JOURNAL OF VIROLOGY, Sept. 1986, p. 755–758
0022-538X/86/090755-04$02.00/0

Vol. 59, No. 3

Localization of Conserved and Nonconserved Epitopes within the Rous Sarcoma Virus-Encoded *src* Protein

SARAH J. PARSONS,* DEBORAH J. McCARLEY, VICTORIA W. RAYMOND, AND J. THOMAS PARSONS

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

Received 10 March 1986/Accepted 10 June 1986

We investigated the location of binding sites of pp60^{src}-specific monoclonal antibodies by immunoprecipitating a panel of structurally altered *src* proteins. Two families of antibodies which recognized epitopes mapping to either amino acid residues 28 to 38 or 92 to 128 were identified. The highly conserved nature of the epitope defined by residues 92 to 128 suggests that it may represent an important functional region of the cellular *src* protein.

The oncogene of Rous sarcoma virus (RSV), the *src* gene, encodes a 60-kilodalton tyrosine protein kinase, pp60^{src}, the expression of which is required for the establishment and maintenance of transformation (2, 5, 7, 12, 14, 16). Biochemical analysis of the *src* protein and of cells transformed by mutant and wild-type RSV has clearly shown that the expression of pp60^{src} and the concomitant appearance of phosphotyrosine-modified proteins are prerequisite steps in transformation (2, 8, 9, 10, 13). Such experiments have also served as models for the analysis of other tyrosine protein kinases whether of cellular or viral origin (1, 13). Analysis of the structure and function of the *src* protein has been facilitated by the derivation of monoclonal antibodies (MAbs) directed against the *src* protein (17, 20, 21, 26). We have previously described the properties of 23 MAbs, all of which recognize conserved and nonconserved epitopes within the amino-terminal 204 amino acid residues of the virally encoded *src* protein (20, 21). The location of the binding sites was determined by testing the capability of the MAbs to immunoprecipitate a bacterial fusion protein containing the amino-terminal 204 amino acids of pp60$^{v\text{-}src}$ fused to β-galactosidase. Among these antibodies, 10 react with the avian cellular homolog of *src*, pp60$^{c\text{-}src}$, and 2 react with the cellular homolog of *src* encoded by rodent, primate, bovine, and human cells (19, 21). To map more precisely the epitopes recognized by this panel of *src*-specific MAbs and to define the location of the highly conserved cross-species epitope, we have examined by immunoprecipitation, polyacrylamide gel electrophoresis, and autoradiography (15, 21) the binding of individual MAbs to structurally altered *src* proteins encoded by deletion mutants of RSV. These mutants have been generated by site-directed deletion of nucleotide sequences within the *src* gene of RSV by conventional techniques (6, 11, 18; J. T. Parsons, V. Wilkerson, and S. J. Parsons, *in* T. S. Papas and G. F. Vande Woude, ed., *Gene Amplification and Analysis*, vol. 4, in press).

The structure and biological properties of the RSV mutants used in these experiments are summarized in Fig. 1. Ten representative MAbs were chosen for the mapping studies. EB7, GF3, HC5, and R2D2 had been shown to react only with pp60$^{v\text{-}src}$, whereas EC10, FG1, FC6, and GB6 recognize both pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ of avian origin (21). GD11 and EB8 exhibit a broad reactivity with pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ from avian, rodent, bovine, primate, and human origins (19, 21).

Initial mapping of the binding sites recognized by the individual antibodies was carried out with a series of mutants bearing large deletions within the amino-terminal 225 residues of pp60^{src}. All of the antibodies immunoprecipitated the pp54^{src} encoded by CH*dl*120, a mutant of the Prague A strain of RSV (Pr-A-RSV) containing a deletion of residues 169 to 225 (Fig. 2A, lanes 3, 4, and 6 to 13). In contrast, the antibodies GD11 and EB8 failed to efficiently immunoprecipitate either the pp52^{src} encoded by CH*dl*121, a mutant containing a deletion of residues 82 to 169 (Fig. 2B, lanes 3 and 4), or the pp45^{src} encoded by CH*dl*129, a mutant

