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Evidence that There Exist Four Classes of RNA Tumor Viruses Which Encode Proteins with Associated Tyrosine Protein Kinase Activities

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The transforming protein of Rous sarcoma virus, p60^{src}, the Abelson virus protein, p120, and the Y73 virus protein, p90, all have associated tyrosine protein kinase activities in vitro. Possible structural homology between these functionally related proteins was investigated by two-dimensional analysis of both methionine-containing and phosphate-containing tryptic peptides derived from biosynthetically labeled proteins. Marked differences were found between the maps of both [^{35}S]methionine-labeled and ^{32}P-labeled tryptic peptides. This suggests that the transforming gene of Rous sarcoma virus and the putative transforming genes of Abelson virus and Y73 virus are different. In addition, each of these genes has been shown previously to be unrelated to the putative transforming gene of Fujinami sarcoma virus, another virus which encodes a protein with associated tyrosine protein kinase activity. Therefore, it appears that there exist at least four distinct classes of functionally related RNA tumor viruses. Analysis of phosphorylated tryptic peptides did, however, reveal homology between one of the two phosphotyrosine-containing tryptic peptides of p90 of Y73 virus and the single phosphotyrosine-containing tryptic peptide of p60^{src} of Rous sarcoma virus. Comigration of these two peptides in several different buffers and the identical mobility of their phosphorylated cleavage products after secondary digestion with protease V8 of *Staphylococcus aureus* indicated that p60^{src} and p90 contain an identical site of tyrosine phosphorylation in vivo. The results are discussed with respect to the evolution of RNA tumor viruses which encode proteins with associated tyrosine protein kinase activities and the limitations of analysis of detecting homology between genes by both molecular hybridization and peptide mapping.

The biochemical basis of cellular transformation by a tumor virus is best understood in the case of Rous sarcoma virus (RSV). The transforming functions of RSV are encoded by a single gene, *src* (21, 50), which is unrelated to the genes encoding the viral structural proteins and is not involved in viral replication. The *src* gene product, p60^{src} (7, 35), is a phosphoprotein (8, 29) which has an associated protein kinase activity (9, 29) that phosphorylates tyrosine in vitro (25). There is strong evidence that this in vitro activity is an intrinsic property of p60^{src} itself: (i) tyrosine protein kinase activity is associated with p60^{src} produced by in vitro translation (11, 40); (ii) this activity is retained in partially purified p60^{src} (10, 12, 30); and (iii) partially purified p60^{src} from a mutant of RSV temperature sensitive for transformation has an associated protein kinase activity which is seven times more heat labile than that of wild-type p60^{src} (12, 30). In addition, the fact that all cells transformed by RSV contain an approximately 10-fold-elevated level of phosphotyrosine in protein which is immediately temperature dependent in cells infected by mutants temperature sensitive for transformation suggests both that p60^{src} acts as a tyrosine protein kinase also in vivo and that phosphorylation of tyrosine by p60^{src} is an important step for transformation by RSV (42).

There is now evidence that this unusual modification of cellular proteins may also be important for cellular transformation by several other retroviruses. Three independently isolated avian sarcoma viruses (Fujinami sarcoma virus [FSV], PRCII virus, and Y73 virus), two independently isolated feline sarcoma viruses (Snyder-Theilen sarcoma virus [ST-FeSV] and Gardner-Arnstein sarcoma virus [GA-FeSV]), and the Abelson murine leukemia virus (A-MuLV) all encode phosphoproteins which have associated tyrosine protein kinase activities when assayed in vitro (2, 14, 26, 32, 33, 37, 48, 53; K. Beemon, Cell, in press). Additionally, transfor-

mation of cells with FSV, PRCII virus, Y73 virus, A-MuLV, and ST-FeSV has been shown to lead to a significant increase in the level of phosphotyrosine in cellular protein (2; Beemon, in press; B. Sefton, T. Hunter, and W. Raschke, Proc. Natl. Acad. Sci. U.S.A., in press).

The genomes of all of these viruses resemble each other in that they are chimeric; a portion is homologous to that of a leukemia virus, and a portion is homologous to a gene or genes present in uninfected cells (16, 19, 44, 45, 55). This has led to the idea that these transforming viruses arose through transduction of a cellular gene by a nontransforming virus. It is thought that this transduced cellular genetic information is responsible for both the ability of these viruses to transform fibroblasts and the tyrosine protein kinase activities associated with the proteins they encode. Except in the case of RSV, the acquired cellular information is expressed as part of a hybrid protein: the amino terminus is encoded by a part of the gene for the precursor to the internal structural proteins of the parental leukemia virus, and the C-terminal domain is encoded by the acquired cellular sequences (3, 22, 26, 28, 31, 47, 54).

The functional similarity of the proteins encoded by this diverse group of tumor viruses suggested that the acquired transformation-specific information of these viruses might be structurally related. That this is indeed the case for four of these viruses has recently been demonstrated. Shibuya et al. (43) have shown by molecular hybridization that the cell-derived sequence of FSV is related to those of PRCII virus, ST-FeSV, and GA-FeSV. The relationship of the transformation-specific sequences of this group of viruses is also demonstrated by the similarity of the tryptic peptide maps of the proteins encoded by these viruses (3, 47; Beemon, in press; J. Neil, J. Delamarter, and P. K. Vogt, Proc. Natl. Acad. Sci. U.S.A., in press). It is also clear, however, that not all of the viruses which encode proteins that have associated tyrosine protein kinase activities contain transformation-specific sequences which are obviously related at the level of nucleic acid sequence. Both molecular hybridization and analysis of oligonucleotides have demonstrated that the *src* gene of RSV and the acquired sequence of Y73 virus are unrelated both to the transformation-specific sequences of the aforementioned group of viruses (22, 28, 55) and to each other (26, 55). These results suggest that at least three classes of functionally homologous viruses exist. Although Shibuya et al. (43) have shown that A-MuLV is not obviously related to FSV, it is not clear whether A-MuLV defines yet another class or is related to RSV or Y73 virus. To address this question, we have analyzed two-dimensional maps of both methionine-containing and phosphate-containing tryptic peptides of biosynthetically labeled p120 of A-MuLV (36, 54), p90 of Y73 virus (26), and $p60^{src}$ of RSV. We have chosen peptide mapping for this comparison, in part because homologies between genes and gene products are sometimes not apparent from molecular hybridization. For example, molecular hybridization demonstrates that the gene for human $p60^{proto\text{-}src}$ differs significantly from that for chicken $p60^{proto\text{-}src}$ (44, 45), whereas peptide mapping demonstrates that the polypeptides are nearly identical (41). Our results show that p120, p90, and $p60^{src}$ are in large part structurally unrelated and thus indicate that these proteins are encoded by different genes. Apparent homology between p90 of Y73 virus and $p60^{src}$ of RSV was found, however, at a site of tyrosine phosphorylation.

