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Comparison of the Expression of the *src* Gene of Rous Sarcoma Virus In Vitro and In Vivo

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We have compared the polypeptide products of the *src* gene of several strains of Rous sarcoma virus produced by in vitro translation of heat-denatured 70S virion RNA in the nuclease-treated reticulocyte lysate with those present in chick cells transformed by these viruses. We have done this by immunoprecipitation, using sera from rabbits injected at birth with Schmidt-Ruppin Rous sarcoma virus. In vitro translation results in the synthesis of at least nine polypeptides which appear to be encoded by the *src* gene. These range in size from 17,000 to 60,000 daltons. The sera from tumor-bearing rabbits precipitated these polypeptides arising from the in vitro translation of RNA from Schmidt-Ruppin Rous sarcoma virus of both subgroup A and subgroup D and from one stock of Prague Rous sarcoma virus of subgroup C. In each case, all of this family of related polypeptides could be precipitated except the smallest, the 17,000-dalton polypeptide. No precipitation of analogous polypeptides resulting from the translation of RNA from other strains of Rous sarcoma virus was observed. Cells transformed by these three strains of Rous sarcoma virus contain easily detectable amounts of a polypeptide, $p60^{src}$, essentially identical to the 60,000-dalton in vitro product. With one exception, they do not contain significant amounts of polypeptides analogous to the smaller in vitro products which can be precipitated by these sera. Cells transformed by one stock of Schmidt-Ruppin Rous sarcoma virus of subgroup A did contain a 39,000-dalton polypeptide, which was related, by peptide mapping, to the 60,000-dalton polypeptide and was similar in size to a precipitable in vitro product. The 60,000-dalton polypeptide present in transformed cells appeared to be phosphorylated 10 to 25 min after its synthesis, metabolically very stable, and not derived from a precursor polypeptide. All immunoprecipitates from transformed cells which contained $p60^{src}$ also contained an 80,000-dalton phosphoprotein. This polypeptide is unrelated to $p60^{src}$, as determined by peptide mapping, and may well be a host cell polypeptide which is specifically associated with $p60^{src}$.

Both transformation of fibroblasts in tissue culture and the formation of solid tumors in birds require the function of the *src* gene of avian sarcoma viruses. It is now clear that this gene encodes a protein(s), and it is reasonable to infer that this protein(s) both initiates and maintains cellular transformation.

In vitro translation of small polyadenylated RNA molecules present in the 70S virion RNA of avian sarcoma viruses results in the synthesis of a family of related polypeptides, the largest of which is approximately 60,000 daltons in size, which are almost certainly encoded by the *src* gene. The most compelling evidence that this group of related polypeptides is produced by translation of sequences within the *src* gene is that in vitro translation of genomic RNA from viruses carrying deletions in the *src* gene does not result in the synthesis of any of these polypeptides (2, 3, 8, 9, 11, 12).

Brugge and Erikson have found that rabbits carrying tumors induced by neonatal injection of the Schmidt-Ruppin (SR) strain of Rous sarcoma virus (RSV) possess antibodies which will precipitate a 60,000-dalton polypeptide from both chick cells and mammalian cells transformed by SR-RSV (5). Purchio et al. have shown by peptide map analysis that this polypeptide is apparently identical to the 60,000-dalton in vitro translation product of the *src* gene (11). It seems very likely, therefore, that this 60,000-dalton polypeptide, $p60^{src}$, is involved in neoplastic transformation.

We have used similar antiserum from rabbits with SR-RSV-D-induced tumors to compare in some detail the expression of the *src* gene in

vitro and in vivo. In addition, we have performed a preliminary biochemical characterization of the 60,000-dalton product of the *src* gene which is present in transformed chick cells.

MATERIALS AND METHODS

Cells and viruses. Embryonated eggs were obtained from SPAFAS, Norwich, Conn., and primary cultures were prepared from them as described by Rein and Rubin (13). The SR-RSV-A used for most of the experiments here was originally from the laboratory of H. Hanafusa (The Rockefeller University). A second stock of SR-RSV-A was obtained from S. Martin, University of California, Berkeley. Two stocks of SR-RSV-D were used with indistinguishable results. One was originally from the laboratory of P. Vogt (University of Southern California), and the other was a gift from M. Linial (The Fred Hutchinson Cancer Center). Two stocks of Prague (PR)-RSV-C were used here. The one which encoded an immunoprecipitable $p60^{src}$ was originally obtained from M. Stone (Duke University). The one which did not encode an immunoprecipitable $p60^{src}$ was obtained through the Viral Oncology Program of the National Cancer Institute. The td-SR-RSV-D (a transformation-defective virus with a deletion in the *src* gene), clone 21, was obtained from M. Lai, University of Southern California.

Purification of viruses, extraction of RNA, and in vitro translation. The procedures used for the growth and purification of the viruses have been described (3). Similarly, the procedure for the extraction and isolation of 70S RNA from purified virions has been described (3). Figure 1 displays the products of translation of heat-denatured 70S RNA in the messenger-dependent rabbit reticulocyte lysate. The preparation of the lysate and the incubation conditions have been described previously (2, 3).

Preparation of antisera. Antisera were prepared essentially as described by Brugge and Erikson (5). SR-RSV-D was partially purified by sedimentation onto a cushion of 55% sucrose. Rabbits, 1.5 days old, were then injected once subcutaneously and once intramuscularly with a total of 10^6 focus-forming units of the purified virus. The titer of the virus was determined after purification. At least four of the six rabbits developed palpable tumors. All the tumors eventually regressed. The sera used here were taken 6 weeks after injection of the virus.

Labeling of cells and immunoprecipitation. Cells to be labeled were transferred to 35-mm dishes at a density of approximately 1.2×10^6 cells per dish 18 to 24 h before labeling. Incubation was at 37°C. Transformed cells were usually used as secondary cultures. Cells infected with td-SR-RSV-D were usually used as tertiary cultures. Labeling was in 0.5 to 1.0 ml of medium for intervals of 10 min to 20 h at 37°C. The labeling medium typically consisted of Dulbecco modified Eagle medium containing methionine at a concentration of 1.5 mg/liter, 4% undialyzed calf serum, and 100 to 200 μCi of [^{35}S]methionine (Amersham Corp.; >500 Ci/mmol). Phosphoproteins were labeled in phosphate-free Dulbecco modified Eagle medium containing 4% dialyzed (against phosphate-free saline) calf serum and 0.3 to 0.5 mCi of $^{32}P_i$ (ICN, carrier-free). Glycoproteins were labeled in Eagle medium containing 4% calf serum and 100 to 250 μCi of [2-^{3}H]mannose (Amersham Corp.; 2 Ci/mmol) or 100 μCi of [6-^{3}H]glucosamine (New England Nuclear; 9.8 Ci/mmol) or 100 μCi of [1-^{3}H]galactose (New England Nuclear; 12.2 Ci/mmol).

Labeled cells were washed once with cold Tris-buffered saline and then dissolved in 0.4 to 1.5 ml of cold 1% Nonidet P-40 (Particle Data Laboratories), 1% sodium deoxycholate (Calbiochem), 0.1% sodium dodecyl sulfate (SDS; BDH), 0.15 M NaCl, 0.01 M sodium phosphate (pH 7.2), and 1% Trasylol (FBA Pharmaceuticals). This buffer is referred to below as RIPA buffer and is essentially the same as that used by Brugge and Erikson (5), except that it contains sodium phosphate instead of Tris buffer. This buffer was used in all steps of the immunoprecipitation. To ensure quantitative solubilization, the cells were scraped from the dish with a rubber policeman and incubated at 4°C for at least 20 min. The lysate was clarified by centrifugation for at least 60 min at 21,000 × *g* at 4°C, and then suitable portions of each lysate were mixed with the antibody. In most experiments, 2 μl of antiserum was used.

