Proc. Natl. Acad. Sci. USA
Vol. 76, No. 10, pp. 5212–5216, October 1979
Cell Biology

Transformation by Rous sarcoma virus: Effects of *src* gene expression on the synthesis and phosphorylation of cellular polypeptides

(transformation-defective mutants/temperature-sensitive mutants/two-dimensional gel electrophoresis/36,000 M_r phosphoprotein/acute leukemia viruses)

KATHRYN RADKE AND G. STEVEN MARTIN

Department of Zoology, University of California, Berkeley, California 94720

Communicated by A. Harry Rubin, July 17, 1979

ABSTRACT **Infection of chicken embryo fibroblasts by Rous sarcoma virus induces a variety of alterations in cellular growth and morphology. We have used two-dimensional polyacrylamide gel electrophoresis to examine the effects of viral transformation on the pattern of synthesis and phosphorylation of cellular polypeptides. Infection by Rous sarcoma virus does not appear to induce the *de novo* synthesis, or the complete suppression, of any of the [^{35}S]methionine-labeled cellular polypeptides that can be resolved with this technique; however, there are quantitative changes in a minor fraction (approximately 4%) of the [^{35}S]methionine-labeled polypeptides. When cells labeled with [^{32}P]orthophosphate were examined, a phosphorylated polypeptide, M_r 36,000, was detected in transformed cells: this polypeptide appears within 20 min when cells infected by a temperature-sensitive mutant of Rous sarcoma virus are shifted from the nonpermissive to the permissive temperature. Phosphorylation of the 36,000 M_r polypeptide thus represents an early event in the process of transformation, and it is possible that this polypeptide is a target for the kinase activity associated with pp60^{src}.**

Transformation of fibroblasts by DNA or RNA tumor viruses results in many changes in cellular morphology and metabolism (1). In Rous sarcoma virus (RSV), these changes occur in response to the expression of a single viral gene, *src*. The product of this gene has recently been identified as a phosphorylated polypeptide, M_r 60,000, denoted pp60^{src} (2–4). The finding that pp60^{src} is associated with protein kinase activity (3, 4) suggests that some or all of the effects of the *src* gene may be mediated by the phosphorylation of cellular polypeptides. Several proteins, such as fibronectin, collagen, glucose transport proteins, and hyaluronic acid synthetase, are present in altered quantities in transformed cells (1). This suggests that the initial phosphorylation event leads to an altered pattern of polypeptide synthesis in the transformed cell.

To investigate the effects of transformation on the synthesis and phosphorylation of cellular polypeptides, we have used two-dimensional polyacrylamide gel electrophoresis to examine the radiolabeled polypeptides of chicken embryo fibroblasts infected either with nondefective (*nd*) RSV or with transformation-defective (*td*) or temperature-sensitive (*ts*) mutants. Our results indicate that in chicken fibroblasts transformation induces some quantitative changes, but no qualitative changes, in the pattern of synthesis of these polypeptides. Transformation does, however, induce the phosphorylation of a 36,000 M_r polypeptide. The kinetics with which this polypeptide becomes phosphorylated after transformation suggest that it may be directly phosphorylated by the pp60^{src}-associated kinase.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cells and Viruses. C/E chicken helper factor-negative fibroblasts were prepared from embryonated eggs obtained from SPAFAS Inc. (Norwich, CT), or Life Sciences, Inc. (St. Petersburg, FL). Schmidt–Ruppin RSV, subgroup A, (SR-A) and the transformation-defective mutant *td*NY106 (5) were supplied by H. Hanafusa. Prague RSV, subgroup A, (PR-A) and the transformation-defective derivative *td*PR-A originated in P. K. Vogt's laboratory. The temperature-sensitive mutant *ts*LA29PR-A (6) was supplied by J. A. Wyke. K. Bister supplied us with avian myelocytomatosis virus (MC29) and with quail fibroblasts transformed by MC29 (clone 8; see ref. 7) and subsequently superinfected with ringneck pheasant virus (RPV). A Rous-associated virus 1 (RAV 1) pseudotype of avian erythroblastosis virus (AEV), strain ES4, was supplied by T. Graf. The isolation and properties of a clone of quail fibroblasts (UV5-C5) transformed by *nd*PR-A have been described (8). Methods for the culture and infection of chicken embryo fibroblasts have been described (9).

