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Cell-free synthesis of two proteins unique to RNA of transforming virions of Rous sarcoma virus

(*sarc* gene/transformation-specific proteins/micrococcal nuclease/reticulocyte lysate)

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ABSTRACT **We have utilized a reticulocyte lysate system to translate the 35S RNA of Rous sarcoma virus. Autoradiograms of the protein products separated on sodium dodecyl sulfate/polyacrylamide gels reveal a heterogeneous mixture of proteins of sizes ranging from 13,000 to 180,000 daltons. In comparing the translational products from 35S RNA of Prague B Rous sarcoma virus with those formed from the RNA of a transformation-defective deletion mutant derived from Prague B, we have found that two proteins, 25,000 and 18,000 daltons, are missing from the latter. Neither of these proteins is immunoprecipitated by monospecific antisera against the structural proteins of avian RNA tumor viruses. The combined atomic mass of 43,000 daltons corresponds to the amount of genetic coding capacity (40,000–50,000 daltons in terms of protein products) deleted from the RNA of the transformation-defective viruses. We propose that these proteins are coded for by the putative oncogene (*onc*) or *sarc* (*src*) gene and that one or both of them may be responsible for the oncogenic transformation caused by these viruses in infected cells.**

Rous sarcoma virus (RSV) is a potent oncogenic virus that can efficiently transform the fibroblasts it infects in culture within 24 hr (1). This virus also causes sarcomas when injected into chickens (2, 3). The virus contains an RNA genome in the form of a 70S RNA complex that can be dissociated by denaturing conditions into two or three identical 35S subunits of 3.3×10^6 daltons (4–6). Analysis of the 35S RNA has shown that there are two size-classes, the *b* subunits being about 1200 nucleotides (12%) smaller than the *a* subunits (7). In avian leukosis viruses and transformation-defective (*td*) derivatives of sarcoma viruses, only the *b* subunit is found (7, 8). Oligonucleotide mapping studies of RNA from transforming viruses and their *td* deletion mutants have established that the RNA from the transforming viruses contains sequences not present in the RNA of the nontransforming derivatives and that these sequences are located at the polyadenylated 3′ end of the RNA (9–11).

Because the *td* viruses are infective and able to produce infective progeny at a normal rate, the missing portion of RNA might be a gene whose sole function is the generation of the transformed phenotype. This gene has been termed the *onc* gene (12) or *src* (*sarc*) gene. There are also temperature-sensitive RSV mutants that selectively transform cells at the permissive temperature (13, 14). These mutants all appear to map in the *sarc* gene region (15). The existence of temperature-sensitive mutants suggests that there might be protein products of the putative *sarc* gene involved in the oncogenic transformation caused by these viruses.

In an effort to identify the *sarc* gene product we used 35S RNA from nondefective (*nd*) and *td* RSV to compare the proteins synthesized in cell-free systems. We find that RNA from Prague B RSV produced two [^{35}S]methionine-labeled proteins that were missing from cell-free translation assays directed by RNA from a *td* derivative of Prague B RSV. In addition to the putative *sarc* gene products, the individual group-specific (*gs*) viral antigens also appear to be synthesized in these cell-free assays.

Abbreviations: RSV, Rous sarcoma virus; *td*, transformation-defective; *nd*, nondefective; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, (ethylenedinitrilo)tetraacetic acid.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine (500 Ci/mmol) was purchased from Amersham/Searle; spermidine, unlabeled amino acids, and creatine kinase, from Sigma; creatine phosphate, from Boehringer-Mannheim; sodium dodecyl sulfate ($NaDodSO_4$), BDH Chemicals (Poole, England); micrococcal nuclease, from Worthington; NP-40, from Shell Chemical Co. (London, England); ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) and hemin, from Eastman. All reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. Monospecific antisera to avian myeloblastosis virus structural proteins was a generous gift of Dani Bolognesi (Duke). Prague B RSV and transformation-defective (*td*) Prague B RSV stocks were gifts from John Coffin (Tufts). All other chemicals were supplied from vendors as reagent grade.

