Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 4355–4359, June 1988
Cell Biology

Oncogene v-*src* transforms and establishes embryonic rodent fibroblasts but not diploid human fibroblasts

(retroviral vectors/tumorigenesis/carcinogenesis/protein-tyrosine kinase)

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Contributed by J. Michael Bishop, February 22, 1988

ABSTRACT **The conversion of cells from a normal phenotype to full malignancy apparently requires multiple genetic events. Efforts to reconstruct multistep tumorigenesis in cell culture have shown that two types of oncogenes (typified by *HRAS* and *MYC*) can cooperate to elicit complete transformation. Transformation of embryonic rodent cells by single oncogenes is reputed either not to occur or to require specialized circumstances. It has not been known how the large group of oncogenes that encode protein-tyrosine kinases might fit into this scheme. We now report that v-*src*, a prototype for the kinase oncogenes, can convert rat embryo fibroblasts to a fully transformed and tumorigenic phenotype when the gene is expressed vigorously. By contrast, v-*src* had no demonstrable effect on diploid human fibroblasts. Our results sustain the view that it is possible for at least some oncogenes to achieve a potency sufficient for unilateral tumorigenesis.**

Mutations, amplifications, and rearrangements of cellular protooncogenes that create potential oncogenes have been found in a wide variety of tumors (1, 2). In an attempt to implicate these changes in tumorigenesis, various protooncogenes and oncogenes have been tested for their ability to transform primary cultures of embryonic rodent cells. None of the genes tested to date readily elicit full transformation, defined here as a combination of establishment, morphological changes, growth in semisolid medium, and tumorigenicity in experimental animals. Some oncogenes (typified by *MYC*) characteristically elicit established growth of cells that are only partially transformed (3–5), whereas others (typified by *RAS*) transform cells but fail to sustain their growth unless the oncogene product is expressed in exceptional quantities and the cells are propagated in the absence of untransformed cells (6, 7). It has been suggested that these distinctions might reflect different sites of action by the products of the oncogenes, with nuclear products serving primarily to establish growth and cytoplasmic products eliciting the remainder of the transformed phenotype (4). The suggestion has been reinforced by demonstrations that pairs of oncogenes encoding nuclear and cytoplasmic proteins can cooperate to elicit tumorigenic transformation (3, 4, 8). It has not been clear how the large group of oncogenes that encode protein-tyrosine kinases might fit into the scheme of cooperative transformation. To address this question, we have evaluated the ability of the retroviral oncogene v-*src* to transform embryonic rat cells, in comparison to the activity of a mutant and transforming allele of *HRAS* known as EJ-*RAS* (9) and a normal human allele of the protooncogene *MYC* (10).

MATERIALS AND METHODS

Plasmids and Cells. The retroviral expression vector pZAS was constructed by inserting the v-*src* allele of the Schmidt–Ruppin strain of Rous sarcoma virus into the *gpt* vector pZIPVGPT (gift of R. Mulligan, Massachusetts Institute of Technology). The pZAS construct was provided by J. Kaplan (University of California, San Francisco). The EJ-*RAS* construct pEJHSG was prepared by inserting the 6.6-kilobase *Bam*HI *HRAS* fragment of pEJ (a gift from R. Weinberg, Massachusetts Institute of Technology) into the hygromycin-resistance (*hmr*) expression vector pHMR272 (a gift from W. Rowekamp) (11). The pMLVLTRDMmyc construct was prepared as described (10). This construct contains the bacterial neomycin-resistance (*neo*) gene, which makes mammalian cells resistant to the antibiotic G418. The human skin fibroblast cultures GM0537, GM0622, and GM2332 were obtained from the Genetic Mutant Cell Repository (Camden, NJ) of the National Institutes of Health.

Transfections and Infections. For transfections, 5 μg of each plasmid DNA was coprecipitated with calcium phosphate and introduced into cultures of rat embryo fibroblasts (REFs) (3). Precipitates were removed after 8 hr; 48 hr later, cells were dispersed and replated at 4:1 dilution. Half of the plates were subjected to selection with the appropriate cytotoxic protocol (see below), while other plates were allowed to grow as monolayers. Three weeks later, monolayers were scored for focus formation.

