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Formation of a Sindbis Virus Nonstructural Protein and Its Relation to 42S mRNA Function

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Chicken embryo fibroblasts infected with an RNA⁻ temperature-sensitive mutant (*ts*24) of Sindbis virus accumulated a large-molecular-weight protein (p200) when cells were shifted from the permissive to nonpermissive temperature. Appearance of p200 was accompanied by a decrease in the synthesis of viral structural proteins, but [^{35}S]methionine tryptic peptides from p200 were different from those derived from a 140,000-molecular-weight polypeptide that contains the amino acid sequences of viral structural proteins. Among three other RNA⁻ *ts* mutants that were tested for p200 formation, only one (*ts*21) produced this protein. The accumulation of p200 in *ts*24- and *ts*21-infected cells could be correlated with a shift in the formation of 42S and 26S viral RNA that led to an increase in the relative amounts of 42S RNA. These data indicate that p200 is translated from the nonstructural genes of the virion 42S RNA and further suggest that this RNA does not function effectively in vivo as an mRNA for the Sindbis virus structural proteins.

The genome of Sindbis virus, an alphavirus of the togaviridae family, consists of a single strand of RNA of 4.2×10^6 daltons with a sedimentation coefficient of 42S (reviewed in reference 12). This RNA is infectious (3) and can serve as an mRNA in cell-free protein synthesis (6, 19). The genetic coding capacity for 42S RNA is about 400,000 daltons of protein. In tissue culture cells infected with Sindbis virus, a subgenomic RNA species about one-third the size of the genome is detected in amounts exceeding that of the genome RNA by four- to eightfold on a molar basis (2, 4, 23). This smaller RNA, with a sedimentation coefficient of 26S, contains the three genes encoding the three major structural proteins of the virion, and some of these proteins can be detected as products of cell-free protein-synthesizing systems to which 26S viral mRNA has been added (5, 6, 19). The coding capacity of the 26S RNA is about 140,000 daltons of protein, a value close to the sum of the molecular weights of the viral structural proteins. These proteins and their larger precursor forms are readily detected in extracts of most infected tissue culture cells, because Sindbis virus infection effectively shuts off host cell synthesis a few hours postinfection. On the other hand, those gene products exclusively made from 42S mRNA have yet to be identified, although recent reports of experiments with the closely related Semliki forest virus identify several "nonstructural" viral proteins (10). These nonstructural proteins are postulated to form a viral-specific replicase. In a survey of proteins made in cells infected with several temperature-sensitive mutants defective in viral RNA synthesis (RNA⁻ mutants), we observed the appearance of a large-molecular-weight protein. The size of this protein, which was estimated as 200,000 daltons, exceeds the coding capacity of the 26S mRNA, and we postulated that this protein, designated p200, is encoded by genes of the virion RNA not found in the 26S subgenomic species. In this communication, we present two kinds of data to support this hypothesis: (i) the analysis of the methionine-labeled tryptic peptides of p200, and (ii) a correlation between the formation of p200 and changes in the relative synthesis of 42S and 26S viral RNA. We also found that the formation of the viral structural proteins was not coupled to the synthesis of p200, and this result suggests that 42S RNA does not serve in vivo as a messenger for the viral structural proteins.

MATERIALS AND METHODS

Virus samples. The temperature-sensitive mutants were kindly provided by E. Pfefferkorn (Dartmouth University), and the yield of virus grown at 40°C relative to 30°C was 1.5×10^{-7} for *ts*24, 5×10^{-9} for *ts*4, 5×10^{-5} for *ts*6, 6×10^{-6} for *ts*21, and 1.4×10^{-4} for *ts*2.

Preparation and analysis of viral RNA. Monolayers of chicken embryo fibroblasts in 60-mm dishes were infected with wild-type or *ts* Sindbis virus mutants at a multiplicity of 100. Following 1 h of

adsorption, the plates received 8 ml of minimal essential medium containing 3% fetal calf serum (FCS) and 1 μg of actinomycin D per ml and were incubated at 30°C. At 12 to 14 h postinfection, one plate was labeled with [^{32}P]phosphate at 30°C, and several other plates were shifted to 40°C and labeled immediately or at various times after the shift. One hour before adding the isotope, the medium was replaced with a phosphate-free medium containing 1% FCS and actinomycin D. At the time of labeling, this medium was replaced with minimal essential medium lacking FCS and containing 500 μCi of [^{32}P]phosphate (carrier-free) per monolayer, 20% the normal concentration of phosphate, and 1 μg of actinomycin D per ml. After 1 h of labeling, the medium was removed and the cells were lysed with 0.5 ml of 2% sodium dodecyl sulfate (SDS) in 50 mM Tris-hydrochloride, pH 7.4, containing 1% 1,5-naphthalene disulfonate (disodium salt, Eastman Co.) to inhibit RNase activity (8). The cell extracts were briefly sonicated, and 7.5 μl of each sample was mixed with 2.5 μl of a buffer consisting of 10 mM Tris-hydrochloride, pH 7.4, 2% SDS, and 10% sucrose before electrophoresis in slab gels composed of 1.8% acrylamide and 0.5% agarose (22). The electrophoresis was at 100 V for 3 h, and the gel was dried under vacuum and exposed to X-ray film.