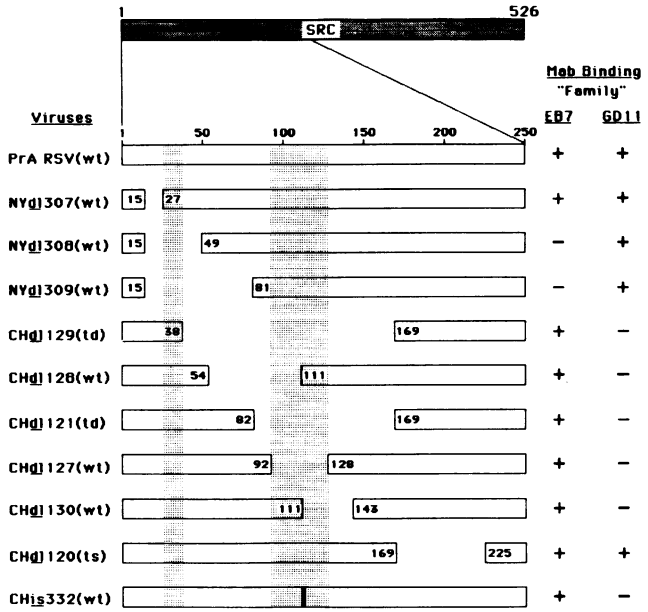

FIG. 1. Reactivity profile of MAbs with *src* proteins encoded by mutants of RSV. The boundaries of the deleted amino acid residues in the *src* protein specified by each of the RSV mutants are indicated by open, horizontal bars. The ability of the MAb families to immunoprecipitate each of the altered *src* proteins is listed in the right-hand column, whereas the putative binding domains of the two families are indicated by stippled, vertical bars. The EB7 family mapped to a region bounded by amino acid residues 28 to 38, and the GD11 family mapped to residues 92 to 128. The closed, vertical bar represents an insertion of four amino acids at position 110.

* Corresponding author.

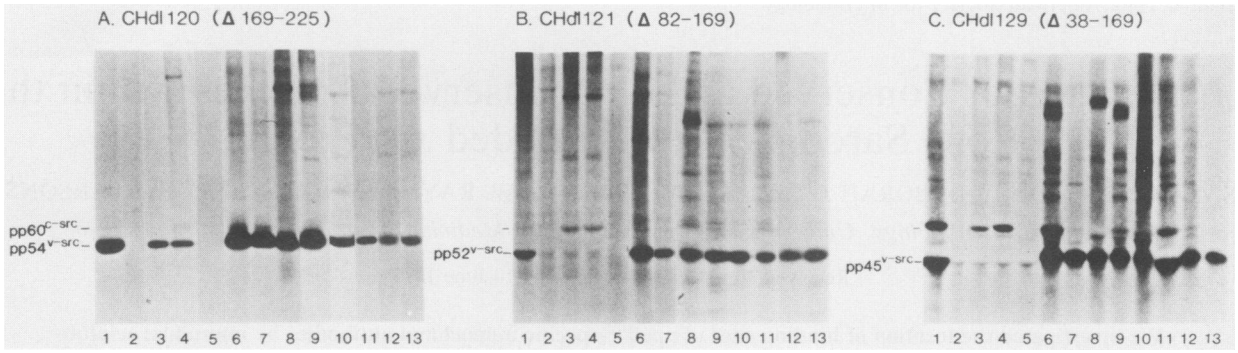

FIG. 2. Immunoprecipitation of structurally altered *src* proteins encoded by Pr-A-RSV mutants CH*dl*120, CH*dl*121, and CH*dl*129. Infected cells were labeled with $^{32}P_i$ for 3 h, and extracts were immunoprecipitated with MAbs or rabbit antibody to bacterial *src* (anti-bp60^{src}) as described previously (21). Lanes: 1, anti-bp60^{src}; 2, normal rabbit serum; 3, GD11; 4, EB8; 5, SP2/0 ascites fluid (control MAb); 6, EC10; 7, EB7; 8, R2D2; 9, FG1; 10, FC6; 11, GB6; 12, GF3; and 13, HC5. The various degrees of efficiency with which the MAbs immunoprecipitated the structurally altered *src* proteins resulted from experimental variability and imply nothing about the nature of the epitopes. The molecular weight of the mutant *src* proteins in this figure and in ensuing figures was determined relative to that of a mixture of known proteins: phosphorylase *a*, 97,000; bovine serum albumin, 66,000; human gamma globulin heavy chain, 55,000; ovalbumin, 45,000; and cytochrome *c*, 12,500. The autoradiograph was exposed for 12 h.

