MOLECULAR AND CELLULAR BIOLOGY, July 1982, p. 875–880
0270-7306/82/070875-06$02.00/0

Vol. 2, No. 7

NOTE

Association of the Transforming Proteins of Rous, Fujinami, and Y73 Avian Sarcoma Viruses with the Same Two Cellular Proteins

LEAH A. LIPSICH, JOHN R. CUTT, AND JOAN S. BRUGGE*

Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794

Received 3 February 1982/Accepted 11 March 1982

Two forms of the transforming proteins of Fujinami (pp140^{fps}) and Yamaguchi 73 (pp94^{yes}) sarcoma viruses were detected in lysates of chicken cells transformed by these viruses; the majority of pp140^{fps} and pp94^{yes} molecules were present as monomers; however, a small percentage of these proteins was associated in a complex with two cellular proteins of M_r 90,000 and 50,000. These cellular proteins were shown to be identical to those previously found to be complexed with the transforming protein of Rous sarcoma virus, pp60^{src}. These results suggest a common role for the interaction of pp90 and pp50 with viral transforming proteins encoding tyrosyl-protein kinases.

Several different classes of sarcoma-inducing retroviruses have been identified (J. M. Bishop and H. Varmus, *in* R. A. Weiss et al., ed., *RNA Tumor Viruses*, in press). The transforming genes of these viruses can be distinguished from one another by molecular hybridization, and the protein products of these genes are immunologically and structurally distinct (Bishop and Varmus, in press). However, all of these viruses are similar in that each unique transforming protein is associated with a protein kinase activity specific for tyrosine (Bishop and Varmus, in press). Furthermore, it has been shown that these various transforming proteins may share cellular targets for tyrosyl phosphorylation. Identical proteins have been found to be phosphorylated at tyrosine after transformation of several different sarcoma viruses (7, 8). This suggests that at least certain aspects of transformation may be common to all of these retroviruses which encode tyrosine-specific protein kinases.

The best-characterized transforming protein of this type is pp60^{src}, which is encoded by Rous sarcoma virus (RSV) (4). It was recently demonstrated that a small percentage of pp60^{src} molecules is associated with two cellular proteins of molecular weights 90,000 (pp90) and 50,000 (pp50) in RSV-transformed cell lysates (3, 15). The precise function of this complex is not understood; however, the pp50 protein contains phosphotyrosine (11) and is thus a candidate substrate of pp60^{src}-mediated phosphorylation. In view of the various common features associated with transformation by the viruses encoding tyrosyl-protein kinases, it was of interest to determine whether other transforming proteins which are distinct from pp60^{src} interact with these two cellular proteins.

In this report, we demonstrate the association of pp90 and pp50 with the transforming proteins of Fujinami sarcoma virus (FSV) and Yamaguchi 73 sarcoma virus (Y73), which represent two unique classes of avian sarcoma viruses. Results suggest that these cellular proteins of M_r 90,000 and 50,000 are involved in a common event mediating transformation by this group of tumor viruses.

To determine whether the cellular proteins, designated pp90 and pp50, coprecipitated with the transforming proteins encoded by FSV and Y73, [^{35}S]methionine-labeled cell lysates were first analyzed by direct immunoprecipitation. Figure 1 shows the proteins precipitated from normal chicken cells (lanes 1 and 2) and chicken cells infected with either transformation-defective RSV (lanes 3 and 4), nondefective Schmidt-Ruppin RSV (lanes 7 through 9), Y73 (lanes 5 and 6), or FSV (lanes 10 and 11), using either normal rabbit serum, antiserum against the structural protein pp19^{gag} (provided by V. Vogt), or serum from rabbits bearing tumors induced by RSV (TBR serum). TBR serum contains antibodies directed against many antigens, both structural and nonstructural, which are expressed in RSV-induced tumor cells. Both TBR and anti-pp19^{gag} sera precipitated structur-

