JOURNAL OF VIROLOGY, Mar. 1982, p. 813–820
0022-538X/82/030813-08$02.00/0

Vol. 41, No. 3

Phosphorylation and Metabolism of the Transforming Protein of Rous Sarcoma Virus

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Received 7 October 1981/Accepted 19 October 1981

p60^{src}, the transforming protein of Rous sarcoma virus, was found to contain 0.5 to 0.9 mol of total phosphate per mol of polypeptide. The protein is known to be phosphorylated at two sites, a serine in the amino-terminal domain and a tyrosine in the carboxy-terminal domain. Because our indirect analysis suggests that the serine is phosphorylated to approximately twice the extent of the tyrosine, we estimate that p60^{src} contains approximately 0.3 to 0.6 mol of phosphoserine and 0.2 to 0.3 mol of phosphotyrosine per mol of polypeptide. p60^{src} was found to represent approximately 0.02% of the total incorporated radioactivity in Rous sarcoma virus-transformed chick cells labeled with [^{35}S]methionine for 48 h. This corresponds to approximately 500,000 molecules of p60^{src} per cell. Pulse-chase experiments revealed that the half-life of p60^{src} ranged from 2 to 7 h, depending on the strain of virus examined. The p60^{src} of the Schmidt-Ruppin strain was significantly more stable than that of the Prague strain.

The transforming gene of Rous sarcoma virus (RSV), *src*, encodes a single polypeptide, p60^{src} (1). This viral phosphoprotein has an associated protein kinase activity (3) which phosphorylates substrates on tyrosine (11). Because this activity is retained through extensive purification (5, 15), and because p60^{src} produced by in vitro translation has the same novel protein kinase activity (11), it is very likely that this is an intrinsic property of the viral protein. There is now considerable evidence that p60^{src} functions as a protein kinase which phosphorylates tyrosine in vivo and that this activity of the viral enzyme is essential to cellular transformation (22).

As yet, our knowledge about the properties of p60^{src} is incomplete. The protein appears to be confined to the cytoplasm of the transformed cell, where a significant fraction of it resides on the cytoplasmic face of the plasma membrane (6, 14, 17, 25). It is phosphorylated at two sites, a serine in the NH_2-terminal domain of the polypeptide (4) and a tyrosine located in the COOH-terminal half (4, 11) at position 419 of the sequence of p60^{src} of Schmidt-Ruppin RSV of subgroup A (SR-RSV-A) (23; T. Patschinsky, T. Hunter, F. S. Esch, J. A. Cooper, and B. S. Sefton, Proc. Natl. Acad. Sci. U.S.A., in press) as deduced by Czernilofsky et al. (7). It is quite possible that the phosphorylation of either or both of these amino acids modulates the enzymatic activity of p60^{src}. To evaluate this possibility properly, one must know the extent to which both of these sites are phosphorylated. We have determined this in two ways: first by calculation of the moles of phosphate present in a measured amount of p60^{src} isolated from cells labeled to steady state with $^{32}P_i$ and second by measurement of the ratio of [^{3}H]isoleucine recovered in the phosphorylated and unphosphorylated forms of the tryptic peptide containing the single site of tyrosine phosphorylation in p60^{src}.

To calculate the extent of phosphorylation of p60^{src}, we had to have an accurate way to measure the number of molecules present in a given preparation of p60^{src}. This was done by labeling cells to steady state with [^{35}S]methionine, determining the specific activity of the cellular protein, and then quantitatively immunoprecipitating p60^{src} with antibody in excess. Since these samples contained a known amount of p60^{src} and were obtained from a known amount of cell lysate, they allowed us also to determine the abundance at steady state of the p60^{src} polypeptide and to measure the turnover number of the associated tyrosine protein kinase activity when it is assayed in the immune complex. Our estimates for the abundance of p60^{src} were significantly lower than those reported by others who used cells labeled for 2 h with [^{35}S]methionine. The discrepancy in these results could be attributed to the differences in experimental design if the half-life of p60^{src} were significantly less than that of bulk cellular protein. Others have reported that the half-life of bulk cellular protein in chick cells is approximately 40 to 50 h. Although we knew that the

p60^{src} of SR-RSV-D was metabolically stable when followed for 2 h (19), the true half-life of the polypeptide was uncertain. We therefore repeated our pulse-chase experiments using much more extended periods of chase.

MATERIALS AND METHODS

Cells and viruses. Cultures of chicken embryo cells were prepared from eggs obtained from SPAFAS, Roanoke, Ill. Prague RSV of subgroup B (PR-RSV-B) and Schmidt-Ruppin RSV of subgroup D (SR-RSV-D) originated in the laboratory of P. K. Vogt, University of Southern California. SR-RSV-A originated in the laboratory of H. Hanafusa, The Rockefeller University.

