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Identification of a polypeptide encoded by the avian sarcoma virus *src* gene

(cell-free protein synthesis/translation of viral RNA/immunoprecipitation/peptide fingerprints)

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ABSTRACT **Two techniques were used to search for the polypeptide encoded by the avian sarcoma virus (ASV) *src* gene. First, antiserum from rabbits bearing ASV-induced fibrosarcomas was used to immunoprecipitate a transformation-specific antigen from ASV-transformed chick embryo fibroblasts. This antigen has an apparent molecular weight (M_r) of 60,000. Second, the 3′ one-third of the ASV genome, selected by oligo(dT)-cellulose chromatography and sucrose gradient sedimentation, was translated in a mRNA-dependent reticulocyte cell-free lysate. This RNA species programmed the synthesis of a polypeptide that comigrated with the transformation-specific antigen of M_r 60,000 immunoprecipitated from transformed cells. The methionine-containing tryptic peptides from the polypeptides of M_r 60,000 obtained from translation *in vitro* and from immunoprecipitation were found to be identical upon two-dimensional fractionation.**

Some RNA-containing tumor viruses are able to cause rapid and efficient transformation of cells in culture as well as the production of sarcomas in animals. The avian sarcoma viruses (ASV) are particularly interesting because they produce tumors in both avian and mammalian species (1, 2). The high efficiency of cell transformation by these viruses, the availability of viral mutants, and the considerable structural information about the viral genome make ASV useful for the study of the molecular events that occur during viral oncogenesis.

Although analyses of viral mutants indicate that a polypeptide encoded by the *src* gene of ASV is likely to be responsible for the maintenance of cell transformation, such a polypeptide had not been identified biochemically or immunologically when we began our studies. Several ASV conditional, temperature-sensitive (ts) mutants, and nonconditional, deletion mutants, termed transformation-defective (td), have been isolated that have an altered capacity to transform cells (3–5). Studies with ts mutant-infected cells indicate that a virus-encoded product affects gross cytoskeletal structure, growth in soft agar, and rate of sugar uptake, characteristics that are phenotypic markers of transformation in culture. Avian or mammalian cells infected with a ts mutant can be reversibly switched to either the transformed or "normal" state within a few hours after the appropriate temperature shift. Inhibitors of protein synthesis do not affect the conversion from the transformed to normal state upon shift to the nonpermissive temperature, but do interfere with the appearance of the transformed phenotype when cultures are shifted from the nonpermissive to permissive temperature. These virus mutants can replicate normally at both temperatures, suggesting that a virus-encoded, heat-labile protein, which is not an essential structural component of the virus, is responsible for cell transformation (6).

All ts transformation mutations occur in a region of the ASV genome that is deleted in td mutants (7). Gel electrophoresis and oligonucleotide mapping indicate that about 20% of the ASV genome is deleted in td mutants (8–10). Although the size of the deletion varies in different mutants, in general it is about 2,000 nucleotides in length, extending from approximately 800 to 3,000 nucleotides from the 3′ end of a nondefective (nd) genome (11).

The *src* gene is transcribed *in vivo* into a mRNA representing the 3′ one-third of the viral genome. Indeed, in ASV-transformed mammalian cells, a 21S polyadenylated RNA is the only ASV-specific mRNA easily detected (12). The RNA sequences in the nd 21S virus-specific message contain enough information to be translated into a protein of molecular weight (M_r) 60,000–70,000.

Because the molecular events leading to transformation are not understood and because there are many levels of control that could be influenced by the *src* product, it is difficult *a priori* to predict its function. Consequently, we have attempted to identify the ASV *src* gene product assuming nothing about its mechanism of action. Two approaches have been used in our search, the translation *in vitro* of the region of the ASV genome that encompasses the *src* gene using the mRNA-dependent reticulocyte lysate system (13), and use of the strategy successful in identification of simian virus 40, polyoma, and adenovirus T antigens, namely, that animals that carry tumors induced by those viruses produce antibody to nonstructural virus-encoded polypeptides (14–18).