Radiolabeling of Cellular Polypeptides. Uninfected cells, or cells infected by *td* mutants, were plated at a density of 1.0–1.2 × 10^5 per 16-mm well; cells transformed by *nd*RSV were plated at 2 × 10^5 per 16-mm well. Cells were fed 12 hr after plating and radiolabeled 12 hr later in 0.25 ml of medium. Under these plating conditions both normal and transformed cells were growing exponentially at the time of labeling. Cells to be labeled with [^{35}S]methionine were washed in methionine-free medium, then labeled for 4 hr in methionine-free medium supplemented with 4% calf serum, 1% chicken serum, and 200 μCi of [^{35}S]methionine per ml (Amersham, 400–900 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels). Cells to be labeled with [^{32}P]orthophosphate were labeled in phosphate-free medium supplemented with 4% calf serum, 1% chicken serum, and 2.5 mCi of [^{32}P]orthophosphate per ml (ICN, carrier-free).

Cell Lysis. [^{35}S]Methionine-labeled cultures were washed twice with phosphate-buffered saline and the cells were lysed directly in the culture wells at 0.5 mg of protein per ml of lysis buffer (10). The contents were scraped with a Teflon scraper,

Abbreviations: RSV, Rous sarcoma virus; *nd*, nondefective; *td*, transformation-defective; *ts*, temperature-sensitive; PR-A, Prague RSV subgroup A; SR-A, Schmidt–Ruppin RSV, subgroup A; *src*, transformation gene of RSV; pp60^{src}, phosphorylated product of *src* gene; MC29, avian myelocytomatosis virus; RPV, ringneck pheasant virus; RAV 1, Rous-associated virus 1; AEV, avian erythroblastosis virus.