Labeling of viral proteins. The protocol for labeling proteins was essentially the same as for RNA, except that the cells did not receive actinomycin D and 1 h before labeling they were starved for amino acids instead of for phosphate. A 60-mm dish of cells was labeled with 10 μCi of L-[^{35}S]methionine (300 Ci/mmol, Amersham-Searle Corp.) in the presence of 10^{-6} M unlabeled methionine, and cells were lysed 1 h later with 2% SDS in 1 M Tris-hydrochloride, pH 9.0. Preparation and analysis of samples by electrophoresis in slab gels has been described previously (16).

Preparation and analysis of tryptic peptides. The [^{35}S]methionine-labeled p200 was obtained from cells infected with *ts*24 according to procedures described above, except that 50 μCi of L-[^{35}S]-methionine was used for 2×10^7 cells. The [^{3}H]-methionine-labeled p140 was obtained from cells infected with the RNA$^+$ mutant *ts*2 (3, 4) at 30°C for 5 h and shifted to 40°C for 1 h. L-[*methyl*-^{3}H]methionine (250 μCi, 6 Ci/mmol, Amersham-Searle Corp.) was added to two 60-mm dishes, and monolayers were harvested 3 h later. The [^{35}S]-methionine-labeled p140 was isolated from cells infected with *ts*2 at 30°C and shifted to 40°C after 5 h. Ten microcuries of L-[^{35}S]methionine and 10^{-6} M unlabeled L-methionine were added to 60-mm dishes at various times after the shift, and cells were harvested 1 h later. Several samples containing p140 were pooled for the final preparation. The [^{35}S]methionine-labeled capsid was obtained from nucleocapsids isolated from 4×10^6 infected cells given 100 μCi of L-[^{35}S]methionine for 1 h at 6 h postinfection. The cells were treated with 1% Triton X-100 in 0.05 M Tris, pH 7.5, 0.1 M NaCl, 1 mM EDTA (TNE), and the extract was centrifuged through a 15 to 30% sucrose gradient at 25,000 rpm for 2.5 h in a Spinco SW27 rotor. Samples from the single radioactive peak were heated with 2% SDS, and the capsid band was recovered after electrophoresis in SDS-polyacrylamide gels (16).

The ^{35}S-labeled proteins were separated on SDS-polyacrylamide slab gels, and bands were located by autoradiography. The p200, p140, and capsid were cut from the dried gel and treated twice for periods of 12 h with 3 ml of 1% NH_4HCO_3 containing 100 μg of TPCK-trypsin (Worthington Biochemical Corp.). The eluates were filtered, lyophilized twice, suspended in 100 μl of water, and applied to Whatman 3MM paper. Electrophoresis was at pH 3.5 for 60 min at 3.5 kV. The ^{3}H-labeled p140 was purified on an SDS-polyacrylamide cylindrical gel, and its position was located by counting samples from 1-mm gel slices that had been incubated with 0.4 ml of water at 37°C for 12 h. Samples of p140 were pooled, filtered, lyophilized, and digested for 12 h at 37°C in 1 ml of 1% NH_4HCO_3 containing 100 μg of TPCK-trypsin. An additional 100 μg of trypsin was added and digestion was continued for 12 h at 37°C.

Samples of ^{3}H-labeled p140 and ^{35}S-labeled p200 treated as above were suspended in 0.5 ml of 0.05 M pyridine acetate, pH 2.5, filtered, and applied to a column (0.9 by 20 cm) containing resin (Beckman AA 15) equilibrated at 55°C. The column was eluted at 40 ml/h with a gradient generated from four chambers containing 250 ml each of pyridine acetate buffers of 0.05 M (pH 2.5), 0.2 M (pH 3.1), 0.5 M (pH 3.75), and 2 M (pH 5.0). An additional 300 ml of the last buffer was used to wash the column after the gradient. Fractions of 4 ml were collected, evaporated to dryness at 60°C, and redissolved in 0.4 ml of water. After 10 min at 37°C, 4 ml of a xylene-Triton X-114 scintillation fluid (1) was added, and samples were counted in a Packard scintillation counter.

