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Multiple forms of *sarc* gene proteins from Rous sarcoma virus RNA

(cell-free translation/peptide maps/two-dimensional electrophoresis/reticulocyte lysate)

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ABSTRACT **In a previous study we were able to identify two proteins of 25,000 and 18,000 daltons that were made from RNA of transforming virions of Rous sarcoma virus (RSV) and that were missing from the translation products of a transformation-defective deletion mutant of RSV. In the present study we have separated RSV virion RNA on sucrose gradients and have determined that the two putative *sarc* gene products are synthesized as doublets from an mRNA of approximately 18 S. There also appear to be several other sizes of virion mRNA that direct the synthesis of other viral proteins. These data are discussed in terms of the structure of the RSV genome. In addition to the 25,000- and 18,000-dalton doublets, there also is a 60,000-dalton protein whose synthesis is directed by 18S viral RNA from transforming virion of RSV. Peptide mapping has shown that the 60,000- and the 25,000-dalton doublet are structurally related. In addition, the use of two-dimensional gel electrophoresis has allowed us to resolve both bands of the 25,000-dalton doublet into several differently charged species.**

The RNA of Rous sarcoma virus (RSV) is believed to contain a gene, termed the *sarc* gene, that is responsible for the cellular transformation of infected fibroblasts in culture and the induction of sarcomas in animals. Deletion mutants that lack this gene can infect cells and can replicate at the normal rate but are not capable of generating the transformed phenotype (1, 2). The existence of temperature-sensitive mutants of RSV for cellular transformation, which map in the *sarc* gene region, suggests that a protein coded for by this gene is specifically involved in cellular transformation (3). Oligonucleotide mapping studies of RNA from transforming RSV and deletion mutants have estimated the size of the *sarc* gene to be 10–15% of the 38S viral RNA (4, 5). Recent electron microscopic analysis of heteroduplexes between viral RNA and cDNA has estimated that the *sarc* gene may comprise 20% of the 38S viral genome, enough information to code for a 65,000-dalton protein (6).

In a previous study (7) we utilized a nuclease-treated reticulocyte lysate system to translate virion RNA of Prague B RSV and compared these translation products to those from RNA of a transformation-defective (*td*) deletion mutant of Prague B RSV. We were able to identify two proteins, 25,000 daltons and 18,000 daltons, on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels that were missing from the translation products of the *td* RSV, lacking the *sarc* gene.

In the present study we separated RSV virion RNA on sucrose gradients and determined that the two putative *sarc* gene products are synthesized as doublets from a mRNA of approximately 18 S. Similar results have been reported by Beemon and Hunter (8). There also appear to be several sizes of virion mRNA that direct the synthesis of other viral proteins. In addition to the 25,000- and 18,000-dalton doublets, there is a 60,000-dalton protein whose synthesis is directed by 18S viral RNA from transforming virions. Peptide mapping has shown that the 60,000-dalton protein and the 25,000-dalton doublet are structurally related. Recently, Brugge and Erickson (9) reported that avian tumor viruses induce a 60,000-dalton transformation-specific antigen in transformed cells. This antigen might be related to our cell-free translation product and further implicates this protein in cellular transformation. In addition, the use of two-dimensional gel electrophoresis has allowed us to resolve both bands of the 25,000-dalton doublet into several species with different isoelectric points.

MATERIALS AND METHODS

Cell Growth and Purification of Viral RNA. Chicken embryo fibroblasts were grown and infected with RSV and the viral RNA was prepared as described (7).

Cell-Free Translation. Reticulocyte lysates were prepared as described by Villa-Komaroff *et al.* (10). Treatment of the lysates with micrococcal nuclease was performed according to Pelham and Jackson (11). The assay procedure for cell-free translation of RSV RNA was carried out as described by Kamine and Buchanan (7).

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. The protein products synthesized in the cell-free reactions were analyzed on $NaDodSO_4$/polyacrylamide slab gels as described by Laemmli (12) with a 5–15% polyacrylamide separation gel. When electrophoresis was performed in two dimensions, gels were prepared and run as described by O'Farrell (13), with a 3–10 pH gradient for the first dimension and 5–15% $NaDodSO_4$/polyacrylamide gel for the second dimension. The gels were prepared for fluorography according to Laskey and Mills (14).

