Coding Assignments of Double-Stranded RNA Segments of SA 11 Rotavirus Established by In Vitro Translation

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The segmented double-stranded (ds) RNA genome of the simian rotavirus SA 11, after denaturation, can be translated in a cell-free protein synthesizing system. Of the 11 genome segments, 9 can be resolved on polyacrylamide gels and thus could be individually isolated and translated, providing a means of identifying the polypeptide encoded by each segment. On the basis of electrophoretic mobility of products in sodium dodecyl sulfate-polyacrylamide gels, the probable gene-coding assignments of dsRNA segments 1 to 6 were determined. RNA segments 1 to 4 code for polypeptides I₁, I₃, I₅, and I₆, respectively; segment 5 codes for a polypeptide very similar in mobility to a minor polypeptide present in SA 11-infected cells, O₁A; and segment 6 codes for the major inner-capsid polypeptide I₅.

Rotaviruses are a common cause of enteritis in the young of many, if not all, mammalian species (8, 10), including humans. Biochemical and biophysical studies have shown that the rotavirus genome consists of 11 segments of double-stranded (ds) RNA (11, 21, 25, 32, 33). The simian rotavirus SA 11 (14, 22, 26) is readily cultivable in cell culture and is thus a useful model rotavirus for detailed laboratory study. Polyacrylamide gel electrophoresis of genomic RNA produces 10 bands, 1 band being composed of two RNA segments (segments 7 and 8) of equal mobility (20). Structural polypeptides of purified SA 11 virus have been well characterized and are similar to those of other rotaviruses (22, 32). Few studies of the virus-coded polypeptides in infected cells have so far emerged (16, 29); however, it appears that in addition to nine structural polypeptides, there are at least three nonstructural polypeptides.

Recently, a method for assigning cognate RNA and polypeptide species for reovirus was published (17). Reovirus also contains a dsRNA segmented genome (24). By isolating individual dsRNA segments, denaturing them, and then adding them to a cell-free translation system, we could analyze the proteins produced, and compare them with true reovirus polypeptides. This enabled coding assignments to be made.

With this method the polypeptides encoded by each of the first six dsRNA segments of SA 11 rotavirus have been determined. We regret that, due to the difficulty of separating segments 7, 8, and 9, and the apparent failure of our translation system to synthesize products related to rotavirus glycoproteins, we are unable to present assignments for the remaining segments at this stage.

MATERIALS AND METHODS

Cells and virus. The fetal rhesus monkey kidney cell line MA104 was a gift from S. Matsuno. Cultures were grown in Eagle minimum essential medium containing nonessential amino acids, 10% fetal calf serum, and antibiotics (penicillin and streptomycin). SA 11 virus was kindly supplied by H. Malherbe and was plaque-purified twice in MA104 cells. A first-passage stock (1.1 × 10⁶ PFU/ml) was used throughout this study. Virus stocks were infected cell lysates prepared by freeze-thawing and were stored at −70°C.

Virus growth and purification. Confluent monolayers of MA104 cells were washed with phosphate-buffered saline and infected with a dilution of plaque-purified virus stock in virus diluent (Hanks balanced salt solution, 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonyl acid [HEPES], 0.02% gelatin) at a multiplicity of infection between 0.1 and 1 PFU/cell.

After an adsorption period of 1 h at 37°C the inoculum was decanted and maintenance medium was added (minimal essential medium, 0.05% bovine serum albumin). The cells and culture fluid were harvested at 3 days postinfection, at which time gross cytopathic effects were evident. Initial fluorocarbon extraction was performed by the method of Rodger et al. (21). The resultant fluid was centrifuged at 24,000 rpm for 2.5 h at 4°C in a Spinco SW25.2 rotor, the virus being pelleted through 8 ml of 35% (wt/wt) sucrose in 0.002 M Tris-hydrochloride (pH 7.5). The concentrated virus was sonicated for 15 s and then banded in CsCl and concentrated by centrifugation as described by Rodger et al. (21).

Plaque assay for SA 11. The method of Matsuno et al. (15) for the plaque assay of Nebraska calf rotavirus was adapted by S. Rodger in this laboratory for SA 11 rotavirus (unpublished data). Briefly, confluent monolayers of MA104 cells in plastic culture dishes were washed once with phosphate-buffered saline, the virus inoculum (suitably diluted in virus diluent) was added, and virus was allowed to absorb for 1 h at 37°C. Then the inoculum was removed and overlay medium (minimal essential medium, 0.06% bovine serum al-
were continued for reservoir was gel buffer, dissolved was gel as reovirus (23).

