSV-REF clones did not change. Six of the 24 clones which originally exhibited no anchorage-independent growth, however, spontaneously changed to the fully transformed phenotype probably due to selection of fast growing cells.

Role of Selection in v-src Transformation

Because the number of G418-resistant colonies was much greater than the number of foci formed by REFs infected with ZSV (Table I), we wished to establish whether elimina-

Table II. Growth Characteristics of G418 Resistant Clones from 3Y1 Cells and REFs Infected with ZSV

Clone activity	Cloning efficiency in soft agar	Doubling time			Src kinase
		0.1%	1%	10% FCS	
3Y1 cells					
Uninfected	0	38.4	23.9	14.1	−
3Y-N	0	40.1	24.7	14.5	−
3Y-T ($n = 5$)	64.0 ± 5.6	32.3 ± 1.2	21.1 ± 0.6	12.5 ± 0.3	+
REFs					
Uninfected	0	61.3	41.2	17.8	−
RF-T(I) ($n = 5$)	55.8 ± 6.1	38.7 ± 1.7	20.2 ± 1.1	12.2 ± 0.4	+
RF-T(II) ($n = 6$)	1.1 ± 0.5	124.3 ± 6.2	53.4 ± 2.4	24.1 ± 1.6	+

Cloning efficiency in soft agar is expressed as percent of inoculated (5×10^3) cells forming colonies >0.5 mm in diameter. Doubling time is expressed in h and determined during culture in liquid medium containing 0.1, 1.0, or 10.0% FCS. When appropriate, values are expressed as means ± SD. Mean values refer to clones grouped according to their morphologic traits (*N*, normal; *T*, transformed) and cloning efficiency in soft agar. In vitro *src* kinase activity is expressed as absent (−) or present (+).

Table III. Foci Formed by ZSV-Transformed REF Clones after Inoculation into Liquid Medium with Uninfected REFs

Clone	Colonies w/o REFs	Foci w/REFs		
	4 d	4 d	8 d	12 d
Capable of growing in soft agar				
RF-TT1	113	99	107	ND
RF-TT2	77	110	120	ND
RF-TT3	109	86	88	ND
RF-TT4	92	91	90	ND
Incapable of growing in soft agar				
RF-MT1	84	7	8	8
RF-MT2	118	3	3	3
RF-MT3	91	2	3	3
RF-MT4	88	4	4	5

One hundred cells from ZSV-transformed REFs either growing (>40%) or not growing in soft agar (<2%) were inoculated into 6-cm dishes with (w/) or without (w/o) 2×10^5 parental REFs. Foci were scored at 4, 8, and 12 d after inoculation. Colonies formed in absence of wild-type REFs were scored at 4 d. Cells from REF clones that grew efficiently in soft agar formed large and confluent foci at 12 d which could not be determined (ND).

tion of uninfected REFs by G418 selection was important in $p60^{v\text{-}src}$ transformation of REFs. To this end, we inoculated 100 cells from various ZSV-REF clones with 5-8 $\times$ 10^5 uninfected REFs. As illustrated in Fig. 2 and summarized in Table III, ZSV-REF clones that were converted to anchorage-independent growth were all able to overgrow under these conditions and promptly produced typical foci similar in number to that of inoculated cells. On the other hand, ZSV-REF clones that exhibited a longer doubling time and no anchorage-independent growth invariably failed to produce clearly visible foci within 14 d from their inoculation.

$p60^{v\text{-}src}$ Expression, Kinase Activity, and Intracellular Distribution

All 3Y1 clones that were morphologically transformed and efficiently grew in soft agar displayed high levels of $p60^{v\text{-}src}$ expression and $p60^{v\text{-}src}$ kinase activity in vitro. 3Y1 clones that were not morphologically transformed failed to do so, as did uninfected 3Y1 cells. Similarly, $p60^{v\text{-}src}$ expression and $p60^{v\text{-}src}$ kinase activity were detected in all morphologically transformed ZSV-REF clones at high levels. Most interestingly, as shown in Fig. 3 for representative ZSV-REF clones, both the expression of $p60^{v\text{-}src}$ and its kinase activity in vitro were the same regardless of whether the ZSV-REF clones were capable of anchorage-independent growth. Results obtained from analysis of tyrosine phosphorylated proteins in cells were consistent with the in vitro kinase data, for a close similarity among all morphologically transformed ZSV-REF clones was observed regardless of their ability to grow in soft agar (Fig. 3). Transformed 3Y1 cells showed patterns of tyrosine phosphorylated proteins in vivo similar to those in ZSV-REF clones, whereas both uninfected 3Y1 cells and REFs displayed minimal levels of protein phosphorylation. Finally, as previously reported for mammalian cells (12, 32), a diffuse intracellular distribution of $p60^{v\text{-}src}$ was observed in all transformed 3Y1 and ZSV-REF clones studied and no substantial differences were observed among ZSV-REF clones regardless of their ability to grow in soft agar (data not shown).