Preparation of infected cultures. For infection, primary cultures were seeded at a density of 10^7 cells per 100-mm dish and secondary cultures were seeded at a density of 2×10^6 cells per 100-mm dish in Dulbecco modified Eagle medium (DMEM) supplemented with 2% tryptose phosphate broth, 1% calf serum, and 1% heat-inactivated chick serum. If the cells were to be infected with PR-RSV-B or SR-RSV-D, Polybrene (2 μg/ml) was included in the medium both before and after infection. As soon as a majority of the cells had attached, the medium was removed and the cells were infected at as high a multiplicity as possible. Adsorption was for 30 min in 1 ml of medium. DMEM (10 ml) supplemented with 2% tryptose phosphate broth, 1% calf serum, 1% heat-inactivated chick serum, and in some cases Polybrene was then added to each dish, and the cells were incubated for 3 to 4 days at 41°C. In most cases, the cells were transferred prior to use. The cells were reseeded at 7×10^5 (uninfected cells) or 1.5×10^6 (RSV-infected cells) cells per 35-mm dish in DMEM supplemented with 2% tryptose phosphate broth and 4% calf serum. The medium was changed once or twice a day, and the cells were used no sooner than 18 h and no later than 72 h after transfer.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The rabbit anti-RSV tumor serum and the procedures for immunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis have been described in detail previously (11, 19).

Determination of the abundance and the extent of phosphorylation of p60^{src}. Chick cells transformed by SR-RSV-D were labeled to steady state with [^{35}S]methionine (50 μCi/ml, >500 Ci/mmol; Amersham/Searle) or $^{32}P_i$ (750 μCi/ml, carrier-free; ICN) for 48 h at 38°C. In both cases, labeling was in DMEM containing 20% of the normal concentration of methionine and supplemented with 4% complete calf serum. At 24 h, the medium was changed to fresh radioactive medium. The specific activity of the phosphate in the labeling medium was calculated from the measured concentration of ^{32}P in the medium at the end of the experiment (determined by scintillation counting performed with Budget-Solv [Research Products International Corp.] as scintillation fluid) and the known concentration of phosphate in DMEM (0.9 mM) and in calf serum (1.5 mM). The specific activity of the cellular protein was determined by dissolving a sister culture, labeled in parallel with [^{35}S]methionine, in Lowry C solution and measuring the protein by the method of Lowry et al. (16) and the incorporated [^{35}S]methionine by precipitation with trichloroacetic acid. Immunoprecipitation of p60^{src} was with antibody in excess, and each sample was subjected to SDS-polyacrylamide gel electrophoresis. The [^{35}S]methionine-labeled p60^{src} bands were excised, dissolved in 0.4 ml of 60% perchloric acid and 0.8 ml of 30% H_2O_2 by incubation at 60°C for 5 h, and then counted by scintillation counting performed with Aquasol (New England Nuclear) as scintillation fluid. The [^{35}S]methionine-labeled trichloroacetic acid precipitates were counted by the same procedure so as to minimize errors due to differences in counting efficiencies. The ^{32}P-labeled p60^{src} bands were excised and counted directly by scintillation counting with 2,5-diphenyloxazole (PPO) dissolved in toluene as scintillation fluid.

Determination of the half-life of the p60^{src} polypeptide. Chick cells transformed by either SR-RSV-D or PR-RSV-B were incubated in methionine-free DMEM supplemented with 4% dialyzed calf serum for 20 to 25 min prior to labeling. This medium was then replaced with 0.75 ml of the same medium (per 35-mm dish) containing [^{35}S]methionine (400 μCi/ml). Labeling was for 60 min at 41°C. The labeled cells were then either lysed immediately or washed twice with at least 2 ml of DMEM supplemented with 2% tryptose phosphate broth and 4% calf serum and then incubated further in 2 ml of this medium. Cell lysis and immunoprecipitation were exactly as described before. The same fraction of each culture was used for immunoprecipitation at each time point. Because the density of the cultures doubled during the 24-h chase period, the amount of p60^{src} in any given fraction of the culture presumably also doubled. The quantity of anti-RSV tumor serum used was such that it was in excess throughout the chase period. Equal fractions of each sample were analyzed by SDS-polyacrylamide gel electrophoresis. Detection of p60^{src} was enhanced by fluorography, and the radioactivity in p60^{src} was quantified by scanning the fluorograms with a homemade gel scanner. Half-lives were calculated from the areas of the peaks which were measured with a Hewlett-Packard digitizer.

Measurement of protein kinase activity. The measurement of p60^{src}-associated protein kinase activity by immunoprecipitation with antibody in excess and the assay in the immune complex were as described (20, 21). The concentration of sodium phosphate, pH 7.0, in the kinase buffer was 0.01 M. (The value of 0.1 M in reference 20 is a printing error.) To minimize inactivation of p60^{src}, we performed cell lysis and washing of the precipitates with phosphate-buffered RIPA buffer, which lacked both sodium deoxycholate and SDS (21). All procedures were performed at 4°C, and the cell lysates were never frozen. The incorporation of radioactivity into the gel-purified heavy chain was determined by scintillation counting with PPO dissolved in toluene as scintillation fluid.

Determination of the half-life of enzymatically active p60^{src}. To measure the turnover of the p60^{src}-associated protein kinase activity, we lysed cells pretreated with emetine (5 to 10 μg/ml) for various periods at 5×10^6 or 10^7 cells per ml of lysis buffer and measured the protein kinase activity remaining by immunoprecipitation and assaying in the immune complex as described before (21). The zero-time value in each experiment was derived from sister cultures to which no emetine was added rather than from cells harvested at the time of addition of emetine to the experimental cultures. This procedure was adopted because it minimized

variations in the reproducibility of the kinase measurements. The cells treated with emetine did not multiply. Correction for this was made by counting the number of cells in each culture, lysing all cultures at the same concentration of cells per milliliter of lysis buffer, and using equal volumes of cell lysate in the assay.

