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Identification of Phosphotyrosine-Containing Proteins in Untransformed and Rous Sarcoma Virus-Transformed Chicken Embryo Fibroblasts

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Phosphorylation on tyrosine residues mediated by pp60^{src} appears to be a primary biochemical event leading to the establishment of the transformed phenotype in Rous sarcoma virus (RSV)-infected cells. To identify the cellular proteins that undergo tyrosine phosphorylation during transformation, a ^{32}P-labeled RSV-transformed chicken embryo cell extract was analyzed by electrophoresis on a polyacrylamide gel. After slicing the gel into approximately 60 slices, phosphoamino acid analyses were carried out on the protein recovered from each gel slice. Phosphotyrosine was found in every gel slice, with two major peaks of this phosphoamino acid around M_r's of 59 and 36 kilodaltons. When the same analysis was performed with cells infected with a transformation-defective *src* deletion mutant of RSV (*td*NY101), significant and reproducible peaks of phosphotyrosine were found in only 2 of 60 gel slices. These gel slices corresponded to M_r's of 42 and 40 kilodaltons. Identical results were obtained with normal uninfected chicken embryo fibroblasts. We conclude from these observations that pp60^{src} or the combined action of pp60^{src} and pp60^{src}-activated cellular protein kinases cause the tyrosine-specific phosphorylation of a very large number of cellular polypeptides in RSV-transformed cells. In addition, untransformed cells appear to possess one or more active tyrosine-specific protein kinases which are responsible for the phosphorylation of a limited number of proteins. These proteins are different from the major phosphotyrosine-containing proteins of the transformed cells.

Infection of chicken embryo fibroblasts (CEF) by Rous sarcoma virus (RSV) leads to the rapid appearance in these cells of a new spectrum of phenotypic properties collectively referred to as the transformed phenotype (14). The finding that the transforming protein of RSV (pp60^{src}) possesses an intrinsic protein kinase activity with tyrosine substrate specificity (8, 9, 11, 13, 16, 20, 21) and the rapid rise in cellular phosphotyrosine levels seen during the first hour of the transformation process (28, 29) strongly suggest that pp60^{src}-mediated phosphorylation of specific cellular targets represents a primary event in the establishment of the transformed phenotype.

We have previously discussed (3, 35) two models with regard to the mechanism of action of pp60^{src}: a single target hypothesis in which the action of pp60^{src} on a single protein could trigger a biochemical cascade leading to the transformed phenotype; and a multitarget hypothesis invoking the action of pp60^{src} on two or more cellular proteins. The isolation of partial transformation mutants of RSV has provided support for the second model (3, 5, 35). More recently (22) we have shown that a prominent cellular protein which becomes phosphorylated on tyrosine during RSV-mediated oncogenic transformation (the 36 × 10^3-molecular-weight ["36K"] protein) is preferentially underphosphorylated in cells infected with a partial transformation mutant of RSV (CU2), providing direct biochemical evidence that the kinase substrate specificity of pp60^{src} must be directed towards one or more additional proteins besides the 36K protein.

In addition to the 36K protein, several other proteins have been shown to become phosphorylated on tyrosine during RSV transformation. Immunoprecipitation has revealed tyrosine phosphorylation in pp60^{src} (13) and vinculin (27). In addition, a 50K protein which co-immunoprecipitates with pp60^{src} is phosphorylated on tyrosine (7, 23). Two-dimensional gel electrophoresis followed by alkali treatment (to partially remove phosphorylations from serine) has revealed several additional phosphotyrosine-containing proteins in transformed cells (10). However, as we will describe, these methods have not revealed the full complexity of transformation-specific phosphorylations on tyrosine.

The identification of phosphotyrosine-containing proteins is a difficult task due to the low levels of cellular phosphotyrosine (1 to 2% of the total phosphorylhydroxyamino acid levels in RSV-transformed cells [22]). In this paper we describe a methodology to identify by molecular weight proteins which are phosphorylated on tyrosine residues. Our approach consists of electrophoresing ^{32}P-labeled whole cell lysates through tubular polyacrylamide gels and analyzing the phosphoamino acid content of the separated phosphoproteins. Analysis of RSV-transformed cell lysates in this fashion revealed a surprisingly large number of phosphotyrosine-containing proteins encompassing a very wide range of molecular weights. Application of the technique to untransformed cells demonstrated that these cells contained reproducibly only two major tyrosine-phosphorylated proteins. Our results suggest that $pp60^{src}$ directly or indirectly triggers a very complex network of tyrosine phosphorylation events involving a large number of cellular proteins.

MATERIALS AND METHODS

Viruses and cells. The wild-type Schmidt-Ruppin A RSV and the Schmidt-Ruppin A RSV-derived transformation-defective mutant *td*NY101 were originally obtained from H. Hanafusa (18). CEF were prepared and subcultured as described (3, 34).

Radiolabeling and cell lysis. Tertiary cultures were plated in 35-mm dishes at a cell density of 3×10^5 cells/dish and labeled 24 h later with 3 mCi of [^{32}P]P_i (New England Nuclear Corp., Boston, Mass., carrier free) in 1 ml of phosphate-free Dulbecco-modified Eagle medium supplemented with 4% calf serum and 1% heat-inactivated chicken serum. Cells were labeled for 12 to 16 h. Cells were washed once with ice-cold phosphate-buffered saline and lysed by scraping in 300 μl of hot Laemmli electrophoresis sample buffer (19). Lysates were incubated in a boiling water bath for 5 min and loaded onto a polyacrylamide gel or frozen at −20°C.

Phosphoamino acid analysis. Gel electrophoresis was carried out as indicated in the legend to Fig. 2. After electrophoresis the separator portion of the gel was frozen over dry ice and sliced into approximately 60 2-mm-thick gel slices. The protein contained in each slice was eluted by incubating the gel slices in 2 ml of 0.05 M NH_4HCO_3, 0.1% sodium dodecyl sulfate (elution buffer) with shaking at 37°C for 24 h. The gel slices were re-eluted an additional 24 h in fresh elution buffer. A 200-μg amount of immunoglobulin G(IgG) was added to the pooled eluants from each slice followed by trichloroacetic acid to a final concentration of 20%. Precipitates were collected by centrifugation at 1,700 × *g*, washed one time each in −20°C ethanol and −20°C ethanol–ether (1:1), and dissolved in 15μ of 0.1 N NaOH. This brief treatment (10 to 15 s) with dilute alkali considerably facilitates the dissolution of the trichloroacetic acid pellets and in control experiments did not result in any detectable change in phosphoamino acid ratios (data not shown). Partial acid hydrolysis was performed in 1 ml of distilled 6 N HCl for 2.0 h at 110°C under nitrogen. After hydrolysis, the HCl was removed under reduced pressure. The dried hydrolysates were dissolved in 20 μl of pH 1.9 high-voltage paper electrophoresis buffer (formic acid–acetic acid–water, 25:87:888) containing unlabeled phosphoserine, phosphothreonine, and phosphotyrosine at 15 μM each. Samples were spotted on 1MM Whatman paper and electrophoresed at 3,000 V for 1.5 h in the pH 1.9 buffer described above. Phosphoserine and the comigrating phosphotyrosine and phosphothreonine were visualized autoradiographically. A strip containing the phosphoamino acids was cut from the first paper, sewn onto a second paper, and run at 1,500 V for 2 h in pyridine–acetic acid–water (1:10:189; pH 3.5). Conventional two-dimensional phosphoamino acid analyses were performed by sewing the paper strip obtained after the pH 1.9 run onto the second paper at a 90° angle with respect to the axis of electrophoretic migration used in the first dimension. At the end of the pH 3.5 run, the standard phosphoamino acids were visualized by ninhydrin staining and the labeled phosphoamino acids were visualized by autoradiography. The radioactivity associated with each phosphoamino acid was determined by liquid scintillation counting of each ninhydrin-stained spot in Aquasol.

