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Translation of 35S and of subgenomic regions of avian sarcoma virus RNA

(cell-free protein synthesis/avian sarcoma virus gene products)

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ABSTRACT **Rabbit antiserum monospecific for an internal structural protein, p27, of avian sacroma viruses (ASV) was found to immunoprecipitate polypeptides with molecular weights (M_r) of 180,000 and 76,000 from cell-free reticulocyte lysates programmed by ASV 35S RNA and also from lysates of ASV-infected cells. In addition, the M_r 180,000 protein was also precipitated by antiserum raised against virion DNA polymerase, suggesting that it is a product of translation of the two genes nearest the 5′ end of virion 35S RNA.**

We have also investigated the ability of subgenomic portions of virion RNA to program cell-free protein synthesis. A 10–12S poly(A)-containing fragment of RNA from both nondefective and transformation-defective ASV directed the synthesis of a polypeptide of M_r 29,000 immunologically unrelated to the gs antigens; 20–24S poly(A)-containing RNA from nondefective ASV directed the synthesis of a polypeptide of M_r 60,000 not found when a similar RNA preparation from transformation-defective ASV was translated, suggesting that it is the product of the ASV *src* gene. These results indicate that internal initiation sites for protein synthesis exist on the 35S RNA genome.

Avian sarcoma viruses (ASV) have been shown to contain at least four genetic elements: a gene for the internal structural proteins, *gag;* a gene for a viral DNA polymerase, *pol;* a gene for the viral envelope glycoprotein, *env;* and a gene for a protein that causes cell transformation, *src*. The order of these genes on the viral RNA is 5′-*gag-pol-env-src*-poly(A)-3′ (1). Although the viral genome is 35 S, virus-specific RNAs of several sizes are found in RNA tumor virus-transformed cells. One has the same size and polarity as the viral genome whereas others consist of discrete subgenomic portions of the 35S RNA (2, 3). If subgenomic RNA does serve as messenger for virus-specific proteins in infected cells, 35S RNA may contain internal initiation sites for protein synthesis.

Intact virion 35S RNA has been reported to program the synthesis of a polypeptide of molecular weight (M_r) 76,000 in cell-free extracts (4–6). According to studies carried out *in vivo*, a polypeptide of M_r 76,000 (Pr76) yields the mature internal structural proteins of the virus (7). Because the *gag* gene has been located at the 5′ end of the ASV genome and several thousand molecules of the *gag* product are found in each virion whereas only a few hundred molecules of other virion proteins are present (8), some mechanism exists to regulate the synthesis of viral structural proteins. For example, perhaps there is a single initiation site at the 5′ end that is functional on 35S RNA and serves as message for the internal structural proteins whereas subgenomic RNA molecules may serve as message for other virus-specific proteins. Such a scenario would help explain the different molar ratios of virion polypeptides produced in infected cells.

In the experiments reported here, we reinvestigated the 35S RNA molecule as a message in cell-free extracts and tested the messenger activity of subgenomic 3′ ends of virion RNA.

MATERIALS AND METHODS

Preparation of Viral RNA. Rous sarcoma virus, nondefective (nd) and transformation defective (td) Prague strain of ASV subgroup C (PrC), was harvested at 8- to 18-hr intervals from the supernatant medium of infected chick embryo fibroblasts (Spafas, Inc., Roanoke, IL) and purified, and 35S RNA was prepared as described (9). Subgenomic fragments were recovered from the same sucrose gradient used to purify 35S RNA, and poly(A)-containing RNA was isolated from these fragments as follows. RNA was precipitated twice with ethanol, dissolved in 0.01 M Tris·HCl, pH 7.4/0.2% sodium dodecyl sulfate ($NaDodSO_4$), and heated to 80° for 3 min. The solution was then quick-chilled, adjusted to 0.5 M NaCl/0.5% $NaDodSO_4$/0.01 M Tris·HCl, pH 7.4, and chromatographed on oligo(dT)-cellulose (T_3 grade, Collaborative Research). The poly(A)-containing material was eluted with 0.01 M Tris/0.05% $NaDodSO_4$, precipitated three times with ethanol, dissolved in 0.01 M Tris/1 mM EDTA, heated to 80° for 3 min, and fractionated by sucrose gradient sedimentation. We believe that subgenomic-sized RNA produced in this manner is derived from 35S RNA because virus harvested at 3-hr intervals yields only 35S RNA. The longer incubation at 41° prior to harvest results in fragmentation of the genome by a nuclease residing in the virion.

