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Identification, Synthesis, and Modifications of Simian Rotavirus SA11 Polypeptides in Infected Cells

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The synthesis and processing of simian rotavirus SA11 polypeptides was investigated after infection of MA104 cells. [^{35}S]methionine- or ^{3}H-amino acid-labeled cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Viral protein synthesis was maximal 3 to 5 h postinfection, and 12 major viral polypeptides were detected. Immunoprecipitation and peptide mapping experiments demonstrated five viral structural proteins (125,000 daltons [125K], 94K, 88K, 41K, and 38K). Three proteins (53K, 35K, and 34K) were identified as nonstructural by comparison of their partial proteolysis maps with those from polypeptides of similar molecular weight synthesized in vitro from viral RNA transcripts. Assignment as to structural or nonstructural status of two other primary gene products (26K and 20K) remains tentative. Pulse-chase experiments and tunicamycin blockage of glycosylation revealed cotranslational or post-translational modifications (or both) and precursor-product relationships of several of the polypeptides. Tunicamycin inhibition of glycosylation identified a 35.5K polypeptide which was proven to be the precursor to the 38K structural glycoprotein by immunoprecipitation and peptide mapping analyses. Tunicamycin treatment of infected cells also resulted in the disappearance of other glycoprotein species (23K to 29K) and in the concomitant build-up of an unglycosylated 20K polypeptide, suggesting a precursor-product relationship between those polypeptides. Labeling with [^{3}H]glucosamine or [^{3}H]mannose suggested that the rotavirus glycoproteins contained high mannose oligosaccharides. The effects of amino acid analogs on rotavirus polypeptide synthesis and processing were also investigated.

Detailed knowledge of the synthesis, processing, and functions of rotavirus polypeptides is required to understand the replication strategies of these gastroenteritis viruses. Studies of polypeptides from rotavirus-infected cells yielded conflicting data concerning the number of virus-specific polypeptides, the relationship of the detected polypeptides to structural polypeptides, and whether the detected polypeptides were primary gene products or polypeptides that had undergone post-translational processing (17–19, 25–27). Definitive information is not available concerning the relationship(s) of the polypeptides in infected cells with the primary gene products synthesized in vitro from virus-specific RNA transcripts or from denatured double-stranded genome RNA (6, 17, 25).

This report describes the polypeptide patterns of cells infected with the simian rotavirus SA11. We analyzed the polypeptides by immunoprecipitation and partial proteolytic peptide mapping and showed that SA11 coded for five structural polypeptides (125,000 daltons [125K], 94K, 88K, and 41K and a glycoprotein of 38K), one precursor to the structural glycoprotein (35.5K), and three nonstructural polypeptides (53K, 35K, and 34K). Two other primary gene products (26K and 20K) were tentatively designated as precursors to the structural polypeptide VP9 and to the nonstructural glycoproteins of ~28K to 29K or to intermediates of these nonstructural glycoproteins of 23K, 26K, and 27K, respectively. Post-translational modifications (glycosylation and proteolytic cleavage of polypeptides) were demonstrated by using sugar labels, by performing pulse-chase experiments, and by examining the effect of amino acid analogs on SA11 polypeptide synthesis and processing. A model of the synthesis and maturation of rotavirus primary gene products is presented.

MATERIALS AND METHODS

Cells and virus. SA11 virus stock was plaque purified three times and propagated at low multiplicity (<0.1 PFU/cell) in MA104 cells and assayed as previously described (9, 24). The SA11 structural glycoproteins (VP7 and VP7a) can exhibit heterogeneity, and independent plaque-purified stocks with phenotypes

containing VP7 alone, VP7a alone, neither glycoprotein, or a glycoprotein with an intermediate migration on gels have been isolated (M. K. Estes, D. Y. Graham, R. F. Raming, and B. L. Ericson, submitted for publication). To simplify characterization of the synthesis and processing of the SA11 polypeptides, the present studies used a stock of plaque-purified virus containing one glycoprotein, VP7 (38K). To produce large quantities of virus, roller bottles of MA104 cells (5×10^7 cells/roller bottle) were washed thoroughly with Eagle minimal essential medium lacking serum (0% MEM) but containing penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and glutamine (0.03%). Before infections, virus stock was treated with trypsin (10 μg/ml, 4 U/ml; twice recrystallized; Worthington Biochemicals Corp., Freehold, N.J.) for 30 min at 37°C, and the cells were inoculated with 5 ml of the trypsin-activated virus (10 PFU/cell). After adsorption (37°C for 90 min), the inoculum was washed off with 0% MEM. Radiolabeled virus was produced in 0% MEM containing one-fourth the normal amount of methionine, actinomycin D (AD; 0.5 μg/ml; P-L Biochemicals, Milwaukee, Wis.), and [^{35}S]methionine (10 μCi/ml, 1,030 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). [^{3}H]mannose-labeled virus was prepared in analogous fashion by adding the isotope ([2-^{3}H]mannose; 5 μCi/ml, 21 Ci/mmol; New England Nuclear Corp., Boston, Mass.) to 0% MEM. Virus was purified by genetron extraction and CsCl density gradient centrifugation as previously described (17).