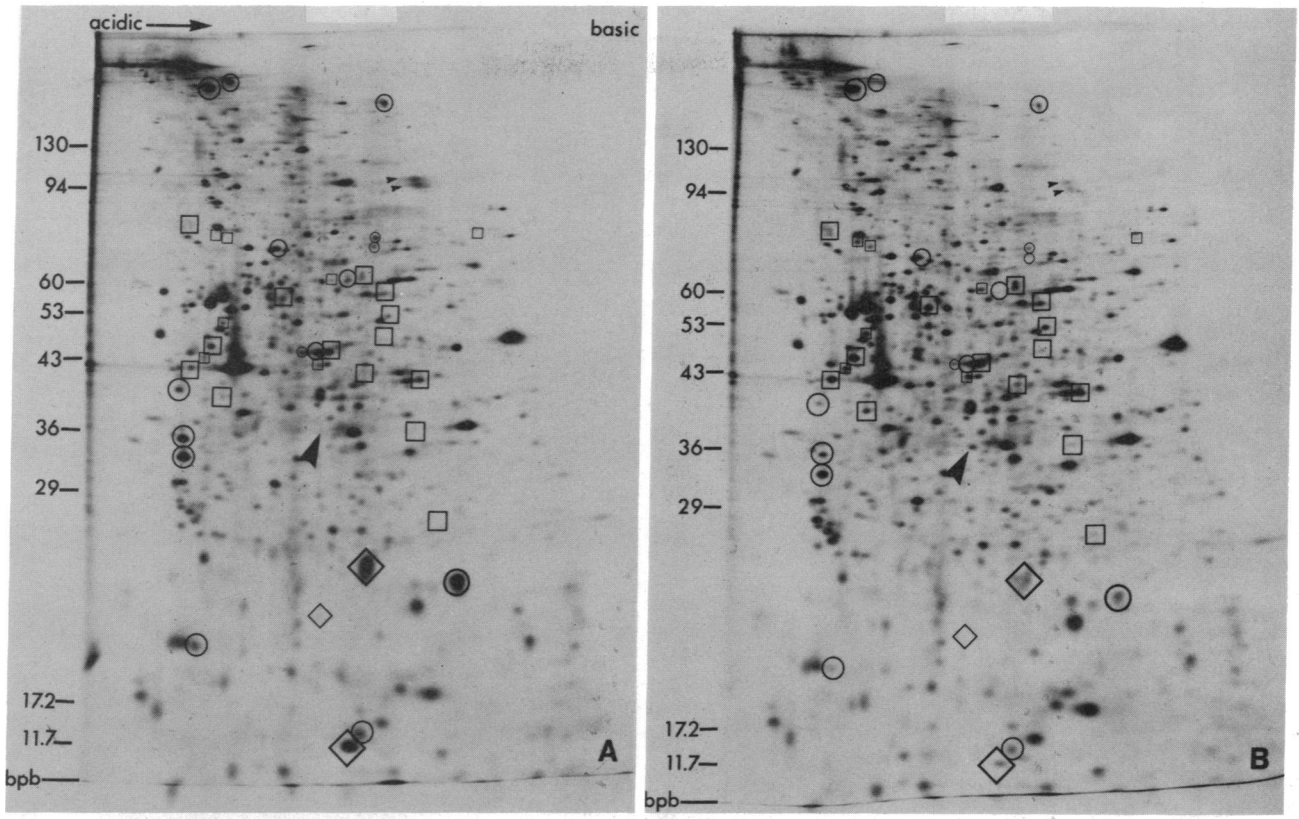

FIG. 1. [^{35}S]Methionine-labeled polypeptides from chicken embryo fibroblasts infected by (*A*) *td*PR-A or (*B*) *nd*PR-A. In this and subsequent figures, nonequilibrium pH gradient electrophoresis is in the horizontal dimension, with the origin, at the acidic end of the gradient, on the left. Electrophoresis in the presence of sodium dodecyl sulfate is in the vertical dimension. The molecular weights of protein standards are indicated as $M_r \times 10^{-3}$. ◇, Virion polypeptides; small arrowheads, unidentified polypeptides appearing in both *td* and *nd*RSV-infected cells (see text); large arrowhead, 36,000 M_r polypeptide; □, polypeptides with increased abundance in transformed cells; ○, polypeptides with decreased abundance in transformed cells. bpb, Bromphenol blue.

removed, and frozen immediately at −70°C. In certain experiments, ^{35}S-labeled cells were lysed as described below.

^{32}P-Labeled cultures were chilled, washed twice with ice-cold phosphate-buffered saline, and then lysed and treated with nucleases by a modification of the method of Garrels and Gibson (11). Cells were lysed at 2 mg of protein per ml in 0.1% Nonidet P-40 (Gallard Schlesinger), 50 μg of *Staphylococcus aureus* nuclease per ml (Boehringer Mannheim), 2 mM $CaCl_2$, and 20 mM Tris·HCl (pH 8.8) by adding the buffer directly onto the monolayer and scraping with a Teflon scraper. The lysate was brought to 0.3% sodium dodecyl sulfate (BDH-Gallard Schlesinger) and 1% 2-mercaptoethanol and scraped again. Nucleases were added to concentrations of 100 μg of deoxyribonuclease I per ml (Worthington) and 50 μg of ribonuclease I per ml (Worthington) in 5 mM $MgCl_2$/50 mM Tris·HCl, pH 