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Completion of the Gene Coding Assignments of SA11 Rotavirus: Gene Products of Segments 7, 8, and 9

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Improved fractionation of double-stranded RNA segments 7, 8, and 9 of simian rotavirus SA11 has permitted their isolation and individual translation in vitro. Segment 7 codes for p31 (NS_2), segment 8 codes for p33 (NS_1), and the segment 9 gene product resembles the gp34 precursor observed in SA11 virus-infected cells. In vitro glycosylation of translation products of segments 5 and 10 was also observed.

Having been recognized as a major cause of enteritis in the young of many mammalian species (10, 17, 23), as well as in human infants (2, 7, 8), rotaviruses are of both medical and veterinary importance. Like other members of the family Reoviridae (14), they possess a segmented double-stranded RNA (dsRNA) genome (10, 21) enclosed within a double-layered protein capsid (8).

Several studies have reported attempts to determine the primary gene products of rotaviruses. Estimates of the number of virus-coded polypeptides observed in rotavirus-infected cells range between 10 and 12 (1, 4, 6), of which between 5 and 9 are considered to be structural (1, 5, 10, 13). In vitro translation studies in which individual dsRNA segments are denatured and directly translated in vitro, as described for reovirus (15), have also been applied to the rotaviruses (1, 4, 16, 22). When all 11 dsRNA segments of United Kingdom bovine rotavirus were individually isolated, 11 primary gene products were detected and assigned (16), although there was cross-contamination between segments 8 and 9. As yet, only eight of the SA11 virus dsRNA coding assignments have been established (4, 22) because of the difficulty of fractionation and isolation of the closely migrating segments 7, 8, and 9 (1, 4, 22).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used to detect differences between rotaviral strains through a comparison of the migration of corresponding genome segments (19, 20), as well as for the isolation of segments (4, 16). SDS-PAGE of the SA11 genome usually reveals only 10 bands of dsRNA, with the seventh and eighth segments migrating together (22). By modifying our electrophoresis system, we were able to establish the coding assignments of segments 7, 8, and 9 of SA11 rotavirus.

SA11 rotavirus (12) was cultivated in MA104 cells as previously described (4). Purification of virus particles was carried out essentially as described by Smith et al. (27), except that sucrose was not involved in the centrifugation step after the initial fluorocarbon extraction. Extraction of dsRNA from virus particles was also performed as described by Smith et al. (22). Genome segments were fractionated by SDS-PAGE on preparative slab gels of 5% acrylamide (36 cm long and 1 mm thick), with the 3% acrylamide stacking gel containing a single 6-cm-wide well. Laemmli sample buffer (11) was added to dsRNA samples, and 100 μg of dsRNA was loaded per gel. Electrophoresis was conducted at room temperature for 21 h at 20 mA constant current.

Gels were briefly stained in ethidium bromide, and dsRNA bands were visualized and excised over UV light. Gel slices were frozen at −20°C before the extraction of dsRNA by electroelution. Dialysis tubing (type 8; Selby Scientific, Ltd.) was boiled for 10 min in 0.01 M Tris-hydrochloride (pH 7.5)–0.001 M EDTA containing 0.1% SDS and cooled to room temperature before use. Frozen gel slices were inserted into sections of dialysis tubing containing 1.5 to 2.0 ml of TASE buffer (0.04 M Tris base, 0.02 M sodium acetate, 0.018 M sodium chloride, and 0.002 M EDTA [pH 8.2]) with 0.1% SDS, and the tubing was sealed. Electroelution was conducted at room temperature in a shallow tank containing TASE buffer with 0.1% SDS, with the tubing at right angles to the current path. After 16 h at a constant current of 5 mA, the current was reversed for 10 min to free the dsRNA attached to the dialysis tubing. The

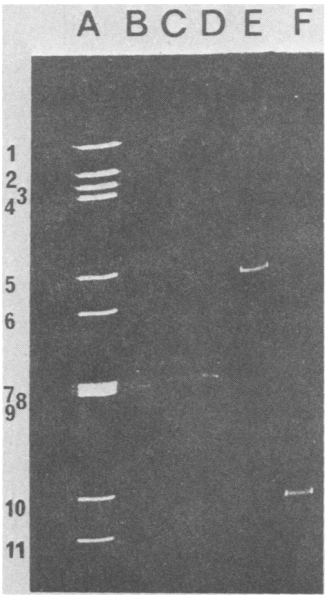

FIG. 1. Analysis of isolated SA11 dsRNA species by SDS-PAGE for purity. After purification and extraction of dsRNA from excised bands of preparative gels, 1/20 of the volume of each of the isolated species was loaded for analysis. Track A, Unfractionated SA11 dsRNA. Isolated segments: 9 (track B); 8 (track C); 7 (track D); 5 (track E); and 10 (track F).

eluate was extracted with chloroform and phenol to remove monomeric acrylamide and repeatedly extracted with isobutanol to reduce the volume of the aqueous phase and to remove ethidium bromide.