This and all subsequent procedures were performed in the cold (0 to 4°C) in glass test tubes (10 by 75 mm). The lysate and antibody were incubated for 60 min, and then the rabbit antibody and the immune complexes were precipitated by the addition of fixed *Staphylococcus aureus* bacteria, usually 0.5 mg, in RIPA buffer. After an additional 30 min, the immune complexes were pelleted by centrifugation at 2,000 × *g* for 5 min, redissolved in 0.5 ml of RIPA buffer, and layered onto 0.5 ml of 10% sucrose in RIPA buffer in a siliconized tube. The precipitates were then pelleted by centrifugation at 2,000 × *g* for 10 min and washed three times with RIPA buffer. The final pellet was dissolved in 2% SDS, 5 mM sodium phosphate (pH 7.0), 0.1 M dithiothreitol, 5% mercaptoethanol, and 10% glycerol (usually 0.05 ml) by boiling, and the bacteria were removed by centrifugation. Normally, 10 μl of the solubilized immunoprecipitates was analyzed.

To prepare the products of in vitro translation for immunoprecipitation, the reticulocyte lysate was diluted at least 10-fold with RIPA buffer, incubated for 20 min at 4°C, and clarified by centrifugation at 21,000 × *g* for 60 min at 4°C. Incubation of the lysate with antibody and purification of the immune complexes were performed as just described.

Immunoprecipitation of polypeptides in the growth medium of transformed cells was as described above, except that the calf serum present in the medium was removed before addition of the antitumor serum. This was accomplished by the addition of a large amount of *S. aureus* bacteria, incubation at 4°C for 15 min, and centrifugation at 2,000 × *g* for 10 min at 4°C.

Absorption of the sera. Absorbed serum was prepared by incubation of the serum with disrupted purified avian sarcoma viruses. In some experiments, virus dissolved in 1% Nonidet P-40–0.15 M NaCl–0.01 M sodium phosphate (pH 7.2) was used. In others, virus dissolved in RIPA buffer was used. Absorption was for 30 min at 4°C with at least 25 μg of viral protein per μl of antiserum. The disrupted virus was not removed after the absorption.

Gel electrophoresis. SDS-polyacrylamide gel

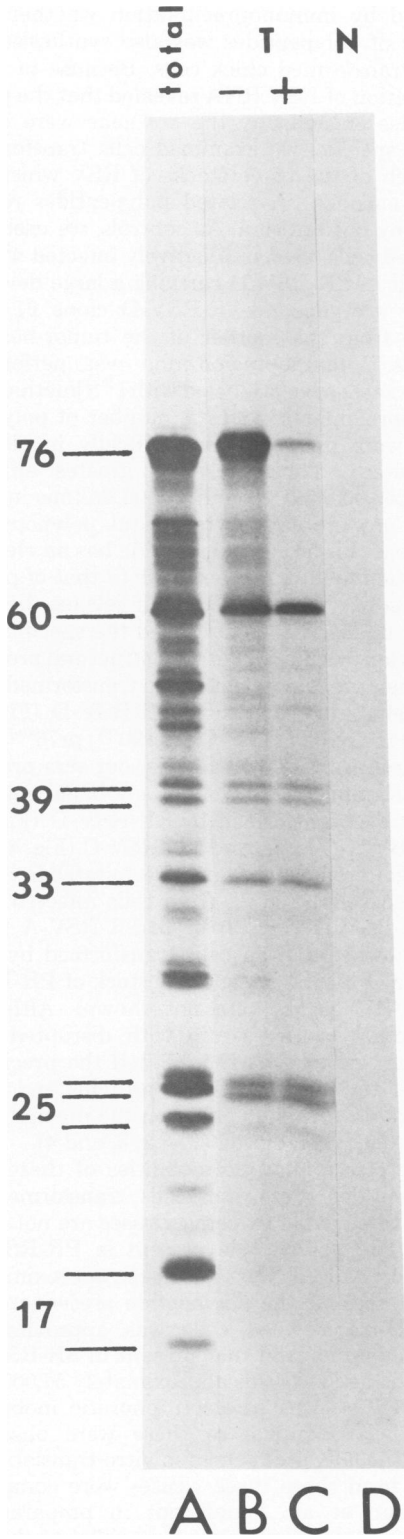

FIG. 1. *Immunoprecipitation of the products of in vitro translation of SR-RSV-D virion RNA. In vitro translation, immunoprecipitation with antitumor serum, and SDS-polyacrylamide gel electrophoresis were performed as described in the text. The gel tracks presented here are an assemblage of nonadjoining tracks from a single slab gel. The track of the unprecipitated material was exposed for a shorter period of time than were the immunoprecipitates. Electrophoresis here and in subsequent gels was from top to bottom. A, Unprecipitated in vitro products; B, polypeptides precipitated by antitumor serum; C, polypeptides precipitated by antitumor serum preabsorbed with disrupted SR-RSV-D; D, polypeptides precipitated by normal serum.*

electrophoresis was performed with slabs which were 13.5 cm long and 1 mm thick and which contained 15% acrylamide, 0.09% methylene bisacrylamide, 0.375 M Tris-hydrochloride (pH 8.8), and 0.1% SDS. The reservoir buffer contained 0.025 M Tris base, 0.192 M glycine, and 0.1% SDS. Electrophoresis was at 18 mA for 6 to 7 h. Radioactive polypeptides were detected by fluorography (10).

Tryptic peptide mapping. Elution of proteins from gels, digestion with trypsin, and two-dimensional separation of the tryptic peptides have been described in great detail previously (3). Exposure of some of the maps was accelerated by dipping the thin-layer plates in molten 2-methylnaphthalene containing 0.4% diphenyloxazole and exposure of the maps to prefogged Kodak XR-5 film at −70°C.

Lactoperoxidase catalyzed iodination. Monolayers of chick cells were labeled with ^{125}I as described before (14).

RESULTS

Immunoprecipitation of in vitro products. It has been shown, by both peptide mapping and comparison with results obtained with td-RSV, that in vitro translation of the *src* gene of RSV results in the synthesis of several related polypeptides approximately 17,000, 25,000, 33,000, 39,000, and 60,000 daltons in size (2, 3, 8, 9). To compare the polypeptide products of the *src* gene of RSV found in transformed cells with those produced by in vitro translation of virion RNA, we prepared antitumor sera as described by Brugge and Erikson (5). These sera caused the specific precipitation of a majority of the products of in vitro translation of heat-denatured 70S RNA from SR-RSV-D virions, including pr180$^{gag\text{-}pol}$, pr76^{gag}, p60^{src}, and a number of polypeptides approximately 39,000, 33,000, 29,000, and 25,000 daltons in size (Fig. 1). No precipitation of the 17,000-dalton polypeptides was observed. Results with six sera from siblings were qualitatively similar. Preabsorption of the sera with disrupted B77 virions greatly inhibited the precipitation of pr180$^{gag\text{-}pol}$, pr76^{gag}, the several polypeptides approximately 29,000 daltons in size, and a number of minor polypeptides (Fig. 1). The precipitation of p60^{src} and of the several polypeptides approximately 39,000, 33,000, and

25,000 daltons in size was unaffected by absorption of the serum (Fig. 1). Absorption of the antiserum with disrupted SR-RSV-D, td-SR-RSV-D, or PR-RSV-C virions gave essentially identical results (data not shown). Our sera therefore recognize antigenic determinants present in the 60,000-, 39,000-, 33,000-, and 25,000-dalton *src*-related polypeptides but not in the 17,000-dalton polypeptides encoded by SR-RSV-D.

We have performed similar experiments with the products of in vitro translation of heat-denatured 70S RNA from SR-RSV-A, PR-RSV-B, two separate stocks of PR-RSV-C, B77 virus, and Bryan RSV(−). Significant precipitation of the in vitro products with serum absorbed with disrupted RSV virions was observed only with SR-RSV-A and one stock of PR-RSV-C, which we have used for all of the experiments reported here. The absorbed serum caused precipitation of polypeptides approximately 60,000, 39,000, 33,000, and 25,000 daltons in size in both cases. To minimize confusion, we have chosen to refer here to all the approximately 60,000-dalton polypeptides as 60K, to all the approximately 39,000-dalton polypeptides as 39K, to all the approximately 33,000-dalton polypeptides as 33K, and to all the approximately 25,000-dalton polypeptides as 25K. The other stock of PR-RSV-C we have tested does encode a similar family of *src*-related polypeptides, but these polypeptides are not precipitable by our antisera (Beemon et al., manuscript in preparation). PR-RSV-B, B77 virus, and Bryan RSV(−) also encode a family of *src*-related polypeptides, but these too are not recognized by our antisera (Beemon et al., manuscript in preparation).