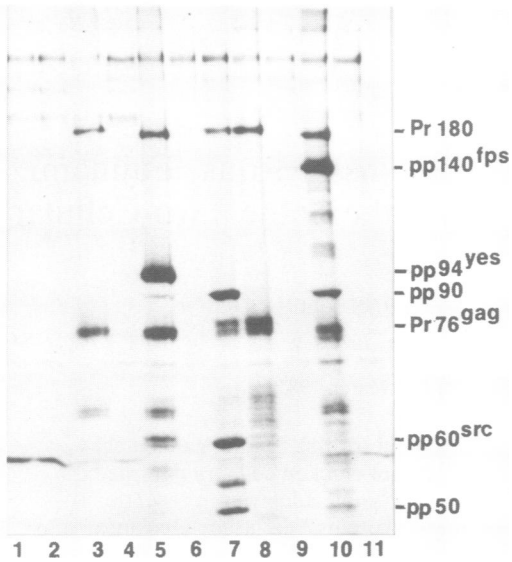

FIG. 1. Proteins immunoprecipitated from [^{35}S]methionine-labeled normal and RSV-, FSV-, and Y73-infected chicken cells with anti-pp19^{gag} or TBR serum. Uninfected chicken cells (Spafas, gs$^-$) were infected with either nondefective or transformation-defective (*td*101) Schmidt-Ruppin, FSV, or Y73 virus as described previously (4). All viruses were obtained from H. Hanafusa. Approximately 5×10^5 cells were labeled with 50 μCi of [^{35}S]methionine per ml for 12 h in methionine-free medium; the lysates were prepared as described (2) and immunoprecipitated with 2 μl of serum, except Y73, which was first incubated with anti-pp27^{gag} (provided by R. L. Erikson). The preclearing with anti-pp27^{gag} was performed to clear the lysate of most of Pr76 so that the anti-pp19^{gag} serum would immunoprecipitate a greater percentage of pp94^{yes}. The immune complexes were suspended in sample buffer and subjected to electrophoresis on a 7.5% polyacrylamide gel as described (2). The types of cells and sera used for immune precipitation were uninfected chicken cells (lanes 1 and 2); chicken cells infected with transformation-defective RSV (lanes 3 and 4); chicken cells transformed with Y73 (lanes 5 and 6); nondefective RSV-transformed chicken cells (lanes 7, 8, and 9); FSV-transformed chicken cells (lanes 10 and 11); normal rabbit serum (lanes 2, 4, 6, 9, and 11); anti-pp19^{gag} serum (lanes 1, 3, 5, 8, and 10); and TBR serum (lane 7).

al proteins from FSV, Y73, transformation-defective RSV, and nondefective RSV lysates. These include Pr180, the translation product of the *gag-pol* genes; Pr76, the *gag* gene precursor protein; and numerous intermediate and mature cleavage products of Pr76.

Three transformation-specific proteins were precipitated by the TBR serum from RSV-transformed cells. The protein of M_r 60,000 has previously been shown to be the product of the RSV *src* gene (pp60^{src}) (2, 4, 17). The two proteins of M_r 90,000 and 50,000 (designated pp90 and pp50, respectively) coprecipitated with pp60^{src} as a consequence of a physical association with pp60^{src} (3, 15). The protein which migrated between pp60^{src} and pp50 appeared to be a cleavage product of pp60^{src}, since its peptides were a subset of those of pp60^{src} (data not shown).

Since the transforming genes of FSV and Y73 are fusion products of the *gag* gene linked to their unique transforming gene, the protein products of these genes can be immunoprecipitated with antiserum to the *gag* gene-encoded protein pp19^{gag}. The protein of M_r 140,000 (designated pp140^{fps}) which was immunoprecipitated from FSV-transformed cell lysates (Fig. 1, lane 10) has previously been shown to be the *gag-fps* fusion product (9, 13), and similarly, the protein of M_r 94,000 (designated pp94^{yes}; Fig. 1, lane 5) has been shown to be the *gag-yes* fusion product (12). Immunoprecipitates of Y73- and FSV-transformed cell lysates incubated with antiserum to pp19^{gag} were also found to contain proteins which comigrated with the pp90 and pp50 proteins, which associate with pp60^{src} in RSV-transformed cell lysates. The pp90 and pp50 proteins were not precipitated from lysates of cells infected with transformation-defective virus, which are deleted in the *src* gene (Fig. 1, lanes 3 and 4), nor from uninfected chicken cells (Fig. 1, lanes 1 and 2). The pp90 and pp50 proteins were also not precipitated from lysates of cells infected with nondefective RSV when anti-pp19^{gag} was utilized (Fig. 1, lane 8), owing to the fact that pp60^{src} is not a *gag* fusion product and is therefore not recognized by the pp19^{gag} antiserum.