RESULTS

Analysis of phosphotyrosine. Because we wished to perform phosphoamino acid analyses on a large number of samples, it was impractical to perform conventional two-dimensional electrophoresis on every sample. On the other hand, it was known that electrophoresis at pH 1.9 would not separate phosphotyrosine from phosphothreonine and that electrophoresis at pH 3.5 would not separate phosphotyrosine from UMP (17). We therefore settled on a procedure in which the samples were electrophoresed on paper at pH 1.9 in the first dimension to separate the phosphoamino acids from unhydrolyzed material, nucleotides, and inorganic phosphate (Fig. 1A). Control experiments demonstrated that, in fact, UMP and other nucleotides migrated well in front of the phosphoserine marker under these conditions (data not shown). After locating the phosphoserine and the comigrating phosphothreonine and phosphotyrosine by autoradiography, the strip of paper containing the phosphoamino acids (shown along arrow in Fig. 1A) was cut out, sewn onto a second sheet of paper, and electrophoresed at pH 3.5 to separate phosphotyrosine from phosphothreonine. By sewing this strip of paper in an orientation which had the phosphoamino acids migrating in the same direction during the second electrophoresis as they did during the first electrophresis, we were able to run as many as 13 samples on a single sheet of paper. We refer to this procedure as a two-stage, one-dimensional separation. A typical separation of this type is shown in Fig. 1B. On the other hand, to perform conventional two-dimensional electrophoretic separations,

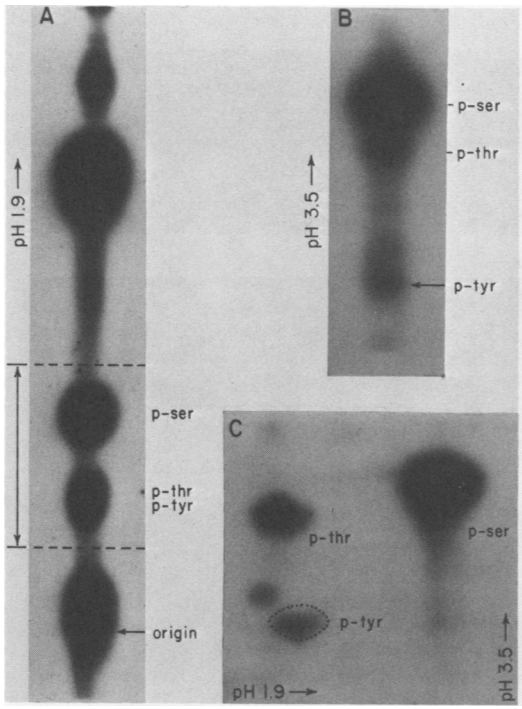


FIG. 1. Separation of phosphoamino acids by high-voltage electrophoresis. The first dimension, at pH 1.9, is shown in panel A. If the second stage, at pH 3.5, is run parallel to the first (two-stage, one-dimensional analysis), data as shown in panel B are generated. If the pH 3.5 electrophoresis is done at right angles to the first electrophoresis (two-dimensional electrophoresis), data of the type shown in panel C are obtained.

the strip of paper containing the phosphoamino acids was sewn onto the second sheet at right angles to its original orientation; electrophoresis at pH 3.5 in this case would give data of the type shown in Fig. 1C.

Since our procedure for phosphoamino acid analysis was slightly unconventional, it was important to determine whether it would resolve phosphotyrosine from other phosphorylated compounds found in cellular macromolecules. One possible source of error would be a phosphorylated compound which could be separated from phosphotyrosine by conventional two-dimensional electrophoresis but which comigrated in a two-stage, one-dimensional separation with phosphotyrosine. Such a contaminant would be found to run parallel to phosphotyrosine at pH 3.5 on a conventional two-dimensional separation. Inspection of Fig. 1C reveals only a modest amount of radioactivity in this region, which is due predominantly to streaking from the phosphoserine. The degree of streaking from phosphoserine has been variable and unpredictable, but has not been of sufficient magnitude to prevent identification of phosphotyrosine except in cases where the level of this phosphoamino acid is extremely low (see below). The streaking can, of course, prevent precise quantitation of the amount of radioactive phosphotyrosine.

Another source of error in assessing the level of phosphotyrosine would be a phosphorylated compound which comigrates with phosphotyrosine even in two-dimensional separations. Such a compound would also have led to an overestimation of phosphotyrosine by other workers (10, 15, 17, 25, 29). Nonetheless, we eluted the phosphotyrosine spot from a conventional two-dimensional electropherogram and chromatographed it in isopropanol–HCl–water (14:3:3). Almost 90% of the radioactivity associated with the phosphotyrosine spot from the electropherogram chromatographed with phosphotyrosine in this solvent; the only additional spot of radioactivity we detected comigrated with P_i. We suspect this phosphate was generated by breakdown of phosphotyrosine during the elution and chromatography. Thus, we believe that essentially all of the radioactively labeled material comigrating with phosphotyrosine during two-dimensional, high-voltage paper electrophoresis is, in fact, phosphotyrosine.

A radioactive spot can be seen running just ahead of phosphotyrosine in Fig. 1B and 1C. This material has been noted by others, and it may contain phosphoserine (10). The presence of this spot is extremely variable. Nonetheless, we do not believe that it makes a significant contribution to our phosphotyrosine estimations, since it is separable from authentic phosphotyrosine under our conditions.

