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Four Different Classes of Retroviruses Induce Phosphorylation of Tyrosines Present in Similar Cellular Proteins

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Chicken embryo cells transformed by the related avian sarcoma viruses PRC II and Fujinami sarcoma virus, or by the unrelated virus Y73, contain three phosphoproteins not observed in untransformed cells and increased levels of up to four other phosphoproteins. These same phosphoproteins are present in increased levels in cells transformed by Rous sarcoma virus, a virus which is apparently unrelated to the three aforementioned viruses. In all cases, the phosphoproteins contain phosphotyrosine and thus may be substrates for the tyrosine-specific protein kinases encoded by these viruses. In one case, the site(s) of tyrosine phosphorylation within the protein is the same for all four viruses. A homologous protein is also phosphorylated, at the same major site, in mouse 3T3 cells transformed by Rous sarcoma virus or by the further unrelated virus Abelson murine leukemia virus. A second phosphotyrosine-containing protein has been detected in both Rous sarcoma virus and Abelson murine leukemia virus-transformed 3T3 cells, but was absent from normal 3T3 cells and 3T3 cells transformed by various other viruses. We conclude that representatives of four apparently unrelated classes of transforming retroviruses all induce the phosphorylation of tyrosines present in the same set of cellular proteins.

Cells transformed by a variety of otherwise unrelated retroviruses have an unusual property in common. Their proteins have a relatively high content of the rare phosphorylated amino acid phosphotyrosine. This phenomenon was first observed for chicken and mouse cells transformed by Rous sarcoma virus (RSV), where there are strong indications that the phosphorylation of tyrosine residues is intimately involved in the mechanism of transformation. The protein product ($p60^{src}$) of the viral transforming gene, *src*, is a protein kinase specific for tyrosine residues in vitro (9, 18, 23), and experiments with temperature-sensitive mutants in vivo show that an active $p60^{src}$ is essential for maintenance of an elevated level of phosphotyrosine, as well as for maintenance of the transformed phenotype (35).

More recently, it has been shown that mink cells transformed by the Gardner-Arnstein and Snyder-Theilen strains of feline sarcoma virus, mouse cells transformed by Abelson murine leukemia virus (A-MuLV), and chicken cells transformed by three defective avian sarcoma viruses, PRC II, Fujinami sarcoma virus (FSV), and Y73, all contain elevated levels of protein phosphotyrosine relative to untransformed control cells (2, 3, 19, 36). In each of these cases, too, the particular transforming protein is associated with a tyrosine-specific protein kinase activity when assayed in vitro (2, 3, 6, 13, 20, 26, 27, 39, 40). However, proof that these transforming proteins are themselves protein kinases is inconclusive because in no case are both conditional mutants and an extensively purified transforming protein available.

All of these retroviruses, except RSV, are defective and require a leukosis virus helper for growth. They contain a fused transforming gene, in which part of the region coding for structural proteins of the helper virus is joined to a region homologous to cellular sequences, presumed to have been acquired by recombination (11). The various chicken viruses are related in their structural genes, but nucleic acid hybridization studies and peptide maps of the transforming proteins show that the "cellular" sequences fall into four distinct classes. The cellular sequences of feline sarcoma virus, PRC II, and FSV are closely related (3, 25, 27) and have been named *fes*, fps^{P}, and fps^{F} (nomenclature proposed by J. M. Coffin and H. E. Varmus, personal communication). However, the cellular sequences of A-MuLV (named *abl*) and of Y73 (*yas*) do not appear to be closely related to each other, to *fes*/*fps*, or to *src*, although some limited homology may exist (3, 25, 37, 43; T. Patschinsky and B. M. Sefton, J. Virol., in press). Their transforming proteins are consequently quite different, yet all may transform cells via unscheduled phosphorylation of tyrosine in cellular proteins.

Not all transforming retroviruses, however, cause a gross increase in cellular protein phosphotyrosine, so there are probably other routes to cell transformation.

In cells transformed by RSV, we have shown that there are a number of proteins which contain elevated levels of phosphotyrosine, including seven proteins of unknown function, identified by two-dimensional gel electrophoresis. These proteins are, potentially, substrates for the $p60^{src}$ protein kinase or for cellular protein kinases activated by $p60^{src}$ (10).

We now report experiments which suggest that FSV, PRC II, Y73, and A-MuLV may cause phosphorylation of some of the same cellular protein substrates as RSV. The same seven phosphotyrosine-containing proteins detected in RSV-transformed chicken embryo cells (CECs) can also be found in CECs transformed by FSV, PRC II, and Y73. Also, mouse cells transformed by RSV or A-MuLV have elevated levels of phosphotyrosine in two phosphoproteins, one of which is clearly homologous to one of the chicken cell proteins.

MATERIALS AND METHODS

Cells and viruses. CECs were prepared as described previously (33) and infected with Prague strain RSV, subgroup A (PR-RSV-A) (10). CECs transformed with PRC II (from P. K. Vogt, University of Southern California), FSV (from H. Temin, University of Wisconsin), and Y73 (from K. Toyoshima, University of Tokyo) were all the kind gift of K. Beemon, of the Salk Institute, and were maintained by adding uninfected CECs at each passage. BALB/c 3T3 cells (A31 [35], from T. Shier, Salk Institute) transformed with Schmidt-Ruppin strain RSV, subgroup D (SR-RSV-D) (from L. Rorschneider, Hutchinson Cancer Center, Seattle, Wash.) were provided by T. Patschinsky, Salk Institute. NIH 3T3 cells (N-f [35], from M.-H. T. Lai, Salk Institute) were infected with Moloney murine sarcoma virus (strain 124 [1], from M.-H. T. Lai). A-MuLV-transformed NIH 3T3 cells, ANN-1 (32), were from W. Raschke, Salk Institute. Simian virus 40-transformed NIH Swiss 3T3 cells (SV3T3 [35]) were from M. Hamilton, Salk Institute. All mouse cells were grown in Dulbecco-modified Eagle medium containing 10% calf serum. Cell cultures were labeled with [^{35}S]methionine or with [^{32}P]orthophosphate as described before (10), except that sparse cultures (2×10^5 cells per 35-mm dish) of NIH 3T3 cells were labeled with 2.4 mCi of ^{32}P per ml.

Two-dimensional gel electrophoresis. Cell cultures were prepared and analyzed as described before (10). Briefly, ^{32}P-labeled proteins were resolved by isoelectric focusing, in pH 6 to 8 range ampholines, for 14,000 V·h, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). The gels were fixed, dried, swollen in 1 M KOH, and incubated at 55°C for 2 h, fixed again, dried, and autoradiographed (10). Phosphoamino acids were isolated and separated as described before (10, 18).

Purification of 39,000-M_r phosphoprotein *p*. Dishes (35 mm) of cells were labeled as above, washed, and lysed at 4°C in 500 μl of 10 mM sodium phosphate (pH 7.8)–0.05% Nonidet P-40–5 mM ethylenediaminetetraacetate–1 mM 2-mercaptoethanol. After centrifugation at 150,000 × *g* for 30 min, the supernatant was passed through a 0.2-ml-bed volume diethylaminoethyl cellulose (Whatman DE52) column equilibrated with the same buffer. A 1.0-ml portion of eluate was collected, and 10 μl of 1 M H_3PO_4 was added and applied to a 0.2-ml carboxymethyl (CM)-cellulose (Whatman CM52) column equilibrated with the same buffer at pH 6.4. Two 1.0-ml fractions were collected, and then the column was eluted with 1.0 ml of the same buffer at pH 7.8. Samples (10 μl) of the fractions were electrophoresed on 15% polyacrylamide gels containing sodium dodecyl sulfate. The remainder of the 1.0-ml, pH 7.8, elution from the CM-cellulose column was similarly electrophoresed after it had been lyophilized and dissolved in 100 μl of 2% sodium dodecyl sulfate–5% 2-mercaptoethanol–50 mM tris(hydroxymethyl)aminomethane(pH 6.8)–10% glycerol. This gel was washed in water, dried, and autoradiographed, and the 39,000-M_r band was extracted, oxidized with performic acid, and digested with trypsin as described previously (4).