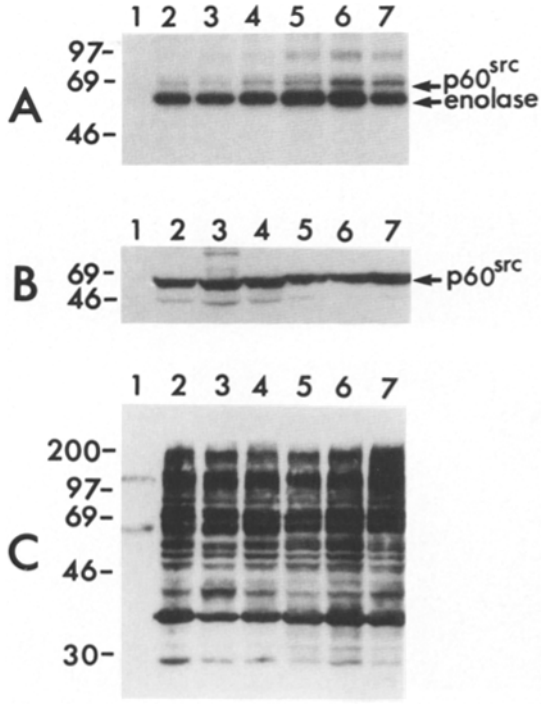

Figure 3. $p60^{v\text{-}src}$ kinase activity in vitro (*A*), $p60^{v\text{-}src}$ expression (*B*), and pattern of tyrosine-phosphorylated proteins (*C*) in parental REFs (lane *1*) and ZSV-REF clones either converted (lanes *2–4*) or not converted (lanes *5–7*) to anchorage-independent growth. Kinase activity in vitro was determined using enolase as substrate after immunoprecipitation of whole cell lysate with anti-$p60^{v\text{-}src}$ antibodies. Autophosphorylated $p60^{v\text{-}src}$ is also shown. $p60^{v\text{-}src}$ expression and tyrosine-phosphorylated proteins were determined by probing Western blots of whole cell lysates with anti-$p60^{v\text{-}src}$ and anti-phosphotyrosine antibodies, respectively. Note the lack of differences among ZSV-REF clones, regardless of their ability to grow in soft agar.

ZSV Infection of Cloned REFs

To determine whether the failure of ZSV to induce anchorage-independent growth in a large fraction of morphologically transformed REF clones reflected a property of the primary cultures, we cloned uninfected REFs and examined the morphological traits and growth rates in liquid medium of several clones during their expansion. Thereafter, these clones were individually infected with ZSV, growth-selected in G418, and inoculated into soft agar. As illustrated in Fig. 4 and summarized in Table IV, the results from these experiments have demonstrated the following. First, there were considerable differences in morphological appearance and growth rates among various clones. Second, upon infection with ZSV, all clones were morphologically transformed but only 2 out of 6 tested were capable of efficiently growing in soft agar. The two REF clones that were permissive for anchorage-independent growth shared a similar morphology—cells were relatively small, flat, somewhat elongated—, and a greater competency of growth in liquid medium. It is highly unlikely that either of these two clones were immortalized before ZSV infection, for long term culture of uninfected REFs in separate experiments always failed to demonstrate the establishment of cell lines.