Separation of the polypeptides immunoprecipitated from the translation products of virion RNA of SR-RSV-A, SR-RSV-D, and PR-RSV-C on long SDS-polyacrylamide gels revealed that the products of the *src* gene were even more numerous than originally thought. Whereas the 60K and 33K products were indeed single polypeptides, the 39K product was a doublet and the 25K product was a triplet. It appears, therefore, that in vitro translation results in the synthesis of seven immunoprecipitable polypeptides which are encoded by the *src* gene. Kamine et al. have also observed this heterogeneity of the 25K in vitro product (9). To complicate matters further, the exact electrophoretic mobilities of most of the immunoprecipitable polypeptides are strain specific.

Immunoprecipitation of polypeptides from transformed cells. Knowing that our sera could precipitate the 60K, 39K, 33K, and 25K polypeptides synthesized in vitro, we examined by immunoprecipitation whether this family of polypeptides was also synthesized in RSV-transformed chick cells. Because in vitro translation of RSV RNA revealed that the polypeptides encoded by the *src* gene were quite strain specific, we examined cells transformed by each of the three stocks of RSV which we knew encoded *src*-related polypeptides recognized by our antisera. As controls, we used uninfected cells, cells productively infected with a mutant of SR-RSV-D carrying a large deletion in the *src* gene, td-SR-RSV-D clone 21, and serum from the mother of the tumor-bearing rabbits. Immunoprecipitation was performed with lysates of cells labeled with [^{35}S]methionine for approximately 18 h. A number of polypeptides were present non-specifically in all our precipitates. Those present in greatest amount were 250,000, 56,000, and 43,000 daltons in size. They are probably cytoskeletal polypeptides. Whereas this 56,000 polypeptide has an electrophoretic mobility quite similar to that of p60^{src}, it is resolved from p60^{src} on our gels (see below).

Our unabsorbed sera caused the specific precipitation of a number of viral structural proteins and their precursors both from transformed cells and cells infected with td-SR-RSV-D (Fig. 2). These included pr180$^{gag\text{-}pol}$, pr90^{env}, pr76^{gag}, p27, p19, and p12/p15. In addition, our sera precipitated significant amounts of a 60K polypeptide from cells transformed by SR-RSV-D (Fig. 2), SR-RSV-A (Fig. 3), and PR-RSV-C (Fig. 4). No analogous polypeptide was precipitated from uninfected cells (Fig. 2), from cells infected with td-SR-RSV-D (Fig. 2) or td-SR-RSV-A (data not shown), or from cells transformed by PR-RSV-A, PR-RSV-B, another stock of PR-RSV-C, or B77 virus (data not shown). Although absorption of the serum with disrupted SR-RSV-D virions greatly inhibited the precipitation of the viral structural proteins and their precursors, it had no effect on the precipitation of the 60K polypeptide (Fig. 2, 3, and 4).

The electrophoretic mobilities of these 60K polypeptides present in cells transformed by these three avian sarcoma viruses are not identical. The polypeptide present in PR-RSV-C-transformed cells was the largest, approximately 60,500 daltons, the polypeptide present in SR-RSV-D-transformed cells was approximately 58,000 daltons, and that present in SR-RSV-A-transformed cells was approximately 57,000 daltons. Differences in electrophoretic mobilities essentially identical to these were observed when the 60K products of in vitro translation of RNA from these three viruses were compared (Beemon et al., manuscript in preparation). However, electrophoresis in parallel of the im-

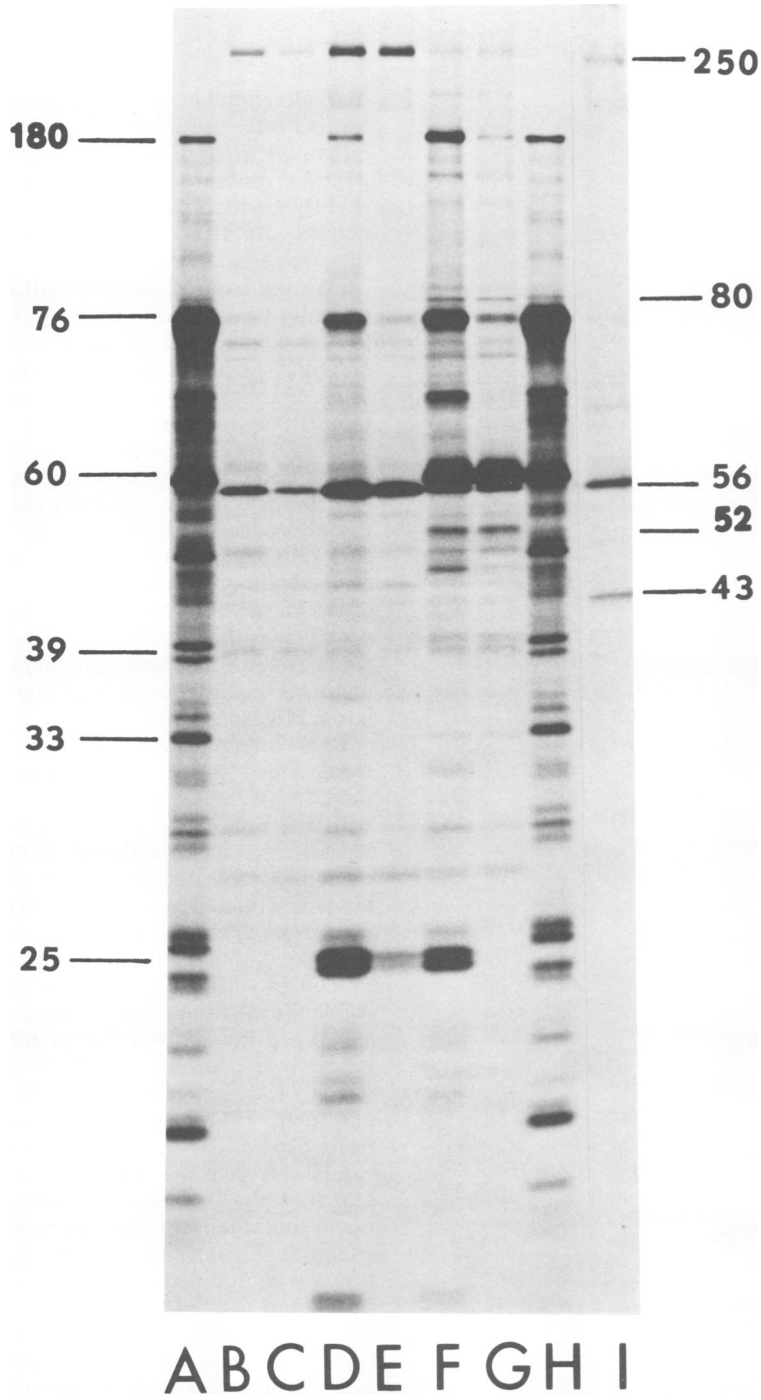

FIG. 2. *Immunoprecipitation of polypeptides present in SR-RSV-D-transformed chick cells. Uninfected chick cells, chick cells infected with td-SR-RSV-D, and chick cells transformed with SR-RSV-D were labeled for 15 h with [^{35}S]methionine. Immunoprecipitates were then prepared from these cells, using both untreated and absorbed antitumor serum and the precipitated proteins separated by SDS-polyacrylamide gel electrophoresis, as described in the text. Track I was prepared with normal serum from SR-RSV-D-transformed cells labeled with [^{35}S]methionine for 4 h. A, Unprecipitated product of the in vitro translation of SR-RSV-D virion RNA; B, uninfected cells precipitated with antitumor serum; C, uninfected cells precipitated with absorbed antitumor serum; D, td-SR-RSV-D-infected cells precipitated with antitumor serum; E, td-SR-RSV-D-infected cells precipitated with absorbed antitumor serum; F, SR-RSV-D-transformed cells precipitated with antitumor serum; G, SR-RSV-D-transformed cells precipitated with absorbed antitumor serum; H, unprecipitated products of the in vitro translation of SR-RSV-D virion RNA; I, SR-RSV-D-transformed cells precipitated with normal serum.*