MATERIALS AND METHODS

Cells and viruses. The preparation and infection, with avian sarcoma viruses, of chicken embryo fibroblasts have been described (39, 41). Y73 virus, with a helper virus of subgroup B, was obtained from K. Toyoshima, University of Tokyo. The ANN-1 line of NIH/3T3 cells nonproductively infected with A-MuLV (38) was from W. Raschke, and NIH/3T3 cells productively infected with Moloney murine leukemia virus (M-MuLV), clone 1, were from D. Donoghue, both of the Salk Institute.

Radioactive labeling and in vitro translation. Schmidt-Ruppin RSV, subgroup A (SR-RSV-A)-transformed chicken embryo fibroblasts in 100-mm petri dishes were labeled overnight with 3 mCi of [^{35}S]methionine (>500 Ci/mmol; Amersham/Searle) or 10 mCi of $^{32}P_i$ (ICN; carrier-free) in 5 ml of medium. Labeling with [^{35}S]methionine was performed in Dulbecco modified Eagle medium (DMEM) containing 5% the normal methionine concentration and 4% calf serum. Labeling with $^{32}P_i$ was in phosphate-free DMEM supplemented with 4% calf serum dialyzed against phosphate-free saline.

Chicken embryo fibroblasts infected with Y73 virus and its associated helper were also grown on 100-mm dishes and were labeled with [^{35}S]methionine or $^{32}P_i$ under the same conditions, except that the labeling time was 3 h.

ANN-1 cells, grown on 50-mm dishes, were labeled for 3 h with 2 mCi of [^{35}S]methionine in 2 ml of methionine-free DMEM supplemented with 10% fetal calf serum dialyzed against saline or with 5 mCi of $^{32}P_i$ in 2 ml of DMEM lacking phosphate and supplemented with 4% dialyzed (against phosphate-free saline) calf serum.

M-MuLV-infected 3T3 cells growing in a 50-mm dish were labeled for 2 h with 2 mCi of $^{32}P_i$ in 2 ml of phosphate-free DMEM supplemented with 10% fetal calf serum.

Heat-denatured viral 70S RNA of M-MuLV, clone 1 (obtained from J. Papkoff, Salk Institute), was translated in the presence of [^{35}S]methionine (10 mCi/ml),

using the messenger-dependent reticulocyte lysate (34) as described (4).

Cell lysis and immunoprecipitation. The procedures of cell lysis with RIPA buffer supplemented with 2 mM EDTA and immunoprecipitation have been described (39, 41).

Rabbit anti-RSV tumor serum was used to isolate $p60^{src}$ and was adsorbed to detergent-disrupted SR-RSV-D virions (2.5 μg of proteins/μl of serum) before use to prevent contamination of the $p60^{src}$ preparation by $Pr60^{gag}$, an intermediate cleavage product of $Pr76^{gag}$ (51). Rabbit antiserum against PR-RSV-B virions was obtained from J. Neil, University of Southern California, Los Angeles, and was used to precipitate Y73 p90 and $Pr76^{gag}$ of the Y73 virus-associated helper virus YAV (26). Rabbit antiserum to PR-RSV-C $gp85^{env}$ (obtained from D. Bolognesi) was used to isolate YAV $Pr92^{env}$. A-MuLV p120 and M-MuLV $Pr65^{gag}$ were precipitated by goat antiserum against Rauscher MuLV $p15^{gag}$.

Gel electrophoresis, electroelution, and tryptic digestion. Immunoprecipitated proteins and in vitro translation products were purified by electrophoresis through 2-mm-thick sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 15% acrylamide and 0.9% bisacrylamide as described (42). The proteins to be analyzed were localized by autoradiography, excised, and electroeluted into 0.01 M NH_4HCO_3, 0.1% SDS, and 2% mercaptoethanol (52).

After addition of 5 to 10 μg of carrier protein, the proteins were precipitated with trichloroacetic acid, oxidized with performic acid, and digested with tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Worthington Biochemicals Corp.) as described in detail (4).

Two-dimensional peptide analysis. Analysis of [^{35}S]methionine-labeled tryptic peptides by electrophoresis at pH 4.7 and ascending chromatography was performed as described (4). Two-dimensional analysis of phosphorylated tryptic peptides was carried out by electrophoresis at pH 8.9 and ascending chromatography (25).

Protease V8 digestion and phosphoamino acid analysis. Tryptic phosphorylated peptides were eluted from thin-layer plates into a buffer consisting of acetic acid-formic acid (88% by volume)-water (78:25:897 by volume), pH 1.9. The buffer was removed by lyophilization, and the peptides were incubated overnight at room temperature with 10 μg of *Staphylococcus aureus* protease V8 (Miles Laboratories, Inc.) in 10 μl of 0.05 M ammonium acetate, pH 4.0. After incubation, 100 μl of water was added, and the samples were lyophilized. The protease V8 digestion products were analyzed as described for the phosphorylated tryptic peptides above.

Partial hydrolysis of eluted phosphorylated peptides was performed in 6 M HCl for 1 h at 110°C under N_2 (25). The hydrolysates were analyzed in the presence of internal markers (phosphoserine, phosphothreonine, and O^4-phosphotyrosine, each 0.5 μg) by electrophoresis at pH 3.5 for 45 min at 1 kV in glacial acetic acid-pyridine-water (50:5:945 by volume). The markers were detected by staining with ninhydrin.

[^{35}S]methionine-labeled peptides were visualized by fluorography (6), whereas detection of ^{32}P-labeled peptides and phosphoamino acids was facilitated by the use of intensifying screens as described (27).