7.0. The lysates were scraped, and the culture dishes were left on ice for 5 min. The lysates were transferred to tubes containing enough urea (Schwarz/Mann, ultrapure) to bring the concentration to 9.5 M. The concentrations of the following components were adjusted: Nonidet P-40 to 2% (wt/vol), 2-mercaptoethanol to 5% (vol/vol), and carrier ampholytes [pH 6–8 Ampholines (LKB)] to 2% (wt/vol). One volume of lysis buffer (10) was added, the urea was dissolved, and the samples were frozen at −70°C.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Cell lysates were subjected to nonequilibrium pH gradient electrophoresis, pH 3.5–10 (12), at 400 V for 5 hr. Equal numbers of trichloroacetic acid-precipitable counts were loaded onto each first-dimension gel (2×10^6 cpm for lysates labeled with [^{35}S]methionine; 5×10^5 cpm for lysates labeled with [^{32}P]-orthophosphate). The second dimension, sodium dodecyl sulfate/polyacrylamide gel electrophoresis, used the buffer system described by Laemmli (13). Transfer to the second dimension was carried out as described by O'Farrell (10), without pre-equilibration of the first-dimension gel. Stacking gels (5 cm) contained 4.4% acrylamide (Bio-Rad) and 0.1% methylene bisacrylamide (Bio-Rad); separating gels (17.5 cm) contained either 12.5% acrylamide and 0.1% methylene bisacrylamide (Fig. 1) or 10% acrylamide plus 0.13% methylene bisacrylamide (Figs. 2–4). Electrophoresis was at 3 W per slab gel. Molecular weight markers were located by staining, as described (8). Radiolabeled polypeptides were located by autoradiography, with Kodak XR-5 film. Autoradiograms of ^{35}S-labeled polypeptides were exposed for 4 days; those of ^{32}P-labeled polypeptides, for 34 hr.

