MOLECULAR AND CELLULAR BIOLOGY, Aug. 1990, p. 4155–4162
0270-7306/90/084155-08$02.00/0
Copyright © 1990, American Society for Microbiology
Vol. 10, No. 8

A Swiss 3T3 Variant Cell Line Resistant to the Effects of Tumor Promoters Cannot Be Transformed by *src*

MUKUND NORI, LAURA K. SHAWVER,† AND MICHAEL J. WEBER*

Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received 20 March 1990/Accepted 24 May 1990

To study the relationship between oncogenesis by v-*src* and normal cellular signalling pathways, we determined the effects of v-*src* on 3T3-TNR9 cells, a Swiss 3T3 variant which does not respond mitogenically to tumor promoters such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). We found that *src* was unable to transform these variant cells, whether the oncogene was introduced by infection with a murine retrovirus vector or by transfection with plasmid DNA. 3T3-TNR9 cells were not inherently resistant to transformation, since infection with similar recombinant retroviruses containing either v-*ras* or v-*abl* did induce transformation. Further analysis of Swiss 3T3 and 3T3-TNR9 cell populations infected with the v-*src*-containing retrovirus revealed that although the amount of v-*src* DNA in each was approximately the same, the level of the v-*src* message and protein and the overall level of phosphotyrosine expressed in the infected variants was much less than in infected parental cells. Cotransfection experiments using separate v-*src* and *neo* plasmids revealed a decrease in the number of G418-resistant colonies when transfections of TNR9 cells occurred in the presence of the *src*-containing plasmid, suggesting a growth inhibitory effect of v-*src* on 3T3-TNR9 cells, as has also been found for TPA itself. Since v-*src* cannot transform this variant cell line, which does not respond mitogenically to the protein kinase C agonist TPA, we suggest that *src* makes use of the protein kinase C pathway as part of its signalling activities.

The *src* oncogene encodes a tyrosine-specific protein kinase, pp60^{src}, whose enzymatic activity is thought to be necessary for transformation (12, 24, 38, 42). Cells in which pp60^{src} is expressed exhibit a very large number of tyrosyl-phosphorylated proteins (13, 34, 38, 49), and this has made it extremely difficult to determine which of these phosphorylations is (are) functionally significant with respect to signal transduction and transformation (reviewed in references 38 and 49). Hence, although the *src* oncogene has been studied in detail for many years, the biochemical steps by which expression of *src* modifies cellular growth control mechanisms are still unclear.

Genetic approaches have often been useful in the analysis of complex biochemical pathways. Since oncogenes appear to be transduced elements of the normal machinery of growth control and subvert that machinery to induce abnormal growth and metabolism, it seemed to us that cellular variants which are altered in their responses to normal mitogenic stimuli might also be altered in their response to some oncogenes. Analysis of such variants could elucidate the signalling pathways used by various oncogenes.

In this study, we examined the effects of v-*src* on 3T3-TNR9 cells. TNR9 cells were derived from Swiss 3T3 cells on the basis of the inability to respond mitogenically to the tumor promoter 12-*O*-tetradecanoyl-13-acetate (TPA) (8), which is an analog of the normal signalling intermediate, diacylglycerol (4, 15, 16, 18, 21). 3T3-TNR9 cells contain normal levels of active protein kinase C (PKC) and respond to TPA stimulation like parental 3T3 cells with respect to most of the measured responses, including phosphorylation of p80 (5; but see reference 3), production of arachidonate metabolites, and elevation of glucose transport (9, 10). However, TPA does not induce activation of pp42-mitogen-activated protein (MAP) kinase, S6 kinase, ornithine decarboxylase, or DNA synthesis in these cells (9, 19; G. L'Allemain, T. W. Sturgill, and M. J. Weber, submitted for publication). It has been reported recently that PKC is defectively down regulated in 3T3-TNR9 cells in response to long-term treatment with TPA (3). Thus, the defect in 3T3-TNR9 cells appears to reside in some early component of the tumor promoter-diacylglycerol signal transduction pathway.

Infection of 3T3-TNR9 cells with the v-*src* oncogene did not result in transformation, whereas infection with either of two other oncogenes, v-*ras* and v-*abl*, did. This refractoriness to *src* was not due to inability of the cells to take up *src* DNA, but rather appeared to be due, at least in part, to v-*src*-induced growth inhibition of these cells. Apparently, the lesion that renders these cells unable to respond mitogenically to TPA also makes them incapable of being transformed by v-*src*, implying that v-*src* activates the PKC signal transduction pathway.

MATERIALS AND METHODS

Cell culture. Swiss 3T3 and 3T3-TNR9 cells were obtained from H. Herschman (8) and grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (GIBCO Laboratories). Virus-producing cell lines were routinely maintained in the same medium with addition of 750 μg of G418 per ml. Cell lines that produce viruses containing no oncogenes or v-*src* were made in our laboratory. Cell lines producing viruses containing v-*myc* or v-*ras* were obtained from K. B. Marcu (46; personal communication).

Immunological reagents. EC10, a monoclonal antibody that has a high affinity for v-*src* and a low affinity for murine c-*src*, was obtained from S. J. Parsons (39). Antiphosphotyrosine antibodies affinity purified from immune rabbit serum by using Affigel-phosphotyrosine columns were provided by A. Rossomando in our laboratory. Rabbit anti-mouse immunoglobulin was obtained from Jackson Laboratory. Radio-

* Corresponding author.
† Present address: Triton Biosciences, Alameda, CA 94501.

active iodinated ^{125}I-labeled protein A was purchased from Amersham Corp.

Viruses and plasmids. *src* or other oncogenes were introduced into cells by either infection or transfection. Infections were performed with murine retrovirus vectors expressing both the oncogene and *neo*, which served as a selectable marker. The *ras*- and *myc*-expressing viruses were harvested from cells obtained from K. B. Marcu (46; personal communication). Abelson murine leukemia virus was obtained from B. Mathey-Prevot and did not carry a *neo* marker. The *src*-expressing retrovirus vector was constructed by us as follows. The *src*-containing plasmid pJD100 (52) was digested with restriction endonucleases *Xho*I and *Eco*RI. The 2.3-kilobase fragment generated by this digest, containing the whole v-*src* gene, including the splice acceptor site, was isolated. This fragment was inserted into fpGV1 (41) at the *Sac*I-*Eco*RI sites of the multiple cloning region downstream of the *neo* gene, thus producing fpGV100, which expresses both *src* and *neo*.