Peptide Mapping of Translation Products. Translation products labeled with [^{35}S]methionine (Amersham; 800–1200 Ci/mmol), equivalent to 10 μl of reticulocyte cell-free translation assay, were separated on 5–15% $NaDodSO_4$/polyacrylamide gels as described. After the gels were dried and exposed for autoradiography for 24 hr, the autoradiograms were used to locate bands to be cut from the gel. The bands were rehydrated and placed in sample wells of a second Laemmli $NaDodSO_4$/polyacrylamide gel (with a 15% acrylamide separating gel) and overlayed with 10 μl of a solution containing either *Staphylococcus aureus* protease (Miles Laboratories) or papain (Sigma), as described by Cleveland *et al.* (15). Dried gels were then subjected to fluorography as described (14). Exposure times were 1–8 days.

RESULTS

In a previous report (7) we described two proteins of 25,000 and 18,000 daltons (on the basis of $NaDodSO_4$/polyacrylamide gels) that were synthesized from 38S RNA of the transforming *nd*

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Abbreviations: RSV, Rous sarcoma virus; *nd*, nondefective; *td*, transformation-defective; $NaDodSO_4$, sodium dodecyl sulfate.

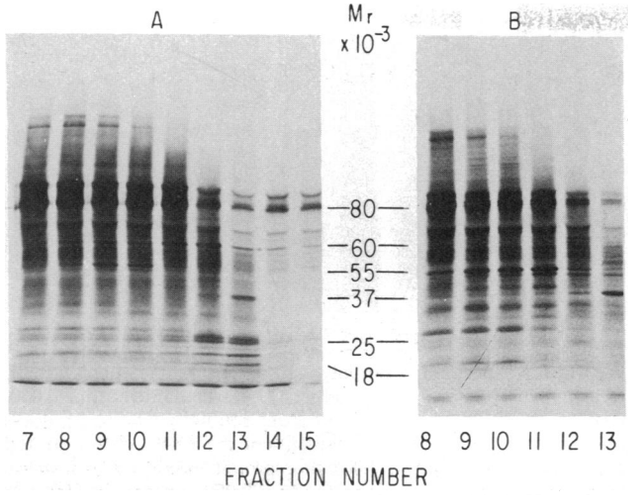

FIG. 1. Cell-free products from sized virion mRNA. Heat-denatured RSV RNA was sedimented through a 15–30% sucrose gradient. Cytoplasmic RNAs from Hela cells were used as markers in a companion gradient. Peak marker fractions: fraction 10, 28S rRNA; fraction 12, 18S rRNA; fraction 15, 4S tRNA. the RSV RNA of each fraction was precipitated with ethanol and 0.5 μg of the RNA of each fraction was used in 25-μl cell-free translation assays. Equal volumes of each translation assay, containing 20,000–100,000 cpm of incorporated [^{35}S]methionine, were applied to a 5–15% NaDodSO$_4$/polyacrylamide slab gel. Autoradiography was for 2 weeks. (*A*) Translation products from Prague B *nd* RSV RNA; (*B*) translation products from Prague B *td* RSV RNA. M_r, molecular weight.