Peptide mapping. Peptides labeled with ^{32}P were separated by electrophoresis on cellulose thin layers in 1% ammonium carbonate (pH 8.9) at 1.4 kV for 18 min in one dimension, followed by chromatography in *n*-butanol–pyridine–acetic acid–water (15:10:3:12, by volume) in the second dimension (18). Peptides labeled with [^{35}S]methionine were separated by electrophoresis in *n*-butanol–pyridine–acetic acid–water (2:1:1:36, by volume) at pH 4.72, followed by chromatography in *n*-butanol–pyridine–acetic acid–water (97:75:15:60, by volume) in the second dimension (16). These plates were impregnated with 2-methylnaphthalene, and fluorescence was detected at −70°C (7).

RESULTS

Phosphoproteins of transformed CECs. We have already shown that alkali treatment of two-dimensional gels containing ^{32}P-labeled phosphoproteins can be useful for identifying proteins which are phosphorylated as a consequence of RSV $p60^{src}$ activity (10). Most phosphoproteins whose ^{32}P label was lost during alkali treatment of the gel contained only phosphoserine; none of them contained phosphotyrosine. However, after alkali treatment, seven phosphoproteins could be detected which were present at higher levels as a result of transformation by RSV, and which were shown to contain phosphotyrosine (10). These are indicated by letters in Fig. 1F. Some of them were detected, at reduced levels, in uninfected CECs (proteins *k*, *m*, *n*, and *q*, Fig. 1F), but others were observed only in transformed cells (proteins *l*, *o*, and *p*).

Parallel cultures of CECs transformed with RSV, FSV, PRC II, and Y73 were labeled with ^{32}P and analyzed by two-dimensional gel electrophoresis, followed by alkali treatment of the gels

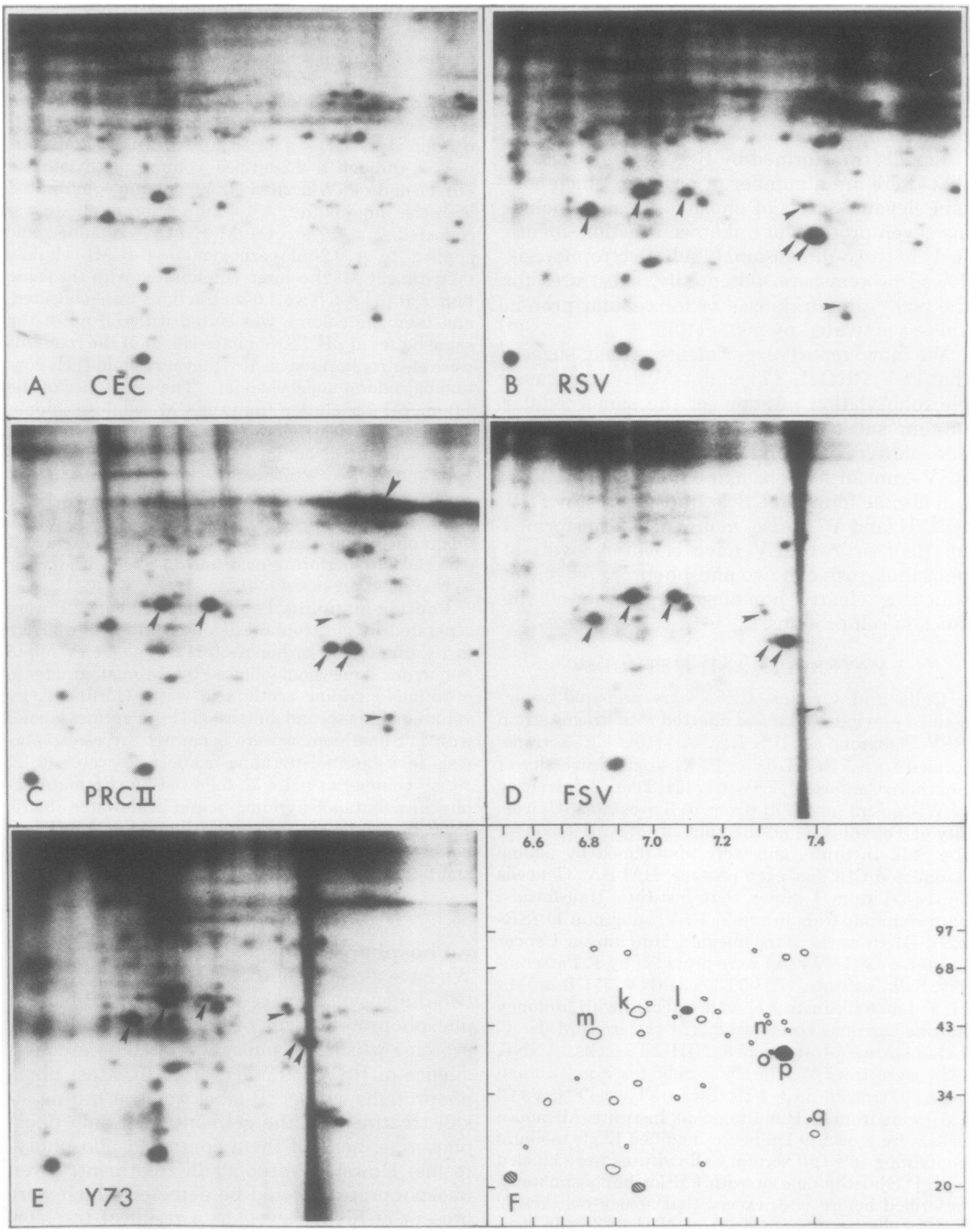

FIG. 1. *Alkali-treated two-dimensional gels of phosphoproteins of normal and transformed CECs. Confluent cultures of uninfected CECs (A) or CECs transformed with PR-RSV-A (B) or PRC II (C) were labeled in parallel as described before (10). In a separate experiment, FSV (D)- and Y73 (E)-transformed CECs were labeled at 36°C. The samples were analyzed by two-dimensional gel electrophoresis, treated with alkali, and autoradiographed with an intensifying screen for 60 (A, B, C) or 21 (D, E) h. The diagram (F) shows the major CEC phosphoproteins with approximate pI's (abscissa) and M_r's ($\times 10^{-3}$, ordinate). Marker proteins included phosphorylase A, bovine serum albumin, ovalbumin, carboxypeptidase A, and soybean trypsin inhibitor. Those phosphoproteins already shown (10) to be elevated by RSV transformation are marked with letters; solid spots are undetectable in normal CECs. These same phosphoproteins are arrowed in (A to E); PRC II p105 is also indicated in (C). Two shaded spots (F) are related to pp19^{src} (10).*