Protein Synthesis. mRNA-dependent reticulocyte lysates were prepared as described (10). The translation reaction was carried out for 50 min at 30° in a total volume of 50 μl containing 100 mM KCl, 20 mM Tris·HCl, (pH 7.4), 2 mM Mg acetate, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.1 mM GTP, creatine phosphate (2 mg/ml), creatine phosphokinase (0.2 mg/ml), unlabeled amino acids except methionine (200 μM each), 25μCi of [^{35}S]methionine (400 Ci/mmol; New England Nuclear), 20 μM hemin, 5 μg of wheat germ tRNA, 1 μg of mRNA, and 25 μl of lysate.

Polyacrylamide Gel Electrophoresis. Products were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis with the buffer systems described by Laemmli (11) and a 15–5% gradient of polyacrylamide in the separation gel. Fluorography was carried out as described (12). Peptide mapping by limited proteolysis and electrophoresis was carried out by the procedure described by Cleveland *et al.* (13).

Immunoprecipitation. Monospecific antibody against the

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Abbreviations: ASV, avian sarcoma virus; M_r, molecular weight; nd, nondefective; td, transformation defective; PrC, Prague strain of ASV subgroup C; $NaDodSO_4$, sodium dodecyl sulfate; p27, virion internal structural protein.

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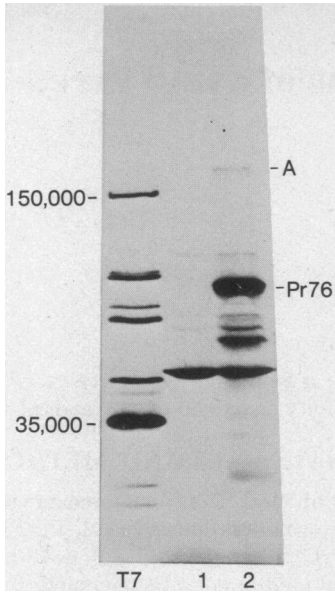

FIG. 1. Autoradiogram of reticulocyte cell-free products after $NaDodSO_4$/polyacrylamide gel electrophoresis. Track 1, no added RNA; track 2, 35S nd PrC RNA. T7 proteins were calibrated against *Escherichia coli* RNA polymerase, ovalbumin, bovine serum albumin, and trypsin.

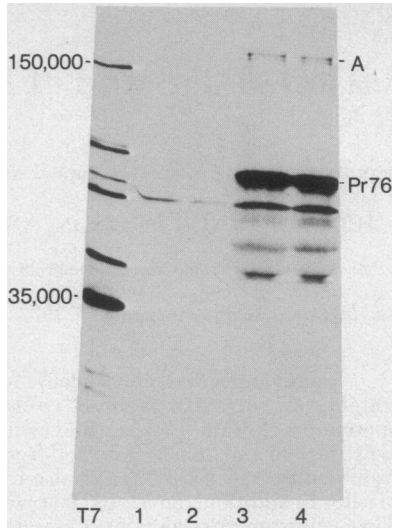

FIG. 2. Autoradiogram of immunoprecipitated cell-free products after $NaDodSO_4$/polyacrylamide gel electrophoresis. The RNA used to program the lysate and the serum used for immunoprecipitation were as follows: track 1, no RNA, anti-p27 serum; track 2, AMV 35S RNA, normal rabbit serum; track 3, 35S nd PrC RNA, anti-p27 serum; track 4, 35S AMV RNA, anti-p27 serum.

virion internal structural protein, p27, was prepared by injecting rabbits with p27 purified by $NaDodSO_4$/gel electrophoresis. Monospecific anti-(RAV-O)-DNA polymerase IgG was the generous gift of Satoshi Mizutani. For immunoprecipitation, a sample of the translation reaction was adjusted to 1% Nonidet P-40 and digested with RNase A (100 μg/ml). Antiserum was added and the mixture was incubated for 1 hr at room temperature; then goat anti-rabbit serum was added at equivalence and the incubation was continued for 1 hr at room temperature and overnight at 4°. The precipitate was washed several times and analyzed by $NaDodSO_4$/gel electrophoresis. Immunoprecipitation of polypeptides from lysates of chick embryo fibroblasts was carried out by the method of Kessl[illegible]14).