In this report we show that a transformation-specific polypeptide synthesized in cell-free extracts programmed by the 3′ one-third of a nd ASV genome and a transformation-specific antigen immunoprecipitated from ASV-transformed chicken cells have the same apparent molecular weight (M_r 60,000) and identical methionine-containing tryptic peptides. These results are consistent with the interpretation that this polypeptide is the ASV *src* gene product.

MATERIALS AND METHODS

Cells and Virus. Chicken embryo fibroblasts were prepared from 11-day-old embryos (Spafas, Inc., Roanoke, IL). The Schmidt–Ruppin (SR) strain of ASV subgroup D was obtained from John Wyke and td SR-ASV was provided by H. Hanafusa.

Preparation of Antiserum and Immunoprecipitation. Tumors were induced in newborn New Zealand rabbits by the

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Abbreviations: ASV, avian sarcoma virus; *src*, designation for an ASV gene responsible for transformation of fibroblasts; ts, temperature-sensitive; td, transformation defective; nd, nondefective; M_r, molecular weight; SR, Schmidt–Ruppin strain of ASV, subgroup D; $NaDodSO_4$, sodium dodecyl sulfate; PrC, Prague strain of ASV, subgroup C.

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subcutaneous inoculation of approximately 10^8 focus-forming units of purified SR-ASV (subgroup D). Within 10–14 days, palpable fibrosarcomas appeared in all animals injected. The serum used in the experiments reported here was obtained 5 weeks after inoculation, and is referred to as TBR serum. TBR serum often contains antibody to all the virion structural proteins (19) as well as to antigens found only in transformed cells (20).

Chick embryo fibroblasts were infected with nd or td SR-ASV and the infected cells were subcultured twice. At this time, more than 80% of the cells infected with nd virus appeared morphologically transformed. Uninfected and nd and td SR-ASV-infected cells were seeded at a density of 8×10^6 cells/100-mm dish and labeled the next day for 3 hr with 20 μCi of [^{35}S]methionine per ml (Amersham, 600 Ci/mmol) in Eagle's medium lacking methionine and supplemented with 5% calf serum (Colorado Serum Co.). Lysates were prepared and immunoprecipitation was carried out as described (20) with the use of the protein A-containing bacterium *Staphylococcus aureus*, strain Cowan I, to adsorb the immune complexes (21). Incubation of lysates of uninfected chicken cells with normal or TBR serum and lysates of td or nd SR-ASV-infected chicken cells with normal serum resulted in the precipitation of 0.02–0.04% of the total ^{35}S label. The percentage of immunoprecipitable counts found with TBR serum and infected cells was 0.05–0.20%. This was reduced to close to background levels when the TBR serum was blocked with disrupted virus.

Preparation of RNA and Cell-Free Protein Synthesis. Large volumes of supernatant were prepared from roller bottles of infected chicken embryo fibroblasts, and virus was pelleted by centrifugation in a Beckman no. 19 rotor at 17,000 rpm for 2.5 hr. The pellet was resuspended and the virus was banded in a sucrose gradient (10–70% wt/vol) by centrifugation for 14 hr at 25,000 rpm in a Beckman SW41 rotor. The RNA was extracted from the banded virus and fractionated by sucrose gradient centrifugation as described (22).

Virion 70S RNA was heated at 80° for 3 min in 0.2% sodium dodecyl sulfate ($NaDodSO_4$) to dissociate the complex, and quick-chilled. The poly(A)-containing RNA was selected by oligo(dT)-cellulose chromatography, precipitated, heated again, and fractionated by sucrose gradient sedimentation as described (22). Fractions of 0.2 ml were collected by bottom puncture. The RNA from each fraction was precipitated with ethanol and redissolved; the absorbance at 260 nm was determined, and the RNA was reprecipitated. For small amounts of RNA, 5 μg of wheat germ tRNA per 0.5 μg of poly(A)-containing RNA was added prior to the second ethanol precipitation. The RNA was heated prior to each fractionation step in order to disrupt any possible aggregates. For translation experiments, the RNA pellet was washed once with ethanol, the tube was dried under reduced pressure, and the RNA was dissolved in water.