To determine whether pp90 and pp50 were associated in a complex with pp140^{fps} and pp94^{yes}, similar to the complex observed with pp60^{src} (3), a [^{32}P]orthophosphate-labeled lysate of FSV- or Y73-transformed chicken cells was sedimented through a 10 to 30% glycerol gradient. Alternating fractions of the gradients were treated with either TBR serum or monoclonal antibody to the cellular pp90 protein, which has been shown to be identical to the pp60^{src}-complexed pp90 protein (Yonemoto et al., unpublished data). Figures 2A and B show the results of electrophoresis of proteins immunoprecipitated from even-numbered fractions with monoclonal antibody to pp90. In Y73-infected cell lysates, the major portion of pp90 sedimented in fractions 42 through 60, with a peak in fraction 46. Two proteins coprecipitated and cosedimented, with trailing portions of pp90 in fractions 34 through 44. These proteins comigrated on the sodium dodecyl sulfate-polyacrylamide gel with the Y73 transforming protein pp94^{yes}

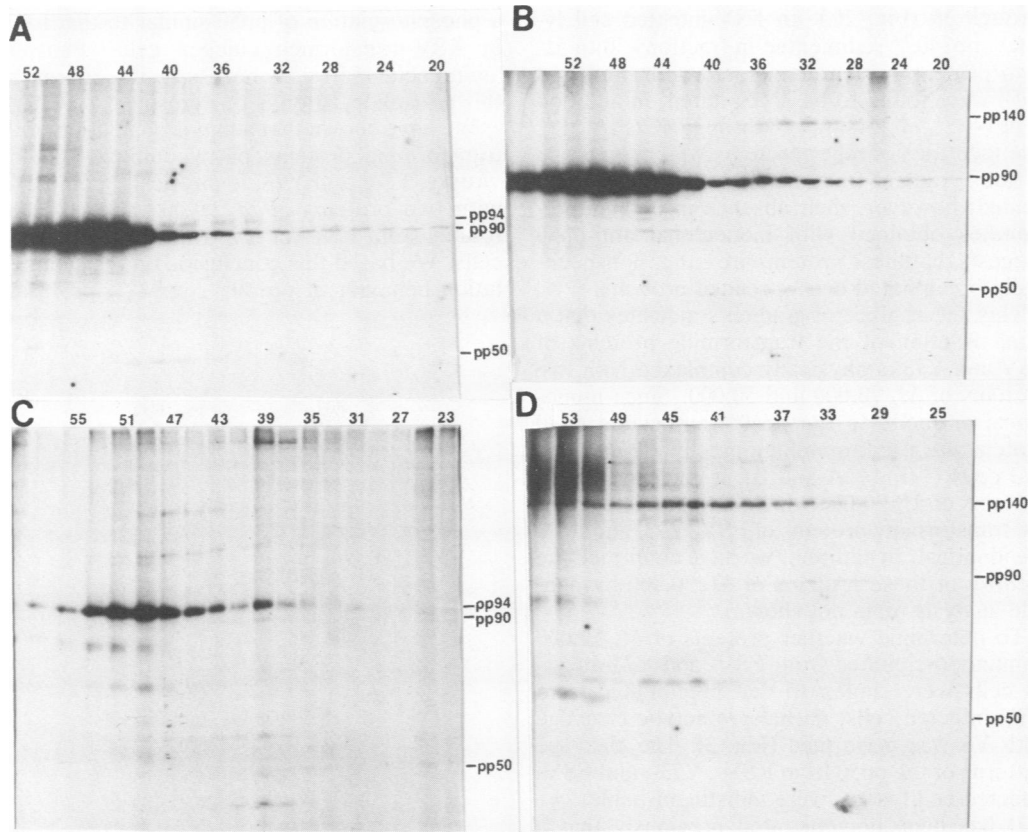

FIG. 2. Glycerol gradient sedimentation of lysates of [^{32}P]orthophosphate-labeled FSV- and Y73-transformed chicken cells. Lysates of FSV- or Y73-transformed chicken cells that were labeled with 1 mCi of [^{32}P]orthophosphate per ml in phosphate-free medium for 12 h were prepared in radioimmune precipitation assay (RIPA) buffer containing 1 mM EDTA and 1 mM EGTA, layered on a 10 to 30% glycerol gradient in RIPA buffer, and centrifuged for 24 h at 40,000 rpm at 4°C in an SW41 rotor. Gradient fractions 20 through 60 (of 70 total fractions) were treated with either 2 μl of anti-pp90 serum (even fractions [A and B]) or 2 μl of anti-pp19^{gag} serum, except Y73, which was first cleared with 2 μl of anti-pp27^{gag} (odd fractions [C and D]), and the immunoprecipitates were subjected to electrophoresis in a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The top of each glycerol gradient is on the left. A and C represent a gradient fractionation of Y73-transformed chicken cells treated with monoclonal antibody to pp90 (A) or anti-pp19^{gag} serum (C). B and D show similar analyses of lysates from FSV-transformed chicken cells with monoclonal antibody to pp90 (B) or anti-pp19^{gag} serum (D). Marker immunoprecipitates containing pp90, pp60^{src}, and pp50 as well as pp140^{fps} or pp94^{yes} were included on the ends of each gel (data not shown). Only the relevant region of each glycerol gradient is shown here. Monoclonal antibody to pp90 was produced as described by Cepko and co-workers (5) for adenovirus 2 proteins, using pp90 purified from chicken brain. This antibody was shown to immunoprecipitate the cellular pp90 protein, which is identical to that bound to pp60^{src} (Yonemoto et al., unpublished data).