Identification of phosphotyrosine-containing proteins in untransformed and RSV-transformed cells. To determine the minimum number of proteins phosphorylated on tyrosine in RSV-transformed cells, ^{32}P-labeled cells were lysed in hot sodium dodecyl sulfate electrophoresis sample buffer and electrophoresed on a tubular polyacrylamide gel under denaturing conditions. The gel was cut into approximately 60 slices, and the phosphoamino acid content of each slice was determined by two-stage, one-dimensional, high-voltage paper electrophoresis at pH 1.9 and then pH 3.5. As shown in the autoradiogram of the second electrophoresis (Fig. 2), phosphotyrosine was found in every slice. Dansylated molecular weight marker proteins were co-electrophoresed in each experiment (see Materials and Methods), and since these fluorescent standards migrated as sharp bands (Fig. 3), we are confident that the broad distribution of phosphotyrosine across the gel was not due to streaking of the radiolabeled proteins during electrophoresis.

We were so surprised to find such an exten-

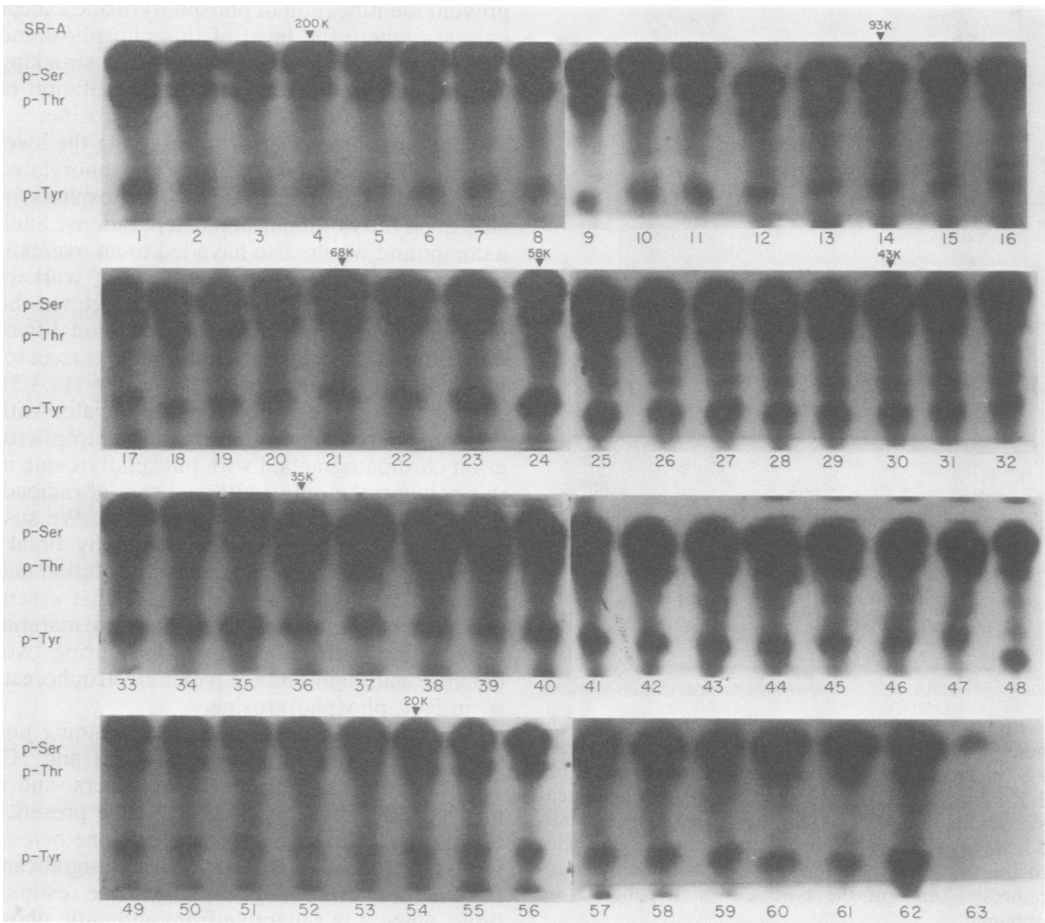


FIG. 2. Phosphoamino acid analysis of the gel-separated phosphoproteins from RSV-infected cells. ^{32}P-labeled whole cell lysates of RSV-infected CEF were electrophoresed on a tubular polyacrylamide gel; the gel was cut into 63 2-mm-thick slices, and the protein from each slice was recovered and subjected to two-stage, one-dimensional phosphoamino acid analysis by high-voltage paper electrophoresis at pH 1.9 and then at pH 3.5 (see text). Electropherograms were autoradiographed with fluorescent screens. Gel slice numbers are indicated beneath each row of autoradiograms. The position on the gel of the dansylated proteins used as internal molecular weight markers is indicated by arrows.

sive molecular weight distribution of phosphotyrosine in RSV-transformed cells that we proceeded to verify using conventional two-dimensional, high-voltage paper electrophoresis the authenticity of the phosphotyrosine spots separated by the two-stage, one-dimensional analyses. To do this, we electrophoresed a ^{32}P-labeled RSV-transformed cell extract on a polyacrylamide tube gel, divided the gel into approximately 30 slices, and analyzed by two-dimensional, high-voltage paper electrophoresis the phosphoamino acid content of the proteins recovered from each gel slice. Autoradiography of the two-dimensional electropherograms confirmed the presence of phosphotyrosine in each one of the 30 gel slices (Fig. 4).

By contrast, when a ^{32}P-labeled extract of cells infected with a *src* deletion mutant of RSV (*td*NY101) was electrophoresed, the overall level of phosphotyrosine was considerably lower, and only four regions of the gel (M_r's of 200, 42, 40, and 22 to 18 kilodaltons) displayed peaks of radioactivity in the phosphotyrosine area of the electropherograms (Fig. 5). Minor quantities of radioactivity appeared in the phosphotyrosine region throughout the gel, although reliable quantitation and unambiguous identification of the phosphotyrosine in these instances was difficult due to the low levels of radioactivity.