The viral RNA prepared in this manner contains subgenome-sized fragments presumably generated from 35S RNA by the action of nuclease(s) resident in the virion (23). In order to prepare subgenomic RNA *in vitro* from 35S molecules, the following procedure was carried out. Virion poly(A)-containing RNA was prepared as described above and that which sedimented at 35S was precipitated with ethanol. This RNA was subjected to two more cycles of heating and sedimentation and 35S RNA was selected a third time (fractions 1–8, Fig. 1). This RNA was digested with T1 ribonuclease (Calbiochem) for 5 min at 25 ° in 0.15 M NaCl/0.05 M Tris·HCl, pH 7.4/1 mM EDTA at a RNA concentration of 10 μg/ml and a RNA:enzyme ratio of 10^4:1. $NaDodSO_4$ was added to 2%; the RNA was extracted three times with phenol, precipitated with ethanol, and heated

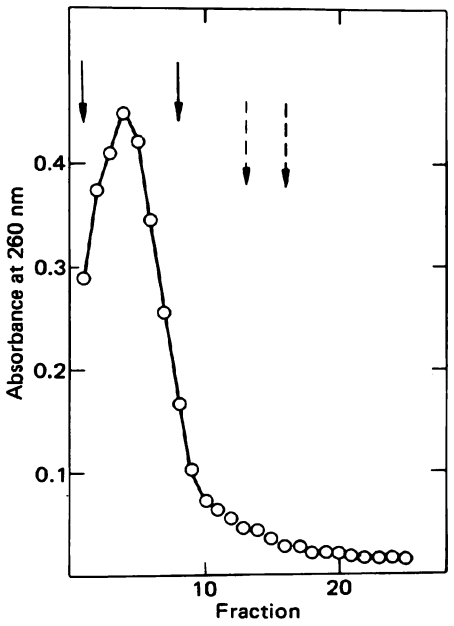

FIG. 1. Sedimentation profile of virion 35S poly(A)-containing RNA selected for degradation by RNase T1. The RNA previously selected from two cycles of heating and sedimentation was dissolved in 10 mM Tris, pH 7.2/1 mM EDTA, heated at 80° for 2 min, quick-chilled, and sedimented at 10° through a 5–20% sucrose gradient containing 0.1 M NaCl for 2 hr at 45,000 rpm in a Beckman SW50.1 rotor. The RNA in fractions 1–8 (solid arrows) was precipitated and degraded with RNase T1. The dashed arrows indicate the fractions of the subsequent gradient from which the degraded RNA 21 S in size was precipitated. This region of the gradient contained 20% of the total RNA recovered from the gradient. Twenty-five percent of the total RNA resedimented in the 35S region.

at 80° for 3 min; and the poly(A)-containing fragments were selected by chromatography on oligo(dT)-cellulose as described above. The resultant RNA fragments were again heated, fractionated by sucrose gradient sedimentation, and prepared for translation as described above.

Reticulocyte lysates were made mRNA-dependent as described (13). Translation reactions were carried out as described (22) with [^{35}S]methionine, except that 0.5 μg of mRNA was added to each 50-μl reaction mixture.

Analysis of Polypeptides. Samples were analyzed by electrophoresis through a discontinuous slab gel system with the buffer systems described by Laemmli (24). Gels were stained and destained as described (25) and dried onto Whatman 3 MM paper, or prepared for fluorography (26) and dried. Radioactivity was located with Du Pont Cronex 4 medical x-ray film, or with Kodak X-Omat R film for fluorography. To locate the polypeptides in preparative gels, we dried a small strip cut from the edge of the gel after it had been destained and exposed it to film, while the remainder of the gel was stored in water at 4°.