Transfections of 3T3 and 3T3-TNR9 cells were performed by cotransfecting a plasmid expressing the oncogene along with plasmid pSV2neo, which allowed selection of transfectants in G418. The plasmids used were pJD100 (*src*) (52), pMC29 (MC29 *gag-myc*) (23), the expression vector fpGV1 (41), and a plasmid containing an ecotropic helper virus, pMOV3, which were all obtained from J. T. Parsons.

Transfection and infection. Transfections of cells were carried out by using the calcium phosphate precipitation method with glycerol shock (51). In cotransfection experiments, the precipitate used contained 1 μg of test DNA mixed with 100 ng of pSV2neo, the selective marker used in these experiments. Control transfections contained either no DNA or only pSV2neo DNA. After about 3 to 4 weeks of selection with 200 μg of G418 (Geneticin; GIBCO) per ml, colonies were picked at random for expansion into cell lines and then the dishes were stained for counting.

Virus-producing cell lines were constructed in essentially the same manner, with the following changes. The target cells used were NIH 3T3 cells. In each case, 1.5 μg of the virus-containing plasmid and 300 ng of pMOV3, the helper virus plasmid, were used to form the precipitates. Cells were selected in medium containing 750 μg of G418 per ml. Culture supernatants were tested for the presence of appropriate viruses by infection into either Swiss or NIH 3T3 cells as described below.

For infection, culture supernatant from the virus-producing cells was harvested and filtered through Millex-GV filters (Millipore Corp.) and 10 ml was added to the target cell culture with Polybrene at a final concentration of 2 μg/ml. Cells were cultured until confluent and then split 1:5 in growth medium supplemented with 200 μg of G418 per ml. Clones were obtained by the limiting dilution method (30).

Genomic DNA analysis. DNAs from various cell lines were extracted from confluent cultures and digested to completion by using at least 2 U of restriction endonuclease *Sac*I per μg of DNA. Ten micrograms of the digested DNA was loaded in each lane and separated by electrophoresis on a 0.8% agarose gel; 0.1 ng of similarly digested plasmid DNA was electrophoresed on the same gel to serve as a positive control. The DNA was transferred to a nylon membrane (Nytran; Schleicher & Schuell, Inc.) and immobilized, and the introduced v-*src* was detected by using a nick-translated probe. Hybridization was performed at 42°C in buffer containing 50% formamide. The filter was rinsed free of excess probe, dabbed dry, and sealed in Saran Wrap, and the results were visualized by autoradiography.

Cytoplasmic RNA analysis. Cells from several confluent 100-mm^2 tissue culture dishes were harvested, and the cytoplasmic RNAs were extracted by the guanidinium isothiocyanate-cesium chloride method (32). Ten micrograms of RNA was loaded into each well and separated by electrophoresis through an agarose-formaldehyde gel (32). The RNA was transferred to a Nytran nylon membrane, immobilized, and hybridized with a DNA probe in buffer containing 50% formamide. The filter was rinsed free of excess probe, dabbed dry, sealed in Saran Wrap, and visualized by autoradiography. The 28S and 18S rRNAs, which were clearly visible under shortwave UV light, were used as internal size markers.

Preparation of DNA probes. The same probe was used to detect the v-*src* DNA that was introduced into the cells by infection or transfection and to detect the RNA expressed from these vectors. Plasmid pJD100, which codes for the complete Rous sarcoma virus genome, was cut with *Eco*RI, and the 3.09-kilobase fragment containing the entire *src* gene was isolated and purified. The fragment was nick translated by using [^{32}P]dCTP at a specific activity of at least 2×10^8 cpm/μg of DNA for use in hybridizations (32).

Protein analysis. For protein analysis, cell extracts were made in boiling electrophoresis sample buffer (29). Extracts of sister cultures were also made in cold RIPA (50 mM Tris hydrochloride [pH 7.4], 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate) and used to determine the protein concentration by the method of Markwell et al. (33). The extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose (7). Western immunoblots to detect tyrosine-phosphorylated proteins were done as described by Kamps and Sefton (27) by using antiphosphotyrosine antibodies made in our laboratory. Western blots to detect v-*src* were done in the same way, except that the blocking buffer contained 3% milk and the primary antibody was EC10 (39).

Other methods. Infected and uninfected cells were cultured in soft agar to test their abilities to grow in an anchorage-independent manner (2). The mitogenic responses of various cell populations to different stimuli were analyzed by using a [^{3}H]thymidine incorporation assay (8). The increase in 2-deoxyglucose uptake was measured (48) as a biochemical parameter of transformation.

RESULTS

Infection of Swiss 3T3 and 3T3-TNR9 cells with retroviruses carrying *src* or other oncogenes. Swiss 3T3 and 3T3-TNR9 cells were infected with recombinant retroviruses carrying the *neo* gene and v-*src*, v-*myc*, or v-*ras*. The infected cell cultures were then transferred into medium containing G418 to select for infected cells expressing *neo*. In all cases, except for v-*src*-infected 3T3-TNR9 cells, more than 50% of the cells survived the initial selection process, as determined by counting a sister culture (data not shown), indicating a high efficiency of infection. However, only 30% of the v-*src*-infected 3T3-TNR9 cells survived, i.e., about half of what was obtained with the other infections. As described below, we suspect that the decreased cell survival in the *src*-infected cultures were due to *src*-induced growth inhibition. In all cases, including v-*src*-infected 3T3-TNR9 cells, there was no evidence of further cell death after two passages in selective medium.

