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Lack of Induction of Neuroretinal Cell Proliferation by Rous Sarcoma Virus Variants That Carry the c-*src* Gene

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Expression of $p60^{v\text{-}src}$ of Rous sarcoma virus in cultured chicken embryo neuroretinal cells was previously shown to result in the transformation and sustained proliferation of normally quiescent cell populations. We show here that Rous sarcoma virus variants that encode $p60^{c\text{-}src}$, the cellular homolog of $p60^{v\text{-}src}$, lack the ability to induce morphological transformation and cell proliferation of cultured neuroretinal cells. Neuroretinal cells infected with c-*src*-containing viruses, however, possess no less p60 protein kinase activity assayed in the immune complex than those infected with the transformation-defective Rous sarcoma virus mutants PA101 or PA104, which do stimulate the growth of these cells.

The viral *src* (v-*src*) gene product of Rous sarcoma virus (RSV), $p60^{v\text{-}src}$ (1), is a tyrosine-specific protein kinase (6, 14) and is responsible for tumor formation and cell transformation (11). This oncogene is derived from the cellular *src* (c-*src*) gene (12, 24), the structure of which has been determined by DNA sequence analysis (25). The c-*src* gene is transcribed in many tissues, and its gene product, $p60^{c\text{-}src}$ (5), has been shown to be expressed at high levels in neural tissues including brain, neuroretina (NR), and spinal ganglia (8, 9, 17, 23). The biological function of $p60^{c\text{-}src}$, however, remains unknown.

We had previously constructed RSV variants that encode $p60^{c\text{-}src}$ instead of $p60^{v\text{-}src}$, and we obtained the following results by infecting chicken embryo fibroblasts (CEF) with these viruses: (i) $p60^{c\text{-}src}$ has no transforming activity even when it is overexpressed (13, 16), and (ii) $p60^{c\text{-}src}$ is low in its protein kinase activity measured both in vivo and in vitro (15). These experiments did not address the question of whether overproduced $p60^{c\text{-}src}$ has any effect on the regulation of cell growth. Changes in growth control caused by $p60^{v\text{-}src}$ expression have been conveniently studied by using differentiating cells from the chicken embryo NR as a host system (2, 3, 22). Infection with wild-type RSV leads not only to morphological transformation but also to sustained proliferation of NR cells, with a growth capacity otherwise restricted in monolayer culture (7). Interestingly, RSV mutants, such as PA101 and PA104, were isolated by their ability to induce NR cell proliferation without causing morphological transformation (3, 4, 19, 20). The p60 protein kinase activities of these mutants were shown to be low by the assays of immunoglobulin G heavy-chain phosphorylation in the immune complex with tumor-bearing rabbit sera or by the levels of phosphotyrosine in total cellular protein or a cellular 34,000-molecular-weight protein (34K protein) in vivo (19). These findings suggest that the mitogenic function of the *src* gene does not require high levels of protein kinase activity of p60. To examine whether $p60^{c\text{-}src}$ can stimulate cell growth, we studied the growth properties of NR cells infected with c-*src*-containing viruses.

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NR cells dissected from 7-day-old chicken embryos were trypsinized and seeded at a cell density of 2.3×10^6 cells per 35-mm plate and were infected with virus as previously described (22). Virus stocks of replication-competent subgroup A RSVs, NYN4, NYHB5, and NY501, were obtained by transfection of viral DNAs into CEF as previously described (16). NYN4 encodes $p60^{v\text{-}src}$ of the wild-type Schmidt-Ruppin A strain, and NY501 and NYHB5 encode chicken $p60^{c\text{-}src}$ (16). Since NYHB5 showed essentially the same biological effect as did NY501, only the results obtained with NY501 are described here. The isolation and characterization of PA101 and PA104 have been described previously (4, 19, 20).