RESULTS

Pattern of viral proteins in shift-up experiments with the RNA$^-$ *ts*24 mutant. We have examined the array of proteins made in cells infected with a Sindbis virus temperature-sensitive mutant (*ts*24) that had been reported not to make viral RNA after infection at the nonpermissive temperature. To detect viral-specific proteins, cells were infected for several hours at the permissive temperature, a procedure that leads to shutoff of host cell synthesis, and then were shifted to the higher nonpermissive temperature. Radioactive amino acid was added immediately before the shift and at intervals thereafter. Under these conditions, a relatively large-molecular-weight protein was detected (Fig. 1). The apparent molecular weight of this protein was 200,000 as measured by comparing its mobility in 5% SDS-polyacrylamide gels with polypeptides of molecular weights 110,000, 153,000, and 210,000 obtained from poliovirus mRNA in vitro (kindly provided by L. Villa-Komaroff, Massachusetts Institute of Technology).

The accumulation of p200 at the nonpermissive temperature was accompanied by a decrease in the amount of labeled low-molecular-

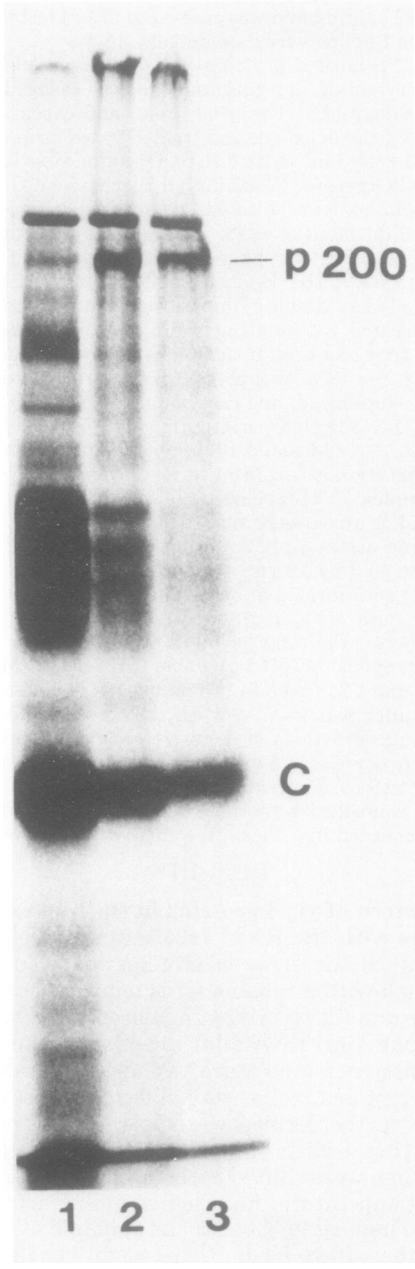

FIG. 1. *Autoradiogram of viral proteins from cells infected with ts24 and shifted from 30°C to 40°C. Sixteen hours after infection, cells were either labeled with [^{35}S]methionine at 30°C (1) or shifted to 40°C and labeled after 2 h (2) and 4 h (3). The amount of isotope incorporated into cell protein as trichloroacetic acid-precipitable cpm was (1) 400,000; (2) 200,000; and (3) 145,000. Two percent of the sample was added to slab gels containing 7.5% polyacrylamide in 0.1% SDS. Refer to Materials and Methods for experimental details. C refers to viral capsid and the radioactive bands in the midregion of the gel are viral envelope glycoproteins.*

weight viral structural proteins (Fig. 1). We considered two possible explanations for this result: (i) p200 is a precursor for viral structural proteins, or (ii) p200 is a nonstructural protein encoded by a gene present only in the 42*S* virion RNA, and translation of this gene is initiated and terminated separately from the translation of the viral structural protein genes that are present in the 42*S* virion RNA as well as in 26*S* viral RNA.