Peptide mapping. Chick cells transformed by SR-RSV-A, growing on 100-mm dishes, were labeled overnight at 41°C with [^{3}H]tyrosine (1 mCi/ml, 53 Ci/mmol; Amersham/Searle) or [^{3}H]isoleucine (2 mCi/ml, 84 Ci/mmol; Amersham/Searle) in 5 ml of DMEM containing 5% of the normal concentration of tyrosine or isoleucine and supplemented with 4% complete calf serum. The procedures for the isolation of p60^{src} and digestion of the protein with trypsin have been described (11; Patschinsky et al., in press). Peptide mapping on cellulose thin-layer plates involved electrophoresis at pH 8.9 in the first dimension and ascending chromatography in modified buffer as described (11). The plates were dipped in molten 2-methylnaphthalene containing 0.4% diphenyloxazole prior to exposure to prefogged Kodak XAR-5 film. After sufficient exposure to allow detection of peptide β, the 2-methylnaphthalene was allowed to sublime completely, and the peptide was recovered by aspiration of the cellulose and elution of the peptide in water. Dephosphorylation of the peptide β labeled with [^{3}H]tyrosine was accomplished by incubation of the peptide in 3 μl of 20 mM Tris-hydrochloride, pH 7.4, and 1 mM EDTA containing 2 U of bacterial alkaline phosphatase (Bethesda Research Laboratories) for 60 min at 45°C. This dephosphorylated peptide [β(-P)] was shown to comigrate in two dimensions with a tyrosine-containing tryptic peptide from p60^{src} synthesized in vitro. To obtain a reasonably dark fluorographic image for the experiment shown in Fig. 1B, we included in the sample not only 100 cpm of the [^{3}H]tyrosine-labeled dephosphorylated peptide β but also 300 cpm of the [^{3}H]tyrosine-labeled peptide identified above, isolated from a tryptic digest of p60^{src} synthesized in vitro. All the radioactivity comigrated with the admixed synthetic peptide (*src* IV) (5 μg) Leu·Ile·Glu·Asp·Asn·Glu·Tyr·Thr·Ala·Arg (corresponding to the tryptic peptide containing the tyrosine phosphorylation site of p60^{src} of SR-RSV-A), which was detected by ninhydrin staining. For the experiment in Fig. 1C, 48,000 cpm of a tryptic digest of SR-RSV-A p60^{src} labeled with [^{3}H]isoleucine were resolved in two dimensions in the presence of 5 μg of *src* IV. After ninhydrin staining and fluorography, the 2-methylnaphthalene was allowed to sublime completely. Areas corresponding to peptides β and β(-P) were aspirated and eluted with pH 1.9 buffer into scintillation vials. After incubation for 30 min at room temperature, the samples were counted in 10 ml of Aquasol.

RESULTS

Abundance of p60^{src}. So as to be able to calculate the amount of p60^{src} in a given preparation, we isolated the polypeptide by immunoprecipitation from cells which had been labeled for 48 h with [^{35}S]methionine. Because these precipitates were prepared in antibody excess, we could also determine the abundance of the polypeptide. p60^{src} constituted 0.01 to 0.08% of total cellular protein in chick cells transformed by SR-RSV-D (10^5 to 8×10^5 molecules per cell). The variation in this value from experiment to experiment probably reflects real variability in the metabolic stability of p60^{src} since quadruplicate determinations in any given experiment gave essentially identical results. This abundance is significantly less than that reported by Collett et al. (2) and by Karess et al. (13). This discrepancy may result in part from the fact that we used cells labeled for 48 h with [^{35}S]methionine as the source of p60^{src}, rather than cells which had been labeled for 2 h, and the fact that the half-life of p60^{src} is noticeably less (see below) than that of total cellular protein in chick cells (9, 24). Alternatively, this discrepancy could be due to differences in the efficiencies of precipitation with different sera. Although such a factor will affect the computation of the absolute abundance of p60^{src} in a transformed cell, it does not affect the calculation of either the turnover number of the kinase activity in the immune complex (see below) or the extent of the phosphorylation of the polypeptide (see below). Finally, because all the estimates of abundance have been made with transformed cultures of primary chick cells rather than cloned cell lines, the possibility exists that the discrepancy can be accounted for by cell variability.

Extent of phosphorylation of p60^{src}. To determine the extent to which p60^{src} is phosphorylated in vivo, we labeled cultures for 48 h with either [^{35}S]methionine or $^{32}P_i$ and isolated p60^{src} by immunoprecipitation. The number of moles of phosphate per mole of p60^{src} was calculated with the assumption that after 48 h the specific activity of the phosphoamino acids in p60^{src} was the same as that of the phosphate in the labeling medium and that the specific activity of the methionine in p60^{src} was the same as that in bulk cellular protein. p60^{src} was found to contain 0.5 to 0.9 mol of phosphate per mol of polypeptide.

p60^{src} is phosphorylated at two sites. The approach used here, however, reveals only the total moles of phosphate at both sites in the polypeptide. We therefore examined the extent of phosphorylation of the single phosphorylated tyrosine residue employing another method that has been used to determine the degree of phosphorylation of a threonine residue in the large T antigen of simian virus 40 (18). This involved estimation of the relative amount of radioactivity in the phosphorylated and unphosphorylated forms of the tryptic peptide corresponding to the tyrosine phosphorylation site in p60^{src} in samples of p60^{src} labeled with ^{3}H-amino acids. We (Patschinsky et al., in press) and Smart and colleagues (23) have deduced that the phosphotyrosine-containing tryptic peptide of p60^{src} of