virus and that were missing from the translation products of *td* RSV, lacking the *sarc* gene. In view of other reports that RNA tumor virus-infected cells contain viral specific RNAs of subgenomic size, we have separated virion RNA on sucrose density gradients for cell-free translation. In Fig. 1 the translation products of the viral RNA from each gradient fraction from *nd* Prague B RSV are compared to those from *td* RNA. As the RNA size decreased in succeeding fractions of the gradient, several bands either disappear or become much more intensely labeled. In particular, two doublets, of 25,000 daltons and 18,000 daltons, were much more evident in fractions 12 and 13; marker ribosomal 18S RNA from HeLa cells sedimented to this position in a companion gradient. Another band at 60,000 daltons also was synthesized maximally from the RNA of fraction 12. In fraction 13, a protein of 37,000 daltons became intensely labeled. In comparison, among the translation products of the *td* mutant of Prague B RSV, the 25,000- and 18,000-dalton doublets were missing and so was the 60,000-dalton protein translated from 18S RNA of the *nd* Prague B RNA. Both *nd* and *td* RSV RNAs appeared to synthesize the 55,000-dalton protein from 28S RNA (fractions 9–11) and the 37,000-dalton protein from an RNA slightly smaller than 18 S (fraction 13). Also, with both viral RNAs the proteins larger than 68,000 daltons appeared to be synthesized from an RNA of 38 S (fractions 7 and 8). Two proteins, 27,000 and 19,000 daltons, also were made from a 38S RNA. These are the sizes of two of the *gs* antigens of the virion, and we have previously shown (7) that both of these proteins as well as those larger than 68,000 daltons are immunoprecipitated with antiserum prepared against the viral *gs* antigens, or *gag* gene products.

By preparing microdensitometer scans of the autoradiograms shown in tracks 8, 12, and 13 of Fig. 1*A*, we were able to compare the relative amounts of the various proteins synthesized from 38S and 18S RNA of *nd* Rous sarcoma virus (Fig. 2). A similar comparison of the corresponding fractions for the RNA of the *td* virus is shown in Fig. 3. A 37,000-dalton protein be-

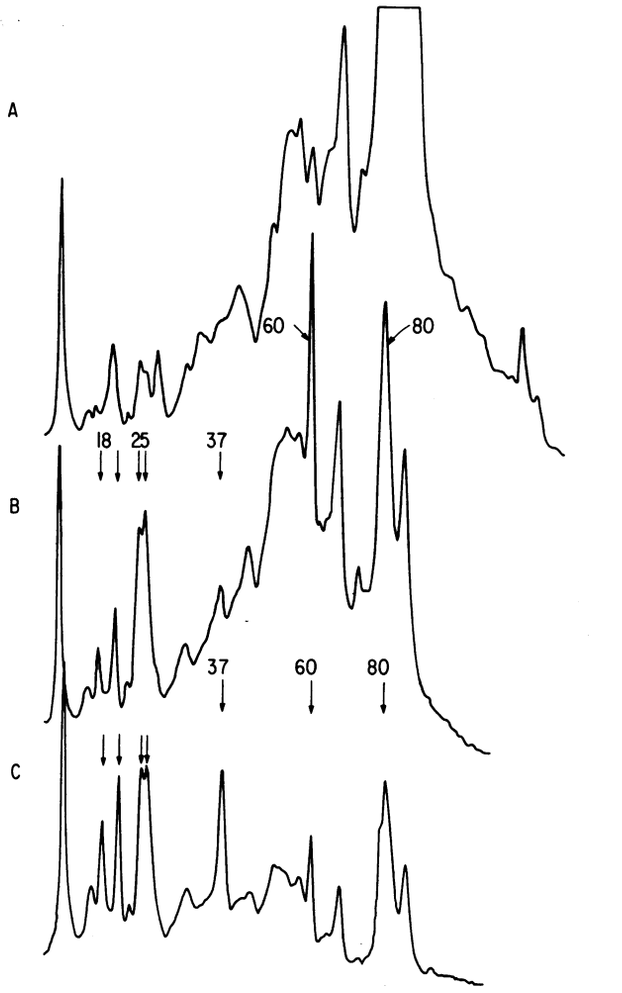

FIG. 2. Densitometer scans of tracks 8, 12, and 13 of Fig. 1*A*. (*A*) Track 8; (*B*) track 12; (*C*) track 13. Numbers and arrows show molecular weights $\times 10^{-3}$.

came heavily labeled in fraction 13 in the cases of both the *nd* and *td* RNAs. However, the 60,000-dalton protein appeared to be heavily labeled in fraction 12 of the *nd* RNA but not of the *td* RNA. Our failure to detect a *sarc*-specific protein of 60,000 daltons in previous experiments (7) resulted from our use of 38S viral RNA. Only in the translation products of lower molecular weight RNAs can the striking appearance of this protein be observed.