and the pp60^{src}-associated pp50 protein. In FSV-infected cell lysates, pp90 sedimented with a peak in fraction 50. Coprecipitating with the trailing fractions of pp90 were two proteins which comigrated with pp140^{fps} and pp50 in fractions 26 through 40 (Fig. 2B). No proteins with electrophoretic mobilities similar to those of pp50, pp94^{yes}, or pp140^{fps} were detected when lysates of uninfected chicken cells were precipitated with antibody to pp90 as described above (data not shown). The precipitation of pp50, pp90, and either pp94^{yes} or pp140^{fps} strongly supports the possibility that the transforming proteins of FSV and Y73 are associated in a complex with pp90 and pp50.

Figures 2C and D show the results of analysis of immunoprecipitates obtained from the odd-numbered fractions with anti-pp19^{gag} serum. In Y73-infected cell lysates, the major portion of pp94^{yes} sedimented in fractions 41 through 57, with a smaller proportion cosedimenting with proteins of M_r 90,000 and 50,000 in fractions 31

through 39 (Fig. 2C). In FSV-infected cell lysates, pp140^{fps} sedimented in fractions 30 to 51. Two proteins which comigrated with pp90 and pp50 were found to be precipitated, in addition to pp140^{fps}, in fractions 28 through 41 (Fig. 2D). The identities of other proteins which appear in various gradient fractions have not been investigated; however, their absence in immunoprecipitates obtained with monoclonal anti-pp90 suggests that these proteins are either nonspecifically precipitated or *gag*-related proteins.

This set of glycerol gradients indicates that a small fraction of the transforming proteins of FSV and Y73 is physically complexed with two proteins of M_r 90,000 and 50,000. Since monoclonal antibody to the pp60^{src}-associated pp90 protein was used to immunoprecipitate pp140^{fps} and pp94^{yes} (Fig. 2A and B), it is clear that the proteins of M_r 90,000 which are associated with the transforming proteins of FSV, Y73, and RSV are identical. In addition, we have confirmed the identity of these proteins of M_r 90,000 by peptide analysis (data not shown).

