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Changes in Protein Phosphorylation in Rous Sarcoma Virus-Transformed Chicken Embryo Cells

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Rous sarcoma virus encodes a tyrosine-specific protein kinase ($p60^{src}$) which is necessary for cell transformation. To identify substrates for this kinase, we set out to detect phosphotyrosine-containing proteins in Rous sarcoma virus-transformed chicken embryo cells, making use of the known alkali stability of phosphotyrosine. ^{32}P-labeled phosphoproteins were separated by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were then incubated in alkali. Using this procedure with normal cells, we detected a total of about 190 alkali-resistant phosphoproteins. In Rous sarcoma virus-transformed cells, five phosphoproteins were found which were not detectable in normal cells. Two of these are probably structural proteins of the virus. The other three transformation-dependent phosphoproteins, and four other phosphoproteins which were elevated by transformation, all contained phosphotyrosine. Increased phosphorylation of these proteins did not occur with cells infected with a mutant Rous sarcoma virus, temperature sensitive for transformation, grown at the restrictive temperature. We conclude that these seven proteins are probably substrates of $p60^{src}$, although they may be substrates for other tyrosine-specific protein kinases activated by $p60^{src}$.

The transforming protein of Rous sarcoma virus (RSV), $p60^{src}$, is a protein kinase which is specific for tyrosine residues in acceptor proteins when assayed in vitro (5, 11). Moreover, the proteins of chicken or mammalian cells transformed by RSV contain up to 10 times more phosphotyrosine than do the proteins of uninfected cells (27). This elevation is probably due to the protein kinase activity of $p60^{src}$, because cells infected with a mutant RSV which encodes a thermolabile $p60^{src}$ exhibit a normal morphology and contain a low level of phosphotyrosine when grown at the nonpermissive temperature (27). The level of phosphotyrosine in mutant-infected cells changes rapidly when the incubation temperature is altered, and this change precedes the changes in morphology which characterize the transition between normal and transformed phenotype. Thus, it seems likely that transformation by RSV is due to unscheduled phosphorylation of tyrosine in protein.

The observation that $p60^{src}$ activity in vivo is manifested as a net increase in tyrosine phosphorylation has stimulated the search for similar protein kinase activities in cells infected with other transforming viruses (14, 27). Cells transformed by chemical carcinogens, deoxyribonucleic acid tumor viruses, and several retroviruses do not contain elevated phosphotyrosine; however, Abelson murine leukemia virus (31; B. M. Sefton, T. Hunter, and W. C. Raschke, in press), feline sarcoma virus (Snyder-Theilin strain; 1), and the avian sarcoma viruses PRC II, Y73, and Fujinami sarcoma virus (K. Beemon, personal communication) all do elevate phosphotyrosine levels. These viruses, like RSV, probably arose by recombination between nontransforming retroviruses and cellular genes. Various lines of evidence suggest that at least three different cellular genes have been acquired by these viruses (28; K. Beemon, personal communication). Thus, it is likely that untransformed cells contain genes for several different tyrosine-specific protein kinases (13). Clearly, a knowledge of the cellular proteins which are substrates for these viral and cellular tyrosine protein kinases would aid our understanding of transformation.

The detection of proteins containing phosphotyrosine is difficult, however, because even in RSV-transformed cells only 0.2 to 0.6% of the acid-stable protein phosphate is in phosphotyrosine, the remainder being in phosphoserine and phosphothreonine. Accordingly, we have developed a procedure which increases the chances of detecting phosphotyrosine-containing proteins, based on the relative stability of the phosphate ester bond of phosphotyrosine under alkaline conditions (21). Using this technique, we have identified several phosphotyrosine-containing proteins that are candidate substrates for $p60^{src}$.

MATERIALS AND METHODS

Cells and viruses. Chick cells were prepared as described (26). Confluent monolayers of primary cells were trypsinized, and the cell suspension was infected

with Prague RSV subgroup A (PR-RSV-A) or PR-RSV-A mutant *ts*LA29 (33) at a cell density of about 5×10^6 to 10×10^6 per ml in Dulbecco-modified Eagle medium supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1% heat-inactivated chicken serum. After 30 min at 25° C, the cells were plated at 3×10^5 cells per 35-mm plastic petri dish (Falcon). Cells infected with PR-RSV-A were grown at 41°C. Cells infected with *ts*LA29 were incubated at 36°C, until judged to be fully transformed. They were then trypsinized and reseeded as described (27). These dishes were incubated for a further 18 h at 36 or 41°C before labeling.

Labeling. Cells were labeled with [^{35}S]methionine (Amersham Searle) at 300 μCi/ml of Dulbecco-modified Eagle medium containing 5% of the standard methionine concentration and 4% calf serum. ^{32}P-labeling of 35-mm dishes of cells was in 1 ml of phosphate-free Dulbecco-modified Eagle medium with 4% calf serum. For analytical purposes, 600 μCi of [^{32}P]-orthophosphate (ICN; carrier free) was used. For preparing phosphoproteins for phosphoamino acid analysis, 2.4 mCi was used.

Sample preparation. Cells were washed with Ca^{2+}-Mg^{2+}-free tris(hydroxymethyl)aminomethane-buffered saline and lysed for two-dimensional gel electrophoresis as described (9), except that the staphylococcal nuclease solution also contained 0.1% Nonidet P-40 (23). A 125-μl volume of this was used per 35-mm dish containing 450 to 600 μg of cellular protein (1×10^6 to 1.5×10^6 cells).

Two-dimensional gel electrophoresis. Samples were analyzed by isoelectric focusing with pH 5 to 7 or pH 6 to 8 ampholines (LKB), using narrow tube gels, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) with the following modifications. The urea concentration in the isoelectric focusing gel was 9.15 M. The first-dimension gels were 15 cm long; the dye front was run 11.5 cm in the second dimension. The second-dimension gel contained 15% acrylamide and 0.087% bis-acrylamide. Samples of 10 μl were analyzed.

The pH profile was measured by slicing a 3-mm-diameter tubular isoelectric focusing gel run in parallel with the first dimensions. Slices were equilibrated with 9.15 M urea, and the pH was measured with a Radiometer electrode. The pH gradient was very shallow at the alkaline end of the gel (4). Molecular weights were estimated by overlaying the second-dimension gel with a solution of unlabeled marker proteins after the first-dimension gel had been equilibrated and fitted in place. After electrophoresis, the gel was stained, destained, and then processed as described below.

Alkali treatment. After electrophoresis, two-dimensional gels were soaked in two changes of 10% acetic acid–10% isopropanol and then dried. ^{32}P was detected by indirect autoradiography (16), using a fluorescent screen and preexposed film at −70°C. ^{35}S radiation was prevented from exposing the film by four layers of Reynolds 656 aluminum foil (M. A. Hutchinson, personal communication). ^{35}S was detected by direct autoradiography at room temperature under conditions where ^{32}P detection was inefficient (16). The gel was swollen in approximately 15 volumes of 1 M KOH and incubated at 55°C for 2 h. It was then neutralized and washed with gentle agitation with four changes of 10% acetic acid–10% isopropanol for at least 2 h. During this time, it shrank back to a final size slightly larger than before alkali treatment. It was then dried again and exposed as before. Subsequent ^{32}P exposures were adjusted to allow for radioactive decay.