The *sarc* gene is probably only large enough to code for a protein of approximately 65,000 daltons. Therefore, to determine the relationship between the 60,000- and the 25,000- and 18,000-dalton doublets, we digested each of the proteins with proteases and analyzed the pattern of the polypeptide fragments on NaDodSO$_4$/polyacrylamide gels according to the peptide mapping procedure of Cleveland *et al.* (15). After cleavage by the *S. aureus* protease, three of the peptide fragments of the 25,000-dalton doublet exactly matched polypeptide fragments of the 60,000-dalton protein, including the major cleavage product (Fig. 4*A*). In contrast, the 80,000-dalton protein, used as a control, showed a completely different pattern of cleavage products compared to either the 25,000- or 60,000-dalton cleavage patterns. Somewhat surprisingly, the 18,000-dalton doublet had only one fragment in common with either the 60,000- or 25,000-dalton proteins (cf. ref. 8). The pattern of polypeptide fragments from the 80,000-dalton protein was strikingly similar to that from the 37,000-dalton

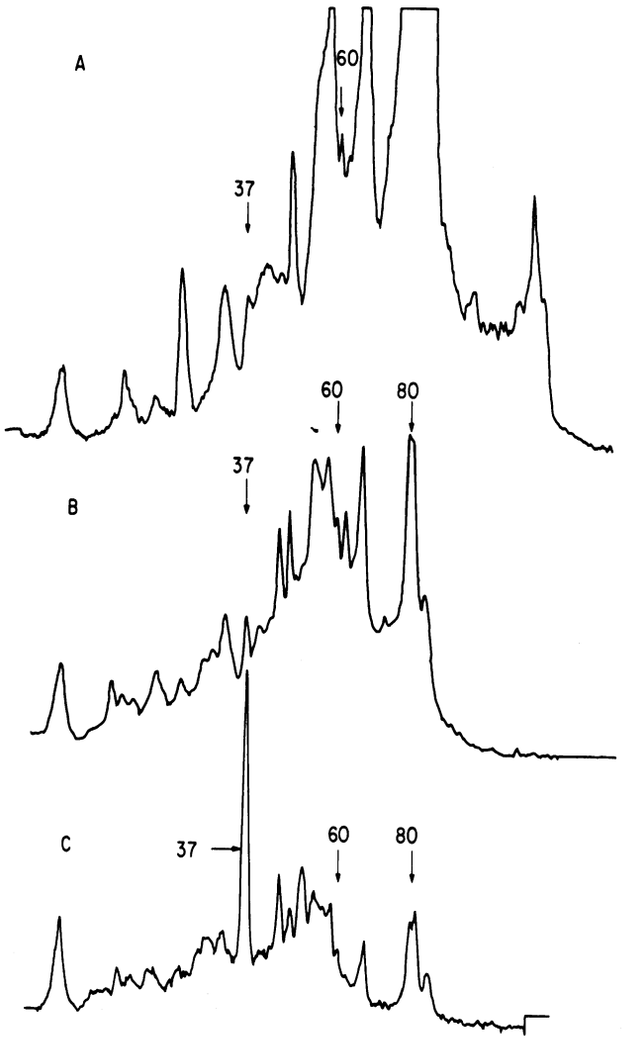

FIG. 3. Densitometer scans of tracks 8, 12, and 13 of Fig. 1*B*. (*A*) Track 8; (*B*) track 12; (*C*) track 13. Numbers and arrows show molecular weights $\times 10^{-3}$.

protein, with only one fragment of the 37,000-dalton protein missing from the 80,000-dalton pattern (Fig. 4*B*). After digestion with papain, the patterns of the polypeptide fragments of the 37,000- and 80,000-dalton proteins were similar to each other (Fig. 4*C*). Also, the patterns of the polypeptide fragments of the 60,000- and 25,000-dalton proteins were nearly identical to each other, whereas the 18,000-dalton protein peptide map was quite different from any of the others. However, the peptide maps of each of the five proteins appeared to be quite different from those obtained when the *Staphylococcus* protease was used. We conclude that the 60,000- and 25,000-dalton proteins are similar and are probably coded for by the same nucleotide sequence; the 80,000- and 37,000-dalton proteins also are similar to each other and are probably both precursors of the viral *gs* antigens.