SR-RSV-A, which we have termed β, has the sequence NH_2-Leu·Ile·Glu·Asp·Asn·Glu·Tyr·Thr·Ala·Arg-COOH. The validity of this conclusion was demonstrated by the fact that a synthetic peptide with this sequence (T. Hunter, J. Biol. Chem., in press) comigrated with [^{3}H]tyrosine-labeled peptide β which had been dephosphorylated in vitro with bacterial alkaline phosphatase (Fig. 1B). This synthetic peptide could therefore be used to identify the unphosphorylated form of peptide β. For this experiment we analyzed a mixture of a tryptic digest of p60^{src} labeled with [^{3}H]isoleucine and the synthetic peptide. This was done because labeling of p60^{src} with [^{3}H]isoleucine was more efficient than labeling with [^{3}H]tyrosine and because the resulting peptide map was simpler and thus the chance of contamination of peptides was reduced. Peptide β(-P) contained approximately nine times as much [^{3}H]isoleucine (1,060 cpm) as did its phosphorylated form, peptide β (120 cpm). This suggested that this site was phosphorylated in vivo to an extent of at least 0.1 mol of phosphate per mol of polypeptide.

Efficiency of the kinase assay in the immune complex. Knowledge of how many moles of p60^{src} were present in a given immunoprecipitate allowed us to measure the efficiency of the protein kinase assay in the immune complex. Because p60^{src} is immobilized when assayed in this manner, it seemed unlikely that any given molecule could phosphorylate many heavy chains. The efficiency of the assay in the immune complex was even lower than anticipated; 0.008 to 0.09 mol of phosphate was incorporated per mol of p60^{src}. This value is similar to that which we estimated previously (12) for the efficiency of the phosphorylation of the heavy chain by p60^{src} produced by in vitro translation.

Half-life of the p60^{src} polypeptide. The apparent abundance of the p60^{src} polypeptide which we determined here by isolation of the protein from cells labeled for 48 h is significantly less than that reported by others who determined the intracellular concentration of p60^{src} with cells labeled for 2 h. The difference in the labeling interval could be the basis of this discrepancy if the half-life of p60^{src} is less than that of bulk cellular protein. We have previously shown that p60^{src} is metabolically quite stable when studied over a period of 2 h (19). To determine more precisely the half-life of this protein, we performed pulse-chase experiments using significantly longer chase periods. The half-life of p60^{src} varied somewhat from experiment to experiment. We consider the variability to be real

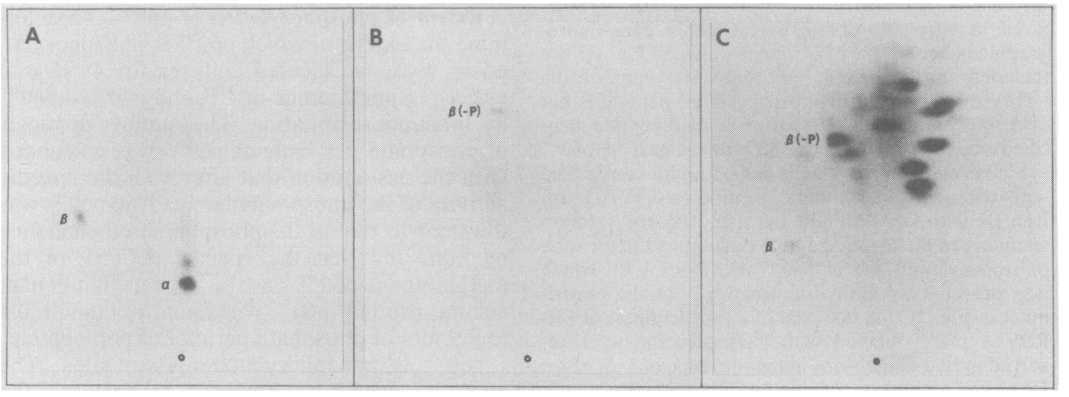

FIG. 1. Identification of the unphosphorylated form of peptide β of p60^{src}. (A) Phosphorylated tryptic peptides of p60^{src} of SR-RSV-A. A tryptic digest of p60^{src} labeled biosynthetically with $^{32}P_i$ was prepared as described in Materials and Methods, and 740 cpm of the digest was spotted on a cellulose thin-layer plate. Separation of the peptides was by electrophoresis at pH 8.9 with the anode at the left and ascending chromatography as described (11). The exposure time was 18 h. (B) Comigration of dephosphorylated peptide β with a synthetic peptide. [^{3}H]tyrosine-labeled peptide β was dephosphorylated in vitro, as described in Materials and Methods, and was mixed with a comigrating peptide from [^{3}H]tyrosine-labeled p60^{src} synthesized in vitro; 400 cpm of this mixture was analyzed as above in the presence of 5 μg of a synthetic peptide, *src* IV, which has an amino acid sequence corresponding to that deduced for peptide β (Patschinsky et al., in press). The position of *src* IV was identified by staining with ninhydrin. The fluorographic image of the dephosphorylated peptide, β(-P), is shown here. The exposure time was 28 days. The two peptides comigrated. (C) Identification of unphosphorylated peptide β. A tryptic digest of p60^{src} of SR-RSV-A labeled biosynthetically with [^{3}H]isoleucine was prepared as described in Materials and Methods; 48,160 cpm of this digest was analyzed as described above in the presence of 5 μg of the *src* IV peptide. The unphosphorylated peptide was located by staining the synthetic peptide with ninhydrin. The exposure time was 27 days. The difference in the chromatographic mobility of β(-P) in panels B and C is most probably due to differences in the thickness of the thin-layer plates. In all panels the origin is indicated (o).

and to reflect perhaps the health of the transformed cells. Additionally, p60^{src} of SR-RSV-D was noticeably more stable than p60^{src} of PR-RSV-B. p60^{src} of SR-RSV-D had a half-life which ranged from 7 to 11 h at 41°C, 7 h being a typical value (Fig. 2). The half-life of p60^{src} of PR-RSV-B was somewhat more variable and ranged from 1.8 to 3 h at 41°C (Fig. 3), 2 h being a typical value.