Because of the low phosphotyrosine content of untransformed cells and the high background surrounding the phosphotyrosine area in some

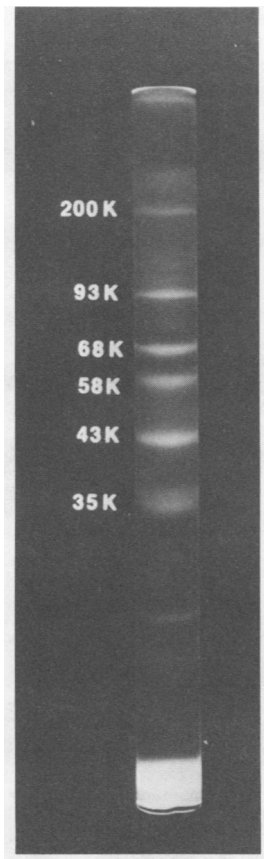


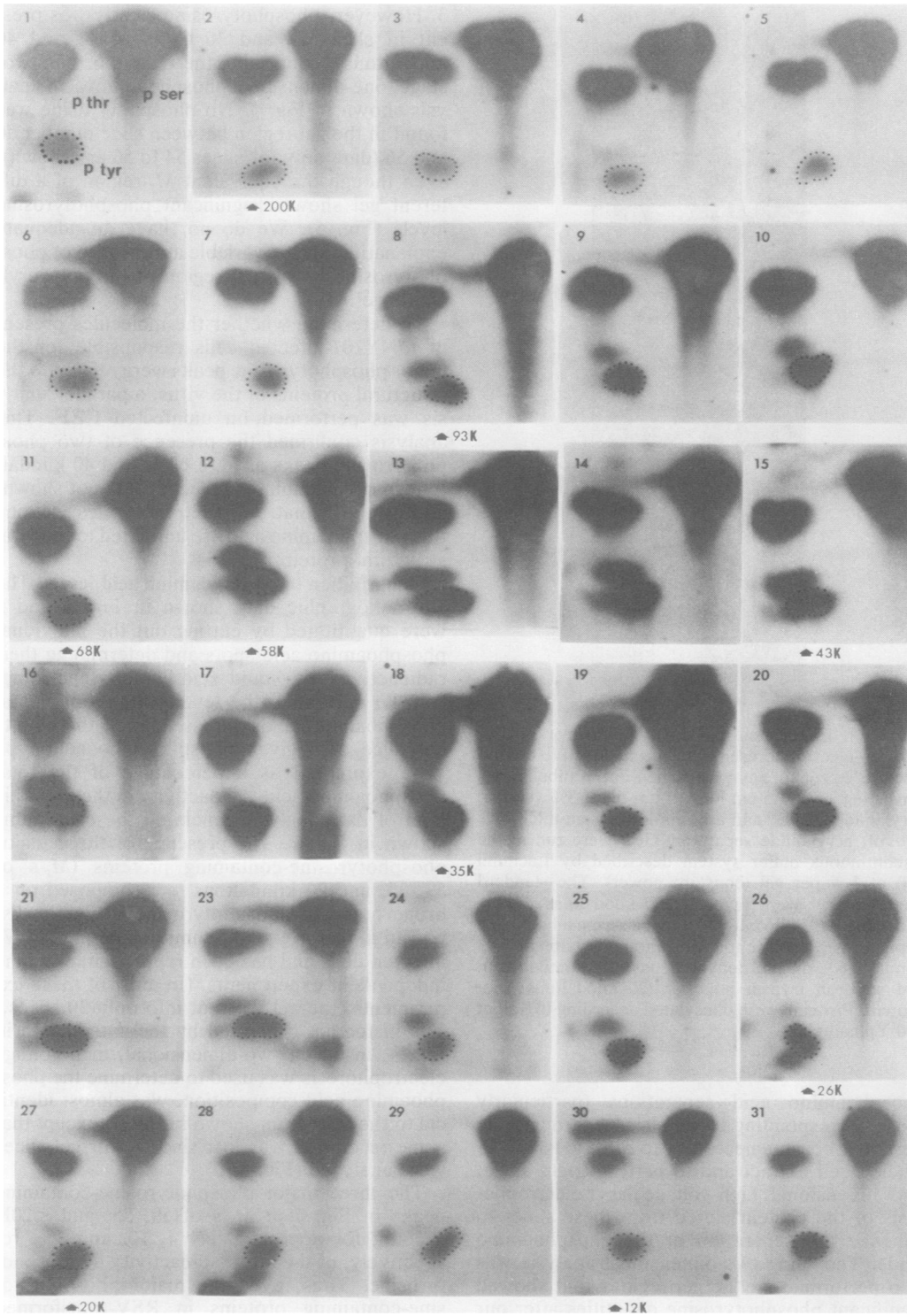
FIG. 3. Dansylated molecular weight marker proteins co-electrophoresed with the radioactive cell lysates. Electrophoresis was carried out in tubular gels consisting of a 1.5-cm-long, 1-cm-thick 3% (wt/vol) acrylamide stacker and a 12-cm-long, 1-cm-thick 10% (wt/vol) acrylamide separator. Gels were run in the discontinuous buffer system described by Laemmli (19) at 1-w per gel (constant power). The standard proteins used were myosin (200 kilodaltons), phosphorylase b (93 kilodaltons), bovine serum albumin (68 kilodaltons), catalase (58 kilodaltons), ovalbumin (43 kilodaltons), malate dehydrogenase (35 kilodaltons), and soybean trypsin inhibitor (20 kilodaltons) (not shown). Proteins were dansylated according to Talbot and Yphantis (32).

phosphoamino acid separations (particularly those corresponding to gel slices 1 through 3 in Fig. 5), it became necessary to run a gel of a ^{32}P-labeled cell extract and to perform right-angle, two-dimensional, high-voltage paper electrophoresis of the protein eluted from these slices to enhance the separation of the phosphoamino acids. The same two-dimensional analysis was also performed on the gel regions that showed significant phosphotyrosine quantities after our standard two-stage, one-dimensional analysis. The results of these experiments are shown in Fig. 6. It is clear that there is little if any detectable phosphotyrosine in gel slices 1, 2, or 3. However, phosphotyrosine clearly was present in slices 28 and 30 (M_r's of 42 and 40 kilodaltons), confirming the results of our two-stage, one-dimensional phosphoamino acid analysis shown in Fig. 5. No phosphotyrosine was found in the gel region between slice number 50 and 56 (data only for slices 54 to 56 are shown), even though the equivalent M_r region of a different gel showed significant phosphotyrosine levels (Fig. 5). We do not have an adequate explanation for the variable appearance of phosphotyrosine in this M_r region of the polyacrylamide gel.

To determine whether the molecules present in *td*NY101-infected cells responsible for the major phosphotyrosine peaks were related to the structural proteins of the virus, a parallel analysis was performed on uninfected CEF. This analysis confirmed the presence of two phosphotyrosine peaks at M_r's of 42 and 40 kilodaltons in normal uninfected cells (data not shown) and establish that the macromolecules yielding this phosphoamino acid are unrelated to the viral structural proteins.

Quantitation of phosphoamino acid levels. The autoradiographic data shown in Fig. 2 and 5 were quantitated by cutting out the individual phosphoamino acid spots and determining their radioactivity by liquid scintillation spectrometry. To determine the levels of the individual phosphoamino acids across the gel, the counts per slice of a given phosphoamino acid type were expressed as a percentage of the total phosphoamino acid radioactivity in the gel. This kind of data analysis generated the histograms shown in Fig. 7. The presence of three major phosphotyrosine-containing proteins (M_r's of 59, 35, and 20 kilodaltons) superimposed on a broad hump of phosphotyrosine is clearly displayed in the profile of Schmidt-Ruppin A RSV-infected cells and has been reproducible in nine independent experiments. (In most of these experiments, the gel was cut into only 30 slices.) The histogram generated by the data shown in Fig. 4, in which two-dimensional, high-voltage electrophoresis was used to determine the phosphoamino acid composition, was almost identical to the histogram shown in Fig. 7, except that some resolution was lost since fewer gel slices were analyzed (Fig. 8).