After six passages in selective medium, the Swiss 3T3 cell population infected with the v-*src*-carrying virus was mor-

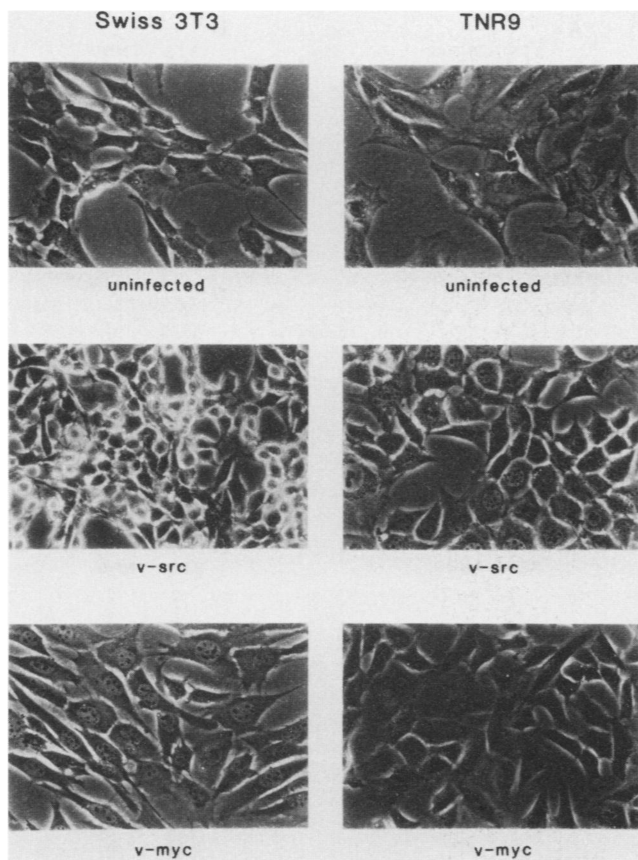

FIG. 1. Morphology of uninfected and recombinant retrovirus-infected Swiss 3T3 and 3T3-TNR9 cells. The recombinant retroviruses used carried either the *neo* gene alone under control of the simian virus 40 promoter or the v-*src* or v-*myc* oncogene under control of the Moloney murine leukemia virus long terminal repeat in a colinear manner.

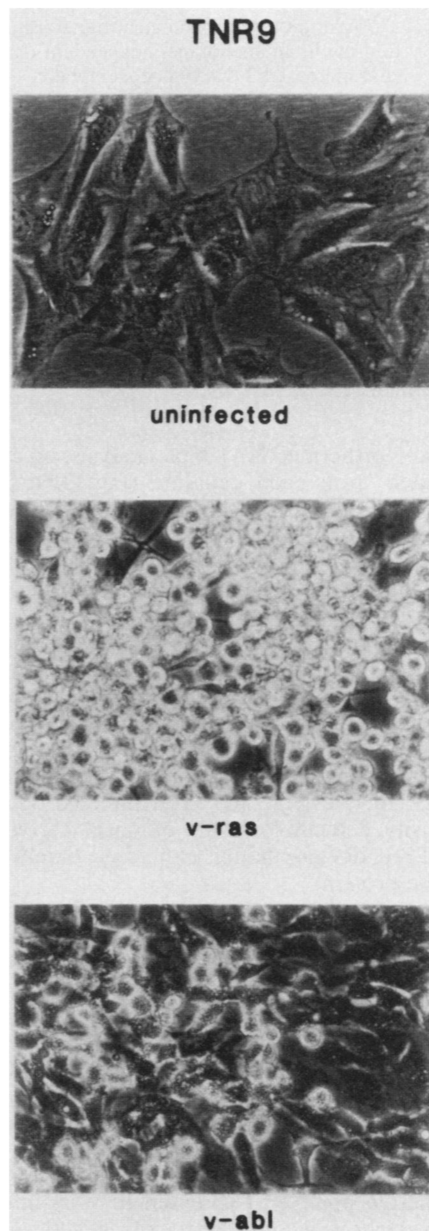

FIG. 2. Morphology of uninfected and v-*ras*- or v-*abl*-infected 3T3-TNR9 cells.

phologically transformed (Fig. 1) compared with uninfected cells. At the same passage number, the 3T3-TNR9 cell population was indistinguishable from its uninfected control. There was no change in the morphology of these cells, even after more than 11 passages (data not shown). Infection with a retrovirus carrying the v-*myc* gene did not transform either cell line (Fig. 1).

To test the possibility that 3T3-TNR9 cells were intrinsically resistant to transformation, they were infected with a recombinant retrovirus carrying the v-*ras* oncogene or with Abelson murine leukemia virus, which expresses the v-*abl* oncogene. Both of these oncogenes were able to transform the 3T3-TNR9 cells (Fig. 2), indicating that these cells are not completely refractory to transformation.

The populations of v-*src*-infected Swiss 3T3 and 3T3-TNR9 cells which had been selected in G418 were cloned by limiting dilution. Of the 24 clones isolated from the 3T3–v-*src* population, 23 showed morphological transformation, whereas of the 48 clones isolated from the 3T3-TNR9–v-*src* population, none were morphologically transformed.

The infected populations and clones were tested for two other parameters of transformation, (i) ability to grow in an anchorage-independent manner in soft agar and (ii) increased 2-deoxyglucose uptake. In all cases, morphological transformation correlated extremely well with growth in soft agar and 2-deoxyglucose uptake (Table 1).

Molecular characteristics of Swiss 3T3 and 3T3-TNR9 cells infected with v-*src*. The presence or proviral DNA in each of the infected cell populations was determined by Southern blot analysis of the respective genomic DNAs (Fig. 3A). Relative proviral copy numbers were determined from the intensities of the proviral bands on the autoradiogram. The data show that the intensities of the proviral bands in the Swiss 3T3–v-*src* (lane 2) and the 3T3-TNR9–v-*src* (lane 4) populations were comparable, indicating that both cell lines were infected to roughly the same extent and that the failure of TNR9 cells to become transformed was not due to failure to take up virus. DNAs from uninfected Swiss 3T3 cells (lane 1) and 3T3-TNR9 cells (lane 3) exhibited no v-*src* DNA.