Preparation of [3S]methionine-labeled SA 11 virus. Cells were infected as described above. At 6 h postinfection, the maintenance medium was replaced by medium containing only 10% of the usual methio-
nine concentration and 5 μCi of [3S]methionine (1,300 Ci/mmol; Amersham Corp.) per ml. At 9 days postinfection the infected cultures were harvested, and the virus was purified as described.

Preparation of labeled intracellular rotavirus-coded polypeptides. Confluent monolayers of MA104 cells in 35-mm plastic petri dishes were washed with phosphate-buffered saline and infected with SA 11 virus at a multiplicity of 10 PFU/cell. Virus was allowed to adsorb for 1 h at 37°C, and then the inoculum was removed and maintenance medium was added. At 10 or 12 h postinfection, the medium was changed to maintenance medium without methionine. After 30 min the medium was again changed to main-
tenance medium containing methionine at 10% of its normal concentration and 10 μCi of [3S]methionine per ml. Incubation was continued for 2 h, after which the cells were washed twice with ice-cold phosphate-buffered saline and dissolved in 200 μl of Laemmli sample buffer (12). DNA was sheared by passage through a 25-gauge needle.

Extraction of dsRNA. Purified SA 11 virus in 0.002 M Tris-hydrochloride (pH 7.5) was made up to 1 ml with STE (0.15 M NaCl, 0.05 M Tris-hydrochloride, 1 mM EDTA, pH 7.5) buffer, and sodium dodecyl sulfate (SDS) was added to a final concentration of 1% (wt/vol). After 30 min at 37°C the solution was ex-
tacted three times at room temperature with an equal volume of water-saturated phenol. The aqueous phase was made 0.3 M with respect to sodium chloride, residual phenol was removed, and RNA was precipi-
tated at −20°C by the addition of 3 volumes of ethanol. The precipitate was collected by centrifugation, washed three times with 90% ethanol, dried in air, and dissolved in 50 μl of distilled water. The amount of RNA was estimated by using the relation: one unit of optical density at 260 nm = 50 μg of dsRNA per ml for reovirus (23). Convenient amounts (50 or 100 μg) were stored as ethanol precipitates at −70°C.

Fractionation of individual dsRNA species. Preparative polyacrylamide slab gels (10%, 1.5 mm thick) were prepared by the method of Laemmli (12) but without a stacking gel. Before loading, the RNA was dissolved in Laemmli sample buffer and heated to 70°C for 2 min to resolve RNA aggregates (27). The gel was preelectrophoresed for 1 h at 40 mA with lower gel buffer, then the lower gel buffer in electrode reser-
voirs was replaced with Laemmli reservoir buffer, the RNA sample was loaded, and electrophoresis con-
tinued for 2 h (room temperature, 25 mA). The gel was stained with 0.005% ethidium bromide in 20 mM sodium acetate (pH 7.8) (27) for 15 min, and the bands were visualized under UV light. The bands were ex-
cised with a scalpel blade and eluted by diffusion as follows. The gel pieces were crushed through an 18-
gauge syringe needle into 2 ml of STE buffer. After being shaken at room temperature overnight, the gel pieces were extracted again with a further 1 ml of STE buffer. The buffer phases were pooled and processed as described below.

Preparation of RNA segments for translation. The RNA segments eluted from gels were treated as described by McCrae and Joklik (17). Briefly, this involved extraction with isoaamyl alcohol to remove ethidium bromide, then phenol extraction to remove monomeric acrylamide, and finally ethanol precipita-
tion. The precipitate was washed twice in 80% ethanol, washed once in 100% ethanol, dried in air, and dis-
solved in 6 μl of 90% (vol/vol) dimethyl sulfoxide (Me2SO) in water. The RNA was stored at −20°C until required. For translation, 2 μl of solution was removed and heated to 30°C for 5 min, and then the translation reaction mixture was added.

In vitro translation. Denatured dsRNA was translated in a wheat germ system prepared essentially as described by Roberts and Patterson (19). The reaction mixture contained: 20 mM HEPES (pH 7.6), 36 mM KCl, 1 mM magnesium acetate, 2 mM dithiothre-
tol, 1 mM ATP, 20 μM GTP, 8 mM creatine phos-
phate, 30 μg of creatine phosphokinase per ml, 0.2 mM Spermidine, to each of 19 unlabeled amino acids at 20 to 30 μM, 5 to 20 μCi of [3S]methionine (1,300 Ci/ mmol Amersham), 10 μl of wheat germ (S-30) extract, and about 2 μg of individual denatured dsRNA seg-
ments. The final volume was 50 μl. Reactions were incubated for 60 min at 30°C; then the reaction was terminated by chilling to 0°C. Samples of 5 μl were withdrawn, spotted onto glass fiber disks (Whatman, GP/A), and processed for trichloroacetic acid-precipitable radioactivity by the method of Roberts and Patterson (19).

