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

Nicola Tavoloni, Hirokazu Inoue, Hisataka Sabe, and Hidesaburo Hanafusa
Laboratory of Molecular Oncology, The Rockefeller University, New York, New York 10021

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\(^{\text{src}}\), 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\(^{\text{src}}\) reflects the heterogeneity of the primary cultures. REFs can be morphologically transformed by p60\(^{\text{src}}\) 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), 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\(^{\text{ras}}\) 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-
sis, therefore, is still widely accepted and the multistage nature of cancer remains to be challenged.

p60^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^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 cultures transfected 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 p60^src gene, into the V2 packaging cell line as previously described (26). REFs were transfected with 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 cultures 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.

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 V2 packaging cell line as previously described (26). REFs were transfected with 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 cultures 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 V2 packaging cell line as previously described (26). REFs were transfected with 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 cultures 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.

Cloning of REFs

REFs which were grown for ~48 h (2-3 divisions) and stored, were reinfected 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 semi-clonality. Each clone was then infected with ZSV (~10^5 fpi) 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 x 10^3 cells were plated in upper layer of 0.5% Bacto-Agar (Difco Laboratories, Detroit, MI) in DMEM containing 10% bovine calf serum. 5 x 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^src and the organization of actin cables. Cells (2 x 10^4) were incubated for 30 rain with 250 pl of a phalloidin-FITC solution (Molecular Probes Inc., Eugene, OR) as previously described (49). Cultures 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-phenylenediamine solution in PBS and inspected under a fluorescent microscope. For p60*~ kinase activity, cells were prepared, washed, fixed, and permeabilized as above. Anti p60*~ antibodies were then added at 1:25-250 dilutions and cultured 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*~ Kinase Activity

The procedure for determining p60*~ kinase activity in vitro was essentially that described by Sabe et al. (41). Cells (~10^6/cm dish) were lysed with RIP 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 Na2MoO4; 1 mM PMSF; 100 Kallikrein inactivator units of aprotinin/ml (1%); 20 μg/ml leupeptin; 1 mM Na3VO4; and 5 mM benzamidine], and lysate containing 20-50 μg of total protein was immunoprecipitated in 0.5 ml of RIPA buffer with ~1/μl of monoclonal antibody against p60*~. 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 Na3VO4; 5 mM MgCl2; 5 μg of acid-denatured enolase; and 10 μCi of [γ-32p]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; 10 mM EDTA; 2 mM Na3VO4; 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*~. Lysates were prepared using RIP buffer described above and SDS-PAGE was performed either after loading whole cell lysate (20-40 μg total protein) onto gel or after immunoprecipitation of the desired protein from cell lysate (50-300 μg total protein) with the specific antibody. Separated proteins were then transferred onto a membrane (Millipore, Bedford, MA) which was blocked for 30 min with 5% 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 x 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 colonies." 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.

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(II)) 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 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.

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(II)) 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...
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 × 10⁴) 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 x 10^6 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).

Expression, Kinase Activity, and Intracellular Distribution

All 3Y1 clones that were morphologically transformed and efficiently grew in soft agar displayed high levels of p60^src expression and p60^src kinase activity in vitro. 3Y1 clones that were not morphologically transformed failed to do so, as did uninfected 3Y1 cells. Similarly, p60^src expression and p60^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^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^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).

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 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 3. p60^src kinase activity in vitro (A), p60^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^src antibodies. Autophosphorylated p60^src is also shown. p60^src expression and tyrosine-phosphorylated proteins were determined by probing Western blots of whole cell lysates with anti-p60^src- and anti-phosphotyrosine antibodies, respectively. Note the lack of differences among ZSV-REF clones, regardless of their ability to grow in soft agar.
Table V. Morphologic Traits and Growth Properties of Parental and Hybrid Cells

| Clone       | Cell morphology | Doubling time (h) | Cloning efficiency |
|-------------|-----------------|-------------------|--------------------|
| Parental    |                 |                   |                    |
| RF-MT2      | A               | 11.4              | 64.8               |
| RF-TT3      | B               | 26.2              | 0.9                |
| Hybrid      |                 |                   |                    |
| RF-H1       | B               | 21.4              | 1.7                |
| RF-H2       | B               | 25.3              | 0.7                |
| RF-H3       | B               | 19.8              | 1.5                |
| RF-H4       | B               | 26.7              | 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 × 10³) that formed colonies >0.5 cm in diameter 21 d after inoculation.