RESULTS

Translation of 35S RNA. When virion 35S RNA was used to program a mRNA-dependent reticulocyte lysate, polypeptides of M_r up to 180,000 were synthesized (Fig. 1). One polypeptide of M_r 45,000 was synthesized in the unprogrammed lysate as shown in Fig. 1 (track 1). The specificity of translation could be monitored with monospecific antiserum raised against the ASV protein p27. As shown in Fig. 2, in addition to some premature termination products two polypeptides of M_r 76,000 and 18,000 were specifically precipitated by anti-p27 serum. These are referred to as Pr76 and *A*, respectively, in Figs. 1 and 2. Two-dimensional fingerprint analyses confirmed that the polypeptide referred to as Pr76 contained tryptic peptides related to virion structural proteins as did the other lower molecular weight virus-specific polypeptides, indicating that some premature termination of protein synthesis occurred in the reticulocyte lysates.

A polypeptide that comigrated with *A* could also be immunoprecipitated by anti-p27 serum from lysates of PrC-infected chick cells. Although there was a limited amount of radioactivity, a peptide map of these polypeptides could be prepared by partial enzymatic proteolysis of gel slices containing the labeled polypetide and subsequent analysis on another polyacrylamide gel (13). The cleavage products generated from *A* labeled *in vitro* and *in vivo* were identical (Fig. 3).

The identity of *A* was further investigated by immunoprecipitation with antiserum raised against virion DNA polymerase. When anti-DNA polymerase IgG was used to precipitate *in vitro* polypeptide products, some Pr76 was usually present in the precipitate in addition to *A*, but *A* could be specifically immunoprecipitated from lysates of infected cells with anti-polymerase IgG (Fig. 4). Furthermore, the immunoprecipitation of *A* by anti-DNA polymerase IgG could be

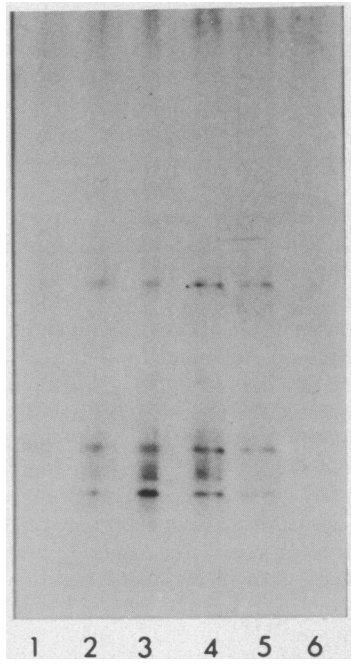

FIG. 3. Autoradiogram of the cleavage products of *A* digested with *Staphylococcus aureus* V8 protease during re-electrophoresis. Gel slices containing *A* were cut from a freshly stained and destained gel, placed in the sample wells of a second $NaDodSO_4$/polyacrylamide gel (20–12% polyacrylamide), and digested and analyzed as described (13). Tracks 1–3, *A* produced in reticulocyte lysates and digested with 0.025, 0.5, and 2 μg of protease, respectively. Tracks 4–6, *A* immunoprecipitated from lysates of infected cells and digested with 2, 0.5, and 0.25 μg of protease, respectively.