containing a deletion of residues 38 to 169 (Fig. 2C, lanes 3 and 4). Antibodies EB7, EC10, R2D2, FG1, FC6, GB6, GF3, and HC5, on the other hand, readily immunoprecipitated the altered forms of *src* protein from cells infected with CH*dl*121 (Fig. 2B, lanes 6 to 13) or CH*dl*129 (Fig. 2C, lanes 6 to 13). This experiment indicated that the antibodies EB7, EC10, R2D2, FG1, FC6, GB6, GF3, and HC5 (the EB7 family) recognized an "epitope(s)" within the first 38 amino acids of pp60^{src}, whereas GD11 and EB8, (the GD11 family) recognized an "epitope(s)" residing within amino acid residues 82 to 169 (Fig. 1).

To map more specifically the "epitopes" recognized by the EB7 family of antibodies, each of the antibodies was used to immunoprecipitate extracts prepared from cells infected with RSV mutants containing deletions within the distal amino-terminal portion of the *src* gene. All of the members of the EB7 family of MAbs failed to immunoprecipitate the *src* proteins from cells infected with either NY*dl*309 (deletion of residues 15 to 81) (Fig. 3C, lanes 6 to 13) or NY*dl*308 (deletion of residues 15 to 49) (Fig. 3B, lanes 6 to 13). In contrast, immunoprecipitation of extracts from cells infected with NY*dl*307 (deletion of residues 15 to 27) revealed that each of the antibodies of the EB7 family (with the exception of R2D2) reacted strongly with this form of the *src* protein (Fig. 3A, lanes 6 to 13), as well as with pp60^{src} from the wild-type Schmidt-Ruppin strain of RSV (SR-RSV)-infected cells (data not shown). Figure 3 also shows that the antibodies GD11 and EB8 immunoprecipitated the structurally altered forms of *src* protein from cells infected with each of the NY-RSV mutants, thus confirming that the GD11 and EB8 epitope(s) resides carboxyterminal to amino acid residue 81. From the data illustrated in Fig. 1, 2, and 3, we conclude that the antibodies belonging to the EB7 family recognize "epitopes" residing within amino acid residues 28 to 38. Previous experiments (21) have demonstrated that MAbs of the EB7 family bind pp60$^{v\text{-}src}$ in a Western immunoblot with various degrees of efficiency. These data are consistent with the interpretation that the epitope(s) recognized by this family may be sequential in nature. Localization of the amino-terminal epitope recognized by R2D2, however, remains somewhat ambiguous, since this antibody did not efficiently immunoprecipitate pp60$^{v\text{-}src}$ encoded by SR-RSV (Fig. 3 and unpublished data) and appears to bind weakly a region in the carboxy half of pp60$^{v\text{-}src}$ encoded by Pr-A-RSV (D. J. McCarley, J. T. Parsons, D. C. Benjamin, and S. J. Parsons, submitted for publication).

In an attempt to more clearly map the epitopes recognized by the GD11 and EB8 antibodies, extracts from cells infected with RSV mutants encoding *src* proteins containing small deletions within the region 54 to 143 were immunoprecipitated and analyzed by polyacrylamide gel electrophoresis. Neither of the antibodies recognized the *src* proteins specified by two contiguous deletion mutants, CH*dl*128, containing a deletion of residues 54 to 111 (Fig.