Analysis of translation products and viral polypeptides. Translation products and viral pro-
teins were analyzed in SDS-polyacrylamide slab gels by using the Laemmli (12) Tris-glycine discontinuous buffer system. The acrylamide/bisacrylamide ratio was 30:0.8. SDS was not present in gels but only in reservoir buffer (0.6%) and loaded samples (1%) before commencement of electrophoresis, as suggested by Wyckoff et al. (34). Protein samples to be analyzed were treated with Laemmli sample buffer (12), (1% SDS, 2% 2-mercaptoethanol, final concentrations) and heated to 100°C for 5 min before being loaded onto the gel. Electrophoresis was performed at room tem-
perature with a current of 25 mA per gel. Fluorographs were prepared as described by Bonner and Laskey (1) and Laskey and Mills (13).

Molecular weight determinations. The molecu-
lar weights of SA 11 polypeptides were determined by comparison with standard protein molecular weight markers run on the same slab gel. The protein stand-
ards used were: β-galactosidase (Escherichia coli, mo-
cellular weight 130,000), phosphorylase b (rabbit mus-
cle, molecular weight 94,000; Worthington Biochemi-
cals Corp.), bovine serum albumin (molecular weight 68,000; Commonwealth Serum Laboratories, Aus-
tralia), catalase (bovine liver, molecular weight 60,000), immunoglobulin G (heavy chain) (rabbit, mo-
lecular weight 53,000; D. Jackson), L-lactic dehydrogenase (bovine heart, molecular weight 36,000), carbonic anhydrase (bovine erythrocytes, molecular weight 29,500), a-chymotrypsinogen A (bovine pancreas, 25,700), myoglobin (whale skeletal muscle, 17,200). All except immunoglobulin G, phosphorylase a, and bovine serum albumin were obtained from the Sigma Chemical Co.

RESULTS

Translation of unfractionated dsRNA. When suitably denatured, the dsRNA segments of the SA 11 genome stimulated the incorporation of [35S]methionine into trichloroacetic acid-precipitable material in a wheat germ cell-free protein synthesizing system. Figure 1 presents several parameters of this reaction. Addition of undenatured dsRNA up to 100 μg/ml did not inhibit endogenous activity (amino acid incorporation in the absence of any added mRNA) (9); however, no stimulation of [35S]methionine incorporation occurred either. Dimethyl sulfoxide (Me2SO) at the concentration used in this study (3.6%), did not inhibit endogenous activity (results not shown), in agreement with the findings of McCrae and Joklik (17).

Denaturation with 90% Me2SO did not require heating to 50°C, as was necessary for reovirus dsRNA (17). In fact, normal room temperatures were adequate, although equilibration to 30°C was chosen for convenience, because this was the temperature at which the wheat germ system was incubated. Once optimal concentrations of KCl and Mg were determined (39 and 1 mM, respectively) they were used throughout further experiments.

An RNA concentration (80 μg/ml) sufficient to saturate the translation system was selected on the basis of preliminary studies (results not shown) for the optimization and time course experiments.

Although high levels of incorporation were observed upon addition of rotavirus RNA, the specific stimulation of incorporation (i.e., the ratio of the incorporation in a system with added RNA to that in a system with no added RNA) was not as high as expected (maximum of about eightfold) due to significant endogenous incorporation by the particular wheat germ preparation used in this study.

Polyacrylamide gel analysis of rotavirus-coded polypeptides. Since identification of

![Figure 1](http://jvi.asm.org/)
\[^{35}S\]methionine-labeled translation products of denatured dsRNA was to be made on the basis of electrophoretic mobility, it was first necessary to establish the migration patterns of rotavirus polypeptides in the SDS-polyacrylamide slab gel system employed.

This gel system gave good resolution of all known rotavirus polypeptides. Figure 2 shows typical patterns of SA 11 virion (structural) proteins, and also virus-specific proteins that appear in \[^{35}S\]methionine-labeled infected cells. Nomenclature of these polypeptides is based upon the system of Thouless (29), in which viral proteins are designated by whether they are inner (I) capsid structural, outer (O) capsid structural, or nonstructural (NS) proteins. They are numbered from highest to lowest molecular weight. SA 11 virus possesses five inner (I1 through I5) and four outer (O1 through O4) capsid polypeptides. In our modified system, I4 and I5 are equivalent to I3 and I4, respectively, in the system of Thouless (29) (see Discussion). The number and pattern of the proteins observed in preparations of purified virus are essentially the same as described by Rodger et al. (22). In addition to structural proteins, virus-infected cells display two polypeptides not seen in purified virus and thus considered nonstructural (NS1 and NS2).