For infections, the amphotropic producer cell line PA317 (12) was transfected as above with the retroviral constructs pZAS or pZIPVGPT. Clones of *gpt*-positive cells were tested for virus production by titration on Rat-1 cells. Both mycophenolic acid resistance and focus formation were scored. Two to three thousand focus-forming units were used for experiments in which human skin fibroblasts were infected.

Cytotoxic Selections. Selection for cells transfected with *neo*, *hmr*, or *gpt* were carried out in medium containing crude G418 (500 μg/ml), hygromycin (200 μg/ml), or mycophenolic acid (20 μg/ml), respectively. Plates transfected with two plasmids were subjected to selection for both markers. Hypoxanthine/aminopterin/thymidine (HAT) medium was as described (13).

Growth in Soft Agarose and Tumorigenesis. For assays in agarose, cultures of transfected or infected cells were expanded and cells were plated onto 60-mm dishes (10^4 cells per dish) in 0.7% low-melting-point agarose. Plates were scored for colonies after 14 days. For assays of tumorigenesis, each clone or pool of clones was injected subcutaneously into the flanks of 4- to 6-week-old athymic nude (BALB/c *nu/nu*) mice (10^6 cells per site). Mice were observed at approximately 3- to 5-day intervals for development of tumors.

Protein Kinase Assays and Immunoprecipitations. Single foci, antibiotic-selected single colonies, or pools of 50–200

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Abbreviations: HAT, hypoxanthine/aminopterin/thymidine; REF, rat embryo fibroblast.

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colonies of cells were expanded and used to prepare cytoplasmic extracts. Extracts were normalized for protein content (14) and the v-*src* gene product and pp60^{v-src} was precipitated with antibody from tumor-bearing rats as described (15, 16). After incubation with [γ-^{32}P]ATP, the labeled immunoglobulin heavy chain was resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis and detected by autoradiography.

Labeling and immunoprecipitation of pp64^{c-myc} and p21^{ras} were performed according to published methods (17, 18).

RESULTS

Transformation of REF Cells by v-*src*. We transfected v-*src* alone (in the form of pZAS) or in combination with either *MYC* (pMLVLTRDMmyc) or a mutant allele of *HRAS* (pEJ-HSG) into cultured REFs (3). The cells were then propagated in the absence or presence of an independent selection. After 3 weeks, plates grown without selection were scored for the presence of foci of morphologically transformed cells. We found that transfection with pZAS, either alone or in combination with pEJ-HSG or pMLVLTRDMmyc, readily produced foci, whereas mock-transfected control plates had no foci (Table 1). The plates that were cotransfected with pEJ-HSG or pMLVLTRDMmyc did not have more foci than plates transfected with pZAS alone (Table 1). By contrast, the combination of EJ-*RAS* and *MYC* appeared more potent for transformation (Table 1).

The finding that v-*src* alone was sufficient to produce foci in the REF assay prompted us to determine whether the pZAS transfectants displayed other properties of transformed cells. Fig. 1 describes the morphology of several pZAS foci after expansion to confluence. The foci ranged in appearance from floridly transformed (round, clumped, and very refractile) to only slightly disorganized and refractile. All clones tested were capable of growth in soft agarose, and all clones elicited tumors when injected into nude mice (Table 2). We also found that REF cells that were transfected with pZAS and subjected to selection for the linked marker *gpt* (rather than for morphological transformation) would grow in soft agarose and form tumors in nude mice. Some of these tumorigenic clones were morphologically transformed, but others were not (data not shown).

Indefinite Growth of Cells Transformed by v-*src*. Previous studies have determined that *MYC* and the related gene *NMYC* can readily convert primary REFs into established and apparently immortalized cell lines without ancillary manipulations (refs. 3 and 5; M. Schwab and J.M.B., unpublished observations), whereas EJ-*RAS* can establish REFs only when coselection with antibiotic markers is used (6, 7). We tested six REF foci induced by v-*src* in the absence of coselection and found that all gave rise to established lines. These lines were passaged twice per week at dilutions of 1:10 for 4 months without any signs of senescence. Normal REFs never survived a passage schedule of this sort.