The three major phosphotyrosine-containing peaks in Fig. 7 at M_r's of 59, 35, and <20K account for approximately 4, 10, and 3%, respectively, of the total radioactivity in phosphotyrosine. Thus, even the major phosphotyrosine-containing proteins in RSV-transformed cells represent a minority of the total phosphotyrosine. In addition, in the two experiments in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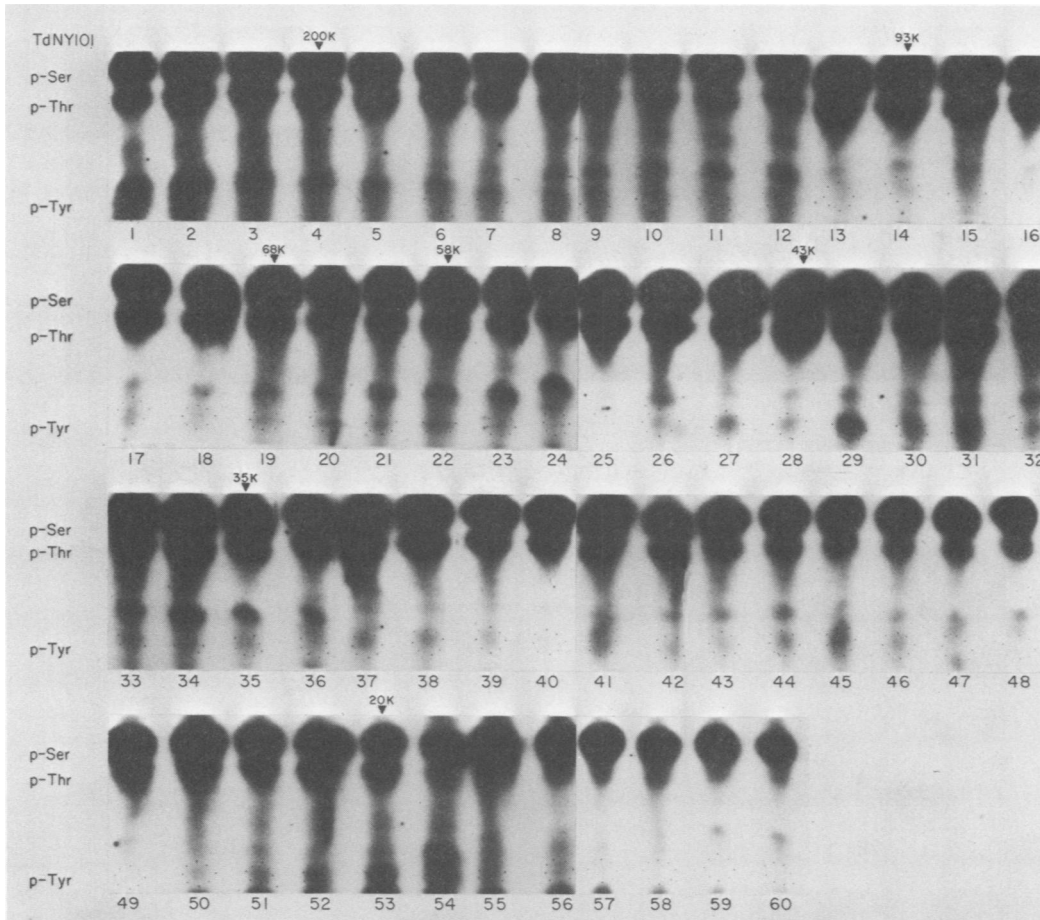

FIG. 5. Two-stage one-dimensional phosphoamino acid analysis of the gel-separated phosphoproteins from cells infected with the transformation-defective mutant of RSV *td*NY101. A ^{32}P-labeled whole cell lysate of *td*NY101-infected CEF was subjected to the same experimental procedure described for SRA RSV-infected cells in the legend to Fig. 2. In this experiment the polyacrylamide gel was cut into 60 2-mm-thick slices. Autoradiography using fluorescent screens was for 48 h. Arrows show the position on the gel of the dansylated molecular weight markers, and dotted circles indicate the ninhydrin-stained phosphotyrosine standards. The light background in this autoradiogram (compared to that shown in Fig. 2) is due to differences in flashing the film.

which the polyacrylamide gel was divided into 60 slices, moderate-sized peaks of phosphotyrosine became evident at M_r's of approximately 94, 80, 68, 50, 42, 30, 27, and 22 kilodaltons. Presumably, increasing the resolution of the analysis still further would reveal additional individual peaks of phosphotyrosine.

Preliminary evidence suggests that the 59- and

FIG. 4. Two-dimensional phosphoamino acid analysis of the gel-separated phosphoproteins from RSV-infected cells. A ^{32}P-labeled transformed cell extract was electrophoresed on a polyacrylamide tube gel as described in the legend to Fig. 3. The gel was sliced into 31 slices, and the protein from each slice was recovered as described in the text. The eluted protein was acid hydrolyzed, and the hydrolysates were subjected to two-dimensional, high-voltage paper electrophoresis at pH 1.9 and 3.5. The electropherograms were autoradiographed with intensifying screens for 23 h. Gel slice numbers appear on the upper left corner of each autoradiogram (material corresponding to slice number 22 was lost during the procedure). The identity of each phosphoamino acid spot is shown in the autoradiogram corresponding to slice number 1. Dotted circles show the position of the ninhydrin-stained phosphotyrosine standard. Arrows indicate the position of the fluorescent molecular weight markers used during gel electrophoresis. In addition to the dansylated proteins used in the experiments corresponding to Fig. 2, two more fluorescent markers were included in this experiment, chymotrypsinogen (26 kilodaltons) and myoglobin (17 kilodaltons).