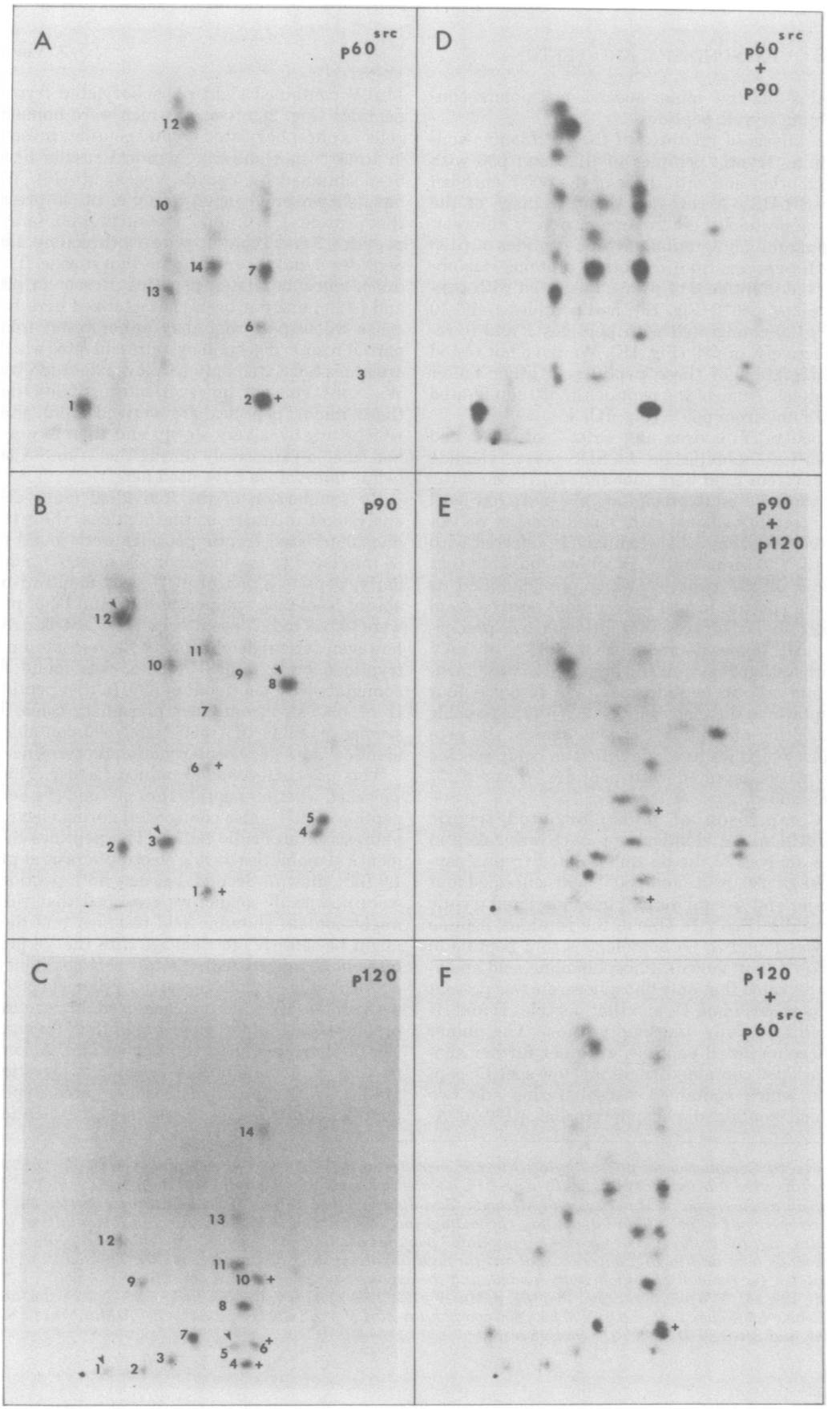
RESULTS

Comparison of methionine-containing tryptic peptides. The putative transforming proteins of A-MuLV (p120) and of Y73 virus (p90) differ from $p60^{src}$ in that they are hybrid proteins. Their amino-terminal domains are closely related to those of the precursors to the internal structural proteins of M-MuLV and of some unidentified avian leukosis virus, respectively. To be able to identify the tryptic peptides specific to p120 and p90, we prepared tryptic peptide maps of $Pr65^{gag}$ of M-MuLV, produced by in vitro translation of virion RNA, and of $Pr76^{gag}$ of the helper virus, YAV, present in the stock of Y73 virus used for the experiments reported here. The latter was done by immunoprecipitation of biosynthetically labeled material.

The map of [^{35}S]methionine-labeled p120 of A-MuLV contained 14 peptides (Fig. 1C). Peptides 1 and 5 (arrows in Fig. 1C) corresponded to peptides found in $Pr65^{gag}$ (not shown). p120 contained, therefore, 12 specific methionine-containing tryptic peptides. Quite similar results have been obtained by Van de Ven et al. (49).

p90 of Y73 virus contained seven major and five minor methionine-containing tryptic peptides (Fig. 1B). Three peptides indicated with arrows comigrated with peptides from $Pr76^{gag}$ of the YAV helper virus (not shown) and were derived from $p19^{gag}$ (40; Beemon, in press; Neil et al., in press). Therefore p90 contained four

FIG. 1. *Comparison of methionine-containing tryptic peptides of SR-RSV-A $p60^{src}$, Y73 p90, and A-MuLV p120. The proteins were labeled biosynthetically with [^{35}S]methionine, isolated by immunoprecipitation, and digested with TPCK-trypsin as described in Materials and Methods. The digests were separated on cellulose thin-layer plates by electrophoresis at pH 4.7 in the first dimension and ascending chromatography in the second. The origin is at the lower left, and the cathode is at the right. Arrows indicate gag-related peptides in p90 and p120, respectively. Peptides which were found to comigrate in mixtures of digests are indicated by +. The numbers for the $p60^{src}$ peptides were adapted from Beemon et al. (5). The source of the peptides, the amount of radioactivity loaded, and the exposure times are as follows: SR-RSV-A $p60^{src}$, 6,760 cpm, 7 days (A); Y73 p90, 5,980 cpm, 4 days (B); A-MuLV p120, 1,850 cpm, 7 days (C); SR-RSV-A $p60^{src}$, 9,900 cpm, and Y73 p90, 1,700 cpm, 14 days (D); Y73 p90, 2,530 cpm, and A-MuLV p120, 2,690 cpm, 13 days (E); A-MuLV p120, 2,000 cpm, and SR-RSV-A $p60^{src}$, 3,400 cpm, 11 days (F).*


A
p60 src
12
10
14
7
13
6
3
1
2
+
D
p60 src
+
p90
B
p90
12
11
10
9
8
7
6
+
5
4
2
3
1
+
E
p90
+
p120
+
+
C
p120
14
13
12
11
9
10
+
8
7
5
6
+
3
4
+
2
1
F
p120
+
p60 src
+

major and five minor specific methionine-containing tryptic peptides.