Expression of the v-*src* gene from these proviruses was

TABLE 1. Correlation of morphological transformation with ability to grow in an anchorage-independent manner and increased 2-deoxyglucose uptake

Oncogene	Morphology[a]		Colony formation in soft agar[b]		2-Deoxyglucose uptake[c]	
	3T3	TNR9	3T3	TNR9	3T3	TNR9
v-*src*	T	NT	+	−	3.9	1.2
v-*ras*	T	T	+	+	3.5	3.1
v-*myc*	NT	NT	−	−	1.0	0.8

[a] T, Transformed; NT, not transformed.
[b] +, Colonies formed in soft agar; −, colonies not formed in soft agar.
[c] Fold increase in [^{3}H]2-deoxyglucose uptake compared with that of corresponding uninfected cells.

tested in a Northern (RNA) blot analysis of cytoplasmic RNA isolated from each cell line (Fig. 3B). There was considerably less v-*src* mRNA in the 3T3-TNR9-derived population (lane 4) than in the corresponding one from Swiss 3T3 cells (lane 2), although both cell populations were apparently infected to the same extent (Fig. 3A, lanes 2 and 4).

The abilities of the various infected cell populations to stably express the pp60$^{v\text{-}src}$ protein were determined by Western blot analysis. Whole-cell extracts were separated by polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose, and the relative amount of pp60$^{v\text{-}src}$ expressed was detected by using p60^{src}-specific monoclonal antibody EC10 (39; Fig. 3C). The infected 3T3 cells expressed easily detectable levels of pp60$^{v\text{-}src}$, whereas the infected 3T3-TNR9 population expressed barely detectable levels of the protein.

To determine the in vivo tyrosyl protein kinase activity of the pp60$^{v\text{-}src}$ expressed in these cells, total cell lysates were electrophoresed and analyzed by Western blots using affinity-purified antiphosphotyrosine antibodies (Fig. 3D). Both v-*src*-infected cell populations exhibited increased levels of tyrosyl-phosphorylated proteins compared with uninfected cells. The extent of this increase correlated with the level of pp60$^{v\text{-}src}$ expressed by the individual cell line (Fig. 3C). Hence, the pp60$^{v\text{-}src}$ expressed in all of these infected cell lines was enzymatically active.

It seemed possible that the extremely low level of enzymatically active pp60$^{v\text{-}src}$ expressed in v-*src*-infected 3T3-TNR9 cells might be a consequence of a genetic defect in the proviruses in these cells, affecting *src* transcription and/or activity. If, as we suspect (see below), *src* expression is growth inhibitory in 3T3-TNR9 cells, then the only cells which would grow in a TNR9 population infected with *src* would be those infected with defective viruses. To test whether the infected but untransformed TNR9-*src* cells contained functional proviruses, genomic DNA from v-*src*-infected cells was digested with restriction endonuclease *Sac*I, which excises the complete provirus. The digested DNA was then transfected into fresh Swiss 3T3 cells, and transfectants were selected by using G418. Clones were picked at random and observed for morphological transformation. The number of G418-resistant transfectants per plate was approximately the same whether DNA was obtained from cells infected with viruses carrying either the *neo* gene alone or colinear v-*src* and *neo* (Table 2). No G418-resistant transfectants were obtained when DNA from uninfected Swiss 3T3 or 3T3-TNR9 cells was used. The percentage of morphologically transformed clones observed in the population transfected with DNA from 3T3-TNR9–v-*src* cells was

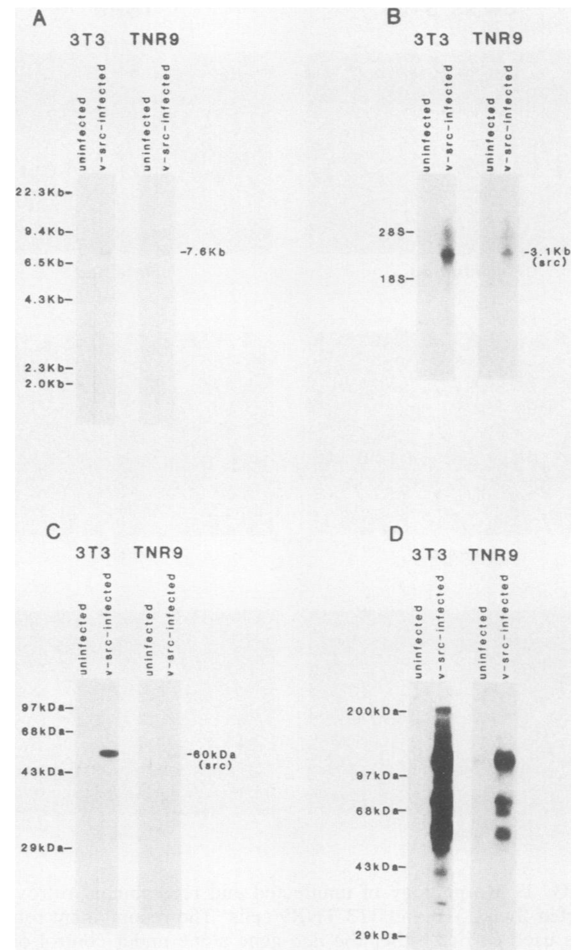

FIG. 3. Analyses of uninfected and v-*src*-infected Swiss 3T3 and 3T3-TNR9 populations. DNA (10 μg) from each cell population was digested with *Sac*I and subjected to Southern blot analysis (A); RNA (10 μg) was subjected to Northern blot analysis (B). The membranes were probed with a piece of ^{32}P-labeled v-*src* DNA isolated from plasmid pJD100. Whole-cell extracts from uninfected and v-*src*-infected Swiss 3T3 and 3T3-TNR9 populations were subjected to Western immunoblot analyses with either an anti-*src* monoclonal antibody (EC10) to detect the level of p60^{src} expression (C) or affinity-purified polyclonal antiphosphotyrosine antibodies to detect tyrosyl-phosphorylated proteins as a measure of *src* activity (D). Kb, Kilobases; kDa, kilodaltons.

the same as that seen in the population transfected with DNA from Swiss 3T3–v-*src* cells. These results indicate that infected 3T3-TNR9 cells, even though they were untransformed, carried proviruses capable of transforming 3T3 cells.