Extraction of phosphoproteins. Phosphoproteins were extracted by homogenization and trichloroacetic acid precipitation (2) from pieces of untreated gels which had simply been washed with water containing Amberlite MB3 resin and dried. Acid hydrolysis (11) was for 1 h at 110°C (12). Material extracted from alkali-treated gels was largely trichloroacetic acid soluble, so a modified procedure was used. Gel pieces were swollen in 3 ml of 50 mM NH_4HCO_3–1% 2-mercaptoethanol by heating at 100°C for 1 min, but were not homogenized. A 5-μl volume of 50-mg/ml pronase (Sigma, exhaustively self-digested) was added, and the pieces were incubated at 37°C for 18 h in a shaking water bath. The liquid was pipetted into a centrifuge tube, and the gel pieces were incubated twice more for 1 h with 2 ml of water each. The pooled extracts (30 to 70% of radioactivity) were clarified and then lyophilized in a hard glass tube. A 0.5-ml volume of 6 M HCl was added, and hydrolysis was peformed at 110°C for 1 h. The HCl was removed under reduced pressure, and the residue was dissolved in 0.5 ml of water containing 0.5 μg each of phosphoserine, phosphothreonine, and phosphotyrosine. The phosphoamino acids were then purified according to Flavin (8). A 0.1-ml volume of a 50% slurry of Dowex 50WX8 (H^+ form) was added and mixed for 10 min. The unbound material was adjusted to 1 mM HCl and mixed with 0.2 ml of a 50% slurry of Dowex AG1X8 (Cl^- form) for 30 min. The resin was washed twice with 1 mM HCl, and the bound radioactivity was eluted in 50 mM HCl and lyophilized. This procedure recovered 15 to 20% of the radioactivity and quantitatively purified the phosphoamino acids.

Thin-layer electrophoresis. Phosphoamino acids were separated on cellulose thin-layer plates by electrophoresis at pH 1.9 for 20 min at 1.5 kV in one dimension and at pH 3.5 for 16 min at 1.0 kV in the second dimension (buffer composition, see reference 11). Under these conditions inorganic phosphate moves 6 cm in each dimension, so four separate samples can be analyzed on a single plate, using four origins arranged in a 8-cm square, without cross-contamination. Markers were detected by staining with ninhydrin.

Interpretation of autoradiographs. Since there is no straightforward procedure for measuring the amount of radioactivity in a ^{32}P-labeled cell lysate which is in phosphoprotein, we attempted to load equivalent amounts of cell protein on the two-dimensional gels. Even so, some variability in loading was evident. Those spots which appeared to be unaffected by transformation were used as standards. Changes in phosphorylation in transformed cells were estimated by making different autoradiographic exposures. Reproducibility of separations was confirmed by running ^{35}S-labeled proteins as internal standards on most gels.

RESULTS

Alkali stability of ^{32}P-labeled cellular proteins. Phosphotyrosine is the most stable of the three phosphorylhydroxyamino acids when incubated at high pH (21). This stability has also been observed when phosphotyrosine is contained in protein. For example, 60% of the [^{32}P]phosphotyrosine of immunoglobulins, labeled in vitro by p60^{src}, could be precipitated with trichloroacetic acid after incubation in 1 M KOH at 55°C for 2 h, whereas less than 10% of the ^{32}P radioactivity could be recovered from a phosphoserine-containing protein (12). However, these measurements may be influenced by alkaline hydrolysis of peptide bonds, which could prevent trichloroacetic acid precipitation of the protein. To test whether the phosphotyrosine in the phosphoproteins of RSV-transformed cells is as alkali resistant as the phosphotyrosine in immunoglobulins, we performed the following experiment, in which trichloroacetic acid precipitation of alkali-degraded proteins was avoided (Table 1).

A sample of total cellular macromolecules was prepared by trichloroacetic acid precipitation of ^{32}P-labeled RSV-transformed chicken embryo cells (CEC). Partial acid hydrolysis of a control portion of the sample generated mostly inorganic phosphate and nucleotides (14), but a little over 1% of the total cellular acid-insoluble radioactivity was recovered as phosphoserine, phosphothreonine, and phosphotyrosine. Phosphotyrosine constituted 0.2% of the recovered phosphoamino acids, a typical value for RSV-transformed cells (27). On the other hand, when another portion of the sample was incubated in 1 M KOH at 55°C for 2 h before acid hydrolysis, phosphotyrosine constituted 1% of the recovered phosphoamino acids, a fivefold increase (Table 1). The proportion of the total radioactivity recovered as phosphoserine was reduced 70%, but the amounts of phosphothreonine and phosphotyrosine increased 50 and 90%, respectively. These apparent increases will be discussed below. However, this and similar experiments show that the phosphotyrosine and phosphothreonine in proteins from transformed cellas are relatively stable under alkaline conditions.

TABLE 1. *Phosphoamino acid analysis of alkali-treated phosphoproteins*

Phosphoamino acid	% of total sample cpm[a]				Ratio: alkali/control
	Control		Alkali		
Phosphoserine	1.1	(94)[b]	0.35	(78)	0.3
Phosphothreonine	0.064	(5.5)	0.096	(21)	1.5
Phosphotyrosine	0.0023	(0.2)	0.0043	(1.0)	1.9

[a] PR-RSV-A-transformed CEC were labeled with ^{32}P and solubilized (26). Duplicate samples were precipitated with cold trichloroacetic acid, extracted with $CHCl_3$-CH_3OH (1:1, vol/vol), and dried. One precipitate was dissolved in 1 M KOH and incubated at 55°C for 2 h. After neutralization with perchloric acid, the insoluble $KClO_4$ was removed by centrifugation. The control precipitate was treated with a $KClO_4$ suspension. Both supernatants were lyophilized, acid hydrolyzed, and analyzed (see text); 2×10^7 cpm of radioactivity was recovered in each case.

[b] Percentage of the total radioactivity recovered in phosphoserine, phosphothreonine, and phosphotyrosine.

Effect of alkali on proteins separated in polyacrylamide gels. The use of alkali stability as a criterion for identifying proteins which contain phosphotyrosine requires that alkali treatment be performed after the proteins have been fractionated, because strong alkali hydrolyzes peptide bonds. Preliminary experiments suggested that proteins fixed in polyacrylamide gel by acidic isopropanol treatment would remain trapped in the gel matrix during incubation in 1 M KOH at 55°C for 2 h. This was confirmed by an experiment in which we separated [^{35}S]-methionine-labeled proteins from RSV-transformed CEC by high-resolution two-dimensional gel electrophoresis (9, 18).

Samples were prepared under conditions which minimize chemical or enzymatic modification of proteins and were resolved by isoelectric focusing in urea, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For example, the autoradiograph of a fixed and dried gel run using ampholines in the pH 6 to 8 range revealed approximately 200 methionine-containing polypeptides with isoelectric points (pI) in the range of 6.5 to 7.5 and molecular weights (M_r) of 15,000 to 200,000. (The gel was loaded with 2×10^6 trichloroacetic acid-precipitable dpm and autoradiographed for 70 h.) After alkali treatment, the gel was neutralized in acidic isopropanol and dried, and a second autoradiographic exposure was obtained. All the polypeptides were detected with the same intensity as before, but were slightly more diffuse. We did not detect any distortion of the pattern beyond small changes in the overall dimensions of the gel, and the autoradiographs obtained before and after treatment were practically identical (not shown). All detectable proteins with isoelectric points between 5.5 and 6.5 or greater than 7.5 were also retained during alkali treatment of two-dimensional gels run under other ampholine conditions (9, 19) (not shown).