Analysis of mixtures of the methionine-containing tryptic peptides of p120 and p90 with each other and with those of the $p60^{src}$ encoded by SR-RSV-A revealed that the maps of the three proteins were in large part very different. Apparent comigration of three peptides of p120 with peptides from the other proteins was observed. Peptide 6 of p120 comigrated with peptide 2 of $p60^{src}$ (Fig. 1F), and peptides 4 and 10 of p120 comigrated with peptides 1 and 6, respectively, of p90 (Fig. 1E). We have not tested comigration of these peptides in other buffer systems. None of the peptides in p90 comigrated with any from $p60^{src}$ (Fig. 1C).

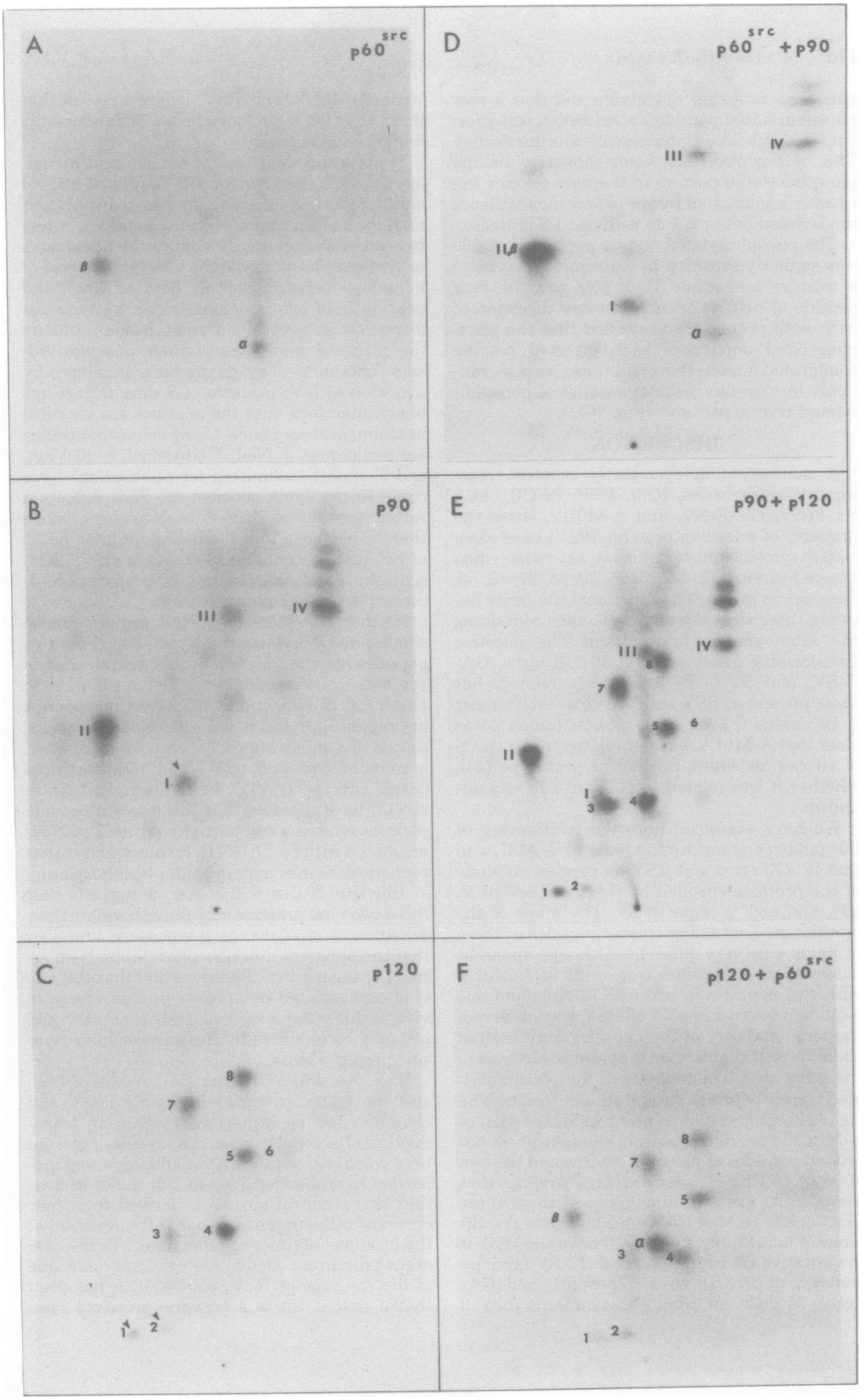
p90 of Y73 virus and $Pr92^{env}$ of YAV had identical mobilities on the SDS-polyacrylamide gel system used here (not shown). Because the serum we used to precipitate p90 had a low level of reactivity against $Pr92^{env}$ and because we isolated p90 from cells productively infected with both Y73 virus and YAV, it was possible that some of the peptides which we identified as being specific to p90 were in fact derived from $Pr92^{env}$. To address this question, we precipitated [^{35}S]methionine-labeled $Pr92^{env}$ of YAV with anti-gp85 serum and prepared a two-dimensional tryptic peptide map. We resolved four peptides in the map of $Pr92^{env}$. With the possible exception of peptide 1 of p90, none of the peptides which we have identified as being specific to p90 comigrated with peptides from $Pr92^{env}$ (not shown).