Metabolic stability of enzymatically active p60^{src}. The fact that only a very small fraction of the p60^{src} molecules which are present in an immunoprecipitate phosphorylate the immunoglobulin when incubated with ATP suggested the possibility that only a subpopulation of p60^{src} had protein kinase activity. If so, the half-life of 2 to 7 h which we determined for the whole population of p60^{src} by labeling with [^{35}S]methionine may not reflect that of those molecules which are enzymatically active.

To determine the half-life of those molecules of p60^{src} which have protein kinase activity, we treated cells with emetine and measured the amount of p60^{src} which was active as a protein kinase in the immune complex as a function of time after the inhibition of protein synthesis. For reasons of convenience this experiment was done by preincubating cells with emetine for various times and preparing the cell extracts from the treated and the control cultures at the same time. Like the half-life of [^{35}S]methionine-labeled p60^{src}, the half-life of the protein kinase activity of p60^{src} was variable. This variation was more pronounced than that observed with biosynthetically labeled p60^{src}. This may well be a result of toxicity of the emetine. Strikingly, however, the half-life of the protein kinase activity of p60^{src} was very much greater than that which we had determined for the whole population of the polypeptide (Fig. 4). The half-life of the kinase activity of p60^{src} of SR-RSV-D ranged from 16 to 30 h. A typical value was 19 h. The half-life of the protein kinase activity of p60^{src} of PR-RSV-B ranged from 12 to 50 h. This was very much greater than the 2-h half-life of the p60^{src} polypeptide of PR-RSV-B. The protein kinase activity of the cellular homolog of viral p60^{src}, p60$^{c\text{-}src}$, had a half-life of approximately 22 h in uninfected chick cells. Similar results were obtained with cycloheximide (data not shown).

Why are these half-life values so much greater than those determined by analysis of [^{35}S]methionine-labeled p60^{src}? Apparently, the inhibition of cellular protein synthesis with emetine inhibits the turnover of p60^{src}. When cells pulse-labeled with [^{35}S]methionine were subjected to a chase in the presence of emetine, the apparent half-life of the [^{35}S]methionine-labeled p60^{src} was increased two- to fivefold (Fig. 2). Stabilization in the presence of emetine is not unique to p60^{src}. Pr76^{gag} is also rendered more stable by the inhibition of protein synthesis with emetine.

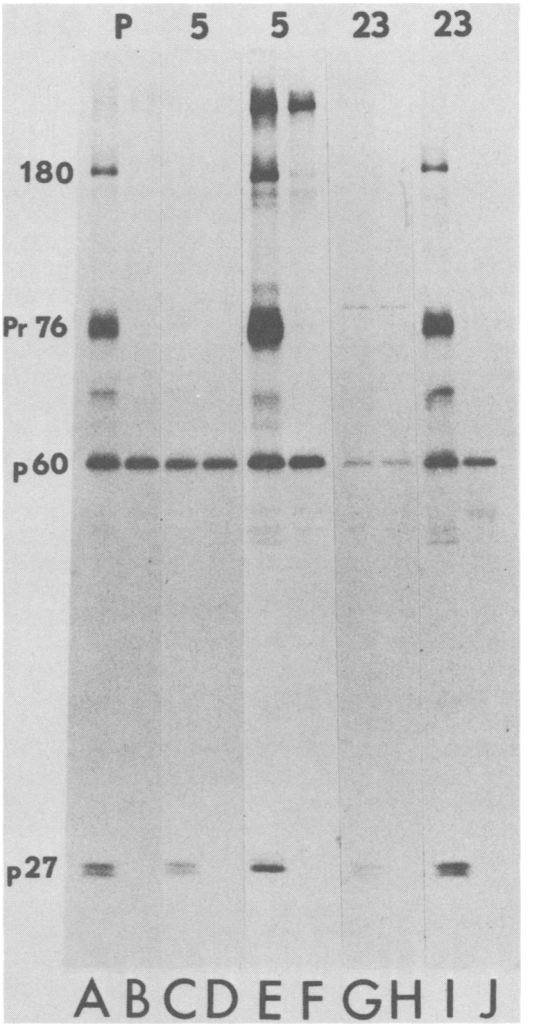

FIG. 2. Half-life of p60^{src} of SR-RSV-D. SR-RSV-D-transformed chick cells were labeled with [^{35}S]methionine for 60 min at 41°C and then were incubated for 5 h or 23 h in unlabeled medium. Immunoprecipitation was with rabbit anti-RSV tumor serum and anti-tumor serum preabsorbed with disrupted SR-RSV-D virions. Some of the cultures were chased in the presence of emetine (12.5 μg/ml). Presented here is a fluorogram of analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis. Other experimental details are described in Materials and Methods. The number 180 indicates the *gag-pol* read-through product. A: 60-min label, no chase. B: 60-min label, no chase, absorbed serum. C: 5-h chase. D: 5-h chase, absorbed serum. E: 5-h chase, emetine present. F: 5-h chase, emetine present, absorbed serum. G: 23-h chase. H: 23-h chase, absorbed serum. I: 23-h chase, emetine present. J: 23-h chase, emetine present, absorbed serum.