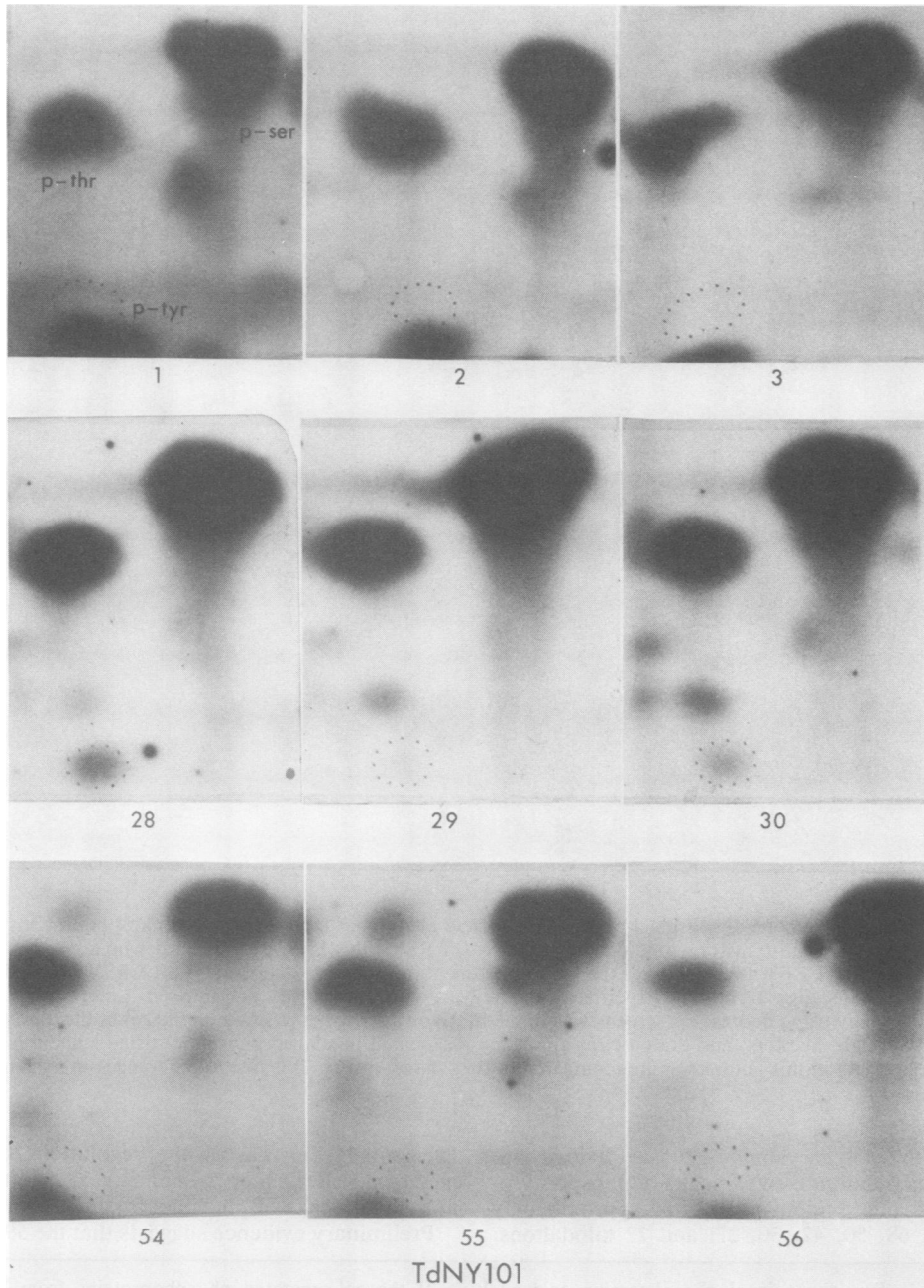

FIG. 6. Two-dimensional phosphoamino acid analysis of the phosphoproteins recovered from selected M_r regions of a gel used to fractionate a *td*NY101-infected whole cell lysate. The lysate was electrophoresed as described in the text. Selected M_r regions of the gel were located using a standard curve constructed with the aid of internal dansylated molecular weight marker proteins. The phosphoproteins from the chosen gel slices were recovered and subjected to right-angle, two-dimensional, high-voltage paper electrophoresis at pH 1.9 and 3.5. The separated phosphoamino acids were autoradiographed for 72 h with intensifying screens. Slice numbers are indicated below each row of autoradiograms. Note that in this experiment the phosphotyrosine in slices 28 and 30 corresponds to slices 29 and 31 in Fig. 5. The position of the phosphoamino acid spots is indicated in the autoradiogram corresponding to slice number 1. Dotted circles show the position of the ninhydrin-stained internal phosphotyrosine standards.