Comparison of phosphorylated tryptic peptides. An identical approach was taken in the analysis of the phosphorylated tryptic peptides of p90, p120, and $p60^{src}$. p90 contained four major and several minor phosphorylated tryptic peptides (Fig. 2B). One of the peptides, peptide I, was found to be homologous to a peptide in $Pr76^{gag}$ (not shown). Phosphoamino acid analysis revealed that only phosphoserine was present in peptides I and IV and that peptides II and III contained only phosphotyrosine. The minor phosphorylated peptides were not further analyzed. p90 contained, therefore, one specific peptide which contained phosphoserine and two which contained phosphotyrosine. p120 of A-MuLV contained eight phosphorylated tryptic peptides (Fig. 2C), two of which were homologous to phosphorylated tryptic peptides present in $Pr65^{gag}$ (not shown). Identical results have been obtained by Van de Ven et al. (48). We have shown previously (Sefton et al., in press) that peptides 1, 2, 6, and 7 contain phosphoserine peptides, 3 and 5 contain phosphothreonine, and peptides 4 and 8 contain phosphotyrosine. The minor phosphorylated peptides present in p90 and p120 have not been characterized here because we suspect that they either result from partial tryptic digestion or represent sites which are phosphorylated only to a low extent in vivo. We cannot rule out, however, the possibility that these minor peptides are derived from sites which turn over very slowly and therefore are not labeled extensively during the 2- to 3-h labeling interval we have used here.

By comparison of the individual maps and analysis of mixtures of the peptides, the p120 phosphorylated tryptic peptides were found to be unrelated to any in either p90 or $p60^{src}$. Similarly, peptide alpha of $p60^{src}$ was found to be absent from p90, and peptides III and IV of p90 were found to be absent from $p60^{src}$. Strikingly, however, the single phosphotyrosine-containing tryptic peptide of $p60^{src}$, beta, was found to comigrate with peptide II of p90. In that peptide II of p90 also contained phosphotyrosine, it seemed possible that $p60^{src}$ and p90 contained identical sites of phosphorylation on tyrosine.

This possibility was examined further. First, we asked whether peptide II of p90 and the beta peptide of $p60^{src}$ also comigrated during electrophoresis in an acidic buffer. The peptides had identical mobilities during electrophoresis at pH 1.9 (not shown). Second, because both peptides were noticeably acidic, we reasoned that they might contain glutamic acid residues, and thus might be sensitive to cleavage with the V8 protease of *S. aureus* (23). To test this possibility, we incubated a tryptic digest of ^{32}P-labeled $p60^{src}$ with protease V8 and then analyzed the reaction products under our standard conditions for analysis of phosphorylated tryptic peptides. A comparison of a map of the secondary digestion products with the map of phosphorylated tryptic peptides of $p60^{src}$ (Fig. 3A) revealed that peptide

FIG. 2. *Comparison of phosphorylated tryptic peptides of RSV $p60^{src}$, Y73 p90, and A-MuLV p120. The proteins were labeled biosynthetically with $^{32}P_i$, isolated by immunoprecipitation, and digested with TPCK-trypsin as described in Materials and Methods. Two-dimensional analysis of the digests was carried out by electrophoresis at pH 8.9 and ascending chromatography. The origin is approximately in the middle of the plates, and the cathode is at the right. gag-related peptides are indicated by arrows. ^{32}P-labeled peptides in the maps here and in Fig. 3 were visualized through the use of intensifying screens (27). The source of the peptides, the amount of radioactivity loaded, and the exposure times are as follows: SR-RSV-A $p60^{src}$, 1,200 cpm, 18 h (A); Y73 p90, 5,200 cpm, 18 h (B); A-MuLV p120, 2,590 cpm, 18 h (C); SR-RSV-A $p60^{src}$, 320 cpm, and Y73 p90, 1,860 cpm, 2 days (D); Y73 p90, 800 cpm, and A-MuLV p120, 400 cpm, 7 days (E); A-MuLV p120, 630 cpm, and SR-RSV-D $p60^{src}$, 310 cpm, 18 h (F).*


A
p60 src
β
α
D
p60 src + p90
III
IV
II,β
I
α
B
p90
III
IV
II
I
C
p120
8
7
5
6
3
4
1
2
E
p90 + p120
III
IV
8
7
5
6
II
I
3
4
1
2
F
p120 + p60 src
8
7
5
β
α
3
4
1
2

beta was no longer detectable and that a new phosphorylated peptide, called beta*, was present (Fig. 3B). The alpha peptide was unaffected. The new peptide has been shown to be the phosphorylated portion of the beta peptide because it is generated by secondary incubation of the isolated beta peptide with the V8 protease.

The phosphorylated tryptic peptide II of p90 was similarly sensitive to digestion. Analysis of a mixture of peptide II of p90 and the beta peptide of $p60^{src}$ after a secondary digestion of each with protease V8 revealed that the phosphorylated portion of each digested peptide comigrated under the conditions we use routinely for two-dimensional analysis of phosphorylated tryptic peptides (Fig. 3C).

DISCUSSION

A number of independently isolated transforming retroviruses, RSV, FSV, PRCII virus, ST-FeSV, GA-FeSV, and A-MuLV, share the property of encoding proteins which have associated tyrosine protein kinase activities when assayed in vitro (2, 14, 25, 26, 32, 33, 37, 48, 53; Beemon, in press). The functional similarity between these viruses is not due to each containing the same genetic information. The putative transforming genes of FSV, PRCII virus, GA-FeSV, and ST-FeSV are clearly related, but those present in RSV and Y73 virus each appear to be unique (22, 26, 28, 43, 55). Although it was clear that A-MuLV was not related to the group of viruses for which FSV is the prototype (43), whether it was related to RSV or Y73 was unknown.

We have examined here the relationship of the putative transforming gene of A-MuLV to that in Y73 virus and RSV by peptide mapping of the proteins encoded by these viruses, p120, p90, and $p60^{src}$, respectively. The maps of the methionine-containing tryptic peptides of these proteins were very different. Although apparent comigration of peptides from p120 of A-MuLV with two peptides in p90 of Y73 virus and one peptide present in $p60^{src}$ of RSV was observed, the large majority of the peptides from each of these three proteins were different from those in the other two. The analysis of the phosphorylated tryptic peptides yielded similar results. The eight phosphorylated tryptic peptides of p120 of A-MuLV were different from the four phosphorylated peptides of p90 of Y73 virus and the two of $p60^{src}$ of RSV. It seems clear, therefore, that the putative transforming genes of these three functionally related RNA tumor viruses are different. Since Shibuya et al. (43) have shown that the putative transforming gene of FSV (and, by analogy, of PRCII virus, ST-FeSV, and GA-FeSV) is different from those present in Y73 virus, A-MuLV, and RSV, it now appears that there exist at least four classes of functionally homologous viruses.