NR cells were infected with NYN4 or NY501 or were mock infected and maintained in F10 medium supplemented with 8% fetal calf serum, 1% chicken serum, and 10% tryptose phosphate broth. NYN4-infected NR cells began changing to a round shape within 2 days, and by day 4 the plates were covered with multilayers of transformed cells, as previously reported for the wild-type RSV-infected NR cells (22). NY501 infection, however, did not result in either any morphological alteration or a significant increase in cell number. Virus replication was examined in these infected NR cells at days 4 and 8 after infection (Table 1). The titers of infectious virus produced by NY501-infected NR cells (measured by establishment of interference in infected CEF) were almost comparable to that of transforming virus produced by NYN4-infected NR cells (measured by focus formation in CEF) at the same day, suggesting that NR cells were infected at similar efficiencies by these two viruses.

One week after infection with NYN4, NY501, PA101, and PA104, each NR cell culture was subcultured into several plates at a low cell concentration for the analysis of growth rates (Fig. 1). NYN4-infected cells grew much faster than did uninfected cells, with a doubling time of 1.2 days, whereas NY501-infected cells exhibited a growth profile identical to that of uninfected cells (doubling time of 2.8 days). The nontransforming viruses PA101 and PA104 induced growth stimulation comparable to NYN4 (Fig. 1) as previously reported (4). The above results demonstrate that NR cells infected with c-*src*-containing virus are neither morphologically transformed nor stimulated for cell growth in culture.

TABLE 1. Virus production by NR cells

Day	Virus titers[a] in NR cells[b] infected with:		
	NYN4 FFU/ml	NY501	
		IU/ml	FFU/ml
0[c]	1.2×10^6	$\sim 10^6$	2.0×10^2
4	2.8×10^5	$\sim 10^5$	6.1×10
8	2.0×10^6	$\sim 3 \times 10^6$	2.4×10^2

[a] FFU, Focus-forming unit. A small number of foci that are detectable in NY501 virus stocks are derived from spontaneous transforming mutants of NY501 (16). IU, Infectious unit (measured by interference assay).

[b] NR cell culture in 35-mm plate was infected with NYN4 or NY501 on day 0; subsequently, culture medium (1.5 ml each) was changed every day. Only NYN4-infected cells were subcultured into the same size of plate at a splitting ratio of 2.5 on day 4 (after virus collection) because of their rapid growth.

[c] Concentration of input virus in 1.5 ml of medium.

Production of p60 in NR cells was examined 12 to 16 days after infection by pulse-labeling these cells with [^{3}H]leucine for 4 h. The p60 proteins were immunoprecipitated with monoclonal antibody 327 (18) (supplied by J. Brugge) and were analyzed as previously described (16) (Fig. 2A). NY501-infected NR cultures produced about 10-fold-higher levels of $p60^{c\text{-}src}$ than did uninfected NR cultures, which produce endogenous $p60^{c\text{-}src}$. Exponentially growing NYN4-, PA101-, and PA104-infected NR cultures, however, appeared to produce p60 at higher relative synthetic rates than did NY501-infected NR cultures. In contrast, each virus stock that was used for NR cell infection could induce uniformly high rates of p60 production in CEF within 4 days after infection (Fig. 2A). Results similar to those found in CEF were also obtained when the rates of p60 production were examined in NR cultures within 5 days after infection (data not shown). These results suggest that the proportion of NR cells expressing p60 in cultures infected with the mitogenic viruses increased with time relative to that of the NY501 cultures. This change in the proportion of cells expressing p60 probably reflects the fact that the stimulation of growth in NR cultures provides a strong selection for cells expressing mitogenic p60 proteins, which eventually overgrow uninfected cells (or cells infected with spontaneous *src* deletion mutants). Such selection would not occur in the case of NY501-infected NR cultures unless the overexpression of $p60^{c\text{-}src}$ could also stimulate cell growth. Therefore, it is probable that the rates of p60 production in individual cells were similar among the NR cultures infected with the different viruses, even though the proportions of cells expressing p60 apparently changed with time in the cultures. It should be noted that previous experiments with CEF indicate that the metabolic half-lives of cellular and viral p60 differ by a factor of three (15). This suggests that individual NR cells infected with NY501 could contain up to threefold-higher steady-state levels of p60 compared with NYN4-infected NR cells.