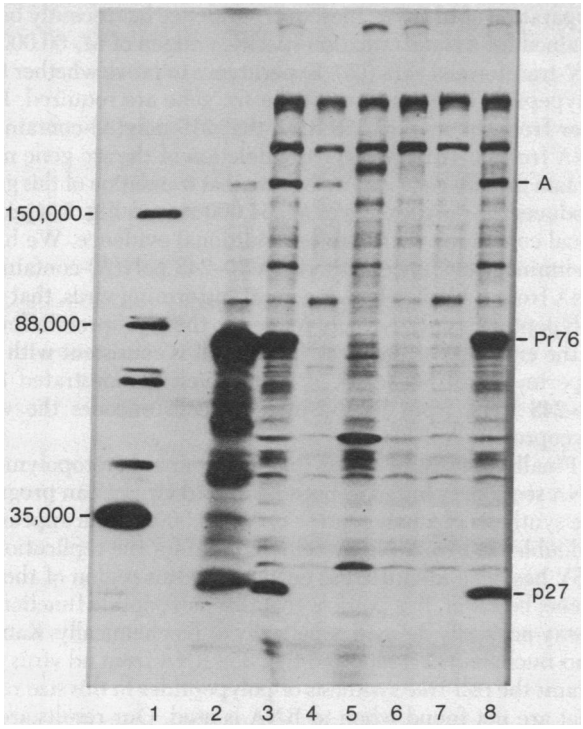

FIG. 4. Autoradiogram of cell-free products and of immunoprecipitates of lysates of nd PrC-infected and uninfected chick embryo fibroblasts after $NaDodSO_4$/polyacrylamide gel electrophoresis. Track 1, reticulocyte lysate, no RNA; track 2, reticulocyte lysate, AMV 35S RNA. Tracks 3–8, peptides immunoprecipitated from cellular lysates. Cells and antisera are as follows: track 3, infected cells, anti-p27 serum; track 4, infected cells, 5 μg of anti-DNA polymerase IgG; track 5, normal cells, anti-p27 serum; track 6, infected cells, preimmune rabbit serum; track 7, infected cells, 5 μg of anti-DNA polymerase IgG blocked with DNA polymerase; track 8, infected cells, anti-p27 serum blocked with DNA polymerase. Infected and normal cells were labeled with [^{35}S]methionine (20 μCi/ml) for 4 hr. The cells were lysed, clarified, and immunoprecipitated as described (14). For the blocking reactions, the antiserum was incubated with 5 μg of purified DNA polymerase for 15 min at 4° prior to the addition of the cell lysate. DNA polymerase was prepared from PrC virions as described (15).

blocked with purified DNA polymerase (Fig. 4, track 7) but DNA polymerase had no effect on the immunoprecipitation of *A* by anti-p27 serum (Fig. 4, track 8). Therefore, the polypeptide of M_r 180,000 appeared to contain antigenic determinants for both p27 and DNA polymerase. One interpretation of the results is that a single functional initiation site near the 5′ end of 35S RNA results in the highly efficient translation of the 5′-terminal gene, *gag*, and that occasionally, both *in vitro* and *in vivo*, ribosomes continue and translate the *pol* gene as well, generating a polypeptide that has an apparent M_r of 180,000. We have not seen any virus-specific products with M_r larger than 180,000. Pr76 appears first during cell-free synthesis and therefore is probably not derived from *A* by proteolytic cleavage.

Translation of Subgenomic Poly(A)-Containing RNA. Because both avian and mammalian cells transformed by ASV contain virus-specific RNAs sedimenting more slowly than 35 S, we investigated the possibility of internal initiation sites for protein synthesis in the 35S molecule. Poly(A)-containing (3′ end) regions of nd and td virion RNA were prepared and further fractionated on sucrose gradients into various size classes ranging from 4 S to 35 S. Two size classes were of particular interest because virus-specific 21S RNA is found in ASV-induced mammalian tumor cells (3) and because 10–12S RNA