Having confirmed that essentially all of the ^{35}S radioactivity remained in the gel during alkali treatment, we examined the fate of ^{32}P radioactivity under the same conditions. A sample was prepared from uninfected CEC labeled for

18 h with [^{32}P]orthophosphate, and an indirect autoradiograph (16) of part of a pH 6 to 8 two-dimensional gel is shown (Fig. 1A). Some discrete spots are evident, but the gel contained many streaks of radioactivity, presumably due to nucleic acids, which were not completely solubilized during sample preparation (25). These streaks were less apparent if the labeling time was shortened, but we used long labeling times to allow complete equilibration of label in the three phosphoamino acids (14).

Another exposure of the same gel after incubation in alkali appears very different (Fig. 1B). Most of the radioactive streaks have been removed, presumably because of alkaline hydrolysis of ribonucleic acid. In addition, many of the major spots are no longer detectable (arrows, Fig. 1A), whereas others are present at reduced levels. A minor fraction of the spots are apparently unaltered in intensity. Note that the autoradiographic exposure of the treated gel was four times longer than that for the untreated gel (Fig. 1A and B). The longer exposure allows some additional spots to be detected in Fig. 1B, which could only be detected in untreated gels when several different autoradiographic exposures of replicate gels were examined. Other workers have experienced similar difficulties with streaks of radioactivity on untreated gels of phosphoproteins (22). The diagram (Fig. 2A) summarizes the pattern of the approximately 90 spots routinely detected in alkali-treated pH 6 to 8 gels of ^{32}P-labeled CEC. We believe that all of the radioactive spots detected after alkali treatment are indeed phosphoproteins, because we have found phosphoamino acids in every spot we have analyzed (see below).

Changes in alkali-stable phosphoproteins after transformation by RSV. CEC were infected with RSV and labeled with [^{32}P]-orthophosphate after 4 to 5 days, when most of the cells were phenotypically transformed. An autoradiograph of part of a pH 6 to 8 two-dimensional gel is shown (Fig. 1C). Some differences between this gel and the one of the parallel uninfected CEC extract (Fig. 1A) are evident. After incubation at high pH (Fig. 1D), many of the spots were destroyed (arrows, Fig. 1C). Comparison of the autoradiograph of the alkali-treated gel of transformed cell phosphoproteins (Fig. 1D) with that of control cells (Fig. 1B) shows that many spots are of similar intensity. However, some are more intense with normal cells (arrows, Fig. 1C), and others are more intense with transformed cells (small arrows, Fig. 2B).

We have also analyzed phosphoproteins with isoelectric points outside the range shown in Fig. 2. Uninfected CEC contained about 60 alkali-stable phosphoproteins with isoelectric points between 5.5 and 6.5, and nonequilibrium pH gradient electrophoresis (19) displayed 40 to 50 phosphoproteins with pI's greater than 7.5. However, there were no qualitative changes nor any major quantitative changes in any of these spots after RSV infection (data not shown). Direct analysis of the phosphoamino acids of several of these acidic and basic proteins did not indicate the presence of phosphotyrosine. Accordingly, we will focus below on phosphoproteins with pI's between 6.5 and 7.5.

The two major spots detected only with RSV-infected CEC, having an apparent M_r of 20,000 to 21,000 and pI's of 6.55 and 6.95, were readily detected in gels which had not been alkali treated (Fig. 1C, proteins *r* and *j*). They contain exclusively phosphoserine (see below). We believe that these spots represent phosphorylation isomers of the structural phosphoprotein of RSV, pp19^{gag}, which has previously been shown to have a pI of 6.9 to 7.0 (6, 24) and which contains phosphoserine (15) at two different sites in the molecule (K. Beemon, personal communication).

The other spots seen only with transformed cells (large arrows, Fig. 2B) were often masked in untreated gels. The 39,000 M_r, 7.33 pI spot (protein *p*) is one of the strongest spots seen after alkali treatment of any of the different two-dimensional separations we have used. Direct radioactive counting of the excised spot suggested that it may contain on the order of 0.05 to 0.1% of the total protein-bound phosphate in RSV-transformed cells. Uninfected cells contain much less than 1/20 of this amount of radioactivity in this region of the gel (data not shown). The small spot, which is slightly more acidic than the major one, was designated 39,000 M_r, 7.31 pI (protein *o*). It contains much less radioactivity than, and may represent a charge variant of, its 7.33 pI partner. The other phosphoprotein that we detect exclusively in transformed cells is about 46,000 M_r and 7.05 pI (protein *l*).

The radioactivity in eight other phosphoproteins was elevated by transformation (small arrows, Fig. 2B). Among these, two major phosphoproteins (46,000 M_r, 6.95 pI [protein *k*] and 43,000 M_r, 6.80 pI [protein *m*]) reproducibility undergo a four- to fivefold increase in transformed cells. Two very minor phosphoproteins (43,000 M_r, 7.32 pI [protein *n*], and 28,000 M_r, 7.38 pI [protein *q*]), which contain much less than 1/10 of the radioactivity of protein *p*, are difficult to detect over the background radioactivity in the gel. However, they do undergo significant quantitative increases upon transformation. The remaining phosphoproteins seen in

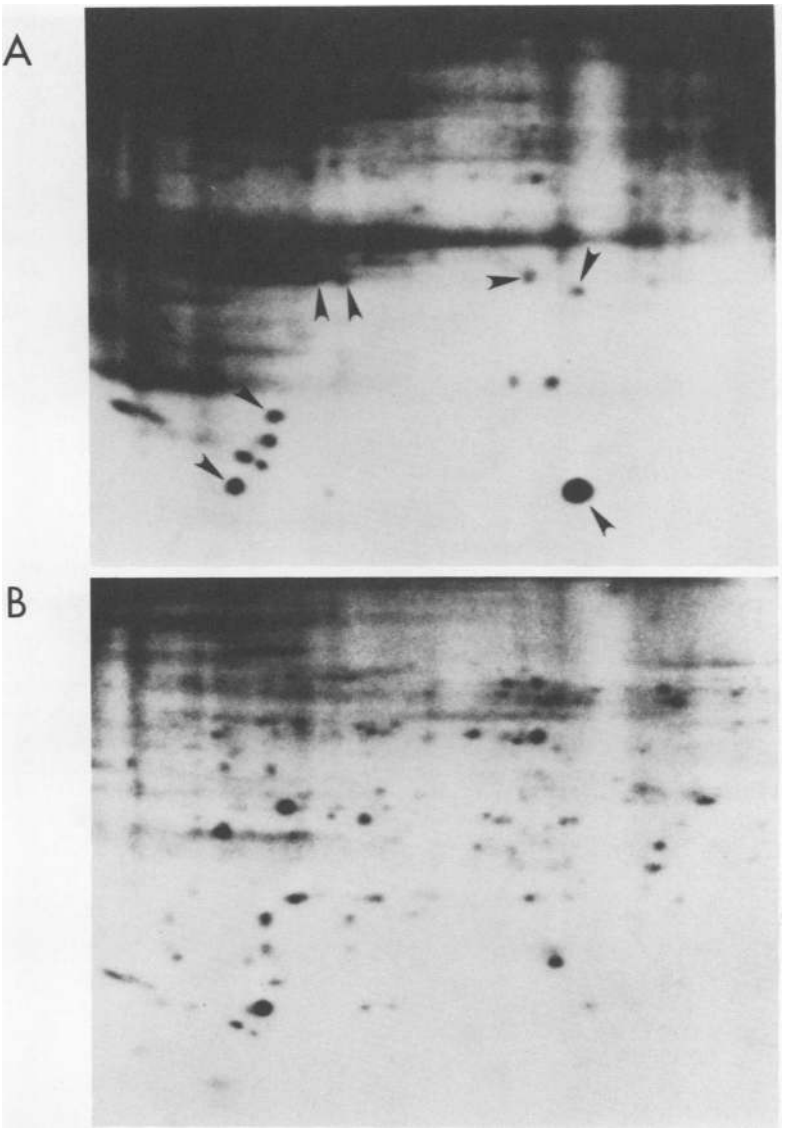

FIG. 1. *Effect of alkali treatment on two-dimensional gels of phosphoproteins from normal and transformed CEC. Parallel cultures of normal (A, B) and PR-RSV-A-transformed (C, D) CEC were labeled with ^{32}P and analyzed by two-dimensional gel electrophoresis using pH 6 to 8 ampholines. The fixed and dried gels were autoradiographed indirectly (with a fluorescent screen [16]) for 16 h (A, C), then incubated in alkali, fixed, dried, and reexposed for 64 h (B, D). Arrows in (A) and (C) point to spots which are totally lost upon alkali treatment [compare with (B) and (D)].*