We examined the in vitro protein kinase activity of the p60 proteins of NY501, PA101, PA104, and NYN4 by measuring casein-phosphorylating activity and autophosphorylation in the immune complex with monoclonal antibody 327. The extent of casein phosphorylation in this assay system was shown to have a very good correlation with the in vivo phosphotyrosine levels of either total cellular protein or the 34K protein in cells infected with various RSV variants (15). p60 proteins were immunoprecipitated from cell lysates containing the same amount of cellular protein, and after the protein kinase reactions, the products were analyzed (Fig. 2B). The extent of casein phosphorylation by the p60 proteins of NY501, PA101, and PA104 was very low, representing only 3.6, 2.2, and 2.0% of that of NYN4 $p60^{v\text{-}src}$, respectively. Similar results were obtained when antiserum raised against $p60^{v\text{-}src}$ expressed in *Escherichia coli* (anti-p60 serum) (10) was used for the immunoprecipitation instead of the monoclonal antibody (data not shown). Autophosphorylation (21) of the p60 proteins is also observed in Fig. 2B and in the immune complex with anti-p60 serum in the presence or absence of exogenous casein (Fig. 3A and data not shown). The trends in p60 kinase activities were similar in this assay: the autophosphorylating activity of NY501 $p60^{c\text{-}src}$ was low compared with $p60^{v\text{-}src}$ but higher than that of the PA101 or PA104 p60.

The above results are in good agreement with the previous observations that total cellular protein phosphotyrosine levels both in CEF infected with NY501 and in NR cells infected with PA101 or PA104 are only slightly increased from those in uninfected cells (15, 19). Moreover, these results show that the NY501-infected NR cells have no less p60 protein kinase activity in vitro per total cell protein than do NR cells infected with PA101 or PA104. This suggests that the level of p60 protein kinase activity does not have a simple correlation with the growth-stimulating activity. If the residual protein kinase activity of the PA101 or PA104 p60 is responsible for the growth stimulation of NR cells, we

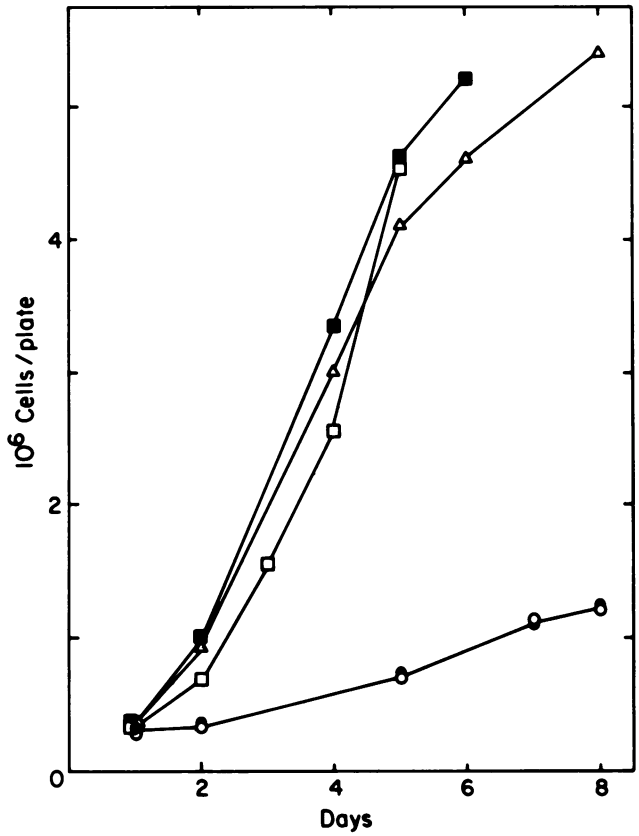

FIG. 1. Growth curves of NR cells infected with RSV variants. One week after infection, NR cells were subcultured into several 35-mm plates at a low cell density. Culture medium was changed every day; on the day indicated, one of the subcultures was trypsinized and the cell number was counted. Because of the rapid cell growth in NYN4-, PA101-, and PA104-infected cells, these cells were subcultured once 4 days after infection. Symbols: ●, uninfected cells;○, NY501-infected cells; ■, PA101-infected cells; △, PA104-infected cells; □, NYN4-infected cells.