![Fig. 2. SDS-polyacrylamide gel analysis of \[^{35}S\]methionine-labeled, purified SA 11 virus (V and V'); virus-infected (IC) and uninfected (UC) MA104 cells; and translation products of a wheat germ system without added RNA (—), or programmed with unfractionated Me2SO-treated SA 11 dsRNA (+). V' is the same as track V except that it is a longer exposure to show the minor outer capsid polypeptides O3 and O4. Analysis was on a 10% slab gel.](image)

**Table 1. Molecular weight estimates of SA 11 rotavirus polypeptides**

<table>
<thead>
<tr>
<th>Polypeptide</th>
<th>mol wt (x10^-4)</th>
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<tbody>
<tr>
<td>I1</td>
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</tr>
<tr>
<td>I2</td>
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</tr>
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<td>I3</td>
<td>88</td>
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</tr>
<tr>
<td>O3</td>
<td>28</td>
</tr>
<tr>
<td>O4</td>
<td>26</td>
</tr>
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* Molecular weight estimates were determined by polyacrylamide gel electrophoresis, using protein standards of known molecular weight.

[Fig. 2]. A protein observed in SA 11-infected cells but not in pure virus was designated O1A. As first suggested by Thouless, this protein is possibly the precursor to O1; however, since O1A is a minor product (see Fig. 2 and 4), the relationship between these two proteins will be difficult to elucidate. The molecular weights of all SA 11 proteins are given in Table 1.

A minor outer capsid protein, O5, was observed only occasionally in virus-infected cells. This protein was also difficult to detect in \[^{35}S\]methionine-labeled cells infected with other rotaviruses (29). However, it was always present in preparations of purified SA 11 virus (Fig. 2).

Virus infection of cells considerably reduced host cell protein synthesis, allowing virus-specific proteins, including those produced in small quantity, to be observed clearly (Fig. 2).

**Identification of the in vitro translation products of unfractionated dsRNA.** The translation products of denatured rotavirus dsRNA were analyzed on polyacrylamide slab gels as described above, and the results are shown in Fig. 2. Products identical in mobility to I1, I2, I3, I4, I5, and NS2 were observed. A protein corresponding in electrophoretic mobility to NS1 was only occasionally seen (not shown in Fig. 2). Proteins of equal mobility to the major (O3) and minor (O2 and O4) outer capsid proteins were not produced. A major translation product (54,000 molecular weight) migrated slightly ahead of O1A.

The fairly high endogenous incorporation of \[^{35}S\]methionine in the wheat germ system produced densely labeled bands near the gel front, which made translation products of low molecular weight difficult to observe.

**Translation of individual species of denatured dsRNA.** Total genomic dsRNA was fractionated by polyacrylamide gel electrophor-
resis on a preparative scale as described in the text. A typical preparative scale gel is shown in Fig. 3. Not all RNA segments could be resolved, because segments 7 and 8 have identical mobilities in this gel system (20) and run together as a heavily staining band (Fig. 3). Bands were excised, and the RNA was eluted. The individual RNA segments (segments 1 to 6) were then denatured and translated as described in the text.

Figure 4 shows the translation products of genome segments 1 to 4. The translation product of the denatured dsRNA segment 1 is identical in electrophoretic mobility to the virion structural polypeptide I₁. Similarly, dsRNA segments 2, 3, and 4 produce labeled polypeptides corresponding to I₂, I₃, and I₄, respectively. Although there are endogenous bands in this region which migrate to positions close to the translation products of segments 2 and 4, they clearly do not interfere with the assignments.

The translation of denatured dsRNA segment 5 produces a labeled protein of similar electrophoretic mobility to O₁A (Fig. 5). However, whereas segment 5 appears to be translated very efficiently in vitro, O₁A is quite difficult to observe in SA 11-infected cells.

Genome segment 6 codes for the major inner capsid polypeptide I₅. This segment appears to

![Fig. 3](http://jvi.asm.org/)

**Fig. 3.** Preparative gel electrophoresis of SA 11 rotavirus dsRNA. RNA was extracted from purified virus and analyzed on 10% SDS-polyacrylamide (Laemmli) slab gels. The gel was stained with ethidium bromide and photographed under a UV light box. The dsRNA segments are numbered in order of decreasing molecular weight.