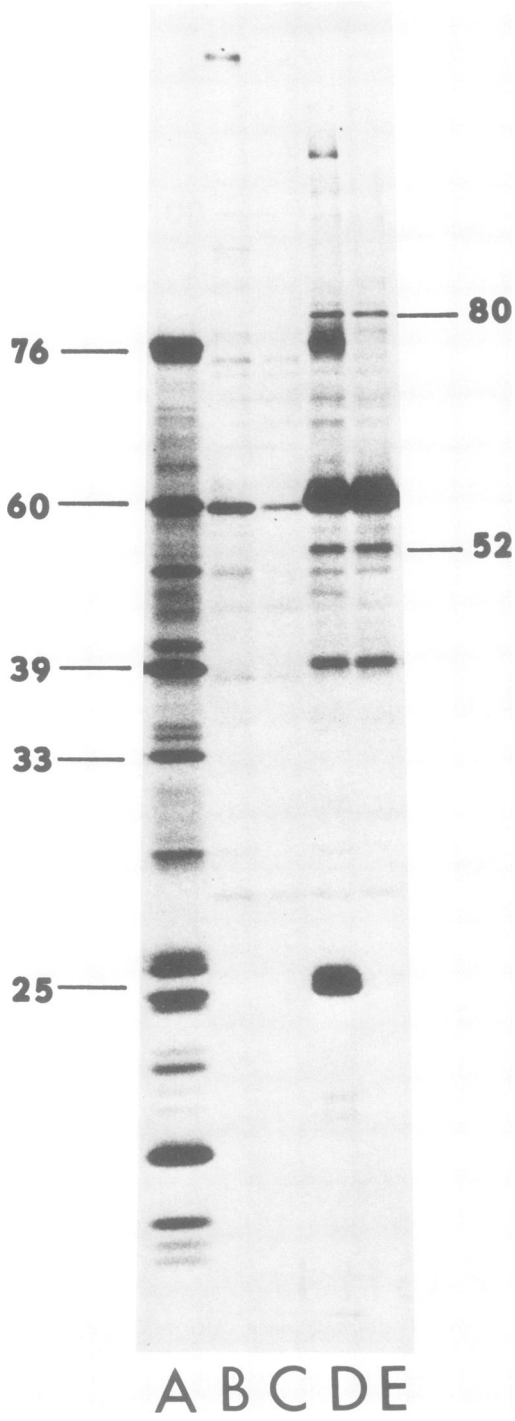

FIG. 3. *Immunoprecipitation of polypeptides present in cells transformed by SR-RSV-A. The experiment was performed exactly as described in the legend to Fig. 2. These samples are from sister cultures to those used in Fig. 2. A, Unprecipitated products of the in vitro translation of SR-RSV-A virion RNA; B, uninfected cells precipitated with antitumor serum; C, uninfected cells precipitated with absorbed antitumor serum; D, SR-RSV-A-transformed cells precipitated with antitumor serum; E, SR-RSV-A-transformed cells precipitated with absorbed antitumor serum.*

munoprecipitable 60K polypeptides from transformed cells and from in vitro translation demonstrated with all three viruses that the polypeptide present in transformed cells had a slightly greater apparent molecular weight (data not shown). This difference in apparent molecular weight was less than 500.

Comparison of the precipitated polypeptides by two-dimensional peptide mapping. We have compared by two-dimensional peptide mapping the methionine-containing tryptic peptides of these three 60K polypeptides precipitated from transformed cells with those of the three 60K polypeptides produced by in vitro translation of viral RNA. All six maps were very similar (Fig. 5). The in vitro products appeared to be essentially identical to the corresponding 60K polypeptide present in transformed cells. Some strain-specific differences among the maps of the three viruses were also apparent. It thus seems clear that these antisera recognize within transformed chick cells a polypeptide essentially identical to that arising from the in vitro translation of virion RNA.

It is becoming apparent that some virally transformed cells contain tumor-specific polypeptides which share antigenic determinants with the product of the *gag* gene of leukemia viruses (15, 17, 18). Although Brugge et al. have demonstrated that $p60^{src}$ and $pr76^{gag}$ are not obviously related to each other (4), we felt it was important to examine in more detail the question of whether $p60^{src}$ shared any amino acid sequences with the products of the *gag* gene of RSV. We therefore have compared two-dimensional peptide maps of the methionine-containing tryptic peptides of $pr76^{gag}$ synthesized in vitro by translation of genomic SR-RSV-D RNA and $p60^{src}$ obtained by immunoprecipitation from SR-RSV-D-transformed cells (Fig. 6). It can be seen that the two maps are very different and that the two proteins have none of their major methionine-containing tryptic peptides in common. This is clearly shown in a mixture of the two tryptic digests (Fig. 6), where the peptides from $p60^{src}$ are marked. Two minor peptides from $p60^{src}$ and one minor peptide from $pr76^{gag}$ were not well resolved from major peptides of the other protein. We conclude that at this level of resolution there is no relationship

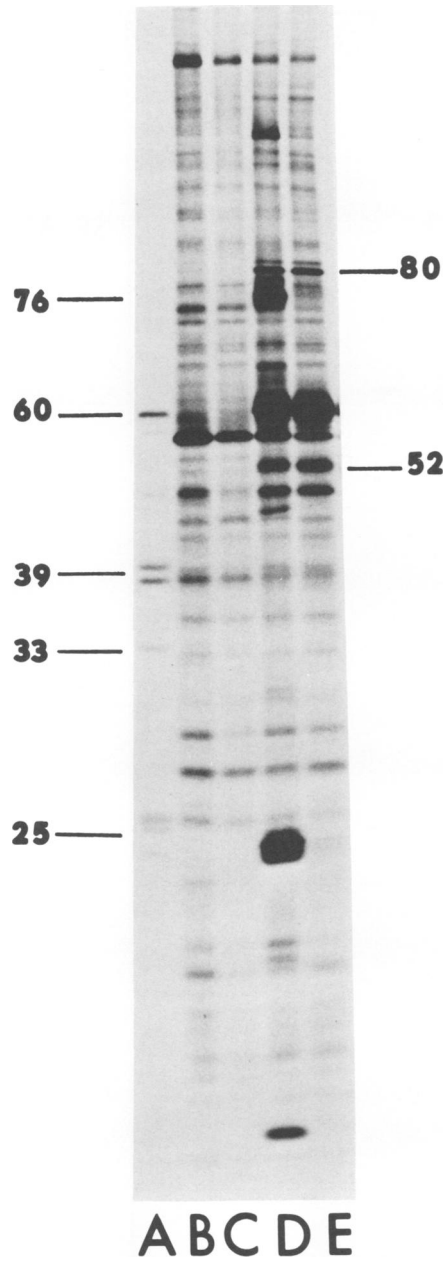

FIG. 4. *Immunoprecipitation of polypeptides present in cells transformed with PR-RSV-C. The experiment was performed as described in the legend to Fig. 2 with sister cultures to those used in Fig. 2 and 3. A, Products of the in vitro translation of PR-RSV-C RNA precipitated with absorbed antitumor serum; B, uninfected cells precipitated with antitumor serum; C, uninfected cells precipitated with absorbed antitumor serum; D, PR-RSV-C-transformed cells precipitated with antitumor serum; E, PR-RSV-C-transformed cells precipitated with absorbed antitumor serum.*

between the two proteins. In particular, the methionine-containing peptides of p19 (marked with arrows in Fig. 6) are not found in $p60^{src}$. Since p19 is at the N-terminus of $pr76^{gag}$, this rules out the possibility that $p60^{src}$ is a fusion product containing the N-terminal sequence of the *gag* gene.

All the precipitates from cells transformed by the three viruses we studied contained two other polypeptides which were specifically precipitated and which were not obviously analogous to polypeptides synthesized in vitro. The larger of these was approximately 80,000 daltons (80K) in size. We found this polypeptide in all precipitates which contained $p60^{src}$. Precipitates from cells transformed by strains of avian sarcoma virus which do not encode a cross-reacting $p60^{src}$ did not contain the 80K polypeptide. In contrast to what was observed with $p60^{src}$, the electrophoretic mobilities of the 80K polypeptides precipitated from cells transformed by SR-RSV-A, SR-RSV-D, and PR-RSV-C were identical. We have compared, by two-dimensional peptide mapping, the methionine-containing tryptic peptides of the 80K polypeptide from cells transformed by SR-RSV-A with those of $p60^{src}$ from the same cells. The two polypeptides appear to be unrelated (Fig. 7). We have not prepared tryptic peptides from the 80K polypeptides precipitated from SR-RSV-D- and PR-RSV-C-transformed cells. In vitro translation of SR-RSV-D RNA occasionally results in the synthesis of a polypeptide approximately 80K in size (Fig. 1 and 2). This polypeptide is not immunoprecipitable with our antitumor serum, does not have the same electrophoretic mobility as the 80K polypeptide precipitated from transformed cells, and is also produced by in vitro translation of td-SR-RSV-D RNA. We have not further characterized this in vitro product.