(10). Autoradiographs of typical gels are shown (Fig. 1B to E). In all cases, phosphoproteins *l*, *o*, and *p* were detected, but these were not labeled in untransformed CECs (Fig. 1A), even when growing rapidly in sparse culture (10). Quantitative increases in the phosphorylation of *k*, *m*, *n*, and *q* were observed in several experiments in which virus-transformed cells were labeled under the same conditions as uninfected control CECs. In some cases, there were quantitative differences in the increases in different phosphoproteins. For example, in PRC II-infected cells, there was significantly higher phosphorylation of spot *o* than of spot *n* (Fig. 1C), whereas in FSV-infected cells, spot *o* was phosphorylated no more than spot *n* (Fig. 1D). With RSV (Fig. 1B) and Y73 (Fig. 1E), spot *p* is much more heavily phosphorylated than spot *l*, but with PRC II and FSV, *p* and *l* were phosphorylated almost equally. Increased phosphorylation of *m* was not detected in PRC II-transformed cells. Such differences may indicate that protein kinases of different specificities, or having different subcellular localizations, are active in CECs transformed by RSV, FSV, and Y73. Some additional differences are clearly dependent on the transforming virus. The streak of radioactivity, at 105,000 M_r and a pI >7.3, in PRC II-transformed CECs (arrowed, Fig. 1C) is the transforming protein ($P105^{gag\text{-}fps}$) itself, as shown by two-dimensional gel electrophoresis of the purified protein (J. A. Cooper and K. Beemon, data not shown). This transforming protein contains several phosphotyrosine residues (K. Beemon, personal communication). Variations in the intensity of phosphoserine-containing spots of about 20,000 M_r and pI 6.55 or 6.95, which are probably $pp19^{gag}$ related (10), presumably reflect different efficiencies of infection by helper viruses.

The stock of FSV we used is known to encode a transforming protein which has a thermolabile protein kinase activity (27), and correspondingly only at the permissive temperature do infected cells show transformed morphology (27) and elevated phosphotyrosine (3). To test whether the phosphorylation of proteins *k* through *q* is similarly dependent on the culture temperature, we labeled FSV-transformed CECs at 36 and 41°C and analyzed their phosphoproteins by two-dimensional gel electrophoresis and alkali treatment of the gels (Fig. 2). Phosphorylation of three transformation-dependent phosphoproteins (*l*, *n*, and *p*) was markedly reduced at 41°C (arrowed, Fig. 2).

Proteins phosphorylated at increased levels contain phosphotyrosine. To see whether phosphoproteins of cells transformed by viruses other than RSV contained phosphotyrosine, we purified various ^{32}P-labeled phosphoproteins, from CECs transformed with FSV or Y73, by two-dimensional gel electrophoresis. The gels were treated with alkali to remove background radioactivity and alkali-labile phosphoproteins, and six phosphoproteins previously shown to contain phosphotyrosine in RSV-transformed cells were located by autoradiography, purified, and partially hydrolyzed with acid as described previously (10). The phosphoamino acids were separated by two-dimensional elec-

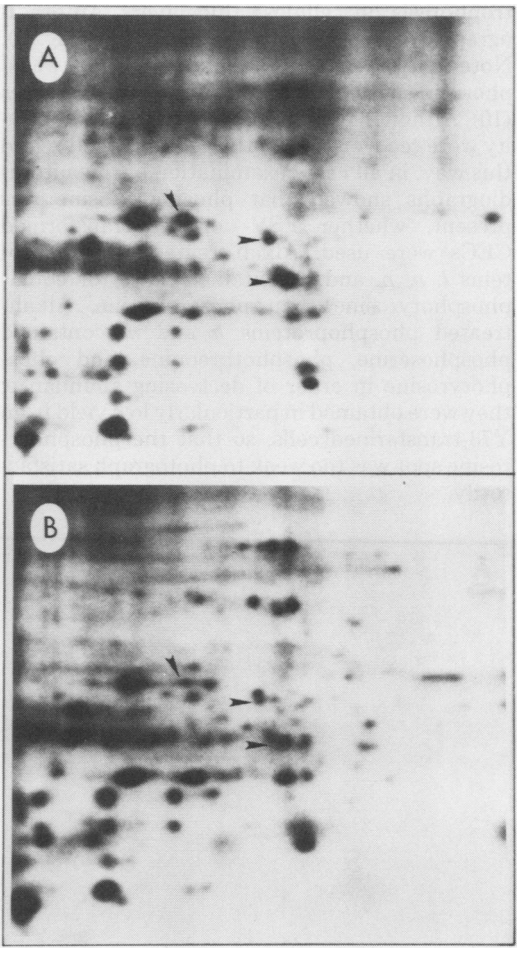

FIG. 2. *Temperature dependence of protein phosphorylation in FSV-transformed CECs. FSV-transformed CECs, maintained at 37°C, were incubated at 36°C (A) or 41°C (B) for 20 h in the presence of 600 μCi of ^{32}P per ml of low-phosphate medium (10). Samples were prepared and analyzed as before (10). Forty-hour indirect autoradiographs of alkali-treated gels are shown. The arrows point to regions of the gel containing phosphoproteins present at reduced levels at 41°C.*

trophoresis on cellulose thin layers. Autoradiographs of some analyses are shown (Fig. 3). Note that the alkali treatment means that phosphoserine is underestimated in these analyses (10). Although only small amounts of radioactivity were recovered from the gels and analyzed in this way, in all cases examination of the autoradiographs showed that phosphotyrosine was present, whether FSV- or Y73-transformed CECs were used. Alkali-treated phosphoproteins *l*, *n*, *p*, and *q* contained more (or equal) phosphotyrosine than phosphoserine. Alkali-treated phosphoproteins *k* and *m* contained phosphoserine, phosphothreonine, and phosphotyrosine in order of decreasing abundance; they were obtained in particularly low yield from Y73-transformed cells, so that the phosphotyrosine spot was too weak to photograph satisfactorily.

The phosphoamino acid compositions of these phosphoproteins, purified from FSV- or Y73-transformed CECs, are thus very similar to those shown before for the equivalent phosphoproteins purified from RSV-transformed CECs (10). Individual phosphoproteins from PRC II-transformed CECs were not examined in this way, but we have shown that phosphoprotein *p* does contain phosphotyrosine and phosphoserine when purified by an alternative procedure, described below (data not shown). The observation that *k* and *m* contain so much phosphoserine implies that much of the increased phosphorylation of these proteins must be due to serine-specific protein kinases and may be secondary to increased tyrosine phosphorylation. Phosphoproteins *p* and *l* are undetectable in uninfected CECs, so it is clear that their phosphotyrosine is elevated by transformation; however, their phos-