Fig. 2B were not found to be reproducibly affected by transformation.

Changes in phosphoproteins which result from *src* gene expression. To show which of the changes in alkali-stable phosphoproteins were due to *src* gene expression, we infected cells with *ts*LA29, a mutant of PR-RSV-A which is temperature sensitive for transformation but largely temperature independent for replication (32, 33). This virus has been shown to encode a thermolabile $p60^{src}$, so that cells transformed by this mutant have elevated phosphotyrosine at the permissive, but not at the restrictive, temperature (27).

Comparison of alkali-treated two-dimensional gels of phosphoproteins from *ts*LA29-infected cells incubated at 41°C (Fig. 3A) with uninfected CEC labeled at the same temperature (Fig. 1B) shows some additional spots which presumably are the products of viral structural genes (small

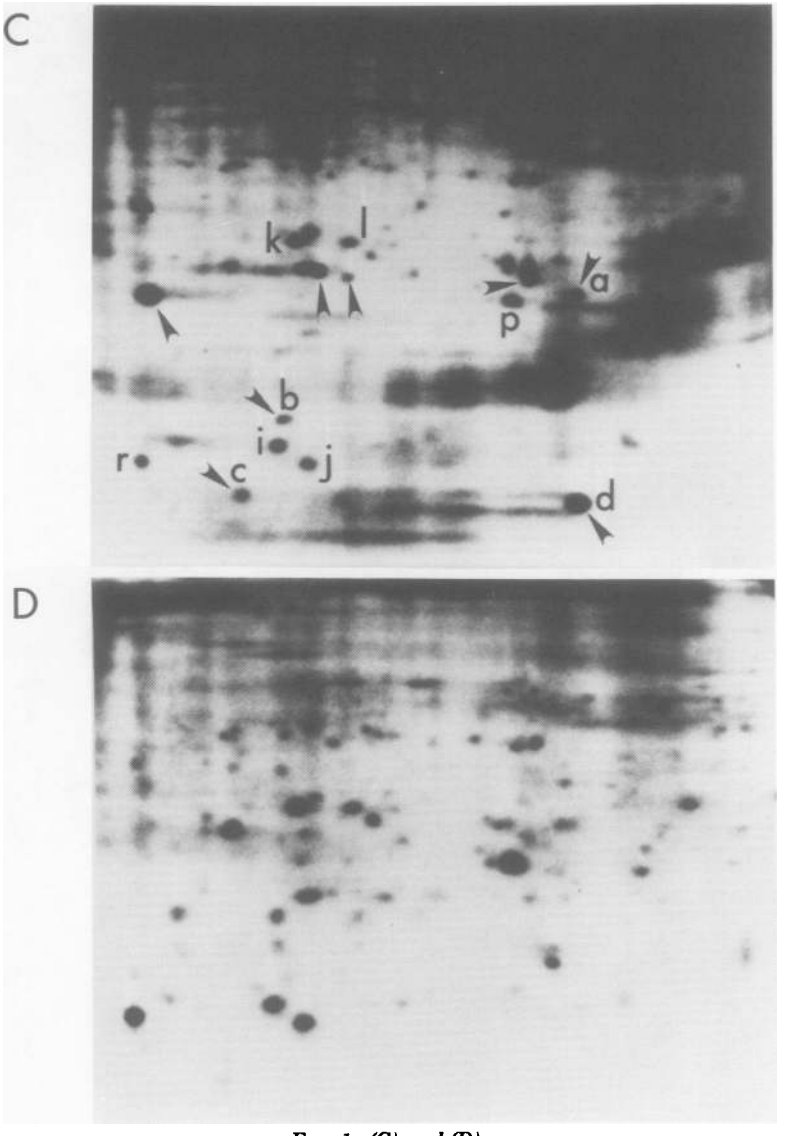

FIG. 1. *(C) and (D).*

arrows, Fig. 3). Two spots of about 22,000 M_r and 6.95 and 7.08 pI were detected in *ts*LA29-infected cells (Fig. 3A). These are apparently about 2,000 daltons larger than the presumed pp19^{gag} species detected in wild-type PR-RSV-A-infected cells (Fig. 1D) and may represent altered *gag* gene products. A structural gene mutation has not been reported for this virus, but it may account for the slight temperature sensitivity of replication of the mutant (32, 33), or may be functionally neutral. *ts*LA29-infected cells also contain three phosphoproteins of about 70,000 M_r and pI 6.75, 6.85, and 6.96, which have slightly different mobilities from minor phosphoproteins detected in wild-type PR-RSV-A-infected cells (Fig. 1D) and may represent phosphorylated, unprocessed precursor Pr76^{gag}, which contains pp19^{gag}. Most importantly, these various phosphorylations are not markedly affected by the growth temperature of *ts*LA29 (Fig. 3).

Comparison of alkali-stable phosphoproteins from *ts*LA29-infected cells labeled under permissive (Fig. 3B) and nonpermissive (Fig. 3A) conditions reveals several quantitative and qualitative differences. Some changes are apparent in phosphoproteins which are not affected by infection with wild-type virus (Fig. 1); for ex-