The second polypeptide present in all the precipitates from transformed cells was approximately 52,000 daltons (52K) in size (Fig. 2, 3, and 4). The electrophoretic mobility of this polypeptide was specific to the strain of virus which had transformed the cell. We have compared by two-dimensional peptide mapping the methionine-containing tryptic peptides of the 52K polypeptide from SR-RSV-D-transformed cells with those of $p60^{src}$ from the same cells (Fig. 8). The two polypeptides are clearly related. We have not similarly analyzed the 52K polypeptides from cells transformed by SR-RSV-A and PR-RSV-C.

In none of the precipitates (Fig. 2, 3, and 4) did we detect polypeptides which were analogous to the 33K and 25K *src*-related in vitro products. In SR-RSV-A-transformed cells, how-

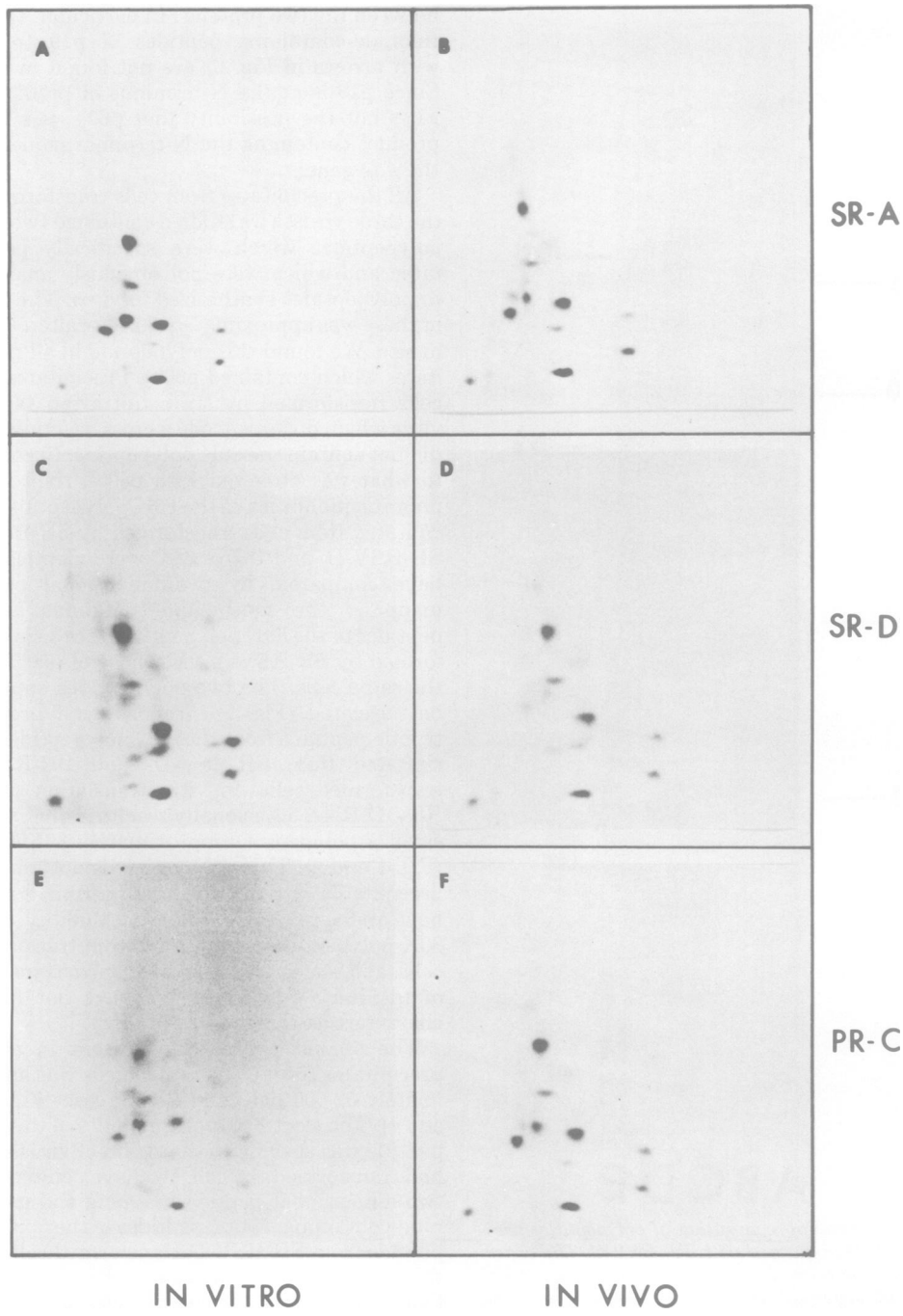

FIG. 5. *Comparison of the methionine-containing tryptic peptides of the 60K polypeptides produced by in vitro translation and the 60K polypeptides present in transformed cells. The preparation of the tryptic peptides is described in the text. Here and in the three subsequent figures, electrophoresis was from left to right, and chromatography was from bottom to top. (A) SR-RSV-A 60K in vitro product; (B) SR-RSV-A 60K precipitated from transformed cells; (C) SR-RSV-D 60K in vitro product; (D) SR-RSV-D 60K precipitated from transformed cells; (E) PR-RSV-C 60K in vitro product; (F) PR-RSV-C 60K precipitated from transformed cells.*

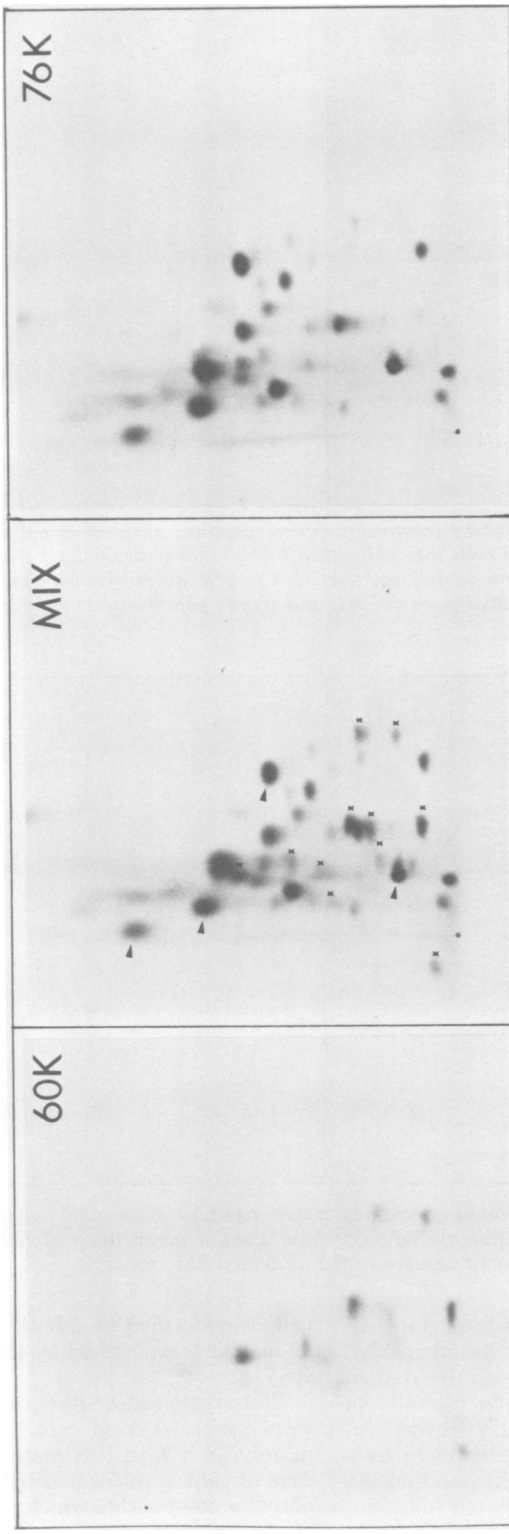

FIG. 6. *Comparison of the methionine-containing tryptic peptides of p60^{src} and pr76^{gag}. The methionine-containing tryptic peptides of p60^{src} precipitated from SR-RSV-D-transformed chick cells and of the 76K polypeptide produced by in vitro translation of SR-RSV-D virion RNA were prepared and separated as described in the text. In the map containing the mixture of the two sets of peptides, there is an x to the right of the peptides obviously derived from p60^{src}, and there are arrows indicating those peptides found in p19.*