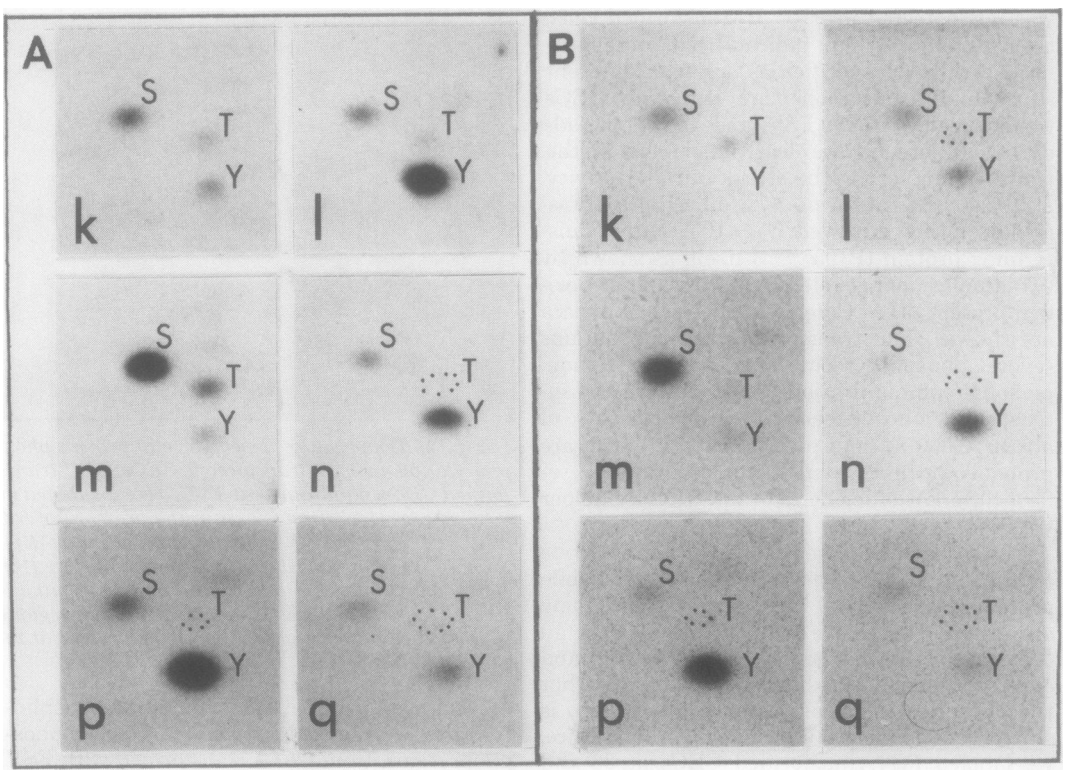

FIG. 3. *Phosphoamino acids of individual phosphoproteins. FSV (A)- and Y73 (B)-transformed CECs were labeled in parallel with 2.4 mCi of ^{32}P per ml at 36°C. Each sample was run on eight replicate two-dimensional gels, which were treated with alkali and autoradiographed. The regions corresponding to phosphoproteins k, l, m, n, p (including o), and q were cut out, pooled, extracted, and hydrolyzed at 110°C for 1 h in 6 N HCl, and the phosphoamino acids were separated by two-dimensional electrophoresis in cellulose thin layers (10, 18). Marker phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) were located by staining. Portions of 8-day indirect autoradiographs are shown. The amounts of radioactivity analyzed in each case (including inorganic phosphate) were: (A, FSV-transformed CECs)—phosphoprotein k, 210 cpm; l, 170 cpm; m, 150 cpm; n, 105 cpm; p (plus o), 130 cpm; q, 115 cpm. (B, Y73-transformed CECs)—phosphoprotein k, 245 cpm; l, 60 cpm; m, 95 cpm; n, 65 cpm; p (plus o), 75 cpm; q, 95 cpm.*

phoserine is elevated also. The apparent interdependence of tyrosine and serine phosphorylation for some proteins has yet to be explained (10, 12).

Characterization of phosphoprotein *p* in transformed CECs. To seek further similarities between the phosphoproteins of CECs transformed by RSV, FSV, Y73, and PRC II, we have purified a major phosphotyrosine-containing protein (*p*; also known as "36K" [21, 27, 29] or "34K" [12]) by a procedure which gives a high yield of protein suitable for peptide mapping.

The basic pI and high abundance of the nonphosphorylated progenitor to phosphoprotein *p* (10, 12, 28), allowed us to assay it by sodium dodecyl sulfate-gel electrophoresis, once it was separated from more acidic proteins by diethylaminoethyl cellulose chromatography (Fig. 4, tracks 1 to 5). We found it necessary to purify the protein further by binding to CM-cellulose at pH 6.4 and eluting at pH 7.8. This fraction contained two proteins of similar M_r (39,000) and similar peptide maps (data not shown). When purified from ^{32}P-labeled cells, a phosphoprotein nearly comigrated with the upper band of the doublet (Fig. 4, tracks 5 and 6). This phosphoprotein had the same mobilities on two-dimensional gels as phosphoprotein *p*, contained phosphotyrosine and phosphoserine, and was not phosphorylated in uninfected CECs. We suspect that phosphoprotein *o* is also present in the material purified by chromatography since its pI is similar to that of phosphoprotein *p*; however, two-dimensional gel analysis of the CM-cellulose fraction did not provide adequate resolution to confirm this.

CECs transformed with RSV, PRC II, FSV, or Y73 were labeled with ^{32}P, and extracts were chromatographed on diethylaminoethyl and carboxymethyl-cellulose columns. The fraction containing phosphoprotein *p* was further purified by preparative sodium dodecyl sulfate-gel electrophoresis. The 39,000-M_r band was located by autoradiography, the phosphoprotein was extracted and digested with trypsin, and the peptides were separated by electrophoresis and chromatography in cellulose thin layers. Autoradiography showed that the 39,000-M_r phosphoprotein contained a common major hydrophobic phosphorylated peptide whether it was purified from RSV-, PRC II-, FSV-, or Y73-transformed cells (Fig. 5A to D). Mapping of mixtures showed that this was the same peptide in all cases (Fig. 5E to G). A number of additional peptides were present in some cases; these peptides also had the same electrophoretic and chromatographic properties independent of the virus used, but the degree of phosphorylation varied

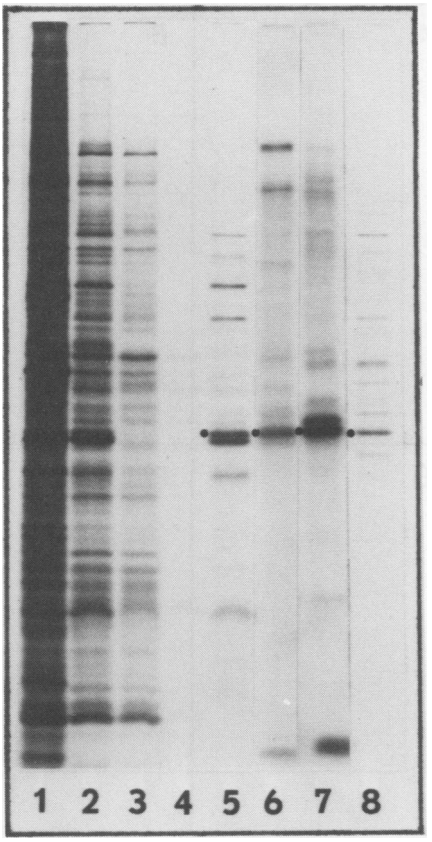

FIG. 4. *Purification of 39,000-M_r phosphoprotein. Sodium dodecyl sulfate-polyacrylamide gel (15% acrylamide, 0.087% bisacrylamide) analysis of samples taken at various steps in the purification of the 39,000-M_r phosphoprotein from RSV-transformed CECs (tracks 1 to 6) or SR-3T3 cells (tracks 7 and 8). The cells were labeled with [^{35}S]methionine (tracks 1 to 5, 8) or ^{32}P (tracks 6 and 7). Samples were from the high-speed supernatant fraction (track 1), diethylaminoethyl cellulose flow-through fraction (track 2), CM-cellulose flow-through fraction (track 3), CM-cellulose wash (track 4), and CM-cellulose, pH 7.8, elution (tracks 5 to 8). The gel was autoradiographed for 1 (tracks 1 to 5, 8) or 5 (tracks 6 and 7) days. The 39,000-M_r phosphoprotein is indicated.*