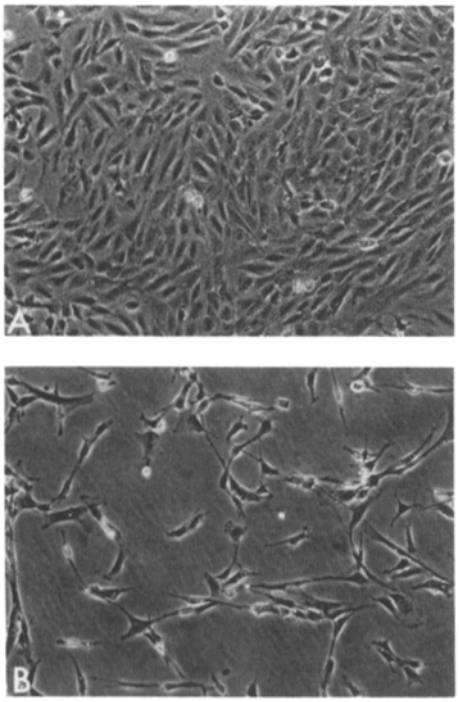

Figure 4. Morphology of two clones of uninfected REFs 5 d after cells were inoculated at low density (200 cells/25-cm dish). As shown in Table IV, the clone shown in *A* (*REF2*) grew faster than the clone shown in *B* (*REF5*) when cultured in 10% FCS-DMEM. After ZSV infection, the clone in *A* grew efficiently in soft agar, whereas the clone in *B* failed to do so. See Table IV for more details. Original magnification ×100.

Cell Fusion Experiments

To determine whether the poor capacity of REF clones to be converted to anchorage-independent growth by ZSV involved a dominant phenotype, a typical ZSV-REF clone which failed to grow in soft agar was fused to a ZSV-REF clone which grew highly efficiently in soft agar. As summarized in Table V, all hybrid clones studied displayed the phenotype of the ZSV-REF parental clone which was not converted to anchorage-independent growth. Hybrid cells were elongated and larger in size than the fully converted ones, exhibited a relatively low growth rate in liquid medium, did not overgrow after reaching confluence, and failed to form colonies in soft agar. Fusion between two ZSV-REF clones that grew efficiently in soft agar resulted in hybrid cells that displayed the same phenotype as the parental cells.

Table IV. Relationship between Growth Rate in Liquid Medium of REF Clones before Virus Infection and Their Growth in Soft Agar after ZSV Infection

Clone	Doubling time (*h*)	Cloning efficiency in soft agar (%)
REF1	15.4	58.9
REF2	16.3	62.1
REF3	20.4	7.2
REF4	24.8	1.3
REF5	25.6	0.6
REF6	23.5	1.7

Growth in soft agar was measured after inoculation of 5×10^3 cells into 0.4% Noble agar suspension. Doubling time was determined during exponential growth in liquid medium containing 10% FCS. Morphology of representative clones is shown in Fig. 5.

Table V. Morphologic Traits and Growth Properties of Parental and Hybrid Cells

Clone	Cell morphology	Doubling time (*h*)	Cloning efficiency: Liquid medium	Cloning efficiency: Soft agar
Parental				
RF-MT2	A	11.4	93	64.8
RF-TT3	B	26.2	87	0.9
Hybrid				
RF-H1	B	21.4	88	1.7
RF-H2	B	25.3	85	0.7
RF-H3	B	19.8	89	1.5
RF-H4	B	26.7	87	4.2

The two parental REF clones (RF-MT2 and RF-TT3) isolated under G418 selection after ZSV infection are the same as those shown in Table III. These two types of cells were fused as described in Materials and Methods. Cell Morphology was defined as follows: *A*, cells were small, refractile, and formed clumps; *B*, cells were elongated, larger in size, and failed to overgrow. Doubling time was determined in 10% FCS. Cloning efficiency in liquid medium represents the number of colonies scored 4–6 d after 100 cells were inoculated in a 10-cm dish containing 10% FCS-DMEM. Cloning efficiency in soft agar is expressed as the percent of inoculated cells (5×10^3) that formed colonies >0.5 cm in diameter 21 d after inoculation.

Organization of F-Actin Cables

It is well known that actin filaments become disorganized after in vitro transformation by a number of oncogenes, including v-*src* (6, 8, 38). The present finding that only a small fraction of the morphologically transformed REF clones were converted to anchorage-independent growth prompted us to study the organization of actin filaments in the attempt to establish a relationship between stress fiber assembly and morphological transformation on one hand and anchorage-independent growth on the other. The results from these studies are shown in Fig. 5 and Table VI. In both parental

Table VI. F-Actin Cable Organization in Normal and ZSV-Infected Clones of 3Y1 Cells and REFs