To determine whether proteins of M_r 50,000 immunoprecipitated from FSV- and Y73-infected cells were similar to that precipitated from RSV-infected cells, partial proteolytic cleavage with V8 was performed (Fig. 3). The cleavage patterns of the pp50 from RSV-, Y73-, and FSV-infected cell lysates were indistinguishable.

It has been demonstrated previously that a phosphoserine-containing form of pp50 is present in uninfected chicken cells, and upon transformation with RSV, a phosphotyrosine-containing form of pp50 is also present (1). These two species of pp50 can be identified by two-dimensional analysis as two distinct spots. Since the results in Fig. 1, 2, and 3 suggest that the Y73- and FSV-transforming proteins are associated with pp50, [^{32}P]orthophosphate-labeled lysates from Y73- and FSV-transformed chicken cells were subjected to such an analysis to examine the phosphorylation states of the cellular pp50 protein (Fig. 4). Uninfected and Schmidt-Ruppin RSV-infected cells were included as negative and positive controls. Uninfected chicken cells contained only one pp50 species, designated pp50B (Fig. 4A). However, RSV-, Y73-, and FSV-transformed cells contained an additional pp50 spot (Fig. 4B, C, and D), designated pp50A. Brugge and Darrow (1) have previously demonstrated that the pp50B species present in RSV-transformed cells contains phosphoserine, whereas the pp50A species contains phosphotyrosine in addition to phosphoserine. This additional phosphorylation could be responsible for the shift in the migration of the pp50 which is apparent in transformed cells. Thus, the above results suggest that the interaction of pp140^{fps} and pp94^{yes} with pp50 results in a phosphorylation of pp50 similar to that found in RSV-transformed chicken cells. Phosphoamino acid analysis is necessary to confirm that this phosphorylation is on tyrosine.

We have shown that a minor population of the transforming proteins of two unique sarcoma viruses, FSV and Y73, is physically complexed with two proteins of M_r 90,000 and 50,000 in lysates from FSV- or Y73-transformed chicken cells. We based this conclusion on the sedimentation behavior of pp140^{fps}, pp94^{yes}, pp90, and

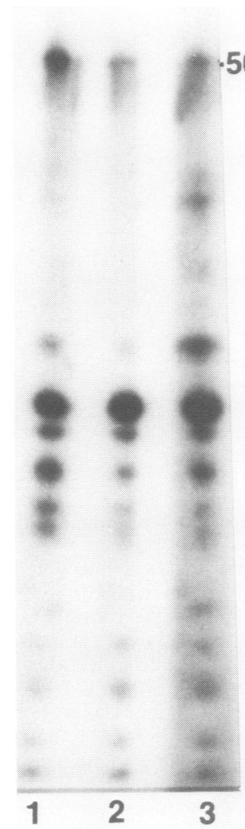

FIG. 3. Partial proteolytic digestion of the 50,000-dalton proteins immunoprecipitated from RSV-, Y73- and FSV-transformed cells. RSV-, Y73- and FSV-transformed chicken cells were labeled with 1 mCi of [^{32}P]orthophosphate per ml in phosphate-free medium for 12 h. Lysates were prepared in radioimmune precipitation assay (RIPA) buffer, and immunoprecipitation, utilizing TBR antiserum for RSV and anti-pp19^{gag} for FSV and Y73, was performed as described in the legend to Fig. 1. The gel represents the pp50 excised from the gel and re-electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel with 25 ng of V8 (Miles Laboratories, Inc.) by the method of Cleveland and co-workers (6). Lane 1, pp50 from Y73-transformed chicken cells; lane 2, pp50 from FSV-transformed chicken cells; lane 3, pp50 from RSV-transformed chicken cells.