Despite the clear lack of nucleic acid homology between the gene for $p60^{src}$ and p90 (26, 55) and the marked differences in the maps of their methionine-containing tryptic peptides, these two proteins apparently contain identical sites of tyrosine phosphorylation. The large degree of homology between peptide beta of $p60^{src}$ and peptide II of p90 is demonstrated by their comigration in several different buffers and by the identical mobilities of their phosphotyrosine-containing cleavage products generated by digestion with V8 protease of *S. aureus*. It seems likely, therefore, that the peptides are identical in amino acid sequence. Using somewhat different techniques, J. Neil, J. Ghysdael, P. K. Vogt, and J. Smart (submitted for publication) have come to the same conclusions. This reflects a rather significant degree of homology, since there is evidence that the beta peptide of $p60^{src}$ of SR-RSV-A contains 10 amino acids (T. Patschinsky et al., manuscript in preparation; J. Smart, personal communication).

Neither the identity of the protein kinase which phosphorylates the tryosine in these two peptides nor the function of this modification is yet clear. Both peptide beta (T. Patschinsky, T. Hunter, F. S. Esh, and B. M. Sefton, manuscript in preparation) and peptide II (Neil et al., submitted for publication) become labeled when immunoprecipitated $p60^{src}$ and p90 are incubated with [γ-^{32}P]ATP. In addition, Erikson et al. (13) have reported that this site can become phosphorylated when partially purified $p60^{src}$ is incubated with [γ-^{32}P]ATP. It thus appears that both proteins may undergo autophosphorylation at this site. If this is the case, it suggests that these two viral proteins may phosphorylate their substrates at similar sites. It is possible, however, that the apparent autophosphorylation is an artifact of the in vitro conditions and that this site is phosphorylated by another protein kinase in vivo. If this is the case, it suggests that $p60^{src}$ and p90 may be modified by the same cellular tyrosine protein kinase.

RSV (44, 45), Y73 virus (54), A-MuLV (19), and the feline sarcoma viruses ST-FeSV and GA-FeSV (16) each presumably arose by transduction of a cellular gene. The results reported here combined with previous analysis using molecular hybridization (26, 43, 55) make it clear that this group of viruses is derived from four different cellular genes. What is the function of the products of these cellular genes? In the case of the protein encoded by the cellular homologue of the *src* gene of RSV, $p60^{proto\text{-}src}$, it has been shown that it too is a tyrosine protein kinase

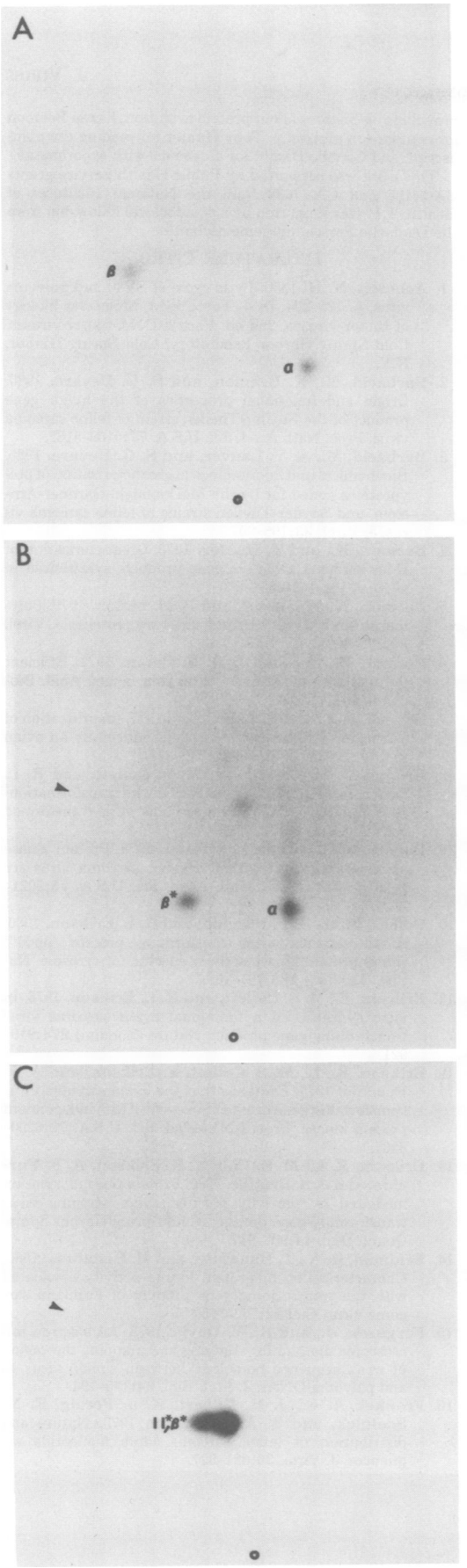


FIG. 3. *Comparison of the protease V8 digestion products of phosphorylated tryptic peptides of SR-RSV-A p60^{src} and Y73 p90. (A) Phosphorylated tryptic peptides of p60^{src}. A tryptic digest of ^{32}P-labeled p60^{src} was prepared and analyzed in two dimensions as described in the legend to Fig. 2. A total of 830 cpm was analyzed, and the exposure time was 18 h. (B) Phosphorylated peptides of p60^{src} after secondary incubation with protease V8. A tryptic digest of ^{32}P-labeled p60^{src} was heated for 10 s in a boiling water bath to inactivate residual trypsin activity, lyophilized, and then incubated with protease V8 as described in Materials and Methods. The secondary digestion products (1,900 cpm) were analyzed as described in (A). The exposure time was 18 h. In addition to peptide beta*, an unmarked peptide in the upper center of the map was generated by the protease V8 digestion. The origin of this peptide is unknown. It was not seen in digests of isolated peptide beta (see panel C). Arrows in (B) and (C) indicate the position of uncleaved p60^{src} peptide beta. (C) Mixture of protease V8 cleavage products of peptide beta of p60^{src} and peptide II of p90. The ^{32}P-labeled tryptic peptides were eluted from thin-layer plates and incubated with protease V8 as described in Materials and Methods. Approximately 300 cpm of each of the secondary reaction products was analyzed together as in (A). The exposure time was 3 days. The origins in all three panels are marked with "o."*