Clone	Cloning efficiency in soft agar (%)	Filament organization: +	±	−
3Y1 Cells				
Uninfected (n = 1)	0	66	23	11
3Y-T (n = 2)	62.8 ± 5.7	6	7	87
REFs				
Uninfected (n = 1)	0	79	13	8
RF-MT (n = 4)	0.9 ± 0.4	76	13	11
RF-TT (n = 4)	54.6 ± 6.3	6	12	82

F-Actin network was scored as highly organized (+, >>20 filaments/cell), poorly organized (±, 5-20 filaments/cell), and disorganized (−, <5 filaments/cell). At least 400 cells were examined in each experiment (n indicates number of experiments) and values are means and indicate % of cells falling into these categories. Clones were grouped according to their cloning efficiency in soft agar. All REF clones were morphologically transformed.

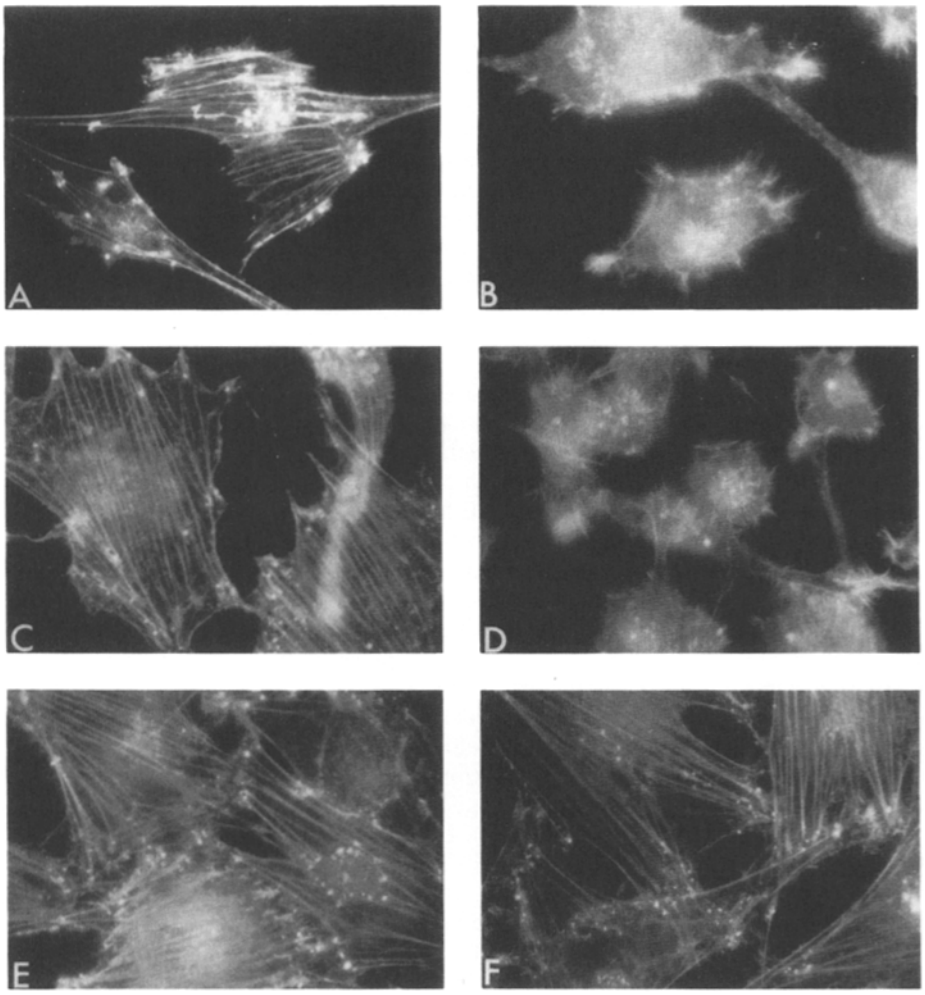

Figure 5. Actin filament organization in 3Y1 cells (*A* and *B*) and REFs (*C, D, E,* and *F*) before (*A* and *C*) and after (*B, D, E,* and *F*) ZSV infection. Clones shown here are the same as those shown in Fig. 1. ZSV-REF clones shown in *E* and *F*, which are morphologically transformed but not converted to anchorage-independent growth, exhibit a highly organized actin filament network as in normal REFs (*C*). Clones that grew efficiently in soft agar (*B* and *D*) contain actin cables nearly entirely disrupted. Original magnification ×100.