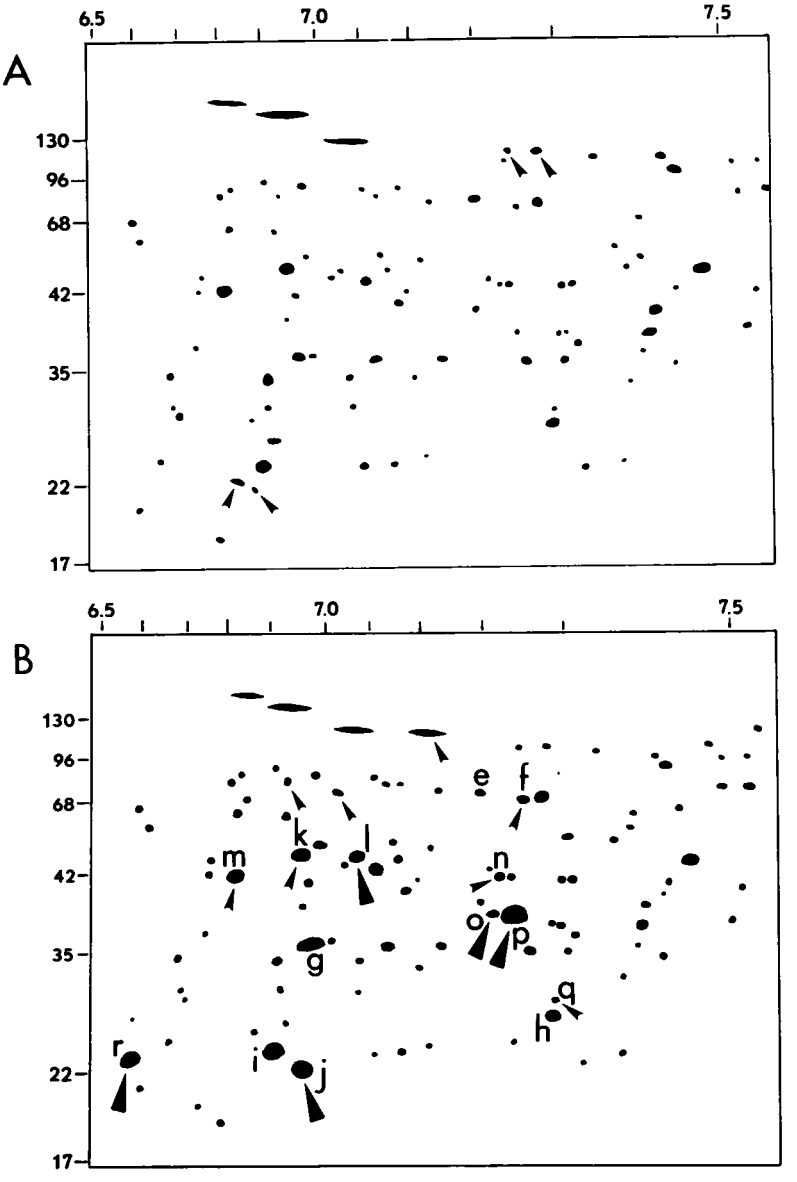

FIG. 2. *Phosphoproteins of normal and transformed CEC. Schematic diagrams of autoradiographs of alkali-treated two-dimensional gels of phosphoproteins of normal (A) and transformed (B) CEC. Arrows in (A) point to alkali-resistant phosphoproteins which are elevated in normal CEC relative to transformed CEC (B). Large arrows in (B) point to alkali-resistant phosphoproteins not detected in uninfected cells, and small arrows indicate phosphoproteins elevated by transformation. Letters refer to phosphoproteins described in the text and Table 2.*

ample, three phosphoproteins of about 35,000 M_r are attenuated at 36°C (large arrows, Fig. 3A). These were also found to be temperature dependent in cells infected with wild-type PR-RSV-A (not shown). Presumably such phosphorylations result from the activity of cellular protein kinases and phosphatases which are affected by growth temperature. On the other hand, phosphorylation of some proteins, including five of the proteins already observed in PR-RSV-A-infected cells (proteins *k, l, m, n, p*; Fig. 2B), was elevated in *ts*LA29-infected cells at 36°C (large arrows, Fig. 3B). This suggests that their phosphorylation results, directly or indirectly, from

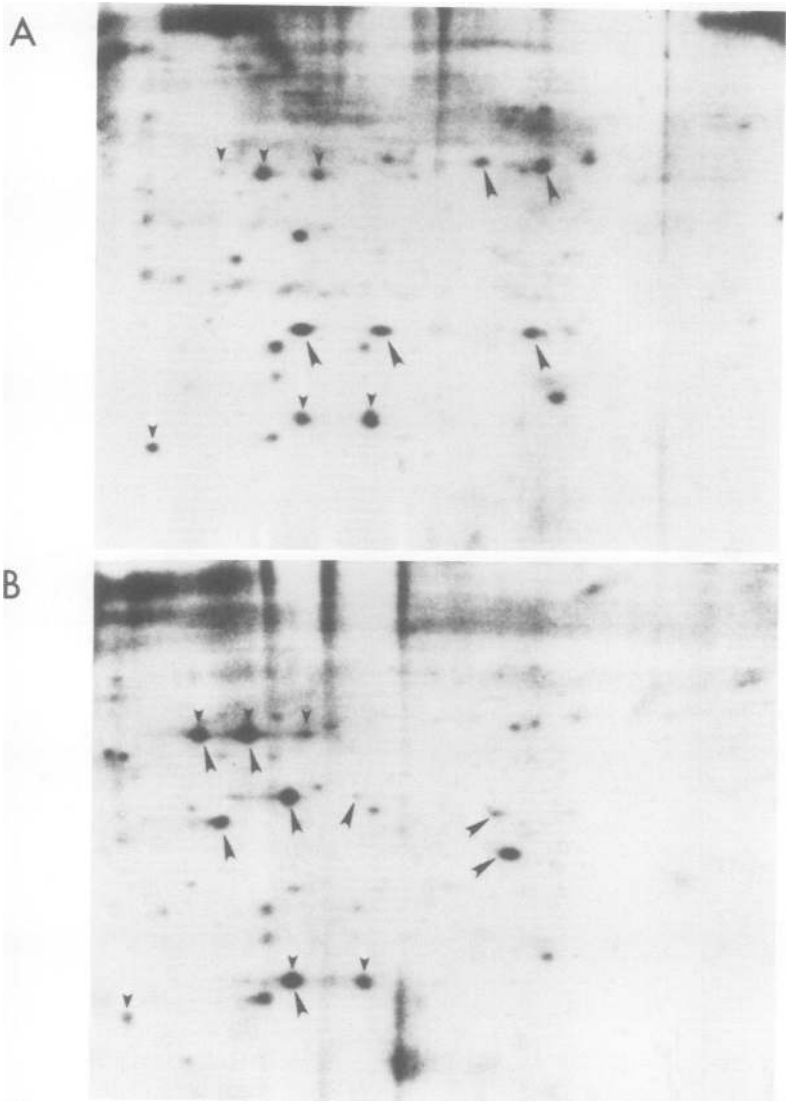

FIG. 3. *Alkali-stable phosphoproteins of CEC infected with tsLA29 (PR-RSV-A). CEC were infected with tsLA29 at 36°C, then transferred to 41°C (A) or 36°C (B) and labeled with ^{32}P. Alkali-treated gels were autoradiographed indirectly for 60 h. Small, downward-pointing arrows (A and B) mark phosphoproteins not detected in uninfected CEC grown at either temperature. Large arrows in (A) point to phosphoproteins present in increased amounts at 41°C. Large arrows in (B) indicate phosphoproteins present in increased amounts at 36°C.*

the activity of thermolabile $p60^{src}$. We have confirmed that phosphorylation of these proteins is independent of culture temperature in cells infected with wild-type PR-RSV-A (not shown). They are not found when the phosphoproteins of uninfected CEC, growing rapidly in sparse culture, are analyzed (not shown), suggesting that these phosphorylations do not correlate with growth rate per se. In addition, labeling of the 39,000 M_r, 7.33 pI phosphoprotein (*p*) in *ts*LA29-infected cells responds rapidly to temperature changes; it is practically undetectable by 1 h after a shift to the restrictive temperature and is detected at maximal level 1 h after lowering the temperature from 41 to 36°C (not shown). The effects of temperature shift on the other minor transformation-dependent phosphoproteins were difficult to determine. However, we believe that the 39,000 M_r protein (*p*), and probably the minor proteins, may be im-