The v-*src* Product Is Expressed in Transformants. We used immunoprecipitation to demonstrate the expression of v-*src* in transfected cells. Confluent 60-mm dishes of clones elicited by transformation with v-*src* were analyzed with an immune-complex kinase assay, in which the v-*src*-encoded protein (pp60^{v-src}) is precipitated with antibody and the immune complex is then incubated with [γ-^{32}P]ATP (15, 16, 19). The v-*src* protein catalyzes transfer of labeled phosphate to the IgG heavy chain at tyrosine. Fig. 2*A* shows that pZAS foci had pp60^{v-src} kinase activity, as did pZAS-transfected clones selected for *gpt* expression (single colonies or pools) and cotransfectants that had received either pZAS/pMLVLTRDMmyc or pZAS/pEJ-HSG. By contrast, parental REF cells had no detectable *src*-specific kinase activity; the low level of kinase activity expected from the cellular *src* product (20–23) was not detectable by the antiserum we used. In addition, cells cotransfected with pZAS and either pMLVLTRDMmyc or pEJ-HSG vectors produced pp64^{c-myc} or p21^{ras}, respectively (Fig. 2 *B* and *C*).

Table 1. Focus formation by v-*src* alone and in concert with EJ-*RAS* or *MYC*

Transfected gene(s)	Foci per plate	Focus morphology
v-*src*	60	Variable (see text)
v-*src* + EJ-*RAS*	70	Round and refractile
v-*src* + *MYC*	55	Round and refractile
EJ-*RAS* + *MYC*	220	Round and refractile
None (mock)	0	Not applicable

Effect of Cotransfection of v-*src* with *HRAS* or *MYC*. Combining v-*src* with either *MYC* or EJ-RAS had only subtle consequences. In particular, all of the transformants obtained with a combination of pZAS and pMLVLTRDMmyc or pZAS and pEJ-HSG appeared floridly transformed by morphological criteria, in contrast to the diversity of foci produced by pZAS alone (data not shown). The cotransfectants, like pZAS transfectants, grew with high efficiency in soft agarose and produced tumors in nude mice (Table 2). All cotransfectants tested grew as established lines.

Human Fibroblasts Are Not Transformed by v-*src*. The finding that v-*src* expression is sufficient to transform and establish primary rodent cells encouraged us to test the oncogene in human diploid skin fibroblasts. The pZAS vector was particularly useful in this experiment because it can be packaged as an amphotropic retrovirus and thus introduced into human cells with high efficiency (12). We used skin fibroblasts from a patient with Lesch–Nyhan syndrome (GM0537) as recipient cells because these cells are deficient in the enzyme hypoxanthine phosphoribosyltransferase (HPRT) and will not grow in HAT medium unless an exogenous *gpt* gene (such as that found in pZAS) is provided as a substitute for the *HPRT* gene.

We produced amphotropic stocks of ZAS virus as described (12) and stocks of virus from the ZIPVGPT construct, which is identical to ZAS except that it lacks v-*src*. The ZAS virus produced foci and *gpt*-positive colonies readily on Rat-1 cells. Infection of GM0537 cells with ZAS virus produced no foci, however. Of 14 HAT-resistant GM0537 clones picked after infection with the ZAS virus, none was capable of more doublings before senescence than any of several clones produced by infection with the ZIPVGPT virus. No ZAS virus-infected human clone produced any colonies in soft agarose or any tumors (Table 2). The morphology of GM0537 cells expressing v-*src* was not significantly altered when compared with the parent cell (data not shown). Several clones produced more v-*src* kinase activity than did some of the pZAS-transfected REF clones that were fully transformed and established (Fig. 3).

We also used the ZAS virus to infect skin fibroblast cultures GM2332 and GM0622, from individuals with tuberous sclerosis and neurofibromatosis, respectively. Individuals with these conditions are known to develop mesenchymal tumors with high frequency (24, 25), suggesting that their fibroblasts might be more easily transformed than other human fibroblasts. Four GM2332 and five GM0622 gpt-positive clones were found to express pp60^{v-src} kinase activity and yet were not altered morphologically and were unable to grow in soft agarose or produce tumors in nude mice (data not shown).