Tryptic peptide analysis of p200. To determine whether p200 was a precursor for viral structural proteins, we compared the electrophoretic mobilities of [^{35}S]methionine-labeled tryptic peptides from p200 with those obtained from a 140,000-molecular-weight protein (p140) that has been shown previously to contain the amino acid sequences of the structural proteins of the virion (17). The patterns of tryptic peptides from p140 and p200 are distinct (Fig. 2), and very few of the p140 peptides have mobilities comparable to those of p200. In addition, a large amount of the [^{35}S]methionine remains at the site of application in the electropherogram of the p140, whereas little is found in this region from a tryptic digest of p200. This material probably represents large-molecular-weight tryptic peptide "cores" of the protein. We also compared the electrophoretic mobilities of [^{35}S]methionine tryptic peptides from viral capsid with those of p200 and p140, and Fig. 2 shows that several of the p140 bands, but not those of p200, can be aligned with capsid peptides.

Co-chromatography of [^{35}S]methionine tryptic peptides from p200 with [^{3}H]methionine tryptic peptides from p140 on a Beckman AA15 resin provided additional evidence that the sequences of these proteins are different (Fig. 3). Thus, p200 appears to be encoded by a nonstructural gene of the viral genome.

RNA synthesis in cells infected with *ts*24. The translation of nonstructural genes at 40°C might be related to the abnormal synthesis of viral RNA that has been reported for the *ts*24 mutant. After a shift from the permissive to a nonpermissive temperature, *ts*24-infected cells showed a cessation of 26*S* RNA synthesis but continued formation of 42*S* RNA (13). We therefore measured the rates of synthesis of viral 42*S* RNA and 26*S* RNA under conditions that led to p200 formation. For rapid analysis of several samples, we labeled infected-cell monolayers with pulses of [^{32}P]phosphate, extracted RNA from the monolayer with SDS, and separated the RNA by electrophoresis in thin polyacrylamide slab gels. A densitometric tracing of a gel autoradiogram showed both a decline in total viral RNA synthesis and a significant

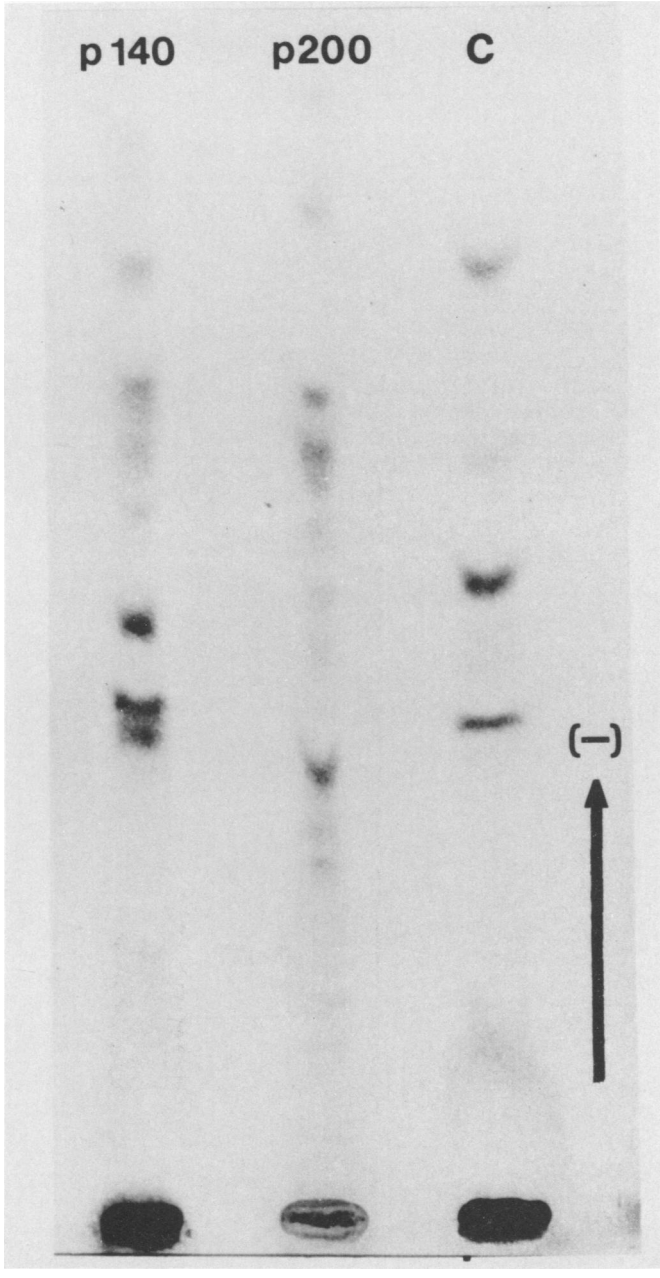

FIG. 2. *Autoradiogram of [^{35}S]methionine tryptic peptides separated by high-voltage electrophoresis. Preparation of samples is described in Materials and Methods; 40,000 cpm was used for C (viral capsid) and p140; 20,000 cpm was used for p200. Spots at the bottom are the site of application. The electropherogram was exposed for 15 days.*