Inhibition of 3T3-TNR9 cell colony formation by *src* expression. To determine whether *src* expression was growth inhibitory in 3T3-TNR9 cells, we ascertained whether cotransfection with *src* would reduce the ability of *neo* to generate G418-resistant colonies in 3T3 and 3T3-TNR9 cells. We reasoned that if *src* was indeed growth inhibitory in 3T3-TNR9 cells, its presence should reduce the ability of *neo* DNA to give rise to G418-resistant colonies in TNR9 cells but not in 3T3 cells. The results of this study are shown in Table 3. Swiss 3T3 and 3T3-TNR9 cells were either transfected with the *neo* gene alone or cotransfected with *neo* and v-*src*. Cotransfection with v-*myc* served as a control for

TABLE 2. Analysis of the abilities of DNAs from uninfected and v-*src*-infected Swiss 3T3 and 3T3-TNR9 populations to transform Swiss 3T3 cells

DNA source	No. of Neo[r] colonies/plate[a]	No. of transformants/ total (%)
Swiss 3T3	0	NA[b]
Swiss 3T3-*neo*	217	0/22 (0.0)
Swiss 3T3-*src*	239	24/35 (68.6)
3T3-TNR9	0	NA
3T3-TNR9-*neo*	184	0/17 (0.0)
3T3-TNR9-*src*	206	17/25 (68.0)

[a] Mean of two experiments.
[b] NA, Not applicable.

nonspecific effects of cotransfection. Transfectants were selected by using G418, and the number of G418-resistant colonies was determined. In transfections with *neo* alone, the number of G418-resistant colonies per plate obtained from the 3T3-TNR9 population was the same as that obtained from the Swiss 3T3 population, indicating that both the parental and the variant cells were equally capable of being transfected and expressing the *neo* gene. Approximately the same numbers of G418-resistant colonies were obtained when Swiss 3T3 cells were cotransfected with *neo* and v-*src*, and some of these colonies were morphologically transformed, indicating that *src* does not have a toxic or growth-inhibitory effect in 3T3 cells. However, there was a drastic reduction in the number of G418-resistant colonies obtained when 3T3-TNR9 cells were similarly cotransfected with *neo* and v-*src*. Furthermore, none of these colonies were morphologically transformed nor did they contain any v-*src* DNA (as determined by Southern blot analysis [data not shown]). This reduction in the number of G418-resistant colonies observed in 3T3-TNR9 cells was not a general phenomenon, since it was not seen when the cells were cotransfected with *neo* and v-*myc*, thus suggesting that it was specific to the effects of v-*src*. Moreover, a transformation-defective mutant of *src* (*dl*155 [38]) did not cause a reduction in the number of Neo[r] colonies of 3T3-TNR9 cells and did result in the generation of colonies containing *src* DNA, indicating that these effects are dependent on functionally active *src*. These data provide strong evidence that v-*src* expression is growth inhibitory in 3T3-TNR9 cells.

Growth inhibition by TPA. If the phenotypic effects of *src* in TNR9 cells result from the same genetic change which affects their responsiveness to TPA, one would expect that TPA, like *src*, would also be growth inhibitory in these cells. Although the 3T3-TNR9 line was derived on the basis of its mitogenic nonresponsiveness to TPA (8), we found that TPA was, in fact, growth inhibitory in these cells (Table 4),

TABLE 3. Analysis of cotransfection of *neo* and v-*src* into Swiss 3T3 and 3T3-TNR9 cells

Plasmid content(s)	Mean ± SD no. of Neo[r] colonies	
	Swiss 3T3	TNR9
None (control [no plasmid])	0	0
neo	120 ± 5	115 ± 20
neo + v-*src*	105 ± 18	12 ± 8
neo + v-*myc*	130 ± 20	120 ± 13
neo + v-*src*–*dl*155	95 ± 10	128 ± 17

TABLE 4. Fold increase in [^{3}H]thymidine incorporation in response to TPA and other mitogens

Cell line	Fold increase in [^{3}H]thymidine incorporation caused by:		
	TPA	Epidermal growth factor	Serum
Swiss 3T3	5–8	8–10	8–15
3T3-TNR9	0.2–0.5	4–6	5–12

reducing the rate of thymidine incorporation into DNA by two- to threefold in the variant while, under identical conditions, stimulating thymidine incorporation in the parent cells. In fact, the original report of Butler-Gralla and Herschman (8), which described 3T3-TNR9 cells, presented evidence that TPA inhibited DNA synthesis in those cells. Thus, these findings strengthen the suggestion that *src* expression is growth inhibitory in 3T3-TNR9 cells and that the inability of the cells to be transformed by *src* is related to their defective responsiveness to TPA.

DISCUSSION

Signalling pathways used by *src*. To identify which of the cellular signalling systems involved in normal growth control are altered by pp60$^{v\text{-}src}$, we tested the effects of v-*src* on cellular variants defective in normal mitogenic responses. We have reported previously that *src* is able to transform 3T3-NR6 cells (47), which lack epidermal growth factor receptors (40), indicating that these receptors are not necessary for *src*-induced transformation. In this study, we examined the effects of *src* on 3T3-TNR9, a variant cell line which was isolated on the basis of its inability to respond mitogenically to TPA and which thus appears to be defective in some component of the PKC signalling pathway. We found that v-*src* was unable to transform these cells, and we interpreted these results as implying that pp60$^{v\text{-}src}$ activates the TPA-diacylglycerol signalling pathway as part of the transformation process. This conclusion is consistent with earlier results demonstrating that *src* expression stimulates phosphatidylinositol turnover (17, 26, 35, 50) and that inhibition of PKC blocks induction of a transformation-related gene by v-*src* (44). To analyze these results further, it is necessary to review what is currently known about the 3T3-TNR9 variant.

Although the genetic defect in the 3T3-TNR9 variant has not been identified, the cells have been characterized extensively at the physiological and biochemical levels (8–10, 19, 20; L'Allemain et al., submitted). 3T3-TNR9 cells contain normal levels of functional PKC (6), and many of the early responses to TPA which occur in the parental 3T3 cells also occur in the variant, including phosphorylation of p80 and p22 (5; but see reference 3) and increased prostaglandin biosynthesis (10) and glucose transport (9). Furthermore, several genes that are specifically inducible by TPA in Swiss 3T3 cells are also expressed in the same inducible manner in 3T3-TNR9 cells (31). The earliest known TPA-induced response which appears to be defective in TNR9 cells is phosphorylation and activation of MAP kinase, a serine-threonine protein kinase which is activated by tyrosine phosphorylation within 5 to 10 min of treatment of 3T3 cells with TPA or other mitogenic agents (L'Allemain et al., submitted). MAP kinase is present in TNR9 cells and can be activated by platelet-derived growth factor, indicating that the defect in the variant cells resides specifically in the regulation of this activation by TPA. MAP kinase, when

phosphorylated and activated, can, in turn, phosphorylate and activate a ribosomal S6 kinase in vitro (45). TPA is unable to induce activation of S6 kinase in 3T3-TNR9 cells (as it can in parental 3T3 cells) (19), consistent with the notion that MAP kinase participates in a kinase cascade that regulates protein synthesis.