RESULTS AND DISCUSSION

Methionine-Containing Polypeptides. Cells from a single chicken embryo were infected with *nd* or *td* RSV; both Prague and Schmidt–Ruppin strains were examined. The infected cells, plus parallel cultures of uninfected cells, were plated under conditions where all cell types would grow exponentially. The cellular polypeptides were pulse-labeled with [^{35}S]methionine and the labeled polypeptides were analyzed by two-dimensional gel electrophoresis. The results obtained with *nd*PR-RSV and *td*PR-RSV are shown in Fig. 1. The virion polypeptides p27, p15, and p19 were identified by comparison with disrupted virions of PR-RSV: these polypeptides represent approximately 0.5% of the total cellular polypeptides (14) and could be detected in *td*PR-A-infected cells, and, in lesser

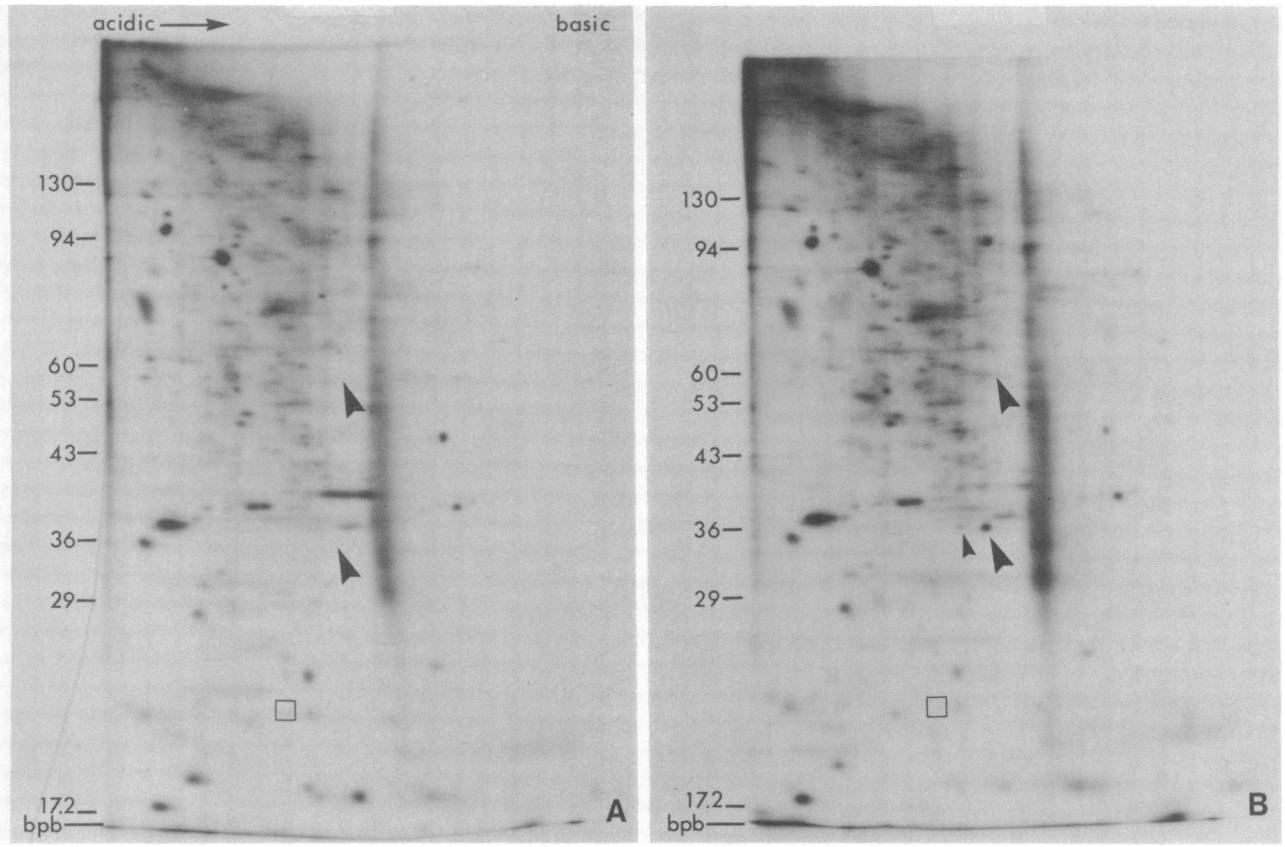

FIG. 2. [^{32}P]Orthophosphate-labeled polypeptides from chicken embryo fibroblasts infected with (*A*) *td*PR-A or (*B*) *nd*PR-A. Large arrowheads, positions of the 36,000 and 60,000 M_r phosphorylated polypeptides; small arrowhead, position of the minor 36,000 M_r species. □, 22,000 M_r phosphorylated polypeptide.

quantities, in *nd*PR-A-infected cells. In addition, two basic polypeptides, of 97,000 and 103,000 M_r (small arrowheads, Fig. 1), appear in lysates of infected cells but not in lysates of uninfected cells; whether these are of viral or cellular origin is unknown.

The distribution of labeled polypeptides is qualitatively very similar in lysates of *td*RSV-infected and *nd*RSV-infected cells (Fig. 1). With the exception of the 36,000 M_r polypeptide discussed in the next section, transformation is not characterized by the appearance or disappearance of any of the polypeptides that can be resolved by this technique. These results are similar to those recently obtained with Rat-1 cells transformed by Kirsten sarcoma virus or Abelson leukemia virus (B. Ozanne and T. Wheeler, personal communication). They contrast, however, with the results of Strand and August (15, 16), who reported that 30% of the polypeptides of 3T3 cells transformed by DNA or RNA tumor viruses differ, either by size or by charge, from those of normal cells. It is likely that the alterations observed in transformed 3T3 cells are secondary changes that take place, after the initial transformation event, during the growth of the transformed clones.

To estimate the fraction of the cellular polypeptides whose synthesis is increased or decreased after transformation, we examined the effects of transformation on the relative labeling intensity of 983 well-resolved polypeptides. Two separate infections were carried out with *nd* and *td*PR-A, and two with *nd* and *td*SR-A; two separations were carried out with each lysate. Inspection of autoradiograms from these experiments indicates that of these polypeptides, 25, or 2.5%, showed increases in relative labeling intensity in both PR-A- and SR-A-infected cells, while 17, or 1.7%, showed decreases in relative labeling intensity. Examples of these changes are shown by circles or squares in Fig. 1. The same alterations were observed in lysates prepared by presolubilization of proteins with sodium