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.

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 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 × 10³ 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 VI. F-Actin Cable Organization in Normal and ZSV-Infected Clones of 3Y1 Cells and REFs

| Clone      | Filament organization | Cloning efficiency in soft agar (%) |
|------------|-----------------------|------------------------------------|
| 3Y1 Cells  |                       |                                    |
| Uninfected (n = 1) | 0                  | 66                                 |
| 3Y-T (n = 2)       | 62.8 ± 5.7           | 6                                  |
| REFs         |                       |                                    |
| Uninfected (n = 1) | 0                  | 79                                 |
| RF-MT (n = 4)      | 0.9 ± 0.4            | 76                                 |
| RF-TT (n = 4)      | 54.6 ± 6.3           | 6                                  |

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 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.

Figure 5. Organization of F-Actin Cables

Three representative ZSV-REF clones are shown with their respective parental cells. As described in Materials and Methods, 3Y1 cells do not develop stress fibers like REFs. Cloning efficiency in soft agar for these clones is as follows: 3Y1, 4%; 3Y-T, 23%; RF-MT, 66%; RF-TT, 79%. The results from these studies are summarized in Table VI. In both parental...
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\textsuperscript{v-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\textsuperscript{v-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\textsuperscript{v-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^{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^{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^{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^{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^{src} transformation of REFs.

The heterogeneous response of REFs to anchorage-independent transformation by p60^{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^{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^{src} expression was observed between the two parental clones, this finding suggests that some REFs exhibit a factor that suppresses the ability of p60^{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^{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^{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^{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^{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^{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 p25 focal adhesion kinase, paxillin, p-class actinin, V third 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 which since is well known that cells lose their actin stress fibers upon transformation by various oncogenes including the src gene family (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^{src} localizes on cytoskeletal structures (7, 18), and a large number of proteins which are phosphorylated by p60^{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^{src} conversion to anchorage-independent growth.

We wish to express our gratitude to J. Brugge for her generous gift of antibodies against p60^{src}, to H. Greulich, A. Hata, and G. Scholz for their support, and to Drs. Y. Yamada, G. Hynes, and J. Brugge for their helpful discussions.
References

1. Atkins, B., A. Leutz, and T. Graf. 1984. Autocrine growth induced by src-related oncoproteins in transformed chicken myeloid cells. Cell. 39:439-445.

2. Armentano, S. F., T., and D. Boettiger. 1985. Myc and src oncoproteins have complementary effects on cell proliferation and expression of specific extracellular matrix components in defined chondroblasts. Mol. Cell. Biol. 5:538-544.

3. Anthony, P. B. 1976. Precursor lesions for liver cancer in humans. Cancer Res. 36:2379-2583.

4. Balmain, A., M. Ramden, G. T. Bowden, and J. Smith. 1984. Activation of the mouse skin cellular Harvey-ras gene in chemically induced benign skin papillomas. Nature (Lond.) 307:658-660.

5. Bednar, M. J., and J. E. Varman. 1984. Functions and origins of retroviral transforming genes. In RNA Tumor Viruses. Vol. 1: R. Weiss, N. Teich, H. Varman, and J. Coffin, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 999-1108.

6. Boschek, C. B., M. J. Oshimura, and J. C. Barrett. 1985. Neo- and kidney diseases. Proc. Natl. Acad. Sci. USA. 82:4625-4628.