Our suggestion that pp60$^{v\text{-}src}$ activates a TPA-diacylglycerol signalling pathway is consistent with the fact that transformation by *src* induces increased activation of MAP kinase (A. Rossomando and M. J. Weber, unpublished data) and of S6 kinase (19), as does treatment with TPA. Moreover, these same TPA-induced responses are the ones which are defective in 3T3-TNR9 cells, which cannot be transformed by *src*.

The correlation between mitogenic nonresponsiveness to TPA and resistance to transformation by *src* in 3T3-TNR9 cells is probably not adventitious: Colburn and collaborators have isolated a variant of JB6 epithelial cells which is resistant to TPA-induced transformation (11), and these cells also cannot be transformed by *src* (personal communication).

Transformation by other oncogenes. Although 3T3-TNR9 cells were not transformed by *src*, they were transformed by *ras* or *abl*, thus indicating that the specific transformation mechanisms used by *src* are blocked in TNR9 cells and that there is no intrinsic block to transformation in these cells. The finding that *ras* can transform these cells is consistent with experiments by Stacey and colleagues (43) which placed *ras* downstream from *src* in the transformation process. Presumably, the defect in 3T3-TNR9 cells lies upstream of *ras* and downstream of *src*. The ability of 3T3-TNR9 cells to be transformed by *abl* indicates that this tyrosyl protein kinase oncogene uses a signalling pathway different from that of *src*. This agrees with earlier results showing that 3T3-NR6 cells (which lack epidermal growth factor receptors) (40) could be transformed to a tumorigenic state by *src* but not by *abl* (47). Interestingly, we have obtained preliminary evidence that the *fps* oncogene, which is closely related to *src*, is also unable to transform 3T3-TNR9 cells (Rossomando and Weber, unpublished data). These findings indicate that these variant cells are specifically refractory to transformation by members of the *src* family of oncogenes.

Mechanism of resistance to transformation. What is the underlying mechanism which prevents the derivation of *src*-transformed 3T3-TNR9 cells? It is clear that the variant cells are capable of being infected with retrovirus vectors that carry the *src* gene, and thus the defect is not at the level of uptake and integration of oncogene DNA. However, expression of the *src* gene was drastically lower in infected 3T3-TNR9 cells than in infected 3T3 cells. This could occur because the variant cells have some mechanism which inhibits expression of the *src* gene—a result which would be surprising, since no such inhibition occurred when the *ras* oncogene was introduced by using a very similar vector. An alternative possibility is that *src* expression is growth inhibitory in 3T3-TNR9 cells. In this case, *src* serves as a negative selective agent, and the only cells which grow in the culture are the ones with low *src* expression. To test this hypothesis, we cotransfected *src* and *neo* on separate plasmids into 3T3-TNR9 cells and found that *src* reduced the number of G418-resistant colonies obtained and that the antibiotic-resistant colonies obtained did not have *src* DNA. This result can best be explained if the cells which took up *src* DNA were inhibited in their growth. Presumably, the reason why infection with a retrovirus vector containing *src* and *neo* did give rise to cells containing *src* DNA was because of the higher efficiency of DNA uptake from the retrovirus vector, combined with the fact that the oncogene and the antibiotic resistance gene were linked in the same DNA in the viral vector. Thus, infection with the retroviral vector forced all of the cells to take up viral DNA and the only survivors were ones which expressed the *src* oncogene poorly. This was likely the basis for the fact that fewer cells were observed in 3T3-TNR9 cultures following infection with the *src*-carrying virus than in 3T3 cells infected with the same virus or in either culture infected with *ras*- or *myc*-containing viruses.

The most direct way to test the growth-inhibitory effects of *src* in 3T3-TNR9 cells would be to generate a derivative of this variant cell containing a conditionally regulated *src* gene. One could then, in theory, grow such cells under restrictive conditions and shift them to permissive conditions to observe the phenotypic effects of *src* expression in this cell background. However, our attempts to perform such experiments by using either *src* under the control of the mouse mammary tumor virus promoter or by using temperature-conditional *src* mutants (*ts*LA24 and *ts*LA29 [53]) have been unsuccessful. The regulated *src* gene behaves essentially like the wild type. We suspect that even low levels of leakiness in the regulation are sufficient to cause *src*-induced growth inhibition. Indeed, we have found substantial tyrosine phosphorylation induced by *ts*LA29*src*, even at the restrictive temperature (28; data not shown). However, it is important to point out that the tyrosine kinase activity of p60$^{v\text{-}src}$ may not be the sole determinant of its growth inhibitory properties.

Growth inhibition or toxicity is not unique to the *src* gene; other oncogenes have been shown to be inhibitory or lethal in specific cell types. For example, v-*abl* is lethal to some BALB/c 3T3 cell lines (54), high concentrations of *mos* have been shown to coincide with cell mortality in Moloney murine sarcoma virus-infected NIH 3T3 cells (37), and overexpression of Ha-*ras* causes growth arrest in some cases (22). Expression of *src* does not transform myocytes (25, 36) and induces differentiation rather than transformation in PC12 pheochromocytoma cells (1). Recently, it has been reported (14) that C127 cells could not be transformed by v-*src* and other tyrosine kinase oncogenes, v-*fms* and *trk*, although they could be transformed by v-*ras* and by serine-threonine kinase oncogenes, v-*mos* and v-*raf*. However, C127–v-*src* cells, unlike the 3T3-TNR9–v-*src* cells reported here, acquire a transformed phenotype upon continued culture. Furthermore, the response of C127 cells to phorbol ester treatment is unknown, as is the relationship, if one exists, between this response to TPA and resistance to transformation by v-*src*. Although growth inhibition by oncogenes has been reported before, this is the first case in which growth inhibition by an oncogene correlated with growth inhibition in response to a defined growth-regulatory agent, namely, the tumor promoter TPA. The most parsimonious explanation for these results is that *src* expression stimulates functionally significant activity in the PKC signalling pathway.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA40042, CA47815, and CA39076 from the National Cancer Institute.