Cell Growth and Purification of Viral RNA. Chick embryo fibroblasts from primary cultures were trypsinized and infected with the viral stock at a multiplicity of infection of 1. Infected cells were grown in medium 199 containing 5% (vol/vol) tryptose phosphate broth, 3% (vol/vol) calf serum, and 1% (vol/vol) chicken serum in plastic roller bottles. The virus-containing medium was collected from the cultures at either 7- or 12-hr intervals as indicated. After clarification of the medium, the virus was pelleted by centrifugation at 21,000 × *g* for 5 hr and resuspended in 2 ml of standard buffer [0.1 M NaCl/0.001 mM (ethylenedinitrilo)tetraacetic acid (EDTA) 0.01 M Tris·HCl, pH 7.2] with sonication for 5 sec. Virus was then banded in a 15–60% sucrose gradient in standard buffer overnight by centrifugation at 286,000 × *g*. The band of virus was collected and diluted with 3 volumes of standard buffer. The virus was then repelleted at 286,000 × *g* for 45 min and resuspended with sonication. Viral RNA was isolated by the method of Singer and Penman (16). To denature the 70S viral RNA complex, the RNA was resuspended in standard buffer containing 0.2% (wt/vol) $NaDodSO_4$ and heated for 2 min at 76°. The 35S RNA was isolated by centrifugation through a 15–30% sucrose gradient in standard buffer containing 0.15% $NaDodSO_4$ for 4 hr (22°) at 36,000 rpm.

Cell-Free Translation. Reticulocyte lysates were prepared as described by Villa-Komaroff *et al.* (17). Treatment of lysates with micrococcal nuclease (nucleate 3′-oligonucleotidohydrolase, EC 3.1.4.7) was performed according to Pelham and Jackson (18). To 100 volumes of reticulocyte lysate were added

1 volume of hemin solution (4 mg/ml in ethylene glycol) and 2 volumes of 50 mM $CaCl_2$. After mixing, 0.05 volume of micrococcal nuclease (150,000 units/ml in 50 mM glycine, pH 7.2/5 mM $CaCl_2$) was added and the solution was incubated for 15 min at 20°. The nuclease was then inactivated by adding 2 volumes of 100 mM EGTA followed by 0.4 volume of creatine phosphate kinase (40 μg/ml in 50% glycerol). The solution was then divided into several parts and stored in liquid nitrogen.

Each 25 μl of reaction mixture for cell-free translation contained 14.5 μl of nuclease-treated reticulocyte lysate and the following assay components in the indicated concentrations: 140 mM potassium acetate, 0.8 mM magnesium acetate, 184 μM spermidine, 1 μM [^{35}S]methionine (about 500 Ci/mmol), 11.5 mM creatine phosphate, creatine kinase at 46 μg/ml, 4.6 mM dithiothreitol, 90 μM of each of 19 unlabeled amino acids, and 1 mM EDTA. Viral RNA concentrations were as stated. Reactions were at 30° for the indicated times.

Incorporation of [^{35}S]methionine into acid-insoluble material was determined by spotting 1 μl of the reaction mixture on Whatman 3MM filter paper that then was dried and incubated in 10% (wt/vol) trichloroacetic acid containing 3% (wt/vol) casamino acids for 30 min. The filters were then placed in 5% trichloroacetic acid at 100° for 10 min, washed with 5% trichloroacetic acid, and finally washed with 95% ethanol. The radioactivity of the dried filters was measured in a Beckman liquid scintillation counter.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. The protein products synthesized in the cell-free reactions were analyzed on NaDodSO$_4$/polyacrylamide slab gels as described by Laemmli (19). Samples (1–5 μl) were diluted to 20 μl in sample buffer (2% NaDodSO$_4$/0.7 M 2-mercaptoethanol/0.06 M Tris, pH 6.8/10% glycerol). The samples were heated at 100° for 2 min before application to the 3% acrylamide stacking gel. The separating gel, which was 10.5 cm in length, consisted of a 5–15% gradient of acrylamide and separated proteins in the molecular weight range 10,000–300,000. The slab gels were subjected to electrophoresis until the bromophenol blue marker reached the bottom of the gel. They were then dried and subjected to autoradiography on Kodak NS-2T no-screen medical x-ray film for the indicated times.