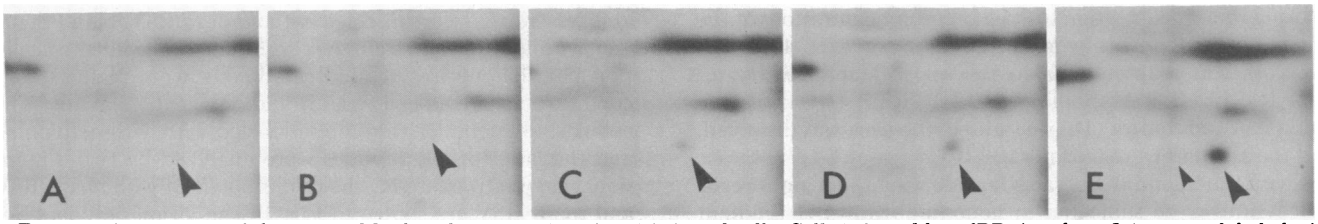

FIG. 3. Appearance of the 36,000 M_r phosphoprotein in *tsLA29*-infected cells. Cells infected by *td*PR-A or by *tsLA29* were labeled with [^{32}P]orthophosphate at 41°C for 4 hr, shifted to 35°C, and lysed at the times indicated. The relevant section of each autoradiogram is shown. (*A*) *td*PR-A-infected cells, shifted to 35°C for 40 min; (*B–E*) *tsLA29*-infected cells at the time of the temperature shift (*B*), after 20 min at 35°C (*C*), after 40 min at 35°C (*D*), and after 4 hr at 35°C (*E*). Large arrowheads show the position of the major 36,000 M_r phosphorylated polypeptide; the small arrowhead, the position of the minor 36,000 M_r species.

dodecyl sulfate. Additional differences in a small fraction of the polypeptides were observed in certain experiments; these changes appear to reflect the strain of virus used or the experimental conditions. These estimates are subject to a number of reservations. The technique resolves only a limited number of the most abundant cellular polypeptides, and only alterations in relative abundance that are apparent by inspection of autoradiograms were identified. Furthermore, some of the alterations observed may result from post-translational modification or changes in rates of degradation. With these reservations, however, we estimate that transformation affects the relative rate of synthesis of 4% of the polypeptides of exponentially growing cells.

Phosphorylated Polypeptides. The finding that the *src* gene product, pp60^{src}, is associated with protein kinase activity (3, 4) led us to examine the effects of transformation on the phosphorylation of cellular polypeptides. Cells infected with *nd* or *td*RSV were labeled with [^{32}P]orthophosphate and then lysed in the presence of nucleases. The phosphorylated polypeptides were then resolved by two-dimensional polyacrylamide gel electrophoresis. In *td*RSV-infected cells, a 22,000 M_r polypeptide with the electrophoretic properties of the phosphorylated form of p19 (17) can be detected (square, Fig. 2*A*); the pattern of labeling is otherwise similar to that of uninfected cells. When the phosphorylated polypeptides of *nd*RSV-transformed cells were examined, the overall pattern of phosphorylation was similar to that of nontransformed cells; as expected from the results obtained with [^{35}S]methionine, a fraction of these polypeptides (approximately 6%) showed changes in relative labeling intensity. However, two sets of phosphorylated polypeptides appear either *de novo* or in greatly increased quantities in *nd*RSV-infected cells: these are indicated by arrowheads in Fig. 2*B*.

One of these phosphorylated polypeptides appears as a streak at 60,000 M_r and covers a region containing several minor polypeptides in uninfected cells. Several pieces of evidence, which will be presented elsewhere (18), suggest that this 60,000 M_r streak represents pp60^{src}. The other phosphorylated polypeptide that is present in greatly increased quantities in *nd*RSV-transformed cells appears as a spot of 36,000 M_r (large arrowhead, Fig. 2*B*). This species comigrates with a 36,000 M_r polypeptide present in [^{35}S]methionine-labeled lysates of *nd*RSV-transformed cells (large arrowhead, Fig. 1*B*). The 36,000 M_r polypeptide cannot be immunoprecipitated by rabbit tumor sera that recognize pp60^{src} (8), implying that it is not a cleavage product of pp60^{src}. When autoradiograms of the phosphorylated polypeptides of uninfected cells were exposed for longer periods, a faint spot could be detected at this position, suggesting that the phosphorylated polypeptide is present at low concentrations in uninfected cells and that it is of cellular rather than viral origin. Phosphorylation of a polypeptide involves the addition of an acidic group and thus alters its mobility in the first dimension (10, 19, 20). We presume, therefore, that the 36,000 M_r polypeptide detected by [^{32}P]-orthophosphate or [^{35}S]methionine labeling results from phosphorylation of a more basic polypeptide. In addition, a less intensely labeled 36,000 M_r species appears at a more acidic position in lysates of ^{32}P-labeled transformed cells (small arrowhead, Fig. 2*B*); this may represent a modification of the major 36,000 M_r species.