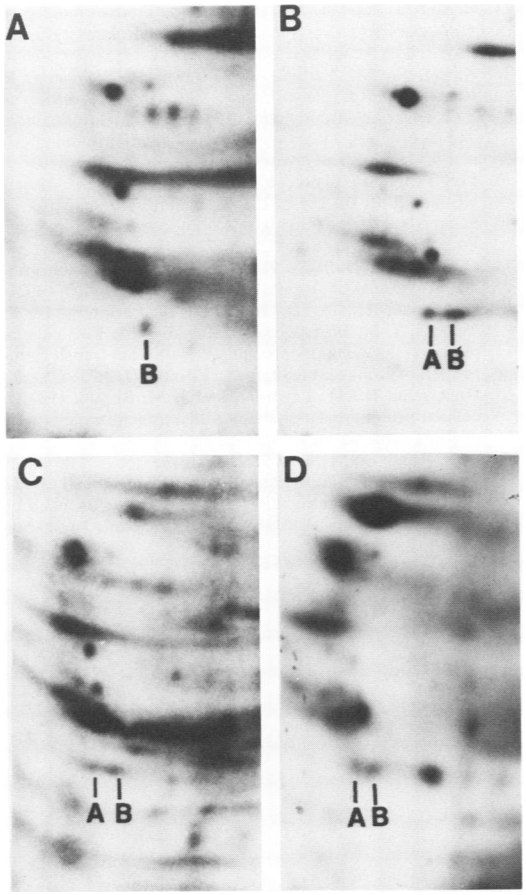

FIG. 4. Two-dimensional analysis of phosphoproteins from normal and RSV-, Y73-, and FSV-transformed chicken cells. Normal and RSV-, Y73-, and FSV-transformed cells were labeled for 12 h with 1 mCi of [^{32}P]orthophosphate per ml in phosphate-free medium. The cells were processed by the Garrels modification (10) of the O'Farrell procedure (14) for two-dimensional analysis of protein as described previously (1). Samples of 15 μl (approximately 10^5 cells) were run in the first dimension on 17-cm cylindrical gels, and the gel sections from 3 to 8 cm from the acidic end were then subjected to electrophoresis in the second dimension in a 7.5% sodium dodecyl sulfate-polyacrylamide gel. The gels were dried immediately and exposed to film for 16 h, using a Lightning-Plus (Kodak) intensifying screen. (A) Lysate prepared from normal chicken cells; (B) lysate from RSV-transformed chicken cells; (C) lysate from Y73-transformed chicken cells; (D) FSV-transformed chicken cells. Spot A indicates pp50A; spot B indicates pp50B.

pp50 on glycerol gradients. The pp90 and pp50 proteins sedimented with the transforming proteins as a complex similar to that seen with pp60^{src}, and these complexes were distinct from free pp140^{fps} or pp94^{yes}. Antisera directed against either the *gag* protein pp19 or the pp90 protein were able to immunoprecipitate the complex of proteins in the same gradient fractions. We concluded that the pp90 and pp50 proteins are precipitated from transformed cell extracts because of their association with pp140^{fps} or pp94^{yes}. The pp90 and pp50 proteins are identical to the proteins associated with pp60^{src}, based on the following criteria: (i) monoclonal antibody directed against the pp90 protein which is pp60^{src} associated was found to immunoprecipitate both pp94^{yes} and pp140^{fps}, and (ii) the peptide map of the pp50 protein bound which coprecipitated with pp94^{yes} and pp140^{fps} is identical to that of the pp50 protein associated with pp60^{src}.

These results strongly suggest that the cellular pp90 and pp50 proteins described here may have a common role in the mechanism of transformation induced by the sarcoma-inducing viruses which encode tyrosine-specific protein kinases. The functional role of this complex in transformation is not known. Since pp50 has been shown to be phosphorylated on tyrosine after transformation by RSV (1, 11), one can speculate that pp50 is a substrate of the tyrosyl-phosphorylating activity of this class of transforming proteins. The pp90 protein, however, does not contain phosphotyrosine and therefore does not appear to be a substrate of the viral protein kinases. pp90 is an abundant cellular protein (3) which has been shown to be identical to one of several "stress" proteins which are induced after heat or arsenite treatment of chicken cells (16). However, no function has been assigned to these stress proteins, and the role of pp90 in the pp90:pp60^{src}:pp50 complex is unclear. Further studies are in progress to elucidate the functional nature of this interaction.