Immunoprecipitations. All sera were clarified by centrifugation at 8000 × *g* for 5 min before use. Samples (10 μl) of the cell-free reaction mixtures were combined with 10 μl of standard buffer containing 1% NP-40 detergent and 5 μl of antiserum. After 15 min at 22°, 100 μl of a suspension of staphylococcal protein A-antibody adsorbent was added. The adsorbent had been prepared as described by Kessler (20). After 15 min at 4° the adsorbent was washed three times. The antigens were then eluted from the adsorbent by heating at 100° for 3 min in 60 μl of a solution containing 6 M urea and 3% NaDodSO$_4$ (20). Aliquots of each sample were diluted with sample buffer and heated at 100° for 2 min before analysis on NaDodSO$_4$/polyacrylamide gels.

RESULTS

Our objective was to compare the protein products made from virion RNA of transformation-defective (*td*) and nondefective (*nd*) RSV in a cell-free translation system. For this purpose we used a recently described reticulocyte lysate procedure in which endogenous RNA is degraded by prior incubation with micrococcal nuclease. The nuclease, which is Ca^{2+}-dependent, can then be inactivated by adding EGTA to chelate the Ca^{2+}. Fig. 1 shows a time course of incorporation of [^{35}S]methionine into acid-insoluble material by the nuclease-treated reticulocyte lysate with and without added 35S RSV RNA. The level of

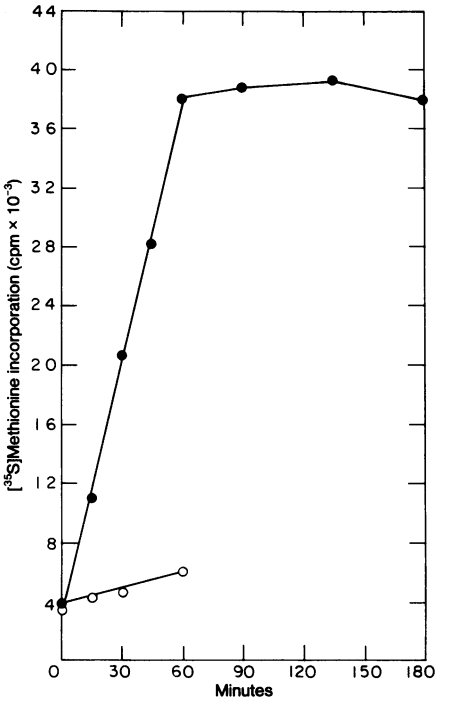

FIG. 1. Time course of [^{35}S]methionine incorporation into acid-insoluble material. Cell-free translation assays were done as described in *Materials and Methods*. Assays contained either no added viral RNA (○) or 35S RNA from Prague B RSV, 18 μg/ml (●). Viruses had been purified from culture medium collected at 7-hr intervals.

endogenous incorporation in these assays was decreased to 1–2% of that observed before nuclease treatment. Only one-third of this radioactivity represents incorporation of [^{35}S]methionine into protein; the rest results from incomplete washing of the precipitates. Upon the addition of 35S RSV RNA, the incorporation of [^{35}S]methionine was rectilinear for 60 min and increased by 20- to 30-fold over that in the control assays. The concentrations of assay components, described in *Materials and Methods*, were optimized for maximal incorporation of [^{35}S]methionine with RSV RNA.

The proteins synthesized from the 35S RNA of *nd* RSV and *td* RSV showed a wide range of sizes, from 13,000 to 180,000 daltons, when analyzed by autoradiography of NaDodSO$_4$/polyacrylamide gradient gels (Fig. 2, slots 1 and 4, respectively). In addition, some autoradiograms showed faint bands of labeled protein at 250,000 daltons with both RNAs. Because the maximal coding capacity of the 35S RNA subunit is between 250,000 and 300,000 daltons of protein, this would be close to a full-length translation product. The important feature of this autoradiogram is that the two samples show identical bands of labeled protein, with two exceptions: two labeled proteins are clearly missing from the cell-free translation assays directed by the RNA from the *td* virus. One protein is molecular weight 25,000 and the other is 18,000. These are invariant patterns; we have not found any assay conditions that qualitatively change the spectrum of proteins synthesized from these RNAs. Without the addition of viral RNA (slot 3) only a few bands of labeled protein were detected on these gels: two faint bands between 15,000 and 19,000 and three bands between 19,000 and 25,000. More heavily labeled bands of endogenously produced proteins comigrated with virally coded proteins at 55,000 and 105,000 molecular weight. Another interesting feature of the autoradiograms shown in slots 1, 2, and 4 is the presence of low-molecular-weight bands, at 29,000, 19,000, 15,000, 13,000. The band at 15,000 is slightly displaced in Fig. 2 by globin but is