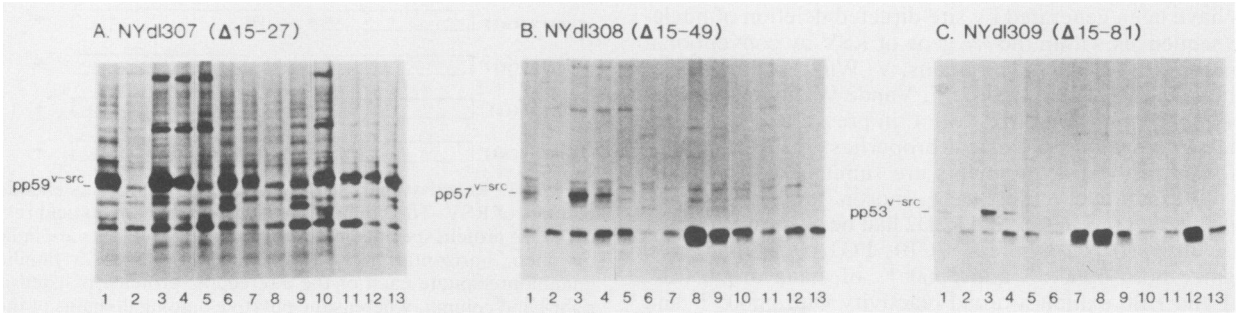

FIG. 3. Immunoprecipitation of structurally altered *src* proteins encoded by the SR-RSV mutants NY*dl*307, NY*dl*308, and NY*dl*309. CE cells infected with each of the SR-RSV deletion mutants were labeled for 6 h with [^{35}S]methionine, and extracts were immunoprecipitated as described in the legend to Fig. 2. Lanes: 1, anti-bp60^{src}; 2, normal rabbit serum; 3, GD11; 4, EB8; 5, SP2/0 ascites fluid; 6, EC10; 7, EB7; 8, R2D2; 9, FG1; 10, FC6; 11, GB6; 12, GF3; and 13, HC5. The autoradiograph was exposed for 18 h.

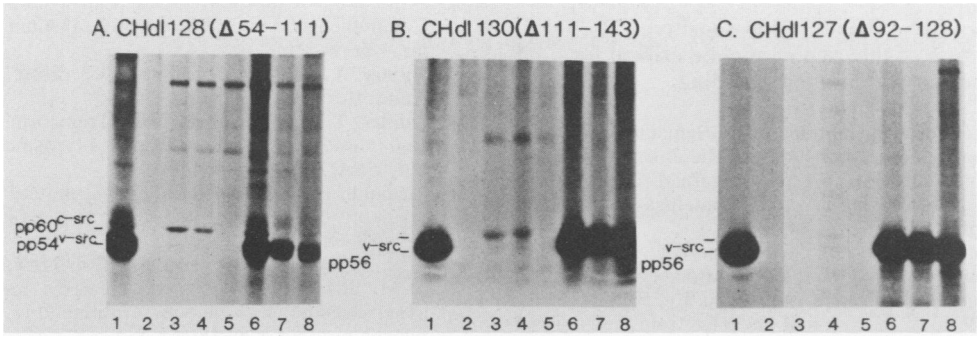

FIG. 4. Immunoprecipitation of *src* proteins encoded by the Pr-A-RSV deletion mutants CH*dl*128, CH*dl*130, and CH*dl*127. CE cells infected with each of the respective deletion mutants were labeled for 2 h with $^{32}P_i$ and immunoprecipitated as described in the legend to Fig. 2. Lanes: 1, anti-bp60^{src}; 2, normal rabbit serum; 3, GD11; 4, EB8; 5, SP2/0 ascites fluid; 6, EC10; 7, EB7; and 8, R2D2. The autoradiograph was exposed for 18 h.

4A, lanes 3 and 4), CH*dl*130, containing a deletion of residues 11 to 143 (Fig. 4B, lanes 3 and 4), or by the deletion mutant CH*dl*127, containing a deletion of residues 92 to 128 (Fig. 4C, lanes 3 and 4). These results appear to indicate that the "epitope(s)" recognized by the GD11 and EB8 antibodies resides either wholly or in part within residues 92 to 128 (Fig. 1). This latter conclusion is further supported by the observation that the *src* protein encoded by CH*is*332, a linker insertion mutant containing a four-amino-acid insertion between residues 110 and 111 (nucleotide 332 of the *src* gene squence [23]), was not immunoprecipitated by GD11 (Fig. 5, lanes 1, 2, and 3) or EB8 antibodies (data not shown).