DISCUSSION

Transformation of Rat Embryo Cells by v-*src*. We have found that the retroviral oncogene v-*src* can elicit full neoplastic transformation of rat embryo cells when expressed abundantly through the agency of the transcriptional promoter and enhancer of the Moloney murine leukemia virus. We have not explored the biological capabilities of v-*src* at

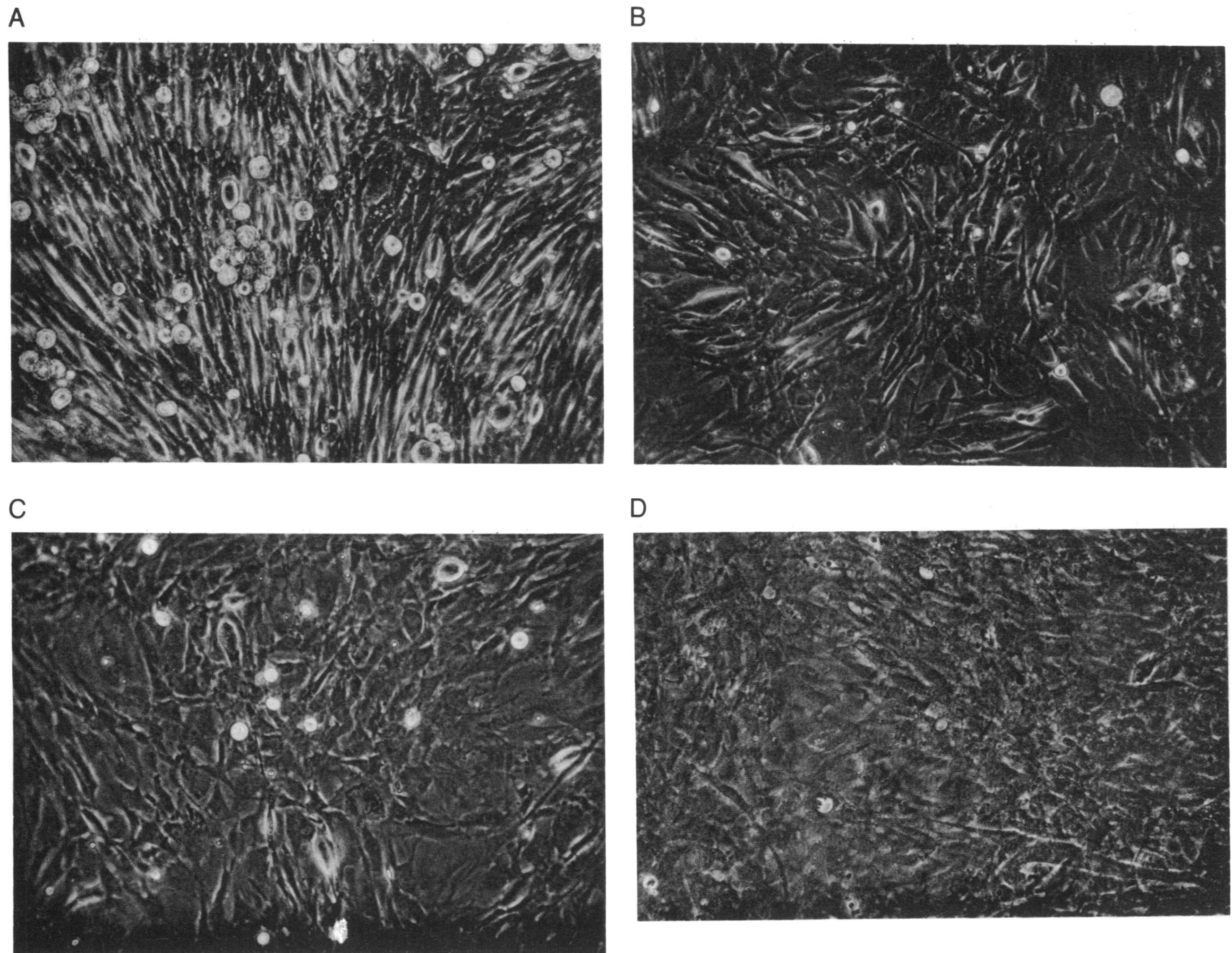

FIG. 1. Morphology of rat embryo cells transformed by v-*src*. Individual foci elicited by pZAS transfection were grown to confluence, and representative clones were photographed. (*A–C*) Three foci induced by v-*src*. (*D*) Parental REF cells.

lower levels of expression. We presume that a threshold of expression must be exceeded if transformation is to be achieved (26), and it is likely that the abundant expression utilized here is essential to full transformation, as reported for mutant alleles of *RAS* (6, 7).