(Fig. 5). These other peptides could come from phosphoprotein *p* or *o* or possibly from contaminating phosphoproteins, which could vary between different purifications. The major phosphopeptide common to all of the peptide maps (arrowed) contained only phosphotyrosine (not shown). The other peptides were not analyzed. However, acid hydrolysis of the trypsin-digested material showed that it contained a low proportion of phosphoserine, whereas phosphoprotein *p* contained almost equal amounts of phosphoserine and phosphotyrosine when purified from

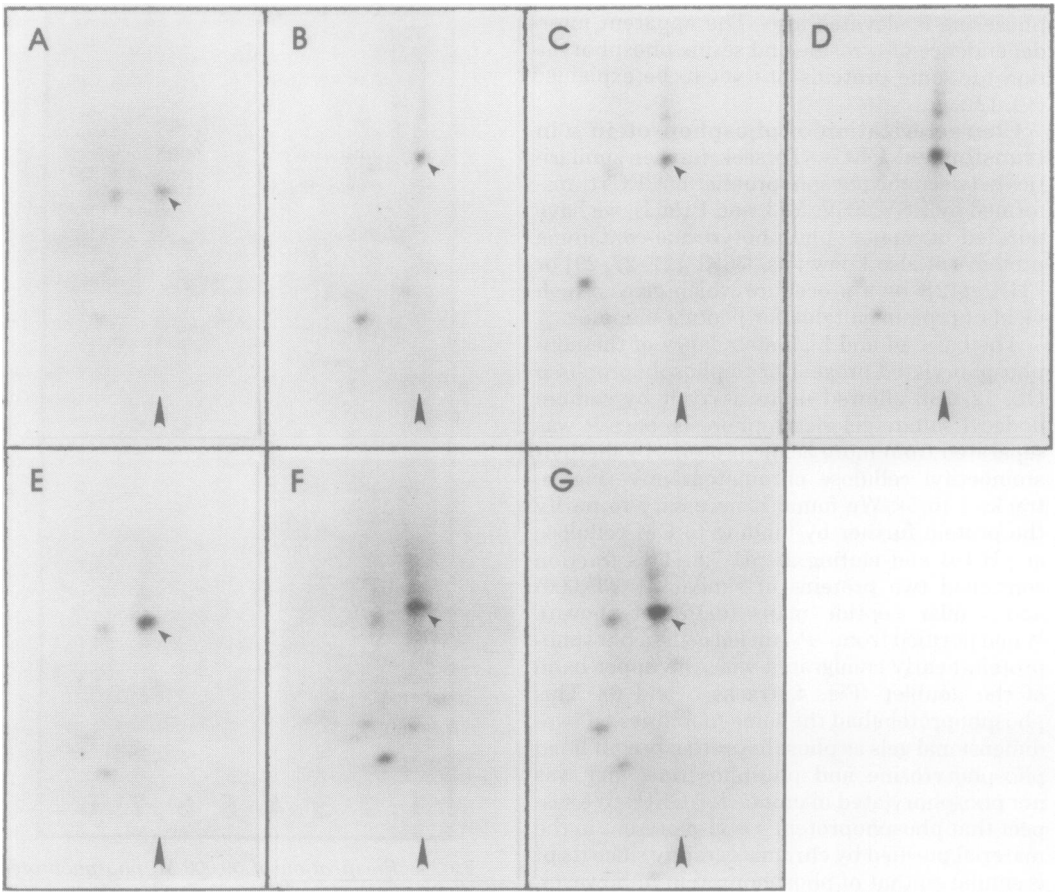

FIG. 5. *Phosphopeptide maps of phosphoprotein p from transformed CECs. CECs transformed by RSV (A), PRC II (B), Y73 (C), and FSV (D) were labeled with 2.4 mCi of ^{32}P per ml, and the 39,000-M_r phosphoprotein was purified by ion-exchange chromatography and preparative gel electrophoresis. After performic acid oxidation and trypsin digestion, 250 to 1,000 cpm of radioactivity were recovered. Phosphopeptides were separated by thin-layer electrophoresis at pH 8.9 (anode on the left) and ascending chromatography. Ten-day indirect autoradiographs of thin-layer plates loaded with 100 cpm are shown. A total of 100 cpm of FSV phosphopeptides were also mixed with 100 cpm of RSV (E), PRC II (F), and Y73 (G) phosphopeptides and mapped under the same conditions. Four-day indirect autoradiographs are shown. The large arrows mark the points of sample application. The small arrows mark a common, phosphotyrosine-containing peptide.*

untreated two-dimensional gels (10). Presumably the phosphoserine is more labile during the preparation for peptide mapping. Others have found it difficult to identify the phosphoserine-containing peptide(s) of this protein (12, 28).

Phosphoproteins of transformed mouse cells. We wished to examine cells transformed with A-MuLV for proteins containing phosphotyrosine, using two-dimensional gel electrophoresis. A-MuLV can transform mouse fibroblasts in culture, but has not yet been shown to transform chicken cells. Since the pI's and M_r's of some mouse and chicken proteins have diverged considerably during evolution, we compared a line of BALB/c mouse 3T3 cells, transformed with SR-RSV-D (SR-3T3) [35]), with a nonproducer line of A-MuLV-transformed NIH 3T3 cells, ANN-1 (32). Both of these cell lines have elevated levels of phosphotyrosine (35, 36). As controls, we tested uninfected NIH 3T3 and BALB/c 3T3 cells, either growing rapidly in sparse culture or resting in confluent culture, as well as NIH 3T3 cells transformed with Moloney murine sarcoma virus and BALB/c 3T3 cells transformed with simian virus 40. All of these control cells have low levels of phosphotyrosine (35, 36).

Cell cultures were grown in parallel and labeled with ^{32}P. Lysates were prepared and phosphoproteins were separated on two-dimensional

gels, using isoelectric focusing with pH 5 to 7 or pH 6 to 8 range ampholines, or non-equilibrium pH gradient electrophoresis. The gels were treated with alkali and autoradiographed. The only major differences between ANN-1 or SR-3T3 cells and their normal cell controls were observed when using isoelectric focusing with pH 6 to 8 range ampholines (Fig. 6 A to C). The levels of three phosphoproteins were characteristically lowered by transformation, proteins *y* and *z* in ANN-1 cells and proteins *x* and *z* in SR-3T3 cells (Fig. 6F), whereas two other phosphoproteins were only detected in the RSV- or A-MuLV-transformed cells. One of these latter phosphoproteins (π, which on some gels was partially resolved into two spots with the same 39,000 M_r but with slightly different pI's) is the same size as, but is more basic than, the chicken cell phosphoprotein *p*. The more minor phosphoprotein (ψ) is of approximately 29,000 M_r and 7.38 pI and is similar, but not identical, in mobility to phosphoprotein *q* of transformed CECs. It was more heavily phosphorylated in ANN-1 than in SR-3T3 cells. None of these changes was observed in 3T3 cells transformed by Moloney murine sarcoma virus or simian virus 40 (Fig. 6D and E), viruses which do not elevate the total cellular phosphotyrosine content. Mouse 3T3 cells transformed by polyoma virus (Py 6 [5]) or by Harvey sarcoma virus likewise did not contain either π or ψ (data not shown). Polyoma virus-transformed rodent cells have low levels of phosphotyrosine (35).

The two phosphoproteins observed in A-MuLV- and RSV-transformed 3T3 cells were purified from alkali-treated gels, and their phosphoamino acids were analyzed (Fig. 7). Both contain phosphotyrosine and phosphoserine.