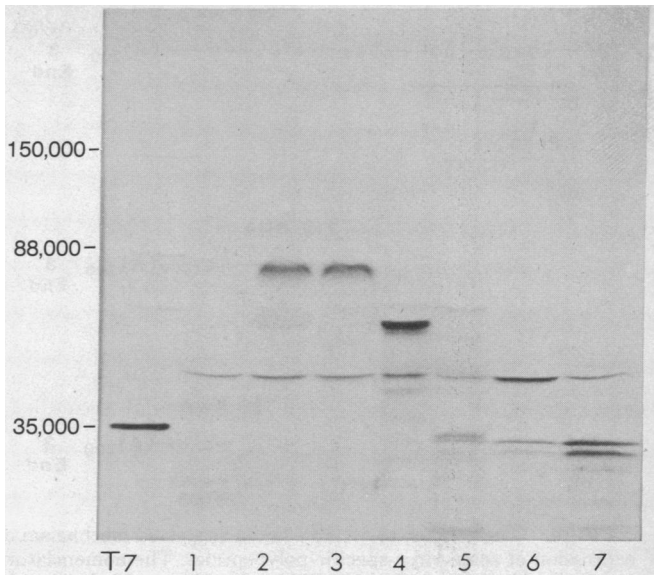

FIG. 5. Autoradiogram of reticulocyte cell-free products after $NaDodSO_4$/polyacrylamide gel electrophoresis. The RNA translated was: track 1, no RNA; track 2, nd PrC 35S; track 3, td PrC 35S; track 4, nd PrC 20–24S poly(A)-containing; 5, td PrC 20–24S poly(A)-containing; 6, nd PrC 10–12S poly(A)-containing; 7, td PrC 10–12S poly(A)-containing. It should be noted that the RNA used here was heated prior to fractionation by sucrose gradient sedimentation in order to disrupt any possible aggregates and that 1 μg of RNA was added to each reaction mixture.

stimulated a dramatic increase in the incorporation of [^{35}S]-methionine in wheat germ cell-free extracts (data not shown).

The Pr76s synthesized in response to nd or td virion 35S RNA had identical mobility upon electrophoresis (Fig. 5, tracks 2 and 3); the polypeptide of M_r 180,000 was present in both cases but the exposure used here did not demonstrate it. As shown in Fig. 5, regions from the 3′ end of virion RNA did not program the synthesis of Pr76 in cell-free extracts but resulted in the synthesis of discrete polypeptides that were not immunoprecipitated by anti-p27 serum (data not shown). Both nd and td 10–12S RNA, which were apparently derived from heteropolymeric RNA sequences common to both nd and td virion RNA (16, 17), programmed the synthesis of a doublet with a M_r of 29,000 (Fig. 5, tracks 6 and 7).

The results found upon translation of 20–24S RNA were different, however. The nd RNA programmed the synthesis of a large amount of polypeptide of M_r 60,000 absent from the translation products of td 20–24S RNA. In both the nd and td translation products, a polypeptide of M_r 64,000 was found (Fig. 5, tracks 4 and 5). The polypeptide of M_r 60,000 could not be immunoprecipitated by antibody directed against virion components and, because it was absent from the translation products of td RNA that contains a deletion in the *src* gene, it may be the polypeptide encoded by the *src* gene of nd ASV. A large amount of virus was required to prepare nd RNA fragments, and consequently this RNA no doubt contained some RNA from deletion mutants which arise spontaneously during growth of nd virus (18, 19) and which may result in the two polypeptides (M_r 60,000 and 64,000) in Fig. 5, track 4.

DISCUSSION

A model based on the results included here is presented in Fig. 6 and partially explains the translation of the ASV genome. Protein synthesis is initiated at the 5′ end of 35S RNA and most

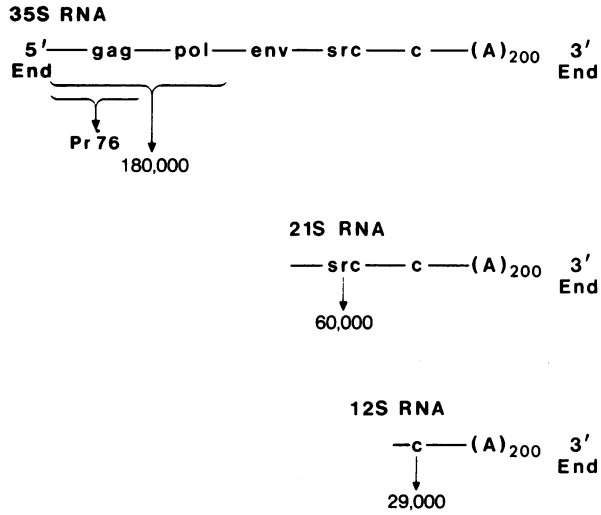

FIG. 6. The genome of nd ASV and a suggested mechanism of expression of some virus-specific polypeptides. The nomenclature is described in the *text*. The 35S RNA is found in purified virions in the form of a heat-denaturable dimer and in infected cells it is associated with polyribosomes. The 3′ one-third of the genome is expressed in virus-transformed mammalian cells as a virus-specific 21S mRNA (3). The region designated c is common to virion RNA of both td and nd virus but has not been reported to be expressed as a virus-specific mRNA. Both subgenomic poly(A)-containing regions of the genome were selected here from degraded virion RNA. The evidence for the expression of the virus-specific polypeptides Pr76, 180,000, 60,000 and 29,000 is discussed in the *text*. No attempt was made to represent the relative lengths of the ASV genes.