change in the ratio of the 42*S* to the 26*S* species after the *ts*24-infected monolayer had been shifted to 40°C (Fig. 4). Quantitative measurements of the radioactivity incorporated into cells at 40°C showed that the rate of 42*S* RNA plus 26*S* RNA synthesis decreased by 46%, and the ratio of ^{32}P-labeled 42*S* to 26*S* changed from a value of 0.76 before the shift to a value of 2.5 4 h after the shift. The coincidental appearance of p200 with a marked increase in the ratio of 42*S* to 26*S* RNA is consistent with our hypothesis that only the 42*S* RNA encodes the structure for the p200.

Protein and RNA synthesis in cells infected

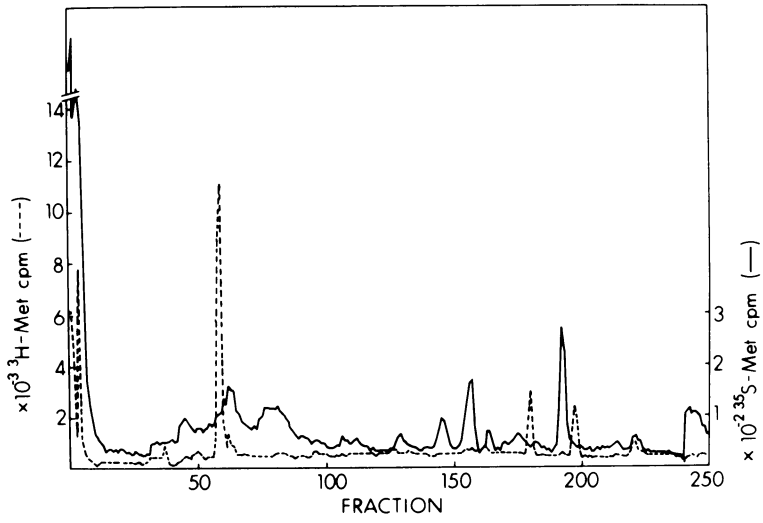

FIG. 3. *Pattern of tryptic peptides from [^{35}S]methionine p200 and [^{3}H]methionine p140 separated by chromatography on Beckman AA15 resin.*