Homology between chicken phosphoprotein p and mouse phosphoprotein π. When extracts of [^{35}S]methionine-labeled SR-3T3 cells were subjected to ion-exchange column chromatography, a 39,000-M_r protein was detected with properties similar to those of the protein purified from transformed chicken cells (Fig. 4, track 8). Peptide mapping of this mouse protein showed that it is very similar to the CEC 39,000-M_r protein (Fig. 8). Some of the peptides apparently unique to either the chicken or the mouse protein were found in both proteins in other experiments, although the yields were lower. Some differences between the species were expected since the chicken protein has a pI of 7.5, but the unphosphorylated mouse protein is too basic to enter pH 6 to 8 range isoelectric focusing gels (pI > 7.5) (data not shown). Preparations purified from ^{32}P-labeled 3T3 cells, transformed with RSV or with A-MuLV, contain two phosphoproteins (Fig. 4, track 7), the faster migrating of which almost comigrates with the ^{35}S-labeled 39,000-M_r protein and contains phosphoserine and phosphotyrosine. After trypsin digestion, the same major phosphotyrosine-containing peptide was found (Fig. 9A and B); it had electrophoretic and chromatographic properties similar, but not identical, to those of the major phosphopeptide of protein *p* purified from FSV-transformed CECs (Fig. 9C to F). The upper member of the ^{32}P doublet was not always detected in such large amounts as in the experiment illustrated (Fig. 4, track 7). It has a distinct phosphopeptide map and contains mostly phosphoserine (data not shown).

DISCUSSION

CECs transformed by RSV, FSV, PRC II, or Y73 contain elevated levels of the same seven alkali-resistant phosphoproteins. Direct analysis of these phosphoproteins, purified by two-dimensional gel electrophoresis from CECs transformed by RSV, FSV, or Y73, shows that they all contain phosphotyrosine. For one of these phosphoproteins (M_r 39,000 [12, 21, 28, 29]), the same tyrosine residue is probably the major site of tyrosine phosphorylation in cells transformed by the different viruses. Mouse 3T3 cells, transformed by RSV, have elevated levels of a phosphoprotein which appears to be homologous to the 39,000-M_r transformation-dependent CEC phosphoprotein. Mouse 3T3 cells transformed by A-MuLV likewise contain elevated levels of this phosphoprotein, probably phosphorylated on the same tyrosine residue. One other mouse cell phosphoprotein (M_r 29,000) is also phosphorylated, on a tyrosine residue, in cells transformed with either RSV or A-MuLV. It may be similar to a transformation-dependent CEC phosphoprotein. Additional phosphoproteins, homologous to those observed in transformed CECs, may also be present in 3T3 cells, but we did not detect them, and the genetic differences between mice and chickens make prediction of the mobilities of such phosphoproteins impossible. It is also important to note that specific increases in proteins containing phosphotyrosine were not detected in cells transformed by viruses which do not elevate the gross level of protein phosphotyrosine. Thus, phosphorylation of these particular proteins at tyrosine residues is not a necessary consequence of the transformed state, but rather may be associated with the mechanism of transformation by certain viruses. We do not know, however, whether any of the proteins that we have detected are actually involved in any cell function affected by transformation.

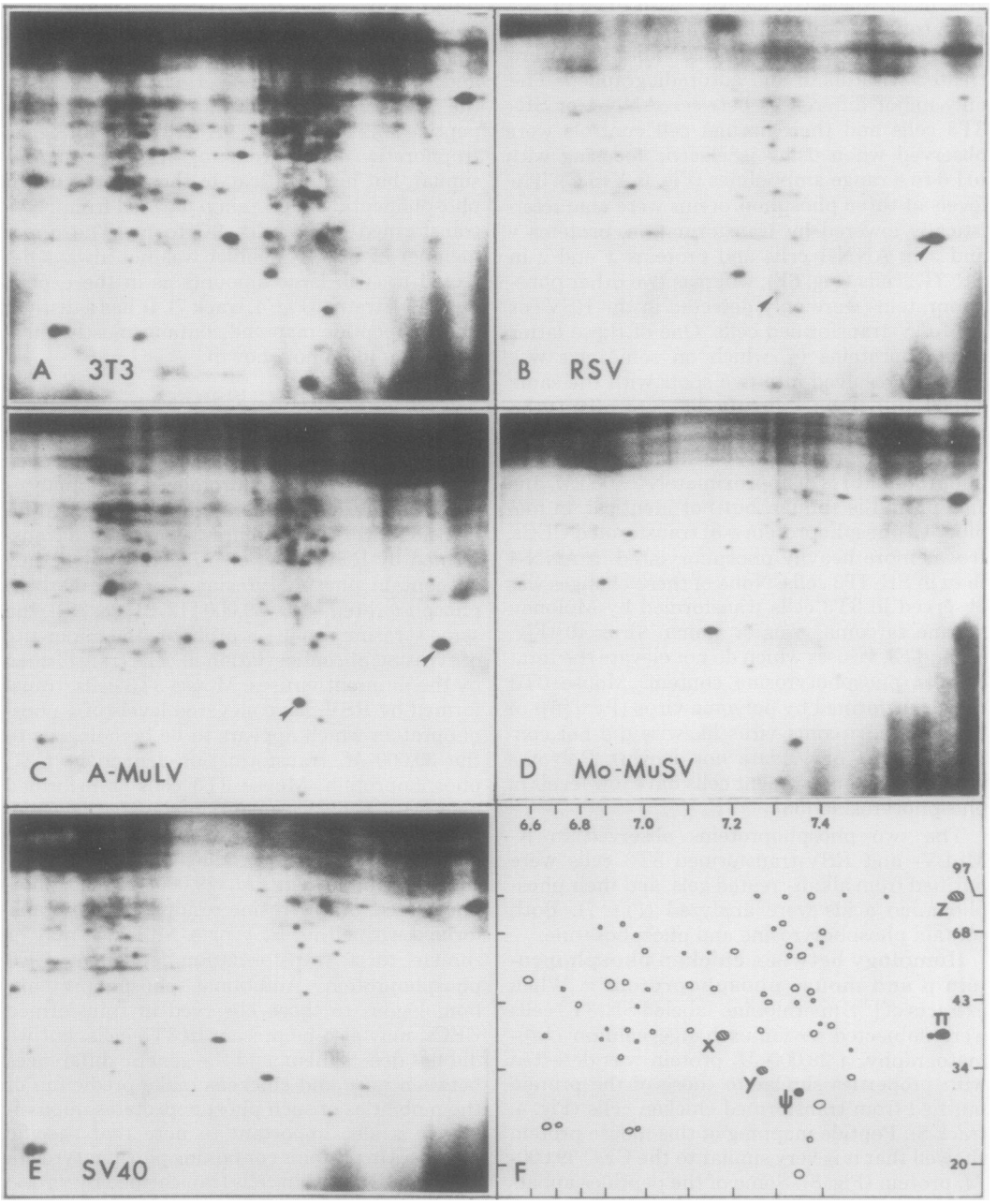

FIG. 6. *Alkali-treated two-dimensional gels of phosphoproteins of normal and transformed mouse 3T3 cells. (A) Uninfected NIH 3T3 cells (2×10^5 cells) and 3T3 cells transformed with (B) RSV (6×10^5 SR-3T3 cells), (C) A-MuLV (9×10^5 ANN-1 cells), (D) Moloney murine sarcoma virus (7×10^5 cells), and (E) simian virus 40 (6×10^5 SV3T3 cells) were labeled in parallel for 17 h with 2.4 (A) or 0.6 (B to E) mCi of ^{32}P per ml. The samples were analyzed by two-dimensional gel electrophoresis, treated with alkali, and autoradiographed with an intensifying screen for 68 h. The diagram (F) indicates the approximate pI's (abscissa) and M_r's ($\times 10^{-3}$, ordinate) of major mouse 3T3 cell phosphoproteins. Three phosphoproteins depressed by RSV or A-MuLV transformation are shaded and lettered x, y, z. Two phosphoproteins detected only in RSV- or A-MuLV-transformed cells are shown as solid spots and are lettered π and ψ; they are arrowed in (B) and (C).*