of the ribosomes terminate synthesis with generation of Pr76 but a small number continue and translate the *pol* gene as well. The resultant polypeptide of M_r 180,000 could be cleaved by a protease in the infected cell to yield a functional polymerase and additional Pr76. This result suggests a mechanism for the generation of unequal amounts of polymerase and internal structural proteins and circumvents the need for a mRNA for polymerase in the infected cell. Support for this conclusion has also been obtained in studies with murine oncornavirus (5, 20, 21).

We believe that the translation of the subgenomic regions of the ASV genome selected here is a valid approach for several reasons. These RNA species probably lack 5′-terminal 7-methylguanosine because they are derived by nuclease clevage of 35S RNA; however, recent results illustrate that RNA lacking a 5′-terminal 7-methylguanosine is correctly translated in reticulocyte lysates (22) although this terminus may be required in other cell-free extracts (23). *N*-Formylmethionine from *N*-formyl-[^{35}S]methionyl-tRNA$_f^{Met}$, which donates methionine at the NH_2 terminus of proteins, is incorporated into the major polypeptides shown in Fig. 5 (A. Siddiqui and A. F. Purchio, unpublished data), indicating that correct initiation has occurred. The 3′ one-third of the ASV genome is expressed in transformed cells as virus-specific 21S mRNA (3). Furthermore, there is precedent for internal sites for the initiation of protein synthesis based on the genome of another virus (24). Throughout the course of this work, we have assumed that no significant amount of cellular mRNA is present in the 70S RNA prepared from virions, but that possibility is now being tested.

The 20–24S RNA from nd virus selected and translated in these studies is illustrated under 21S in Fig. 6. Our results demonstrate that nd 21S RNA programs the synthesis of a polypeptide with a M_r of 60,000 not programmed by a similar preparation of td RNA. Moreover, evidence has recently been obtained for a transformation-specific antigen of M_r 60,000 in ASV-transformed cells (25). Experiments to prove whether this polypeptide is the product of the *src* gene are required. It is clear from the map of 35S RNA that 21S poly(A)-containing RNA from td virus which has a deletion of the *src* gene may contain the *env* gene and we believe that translation of this gene produces the polypeptide of M_r 64,000 seen in Fig. 5, a provisional conclusion that requires additional evidence. We have preliminary evidence with virion 20–24S poly(A)-containing RNA from RAV-2, which is a nontransforming virus, that this polypeptide is antigenically related to the glycoprotein found in the envelope of the virus. This result is consistent with the experiments of Stacey *et al.* (26) which demonstrated that 20–24S RNA from RAV-2-infected cells encodes the viral glycoprotein.

Finally, it must be noted that 3′-terminal heteropolymeric RNA sequences found in both nd and td viruses can program the synthesis of a polypeptide of M_r 29,000, which appears as a doublet in Fig. 5. No protein essential for the replication of ASV has been identified as encoded by this region of the genome; however, it is possible that this polypeptide functions in a way not easily defined genetically or biochemically. Kamine and Buchanan (27) reported that 35S RNA from nd virus programs the cell-free synthesis of polypeptides in this size range that are not found when td RNA is used. Our results are inconsistent with this claim because we find that the only polypeptide uniquely produced by RNA from transforming virus has a M_r of 60,000. Because the strategy used here is completely different from the one they used, additional experiments are required to resolve this apparent discrepancy.

Whether or not a specific polypeptide product can be assigned to a specific ASV gene at this time, we feel that the approach outlined in this communication illustrates the usefulness of subgenomic regions of ASV virion RNA in cell-free translation experiments and may provide a means to study the polypeptides encoded by specific ASV genes.

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