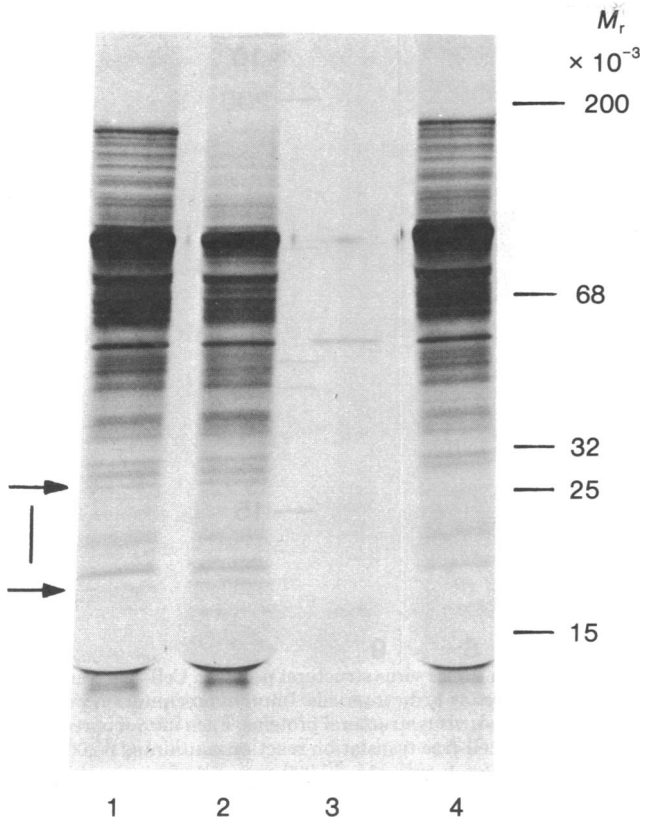

FIG. 2. Autoradiogram of $NaDodSO_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine labeled cell-free translation products. Slot 1: cell-free translation of 35S RNA from Prague B RSV. RNA was assayed at 18 μl/ml, then 3 μl of the 25-μl reaction mixture containing 99,000 cpm of [^{35}S]methionine was applied to the gel. Virus had been purified from culture medium collected at 7-hr intervals. Slot 2: same as slot 1 except RNA was purified from virus collected at 12-hr intervals. Slot 3: control, without viral RNA; 3 μl of a 25-μl reaction mixture, containing 15,000 cpm of [^{35}S]methionine was applied to gel. Slot 4: cell-free translation of 35S RNA from *td* Prague B RSV. RNA was assayed at 20 μg/ml and then 3 μl of the 25-μl reaction mixture containing 99,000 cpm of [^{35}S]methionine was applied to gel. Translation reactions were all for 60 min at 30°. Molecular weight (M_r) markers were: heavy subunit of myosin, 200,000; bovine serum albumin, 68,000; α-thrombin, 32,000; chymotrypsinogen, 25,000; and globin, 15,000. Film was exposed for 10 days. Arrows indicate position of proteins unique to cell-free assays containing RNA from *nd* RSV; vertical bar indicates positions of endogenously labeled proteins.

clearly at 15,000 when viral proteins have been immunoprecipitated and are free of globin. These four components have approximately the molecular weights of the group-specific structural proteins of the virus particle.

To quantify the amounts of [^{35}S]methionine incorporated into the individual labeled proteins seen on the autoradiogram of Fig. 2, densitometer tracings were performed (Fig. 3). Less than 1% of the total [^{35}S]methionine label on this autoradiogram appeared to be incorporated into the two *sarc* gene products, whereas most of the [^{35}S]methionine was incorporated into proteins of 50,000 molecular weight or larger. However, preliminary experiments have indicated that increasing the concentration of the RNA in the cell-free translation assays can selectively increase the amounts of some of the labeled proteins smaller than 90,000, mostly the 18,000 *sarc* gene protein with RNA from *nd* virus. With some preparations of RNA, a decreased amount of [^{35}S]methionine was incorporated into bands above molecular weight 100,000 (Fig. 2, slots 1 and 2). This