We thank Anita Lewis, Mark Darrow, Teresa Tang, and Wes Yonemoto for help in the preparation of monoclonal antibody to pp90; Diane Darrow for two-dimensional analysis of FSV-transformed cell lysates; Volker Vogt and Ray Erickson for the generous gift of antiserum to pp19^{gag} and pp27^{gag}; and H. Hanafusa for the generous gift of the Y73 and FSV viral stocks.

This research was supported by Public Health Service grants CA-27951 and CA-2814601 from the National Cancer Institute.

LITERATURE CITED

1. **Brugge, J. S., and D. Darrow.** 1982. Rous sarcoma virus induced phosphorylation of a 50,000 dalton cellular protein. Nature (London) **295**:250–253.
2. **Brugge, J. S., E. Erikson, and R. L. Erikson.** 1978. Antibody to virion structural proteins in mammals bearing avian sarcoma virus-induced tumors. Virology **84**:429–433.
3. **Brugge, J. S., E. Erikson, and R. L. Erikson.** 1981. The specific interaction of the Rous sarcoma virus transforming protein, pp60^{src}, with two cellular proteins. Cell **25**:363–372.

4. **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.
5. **Cepko, C. L., P. S. Changelian, and P. A. Sharp.** 1981. Immunoprecipitation with two-dimensional pools as a hybridoma screening technique: production and characterization of monoclonal antibodies against adenovirus-2 proteins. Virology **110**:385–401.
6. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl-sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
7. **Cooper, J. A., and T. Hunter.** 1981. Four different classes of retroviruses induce phosphorylation of tyrosines present in similar cellular proteins. Mol. Cell. Biol. **1**:394–407.
8. **Erikson, E., R. Cook, G. J. Miller, and R. L. Erikson.** 1981. The same normal cell protein is phosphorylated after transformation by avian sarcoma viruses with unrelated transforming genes. Mol. Cell. Biol. **1**:43–50.
9. **Feldman, R., T. Hanafusa, and H. Hanafusa.** 1980. Characterization of protein kinase activity associated with the transforming gene product of Fujinama sarcoma virus. Cell **22**:757–765.
10. **Garrels, J. I.** 1979. Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. J. Biol. Chem. **254**:7961–7977.
11. **Hunter, T., and B. M. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77**:1311–1315.
12. **Kawai, S., M. Yoshida, K. Segawa, H. Sugiyama, R. Ishizaki, and K. Toyoshima.** 1980. Characterization of Y73, an avian sarcoma virus: a unique transforming gene and its product, a phosphopolyprotein with protein kinase activity. Proc. Natl. Acad. Sci. U.S.A. **77**:6199–6203.
13. **Lee, W.-H., K. Bister, A. Pawson, T. Robins, C. Moscovici, and P. H. Duesberg.** 1980. Fujinami sarcoma virus: an avian RNA tumor virus with a unique transforming gene. Proc. Natl. Acad. Sci. U.S.A. **77**:2018–2022.
14. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
15. **Opperman, H., A. D. Levinson, L. Levintow, H. E. Varmus, J. M. Bishop, and S. Kawai.** 1981. Two cellular proteins that immunoprecipitate with the transforming protein of Rous sarcoma virus. Virology **113**:736–751.
16. **Opperman, H., W. Levinson, and J. M. Bishop.** 1981. A cellular protein that associates with a transforming protein of Rous sarcoma virus is also a heat-shock protein. Proc. Natl. Acad. Sci. U.S.A. **78**:1067–1071.
17. **Purchio, A. F., E. Erikson, J. S. Brugge, and R. L. Erikson.** 1979. Identification of a polypeptide encoded by the avian sarcoma virus *src* gene. Proc. Natl. Acad. Sci. U.S.A. **75**:1567–1571.