Although it is likely that unscheduled phosphorylation of tyrosine by p60^{src} mediates transformation by RSV, the evidence for a similar mechanism of transformation by other viruses is circumstantial. For RSV, it is known that the purified transforming protein (p60^{src}) has tyrosine-specific protein kinase activity and that cells infected with a mutant virus, temperature sensitive for transformation, contain a thermolabile p60^{src} (9, 18, 35). The gross level of phosphotyrosine contained in proteins of cells transformed with such a mutant RSV is temperature sensitive and correlates with transformed morphology.

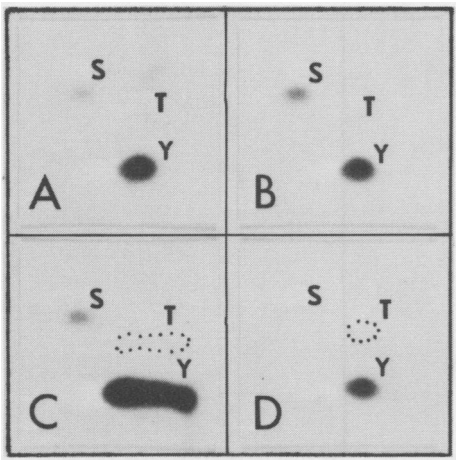

FIG. 7. *Phosphoamino acids of individual phosphoproteins. SR-3T3 cells (A and B) and ANN-1 cells (C and D) were labeled in parallel with 2.4 mCi of ^{32}P per ml at 37°C. Phosphoproteins π (A and C) and ψ (B and D) were prepared and extracted from replicate alkali-treated two-dimensional gels, and the phosphoamino acids were analyzed (see legend to Fig. 3). Portions of 8-day indirect autoradiographs of thin-layer plates loaded with 900 (A), 900 (B), 380 (C), and 110 (D) cpm (including inorganic phosphate) are shown.*

All of the other viruses considered here are defective and require a nondefective "helper" virus for replication. These helper viruses do not contain the cell-related sequences and do not transform. Thus, it is presumed that transformation by these defective viruses is due to the cell-related sequences. In each case, these are probably expressed as a single polypeptide, containing viral structural protein determinants fused to the cellular protein determinants (17, 20, 22, 24, 30, 42).

For A-MuLV, it has been shown that the transforming protein, P120$^{gag\text{-}abl}$, is associated with a tyrosine-specific protein kinase in immunoprecipitates (40) and in preparations partially purified by conventional procedures (40). Unconditional mutants encoding altered transforming proteins have diminished kinase activity in vitro (31, 41). In the experiments reported here, we used a nonproducer cell line in which the helper virus was absent, so the altered phosphorylation presumably was due to the action of

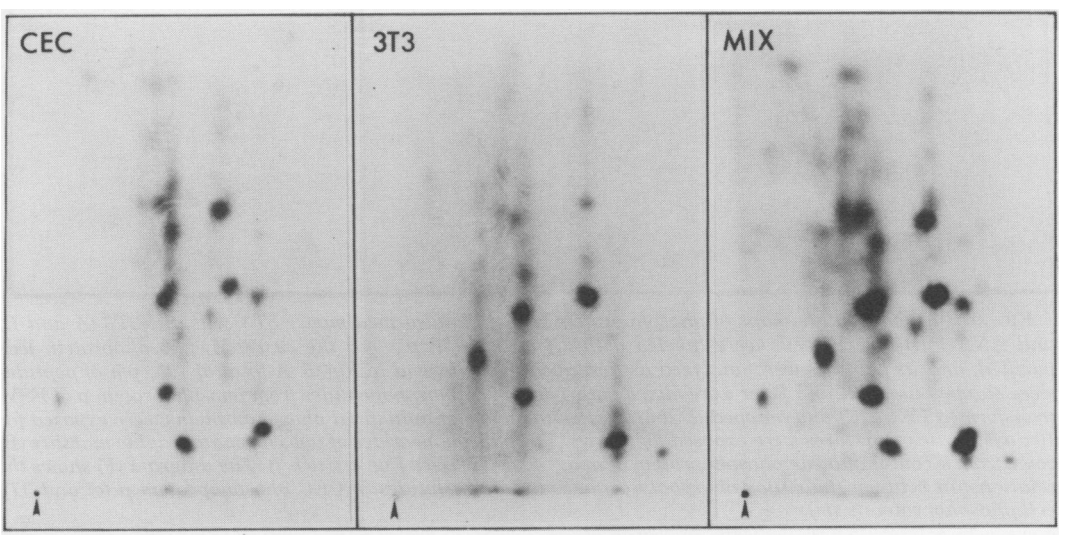

FIG. 8. *Peptide maps of 39,000-M_r protein from CECs and mouse 3T3 cells. RSV-transformed CECs and SR-3T3 cells were labeled in parallel with 500 μCi of [^{35}S]methionine per ml of low-methionine medium (10) for 18 h at 37°C, and the 39,000-M_r protein was purified by ion-exchange chromatography and preparative gel electrophoresis. After performic acid oxidation and trypsin digestion, approximately 2×10^5 cpm of each were recovered. A total of 18,000 cpm of CEC peptides and 14,000 cpm of 3T3 peptides were analyzed, alone or in combination, by electrophoresis at pH 4.7 (cathode at the right) and ascending chromatography. Twelve-day fluorographs are shown. The arrow marks the point of sample application.*