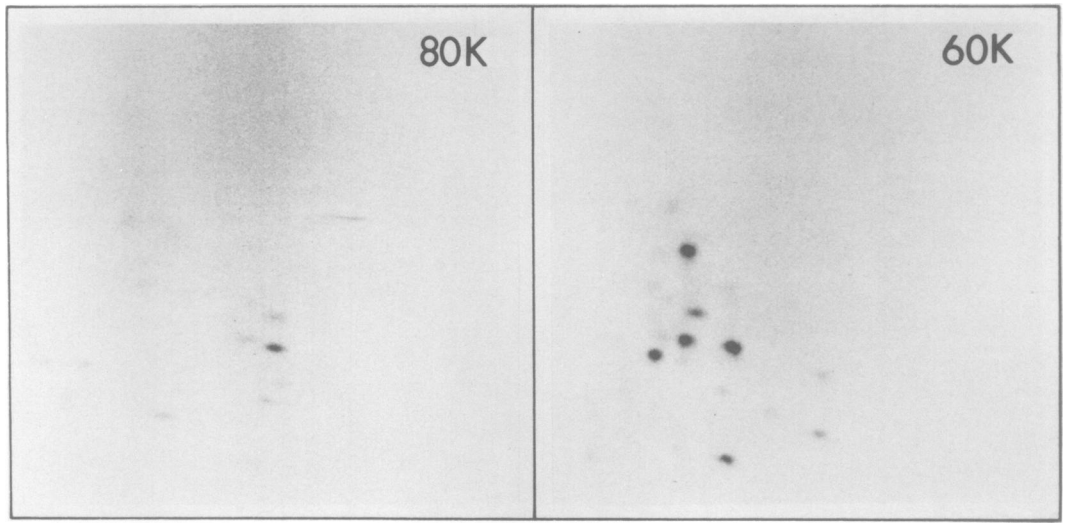

FIG. 7. *Comparison of the methionine-containing tryptic peptides of the 80K polypeptide and of p60^{src}. Numerous immunoprecipitates from cells infected with SR-RSV-A or with the temperature-sensitive mutant derived from this virus, tsNY68, were pooled and subjected to SDS-polyacrylamide gel electrophoresis. The 80K polypeptide and p60^{src} were eluted from the gel, and tryptic peptides were prepared and separated as described in the text.*

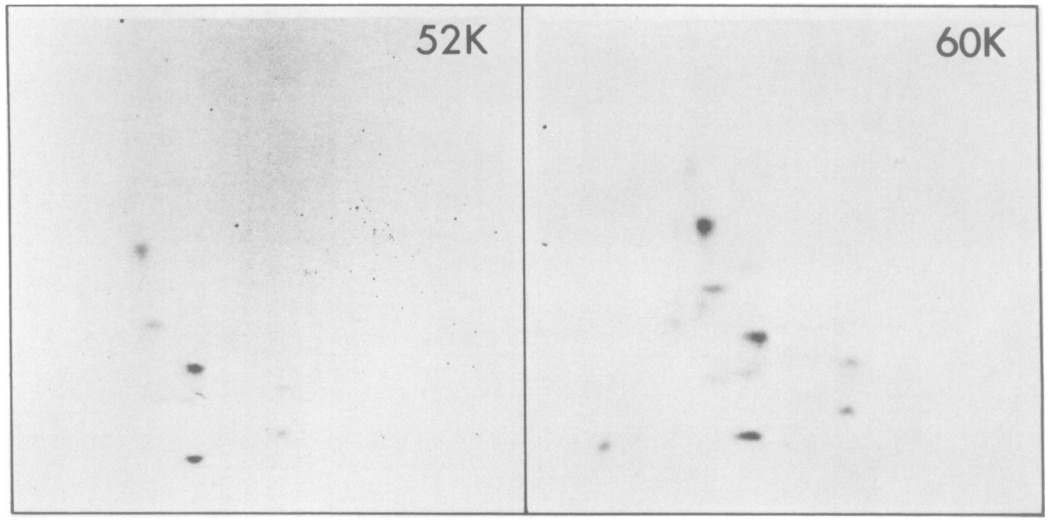

FIG. 8. *Comparison of the methionine-containing tryptic peptides of the 52K polypeptide and of p60^{src}. Tryptic peptides of the 52K polypeptide and of p60^{src} from a single preparation of methionine-labeled, SR-RSV-D-transformed cells were prepared and separated as described in the text.*

ever, we did detect a polypeptide of approximately 39K which appeared to be an in vivo analog of the 39K polypeptide synthesized in vitro (Fig. 3). The electrophoretic mobility of this polypeptide from transformed cells was similar to that of the in vitro product, and two-dimensional peptide mapping indicated that the two polypeptides were closely related (data not shown). We did not detect a 39K polypeptide in cells transformed by SR-RSV-D or PR-RSV-C or in cells transformed by another stock of SR-RSV-A.

The precipitates presented in Fig. 2, 3, and 4 were prepared from cells labeled for approximately 18 h with [^{35}S]methionine. Only in the case of cells transformed by SR-RSV-A did we detect a polypeptide which corresponded to any of the *src*-related in vitro products which are

smaller than 60K. It was possible that these smaller polypeptides were synthesized in transformed cells but were metabolically unstable and thus could not be detected after prolonged labeling. This does not appear to be the case. SR-RSV-D-transformed cells labeled for as briefly as 10 min contained no detectable 33K or 25K polypeptides (Fig. 9). Indeed, detection of even the *src*-related 52K and 39K polypeptides in transformed cells was difficult if the cells were labeled for less than 4 h.

Characterization of p60^{src} in cells transformed by SR-RSV-D. We have studied in some detail the synthesis and the post-translational modification of p60^{src} in SR-RSV-D-transformed chick cells. We knew from preliminary experiments that maximal yields of radioactive p60^{src} were obtained from cells labeled for longer than 14 h. This suggested that this polypeptide was metabolically stable. We examined this question more precisely by labeling cells for 10 min with [^{35}S]methionine and then chasing for 2 h in nonradioactive medium. Immunoprecipitates prepared at the end of the labeling period and at various intervals during the chase are displayed in Fig. 9. We used both untreated serum and serum absorbed with disrupted SR-RSV-D virions so we could distinguish p60^{src} from precursors to the virion structural proteins. Three polypeptides approximately 60K in size were precipitated from the cells labeled for 10 min. Two were present non-specifically. The smallest of these was the host cell polypeptide, which was present even in precipitates from uninfected cells (see Fig. 2). The largest of these must be related to a virus structural protein because its precipitation was prevented by absorption of the serum with disrupted SR-RSV-D virions.

The third precipitated polypeptide of approximately 60K appeared to be p60^{src}. Its precipitation was not affected by absorption of the serum with disrupted RSV virions. The electrophoretic mobility of this labeled polypeptide was not, however, exactly the same as that of p60^{src} precipitated from cells labeled for longer intervals. The pulse-labeled p60^{src} had an apparently lower molecular weight. Since mature p60^{src} is phosphorylated (4; see below also), it seems likely that this difference in electrophoretic mobility is due to the fact that the newly synthesized polypeptide is not yet phosphorylated. The apparent molecular weight of p60^{src} increased to that seen in samples from cells labeled for longer periods after a chase of 15 min and remained unchanged thereafter. The amount of label in p60^{src} during the chase increased slightly during the first 30 min and thereafter was essentially constant. We feel that much of this increase in labeled p60^{src} can be attributed to the completion of radioactive nascent chains and to the fact that the dilution of the radioactive methionine pool at the beginning of the chase was not instantaneous. There was no obvious precipitation of higher-molecular-weight precursors to p60^{src}. The fact that labeled p60^{src} can be detected after 10 min of labeling demonstrates that it does not have a precursor with an appreciable half-life. We cannot exclude the possibility of a precursor which is not reactive with our antisera, but we consider this possibility to be unlikely. The absence of an obvious precursor together with the constancy of the amount of label in p60^{src} during the 2-h chase period suggests that the polypeptide is metabolically very stable. It can be seen that p60^{src} is clearly much more stable than pr76^{gag}. It should be noted that very little labeling of the 80K and 52K polypeptides seen in Fig. 2 was observed here in a 10-min pulse.