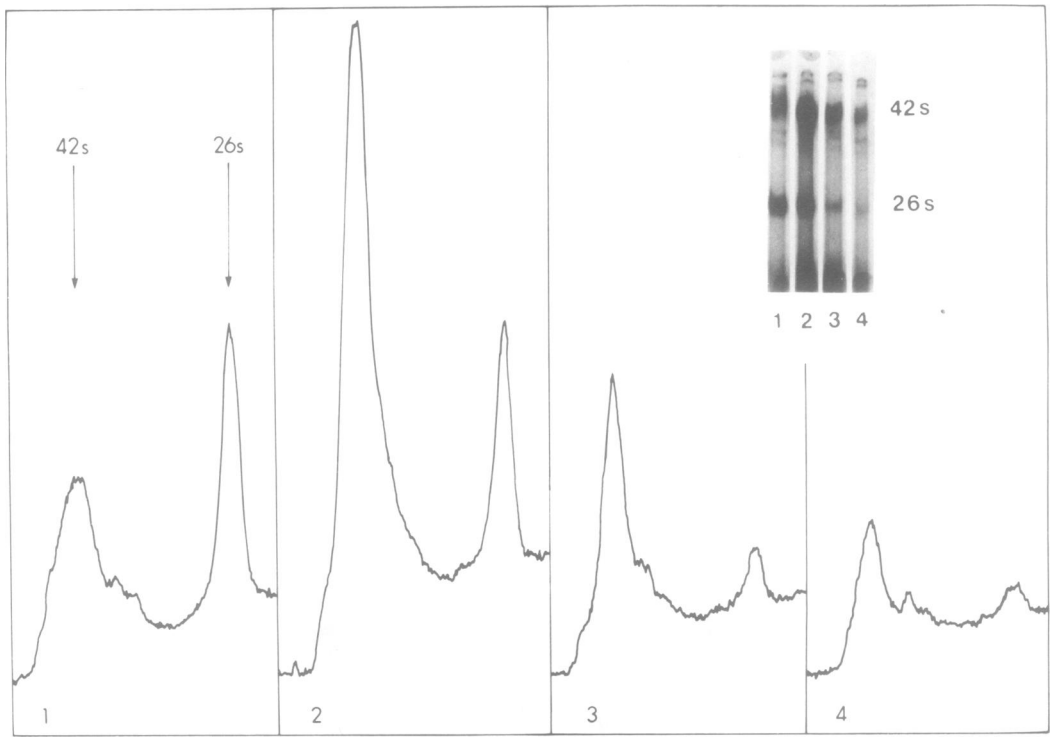

FIG. 4. *Pattern of viral RNA synthesis from cells infected with ts24 after a temperature shift. Cells infected at 30°C were shifted after 14 h to 40°C and labeled with [^{32}P]phosphate at various times for 1 h. Inset shows autoradiogram of the slab-gel electropherogram, and curves are densitometric scans of the autoradiogram. Sample 1 was labeled at 30°C, and samples 2 to 4 at 0, 2.5 h, and 4 h, respectively, after the shift.*

with different RNA⁻ mutants. Several other RNA⁻ mutants of Sindbis virus were tested for their ability to accumulate p200 in infected cells that were shifted to 40°C. The results from four representative samples of RNA⁻ mutants are presented in Fig. 5. Only cells infected with *ts*24 and *ts*21 yielded a p200 polypeptide, and the amount of p200 from *ts*21 was significantly less than that detected in cells infected with *ts*24. Cells infected with *ts*21 also accumulated

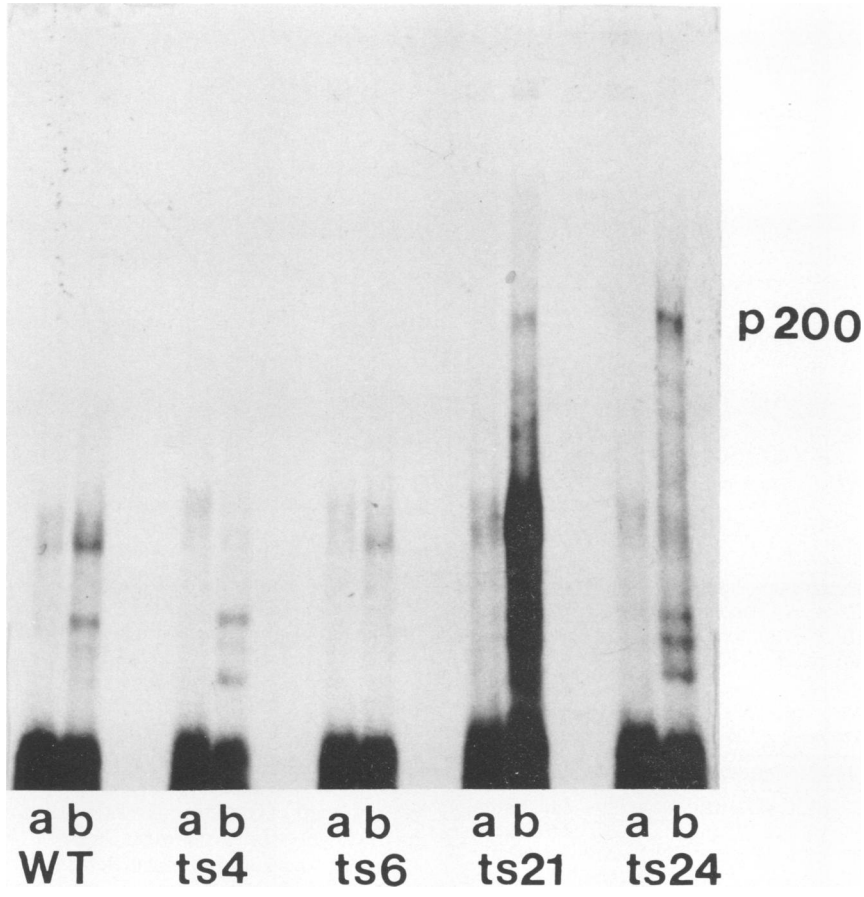

FIG. 5. *Autoradiogram of viral proteins formed in cells infected with various RNA⁻ ts mutants and shifted to 40°C after a 12-h incubation at 30°C. Samples were labeled either at 30°C immediately before the shift (a) or at 3 h after the shift to 40°C (b) with [³⁵S]methionine. A 5% polyacrylamide gel was used to resolve the high-molecular-weight proteins. The radioactive material at the bottom of the lanes consists of viral structural proteins that do not separate in the 5% gel. A total of 50,000 cpm was added to each well of the gel.*

large amounts of a protein of molecular weight 110,000 and failed to convert the viral PE-2 precursor glycoprotein to E2 at nonpermissive temperatures (data not shown). Thus, this strain probably contains two mutations, one of them leading to a defect that has been detected in several RNA⁺ mutants (M. Bracha and M. J. Schlesinger, Virology, in press). Several other proteins of molecular weights in the range of 70,000 to 90,000 were detected after the shiftup, and some of these may represent subunits of a viral replicase-transcriptase complex that could be formed by proteolytic processing of p200.