7. Hamaguchi, M., H. Xiao, Y. Uehara, Y. Onishi, and Y. Nagal. 1993. Hereditary tyrosinemia in pigs: Identification of novel mutations in the phenylalanine hydroxylase gene. Proc. Natl. Acad. Sci. USA. 77:4142-4146.

8. Tavoloni, E. A., B. A. Birgel, and C. Basil. 1988. Cell transformation by the viral src gene. Annu. Rev. Cell Biol. 3:531-56.

9. Kamps, M. P., B. M. Sefton. 1988. Identification of multiple novel polypeptide substrates of the src-v, v-ras, v-fos, v-ros, and v-hev oncogenes. J. Cell Biol. 129:903-914.

10. Houghta, E., M. Avrin, and L. C. Andersson. 1993. Polymyomas are essential for cell transformation by p60src. delineation of two necrotic events relevant for the transformation. Oncogene. J. Cell Biol. 122:903-914.

11. Houghta, T. 1991. Cooperation between oncogenes. Cell. 64:249-270.

12. Inoue, H., M. Isaka, S. I. Takeda, and A. Hakura. 1991. Simple system for isolation of cellular and viral mutants for transformation by retrovirus. J. Med. Virol. 44:256-249.

13. Inoue, H., M. K. Owada, Y. Matsu, and A. Hakura. 1989. A rat mutant cell line showing temperature-dependent phenotypes with functional expression of the src gene product. Virology. 168:57-66.

14. Jove, R., and H. Hanafusa. 1987. Cell transformation by the viral src gene. Annu. Rev. Cell Biol. 3:531-56.

15. MacAuley, A., and T. Dawson. 1988. Cooperative transforming activities of src, v-src, and src v-onc proteins in nontransduced rat adrenergic cells. J. Virol. 62:4712-4721.

16. Marshall, C. J. 1991. Tumor suppressor genes. Cell. 64:313-326.

17. Morgenstern, J., J. W. Eng. and L. H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18:357-359.

18. Nigg, E. A., B. E. H. Serfson, S. J. Singler, and P. K. Vogt. 1993. Cytoskeletal organization, vinculin-phosphorylation, and fibroblast organization in transformed fibroblasts with different cell morphology. Virology. 151:10-35.

19. Parsons, J. M., and J. M. Weber. 1989. Genetics of src: structure and functional organization of a protein tyrosine kinase. Curr. Top. Microbiol. Immunol. 147:79-127.

20. Ruley, H. E. 1983. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (Lond.) 304:602-606.

21. Sable, H., M. Okada, H. Nakagawa, and H. Hanafusa. 1992. Activation of c-Src in cells bearing v-Crk and its suppression by Csk. Mol. Cell. Biol. 11:609-616.

22. Schaller, M. D., C. A. Borgman, B. J. Cobb, R. Vines, A. B. Reynolds, and J. T. Parsons. 1992. srcp25AK, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. Proc. Natl. Acad. Sci. USA. 89:5192-5196.

23. Seidel-Dugan, C., B. E. Meyer, S. M. Thomas, and J. S. Brugge. 1992. Effects of SH- and SH+-deletions on the functional activities of wild-type and transforming variants of c-src. Mol. Cell. Biol. 12:1835-1845.

24. Spandolis, D. A., and N. M. Wilkie. 1984. Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature (Lond.) 310:469-475.

25. Turner, C. E., R. D. Glimson, and K. Burridge. 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. J. Cell Biol. 111:1099-1106.

26. Wistner, M. A., P. El lers, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell. 14:725-731.

27. Wu, H., and J. T. Parsons. 1993. Cortactin, an 80/85-kilodalton p60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. J. Cell Biol. 120:1471-1476.

28. Wulf, E., A. Deboren, F. A. Bant, H. Falsite, and T. Wieland. 1979. Flow cytometric flouroscopy, a tool for the visualization of cellular actin. Proc. Natl. Acad. Sci. USA. 76:4498-4502.