Our pulse-labeling data suggested that p60^{src} underwent post-translational modification. We therefore examined whether it was a glycoprotein. No incorporation of radioactive mannose, glucosamine, or galactose into p60^{src} was observed under conditions where pr90^{env}/gp85 was labeled. The p60^{src} is, however, readily labeled when transformed cells are grown in the presence of $^{32}P_i$. Immunoprecipitates from uninfected cells and from cells transformed by SR-RSV-D, labeled for 15 h with $^{32}P_i$, are compared in Fig. 10. A majority of the precipitated label is present in p60^{src}. Precipitation of variable amounts of an 80K and a 50K polypeptide from the transformed cells was also observed. The 80K phosphorylated polypeptide appears to have an electrophoretic mobility identical to that of the 80K polypeptide precipitated from [^{35}S]methionine-labeled cells. The 50K phosphorylated polypeptide had a lower apparent molecular weight than the 52K methionine-labeled polypeptide.

We have examined whether p60^{src} is present on the cell surface by lactoperoxidase-catalyzed iodination of cells transformed by SR-RSV-D. We did not detect any ^{125}I-labeled p60^{src} under conditions where significant iodination of cell surface proteins occurred. We also examined whether p60^{src} was released into the medium of cells transformed by SR-RSV-D. Immunoprecipitation of the medium of SR-RSV-D-transformed chick cells labeled with [^{35}S]methionine failed to reveal significant amounts of immunoprecipitable p60^{src}.

DISCUSSION

Translation of genomic RNA from RSV in the nuclease-treated reticulocyte lysate results in the synthesis of a family of at least nine related

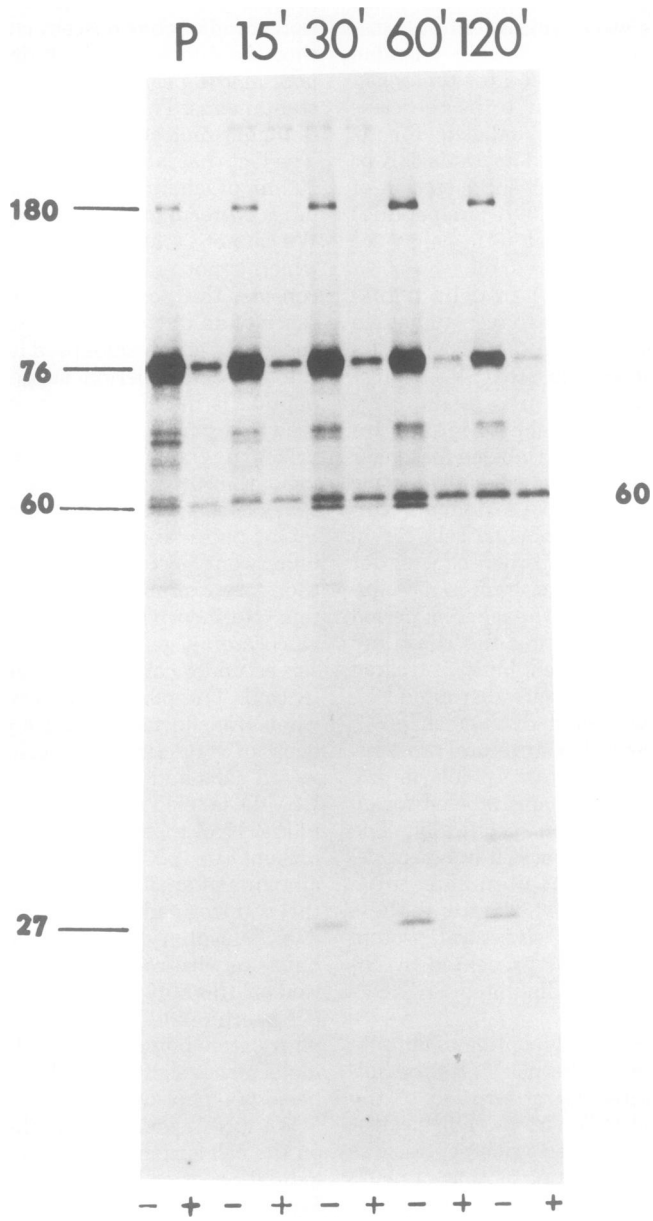

FIG. 9. *Immunoprecipitation of SR-RSV-D-transformed cells labeled for 10 min and chased for 2 h. SR-RSV-D-transformed cells were labeled with [^{35}S]methionine (1.2 mCi/ml in methionine-free Dulbecco modified Eagle medium supplemented with 0.5% calf serum) for 10 min. The cells were then washed three times with, and then incubated for the indicated intervals in, Dulbecco modified Eagle medium supplemented with 5% tryptose phosphate broth and 5% calf serum. Immunoprecipitation was performed with both untreated and absorbed antitumor serum. Other details of the experiment were as described in the text. Two samples from each time point were subjected to SDS-polyacrylamide gel electrophoresis. Those labeled (−) were prepared with untreated antitumor serum; those labeled (+) were prepared wi th absorbed antitumor serum. The two tracks labeled P are from cells labeled for 10 min. The other tracks contain samples which were prepared from cells labeled for 10 min and then chased for the indicated intervals.*

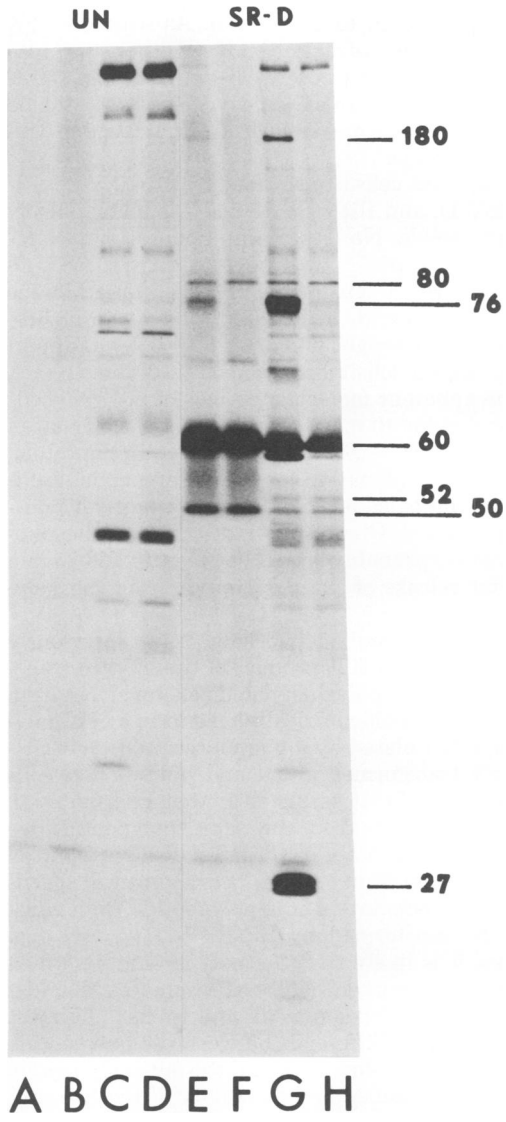

FIG. 10. *Immunoprecipitation of phosphoproteins from cells transformed by SR-RSV-D. Uninfected chick cells and chick cells transformed by SR-RSV-D were labeled for 17 h with either [^{35}S]methionine or $^{32}P_i$ and then immunoprecipitates were prepared from the cells. The details of this experiment are described in the text. The precipitated polypeptides were separated by SDS-polyacrylamide gel electrophoresis. A, Uninfected cells labeled with $^{32}P_i$ and precipitated with antitumor serum; B, uninfected cells labeled with $^{32}P_i$ and precipitated with absorbed antitumor serum; C, uninfected cells labeled with [^{35}S]methionine and precipitated with antitumor serum; D, uninfected cells labeled with [^{35}S]methionine and precipitated with absorbed antitumor serum; E, SR-RSV-D-transformed cells labeled with $^{32}P_i$ and precipitated with antitumor serum; F, SR-RSV-D-transformed cells labeled with $^{32}P_i$ and precipitated with absorbed antitumor serum; G, SR-RSV-D-transformed cells labeled with [^{35}S]methionine and precipitated with antitumor serum; H, SR-RSV-D-transformed cells labeled with [^{35}S]methionine and precipitated with absorbed antitumor serum.*