An analysis of viral RNA synthesis in cells infected with these four RNA⁻ *ts* mutants provided further evidence that the formation of p200 was coupled to an increase in the relative amount of 42*S* RNA. The patterns of [³²P]phosphate-labeled viral RNA produced in cells infected with *ts*21 and *ts*24 were distinct from that of two other *ts* mutants and wild-type virus (Fig. 6). The molar ratio of 42*S* RNA to 26*S* RNA synthesis for both *ts*21 and *ts*24, measured at 4 h after the shift to nonpermissive temperatures, was significantly greater than that determined for wild-type virus (Table 1). The p200 was detected when the molar ratio of 42*S* RNA to 26*S* RNA was greater than 0.5.

DISCUSSION

The results presented here show that the changes in viral RNA synthesis in cells infected with the *ts*24 mutant and shifted from permissive to nonpermissive temperatures were accompanied by two significant changes in the viral-specific protein pattern of the infected cell. First, a protein of about 200,000 molecular weight appeared and, second, the amounts of

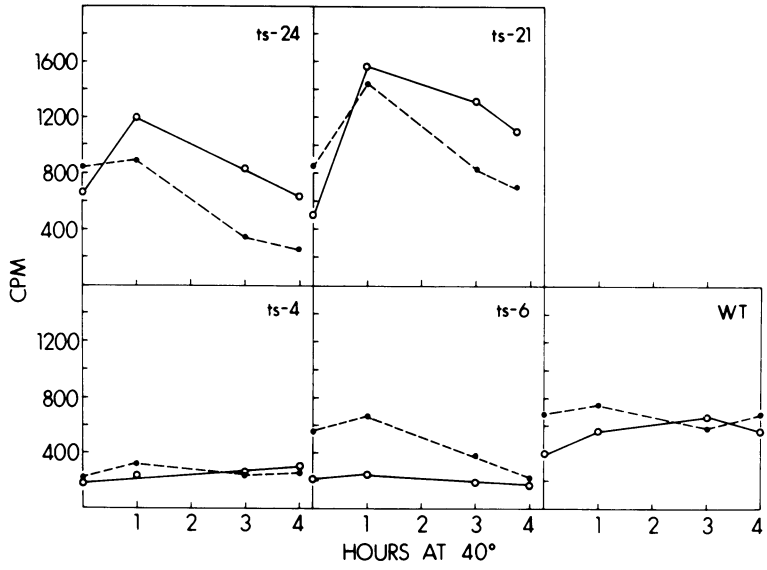

FIG. 6. *Viral rRNA synthesis in cells infected with Sindbis virus RNA⁻ mutants and shifted from 30°C to 40°C. The RNA was separated on slab gels by electrophoresis and bands were located from autoradiograms. Radioactivity in these bands was determined by cutting the gels and counting them in a scintillation counter. Symbols: 42S RNA (○); 26S RNA (●).*

TABLE 1. *Ratio of 42S to 26S RNA synthesis and appearance of p200 in RNA⁻ ts mutants*

Virus strain	42s/26S[a]	Presence of p200
Wild type	0.8 (0.29)[b]	Not detectable
*ts*4	1.2 (0.4)	Not detectable
*ts*6	0.75 (0.25)	Not detectable
*ts*21	1.6 (0.53)	Detectable (0.5)[c]
*ts*24	2.4 (0.80)	Detectable (3.0)

[a] Ratios were obtained by determining radioactivity in bands on slab gels corresponding to the appropriate RNA species. These values were measured on extracts of cells shifted to 40°C for 4 h.

[b] Figures in parentheses represent molar ratios of 42S to 26S.

[c] Figures are percentages of total methionine-labeled viral proteins detected in slab gels that are in the p200 band.

the structural proteins of the virus diminished during the labeling period. Tryptic peptide analyses offer convincing evidence that the large protein does not contain the sequences of the viral structural proteins; thus, we conclude that this protein is encoded by genes found only in the 42S RNA. These are the genes presumed to encode the structure of the viral replicase, and the p200 could represent either a part of the replicase or a precursor to smaller subunits making up the replicase. Preliminary studies on the stability of p200 have shown that 40% of p200 that accumulated in a 1-h pulse disappeared after a 2-h chase at 30°C, but none was chased at 40°C. We are currently searching for those polypeptides that form the Sindbis virus replicase. Semliki forest virus replicase has recently been reported to contain subunits of molecular weights 90,000 and 70,000 (10; S. I. T. Kennedy, personal communication).