(25). That the proteins encoded by the cellular genes homologous with the transformation-specific sequences of FSV, Y73 virus, and A-MuLV will be found also to resemble the viral protein at least in fundamental enzymatic activity seems likely. It is well possible, therefore, that the genome of vertebrates encodes at least four distinct tyrosine protein kinases. Indeed, Spector et al. (M. Spector, S. O'Neal, and E. Racker, J. Biol. Chem., in press) have reported the isolation of three tyrosine protein kinases from apparently uninfected Ehrlich ascites tumor cells.

Although we have emphasized here the marked structural differences between $p60^{src}$, p120, and p90, it must be remembered that neither our data nor the results of the experiments using molecular hybridization exclude the possibility that these proteins have as yet inapparent homology. The limitations of both peptide mapping and molecular hybridization are well exemplified by the analyses of the relatedness of the proteins encoded by simian virus 40 and polyoma virus. Although these two viruses are clearly closely related in structure and in the functions of both their early and late genes (1, 20, 46), homology between any of their genes is difficult to detect by molecular hybridization (15, 24). Additionally, comparison of methionine-containing tryptic peptide maps of both the early and late proteins reveals no homology (T. Hunter, personal communication). The significant homology in primary amino acid sequence between both the tumor antigens and the structural proteins of these viruses becomes apparent only when the predicted amino acid sequences of the proteins are aligned (17, 18, 20). Given the difficulties in detecting homology between the proteins of simian virus 40 and polyoma virus, which are almost certainly functionally equivalent and evolutionarily related, care must be taken not to interpret the apparent lack of homology between $p60^{src}$, p120, and p90 as meaning that these proteins are in no sense structurally related. We found two identical sites of tyrosine phosphorylation and comigration of a few methionine-containing tryptic peptides. Although we have not emphasized comigration of these few peptides because it may be just coincidental, it cannot be excluded that this comigration is a hint of homology between p120, $p60^{src}$, and p90. Thus, although it seems clear that different cellular genes gave rise to A-MuLV, RSV, and Y73 virus, it may be that the cellular genes represent divergent forms of one ancestral tyrosine protein kinase.

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LITERATURE CITED

1. **Acheson, N. H.** 1980. Lytic cycle of SV40 and polyoma virus, p. 125–204. *In* J. Tooze (ed.), Molecular biology of tumor viruses, 2nd ed. Part 2: DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Barbacid, M., K. Beemon, and S. G. Devare.** 1980. Origin and functional properties of the major gene product of the Snyder-Theilen strain of feline sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **77**:5158–5162.
3. **Barbacid, M., A. V. Lauver, and S. G. Devare.** 1980. Biochemical and immunological characterization of polyproteins coded for by the McDonough, Gardner-Arnstein, and Snyder-Theilen strains of feline sarcoma virus. J. Virol. **33**:196–207.
4. **Beemon, K., and T. Hunter.** 1978. Characterization of Rous sarcoma virus *src* gene products synthesized in vitro. J. Virol. **28**:551–566.
5. **Beemon, K., T. Hunter, and B. M. Sefton.** 1979. Polymorphism of avian sarcoma virus *src* proteins. J. Virol. **30**:190–200.
6. **Bonner, W. M., and J. D. Stedman.** 1978. Efficient fluorography of ^{3}H and ^{14}C on thin layers. Anal. Biochem. **89**:247–256.
7. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature **269**:346–348.
8. **Brugge, J. S., E. Erikson, M. S. Collett, and R. L. Erikson.** 1978. Peptide analysis of the transformation-specific antigen from avain sarcoma virus-transformed cells. J. Virol. **26**:773–782.
9. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021–2024.
10. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus-transforming protein, $pp60^{src}$ shows protein kinase activity specific for tyrosine. Nature.(London) **285**:167–169.
11. **Erikson, E., M. S. Collett, and R. L. Erikson.** 1978. In vitro synthesis of a functional avian sarcoma virus transforming-gene product. Nature (London) **274**:919–921.
12. **Erikson, R., L., M. S. Collett, E. Erikson, and A. F. Purchio.** 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic AMP-independent protein kinase. Proc. Natl. Acad. Sci. U.S.A. **76**:6260–6264.
13. **Erikson, R. L., M. S. Collett, E. Erikson, A. F. Purchio, and J. S. Brugge.** 1980. Protein phosphorylation mediated by partially purified avian sarcoma virus transforming-gene product. Cold Spring Harbor Symp. Quant. Biol. **44**:907–917.
14. **Feldman, R. A., T. Hanafusa, and H. Hanafusa.** 1980. Characterization of protein kinase activity associated with the transforming gene product of Fujinami sarcoma virus. Cell **22**:757–765.
15. **Ferguson, J., and R. W. Davis.** 1975. An electron microscopic method for studying and mapping the region of weak sequence homology between simian virus 40 and polyoma DNAs. J. Mol. Biol. **94**:135–194.
16. **Frankel, A. E., J. H. Gilbert, K. J. Porzig, E. M. Scolnick, and S. A. Aaronson.** 1979. Nature and distribution of feline sarcoma virus nucleotide sequences. J. Virol. **30**:821–827.