polypeptides approximately 60K, 39K, 33K, 25K, and 17K in size, which are encoded by the *src* gene of the virus (2, 3, 8, 9, 11, 12). Antiserum from rabbits carrying a tumor induced by SR-RSV-D will precipitate all of these related polypeptides encoded by SR-RSV-A, SR-RSV-D, and PR-RSV-C except the smallest, the 17K doublet. Using such sera, we have found that cells transformed by these three strains of RSV contain large amounts of a polypeptide essentially identical to the 60K in vitro product and variable amounts of a polypeptide analogous to the 39K in vitro product. They contain no polypeptides which resemble the 33K and 25K in vitro products. Thus, translation of phenol-extracted, heat-denatured RSV virion RNA in the nuclease-treated reticulocyte lysate reveals significant mRNA activities which do not appear to be present in transformed chick cells.

The absence of the 33K and 25K polypeptides in RSV-transformed cells is probably due to the fact that they are not synthesized at a detectable rate rather than to the fact that they are metabolically unstable. We failed to detect these polypeptides, even in cells labeled with [^{35}S]methionine for 10 min. We did detect a polypeptide which closely resembles the 39K product of the translation of SR-RSV-A RNA in cells transformed by this virus. An analogous polypeptide was not seen in cells transformed by SR-RSV-D or PR-RSV-C. This 39K polypeptide is most prominent in precipitates from cells labeled with [^{35}S]methionine for longer than 4 h. This property could be due to a low level of synthesis of a metabolically stable 39K polypeptide or to slow proteolytic processing of the 60K polypeptide. We cannot distinguish between these possibilities, but because virions contain messenger activity for this polypeptide, it seems likely that transformed cells do also.

Different strains of RSV encode biochemically (3), immunologically (11), and biologically (1, 16) distinguishable *src* gene products. We have compared the expression of the *src* genes of SR-RSV-D, SR-RSV-A, and PR-RSV-C, both in vitro and in vivo, and have found several significant differences between these viruses (Beemon et al., manuscript in preparation). Most striking are the strain-specific differences in the sizes of the polypeptides encoded by the *src* genes of these three viruses. The 60K polypeptides derived from either in vitro translation or cells

transformed by SR-RSV-A, SR-RSV-D, and PR-RSV-C are clearly distinguishable by SDS-polyacrylamide gel electrophoresis. The 60K polypeptide from PR-RSV-C is approximately 60,500 daltons, that of SR-RSV-D approximately 58,000 daltons, and that from SR-RSV-A approximately 57,000 daltons in size. Side-by-side comparison of each of the 60K in vitro products with the 60K polypeptide from cells transformed by that strain of virus indicates that each of the polypeptides from transformed cells is very slightly larger than that synthesized in vitro. This difference may be due to phosphorylation. One other difference between these three strains of viruses is that cells transformed by one stock of SR-RSV-A contain significant amounts of a polypeptide which appears to be similar to the 39K in vitro product. Cells transformed by SR-RSV-D and PR-RSV-C do not contain an analogous polypeptide.

Two other polypeptides were specifically found in immunoprecipitates from cells transformed by SR-RSV-A, SR-RSV-D, and PR-RSV-C. The largest of these, which is approximately 80K in size, was not precipitated from uninfected cells, cells infected with transformation-defective mutants of SR-RSV-A or SR-RSV-D, or cells transformed by any avian sarcoma virus which encodes a $p60^{src}$ which is not recognized by our antisera. In contrast to $p60^{src}$, the electrophoretic mobility of the 80K polypeptide was identical in precipitates from cells transformed by SR-RSV-A, SR-RSV-D, and PR-RSV-C. Two-dimensional tryptic peptide mapping demonstrated that this polypeptide was not related to the 60K polypeptide. These observations lead us to believe that this polypeptide is in all probability a host cell protein and that it is present in our precipitates because it is stably associated with a polypeptide recognized by our antisera and not because it, itself, is recognized by our antisera. It is probably associated with $p60^{src}$. We do not know the nature or the function of this association.

The other polypeptide specific to precipitates from cells transformed by SR-RSV-A, SR-RSV-D, and PR-RSV-C is approximately 52K in size. The electrophoretic mobility of this polypeptide was different in the precipitates from cells transformed by each of the three different viruses we examined. There is no polypeptide synthesized in vitro which is obviously analogous to this peptide. Two-dimensional tryptic peptide mapping demonstrated that the 52K polypeptide present in SR-RSV-D-transformed cells was related to $p60^{src}$ present in these cells. Like the 39K polypeptide seen in SR-RSV-A-transformed cells, the 52K polypeptide was most prominent in cells labeled with [^{35}S]methionine for periods in excess of 4 h. As with the 39K polypeptide, we do not know whether this property is due to a low level of synthesis of a stable polypeptide or to slow cleavage of $p60^{src}$. We do not know the physiological significance of this polypeptide.

In chick cells transformed by SR-RSV-A, SR-RSV-D, and PR-RSV-C, $p60^{src}$ is metabolically very stable. No loss of immunoprecipitable radioactive $p60^{src}$ was obvious in SR-RSV-D-transformed cells labeled with [^{35}S]methionine for 10 min and then chased for 2 h. No higher-molecular-weight precursor to $p60^{src}$ was apparent in the cells labeled for only 10 min. In fact, the apparent molecular weight of $p60^{src}$ in cells labeled for 10 min was slightly less than that of mature $p60^{src}$. Since $p60^{src}$ is a phosphoprotein, a likely explanation for the difference in size is that the pulse-labeled material is not yet phosphorylated. Our results indicate that phosphorylation probably occurs between 10 and 25 min after release of the polypeptide from the polysome.

Erikson and co-workers have previously shown that a 60K product of the in vitro translation of 21S polyadenylated genomic RNA from SR-RSV is biochemically related to a 60K polypeptide isolated by immunoprecipitation of SR-RSV-transformed chick and mammalian cells (4, 5, 11, 12). It seems clear that Erikson et al. have characterized the same polypeptide we have. On the other hand, Jay et al. have reported that serum from a tumor-bearing rat will specifically precipitate a 56K polypeptide from chick cells transformed by SR-RSV-D (7). We feel that it is likely that the polypeptide described by these workers is not the same as that described by Erikson et al. and by us. The most pronounced difference between the polypeptide described by Jay et al. and the 60K polypeptide we have studied is the stability of the two proteins within transformed cells. The 56K polypeptide has a half-life of approximately 15 min; the 60K polypeptide has a half-life much in excess of 2 h. Another possible difference between these two polypeptides is their behavior during two-dimensional polyacrylamide gel electrophoresis. We have been unable to find conditions under which the 60K polypeptide behaves as a discrete species during isoelectric focusing. Jay et al., however, have demonstrated that the 56K polypeptide has an isoelectric point of 6.3. We unfortunately are not sure whether this difference is technical or is, in fact, due to differences between the two polypeptides.

Results of preliminary experiments directed at localizing $p60^{src}$ within transformed cells have yielded largely negative results. Immunofluorescence gave no evidence of a nuclear location,

lactoperoxidase-catalyzed iodination gave no evidence of a location on the cell surface, and immunoprecipitation failed to demonstrate that $p60^{src}$ was secreted by the cells. Collett and Erikson have demonstrated that immunoprecipitates from chick cells and mammalian cells transformed by SR-RSV contain a protein kinase activity (6). We have since shown that immunoprecipitates which contain the 60K in vitro product of the *src* gene of RSV also contain a protein kinase activity. It seems clear, therefore, that a protein kinase activity is specifically associated with precipitates which contain $p60^{src}$. Of importance now is the localization and identification of the substrates of $p60^{src}$.

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