ADDENDUM

While this report was under review, Biemann and Erikson (3) reported that the p80 PKC substrate was decreased in

3T3-TNR9 cells, in contrast to our earlier results (5). They also reported that 3T3-TNR9 cells, in contrast to parental 3T3 cells, were defective in the ability to down regulate PKC in response to chronic TPA treatment. This defective response was seen only at high cell densities, and the researchers reported that their 3T3-TNR9 cells grew to a very high density, forming multiple cell layers. In our experience, 3T3-TNR9 cells do not ordinarily grow to very high densities or form multiple layers unless they are beginning to become transformed—which occurs spontaneously upon extended passaging. Because p80 phosphorylation and PKC down regulation may well be affected by malignant transformation, it seems possible to us that the results of Biemann and Erikson are secondary consequences of these phenotypic changes.

LITERATURE CITED

1. **Alema, S., P. Casalbore, E. Agostini, and F. Tato.** 1985. Differentiation of PC12 phaechromocytoma cells induced by v-*src* oncogene. Nature (London) **316**:557–559.
2. **Anderson, D. D., R. P. Beckmann, E. H. Harms, K. Nakamura, and M. J. Weber.** 1981. Biological properties of "partial" transformation mutants of Rous sarcoma virus and characterization of their pp60^{src} kinase. J. Virol. **37**:445–458.
3. **Biemann, H.-P. N., and R. L. Erikson.** 1990. Abnormal protein kinase C down regulation and reduced substrate levels in non-phorbol ester-responsive 3T3-TNR9 cells. Mol. Cell. Biol. **10**:2122–2132.
4. **Bishop, R., R. Martinez, K. D. Nakamura, and M. J. Weber.** 1985. A tumor promoter stimulates phosphorylation on tyrosine. Biochem. Biophys. Res. Commun. **115**:536–543.
5. **Bishop, R., R. Martinez, M. J. Weber, P. J. Blackshear, S. Beatty, R. Lim, and H. R. Herschman.** 1985. Protein phosphorylation in a tetradecanoyl phorbol acetate-nonproliferative variant of 3T3 cells. Mol. Cell. Biol. **5**:2231–2237.
6. **Blumberg, P. M., E. Butler-Gralla, and H. R. Herschman.** 1981. Analysis of phorbol ester receptors in phorbol ester unresponsive 3T3 cell variants. Biochem. Biophys. Res. Commun. **102**: 818–823.
7. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
8. **Butler-Gralla, E., and H. R. Herschman.** 1981. Variants of 3T3 cells lacking mitogenic response to the tumor promoter tetradecanoyl-phorobol-acetate. J. Cell. Physiol. **107**:59–67.
9. **Butler-Gralla, E., and H. R. Herschman.** 1983. Glucose uptake and ornithine decarboxylase activity in tetradecanoyl-phorbol-acetate nonproliferative variants. J. Cell. Physiol. **114**:317–320.
10. **Butler-Gralla, E., S. Taplitz, and H. R. Herschman.** 1983. 12-*O*-Tetradecanoyl-phorbol-13-acetate stimulates release of arachidonic acid, prostaglandin E2 and prostaglandin F2-alpha from TPA nonproliferative variants of 3T3 cells. Biochim. Biophys. Res. Commun. **111**:194–199.
11. **Colburn, N. H., E. J. Wendel, and G. Abruzzo.** 1981. Dissociation of mitogenesis and late-stage promotion of tumor cell phenotype by phorbol esters: mitogen-resistant variants are sensitive to promotion. Proc. Natl. Acad. Sci. USA **78**:6912–6916.
12. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. USA **75**:2021–2024.
13. **Cooper, J. A., and T. Hunter.** 1981. Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells. Mol. Cell. Biol. **1**:165–178.
14. **Cuadrado, A., M. Talbot, and M. Barbacid.** 1990. C127 cells resistant to transformation by protein kinase oncogenes. Cell Growth Differ. **1**:9–15.
15. **Diamond, L., S. O'Brien, C. Donaldson, and Y. Shimizu.** 1974. Growth stimulation of human diploid fibroblasts by the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate. Int. J. Cancer **13**:721–730.
16. **Diamond, L., T. G. O'Brien, and W. M. Baird.** 1980. Tumor promoters and the mechanism of tumor promotion. Adv. Cancer Res. **32**:1–74.
17. **Diringer, H., and R. R. Friis.** 1977. Changes in phosphatidylinositol metabolism correlated to growth state of normal and Rous sarcoma virus-transformed Japanese quail cells. Cancer Res. **37**:2979–2984.
18. **Dreidger, P. E., and P. M. Blumberg.** 1980. Specific binding of phorbol diesters in chicken embryo fibroblasts. Cancer Res. **37**:3257–3265.
19. **Erikson, R. L., D. Alcorta, P. A. Bedard, J. Blenis, H.-P. Biemann, S. W. Jones, J. L. Maller, T. J. Martis, and D. L. Simmons.** 1988. Molecular analyses of gene products associated with the response of cells to mitogenic stimulation. Cold Spring Harbor Symp. Quant. Biol. **53**:143–151.
20. **Feinup, V. K., M.-H. Jeng, R. T. Hamilton, and M. Nilsen-Hamilton.** 1986. Relation between the regulation of DNA synthesis and the production of two secreted glycoproteins by 12-*O*-tetradecanoyl-phorbol-13-acetate in 3T3 cells and phorbol ester nonresponsive 3T3 variants. J. Cell. Physiol. **129**:151–158.
21. **Frantz, C. N., C. D. Stiles, and C. D. Scher.** 1979. The tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate enhances the proliferative response of Balb/c 3T3 cells to hormonal growth factor. J. Cell. Physiol. **100**:413–424.
22. **Franza, B. E., Jr., K. Muruyama, J. I. Garrels, and H. E. Ruley.** 1986. *In vitro* establishment is not a sufficient prerequisite for transformation by activated *ras* oncogenes. Cell **44**:409–418.
23. **Heaney, M. L., J. Pierce, and J. T. Parsons.** 1986. Site-directed mutagenesis of the *gag-myc* gene of avian myelocytomatosis virus 29: biological activity and intracellular localization of structurally altered proteins. J. Virol. **60**:167–176.
24. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
25. **Hynes, R. O., G. S. Martin, M. Shearer, D. R. Critchley, and C. J. Epstein.** 1976. Viral transformation of rat myoblasts: effects on fusion and surface properties. Dev. Biol. **48**:35–46.
26. **Johnson, R. M., W. J. Wasilenko, R. R. Mattingly, M. J. Weber, and J. C. Garrison.** 1989. Fibroblasts transformed with v-*src* show enhanced formation of an inositol tetrakisphosphate. Science **246**:121–124.
27. **Kamps, M. P., and B. M. Sefton.** 1988. Identification of multiple novel polypeptide substrates of the v-*src*, v-*yes*, v-*fps*, v-*ros* and *v-erb* B oncogenic tyrosine protein kinases utilizing antisera against phosphotyrosine. Oncogene **2**:305–315.
28. **Kozma, L. M., A. B. Reynolds, and M. J. Weber.** 1990. Glycoprotein tyrosine phosphorylation in Rous sarcoma virus-transformed chicken embryo fibroblasts. Mol. Cell. Biol. **10**: 837–841.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lefkovits, I.** 1979. Limiting dilution analysis, p. 355–370. *In* I. Lefkovits, and B. Pernis (ed.), Immunological methods. Academic Press, Inc., New York.
31. **Lim, R. W., B. C. Varnum, T. G. O'Brien, and H. R. Herschman.** 1989. Induction of tumor promoter-inducible genes in murine 3T3 cell lines and tetradecanoyl phorbol acetate-nonproliferative 3T3 variants can occur through protein kinase C-dependent and -independent pathways. Mol. Cell. Biol. **9**: 1790–1793.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
34. **Martinez, R., K. D. Nakamura, and M. J. Weber.** 1982. Identification of phosphotyrosine-containing proteins in untrans-