For tryptic fingerprinting, the pertinent regions were cut from preparative gels and the polypeptides were eluted, precipitated with trichloroacetic acid after the addition of 100 μg of bovine serum albumin, and oxidized with performic acid (27). After two lyophilizations, the polypeptides were digested with trypsin and the peptides were separated by ascending chromatography followed by electrophoresis as described (25).

RESULTS

Detection of a Transformation-Specific Polypeptide. The [^{35}S]methionine-labeled antigens immunoprecipitated from

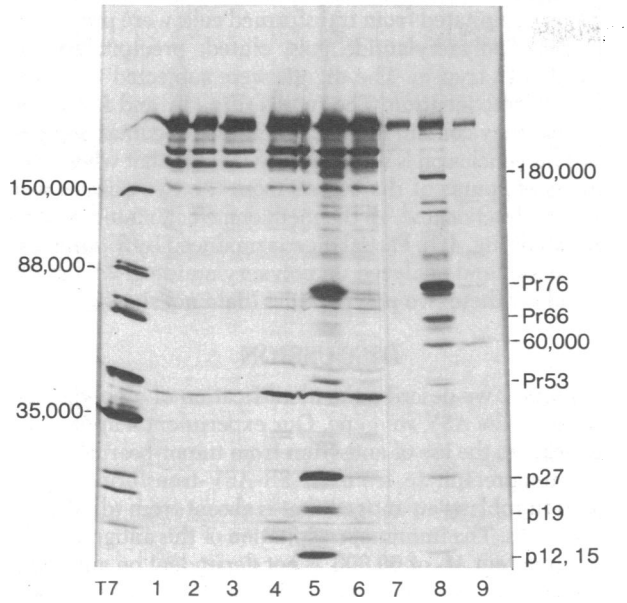

FIG. 2. Autoradiogram of NaDodSO$_4$/polyacrylamide gel analysis of proteins immunoprecipitated from uninfected and td and nd SR-ASV-infected chicken cells. Tracks 1, 2, and 3: normal cells and (1) normal serum, (2) TBR serum, and (3) TBR serum-blocked. Tracks 4, 5, and 6: td SR-ASV-infected cells and (4) normal serum, (5) TBR serum, and (6) TBR serum-blocked. Tracks 7, 8, and 9: nd SR-ASV-infected cells and (7) normal serum, (8) TBR serum, and (9) TBR serum-blocked. For the blocking reactions, the serum was incubated with 125 μg of purified disrupted nd SR-ASV for 20 min before addition of the cellular extract. T7 proteins were calibrated against *Escherichia coli* RNA polymerase, ovalbumin, bovine serum albumin, and trypsin. Labeling and immunoprecipitation were carried out in parallel; however, a longer exposure is shown here for tracks 1–6 in order to increase the possibility of detecting the presence of lower levels of 60,000 M_r polypeptide in the uninfected and td-infected cells.