Our findings conform to the well-established ability of v-*src* to transform embryonic avian cells in culture (27) and to elicit tumors in chickens (27, 28). If anything, v-*src* is more potent in rodent cells: chicken cells transformed by v-*src* retain a finite life-span rather than being converted to established growth. Embryonic rodent fibroblasts are reputed to resist transformation by oncogenes, even when the genes are expressed in abundance comparable to that achieved in the present work. For example, *MYC* elicits established growth of cells that are otherwise not transformed (refs. 3 and 5; unpublished work), and abundantly expressed mutant alleles of *HRAS* fail to sustain the growth of transformed cells unless the cells are propagated in the absence of untransformed cells (6, 7). [A normal allele of *HRAS* carried in a retroviral vector elicits morphological transformation of embryonic rat cells, but other aspects of the transformed phenotype were not evaluated (see ref. 29).] No such constraints appear to apply to v-*src*; abundant expression of the gene suffices to elicit the complete transformed phenotype in embryonic rodent cells, including tumorigenicity.

Origins of the Transforming Potential of v-*src*. The v-*src* oncogene arose by transduction of the protooncogene c-*src* (30) and now differs from its cellular progenitor by the presence of both a genetic substitution and point mutations (31). The mutations work in a combinatorial fashion to create

Table 2. Phenotypes of transfected REFs and infected human fibroblasts

Parental fibroblast	Introduced gene(s)	Growth in agarose*	Cloning efficiency in agarose,† %	Tumorigenicity‡	Tumor latency, days
Rat embryo	v-*src*	4/4	15–50	4/4	4–21
	v-*src* + EJ-*RAS*§	1/1	70	4/4	10–13
	v-*src* + *MYC*	3/3	50–80	4/4	10–25
Human	v-*src*	0/7	—	0/5	—

*Number of cultures that produced colonies in soft agarose (numerator) over the number of cultures tested (denominator).
†Percentage of tested cells that gave rise to colonies in agarose.
‡Number of cultures giving rise to a tumor (numerator) over the number of cultures tested (denominator).
§Pools of transfected clones, rather than single clones, were used to characterize cells transfected by v-*src* and EJ-*RAS*.

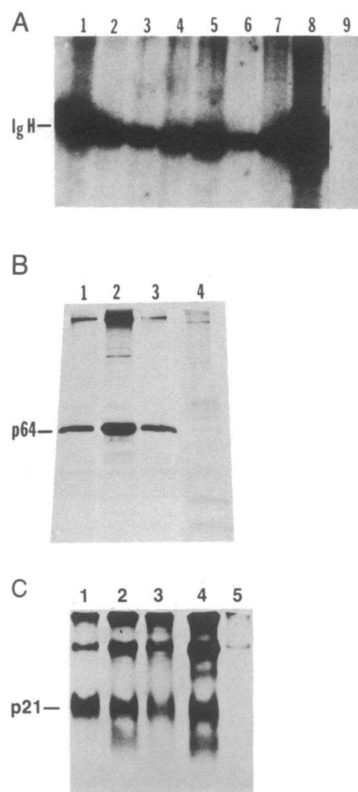

FIG. 2. Expression of oncogene products in transfected rat embryo cells. Single foci, antibiotic-selected single colonies, or pools of 50–200 colonies were analyzed for expression of various oncogene products. (*A*) Protein kinase activity of pZAS transfectants. Cytoplasmic extracts were normalized for protein content (14) and $pp60^{v\text{-}src}$ was precipitated with antibody (15, 16). After incubation with [γ-^{32}P]ATP, the labeled immunoglobulin heavy chain (IgH) was resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis and detected by autoradiography. Lanes 1 and 2, foci transfected with pZAS alone; lanes 3 and 4, foci from pZAS and pEJ-HSG; lanes 5 and 6, foci from pZAS and pMLVLTRDMmyc; lanes 7 and 8, pools of mycophenolic acid-resistant colonies produced by transfection with pZAS; lane 9, parental REF cells. (*B*) Expression of $pp64^{c\text{-}myc}$ in cells transfected with pMLVLTRDMmyc (17). Lanes 1 and 2, foci from cotransfection with pZAS and pMLVLTRDMmyc; lane 3, a pool of antibiotic-resistant colonies from the same transfection; lane 4, REF cells. (*C*) Expression of $p21^{ras}$ in cells transfected with pEJ-HSG (18). Lane 1, a pEJ-HSG/pMLVLTRDMmyc focus; lanes 2 and 3, two foci from cotransfection with pEJ-HSG and pZAS; lane 4, pool of antibiotic-resistant colonies formed by cotransfection with pEJ-HSG/pZAS; lane 5, REF cells.