In order to increase our resolution of the viral proteins synthesized in the cell-free translation assays, we used two-dimensional gel electrophoresis. The first dimension separates proteins on the basis of their charge in an isoelectric focusing pH gradient. The second dimension is a Laemmli Na-DodSO$_4$/polyacrylamide gradient gel, which we have previously used for one-dimensional electrophoresis and which separates proteins on the basis of their molecular weights. Fig. 5 compares the translation products of 38S RNA (fractions 8–10)

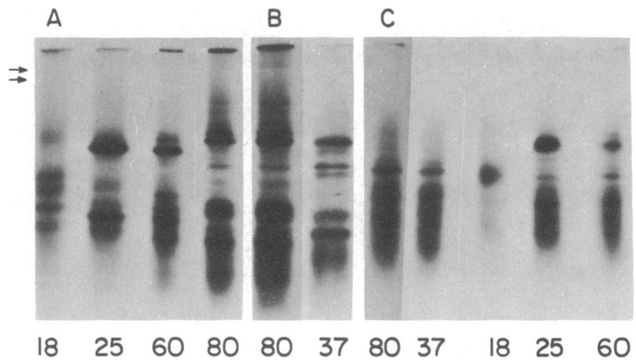

FIG. 4. Fluorographs of polypeptide fragments in peptide mapping analysis of translation products. (*A*) Fragments of the 80,000-, 60,000-, and 25,000- and 18,000-dalton doublet proteins, produced by *S. aureus* protease. (*B*) Fragments of the 80,000 dalton protein compared to those of the 37,000 dalton protein, produced by *S. aureus* protease. (*C*) Fragments of the 80,000-, 37,000-, 18,000-, 25,000-, and 60,000-dalton proteins produced by papain. Bands of the 60,000- and the 25,000- and 18,000-dalton doublet proteins were made from RNA of fraction number 12 of the sucrose gradients. The 37,000-dalton protein was synthesized from RNA of fraction number 13 and the 80,000 dalton protein was synthesized from RNA of fraction number 8. Arrows indicate positions of intact 80,000- and 60,000-dalton proteins on these gels.

of *nd* Prague B RSV and the corresponding fractions of *td* Prague B RSV. There were seven spots on the two-dimensional gel of the translation products from *nd* RSV RNA that were missing from the two-dimensional gel of *td* RSV RNA products. In addition to the 18,000-dalton *sarc* gene protein, a *sarc*-specific spot at 19,000 daltons also was detected. We had not previously observed this protein (7) because it comigrates with the 19,000-dalton *gs* antigen on a one-dimensional NaDodSO$_4$/polyacrylamide gel. The nominal isoelectric points of the 19,000- and 18,000-dalton proteins are 5.4 and 4.5, respectively. There also appeared to be two faint spots at 26,000 daltons on the gels of the translation products of *nd* RSV RNA that were missing from the *td* translation products. The 25,000-dalton protein separated into three species of different charge.

To enhance selectively the amounts of the *sarc* gene products, we analyzed the translation products made from 18S RNA on these gels. Fig. 6 compares the translation products of 38S RNA (fraction 8) and 18S RNA (fraction 12). Open triangles indicate the position of the *sarc* gene products in each fluorograph. All seven spots become darker in the products formed from 18S RNA compared to those from 38S RNA. In addition to the three 25,000-dalton *sarc* proteins, the 26,000-dalton *sarc* protein was resolved into two major spots with isoelectric points of 5.6 and 6.4. As yet, we have been unable to resolve the 60,000-dalton protein into discrete spots on these two-dimensional gels because all of the cell-free translation products of 60,000 daltons or larger streak along the isoelectric focusing dimension of the gels.