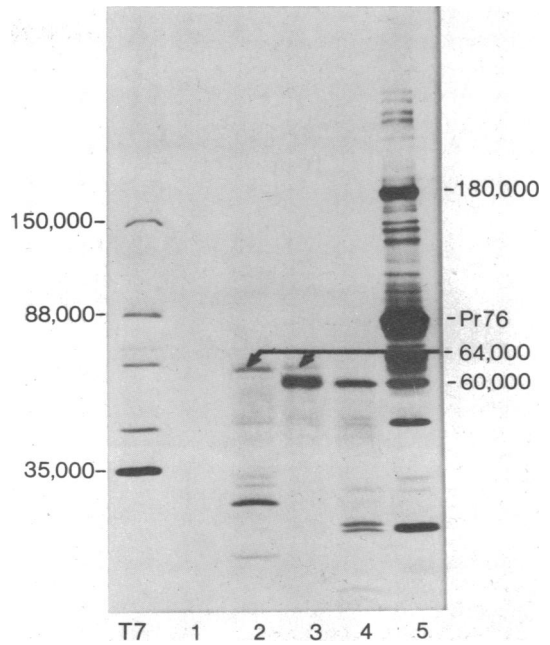

FIG. 3. Autoradiogram of NaDodSO$_4$/polyacrylamide gel analysis of reticulocyte cell-free products. Products synthesized in a mRNA-dependent reticulocyte lysate programmed by: 1, no RNA; 2, 21S poly(A)-containing td SR-ASV RNA; 3, 21S poly(A)-containing nd SR-ASV RNA; 4, 21S poly(A)-containing nd SR-ASV RNA prepared *in vitro* by RNase T1 digestion of 35S poly(A)-containing nd SR-ASV RNA; 5, same as track 8 of Fig. 1.

radiolabeled cell extracts by TBR serum are shown in Fig. 2. Uninfected chick embryo fibroblast lysates yielded few, if any, polypeptides of M_r less than 150,000. On the other hand, immunoprecipitation of extracts from td SR-ASV-infected or nd SR-ASV-transformed chick fibroblasts yielded several virus-specific polypeptides ranging in M_r up to 180,000, as shown in tracks 5 and 8. The polypeptide of M_r 180,000 is believed to be the product of the genes that encode the internal structural proteins and DNA polymerase (22). The proteins of M_r 76,000 (Pr76), 66,000 (Pr66), 53,000 (Pr53), 27,000, 19,000, and 12,000–15,000 are the precursor, intermediate, and mature cleavage products of the major internal structural proteins of ASV (27). In addition, an antigen of M_r 60,000 is also precipitated from nd ASV-transformed cells. When the antiserum was incubated with disrupted virions prior to addition of the labeled cell extract, the immunoprecipitation of all virus-specific antigens, with the exception of the 60,000 M_r one, was greatly reduced or undetectable. Although the amount of 60,000 M_r protein present in track 9 is somewhat less than in track 8, this is not the usual result; an equal amount of 60,000 M_r protein is often seen after the block. Longer autoradiographic exposures did not reveal the 60,000 M_r protein in either uninfected or td SR-ASV-infected cells. The 60,000 M_r antigen is not immunoprecipitated by antibody against virion structural proteins and appears to be transformation-specific (20).

Cell-Free Synthesis of a Transformation-Specific Polypeptide. The *src* gene is transcribed in transformed mammalian and chicken cells into a 21S mRNA (12, 28, 29). Previous results have illustrated that subgenomic portions of the ASV genome can be used to program cell-free reticulocyte lysates and, using this technique, we have previously observed that translation of 21S polyadenylated 3′ ends of nd Prague C (PrC)-ASV RNA yielded a polypeptide of M_r 60,000 not found when a similar preparation of td PrC RNA was translated (22). Results obtained from translation of td and nd SR-ASV RNA are similar to those observed with PrC RNA in that translation of the 21S 3′ end of nd RNA yielded a polypeptide of M_r 60,000 absent from the products of the corresponding td RNA preparation (Fig. 3). 21S polyadenylated RNA from both nd and td virus programmed the synthesis of a polypeptide of M_r 64,000.

Our routine preparations of subgenomic 3′ ends are obtained by heat denaturation of purified virion 70S RNA. It seems possible, although unlikely, that cellular 21S mRNA may be sequestered in the virion, copurified with 70S RNA, released by heat denaturation, and may program the synthesis of the 60,000 M_r protein. To determine whether the RNA coding for this protein was indeed contained in the 35S genome, virion 35S poly(A)-containing RNA was degraded with RNase T1, and poly(A)-containing RNA 21S in size (fractions 13–16, Fig. 1) was selected from the degradation products. Translation of this preparation also yielded the 60,000 M_r protein (Fig. 3, track 4). This result indicates that we have translated only subgenomic regions of the 35S RNA genome of ASV in this series of experiments. The lower molecular weight polypeptides (Fig. 3, track 4) are probably the product of 10–12S RNA, which is also among the degradation products of 35S RNA and which is probably present in the 21S RNA used here because it was precipitated from such a broad region of the sucrose gradient (Fig. 1). These lower molecular weight polypeptides are the only products seen upon translation of RNase T1-digested virion RNA that sedimented more slowly than 21S (data not shown). Similar products are seen upon translation of poly(A)-containing ASV RNAs 10–12S in size (unpublished results and ref. 22).