![Fig. 4](http://jvi.asm.org/)

**Fig. 4.** Wheat germ cell-free protein synthesizing system programmed with denatured dsRNA segments 1 through 4. Individual dsRNA segments were recovered from excised gel bands from a preparative RNA gel and purified by organic solvent extraction and ethanol precipitation. After treatment with Me₂SO they were added to a wheat germ translation system, and the [³⁵S]methionine-labeled products were analyzed on a 10% SDS-polyacrylamide slab gel. IC, SA 11 virus-infected MA104 cells labeled with [³⁵S]methionine; O, wheat-germ system with no added RNA. Translation system was programmed with dsRNA segment 1 (S1), segment 2 (S2), segment 3 (S3), and segment 4 (S4).

![Fig. 5](http://jvi.asm.org/)

**Fig. 5.** Wheat germ cell-free protein-synthesizing system programmed with denatured dsRNA segments 5 and 6. UC, Uninfected [³⁵S]methionine-labeled MA104 cells; IC, SA 11 virus-infected [³⁵S]methionine-labeled MA104 cells; O, no RNA added to the wheat-germ translation system; S5, denatured dsRNA segment 5 added; S6, denatured segment 6 added to the translation system. Products were analyzed on a 12% slab gel.
be translated efficiently both in vitro and in vivo, unlike segment 5.

**DISCUSSION**

McCrae and Joklik (17) found that for the efficient translation of the denatured dsRNA genome of reovirus, a rapid shift in potassium ion concentration, soon after the addition of RNA to their wheat germ system, was required. If this "salt jump" was not performed, none of the high-molecular-weight (lambda) polypeptides were produced. This was not necessary for the translation of the rotavirus genome because all known high-molecular-weight proteins (I1 through I4) were formed.

O2 is known to be a glycoprotein (22). No product equal in mobility to this protein was formed in vitro, which is consistent with other studies (2, 4, 6) in which mRNA's of viral glycoproteins were used to program cell-free, protein-synthesizing systems. Often the unglycosylated form of the viral protein was produced, which migrated significantly ahead of the glycoprotein (i.e. of lower apparent molecular weight) on SDS-polyacrylamide gels. This possibility is currently under investigation in this laboratory.

This method of directly translating virion genetic material clearly indicates primary gene products, and clarifies the published data concerning viral polypeptides. Rodger et al. (22) described four high-molecular-weight rotavirus polypeptides (e.g., p133, p102, p99, and p92 for SA 11), but others have observed only three (16, 18). Thouless (29) described an occasional splitting of bands in the I2 or I3 region and suggested this may be due to cleavage, similar to that of reovirus (36). Our results demonstrate that there are indeed four distinct high-molecular-weight polypeptides and that they are all primary gene products. It is for this reason that we found it necessary to change the designation of the major inner shell polypeptide from I3 (29) to I5.

The assignment of dsRNA segment 5 to O1A is quite firm, although the relationship between O1A and O1 has not been clearly established. Thouless (29) also showed a slight difference in migration between the O1 in infected cells and the corresponding virus structural protein. Since O1A is produced in such small quantity in virus-infected cells, it will be difficult to compare it with the translation product of segment 5 and with O1 from virus particles, but the comparison (for example by limited proteolysis analysis) appears feasible. Unlike Matsuno and Mukoyama (16), neither Thouless (29) nor we were able to detect a polypeptide in infected cells which had the same mobility as the structural polypeptide O1 of purified virus. We consider that the protein designated NCV P1 by Matsuno and Mukoyama (16) may correspond to O1A in our system.

In another recent study (5), translation of transcription products of calf rotavirus appeared to yield only polypeptide I1 and possibly NS1 and NS2. However, no comparison between labeled translation products and virus-infected cell proteins was done, so any lower-molecular-weight bands are of uncertain identity.

Since infectivity and antigenic specificity of rotaviruses depend on polypeptides of the outer capsid (3, 30, 35), it is tantalizing that most of the successful assignments so far are for inner capsid proteins. From other studies in progress in this laboratory (I. Lazdins, unpublished data), it appears probable that O1 is a glycoprotein, as well as O2, and in vitro translation of these may require variations in technique. These assignments, and the identification of the protein carrying type-specific antigenic determinants, are of high priority to assist interpretation of epidemiological investigations based on electropherotypes (7, 11, 20; S. Rodger, manuscript in preparation).

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**LITERATURE CITED**