Isolated segments were analyzed for purity by SDS-PAGE on 5% acrylamide gels (Fig. 1). The unfractionated SA11 rotavirus genome (Fig. 1, track A) was also included for comparison. Within the limits of ethidium bromide sensitivity, no contamination was detected for segments 7, 8, and 9 (Fig. 1; tracks B, C, and D, respectively). The more readily separable segments 5 and 10 (tracks E and F, respectively) were also free from contamination.

In vitro translation was carried out using a commercially available cell-free preparation of rabbit reticulocyte lysate (code N. 90; Amersham Australia, Proprietary Ltd.) according to the recommendations of the manufacturer. Briefly, 1 μl of dimethyl sulfoxide-denatured dsRNA was added to 10 μl of the lysate containing 10 μCi of [^{35}S]methionine. In vitro glycosylation was coupled to the translation reaction by the addition of 1.2 μl of a cell-free preparation of pancreatic membrane vesicles (kindly provided by G. Both, Commonwealth Scientific and Industrial Research Organization, Division of Cellular and Molecular Biology, Sydney, Australia)

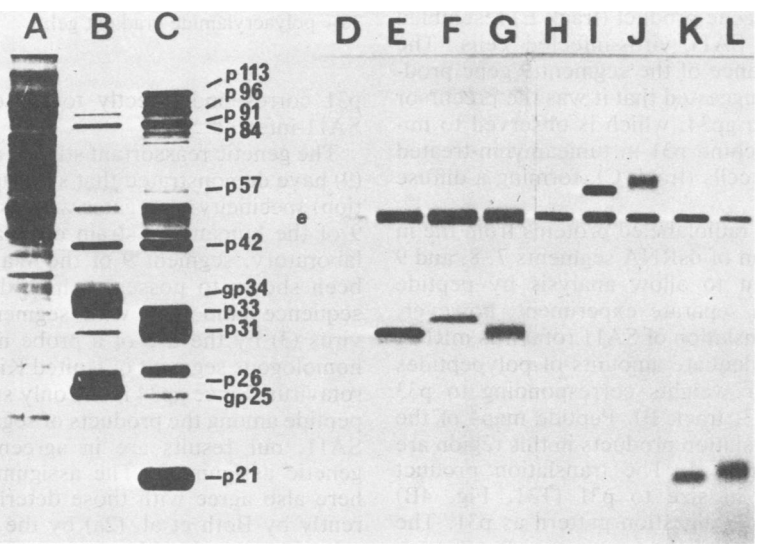

FIG. 2. In vitro translation products, the synthesis of which is directed by dimethyl sulfoxide-denatured dsRNA segments of SA11. Mock-infected MA104 cells (track A) and SA11-infected MA104 cells in the presence (track C) and absence (track B) of tunicamycin were included for comparison. Controls to which no dsRNA was added with (track D) and without (track H) membrane vesicles were also included. Also shown are gene products of segments 7 (track E), 8 (track F), 9 (track G), 5 (track I), and 10 (track K) in the absence of membrane vesicles and segments 5 (track J) and 10 (track L) in the presence of membrane vesicles. e, A polypeptide, the synthesis of which was directed by endogenous message in the lysate.

to the mixture before incubation. All reactions were incubated at 30°C for 1 h and then treated with RNase A (40 μg/ml) in the presence of EDTA (0.01 M) for 15 min at 30°C to remove labeled amino-acyl tRNA species. In vitro synthesis was monitored by trichloroacetic acid-precipitable radioactivity, and the products were analyzed by SDS-PAGE on 13% acrylamide gels, the fluorography (18) of which is shown in Fig. 2.