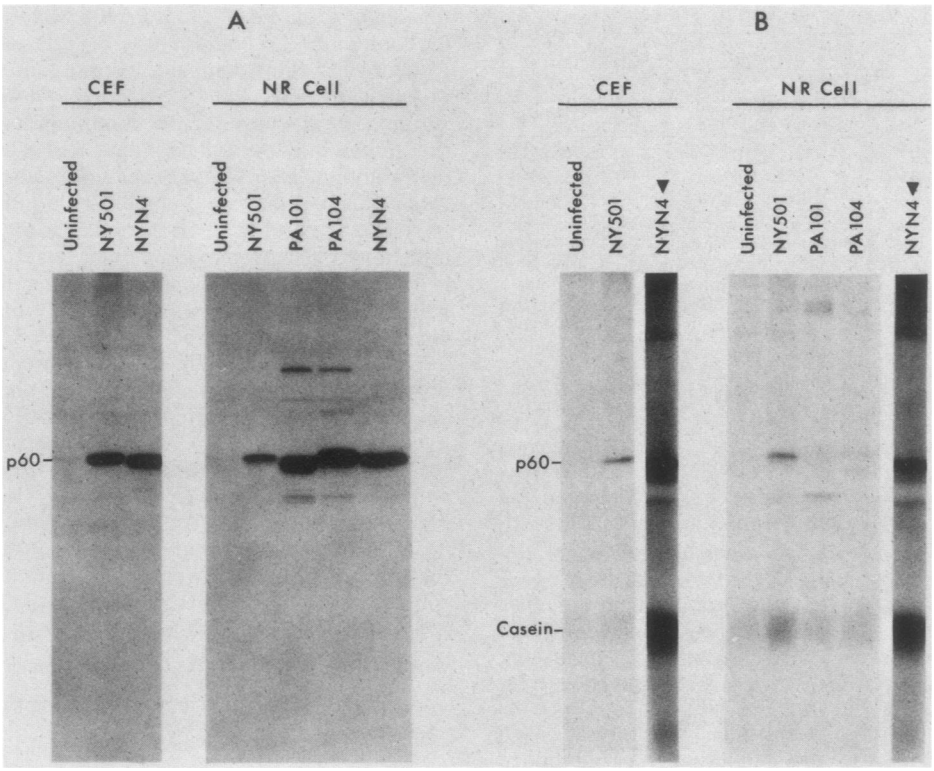

FIG. 2. Synthesis and in vitro protein kinase activity of various p60 proteins in CEF or NR cells. (A) Infected or mock-infected NR cells were grown for 12 to 16 days before labeling with [^{3}H]leucine for 4 h. NYN4-, PA101-, and PA104-infected NR cells were passaged two or three times after infection and were labeled at a cell density of about 10^7 cells per 60-mm plate. Uninfected and NY501-infected NR cells were passaged only once prior to the labeling at a cell density of 4×10^6 cells per 60-mm plate. CEF were infected as previously described (16) and were labeled 4 days after infection with [^{3}H]leucine for 4 h. From the lysates of the ^{3}H-labeled cells containing radioactivity of 2.7×10^7 cpm, the p60s were immunoprecipitated with monoclonal antibody 327, analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel, and detected by fluorography. The ratio of [^{3}H]leucine incorporated per milligram of total cell protein did not vary significantly among the different samples. (B) The cell lysates prepared were normalized for protein content (250 μg) and the p60s were immunoprecipitated for the protein kinase reaction in the presence of casein as an exogenous substrate. Films were exposed for 2 days, except for the lane with NYN4 cell lysate (▼), which was exposed for 5 h.

must assume that some difference in protein properties, such as substrate specificity, distinguishes these growth-stimulating p60 proteins from $p60^{c\text{-}src}$.