DISCUSSION

The RSV genome is believed to be composed of four genes located on a 38S RNA probably in the order: 5′-*gag-polymerase-envelope glycoprotein-sarc*-poly(A)-3′ (4, 5, 16). In addition to the 38S genomic RNA, RSV virions contain variable amounts of subgenomic RNA species ranging in size from 14 to 28 S (16). Corresponding sizes of viral specific RNAs have also been found in cells infected with RNA tumor viruses (17, 18).

In the present report we have shown that the putative *sarc* proteins are preferentially synthesized from a virion RNA

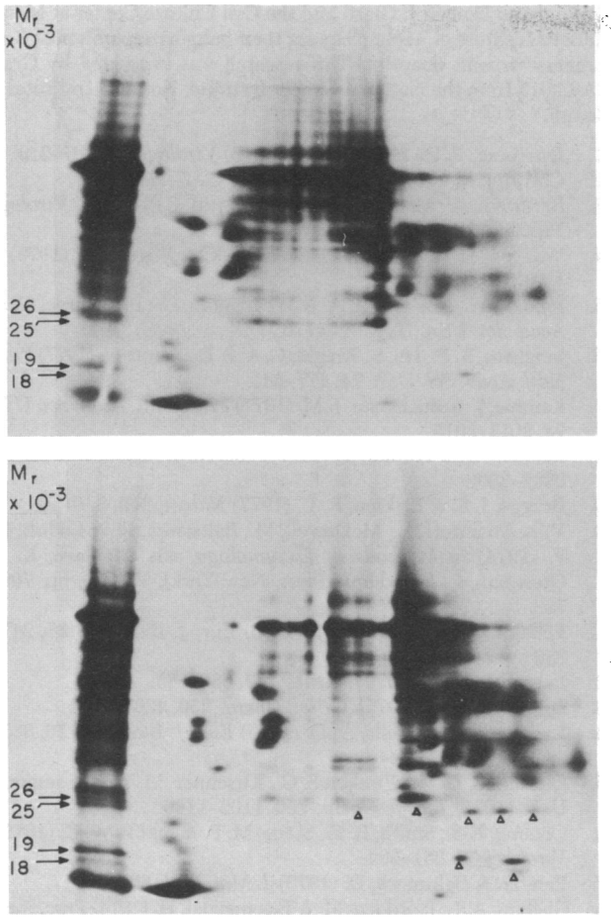

FIG. 5. Fluorographs of [^{35}S]methionine-labeled cell-free translation products separated by two-dimensional electrophoresis; 1×10^5 cpm was applied to each gel and film was exposed for 9 days. (*Upper*) Translation products of 38S RNA from *td* RSV. (*Lower*) Translation products of 38S RNA from *nd* RSV. Open triangles point to proteins unique to the translation products of *nd* RSV RNA.