Assignment of "epitopes" to defined regions of the *src*

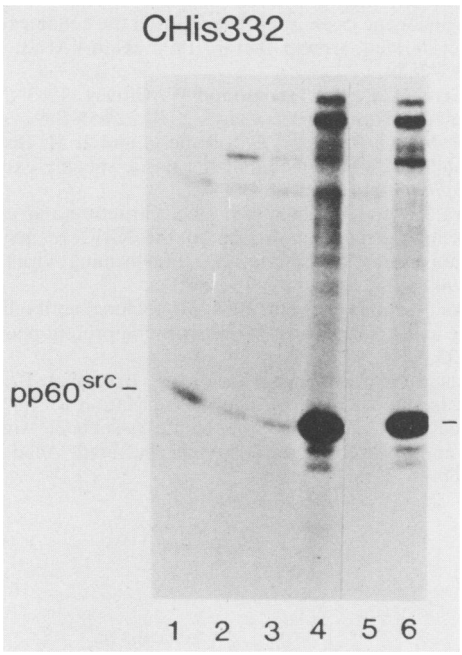

FIG. 5. Immunoprecipitation of *src* proteins encoded by the Pr-A-RSV insertion mutant CH*is*332. CE cells infected with CH*is*332 were labeled for 3 h with $^{32}P_i$ and aliquots of the radiolabeled extract were immunoprecipitated with GD11 (lane 1), EB7 (lane 4), or SP2/0 ascites fluid (lane 5). The supernatant fluid remaining after the removal of the GD11 immune complexes (lane 1) was subjected to second (lane 2) and third (lane 3) immunoprecipitations with GD11 antibody and was finally immunoprecipitated with EB7 antibody (lane 6). The autoradiograph represents an 18-h exposure.

protein was predicated on at least two assumptions. First, we assumed that each of the antibodies had a single, high-avidity binding site within pp60^{src}. Second, we assumed that the lack of reactivity of a given antibody with a structurally altered form of the *src* protein reflected either complete or partial removal of its epitope or perturbation of the framework required for the proper structural presentation of the contact residues. We cannot determine in our analysis whether the actual binding site of the antibody resides within or adjacent (perhaps even distal) to the deleted sequence.

The conserved nature of the "epitope" recognized by the GD11 family of antibodies poses the interesting question of whether this region of the *src* protein bears a functional domain. In the case of pp60$^{v\text{-}src}$, results of deletion mutagenesis (Parsons et al., in press) (Fig. 1) clearly have indicated that the region of GD11-EB8 binding is not required to mediate efficient cellular transformation (V. W. Raymond and J. T. Parsons, manuscript in preparation). Furthermore, in support of this view we have found that microinjection of GD11 into RSV-transformed rat cells does not significantly alter the morphological properties of these cells (D. Stacey and S. Parsons, unpublished observations). However, the role of this region in maintaining the structural or functional integrity or both of cellular pp60^{src} remains unclear. A comparison of the deduced amino acid sequence of the second coding exon (amino acids 81 to 114) of chicken and human c-*src* has revealed extensive sequence homology (25; A. Tanaka, C. P. Gibbs, H.-J. Kung, and D. Fujita, personal communication). Similarly, a comparison of the deduced amino acid sequence of chicken c-*src* with that of the distantly related *Drosophila* c-*src* protein reveals a rather striking homology throughout this region (24, 25); 14 of 19 residues are conserved between positions 89 and 107 (74% homology), and 23 of 40 residues are conserved between positions 89 and 128 (57% homology) as compared with 95 of the first 250 residues (38% homology). Therefore, it remains possible that the highly conserved "epitope" recognized by the GD11-EB8 antibodies may reflect the preservation of a necessary functional domain of the cellular *src* protein.

Recently, several investigators have suggested that phosphorylation of amino-terminal tyrosine residues may serve to activate cellular pp60^{src} in a variety of cell types (3, 4, 22, 27). Potential sites of tyrosine modification in chicken pp60^{src} reside at positions 90 and 92, sites at or near the "epitope" recognized by the GD11 and EB8 MAbs. We speculate that the conserved nature of this portion of pp60^{src} may represent a common site of modification and regulation of the *src* protein. In addition, since the GD11-EB8

"epitope" appears to be denaturation sensitive (21), third-dimensional structure of this region may be critical for both function of the protein and antibody binding.

We are grateful for the contributions of D. Benjamin during the derivation of the v-*src*-specific MAbs, the excellent technical assistance of B. Creasy and C. Ely, and the many helpful discussions with M. Weber. We also thank E. Garber and H. Hanafusa for providing the NY-RSV mutants.

S.J.P. is a Scholar of the Leukemia Society of America, and J.T.P. is a recipient of an American Cancer Society Faculty Research Award. This research was supported by Public Health Service grants CA29243, CA27578, and CA39438 from the National Institutes of Health and grant NP-462G from the American Cancer Society.