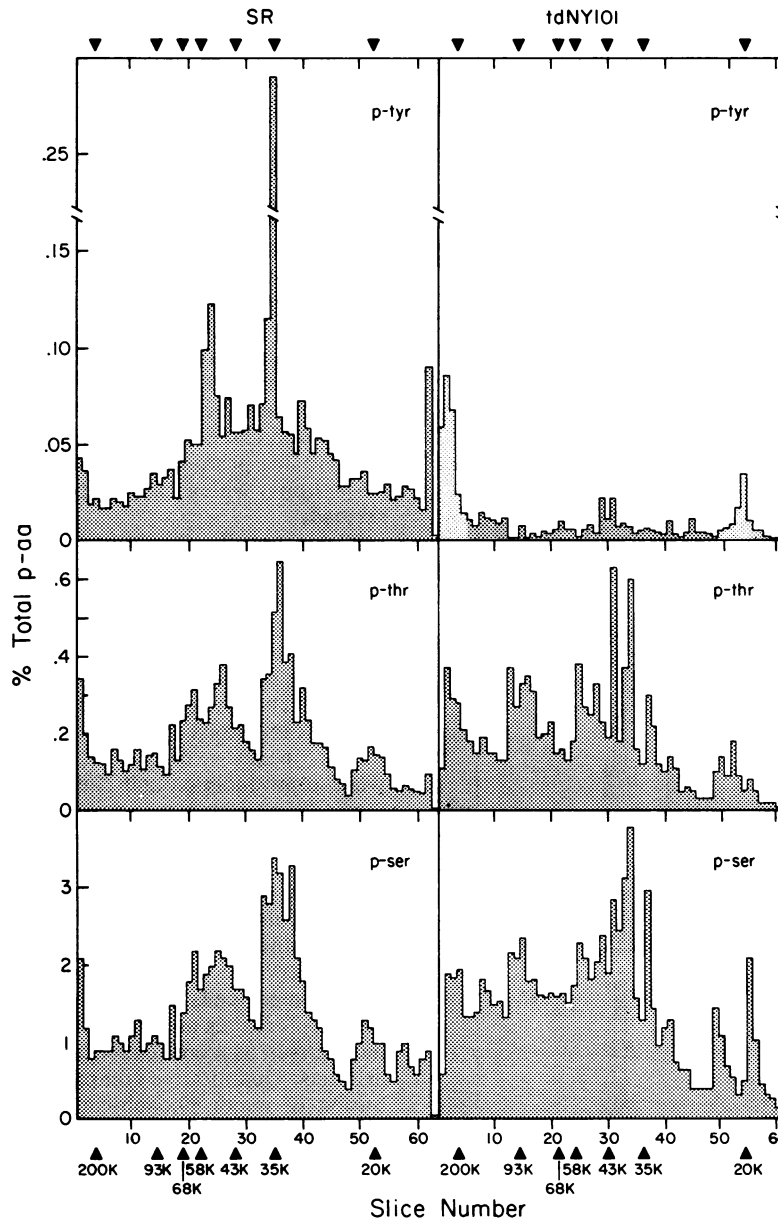

FIG. 7. Quantitative distribution of individual phosphoamino acids across polyacrylamide gels used to separate whole cell lysates of RSV and *td*NY101-infected CEF. The individual ninhydrin-stained phosphoamino acid standard spots corresponding to the autoradiograms displayed in Fig. 3 and 5 were cut out from the paper and placed in scintillation vials containing 0.5 ml of 0.1 N NaOH. Vials were placed at 37°C for 1 h with shaking. After adding 0.1 ml of 1 N HCl to each vial, the radioactivity associated with each phosphoamino acid was determined by liquid scintillation counting in Aquasol. Percentages on the ordinate axis were obtained by dividing the counts per minute associated with each individual phosphoamino acid for each gel slice by the sum of counts per minute of all three phosphoamino acids contained in the entire gel. The abscissa shows the gel slice number for both cell types. Arrows point to the position of the internal standard molecular weight marker proteins on the gel. The total phosphoamino acid counts per minute for the two gels were: 903,357 for the Schmidt-Ruppin gel and 1,781,196 for the *td*NY101 gel. A background baseline of 50 cpm was determined by counting a paper region outside of the axis of electrophoretic migration. Note that the radioactivity in "phosphotyrosine" in the high-molecular-weight region of the *td*NY101 gel has been shown not to be phosphotyrosine (Fig. 6), and the phosphotyrosine in slices 50 to 56 appeared variably; thus, these peaks are shown with lighter shading.

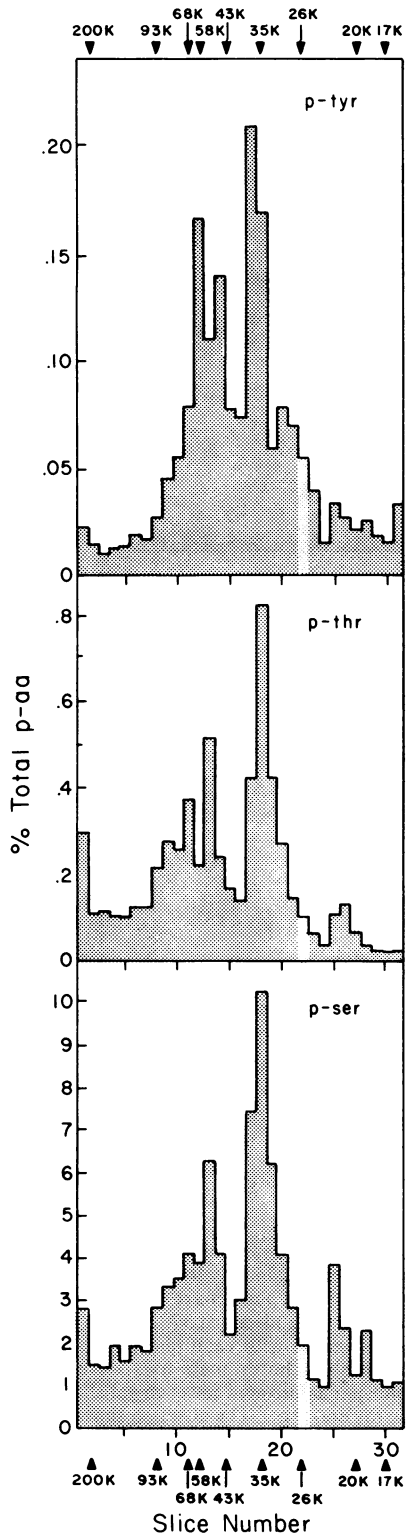

FIG. 8. Quantitative distribution of phosphoamino acids across a polyacrylamide gel used to separate a whole cell lysate of RSV-transformed CEF. The individual ninhydrin-stained phosphoamino acid standard spots corresponding to the autoradiogram in Fig. 4 were cut out from the paper, and radioactivity was determined by scintillation spectrometry. Since the material from slice 22 was lost, this point is shown with lighter shading as the average of slices 21 and 23.

35-kilodalton phosphotyrosine peaks correspond to pp60^{src} and the 36K protein (24), respectively. Immunoprecipitation of pp60^{src} from a transformed cell extract before gel electrophoresis and phosphoamino acid analysis showed a significant lowering of the 59-kilodalton phosphotyrosine peak (data not shown). Evidence for the 36K phosphoprotein being the major tyrosine phosphorylated protein represented by the 35-kilodalton peak was provided by the finding that RSV CU2-infected cells generated a phosphotyrosine profile characterized by a very low 35K peak (data not shown). As indicated before, this partial transformation mutant of RSV is defective in the phosphorylation of the 36K protein (22). Finally, of particular interest is the reproducible appearance of a major phosphotyrosine peak of low molecular weight (<20 kilodaltons) which has not previously been described.

The overall phosphotyrosine profile of *td*NY101-infected cells is strikingly lower than that of the RSV-transformed cells. As discussed above, the radioacivity in "phosphotyrosine" in slices 1 to 3 of this gel was an artifact of streaking during the high-voltage electrophoresis run. The phosphotyrosine peak between 22 and 18 kilodaltons appeared variably. However, the two peaks at M_r's of 42 and 40 kilodaltons were reproducible, and their levels, although lower than seen in transformed cells in equivalent regions of the gel, were highly significant.

Figure 7 also displays profiles of phosphothreonine and phosphoserine for RSV- and *td*NY101-infected cells. Although some differences in the levels of these phosphoamino acids are observable between transformed and untransformed cells, we cannot state whether these differences are significant or whether they have arisen as a consequence of methodological artifacts. Quantitative comparisons in the distribution of major phosphoamino acids (such as phosphoserine and phosphothreonine) between two gels is technically difficult using our current techniques since it is impossible to reproducibly slice up tube gels with the precision needed to generate slices with identical M_r spans.

DISCUSSION

Transformation of CEF by RSV results in a 10- to 20-fold increase in the levels of cellular

phosphotyrosine (16, 22, 29). By performing phosphoamino acid analyses of an entire polyacrylamide gel used to separate the phosphoproteins of a RSV-transformed whole cell lysate, we have shown that the phosphotyrosine of transformed cells is associated with a multiplicity of proteins that span the entire gel. The simplest interpretation of this finding is that neoplastic transformation of CEF by RSV results in the tyrosine-specific phosphorylation of a large number of proteins. Indeed, our data indicate that the major phosphotyrosine-containing protein in the RSV-transformed cells (the 36K protein) represents no more than 10% of the total phosphotyrosine.