3Y1 cells and REFs, actin bundles were highly organized. Conversely, the actin filament network was essentially lost in 3Y1 cells transformed by ZSV. Similarly, ZSV-REF clones which displayed anchorage-independent growth showed very poor or nearly absent actin filament organization. Interestingly, essentially no filament disruption was observed in ZSV-REF clones which were morphologically transformed but failed to grow in soft agar. In these cells the degree of actin filament assembly was indistinguishable from that observed in parental REFs.

Discussion

The objective of the present studies was to determine whether nonestablished rodent cells can be efficiently transformed by a single oncogene. To this end, we have infected REFs with a replication-defective retrovirus containing the v-*src* gene driven by the LTR of the Moloney leukemia virus in order to induce a uniform and abundant expression of $p60^{v\text{-}src}$. For comparison, we have used the established rat 3Y1 cells. When transformation was monitored by the focus assay, a considerable difference was observed between 3Y1 cells and REFs. With the latter, the number of foci averaged only 6% of those observed with 3Y1 cells. On the other hand, when G418 selection was adopted to eliminate non-infected cells, the number of morphologically transformed colonies in REF plates was much higher than the number of foci produced by the same ZSV titer. The efficiency of ZSV to morphologically transform REFs in presence of G418 selection was still lower when compared to 3Y1 cells. Yet, the difference between the results from the focus assay and the G418 selection protocol was considerable and similar in all three infection experiments, thus suggesting that technical artifacts were not at the basis of the divergent results. These findings are in keeping with those previously obtained with the *ras* (33, 35) and v-*src* (17, 35) oncogenes and indicate that, (*a*) compared to the 3Y1 cell line, REFs are resistant to transformation by $p60^{v\text{-}src}$ when assessed by the focus assay, and (*b*) more efficient REF transformation is apparently achieved when adjacent normal cells are eliminated by G418 selection. As discussed below, however, this latter conclusion is incorrect since it does not truly involve a higher susceptibility of REFs to $p60^{v\text{-}src}$ transforming ability.

To clarify the significance of the different susceptibilities of 3Y1 cells and REFs to ZSV transformation, we isolated several ZSV-infected REF and 3Y1 colonies after selection in G418 medium, and studied a number of biological properties relevant to the process of oncogenic transformation. We first assessed whether the morphologically transformed clones were converted to anchorage-independent growth, and, with much surprise, found that while 100% of 3Y1 clones grew highly efficiently in soft agar, only 25% of the

REF clones did so. The failure of ZSV to induce anchorage-independent growth in most REF clones clearly reflected an intrinsic property of REFs. First of all, the expression and intracellular distribution of $p60^{v\text{-}src}$, and its protein tyrosine kinase activity in vitro and in vivo were comparable in all REF clones tested, regardless of their ability to grow in soft agar. Second, although it is known that a mutation in $p60^{v\text{-}src}$ prevents its transforming ability (11, 28, 30, 39), it is highly unlikely that a mutation in the majority of v-*src* gene and/or in the genome of selected REFs occurred as to induce resistance to anchorage-independent transformation. And, since several ZSV-REF colonies were anchorage-independent in growth, the same argument can be raised to rule out a mutational event facilitating conversion to the fully transformed phenotype. Finally, ZSV infection of normal REF clones demonstrated that only 2 out of 6 clones examined were permissive for anchorage-independent growth. Altogether, these results indicate that heterogeneity of REFs is responsible for their different susceptibility to $p60^{v\text{-}src}$ conversion to anchorage-independent growth. At least, more than 50% of REFs are morphologically transformable upon infection by ZSV, but only a small fraction of the REF population can be converted to the fully transformed phenotype.

As mentioned above, previous studies have shown that, under selection conditions, a *ras* oncogene alone can fully convert primary cultures to tumorigenicity, thus suggesting that elimination of adjacent normal cells may be important in *ras* transformation of nonestablished cells (33, 45). Our data showing that REFs are heterogeneous in response to v-*src*-induced anchorage-independent growth provide a different view on the role of normal REF cells in focus formation. Since the majority of ZSV-infected REF cells are not permissive for anchorage-independent growth and grow in liquid medium at a rate slower than that of parental REFs, these infected cells cannot produce visible foci in the presence of outgrowing normal REFs. On the other hand, all ZSV-infected REF cells are immortalized by $p60^{v\text{-}src}$, hence they can expand and be detected as G418 resistant colonies when adjacent parental REFs are eliminated. Therefore, our results do not support the notion that G418 selection plays a direct role in $p60^{v\text{-}src}$ transformation of REFs.