the full potency of the oncogene, perhaps by deregulating and augmenting the tyrosine-specific kinase activity of the gene product (20–23). The combination of tumorigenic mutations in v-*src* may have arisen from intensive selection for rapid transformation and strong tumorigenicity over many years of experimental use. In the present work, we have imposed an additional abnormality on v-*src*: unregulated and abundant expression, similar to that resulting from natural transduction of the gene into a retrovirus (30).

It has been suggested that tumorigenic transformation of embryonic rat cells requires the cooperation of two types of oncogenes—one encoding a protein that acts in the nucleus and the other encoding a cytoplasmic protein (3, 4, 8). A caveat to this proposal has been provided by the finding that a mutant allele of the *HRAS* protooncogene can transform embryonic rat fibroblasts unilaterally if the gene is overexpressed and the transformed cells are propagated in the absence of normal cells (6, 7). The findings reported here add another caveat: the protein encoded by v-*src* appears to act at the plasma membrane and in the cytoplasm (19), yet it is

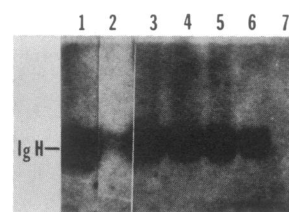

FIG. 3. Expression of v-*src* in human and rat cells. Extracts of cells were analyzed for protein-tyrosine kinase activity as in Fig. 2*A*. Lanes 1 and 2, extracts from two REF foci elicited by pZAS; lanes 3–6, four HAT-resistant GM0537 clones that were produced by infection with the ZAS virus; lane 7, parental GM0537 cells.

sufficiently potent to fully transform rat embryo fibroblasts. Moreover, the transformed cells grew well in the presence of normal cells, in contrast to results obtained with *RAS* (6, 7). We do not know whether the same will be true for any of the other oncogenes encoding protein kinases. We acknowledge that tumorigenesis typically requires abnormalities of more than one genetic function (1). Nevertheless, our findings sustain the view that at least some oncogenes can acquire potency sufficient for unilateral tumorigenesis, by accumulating multiple mutations whose effects are combinatorial.

Human Cells Resist Transformation by v-*src*. Although vigorously expressed in diploid human cells, the v-*src* oncogene had no demonstrable effect on their phenotype. These findings are in accord with the failure of previous efforts to transform diploid human cells with the EJ-*RAS* oncogene (32). Human cells in culture characteristically resist transformation by chemical, physical, or biological agents (33, 34). The mechanism of this resistance has not been found, although karyotypic stability, efficient repair of DNA, and dominant trans-acting inhibitors of transformation have all been invoked as possible explanations (32–34). Since the product of v-*src* elicits no permanent change in the genome of the cell, we presume that the resistance of human cells to transformation by the oncogene cannot be attributed to exceptional stability of the human genome. Instead, it seems possible that human cells lack the substrates whose phosphorylation accounts for the transformation of avian and rodent cells by v-*src* (or contain comparable substrates that are not recognized by the v-*src* product). The resistance could arise from the fact that the rodent and avian cells used for transformation by v-*src* originated from embryos, whereas the diploid human fibroblasts used here and in other studies with oncogenes were derived from differentiated tissue (generally skin). Further exploration of cellular resistance to the action of v-*src* may provide insight into the biochemical mechanisms of tumorigenesis.

We thank J. Kaplan, R. Weinberg, W. M. F. Lee, W. Rowekamp, and R. C. Mulligan for providing plasmids used in this study. We are also grateful to C. Dang, R. Ralston, and E. Scolnick for providing antibodies to the products of *MYC*, v-*src*, and *HRAS*, respectively. Work summarized here was supported by National Institutes of Health Grants CA12705 and CA44338 and by funds from the G. W. Hooper Research Foundation. B.H. was supported by funds from National Cancer Institute Grant 09463.

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