We have found that an in vitro protein-synthesizing system programmed with virion 42S RNA as a messenger can form very large polypeptides (D. Sagher and M. J. Schlesinger, unpublished data)—up to 200,000 molecular weight—and we plan to compare the tryptic peptides of the in vitro products with the p200 isolated and studied here.

Earlier results from an in vitro protein-synthesizing system indicated that Sindbis virion 42S RNA has two potential sites for initiation of translation of polypeptides, presumably one for the replicase genes and the second for the structural genes (7). During viral replication, that portion of the 42S coding for the structural genes is amplified in the form of a 26S mRNA species. The latter functions as a typical eukaryotic polycistronic mRNA in that a single site exists for initiation of translation, and a large precursor polypeptide is produced and undergoes post-translational proteolytic cleavages (7, 12, 15, 17). On the basis of data presented here we suggest that the nonstructural genes of 42S mRNA are expressed according to this general principle. What is not clear is whether the potential internal initiation site—believed to be

analogous to the 26S RNA initiation site—on the 42S RNA can function. Our observation that the synthesis of viral structural gene proteins decreases under conditions in which 42S RNA production and translation continue suggests that this RNA is not serving as an mRNA for structural gene expression. On this basis, we believe that the internal initiation site functions ineffectively or possibly not at all in vivo. Results from in vitro translation of 42S mRNA also indicate that the putative internal initiation site may not function (7, 19). Initiation at internal sites of an mRNA is well documented for prokaryotic mRNA's (11, 20) but has not yet been described in higher organisms. In fact, there are several cases described in which the expression of genes in an mRNA would appear to depend on the formation of subgenomic species of that mRNA (9, 18).

We attempted to correlate the preferential translation of 42S mRNA over the 26S RNA with the relative rates of synthesis of the two species. The molar ratio of 42S RNA to 26S RNA was 0.29 late in the infection of cells by wild-type virus, and most of the 42S RNA was probably in nucleocapsids and unavailable for translation. A doubling in this ratio was detected in cells that also produced p200, indicating that the excess 42S RNA was in polysomes. This extra amount of 42S RNA was apparently able to effectively compete with 26S RNA for initiation factors and ribosomes, and thereby interfere with viral structural protein synthesis. A decrease in the latter would in turn limit the encapsidation of 42S RNA and allow for additional amounts of 42S RNA to bind ribosomes.

It is not known how the defect in *ts*24 and *ts*21 mutants alters the synthesis of the two species of viral RNA. Scheele and Pfefferkorn (13) postulated that a "conversion protein" is required for formation of 26S RNA, and this protein is temperature sensitive in the *ts*24 mutant. After the temperature shift, a decreasing amount of both species of viral RNA was observed; thus, the mutation produced two changes in Sindbis viral RNA synthesis. We can account for the decreased synthesis in total viral RNA and the concomitant appearance of p200 by postulating that the active form of the Sindbis virus replicase-transcriptase complex requires a proteolytic processing of a p200 precursor, and the mutational alteration in *ts*21 and *ts*24 inhibits this processing at the nonpermissive temperature. A similar kind of mutation has been found in Sindbis virus RNA$^+$ mutants and prevents the formation of the individual viral structural proteins (14). The mutational alteration in p200 that affects processing at nonpermissive temperatures may also be present in one of the putative lower-molecular-weight proteins that is derived from p200 and forms a replicase-transcriptase complex when the *ts*24 and *ts*21 mutant viruses are grown in cells at permissive temperatures. If this mutationally altered subunit were the temperature-sensitive conversion protein cited above, then synthesis of 26S RNA would be inhibited after infected cells were shifted from the permissive to nonpermissive temperatures. This model can explain how one mutation can lead to three effects: a defect in total RNA synthesis, a change in RNA species, and the appearance of p200 after the temperature shift.

Another RNA$^-$ *ts* mutant of Sindbis virus (*ts*11) has been shown to accumulate a large-molecular-weight protein (133,000) under conditions that also favored an increase in the relative amount of 42S RNA synthesis (21). Further studies with these mutants and with cell-free replicase complexes should reveal the mechanism for viral 26S RNA formation and the function of subunits in the Sindbis virus replicase.

ACKNOWLEDGMENTS

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