The heterogeneous response of REFs to anchorage-independent transformation by $p60^{v\text{-}src}$ raises the intriguing question as to why some primary cells are susceptible to this effect while others are resistant. Two possible explanations can be considered. Either the permissive cells express appropriate levels of a $p60^{v\text{-}src}$ target required for their conversion, or the nonpermissive ones exhibit a factor suppressing this effect. The results from the cell fusion experiment support the latter possibility, for the nonpermissive phenotype proved to be dominant in the hybrid cells. And, since no difference in $p60^{v\text{-}src}$ expression was observed between the two parental clones, this finding suggests that some REFs exhibit a factor that suppresses the ability of $p60^{v\text{-}src}$ to induce the fully transformed phenotype at the posttranscriptional level. Such a biochemical heterogeneity may stem either from differences in histogenesis of REFs or from the maturation gradient which emerges during embryonic development.

The property of REFs to exhibit different susceptibilities to $p60^{v\text{-}src}$ conversion to anchorage-independent growth is of particular interest since it may lend itself to obtaining new insights into this process. Obviously, studies with normal REF clones could provide the most direct information, but the limited life span of the primary cells prevented a thorough study in this direction. Nevertheless, the information obtained from the present studies suggests that the rate of cell division may play a role in $p60^{v\text{-}src}$ conversion to the fully transformed phenotype. First of all, the two normal REF clones which were converted to anchorage-independent growth by ZSV shared a higher growth rate than the nonpermissive ones. Second, all ZSV-infected REF clones which were capable of efficient growth in soft agar displayed shorter doubling times than the nonconverted counterparts. Third, at each FCS concentration, ZSV-REF clones that efficiently grew in soft agar exhibited growth rates similar to those of transformed 3Y1 clones, whereas ZSV-REF clones that were morphologically transformed but not anchorage independent displayed growth rates even lower than that of the parental cells. Finally, all hybrid cell clones were not converted to anchorage-independent growth and exhibited growth rates comparable to that of the nonpermissive parental clone. Altogether, these observations support the hypothesis that a mechanism leading to the phenotype of a rapid cell growth may be related to the capacity of $p60^{v\text{-}src}$ to transform to anchorage-independent growth. Suppression of the fully transformed phenotype may therefore be related to the ability of such a factor to negatively regulate REF growth.

$p60^{v\text{-}src}$ is a strong protein tyrosine kinase which phosphorylates a large number of proteins both in vivo and in vitro (19, 29, 39). However, the key target(s) and pathway(s) involved in $p60^{v\text{-}src}$ tumorigenic transformation still remain to be identified. In the present investigation, we have exploited the property of ZSV-infected REFs to exhibit varying growth ability in soft agar in order to obtain an insight into the mechanism leading to or suppressing the fully transformed phenotype. We thus studied the expression and/or phosphorylation level of a number of proteins including p125 focal adhesion kinase, paxillin, α-actinin, β_1 integrin, and mitogen-associated kinase, but found no substantial differences among ZSV-REF clones that exhibited varying growth ability in soft agar (data not shown). We also studied the organization of actin filament network since it is well known that cells lose their actin stress fibers upon transformation by various oncogenes including v-*src* (6, 8, 38). We found that REF clones that were morphologically transformed by ZSV displayed considerable differences in actin filament assembly, whereas previous studies have suggested that such a filament derangement is an early event directly related to morphologic transformation (6, 8, 20, 44). Conversely, an inverse relationship existed between actin filament organization and REF growth in soft agar, thus indicating that at least in REFs, loss of actin filament network is expressive of the fully transformed phenotype. Whether such a cytoskeleton change is a casual event remains to be established. However, it is known that $p60^{v\text{-}src}$ localizes on cytoskeletal structures (7, 18), and a large number of proteins which are phosphorylated by $p60^{v\text{-}src}$ are an integral part of the cytoskeleton (13, 31, 43, 46, 48). Thus, it is reasonable to postulate that changes in actin filament organization play a direct role in $p60^{v\text{-}src}$ conversion to anchorage-independent growth.

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