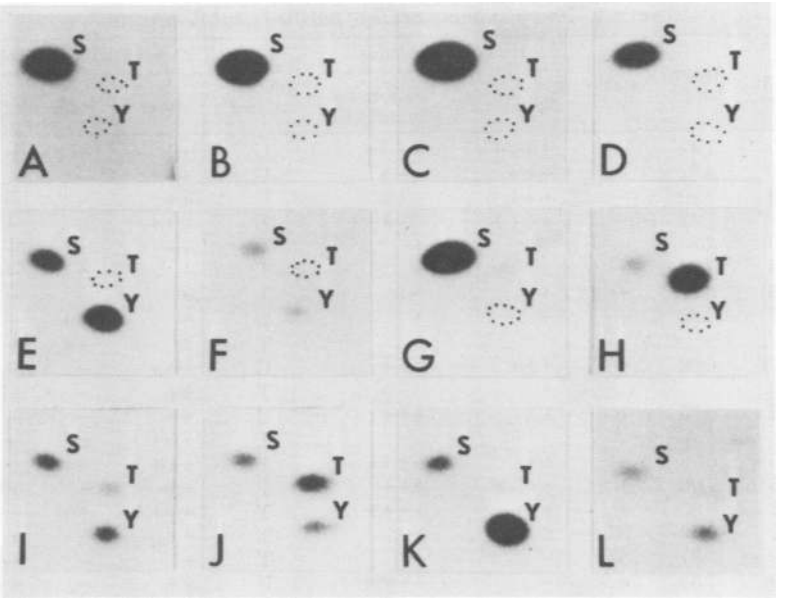

FIG. 4. *Phosphoamino acid composition of purified phosphoproteins.* ^{32}*P-labeled phosphoproteins from PR-RSV-A-transformed CEC were extracted from untreated (A to F) or alkali-treated (G to L) two-dimensional gels and hydrolyzed in 6 N HCl at 110°C for 1 h; the phosphoamino acids were separated by thin-layer electrophoresis. Each panel shows part of an indirect autoradiograph (6 to 8 days of exposure); there was no radioactivity at the origin, which lies off the photograph to the bottom right. The first dimension (pH 1.9) was with the anode at the left; the second dimension (pH 3.5) was with the anode at the top. Marker phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) were located by staining. The phosphoproteins analyzed, and approximate radioactivity, were: (A) 22,000* M_r, *6.92 pI (i), 600 cpm; (B) 26,000* M_r, *6.92 pI (b), 450 cpm; (C) 19,000* M_r, *7.41 pI (d), 830 cpm; (D) 20,000* M_r, *6.95 pI (j), 200 cpm; (E) 39,000* M_r, *7.33 pI (p), 340 cpm; (F) 28,000* M_r, *7.38 pI (q), 85 cpm; (G) 20,000* M_r, *6.95 pI (j), 340 cpm; (H) 27,000* M_r, *7.38 pI (h), 160 cpm; (I) 46,000* M_r, *7.05 pI (l), 120 cpm; (J) 43,000* M_r, *7.32 pI (n), 100 cpm; (K) 39,000* M_r, *7.33 pI (p), 360 cpm; (L) 28,000* M_r, *7.38 pI (q), 60 cpm.*

mediate substrates of the p60^{src} of *ts*LA29, although the phosphorylations could also occur via other tyrosine-specific protein kinases, activated by p60^{src}.

Phosphoamino acids present in purified phosphoproteins. We expected that proteins phosphorylated by p60^{src} would contain phosphotyrosine. Accordingly, we have purified many phosphoproteins from transformed cells, using two-dimensional gels, and analyzed their phosphoamino acids by acid hydrolysis and two-dimensional high-voltage electrophoresis on cellulose thin layers. We were careful to include internal phosphoamino acid markers in each sample, because many hydrolysates were found to contain two minor phosphorylated species of unknown structure, one of which has a mobility similar to phosphotyrosine. These spots are readily detected in acid hydrolysates of total cellular phosphoproteins (see Fig. 1A of reference 27). We have found that they generate only phosphoserine after further incubation in acid or alkali (not shown). Autoradiographs of some representative analyses are shown (Fig. 4), and the data for 17 proteins are summarized in Table 2.

^{32}P-labeled material was purified from alkali-treated gels to analyze the transformation-dependent phosphoproteins, as well as some other phosphoproteins which are frequently obscured by background radioactivity in untreated gels. Clearly, the amounts of phosphoamino acids recovered are not meaningful after alkaline hydrolysis, but some qualitative conclusions can be drawn (Table 2). The transformation-dependent phosphoproteins (*l, n, o, p, q*) all contained phosphotyrosine as well as phosphoserine (Fig. 4I, J, K, and L). The phosphothreonine in the protein of 28,000 M_r, 7.38 pI (*q*) could be partly due to contamination with an adjacent major phosphothreonine-rich protein (*h*) (Fig. 4H). Two major phosphoproteins (46,000 M_r, 6.95 pI [*k*] and 43,000 M_r, 6.80 pI [*m*]), which are elevated several-fold in transformed cells (Fig. 2), contained small amounts of phosphotyrosine in addition to large amounts of phosphoserine and

TABLE 2. *Phosphoamino acids of purified phosphoproteins*[a]

Phosphoprotein	Apparent M_r ($\times 10^3$)	Apparent pI	Alkali stability[b]	Relative abundance[c]		Source of protein analyzed[d]	Phosphoamino acid content[e]			Comments[f]
				Normal CEC	RSV-transformed CEC		P-ser	P-thr	P-tyr	
a	40	7.41	−	++++	++++	U	++	+	−	
b	26	6.92	−	+++	+++	U	+++	−	−	B
c	19	6.80	−	+++	+++	U	++	−	−	
d	19	7.41	−	++++++	++++++	U	+++	−	−	C
e	70	7.30	+	+	+	T	++	±	−	
f	68	7.33	+	±	+	T	+	±	−	
g	36	6.97	+	+	++	T	+	−	−	
h	27	7.38	+	+	+	U	+	++	−	
						T	±	++	−	H
i	22	6.92	+	+++	+++	U	+++	−	−	A
						T	++	−	−	
j	20	6.95	+	−	+++	U	++	−	−	D
						T	+++	±	−	G
k	46	6.95	+	++	++++	T	+++	++	+	
l	46	7.05	+	−	+++	T	++	+	++	I
m	43	6.80	+	++	++++	T	++++	++	+	
n	43	7.32	ND	±	+	T	+	+	+	J
o	39	7.31	ND	−	+	T	+	−	++	
p	39	7.33	+	−	++++	U	++	−	++	E
						T	++	±	++++	K
q	28	7.38	ND	±	+	U	+	−	+	F
						T	+	±	+	L

[a] ^{32}P-labeled phosphoproteins were purifed from two-dimensional gels, and their phosphoamino acids were analyzed after partial acid hydrolysis. The phosphoproteins are indicated in Fig. 1C and Fig. 2B.

[b] Relative alkali stability by comparison of untreated and alkali-treated gels: −, completely alkali labile; +, alkali stable; ND, not detected in untreated gel because of interference from background radioactivity.

[c] Relative phosphate content of protein, estimated from Fig. 1A and C for alkali-labile proteins and from Fig. 1A, B, C, and D for alkali-stable proteins.

[d] Proteins were extracted from untreated (U) or alkali-treated (T) gels.

[e] Relative phosphoamino acid composition of partial acid hydrolysate of phosphoprotein. The symbols − to ++++ represent increasing intensity of the autoradiographic image; however, the amount of radioactivity analyzed did not always reflect the abundance of the phosphoprotein, and this might limit the detection of minor amounts of phosphoamino acids. P-ser, phosphoserine; P-thr, phosphothreonine; P-tyr, phosphotyrosine.