Kinetics of Phosphorylation. To examine the relationship between the appearance of the 36,000 M_r polypeptide and the expression of the *src* gene, we examined the kinetics of appearance of this phosphoprotein in cells infected by a temperature-sensitive mutant *tsLA*29. Cells infected by this mutant are phenotypically normal at 41°C, but transform when shifted to 35°C. Cells infected by *tsLA*29 at 41°C were labeled with [^{32}P]orthophosphate in order to label the ATP pool. The cells were then shifted to 35°C and lysates were prepared at 20 min, 40 min, and 4 hr after the shift. The lysates were analyzed as described previously; the results are shown in Fig. 3.

The phosphorylated 36,000 M_r polypeptide is barely detectable at 41°C (Fig. 3*B*); it is clearly detectable 20 min after the temperature shift (Fig. 3*C*), and the intensity of labeling increases progressively at later times (Fig. 3 *D* and *E*). The minor, more acidic, form of the 36,000 M_r polypeptide is detectable in cells labeled for 4 hr after the temperature shift (small arrowhead, Fig. 3*E*). These changes do not occur in *td*PR-A-infected cells shifted from 41°C to 35°C (Fig. 3*A*), indicating that they do not represent a cellular response to the temperature shift. We conclude that the 36,000 M_r polypeptide becomes phosphorylated at very early times in the process of transformation. Other changes that occur in *tsLA*29-infected cells shifted from 41°C to 35°C, such as the loss of fibronectin or the increase in the uptake of glucose analogs, can be detected only after a lag of several hours (21). The phosphorylation of the 36,000 M_r polypeptide therefore represents the earliest cellular alteration detectable in these temperature-shift experiments.

Transformation by Avian Acute Leukemia Viruses. To determine if the 36,000 M_r polypeptide becomes phosphorylated in cells transformed by agents other than RSV, we examined the ^{32}P-labeled polypeptides of fibroblasts transformed by the acute leukemia viruses, MC29 and AEV. These viruses lack the *src* gene of RSV (22), and fibroblasts transformed by these viruses are phenotypically different from those transformed by RSV (23).

The 36,000 M_r phosphoprotein is not present in quantities greater than in uninfected cells among the ^{32}P-labeled polypeptides of a clone of quail fibroblasts (clone 8) transformed by MC29 and superinfected with RPV (Fig. 4*A*), although it is present in increased abundance in a clone of quail fibroblasts (UV5-C5) producing RSV (Fig. 4*B*). Similarly, the 36,000 M_r phosphoprotein is not present in increased quantities in chicken fibroblasts transformed by MC29(RPV) (data not shown). These results indicate that an increased abundance of the 36,000 M_r phosphorylated polypeptide is not necessary for transformation of avian fibroblasts by MC29.

When we examined the phosphoproteins of AEV(RAV 1)-transformed chicken fibroblasts derived from a colony selected in soft agar (Fig. 4*C*), we found that the 36,000 M_r phosphoprotein is present in greater abundance than in uninfected cells or in RAV 1-infected cells, but in lesser abundance than in cells transformed by RSV. We obtained the same result by using chicken fibroblasts transformed after a mass infection by AEV(RAV 1) (data not shown). Thus, transformation by AEV does result in enhanced levels of phosphorylation of the 36,000

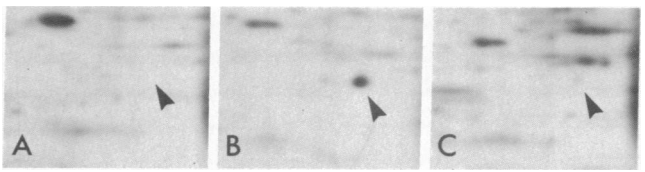

FIG. 4. Presence of the 36,000 M_r phosphoprotein in fibroblasts infected by avian acute leukemia viruses. Transformed fibroblasts were labeled with [^{32}P]orthophosphate for 4 hr at 37°C. Relevant sections of autoradiograms are shown. (*A*) Quail fibroblasts (clone 8) transformed by MC29(RPV); (*B*) quail fibroblasts (clone UV5-C5) transformed by PR-A-RSV; (*C*) chicken fibroblasts infected by AEV(RAV 1) and cloned in soft agar. Arrowheads indicate the position of the major 36,000 M_r phosphorylated polypeptide.