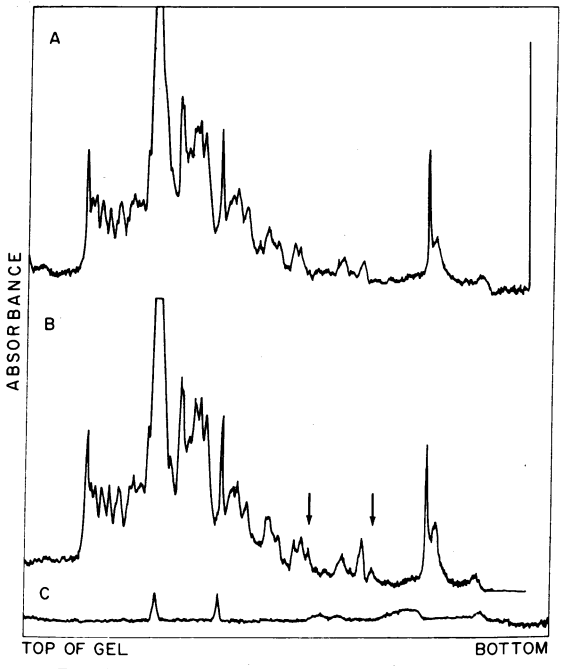

FIG. 3. Densitometer scan of autoradiograms of Fig. 2, made with a Canalco model GII densitometer. *A*, *B*, and *C* are from scan slots 4, 1, and 3, respectively, of the autoradiogram in Fig. 2. Arrows indicate the location of the proteins unique to cell-free assays containing RNA from *nd* RSV.

decrease correlated with longer collection periods of the virus-containing medium from the cells and presumably resulted from degradation of the RNA.

If the 25,000 and 18,000 molecular weight proteins are the putative *sarc* gene products, they should not be immunoprecipitated by antibodies against the structural proteins of the virion. Fig. 4 is an autoradiogram of $NaDodSO_4$/polyacrylamide gel of labeled proteins immunoprecipitated with the monospecific antisera prepared against all of the structural proteins of avian RNA tumor virus. Low levels of immunoprecipitation of labeled proteins occurred even with normal rabbit serum (slot 7); however, the immunoprecipitation of labeled viral protein was much more complete with monospecific antisera against the viral antigens. Only four proteins appeared not to be immunoprecipitated by at least one of the viral antisera, the two *sarc* gene products, a protein at 28,000 and another at 55,000. The monospecific antisera against the *gs* antigens of the virus immunoprecipitated almost all of the other labeled proteins synthesized in these cell-free assays. These included proteins at 29,000, 19,000, 15,000, and 13,000, the sizes of the individual group-specific antigens of the virus (slots 2–6). Antiserum against the large (85,000) surface glycoprotein of the virus particle immunoprecipitated most of the labeled protein above 30,000 but nothing of lower molecular weight (slot 8). It would appear that most of the larger protein products contained antigenic determinants of both the *gs*-antigens and the larger glycoprotein. On the other hand, antiserum against the smaller surface glycoprotein of avian RNA tumor viruses did not precipitate any of the cell-free translation products (slot 9).

Although we used antisera prepared against the individual *gs*-antigens of avian myeloblastosis virus for the immunoprecipitation reactions, a selective precipitation of the individual *gs*-antigens was not observed. In this experiment the antisera were all used undiluted to ensure complete precipitation of the labeled protein. Under this condition, immunospecificity