This interpretation would be weakened by any one of the following three artifacts. (i) The first artifact is proteolysis during cell lysis of a high-molecular-weight phosphotyrosine phosphoprotein generating a number of discrete breakdown products phosphorylated in tyrosine. Our cell lysis, however, is rapidly carried out under denaturing conditions that would minimize such proteolytic activity. (ii) The second artifact is overestimation of phosphotyrosine due to the comigration of a transformation-specific contaminant with this phosphoamino acid. We routinely separate the phosphoamino acids by electrophoresis at pH 1.9 and then at pH 3.5. In addition, we have chromatographed the transformed cell phosphotyrosine separated by two-dimensional, high-voltage paper electrophoresis at pH 1.9 and 3.5 in a solvent consisting of isopropanol, HCl, and water (14:3:3) and shown it to be homogenous. (iii) The third artifact is that O^4-(5'-uridyl) tyrosine linkages are capable of yielding phosphotyrosine upon acid hydrolysis (26); nucleotidyl-O-tyrosine bonds have been found in proteins covalently bound to RNA (2, 26), DNA (23), and in certain enzymes (1, 30). We cannot estimate what proportion of the transformed cell phosphotyrosine derives from protein-nucleotidyl linkages as opposed to phosphoproteins. The ability of $pp60^{src}$ to phosphorylate in vitro the 36K protein (12) and the fact that this cellular protein is recoverable from transformed cells in a tyrosine phosphorylated state (13, 24) lend support to the notion that tyrosine phosphorylation by protein kinases does occur in vivo. In any case, if macromolecules other than phosphoproteins are responsible for some of the phosphotyrosine seen in our experiments, their presence would be transformation specific.

Cooper and Hunter have identified a number of phosphotyrosine-containing proteins in Prague-A RSV-infected chicken cells (10). Their procedure involves separating the phosphoproteins from a transformed cell extract in an isoelectric focusing gel followed by a denaturing polyacrylamide gel in the second dimension. Treatment of these gels with alkali preferentially hydrolyzes the phosphoserine phosphoester bond, revealing the presence of seven transformation-specific phosphotyrosine-containing protein spots on the gel. A variety of factors could account for the inability of their technique to detect the multitude of phosphotyrosine-containing proteins that we observed in denaturing gels. These considerations have been previously discussed in detail by Cooper and Hunter (10) and are cited here only briefly. (i) Low abundance phosphoproteins are difficult to detect due to the low loading capacity of the isoelectric focusing gels. (ii) Some phosphotyrosine-containing proteins do not electrofocus sharply on isoelectric focusing gels (e.g., $pp60^{src}$). (iii) Some proteins contain alkali-sensitive tyrosine-phosphate linkages that are hydrolyzed after KOH treatment. (iv) Finally, even though the alkali treatment enriches the relative quantities of phosphotyrosine about 10-fold, the large proportion of serine phosphorylated proteins containing alkali-resistant phosphoserine linkages may obscure detection of additional tyrosine-phosphorylated proteins.

Although the methodology we have described in this paper does not suffer from these technical limitations, our approach does have considerably less resolving power. Thus, no precise estimate can be given as to the number of phosphoproteins that contribute to the phosphotyrosine recovered from any particular gel slice. Assuming that a single protein would be divided into no more than two 2-mm-thick gel slices, the minimum number for this class of phosphoproteins in RSV-transformed cells would be 30.

Even though phosphorylation on tyrosine is likely to be a primary event in RSV transformation, the biological significance of the observed tyrosine phosphorylation events is still obscure. It can be imagined that $pp60^{src}$ could phosphorylate a large number of exposed tyrosine residues without affecting the biological function of the phosphorylated proteins. However, the fact that a number of different transforming protein kinases encoded by various retroviruses (4, 6) and at least two growth factors (15, 25) can trigger substantial increases in the levels of cellular phosphotyrosine support the notion that some oncogenic and growth factor-responsive biochemical pathways are characterized by a complex network of tyrosine phosphorylation events. Epidermal growth factor treatment of A431 cells increases their total phosphotyrosine content and stimulates the phosphorylation of at least three proteins identifiable on two-dimensional gels (15). Using our methodology, we have detected a large number of phosphotyrosine-containing proteins in epidermal growth

factor-stimulated A431 cells (unpublished results).

The existence of multiple biologically significant primary pp60^{src} targets is supported by our own biological and biochemical evidence (3, 22, 35), as well as that of others (5, 10, 27). Nevertheless, we were surprised to find such a large number of phosphotyrosine-containing proteins. This finding raises the question of whether all of these phosphorylations are catalyzed directly by pp60^{src}. It seems quite possible that at least some of the tyrosine-phosphorylated proteins are themselves the targets of pp60^{src} activated cellular kinases rather than being primary targets of pp60^{src} itself. Several observations have provided evidence for the existence of normal cellular proteins capable of phosphorylating on tyrosine. At least one cellular homolog of a viral transforming gene (c-*src*) encodes a tyrosine-specific protein kinase that is present at low levels in untransformed cells (16). Endogenous protein kinases with tyrosine substrate specificity also appear to mediate the rise in phosphotyrosine levels induced by epidermal growth factor (15).

We have presented data in this paper indicating that untransformed cells contain at least two prominent tyrosine-phosphorylated proteins. Interestingly, the M_r's of these proteins differ from that of the major phosphotyrosine-containing proteins of RSV-transformed cells. This finding raises the possibility that the active tyrosine-specific protein kinase(s) of normal cells has a target specificity different from that of pp60^{src}. This is consistent with recent findings by Smart et al. (31) showing that the endogenous pp60^{src} homolog (pp60^{c-src}) is phosphorylated in vivo in normal cells on a different tyrosine-containing peptide than becomes phosphorylated in vitro.

The methodology we have described in this study appears to be sensitive enough to detect phosphotyrosine-containing proteins in cells characterized by low levels of total phosphotyrosine, such as the untransformed cells. Thus, the techniques described may prove useful for detecting minor tyrosine phosphorylation events that could take place after neoplastic transformation by agents that do not raise the overall phosphotyrosine levels such as papovaviruses, other retroviruses, and chemical carcinogens (29). Such experiments are in progress.

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ADDENDUM IN PROOF

Recent experiments indicate that there are other tyrosine-phosphorylated proteins in normal, uninfected chicken embryo fibroblasts besides the 40- and 42-kilodalton proteins described in this manuscript. The degree of tyrosine phosphorylation of these newly identified proteins relative to the degree of phosphorylation of the 40- and 42-kilodalton species is dependent on conditions that are currently being investigated. One such condition is the availability of serum or of specific growth factors in the culture medium, which results in specific changes in the pattern of tyrosine phosphorylation of proteins in normal chicken embryo cells (manuscript in preparation).

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