[f] Phosphoamino acid analyses shown in Fig. 4 are indicated.

phosphothreonine (Table 2). All the other nine phosphoproteins analyzed from alkali-treated gels run with pH 6 to 8 ampholines (e.g., Fig. 4G, H; Table 2) contained phosphoserine and, frequently, phosphothreonine, but not phosphotyrosine. Five phosphoproteins analyzed from gels run with pH 5 to 7 ampholines similarly lacked phosphotyrosine.

We have also purified various phosphoproteins from two-dimensional gels which had not been treated with alkali, so that the relative amounts of phosphoserine, phosphothreonine, and phosphotyrosine could be estimated. The amount of phosphoserine in the 39,000 M_r, 7.33 pI (*p*) and 28,000 M_r, 7.38 pI (*q*) phosphoproteins was approximately equal to the amount of phosphotyrosine (Fig. 4E, F), whereas it was reduced when these proteins were purified from alkali-treated gels (Fig. 4K, L). The 20,000 M_r, 6.95 pI phosphoprotein (*j*), thought to be pp19^{gag}, contained only phosphoserine (Fig. 4G), as did the 21,000 M_r, 6.55 pI phosphoprotein (*r*). All the alkali-sensitive phosphoproteins tested from two-dimensional gels run with pH 5 to 7 or pH 6 to 8 ampholines contained phosphoserine (e.g., Fig. 4B, C) and, in 5 of 12 cases, lesser amounts of phosphothreonine (Table 2).

Radioactive labeling of the protein moiety of phosphoproteins. To determine whether any of the alkali-stable phosphoproteins correspond to proteins detected by [^{35}S]-methionine labeling, we autoradiographed alkali-treated two-dimensional gels containing mixed ^{32}P- and ^{35}S-labeled proteins under conditions where detection of ^{32}P was inefficient. Gels containing ^{35}S-labeled proteins alone were also autoradiographed. None of the transformation-dependent ^{32}P spots aligned exactly with a major ^{35}S spot, although minor amounts of ^{35}S could be detected in some cases (Fig. 5). However, some of the phosphoproteins are of similar molecular weight to, and are more acidic than,

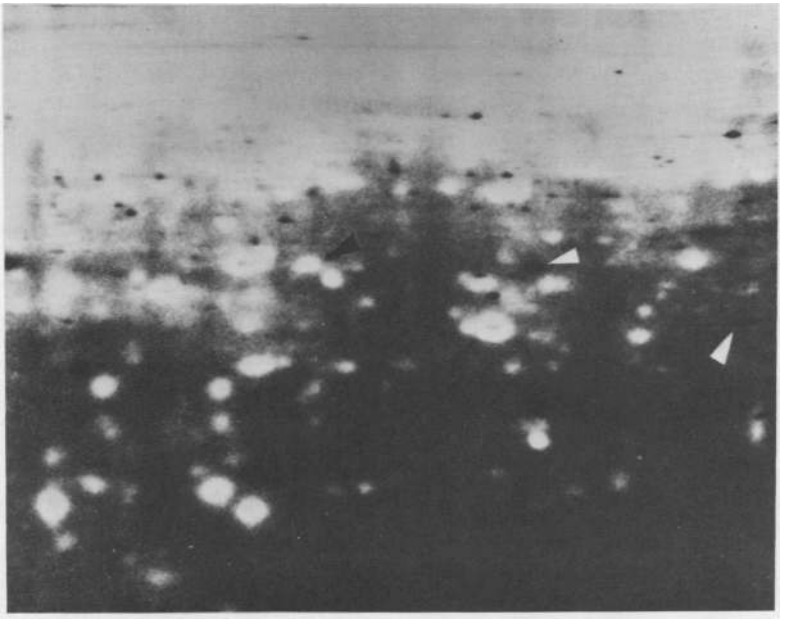

FIG. 5. *Comparison of phosphoproteins and proteins of PR-RSV-A-transformed CEC. Approximately 2 × 10^6 trichloroacetic acid-precipitable dpm of [^{35}S]methionine-labeled cell extract and 7.5 × 10^6 trichloroacetic acid-precipitable dpm of ^{32}P-labeled cell extract (of which an estimated 0.3 × 10^6 to 0.6 × 10^6 dpm was in phosphoproteins) were mixed and subjected to two-dimensional gel electrophoresis. The gel was incubated in alkali and autoradiographed under two different sets of conditions, to detect either ^{35}S or ^{32}P with minimal detection of the alternate isotope. The two autoradiographs are shown in a representation suggested by McConkey (17). A contact negative was made of the [^{32}P] autoradiograph and superimposed on the [^{35}S] autoradiograph. Thus, the white spots represent ^{32}P, and the black spots represent ^{35}S. Black arrows point to two ^{32}P-labeled phosphoproteins (46,000 M_r, 7.05 pI [l] and 39,000 M_r, 7.33 pI [p]). White arrows point to ^{35}S-labeled proteins which may be progenitors of these phosphoproteins.*

major cellular proteins (Fig. 5). Verification of which phosphoprotein is derived from which unphosphorylated protein is complicated, however, because an extra phosphate group on a polypeptide alters its isoelectric point by an amount dependent on the amino acid composition of that protein and may also affect its ability to bind dodecyl sulfate, and thus its apparent molecular weight. One way of testing the effect of phosphorylation on isoelectric point is to alter the protein charge in some other way, for example, by preventing protonation of the ϵ-amino groups of lysine by carbamylation (3). Partially carbamylated cell proteins give rise to complex patterns upon two-dimensional gel electrophoresis, in which each spot generates a series of spots separated by single charge increments (29). Since the second phosphate ionization of phosphotyrosine has an intrinsic pK_a of 6.5 (measured in 9 M urea; data not shown), dephosphorylation of a phosphoprotein with a pI between 6.5 and 7.5 will increase its net charge by 1.5 to 1.9, the exact amount being influenced by the actual pK_a of the phosphate group in the protein. The phosphoprotein thus lies between the first and second carbamylated derivates of its nonphosphorylated progenitor (29).

Comparison of transformation-dependent phosphoproteins with carbamylated derivatives of ^{35}S-proteins (not shown) demonstrated that the isoelectric point of the 39,000 M_r, 7.33 pI phosphoprotein (*p*) is approximately 1.8 charges more acidic than a major cellular protein of the same apparent molecular weight (arrow, Fig. 5). The minor 39,000 M_r, 7.31 pI phosphoprotein (*o*) is a further 0.1 charge units away, too small a difference to represent a second phosphorylation of the 7.33 pI phosphoprotein. It could reflect an alternate phosphorylation site on the same precursor protein. The 46,000 M_r, 7.05 pI phosphoprotein (*l*) is approximately 1.6 charges away from a major cellular protein of 7.40 pI. The 46,000 M_r, 6.95 pI phosphoprotein (*k*) probably is not a phosphorylated derivative of this particular protein, since it is 2.3 charges more acidic.

Confirmation of these relationships requires further analysis, for example by peptide mapping of the phosphoproteins and their presump-

tive precursors, but the quantities of radioactive material obtained and the use of alkali treatment preclude such an analysis at present.