NR tissue has been reported to contain high protein kinase activity of endogenous $p60^{c\text{-}src}$, which is at least partly attributable to a high level of expression of the protein (8, 23). As shown here, however, the relative synthetic rate and the in vitro kinase activity of endogenous $p60^{c\text{-}src}$ in cultured NR cells were almost comparable to those in cultured CEF (Fig. 2 and 3A). Therefore, we compared NR tissues and NR monolayer cultures in their protein kinase activity in vitro per total cell protein. Cell lysates of freshly isolated NR were prepared as previously described (8). For the assay of in vitro protein kinase activity of endogenous $p60^{c\text{-}src}$, we used casein-phosphorylating activity and autophosphorylating activity in the immune complex with monoclonal antibody or anti-p60 serum. The trends in kinase activity were similar among these assays. The result of autophosphorylating activity in the immune complex with anti-p60 serum is shown in Fig. 3B. An NR monolayer culture maintained in vitro for 1 week had $p60^{c\text{-}src}$ kinase activity comparable to that of CEF, but it was fourfold lower than that of NR tissue of 7-day-old embryo from which the monolayer culture was derived. NR tissues from an older embryo (10 days) exhibited a further increase in p60 kinase activity (about 10-fold that of CEF), in good agreement with the previous reports that the levels of $p60^{c\text{-}src}$ expression or its kinase activity reach a peak around day 10 (8, 23). The results presented here show that NR cells reduce the expression (and possibly the specific activity of the tyrosine kinase) of $p60^{c\text{-}src}$ upon culturing in vitro and that artificial induction of $p60^{c\text{-}src}$ expression in these differentiating cells by NY501 infection does not stimulate cell proliferation. To explain the immunocytochemical observation that $p60^{c\text{-}src}$ is expressed in fully differentiated neuronal cells, Sorge et al. (23) suggested that $p60^{c\text{-}src}$ is more important in neuronal differentiation or function than in cell proliferation. Our results support this hypothesis.

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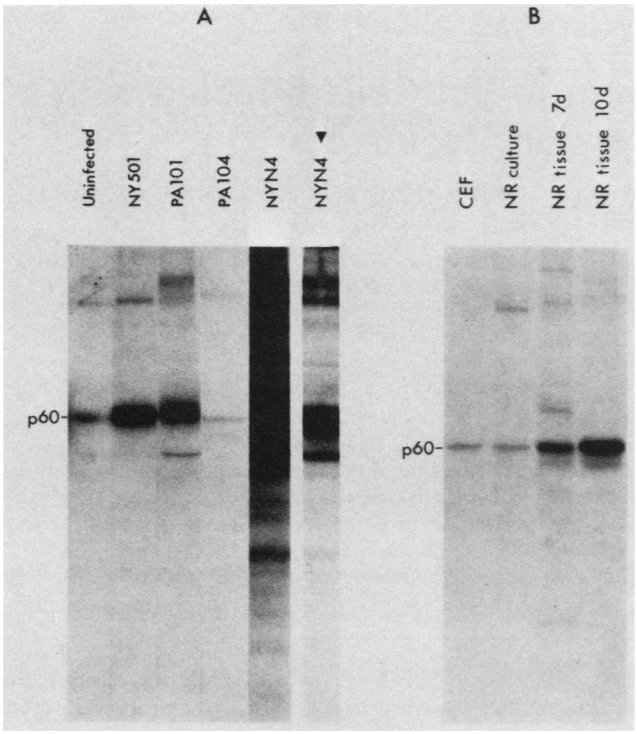

FIG. 3. Analysis of the p60 protein kinase activity in vitro by autophosphorylation. (A) NR cells were infected and labeled as described in the legend to Fig. 2. After normalization by protein content (500 μg), the cell lysates were immunoprecipitated with anti-p60 serum and assayed for protein kinase activity in the absence of exogenous substrate, and the reaction products were analyzed as described in the legend to Fig. 2. Film was exposed for 1 day, except for the lane marked with ▼, which was exposed for 2 h. (B) Cell lysates were prepared from a tertiary CEF culture, an NR monolayer culture (1 week after dissection), and NR tissues freshly isolated from 7- or 10-day-old embryos. After normalization by protein content (250 μg), cell lysates were assayed for p60 autophosphorylation in the absence of exogenous casein as described above.