Having overcome the problem of isolating individual segments and having found a suitable translation system, we now required labeled intracellular virus-coded polypeptides for comparison with in vitro translation products. SA11 polypeptides were prepared from MA104 cells as described by Smith et al. (22). Virus-coded polypeptides from tunicamycin-treated SA11-infected MA104 cells were prepared by the addition of tunicamycin (2.5 μg/ml) to infected monolayers from 30 min before labeling until the time of harvesting. Profiles of mock-infected cells (Fig. 2, track A) and MA104 cells infected with SA11 virus in the presence and absence of tunicamycin (tracks C and B, respectively) are given. The molecular weight estimates used are those previously determined in our laboratory for the SA11 virus (4).

Our results confirm previous suggestions that the segment 7, 8, and 9 gene products migrate in the region between p42 and p26 of SA11 virus-infected cells (1, 4). The segment 8 gene product (track F) corresponded to p33 (NS_1), whereas the segment 7 gene product (track E) resembled p31 (NS_2) of SA11 virus-infected cells. The diffuse appearance of the segment 9 gene product (track G) suggested that it was the precursor of glycoprotein gp34, which is observed to migrate a little behind p31 in tunicamycin-treated SA11-infected cells (track C), forming a diffuse band.

The yield of radiolabeled proteins from the in vitro translation of dsRNA segments 7, 8, and 9 was insufficient to allow analysis by peptide mapping. In a separate experiment, however, the in vitro translation of SA11 rotavirus mRNA (4) provided adequate amounts of polypeptides with molecular weights corresponding to p33 and p31 (Fig. 3, track B). Peptide maps of the two major translation products in this region are displayed in Fig. 4. The translation product corresponding in size to p31 (T31, Fig. 4B) reveals the same digestion pattern as p31. The major translation product corresponding in size to p33 has a peptide profile identical to the sum of the peptide patterns of p33 and gp34 (Fig. 4A). It appeared that in this experiment the glycoprotein precursor migrated closer to p33 than to p31. These results confirm that the in vitro-synthesized products in the region of p33 and p31 correspond directly to those observed in SA11-infected cells.

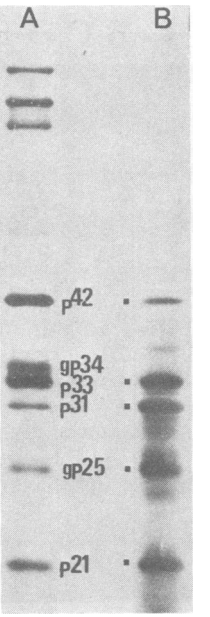

FIG. 3. Analysis of the in vitro translation products of SA11 mRNA. mRNA was extracted from actinomycin D-treated SA11-infected cells at 9 h postinfection as described previously (4). In vitro translation was performed in a wheat germ system as described previously (4). Track A, SA11 virus-infected cell proteins (only the lower-molecular-weight proteins are labeled); track B, in vitro translation products of SA11-infected cell mRNA (major translation products are indicated by dots; the product migrating like gp25 is from segment 11). Analysis was carried out on a 10 to 20% polyacrylamide gradient gel.

The genetic reassortant studies of Kalica et al. (9) have demonstrated that serotype (neutralization) specificity segregates with dsRNA segment 9 of the human Wa strain of rotavirus. In this laboratory, segment 9 of the Wa rotavirus has been shown to possess a high degree of base sequence homology with segment 9 of SA11 virus (3) by the use of a probe made from the homologous segment of United Kingdom bovine rotavirus. Since gp34 is the only structural polypeptide among the products of segments 7 to 9 of SA11, our results are in agreement with the genetic assignment. The assignments reported here also agree with those determined concurrently by Both et al. (2a) by the use of hybrid selection to isolate the segment 9 message from unfractionated SA11 virus transcripts, followed by in vitro translation and glycosylation.

Now that it is established that segment 9 of SA11 virus encodes gp34 and that Northern blot hybridization can be used to identify corresponding genes in other rotaviral strains (3), the