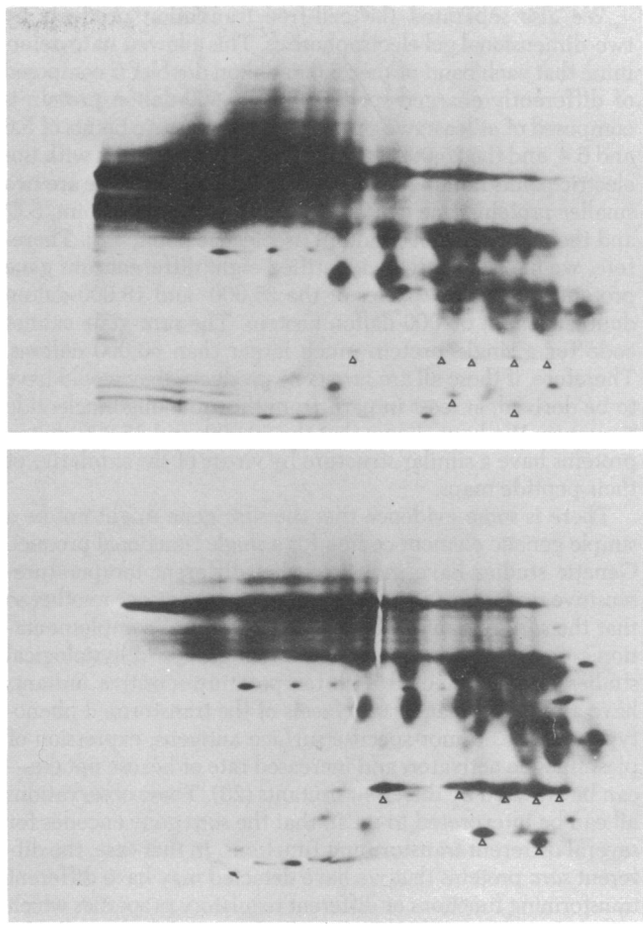

FIG. 6. Fluorographs of [^{35}S]methionine-labeled cell-free translation products of RNA from a temperature-sensitive mutant (ts 28) of Prague B RSV; 1×10^5 cpm was applied to each gel, and film was exposed for 10 days. Open triangles point to *sarc* gene products. (*Upper*) 38S RNA; (*Lower*) 18S RNA.

message of approximately 18 S as two doublets. Similar results have been reported recently by Beemon and Hunter (8). In addition to these proteins, we now show that a 60,000-dalton protein is also synthesized from 18S RNA of *nd* RSV. This protein is absent or is produced in much less amounts from RNA of *td* RSV, and therefore it may also be a *sarc*-specific protein. Estimations of the size of the *sarc* gene indicate it to be large enough to code for a 65,000-dalton protein (6). To determine whether the 60,000-dalton protein is related to the *sarc* gene products of 25,000 and 18,000 daltons, we compared their peptide maps. Regardless of the protease used, the polypeptide fragments of both the 60,000- and 25,000-dalton proteins have patterns that are similar overall, both in the position of the polypeptide fragments on the gels and in their relative amounts, whereas both these patterns are completely different from those of the 80,000- and 37,000-dalton translation products. We conclude that the 25,000- and 60,000-dalton proteins are derived, in part, from the same coding sequence of viral RNA. The polypeptides generated from the 18,000-dalton doublet, however, show only a slight resemblance to either the 60,000- or 25,000-dalton protein cleavage patterns, and we must tentatively conclude that the 18,000-dalton doublet has, in large part, an amino acid sequence that is different from the sequence of either of the other proteins (cf. ref. 8).

Besides 18S mRNA there appear to be three other sizes of viral mRNA found in both *nd* and *td* virions: (*i*) the 38S genomic RNA that yields the 80,000-dalton *gs* antigen precursor (*gag*), the major translation product, and the 27,000- and 19,000-dalton *gs* antigens; (*ii*) a 28S RNA that yields a 55,000-dalton protein; and (*iii*) an RNA slightly smaller than 18 S that yields a 37,000-dalton protein product. Previous studies have shown that cells infected with murine leukemia virus have not only 38S viral specific RNA in their cytoplasm but also small amounts of 28S, 21S, and 14S RNAs (17, 18). Other studies with RSV-infected cells have shown that the viral glycoprotein is synthesized from an RNA 20–30 S in size (19–21). It is possible that in our reticulocyte lysate system the 55,000-dalton protein made from 28S RNA is related to the viral glycoprotein. All this information seems to indicate that proteins corresponding to three of the four RSV genes, *gag*, *envelope*, and *sarc* might be synthesized from the 5′ end of separate mRNAs of subgenomic size. The reverse transcriptase on the other hand could be cleaved from the 180,000-dalton precursor containing the *gag* gene protein. This scheme for the translation of RSV genes is in accord with the belief that translation of eukaryotic mRNA can only be initiated at or near the 5′ end of the RNA (22). There is no evidence of how such viral mRNAs are generated or the significance of the fact that they are found within the virus particle. However, this scheme for the translation of RSV RNA supports our proposal that the protein products we synthesize in cell-free assays from an 18S RNA are in fact *sarc* gene products.