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LITERATURE CITED

1. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
2. **Calothy, G., and B. Pessac.** 1976. Growth stimulation of chick embryo neuroretinal cells infected with Rous sarcoma virus: relationship to viral replication and morphological transformation. Virology **71**:336–345.
3. **Calothy, G., F. Poirier, G. Dambrine, P. Mignatti, P. Combes, and B. Pessac.** 1980. Expression of viral oncogenes in differentiating chick embryo neuroretinal cells infected with avian tumor viruses. Cold Spring Harbor Symp. Quant. Biol. **44**:983–990.
4. **Calothy, G., F. Poirier, G. Dambrine, and B. Pessac.** 1978. A transformation defective mutant of Rous sarcoma virus inducing chick embryo neuroretinal cell proliferation. Virology **89**:75–84.
5. **Collett, M. S., J. S. Brugge, and R. L. Erikson.** 1978. Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product. Cell **15**:1363–1369.
6. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus transforming protein, $p60^{src}$, shows protein kinase activity specific for tyrosine. Nature (London) **285**:167–169.
7. **Combes, P. S., A. Privat, B. Pessac, and G. Calothy.** 1977. Differentiation of chick embryo neuroretina cells in monolayer cultures. An ultrastructural study. I. Seven-day retina. Cell Tissue Res. **185**:159–173.
8. **Cotton, P. C., and J. S. Brugge.** 1983. Neural tissues express high levels of cellular *src* gene product $pp60^{c\text{-}src}$. Mol. Cell. Biol. **3**:1157–1162.
9. **Fults, D. W., A. C. Towle, J. M. Lauder, and P. F. Maness.** 1985. $pp60^{c\text{-}src}$ in the developing cerebellum. Mol. Cell. Biol. **5**:27–32.
10. **Gilmer, T. M., and R. L. Erikson.** 1983. Development of anti-$pp60^{src}$ serum with an antigen produced in *Escherichia coli*. J. Virol. **45**:462–465.
11. **Hanafusa, H.** 1977. Cell transformation. Compr. Virol. **10**: 401–483.
12. **Hanafusa, H., C. C. Halpern, D. L. Buchhagen, and S. Kawai.** 1977. Recovery of avian sarcoma virus from tumors induced by transformation-defective mutants. J. Exp. Med. **146**:1735–1747.
13. **Hanafusa, H., H. Iba, T. Takeya, and F. R. Cross.** 1984. Transforming activity of the *c-src* gene, p. 1–7. *In* G. F. Vande Woude, A. J. Levine, W. C. Topp, and J. D. Watson, (ed.), Cancer cells, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Hunter, T., and B. W. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
15. **Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa.** 1985. Low protein kinase activity of nontransforming overproduced $p60^{c\text{-}src}$. Mol. Cell. Biol. **5**:1058–1066.
16. **Iba, H., T. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants that carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4424–4428.
17. **Levy, B. T., L. K. Sorge, A. Meymandi, and P. F. Maness.** 1984. $pp60^{c\text{-}src}$ kinase in embryonic tissues of chick and human. Dev. Biol. **104**:9–17.
18. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
19. **Poirier, F., G. Calothy, R. E. Karess, E. Erikson, and H. Hanafusa.** 1982. Role of $p60^{src}$ kinase activity in the induction of neuroretinal cell proliferation by Rous sarcoma virus. J. Virol. **42**:780–789.
20. **Poirier, F., P. Jullien, P. Dezelee, G. Dambrine, E. Esnault, A. Benatre, and G. Calothy.** 1984. Role of mitogenic property and kinase activity of $p60^{src}$ in tumor formation by Rous sarcoma virus. J. Virol. **49**:325–332.
21. **Purchio, A. F.** 1982. Evidence that $p60^{src}$, the product of the Rous sarcoma virus *src* gene, undergoes autophosphorylation. J. Virol. **41**:1–7.
22. **Pessac, B., and G. Calothy.** 1974. Transformation of chick embryo neuroretinal cells by Rous sarcoma virus *in vitro*: induction of cell proliferation. Science **175**:898–900.
23. **Sorge, L. K., B. T. Levy, and P. F. Maness.** 1984. $pp60^{c\text{-}src}$ is developmentally regulated in the neural retina. Cell **36**:249–257.
24. **Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt.** 1976. DNA related to the transforming genes of avian sarcoma virus is present in normal avian DNA. Nature (London) **260**:170–173.
25. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the *src* gene of Rous sarcoma virus and the mechanism for the generation of the viral transforming gene. Cell **32**:881–890.