DISCUSSION

We have shown that many phosphoproteins in uninfected chick cells contain significant amounts of phosphate even after incubation in 1 M KOH at 55°C for 2 h. Analysis of some of these proteins, either before or after alkaline hydrolysis, showed that the majority did not contain phosphotyrosine, but phosphoserine or phosphothreonine or both were detected. Chick cells transformed by RSV contain an additional three major and two minor alkali-resistant phosphoproteins, as well as showing quantitative changes in at least four of the phosphoproteins found in uninfected cells. Two of the major additional phosphoproteins (*r* and *j*; Fig. 2) contain only phosphoserine, are not dependent on a functional p60^{src}, and are probably products of structural genes of RSV. The third major phosphoprotein (*p*; Fig. 2) and the minor ones (*k* through *o*, *q*; Fig. 2) contain phosphotyrosine, phosphoserine, and, possibly, phosphothreonine. The tyrosine phosphates probably result from the activity of the tyrosine protein kinase encoded by the RSV *src* gene, because they were phosphorylated in decreased amounts when temperature-sensitive mutant-infected cells were cultured at high temperature. The most simple explanation is that they are subtrates for p60^{src} itself. Alternatively, they could be substrates for other tyrosine-specific protein kinases which are regulated by p60^{src}.

The major phosphotyrosine-containing protein (*p*) can be detected on untreated gels, particularly after short labeling times and with nonequilibrium pH gradient electrophoresis (not shown), and its molecular size and pI suggest that it is the protein first identified by Radke and Martin (23) and recently characterized as a substrate for p60^{src} (7, 22). Our M_r estimate of 39,000 is not inconsistent with previous determinations (36,000, reference 23; 34,000, reference 7), since many phosphoproteins migrate more slowly in sodium dodecyl sulfate-polyacrylamide gels than their unphosphorylated counterparts, and the apparent M_r can vary with gel porosity. Our analysis confirms that this protein is a major tyrosine phosphoprotein in RSV-transformed cells. Measurement of the radioactivity in this spot suggests that it contains a significant proportion of the total cell protein phosphotyrosine.

The proteins (*k* through *o*, *q*) containing more minor amounts of phosphotyrosine have not been described in detail before, although other workers have estimated that 6% of cellular phosphoproteins undergo quantitative changes after transformation (23). We have analyzed the presumptive p60^{src} substrates vinculin (B. M. Sefton, T. Hunter, E. Ball, and S. J. Singer, Cell, in press), the "50K" (50,000 molecular weight) phosphoprotein associated with p60^{src} (11), and p60^{src} itself (11) by two-dimensional gel electrophoresis, and they do not correspond in mobility with any of the spots described here (see below).

This study raises several questions about the value of alkali stability as an indicator of the phosphoamino acid content of a protein. The mildly acidic conditions of gel fixation almost certainly hydrolyze any phosphoramidate, acylphosphate, or thiophosphate bonds in proteins, leaving only the esters of the hydroxyamino acids (20, 30). The ester bond of phosphotyrosine is much more alkali stable than that of phosphoserine and phosphothreonine (21). In that case, why did we detect so many phosphoserine- and phosphothreonine-containing proteins after alkali treatment, and could we have failed to detect some major phosphotyrosine-containing ones?

Even though some phosphoserine-containing proteins are totally hydrolyzed by alkali, others are not (Fig. 1, Table 2). Presumably the 30% of phosphoserine in cell proteins surviving alkali treatment (Table 1) is a result of a wide range of individual phosphoprotein stabilities. In certain cases, pp19^{gag} for example (Fig. 1), the phosphoserine seems to be almost totally alkali stable. The common detection of phosphothreonine was surprising because it is only 1/10 as abundant as, and only slightly more alkali stable than, phosphoserine (21). However, by analogy with model chemical compounds (30), it is likely that phosphothreonine is more stable when part of a polypeptide than when free. Ionic effects of particular adjacent amino acids could increase this. Indeed, we found total threonine phosphoproteins to be quite alkali stable, and more phosphothreonine (and phosphotyrosine) was recovered by acid hydrolysis from alkali-treated proteins than from untreated proteins (Table 1). This apparent increase may be partly because the acid conditions used do not break all peptide bonds (12), so alkali degradation of the polypeptide may more than offset discharge of the phosphate group. This consideration may also increase the sensitivity of detection of phosphothreonine in individual phosphoproteins isolated from alkali-treated gels.

For these reasons, most of the alkali-stable phosphoproteins in normal chick cells appear to contain phosphoserine and phosphothreonine and insignificant amounts of phosphotyrosine, so it is not surprising that transformation by RSV, which raises total phosphotyrosine content 10-fold, only results in the detection of three

additional alkali-stable phosphoproteins and quantitative changes in some others. However, there are several reasons why we have probably failed to detect all tyrosine phosphoproteins.

Low-abundance phosphoproteins are not detected simply because of labeling considerations. We estimate that the weakest spots on our autoradiographs contain approximately 0.001 to 0.01% of the cellular protein phosphate. For example, even though the 130,000 M_r, 7.3 pI cytoskeletal protein vinculin (10) comprises 0.1 to 0.5% of CEC protein (B. Sefton, personal communication), only 1 to 2% of the molecules are phosphorylated at tyrosine in RSV-transformed cells (Sefton et al., in press). Furthermore, it is likely that these molecules are present as a series of charge isomers, so individual spots may be below the level of detection by our technique. Some other tyrosine phosphoproteins may not focus on isoelectric focusing gels. The RSV $p60^{src}$ is known to be intransigent in this respect (22; our unpublished data). Yet other tyrosine phosphoproteins may contain alkali-labile phosphate. For example, the 50K protein found in immunoprecipitates of $p60^{src}$ (26), which contains phosphotyrosine and phosphoserine (11), is highly alkali labile when assayed by trichloroacetic acid precipitation from alkaline solution or by autoradiography of alkali-treated gels (not shown). This unusual sensitivity could be due to the local environment of the phosphotyrosine, causing increased hydrolysis either of the phosphate ester bond or of the polypeptide chain. Even though none of the ^{35}S-proteins we detected was solubilized extensively by alkali treatment, we cannot exclude the possibility that fragments of the polypeptide of some of the phosphoproteins could be selectively solubilized, particularly since β-elimination of phosphoserine and phosphothreonine causes peptide bond breakage (30).

All of the tyrosine phosphoproteins we identified also contain phosphoserine, and some contain phosphothreonine as well. However, all were purified by isoelectric focusing, so presumably each is homogeneous in charge. The 36K protein has already been shown to contain approximately equal amounts of phosphoserine and phosphotyrosine (7, 22) and yet has only one phosphate group per molecule (22). The dependence of both phosphorylations on transformation, and the apparent stoichiometry, have not yet been completely explained (7). Our finding of phosphoserine in the minor tyrosine phosphoproteins suggests that a similar problem may exist for these substrates too. For example, the 46,000 M_r, 7.05 pI phosphoserine- and phosphotyrosine-containing protein seems to be derived from a phosphate-free precursor by one phosphorylation event per polypeptide chain. The major phosphoproteins of 46,000 M_r, 6.95 pI and 43,000 M_r, 6.80 pI are significantly elevated in RSV-transformed CEC, yet apparently contain only a small proportion of phosphotyrosine.

In conclusion, we are confident that this analysis shows that transformation of CEC by RSV causes several different cellular proteins to become phosphorylated at tyrosine, suggesting that $p60^{src}$ could mediate directly several of the different biological changes associated with transformation. However, we have no indication of the functions or subcellular localizations of the proteins we have recognized. Preliminary results show that the same proteins are phosphorylated in CEC transformed by unrelated viruses, although to different extents (J. A. Cooper and T. Hunter, submitted for publication). Thus, it is possible that the different protein kinases of these viruses are similar, not only in the recognition of tyrosine, but also in having the same protein substrates.

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