We also separated the cell-free translation products by two-dimensional gel electrophoresis. This allowed us to determine that each band of the 25,000-dalton doublet is composed of differently charged species. The 26,000-dalton protein is composed of at least two proteins with isoelectric points of 5.6 and 6.4, and the 25,000-dalton band, of three species with isoelectric points ranging from 4.2 to 5. In addition, there are two smaller proteins, one of 19,000 daltons (isoelectric point, 5.4) and the other of 18,000 daltons (isoelectric point, 4.5). Therefore, we have tentatively identified eight different *sarc* gene proteins: the seven species of the 25,000- and 18,000-dalton doublets and a 60,000-dalton protein. The *sarc* gene cannot code for a single protein much larger than 60,000 daltons. Therefore, if these all are *sarc* gene products, they would have to be derived, at least in part, from the same oligonucleotide sequence. We have shown that the 60,000- and 25,000-dalton proteins have a similar structure by virtue of the similarity of their peptide maps.

There is some evidence that the *sarc* gene might not be a simple genetic element coding for a single functional product. Genetic studies have indicated that different temperature-sensitive mutants can functionally complement one another so that the *sarc* region can be divided into four "complementation" or cooperative transformation groups. Physiological studies of cells infected with temperature-sensitive mutants have also found that certain facets of the transformed phenotype—such as tumor-specific surface antigens, expression of plasminogen activator, and increased rate of hexose uptake—can be affected by different mutants (23). These observations all can be interpreted to mean that the *sarc* gene encodes for several different transforming functions. In that case, the different *sarc* proteins that we have detected may have different transforming functions or different regulatory properties which affect their functioning or site of action within the cell. If a putative *sarc* protein has an altered chemical charge and hence an altered location on the two-dimensional gels as a result of a temperature-sensitive mutation in the *sarc* gene, such a finding would confirm that it possesses a transformation-specific function. Data of this kind might allow us to make correlations between the different *sarc* products that we can identify on the two-dimensional gels and the physiological properties of the different temperature-sensitive RSV-infected cells.

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1. Duesberg, P. H. & Vogt, P. K. (1973) *Virology* **54,** 207–219.
2. Coffin, J. M. (1976) *Cancer Res.* **36,** 4282–4288.
3. Bernstein, A., MacCormick, R. & Martin, G. S. (1976) *Virology* **70,** 206–209.
4. Wang, L-H., Duesberg, P., Beemon, K. & Vogt, P. K. (1975) *J. Virol.* **16,** 1051–1070.
5. Joho, R. H., Billeter, M. A. & Weissmann, C. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4772–4776.
6. Junghans, R. P., Hu, S., Knight, C. A. & Davidson, N. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 477–481.
7. Kamine, J. & Buchanan, J. M. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2011–2015.
8. Beemon, K. & Hunter, T. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3302–3306.
9. Brugge, J. S. & Erikson, R. L. (1977) *Nature* **269,** 346–348.
10. Villa-Komaroff, L., McDowell, M., Baltimore, D. & Lodish, H. F. (1974) in *Methods in Enzymology,* eds. Moldave, K. & Grossman, L. (Academic Press, New York), Vol. 30, pp. 709–723.
11. Pelham, P. B. & Jackson, J. (1976) *Eur. J. Biochem.* **67,** 247–256.
12. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
13. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250,** 4007–4021.
14. Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56,** 335–341.
15. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106.
16. Cheung, K-S., Smith, R. E., Stone, M. P. & Joklik, W. K. (1972). *Virology* **50,** 851–864.
17. Fan, H. & Baltimore, D. (1973) *J. Mol. Biol.* **80,** 93–117.
18. Gielkins, A. L. J., Salden, M. & Bloemendal, H. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1093–1097.
19. Pawson, J., Harvey, R. & Smith, A. E. (1977) *Nature* **268,** 416–420.
20. Van Zaane, D., Gielkens, A. L. J., Hesselink, W. G. & Bloemers, H. P. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1855–1859.
21. Stacey, D. W., Allfrey, V. G. & Hanafusa, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1614–1618.
22. Jacobsen, M. F. & Baltimore, D. (1968) *Proc. Natl. Acad Sci. USA* **61,** 77–84.
23. Wyke, J. A. (1975) *Biochim. Biophys. Acta* **417,** 91–121.