formed and Rous sarcoma virus-transformed chicken embryo fibroblasts. Mol. Cell. Biol. 2:653–665.

35. **Martins, T. J., Y. Sugimoto, and R. L. Erikson.** 1989. Dissociation of inositol trisphosphate from diacylglycerol production in Rous sarcoma virus-transformed fibroblasts. J. Cell Biol. **108:** 683–691.
36. **Moss, P. S., N. Honeycutt, T. Pawson, and G. S. Martin.** 1979. Viral transformation of chick myogenic cells: the relationship between differentiation and the expression of the *src* gene. Exp. Cell Res. **123:**95–105.
37. **Papkoff, J., I. M. Verma, and T. Hunter.** 1982. Detection of a transforming gene product in cells transformed by Moloney murine sarcoma virus. Cell **29:**417–426.
38. **Parsons, J. T., and M. J. Weber.** 1989. Genetics of *src*: structure and functional organization of a protein tyrosine kinase. Curr. Top. Microbiol. Immunol. **147:**79–127.
39. **Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons.** 1984. Monoclonal antibodies to Rous sarcoma virus pp60^{src} react with enzymatically active cellular pp60^{src} of avian and mammalian origin. J. Virol. **51:**272–282.
40. **Pruss, R. M., and H. R. Herschman.** 1977. Variants of 3T3 cells lacking mitogenic response to epidermal growth factor. Proc. Natl. Acad. Sci. USA **74:**3918–3921.
41. **Robins, T. S., C. Jhappan, J. Chirikjian, and G. F. Vande Woude.** 1986. Molecular cloning of the intronless EJ *ras* oncogene using a murine retrovirus shuttle vector. Gene Anal. Tech. 3:12–16.
42. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20:**807–816.
43. **Smith, M. R., S. J. DeGudicibus, and D. W. Stacey.** 1986. Requirement for c-*ras* proteins during viral oncogene transformation. Nature (London) **320:**540–543.
44. **Spangler, R., C. Joseph, S. A. Qureshi, K. L. Berg, and D. A. Foster.** 1989. Evidence that v-*src* and v-*fps* gene products use a protein kinase C-mediated pathway to induce expression of a transformation-related gene. Proc. Natl. Acad. Sci. USA **86:** 7017–7021.
45. **Sturgill, T. W., L. B. Ray, E. Erikson, and J. L. Maller.** 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature (London) **334:**715–718.
46. **Wagner, E. F., M. Vanek, and B. Vennstrom.** 1985. Transfer of genes into embryonal carcinoma cells by retrovirus infection: efficient expression from an internal promoter. EMBO J. **4:** 663–666.
47. **Wasilenko, W. J., L. K. Shawver, and M. J. Weber.** 1987. Down-modulation of EGF receptors in cells transformed by the *src* oncogene. J. Cell. Physiol. **131:**450–457.
48. **Weber, M. J.** 1973. Hexose transport in normal and Rous sarcoma virus-transformed cells. J. Biol. Chem. **248:**2978–2983.
49. **Weber, M. J.** 1984. Malignant transformation by Rous sarcoma virus: from phosphorylation to phenotype. Adv. Viral Oncol. 4:249–268.
50. **Whitman, M., D. Kaplan, T. Roberts, and L. Cantley.** 1987. Evidence for two distinct phosphatidylinositol kinases in fibroblasts. Biochem J. **247:**165–174.
51. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eukaryotes. Cell **16:**777–785.
52. **Wilkerson, V. W., D. L. Bryant, and J. T. Parsons.** 1985. Rous sarcoma virus variants that encode *src* proteins with an altered carboxy terminus are defective for cellular transformation. J. Virol. **55:**314–321.
53. **Wyke, J. A., and M. Linial** 1973. Temperature-sensitive avian sarcoma viruses: a physiological comparison of twenty mutants. Virology **53:**152–161.
54. **Ziegler, S. F., C. A. Whitlock, S. P. Goff, A. Gifford, and O. N. Witte.** 1981. Lethal effect of the Abelson murine leukemia virus transforming gene product. Cell **27:**477–486.