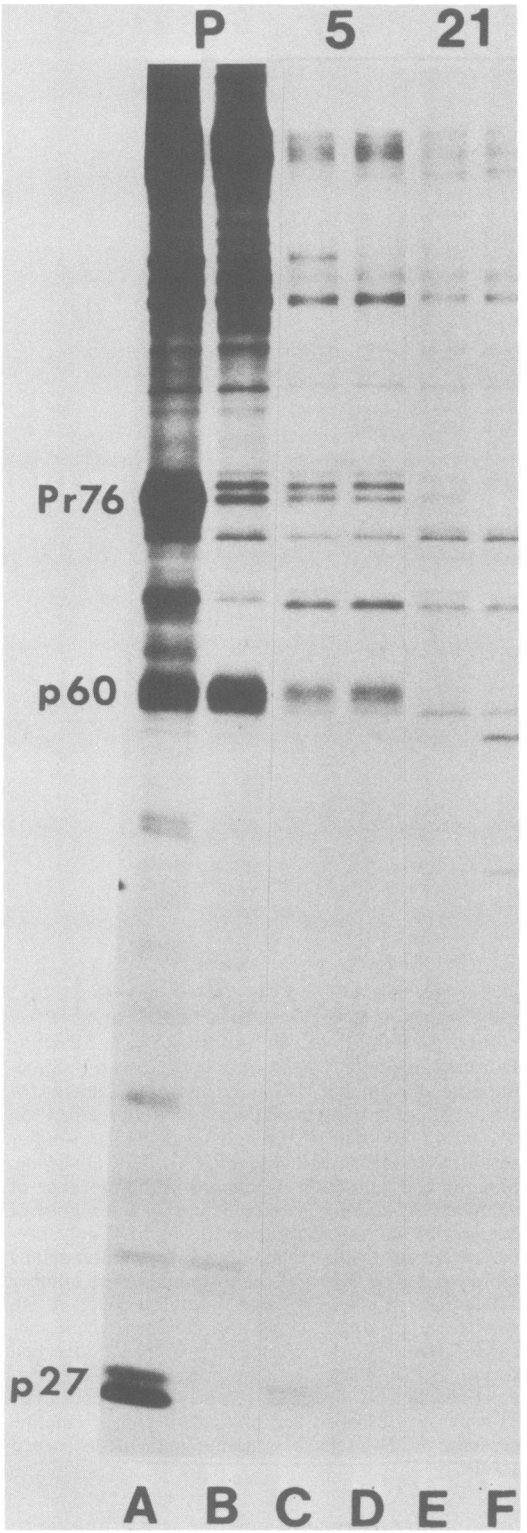

FIG. 3. Half-life of p60^{src} of PR-RSV-B. Chick cells transformed with PR-RSV-B were labeled with [^{35}S]methionine for 60 min and were then incubated for 5 or 21 h in unlabeled medium at 41°C. At each time point immunoprecipitation was done with both neat rabbit anti-RSV tumor serum and with this serum preabsorbed with disrupted SR-RSV-D virions. All other experimental details are presented in Materials and Methods. Presented here is a fluorogram of analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis. A: 60-min label, no chase. B: 60-min label, no chase, absorbed serum. C: 5-h chase. D: 5-h chase, absorbed serum. E: 21-h chase. F: 21-h chase, absorbed serum.

The long half-life of the protein kinase activity of p60^{src} in the presence of emetine is therefore probably not due to a relatively greater stability of an enzymatically active subpopulation but to the greater stability of all molecules. The half-lives of the protein kinase activities reported here must therefore be regarded as overestimates. The extent of overestimation is approximately threefold.

DISCUSSION

We have found p60^{src} to contain between 0.5 and 0.9 mol of phosphate per mol of polypeptide after extraction from chick cells transformed by SR-RSV-D and grown at 41°C. p60^{src} has two major sites of phosphorylation, a serine located somewhere near the NH_2 terminus of the protein (4) and a tyrosine located in the COOH-terminal domain of the protein (4, 11) at position 419 (23; Patschinsky et al., in press) in the numbering system of Czernilofsky et al. (7). The phosphate we have measured here represents the sum of that present at these two sites. We have found, both by peptide mapping and by partial acid hydrolysis (11), that the serine is phosphorylated to approximately twice the extent of the tyrosine. A similar ratio is also obtained when the amounts of radioactivity in the NH_2- and COOH-terminal fragments of ^{32}P-labeled p60^{src} generated by partial proteolysis with *Staphylococcus aureus* V8 protease are measured (data not shown). Although all these methods for determining the ratio of phosphoserine to phosphotyrosine in p60^{src} are subject to some drawbacks, we estimate that p60^{src} contains, on average, 0.3 to 0.6 mol of phosphoserine and 0.2 to 0.3 mol of phosphotyrosine per mol of polypeptide. Because these are aggregate values, we cannot estimate what fraction of the population of p60^{src} molecules is unphosphorylated, what fraction is phosphorylated at only a single site, and what fraction is phosphorylated at both sites.