LITERATURE CITED

1. **Bishop, J. M.** 1983. Cellular oncogenes and retroviruses. Annu. Rev. Biochem. **52**:301–354.
2. **Bishop, J. M.** 1985. Viral oncogenes. Cell **42**:23–38.
3. **Bolen, J. B., N. Rosen, and M. A. Israel.** 1985. Increased $pp60^{c\text{-}src}$ tyrosyl kinase activity in human neuroblastomas is associated with amino-terminal tyrosine phosphorylation of the *src* gene product. Proc. Natl. Acad. Sci. USA **82**:7275–7279.
4. **Brugge, J. S., P. C. Cotton, A. E. Queral, J. N. Barrett, D. Nonner, and R. W. Keane.** 1985. Neurons express high levels of a structurally modified, activated form of $pp60^{c\text{-}src}$. Nature (London) **316**:554–557.
5. **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.
6. **Bryant, D., and J. T. Parsons.** 1982. Site-directed mutagenesis of the *src* gene of Rous sarcoma virus: construction and characterization of a deletion mutant temperature sensitive for transformation. J. Virol. **44**:683–691.
7. **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.
8. **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.
9. **Cooper, J. A., K. D. Nakamura, T. Hunter, and M. J. Weber.** 1983. Phosphotyrosine-containing proteins and expression of transformation parameters in cells infected with partial transformation mutants of Rous sarcoma virus. J. Virol. **46**:15–28.
10. **Cooper, J. A., N. A. Reiss, R. J. Schwartz, and T. Hunter.** 1983. Three glycolytic enzymes are phosphorylated at tyrosine in cells transformed by Rous sarcoma virus. Nature (London) **302**:218–223.
11. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence in the $p60^{src}$ N terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol. Cell. Biol. **4**:1834–1842.
12. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Publishing Corp., New York.
13. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
14. **Hunter, T., and B. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Levinson, A. D., H. Opperman, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561–572.
17. **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.
18. **Parsons, J. T., D. Bryant, V. Wilkerson, G. Gilmartin, and S. J. Parsons.** 1984. Site-directed mutagenesis of Rous sarcoma virus $pp60^{src}$: identification of functional domains required for transformation, p. 36–42. *In* G. F. Vande Woude, A. J. Levine, W. C. Topp, and J. D. Watson (ed.), Cancer cells: oncogenes and viral genes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Parsons, S. J., and C. Creutz.** 1986. $P60^{c\text{-}src}$ activity detected in the chromaffin granule membrane. Biochem. Biophys. Res. Commun. **134**:736–742.
20. **Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons.** 1983. Isolation and characterization of a monoclonal antibody to the Rous sarcoma virus-transforming protein, $pp60^{src}$. J. Virol. **45**:1190–1194.
21. **Parsons, S. J., D. J. McCarley, C. Ely, D. C. Benjamin, and J. T. Parsons.** 1984. Monoclonal antibodies to the Rous sarcoma virus $pp60^{src}$ react with enzymatically active cellular $pp60^{src}$ of avian and mammalian origin. J. Virol. **51**:272–282.
22. **Ralston, R., and J. M. Bishop.** 1985. The product of the proto-oncogene c-*src* is modified during the cellular response to platelet-derived growth factor. Proc. Natl. Acad. Sci. USA **82**:7845–7849.
23. **Schwartz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of Rous sarcoma virus. Cell **32**:853–869.
24. **Simon, M. A., B. Drees, T. Kornberg, and J. M. Bishop.** 1985. The nucleotide sequence and the tissue-specific expression of *Drosophila* c-*src*. Cell **42**:831–840.
25. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. Cell **32**: 881–890.
26. **Tanaka, T., and R. Kurth.** 1984. Monoclonal antibodies specific for the avian sarcoma virus transforming protein $pp60^{src}$. Virology **133**:202–210.
27. **Yonemoto, W., M. Jarvis-Morar, J. S. Brugge, J. B. Bolen, and M. A. Israel.** 1985. Tyrosine phosphorylation within the amino-terminal domain of $pp60^{c\text{-}src}$ molecules associated with polyoma virus middle-sized tumor antigen. Proc. Natl. Acad. Sci. USA **82**:4568–4572.