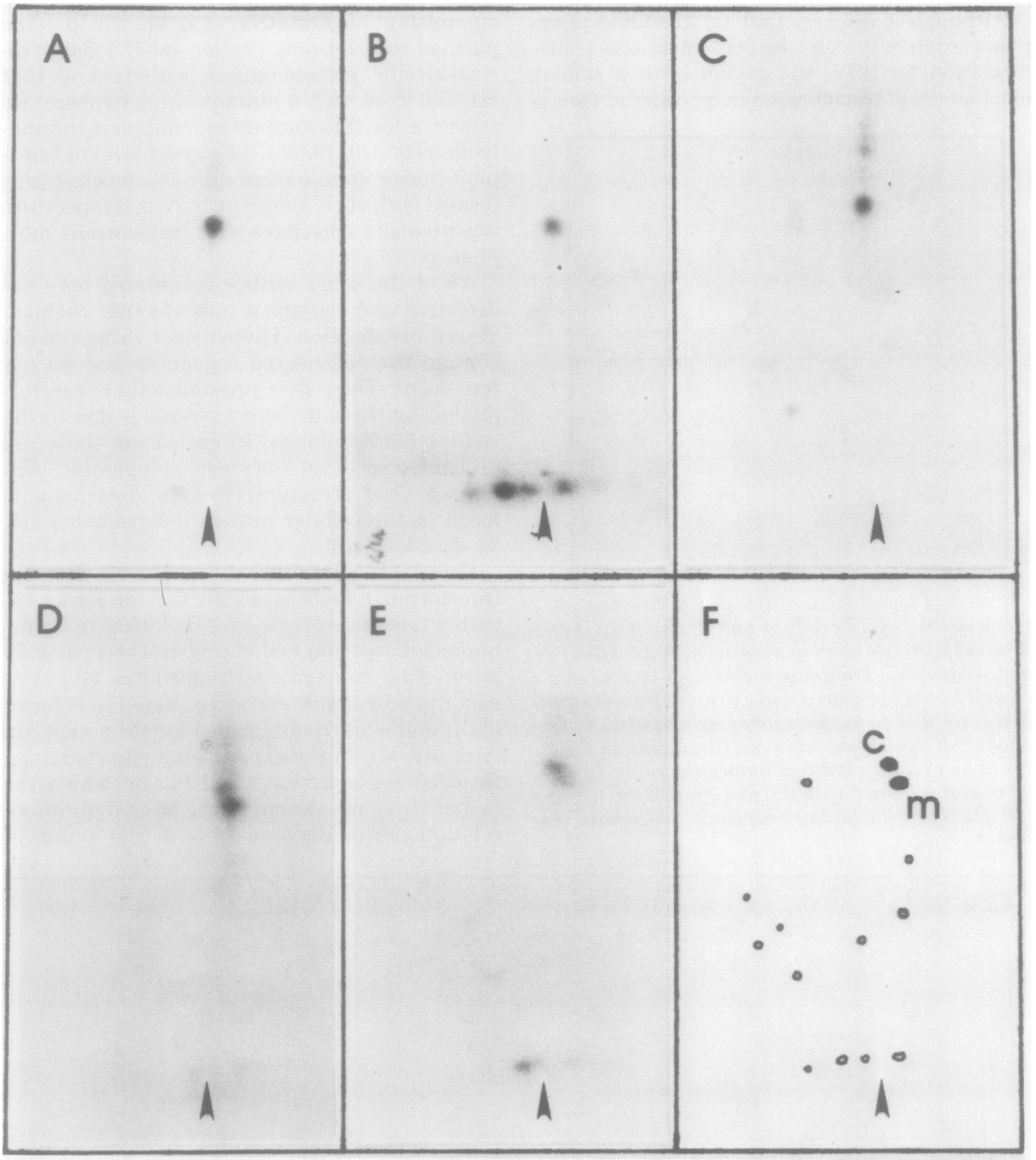

FIG. 9. *Phosphopeptide maps of phosphoprotein π from transformed mouse 3T3 cells. SR-3T3 (A and D) and ANN-1 (B and E) cells were labeled with 2.4 mCi of ^{32}P per ml; the 39,000-M_r phosphoprotein was purified, and its peptides were analyzed as described in the legend to Fig. 5. A total of 100 cpm of peptides were mapped alone (A and B) or were mixed with 100 cpm of phosphopeptides from phosphoprotein p of FSV-transformed CECs (C) and mapped (D and E). Peptide maps of individual phosphoproteins were exposed for 10 days; those of mixtures were exposed for 4 days. The phosphopeptides of low chromatographic mobility (B) come from a contaminating phosphoprotein of about 40,000 M_r (see Fig. 4, track 7). The diagram (F) shows the relationship between the major phosphotyrosine-containing peptides of CEC phosphoprotein p (c) and 3T3 cell phosphoprotein π (m).*

$P120^{gag\text{-}abl}$. Moloney murine sarcoma virus, which has *gag* sequences related to those of A-MuLV, did not have the same effects on phosphorylation.

For the experiments with FSV, PRC II, and Y73, virus stocks containing helper virus were used. However, avian leukosis viruses, for which transformatiom-defective RSV having a deleted

src gene is a model (11), do not elevate total cell protein phosphotyrosine (18). RSV carrying a temperature-sensitive mutation in the *src* gene, cultivated at the nonpermissive temperature, did not cause phosphorylation of the specific proteins described here (10), even though this virus has the same structural genes as are found in leukosis viruses. In cells transformed by RSV, PRC II, and Y73, therefore, the phosphorylation of tyrosine in the specific substrates *k* through *q* is almost certainly due to the transforming virus and not to the helper. In the case of FSV, the temperature-sensitive nature of the virus stock for transformation, but not for replication, correlates with the temperature sensitivity of the tyrosine phosphorylations, providing strong evidence that the transforming protein of this virus ($P140^{gag\text{-}fps^F}$) is responsible for these phosphorylations (27).

The simplest explanation of these results is that these disparate viruses encode different tyrosine-specific protein kinases which have a common spectrum of cellular substrate proteins, some or all of which are involved in maintaining the normal cell phenotype. Phosphorylation may regulate their functions, and increased phosphorylation may result in a transformed phenotype. Alternatively, the different viral protein kinases could phosphorylate one or more cellular protein(s) which is itself a tyrosine-specific protein kinase and which then phosphorylates the proteins we have detected. Such a protein kinase cascade has been detected in Ehrlich ascites tumor cells (38). One argument against this in our case is that different substrates are not phosphorylated to the same degree in cells transformed by different viruses, such as might be expected if the same kinase were responsible. For example, protein *o* is relatively more phosphorylated, and protein *m* less phosphorylated, in PRC II-transformed cells than in RSV-transformed cells. These quantitative differences could be due to differences in the subcellular location of the kinases or to their intrinsic substrate specificities.

We have also found that one other protein, not detected in the two-dimensional gel analysis reported here, is phosphorylated on tyrosine residues in cells transformed by particular viruses. This protein, the 130,000 M_r cytoskeletal protein vinculin (8, 15), contains 6- to 10-fold more phosphotyrosine when immunoprecipitated from CECs transformed by RSV or by Y73, or from mouse 3T3 cells transformed by RSV or A-MuLV, than when purified from normal CECs or from 3T3 cells transformed by simian virus 40 or Moloney murine sarcoma virus (34). However, vinculin from PRC II-transformed CECs contains only low levels of phosphotyrosine, further suggesting that a single common cellular kinase is not activated by the different viral transforming proteins (34).

One question is why the cell has several genes (the cellular equivalents of *src, abl, fes/fps,* and *yas*) which presumably all code for tyrosine-specific protein kinases with overlapping substrate specificities. Since, in many instances, the cellular genes appear to be highly conserved through evolution, these normal cell protein kinases presumably play vital roles in the cell. Their activities may be controlled at the level of gene expression (for example, during differentiation or the cell cycle) or by such post-translational mechanisms as covalent modification and effector binding. We have recently observed that a further, probably unrelated class of tyrosine-specific protein kinase, the epidermal growth factor–receptor-associated kinase, causes phosphorylation of a human cell protein homologous to chicken protein *p* or mouse protein π (Hunter and Cooper, Cell, in press). Thus, there are fascinating parallels between transformation by some retroviruses and the effects of a growth factor.

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ADDENDUM IN PROOF

Other work in this laboratory (B. Adkins and T. Hunter, J. Virol. submitted for publication) has shown that a cellular mRNA is selectively packaged in RSV virions. It now appears that the in vitro translation product of this mRNA may be related to the 39K phosphoprotein (*p*). In addition, both the in vitro product and the 39K protein have some peptides in common with the RSV structural protein, $Pr76^{gag}$. The significance of this is not clear, but it may indicate an evolutionary or functional relationship.

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