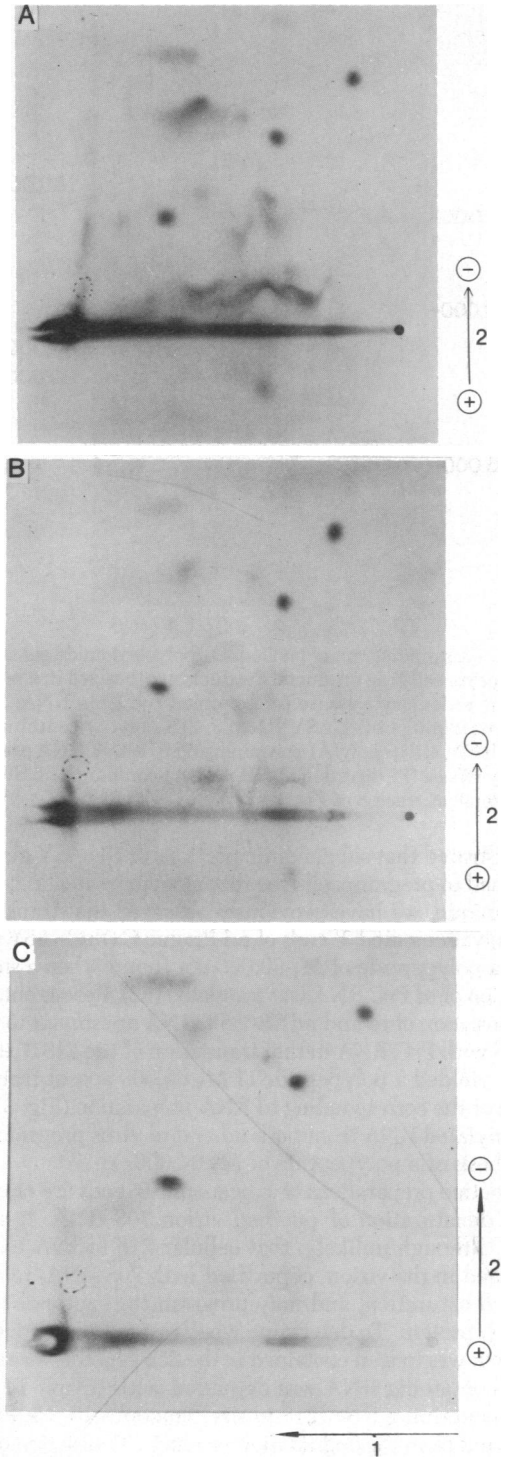

FIG. 4. Two-dimensional fingerprints of tryptic peptides from the 60,000 M_r protein synthesized *in vitro* and that immunoprecipitated from nd SR-ASV-infected cells. Dashed circle denotes the position of ϵ-Dnp-lysine. [^{35}S]Methionine-labeled 60,000 M_r protein was eluted from preparative gels, precipitated, digested with trypsin, and analyzed. (*A*) 60,000 M_r protein synthesized in a reticulocyte cell-free lysate programmed by 21S poly(A)-containing nd SR-ASV RNA. (*B*) 60,000 M_r protein immunoprecipitated with TBR serum from a lysate of nd SR-ASV-infected chicken cells. (*C*) Equal counts of 60,000 M_r protein from the two sources were mixed prior to peptide analysis.