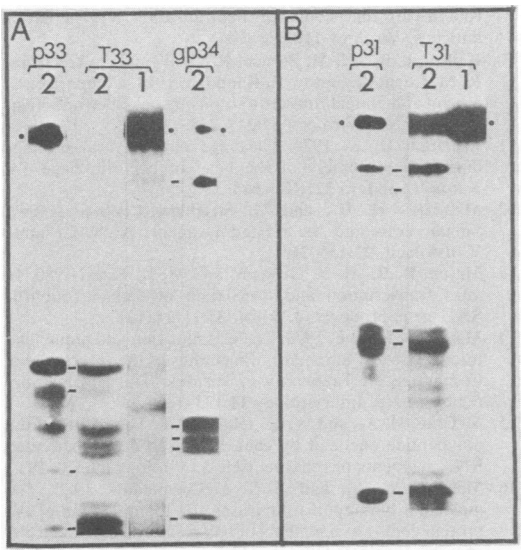

FIG. 4. Limited proteolysis analyses of the in vitro translation products T33 and T31. The in vitro translation products of SA11 virus (infected-cell) mRNA with the same apparent molecular weights as the infected-cell proteins p33 and p31 (T33 and T31, respectively) were isolated from preparative polyacrylamide gels and digested with 0.5 (track 1) or 10 (track 2) μg of *Staphylococcus aureus* V8 protease (30 min at 37°C, as described previously [4]). They were compared to the V8 protease digestion patterns of proteins p33, p31, and gp34, isolated from preparative gels of SA11 virus-infected cell proteins. (A) Infected-cell proteins p33 and gp34 and the in vitro translation product T33. (B) Infected-cell protein p31 and the corresponding translation product T31. Dashes indicate peptides in common, and dots indicate the gel positions of the undigested proteins. Peptides were separated on 15% polyacrylamide gels.

cloning and sequencing of several of these genes from different rotavirus strains will be facilitated. Sequence data will be invaluable for determining the nature and extent of genetic variation in rotaviruses and for locating variable regions within the gene which may be correlated with serotype determinants.

Also shown in Fig. 2 are the in vitro translation products of segments 5 and 10 (tracks I and K, respectively) that correspond to p57 and p21, respectively, in agreement with previous reports (1, 4, 22). For both genes, the addition of pancreatic membrane vesicles to the translation reaction resulted in the synthesis of polypeptides that showed a decrease in mobility and diffuse banding (tracks J and L, respectively). To our knowledge, glycosylation is the only process that could have occurred in vitro in the presence of membrane vesicles which could result in the decreased mobility. The apparent glycosylation of p21 confirms the finding that p21 is the precursor of glycoprotein gp25 (16). Although the change in mobility was not as great as that seen in vivo, the in vitro product may correspond to an intermediate (p23) observed by Ericson et al. (6). Examination of tunicamycin-treated and untreated SA11 virus-infected cell lysates, however, did not reveal any glycosylation of the segment 5 gene product such as apparently occurs in vitro. The labeling studies of Ericson et al. (6) did not reveal the attachment of carbohydrate to p57. It is conceivable that p57 might possess amino acid sequences suitable for glycosylation but lack a hydrophobic leader sequence and that the pancreatic vesicle preparation contained inside-out vesicles which could carry out glycosylation of polypeptides that remained outside them, but this does not seem very likely. This is another mystery which may be clarified by future sequencing studies. We hope that the complete set of genome coding assignments now provided (Fig. 5) will facilitate interpretation of such studies. We have revised our earlier assignment of gene 11 since it is now clear that this gene does not encode gp25, as previously suggested (4). It has recently been demonstrated (1, 6, 16) that gp25 is the glycosylated form of p21, the segment 10 product (4). In SA11-infected cells, the gene 11 product appears to be a minor protein species with an apparent molecular weight slightly less than that of gp25 (1, 16), and it is best observed when the formation of gp25 has been blocked by the glycosylation inhibitor tunicamycin (1, 16; unpublished data).

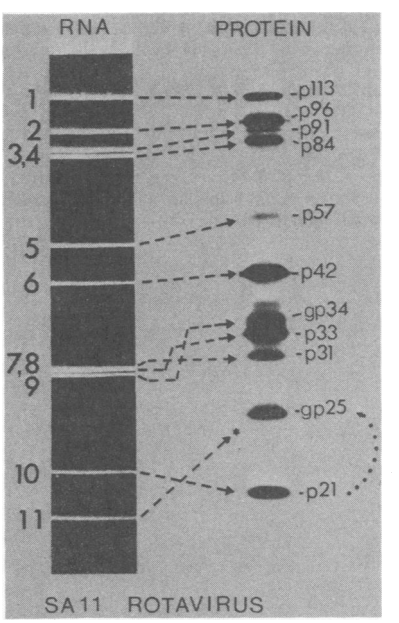

FIG. 5. Coding assignments of all 11 segments of the SA11 rotavirus genome.

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ADDENDUM IN PROOF

After this manuscript was submitted, work by Mason et al. (J. Virol. **46**:413–423, 1983) was published. These authors performed in vitro translations of simian rotavirus SA11 single-stranded RNA transcripts to determine coding assignments and obtained results in agreement with our findings.

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