17. **Friedman, T., R. F. Doolittle, and G. Walter.** 1978. Amino acid sequence homology between polyoma and SV40 tumor antigens deduced from nucleotide sequences. Nature (London) **274**:291–293.
18. **Friedmann, T., A. Esty, P. LaPorte, and P. Deininger.** 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. Cell **17**:715–724.
19. **Goff, S. P., E. Gilboa, O. N. Witte, and D. Baltimore.** 1980. Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: studies with cloned viral DNA. Cell **22**:777–785.
20. **Griffin, B. E.** 1980. Structure and genomic organization of SV40 and polyoma virus, p. 61–123. *In* J. Tooze (ed.), Molecular biology of tumor viruses, 2nd ed. Part 2: DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. P. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Publishing Corp., New York.
22. **Hanafusa, T., L.-H. Wang, S. M. Anderson, R. E. Karess, W. S. Hayward, and H. Hanafusa.** 1980. Characterization of the transforming gene of Fujinami sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **77**:3009–3013.
23. **Houmard, J., and G. R. Drapeau.** 1972. Staphylococcal protease: a proteolytic enzyme specific for glutamoyl bonds. Proc. Natl. Acad. Sci. U.S.A. **69**:3506–3509.
24. **Howley, P. M., M. A. Israel, M.-F. Law, and M. A. Martin.** 1979. A rapid method for detecting and mapping homology between heterologous DNAs. J. Biol. Chem. **254**:4876–4883.
25. **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.
26. **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.
27. **Laskey, R. A., and A. D. Hills.** 1977. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitive film. FEBS Lett. **82**:314–316.
28. **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.
29. **Levinson, A. D., H. Oppermann, 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.
30. **Levinson, A. D., H. Oppermann, H. E. Varmus, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. **255**:11973–11980.
31. **Neil, J. C., M. L. Breitmann, and P. K. Vogt.** 1981. Characterization of a 105,000 molecular weight *gag*-related phosphoprotein from cells transformed by the defective avian sarcoma virus PRCII. Virology **108**:98–110.
32. **Neil, J. C., J. Ghysdael, and P. K. Vogt.** 1981. Tyrosine-specific kinase activity associated with p105 of avian sarcoma virus PRCII. Virology **109**:223–228.
33. **Pawson, T., J. Guyden, T.-H. Kung, K. Radke, T. Gilmore, and S. G. Martin.** 1980. A strain of Fujinami sarcoma virus which is temperature-sensitive in protein phosphorylation and cellular transformation. Cell **22**: 767–775.
34. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
35. **Purchio, A. F., E. Erikson, J. S. Butel, and R. L. Erikson.** 1978. Identification of a polypeptide encoded by the avian sarcoma virus *src* gene. Proc. Natl. Acad. Sci. U.S.A. **75**:1567–1571.
36. **Reynolds, F. H., Jr., T. L. Sacks, D. N. Deobagkar, and J. R. Stephenson.** 1978. Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polyprotein containing structural and nonstructural components. Proc. Natl. Acad. Sci. U.S.A. **75**:3974–3978.
37. **Reynolds, F. H., Jr., W. J. M. Van de Ven, and J. R. Stephenson.** 1980. Feline sarcoma virus P115-associated protein kinase phosphorylates tyrosine. Identification of a cellular substrate conserved during evolution. J. Biol. Chem. **255**:11040–11047.
38. **Scher, C. D., and R. Siegler.** 1975. Direct transformation of 3T3 cells by Abelson murine leukemia virus. Nature (London) **253**:729–731.
39. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vivo and in vitro. J. Virol. **28**:957–971.
40. **Sefton, B. M., T. Hunter, and K. Beemon.** 1979. Product of in vitro translation of the Rous sarcoma virus *src* gene has protein kinase activity. J. Virol. **30**:311–318.
41. **Sefton, B. M., T. Hunter, and K. Beemon.** 1980. Relationship of polypeptide products of the transforming gene of Rous sarcoma virus and the homologous gene of vertebrates. Proc. Natl. Acad. Sci. U.S.A. **77**:2059–2063.
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. **Shibuya, M., T. Hanafusa, H. Hanafusa, and J. R. Stephenson.** 1980. Homology exists among the transforming sequences of avian and feline sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. **77**:6536–6540.
44. **Spector, D. H., H. E. Varmus, and J. M. Bishop.** 1978. Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates. Proc. Natl. Acad. Sci. U.S.A. **75**:4102–4106.
45. **Stehelin, D., H. E. Varmus, J. M. Bishop, and P. K. Vogt.** 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature (London) **260**:170–173.
46. **Topp, W. C., D. Lane, and R. Pollack.** 1980. Transformation by SV40 and polyoma virus, p. 205–296. *In* J. Tooze (ed.), Molecular biology of tumor viruses, 2nd ed. Part 2: DNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Van de Ven, W. J. M., A. S. Khan, F. H. Reynolds, Jr., K. T. Mason, and J. R. Stephenson.** 1980. Translational products encoded by newly acquired sequences of independently derived feline sarcoma virus isolates are structurally related. J. Virol. **33**:1034–1045.
48. **Van de Ven, W. J. M., F. H. Reynolds, Jr., and J. R. Stephenson.** 1980. The nonstructural components of polyproteins encoded by replication-defective mammalian transforming retroviruses are phosphorylated and have associated protein kinase activity. Virology **101**: 185–197.
49. **Van de Ven, W. J. M., F. H. Reynolds, Jr., R. P. Nalewaik, and J. R. Stephenson.** 1979. The nonstructural component of the Abelson murine leukemia virus polyprotein p120 is encoded by newly acquired genetic sequences. J. Virol. **32**:1041–1045.
50. **Vogt, P. K.** 1977. The genetics of RNA tumor viruses, p. 341–455. *In* H. Fraenkel-Conrat and R. P. Wagner (ed.), Comprehensive virology, vol. 9. Plenum Publishing Corp., New York.

51. **Vogt, V. M., R. Eisenman, and H. Diggelmann.** 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor protein. J. Mol. Biol. **96**:471–493.
52. **Welch, W. J., B. M. Sefton, and F. S. Esch.** 1981. Amino-terminal sequence analysis of alphavirus polypeptides. J. Virol. **38**:968–972.
53. **Witte, O. N., A. Dasgupta, and D. Baltimore.** 1980. Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature (London) **283**:826–831.
54. **Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. Baltimore.** 1978. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblasts and lymphoid cells. Proc. Natl. Acad. Sci. U.S.A. **75**:2488–2492.
55. **Yoshida, M., S. Kawai, and K. Toyoshima.** 1980. Uninfected avian cells contain structurally unrelated progenitors of viral sarcoma genes. Nature (London) **287**: 653–654.