There is necessarily some uncertainty in these values. They may be an underestimate if the specific activity of the phosphoamino acids in p60^{src} had not come to equilibrium with that of the phosphate in the labeling medium or if

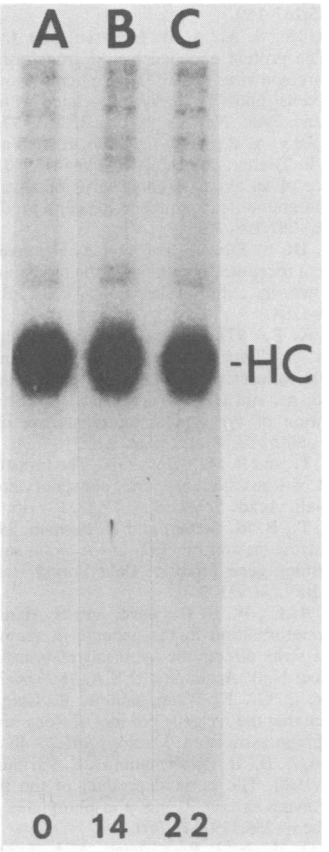

FIG. 4. Stability of the protein kinase activity of p60^{src} of PR-RSV-B. Chick cells transformed by PR-RSV-B were incubated for 0, 14, or 22 h at 41°C in the presence of emetine (5 μg/ml). The amount of p60^{src}-associated protein kinase activity remaining was measured by immunoprecipitation of p60^{src} with rabbit anti-RSV tumor serum in excess and assaying the kinase activity in the immune complex. All other experimental details are described in Materials and Methods. Presented here is an autoradiogram of an analysis of the phosphorylated products of the protein kinase reaction by SDS-polyacrylamide gel electrophoresis. HC indicates the position of the heavy chain of the immunoglobulin. A: No emetine. B: 14-h pretreatment with emetine. C: 22-h pretreatment with emetine.

cellular phosphatases were active during cell lysis. We attempted to minimize these factors by labeling the cells for 48 h and by using a phosphate-buffered RIPA buffer for cell lysis. The fact that inclusion of NaF in the extraction buffer had no effect on the recovery of ^{32}P-labeled p60^{src} (data not shown) suggests that marked enzymatic dephosphorylation was not occurring. These values will tend to be overestimates if phosphorylation occurred after cell lysis. We consider this unlikely because 2 mM EDTA was present in the lysis buffer.

The apparent extent of the phosphorylation of the tyrosine residue in p60^{src} revealed by this approach is somewhat greater than that obtained from tryptic peptide mapping. We found that 10% of the [^{3}H]isoleucine-labeled tryptic peptide which contains the site of tyrosine phosphorylation was phosphorylated. Although this approach does not involve assumptions as to the specific activity of the cellular ATP pool, it too is somewhat uncertain. The apparent extent of phosphorylation measured this way, 0.1 mol of phosphotyrosine per mol of p60^{src}, is probably a minimum estimate. Both chemical dephosphorylation of the tyrosine and simple losses of the phosphorylated peptide may have occurred during oxidation and digestion of the protein.

To estimate the extent of phosphorylation of p60^{src}, we had to label transformed cells to steady state with [^{35}S]methionine. This allowed us to calculate the abundance of the p60^{src} polypeptide in these cells. Typically, p60^{src} of SR-RSV-D constituted approximately 0.02% of total cellular protein in cultures maintained at 41°C. Using the value of 1 mg of cellular protein per 4×10^6 RSV-transformed chick cells, we were able to calculate that this abundance corresponds to approximately 500,000 molecules of p60^{src} per cell. The cellular homolog of viral p60^{src}, p60$^{c\text{-}src}$, is thought to be approximately 50- to 100-fold less abundant than the viral protein (2). This would correspond, therefore, to approximately 5,000 to 10,000 molecules of p60$^{c\text{-}src}$ per uninfected cell.

Our value for the abundance of p60^{src} is somewhat lower than that reported by others (2, 13) who used cells labeled for 2 h. We suspect that this disagreement results in part from differences in the length of labeling of the transformed cells with [^{35}S]methionine. Total cellular protein in chick cells is reported to have an average half-life of 40 to 50 h (9, 24). In contrast, p60^{src} of SR-RSV-D has a half-life of only 7 h at 41°C. p60^{src} of PR-RSV-B is even less stable. In some experiments it had a half-life of 2 h. p60^{src} is therefore noticeably less stable than the average cellular protein in chick cells and may, as a result, appear more abundant in cells labeled for a short time than in cells labeled to steady state.

Because we knew how many molecules of p60^{src} we had immunoprecipitated, we were able to calculate the turnover number of the associated tyrosine protein kinase activity when assayed in the immune complex. The turnover number was very low; 0.008 to 0.09 mol of phosphate per mol of p60^{src}. This inefficiency probably derives in part from the immobilization of the enzyme. It is also possible, however, that only a small minority of the immunoglobulin heavy chains in the immunoprecipitate are suitable substrates for p60^{src}.