M_r polypeptide, but not to the extent found in RSV-transformed cells. When lysates were mixed, the 36,000 M_r polypeptide from AEV-transformed fibroblasts comigrated with the 36,000 M_r polypeptide from RSV-transformed fibroblasts (data not shown), implying that the two are identical. These observations reinforce the conclusion that this polypeptide is of cellular origin and is structurally unrelated to pp60^{src} since AEV lacks the *src* gene and does not induce cellular *src*-related sequences (22).

The findings reported here indicate that changes in protein phosphorylation represent very early events in the process of transformation by RSV. The rapidity with which the 36,000 M_r phosphoprotein appears after transformation by RSV suggests that the polypeptide may be directly phosphorylated by the pp60^{src}-associated kinase activity. Additional observations, which will be reported elsewhere (18), are consistent with this idea: like pp60^{src}, the 36,000 M_r polypeptide is cytoplasmic; it becomes phosphorylated in cells infected by *tsLA29* and shifted from 41°C to 35°C even when protein synthesis is inhibited by cycloheximide; its continued phosphorylation requires continued activity of pp60^{src}. Alternatively, it is possible that the 36,000 M_r polypeptide is phosphorylated as a secondary consequence of pp60^{src} function. Further experiments are necessary to distinguish between these possibilities.

We thank Dan Schleske and Masae Namba for excellent technical assistance. We also thank Tony Pawson for a gift of labeled virions; Patricia O'Farrell, Jim Garrels, and Martin Privalsky for helpful suggestions; Ray Erikson, J. Michael Bishop, and Tom August for stimulating discussions; and Peter Duesberg for critical review of the manuscript. This work was supported by National Institutes of Health Grant CA17542. K.R. was supported by fellowships from the Damon Runyon–Walter Winchell Cancer Research Fund and the National Institutes of Health.

1. Hanafusa, H. (1977) in *Comprehensive Virology*, eds. Fraenkel-Conrat, H. & Wagner, R. R. (Plenum, New York), Vol. 10, pp. 401–483.
2. Brugge, J. S. & Erikson, R. L. (1977) *Nature (London)* **269**, 346–348.
3. Collett, M. S. & Erikson, R. L. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2021–2024.
4. Levinson, A. D., Oppermann, H., Levintow, L., Varmus, H. E. & Bishop, J. M. (1978) *Cell* **15**, 561–572.
5. Kawai, S., Duesberg, P. H. & Hanafusa, H. (1977) *J. Virol.* **24**, 910–914.
6. Wyke, J. A. & Linial, M. (1973) *Virology* **53**, 152–161.
7. Bister, K., Hayman, M. J. & Vogt, P. K. (1977) *Virology* **82**, 431–448.
8. Martin, G. S., Radke, K., Hughes, S., Quintrell, N., Bishop, J. M. & Varmus, H. E. (1979) *Virology* **96**, 530–546.
9. Vogt, P. K. (1969) in *Fundamental Techniques in Virology*, eds. Habel, K. & Salzman, N. P. (Academic, New York), pp. 198–211.
10. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
11. Garrels, J. I. & Gibson, W. (1976) *Cell* **9**, 793–805.
12. O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142.
13. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
14. Vogt, V. M., Eisenman, R. & Diggelmann, H. (1975) *J. Mol. Biol.* **96**, 471–493.
15. Strand, M. & August, J. T. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2729–2733.
16. Strand, M. & August, J. T. (1978) *Cell* **13**, 399–408.
17. Erikson, E., Brugge, J. S. & Erikson, R. L. (1977) *Virology* **80**, 177–185.
18. Radke, K. & Martin, G. S. (1980) in *Cold Spring Harbor Symp. Quant. Biol.* **44**, in press.
19. O'Farrell, P. Z. & Goodman, H. M. (1976) *Cell* **9**, 289–298.
20. Farrell, P. J., Hunt, T. & Jackson, R. J. (1978) *Eur. J. Biochem.* **89**, 517–521.
21. Hynes, R. O. & Wyke, J. A. (1975) *Virology* **64**, 492–504.
22. Stéhelin, D. & Graf, T. (1978) *Cell* **13**, 745–750.
23. Royer-Pokora, B., Beug, H., Claviez, M., Winkhardt, H.-J., Friis, R. R. & Graf, T. (1978) *Cell* **13**, 751–760.