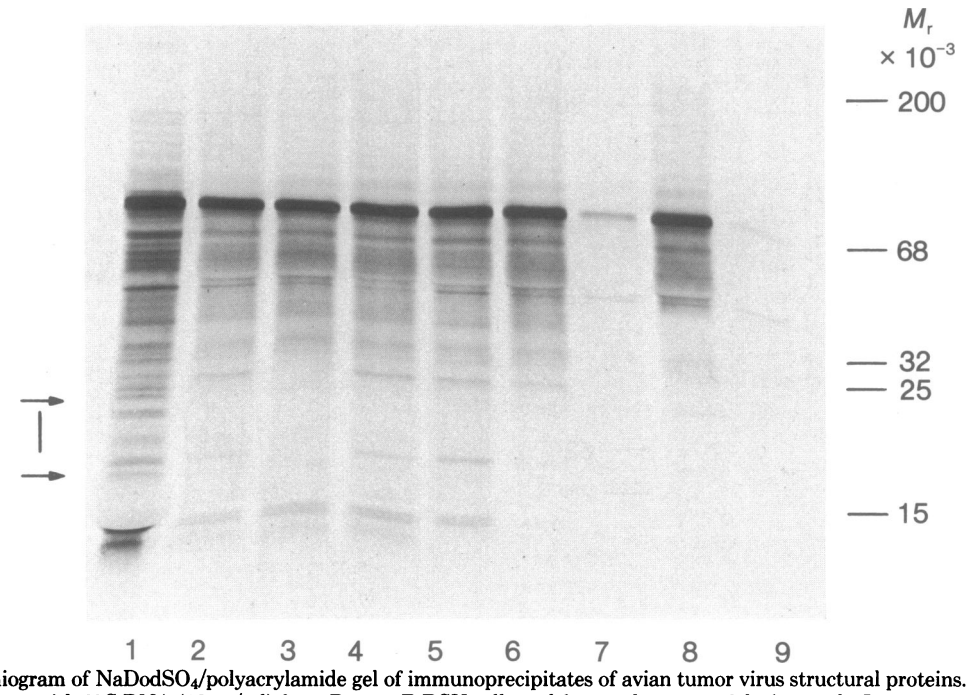

FIG. 4. Autoradiogram of NaDodSO$_4$/polyacrylamide gel of immunoprecipitates of avian tumor virus structural proteins. Cell-free translation assays were carried out with 35S RNA (18 μg/ml) from Prague B RSV collected from cultures at 12-hr intervals. Immunoprecipitation was done as described in *Materials and Methods* with monospecific antisera against avian myeloblastosis virus structural proteins. Each immunoprecipitate represents the equivalence of 2 μl of cell-free reaction mixture. Slot 1: 2 μl of reticulocyte cell-free translation reaction containing 65,000 cpm. Slot 2: anti-p27, 60,000 cpm was applied to gel. Slot 3: anti-p19, containing 54,000 cpm. Slot 4: anti-p15, 57,000 cpm. Slot 5: anti-p12, 51,000 cpm. Slot 6: anti-p10, 49,000 cpm. Slot 7: normal rabbit serum, 9,000 cpm. Slot 8: anti-gp 85, 37,000 cpm. Slot 9: anti-gp 37, 9,000 cpm. Film was exposed for 6 days. Arrows indicate positions of proteins unique to cell-free assays containing RNA from *nd* RSV; vertical bar indicates positions of endogenously labeled proteins.

among antisera against the *gs*-antigens is often not demonstrable because of cross contamination (21).

DISCUSSION

We have utilized a nuclease-treated cell-free reticulocyte lysate to translate the 35S RNA of RSV. The extremely low levels of endogenous incorporation of [^{35}S]methionine in this system and the relatively high level of [^{35}S]methionine incorporation into RSV proteins allowed us to identify two proteins, molecular weights 25,000 and 18,000, made with the RNA of transforming viruses that are not synthesized with RNA from a nontransforming virus. The combined atomic mass, 43,000 daltons, matches the estimated size of the coding capacity of the *sarc* gene, 40,000–50,000 daltons as expressed in terms of protein products. With the exception of those two proteins, the products synthesized from the two RNAs appear to be identical on NaDodSO$_4$/polyacrylamide gels.

We think that the most likely interpretation of our results is that these two proteins represent the protein products of the *sarc* gene. The specific role of these proteins in the oncogenic transformation caused by these viruses is obscure. One can only speculate on their cellular function or site of action. The growth regulation and morphology of chick embryo fibroblasts in culture are especially sensitive to proteases, which cause changes that mimic to some extent the changes resulting from RSV infection (22). Chick embryo fibroblasts transformed by RSV produce a serine protease that has been claimed to be responsible for generating the transformed phenotype of cells (23). The *sarc* gene product might also be identical to the tumor-specific transplantation antigen, which is present on RSV-transformed cells but absent from cells infected with nontransforming RSV (24).