We measured the half-life of the protein kinase activity of p60^{src} by incubating transformed cells in the presence of inhibitors of protein synthesis and observing the decay of the activity. The half-life of the protein kinase activity in such an experiment was three-to fourfold greater than that determined for the polypeptide by pulse-chase analysis. Although this could suggest, in theory, that a stable subpopulation is responsible for the protein kinase activity, it does not. The inhibition of protein synthesis by emetine causes a stabilization of p60^{src}. The half-life of the p60^{src} polypeptide is increased approximately threefold in the presence of emetine. This is not, however, unique to p60^{src}. Pr76^{gag} is also stabilized. Additionally, a less pronounced but similar effect of inhibitors of protein synthesis on the turnover of bulk cellular protein has been observed (8–10).

The stability of the protein kinase activity under these conditions is notable. We measured half-lives of approximately 24 h when protein synthesis was inhibited. This suggests that the protein kinase activity is quite resistant to simple thermal inactivation in whole cells and that the much more rapid rate of turnover normally is due to the activity of some cellular enzyme rather than to spontaneous denaturation of the protein. The stability of the protein kinase activity of endogenous p60$^{c\text{-}src}$ in uninfected chick cells is comparable to that of viral p60^{src} when measured in the presence of emetine. Although the half-life measured this way, approximately 20 h, is almost certainly artifactually large, it does suggest that the protein kinase activities of the viral and the cellular enzymes have similar half-lives.

ACKNOWLEDGMENTS

We thank Jon Cooper for helpful suggestions about the manuscript.

T.E. was supported by Public Health Service training grant CA 05274 from the National Cancer Institute, and T.P. was supported by a fellowship from the Deutsche Forschungsgemeinschaft. This work was supported by Public Health Service grants CA 14195, CA 17096, and CA 17289 from the National Cancer Institute.

LITERATURE CITED

1. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
2. **Collett, M. S., J. S. Brugge, and R. L. Erikson.** 1978. Characterization of a normal avian cell protein related to avian sarcoma virus transforming gene product. Cell **15**:1363–1370.
3. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021–2024.
4. **Collett, M. S., E. Erikson, and R. L. Erikson.** 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. J. Virol. **29**:770–781.
5. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus-transforming protein, pp60^{src}, shows protein kinase activity specific for tyrosine. Nature (London) **285**:167–169.
6. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus (pp60^{src}) and a homologous protein in normal cells (pp60$^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **77**:3783–3787.
7. **Czernilofsky, A. P., A. D. Levinson, H. E. Varmus, J. M. Bishop, E. Tischer, and H. M. Goodman.** 1980. Nucleotide sequence of an avian sarcoma virus oncogene (*src*) and proposed amino acid sequence for gene product. Nature (London) **287**:198–203.
8. **Epstein, D., S. Elias-Bishko, and A. Hershko.** 1975. Requirement for protein synthesis in the regulation of protein breakdown in cultured hepatoma cells. Biochemistry **14**:5199–5204.
9. **Hendil, K. B.** 1977. Intracellular protein degradation in growing, in density inhibited, and in serum-restricted fibroblast cultures. J. Cell. Physiol. **92**:353–364.
10. **Hershko, A., and G. M. Tompkins.** 1971. Studies on the degradation of tyrosine aminotransferase in hepatoma cells in culture. J. Biol. Chem. **246**:710–714.
11. **Hunter, T., and B. M. Sefton.** 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77**:1311–1315.
12. **Hunter, T., B. M. Sefton, and K. Beemon.** 1979. Studies on the structure and function of the avian sarcoma virus transforming gene product. Cold Spring Harbor Symp. Quant. Biol. **44**:931–941.
13. **Karess, R. E., W. S. Hayward, and H. Hanafusa.** 1979. Cellular information in the genome of recovered avian sarcoma virus directs the synthesis of transforming protein. Proc. Natl. Acad. Sci. U.S.A. **76**:3154–3158.
14. **Kreuger, J. G., E. Wang, and A. R. Goldberg.** 1980. Evidence that the *src* gene product of Rous sarcoma virus is membrane associated. Virology **101**:25–40.
15. **Levinson, A. D., H. Oppermann, H. E. Varmus, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. **255**:11973–11980.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Rohrschneider, L. R.** 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **77**:3514–3518.
18. **Scheidtmann, K.-H., A. Kaiser, A. Carbone, and G. Walter.** 1981. Phosphorylation of threonine in the proline-rich carboxy-terminal region of simian virus 40 large T antigen. J. Virol. **38**:59–69.
19. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vivo and in vitro. J. Virol. **28**:957–971.
20. **Sefton, B. M., T. Hunter, and K. Beemon.** 1979. Product of in vitro translation of Rous sarcoma virus *src* gene has protein kinase activity. J. Virol. **30**:311–318.
21. **Sefton, B. M., T. Hunter, and K. Beemon.** 1980. Temperature-sensitive transformation by Rous sarcoma virus and temperature-sensitive protein kinase activity. J. Virol. **33**:220–229.
22. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20**:807–816.
23. **Smart, J. E., H. Oppermann, A. P. Czernilofsky, A. F. Purchio, R. L. Erikson, and J. M. Bishop.** 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60$^{v\text{-}src}$) and its normal cellular homologue (pp60$^{c\text{-}src}$). Proc. Natl. Acad. Sci. U.S.A. **78**:6013–6017.
24. **Weber, M. J.** 1972. Ribosomal RNA turnover in contact inhibited cells. Nature (London) New Biol. **235**:58–60.
25. **Willingham, M. C., G. Jay, and I. Pastan.** 1979. Localization of the ASV *src* gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell **18**:125–134.