Peptide Fingerprints of 60,000 M_r Protein Synthesized *In Vitro* and of That Immunoprecipitated from Transformed Cells. The 60,000 M_r protein synthesized *in vitro* and that immunoprecipitated from transformed cells were purified on $NaDodSO_4$/polyacrylamide gels, eluted, precipitated, and digested with trypsin. The digests were subjected to two-dimensional fractionation. The results (Fig. 4*A* and *B*) demonstrate that they contain identical methionine-containing peptides. This conclusion is supported by the fact that when equal numbers of counts of the digests from the two sources were mixed and fractionated, all the methionine-containing peptides comigrated (Fig. 4*C*). Partial digests produced by *S. aureus* V8 protease (30) and analyzed on polyacrylamide gels were also identical for these two polypeptides (data not shown).

DISCUSSION

In this report we describe the identification of a polypeptide encoded by the ASV *src* gene. Our experimental approaches have included the use of antiserum from tumor-bearing rabbits to immunoprecipitate from nd SR-ASV-transformed chick embryo fibroblasts an antigen that is absent from td SR-ASV-infected cells. The immunoprecipitation of this antigen, which has an apparent M_r of 60,000, is not dependent on antibodies against virion antigens because incubation of the antiserum with disrupted virus prior to the addition of the radiolabeled cell extract failed to block its precipitation. Furthermore, none of the antisera specific for the virion structural proteins precipitated this antigen. Normal rabbit serum did not precipitate the antigen, nor did serum from adult rabbits that had received the same inoculum of virus but that failed to develop tumors. An antigen of identical mobility is present in SR-ASV-induced hamster tumor cells (20). Our recent unpublished experiments show that the 60,000 M_r antigens from both chick and hamster cells yield the same protease degradation products. Under the conditions described here, TBR antiserum does not efficiently immunoprecipitate the 60,000 M_r polypeptide from cells transformed by strains of ASV other than SR, which, in view of the similarity of the nucleic acid sequences in the *src* genes of various strains of ASV (31), suggests that the antibody is directed against only a limited number of antigenic sites. Nevertheless, the pattern of expression of this antigen indicates that it is virus-encoded and not merely a host-cell protein induced as a secondary event during generation of the transformed phenotype.

However, support for the conclusion that the 60,000 M_r protein is encoded by the ASV genome is provided by *in vitro* translation of the 3′ one-third of virion 35S RNA. This is the region of the genome that is the principal virus-specific mRNA in ASV-transformed hamster cells (12) and that is also found in transformed avian cells as a 21S mRNA (28, 29). We previously reported (22) that this region of the genome of nd PrC programmed the synthesis of a polypeptide of M_r 60,000 and that this product was absent when similar RNA from td PrC was translated, therefore identifying *in vitro* a transformation-specific polypeptide. In this report we show that the tryptic fingerprint of the 60,000 M_r protein translated from 21S nd SR-ASV RNA is identical to that of the 60,000 M_r protein immunoprecipitated from SR-ASV-transformed chick cells. The 60,000 M_r protein produced in cell-free extracts is not translated from cellular mRNA sequestered in the virion since 21S RNA selected from virion 35S RNA degraded by RNase T1 also programmed its synthesis. This result and the peptide maps displayed in Fig. 4 lend additional support to the notion that a site for the initiation of protein synthesis exists at the 5′ end of the *src* gene and that the site becomes functional when this region of the ASV genome is selected and translated. These results provide a biochemical identification for the *src* gene-encoded polypeptide.

We anticipate that identification of the product of the ASV *src* gene will permit us to devise a purification procedure for the polypeptide. Purified 60,000 M_r protein may then be used to develop high titer antiserum which, in turn, may be useful in studies of the *in situ* localization of the antigen. Ultimately, the purified native polypeptide may be used for determination of its function. Because normal avian cells have DNA sequences related to those of the *src* gene (32) that are expressed at low levels (31), perhaps a polypeptide related to the *src* gene product is present in untransformed cells under tightly controlled expression. Such a polypeptide might be associated with the regulation of cell growth and proliferation, exerting its control transiently in normal situations, such as cell division, and constitutively in instances of malignant disease.

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