Genetic experiments have sometimes been interpreted to indicate the involvement of more than one *sarc* gene product in the transformation of infected fibroblasts. When certain combinations of temperature-sensitive *td* mutants are used to coinfect cells in culture, there is complementation between them (25). More recently, this complementation has been interpreted to be the result of high levels of recombination that occur in RSV (26). One means to evaluate the importance of these two putative *sarc* gene products in cellular transformation would be to analyze the proteins made from RNA of *nd* RSV and the various temperature-sensitive *td* RSV mutants by two-dimensional gel electrophoresis. If the electrophoretic mobility of one of these proteins changes as a result of a temperature-sensitive mutation, this would be definitive proof of its critical importance in oncogenic transformation.

Previous investigations with other cell-free translation systems have failed to detect differences in the gene products derived from the RNA of nondefective and defective avian sarcoma viruses. Von der Helm and Duesberg (27) achieved a 2-fold stimulation of [^{35}S]methionine incorporation with a 35S RSV RNA in a Krebs ascites cell-free system. Immunoprecipitation with antisera to the viral *gs* antigens was used to detect a 75,000-dalton product. Pawson *et al.* (28) compared the cell-free translation products of 35S RNA from various strains of RSV and found no difference in [^{35}S]methionine-labeled protein produced by RNA from *td* Prague B RSV and *nd* Prague RSV. In both cases, the major product was a 76,000-dalton protein. However, as our study shows, the percentage of [^{35}S]methionine incorporated into the putative *sarc* gene products is low, constituting less than 1% of the total [^{35}S]methionine incorporation into protein. Therefore, in the study reported by Pawson *et al.*, the high levels of endogenous [^{35}S]methionine incorporation in their assays probably would have prevented them from detecting these proteins even if they had been synthesized in their system.

Immunoprecipitation experiments with antisera to avian tumor virus structural proteins have confirmed that the two putative *sarc* gene products are not structural proteins of the virion. In addition to the *sarc* gene, avian sarcoma virus RNA is believed to contain three other genes (12). These genes code for the reverse transcriptase (60,000 daltons), the group-specific (*gs*) viral antigens, and the 85,000-dalton glycoprotein (and possibly a 35,000-dalton glycoprotein) of the virion. The gene coding for the group-specific antigens is believed to be translated in cells as a 76,000-dalton polypeptide that is cleaved to individual proteins of 27,000, 19,000, 15,000, and 12,000 daltons (29). Our results indicate that most of the protein larger than 30,000 daltons synthesized in our translation assays contains both the antigenic determinants of the *gs* antigens and the 85,000-dalton glycoprotein. In addition, there are four proteins smaller than 30,000 daltons that have *gs* antigenic determinants. The atomic masses of those proteins are 29,000, 19,000, 15,000, and 13,000 daltons, corresponding closely to the sizes of the *gs* antigens of the virus particle.

There are other reports that RNA from murine leukemia virus (30) and avian myeloblastosis virus (31) direct the synthesis of proteins that correspond in size to the *gs* antigens. It has been proposed that eukaryotic messenger RNAs contain only one initiation site and one termination site for protein synthesis (32). In the reticulocyte cell-free translation system, either the initial products are subject to cleavage or RSV RNA would appear to have internal initiation sites for both the *sarc* gene and some of the *gs* antigens, which are utilized at low efficiency. There are other reports of the existence of internal initiation sites for viral RNAs (33). In all these cases the sites seem to be relatively inactive.

Antiserum against the 85,000-dalton glycoprotein of the virion precipitates most of the labeled protein larger than 30,000 daltons, whereas antiserum against the 35,000-dalton glycoprotein of avian myeloblastosis virus does not precipitate any [^{35}S]methionine-labeled protein. It would appear that this protein is not coded for by the viral RNA or is not synthesized in these cell-free assays. More unlikely possibilities are that either the carbohydrate moiety, which would be absent from our translation products, is important in immunospecificity of *gp* 35 or this glycoprotein is not immunologically homologous in Prague B RSV and avian myeloblastosis virus.

The *in vivo* significance of the many large translation products we observed is not clear. No virus-specific protein larger than the 76,000-dalton *gs*-antigen precursor protein has been observed in RSV-infected cells. However, murine leukemia virus RNA also directs the synthesis of proteins up to 180,000 daltons in cell-free systems (34, 35). These proteins might represent artifacts of cell-free translation assays or have unrecognized *in vivo* significance as precursors to viral proteins.

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