Radiolabeling of intracellular rotavirus-coded polypeptides. Confluent monolayers of MA104 cells (1×10^6 to 2×10^6 cells) in 35-mm plastic petri plates were incubated in 0% MEM for 12 h before infection. The cells were infected with trypsin-activated SA11 virus at a multiplicity of 20 to 30 PFU/cell or mock infected with trypsin-TBS (10 μg of trypsin per ml in 20 mM Tris-hydrochloride, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 6 mM dextrose, 0.5 mM $MgCl_2$, and 0.7 mM $CaCl_2$). The time of addition of virus to the plates was considered to be time zero (0 h postinfection [p.i.]). After 1 h of adsorption at 37°C, the inoculum was washed off with TBS, and 0% MEM containing 5 μg of AD per ml (MEM-AD) was added. Cells were preincubated in MEM-AD lacking methionine or amino acids for 30 min before the pulse, and [^{35}S]methionine (40 μCi/ml) or ^{3}H-amino acids (50 μCi/ml), respectively, were added. After a 1-h labeling period, the cells were harvested in 100 μl of RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 10 mM Tris-hydrochloride, pH 7.2, and 1% Trasylol; FBA Pharmaceuticals, New York), sonicated for 30 s to shear DNA, and mixed with an equal volume of 2× electrophoresis sample buffer (10 mM Tris-hydrochloride, pH 6.8, 16% glycerol, 2% SDS, 1.0 M urea, 10% 2-mercaptoethanol, and 0.006% phenol red).

For labeling with carbohydrates, the medium was changed to MEM-AD lacking glucose for [2-^{3}H]mannose (25 μCi/ml), [6-^{3}H]fucose (25 μCi/ml, 13.12 Ci/mmol; New England Nuclear Corp.), or [1-^{3}H]galactose (25 μCi/ml, 9.3 Ci/mmol; Amersham). Labeling with [1,6-^{3}H]glucosamine (50 μCi/ml, 39.6 Ci/mmol; New England Nuclear Corp.) was performed in MEM-AD free of glucose with 10 mM fructose (23). Phosphorylation or sulfation of polypeptides was examined in 0% MEM lacking phosphate or sulfate but containing $^{32}PO_4$ (100 μCi/ml, carrier free; New England Nuclear Corp.) or $^{35}SO_4$ (100 μCi/ml, 25 to 40 Ci/mg; Amersham), respectively. Infected cells were harvested and analyzed as above.

Pulse-chase experiments. MA104 cells were grown in 35-mm dishes and infected as above. Thirty minutes before the pulse, the medium was changed to MEM-AD without methionine or without glucose, and the infected cells were pulsed for 10 min with [^{35}S]methionine (40 μCi/ml) or for 15 min with [2-^{3}H]mannose (100 μCi/ml), respectively. Infected cells were either harvested immediately after the pulse or chased for various lengths of time with MEM-AD containing 400× unlabeled methionine, 400× unlabeled methionine and 50 μg of cycloheximide per ml (Sigma Chemical Co., St. Louis, Mo.), or 1 mM unlabeled mannose and 50 μg of cycloheximide per ml. Lysates were harvested and treated as above.

Trichloroacetic acid precipitation. Duplicate 5-μl portions from all samples were dried onto 2.4-cm Whatman filter paper disks. The filters were immersed into ice-cold 10% trichloroacetic acid for 5 min, washed with 5% acid for 5 min, washed twice with 100% acetone (2 min), dried under a heat lamp, and counted in 5 ml of a toluene-based scintillation fluid, using a Beckman LS250 liquid spectrometer.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using 12% polyacrylamide slab gels (0.75 mm thick) and the Laemmli discontinuous buffer system containing 0.5 M urea (16, 17). Samples were suspended in electrophoresis buffer and boiled for 2 min immediately before electrophoresis. The resultant gels were prepared for autoradiography or fluorography (2) and exposed to X-ray film (Kodak X-Omat) at −70°C. Molecular weight determinations were estimated by comparison of the relative mobilities of the viral polypeptides with ^{14}C-labeled markers from New England Nuclear Corp., which included phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), and cytochrome *c* (12,500).

V8 protease and chymotryptic peptide mapping. The procedure for digestion of rotavirus SA11 polypeptides by *Staphylococcus aureus* V8 protease or chymotrypsin (Sigma) in SDS-gels was that described by Cleveland et al. (4). Bands from SDS-gels were identified by autoradiography or fluorography, excised, placed in the sample wells of a second SDS-gel (1.5 mm thick; 5-cm stacking gel), and soaked (30 min) in 10 μl of soaking buffer (2% 2-mercaptoethanol, 0.125 M Tris-hydrochloride, pH 6.8, 0.1% SDS, and 1.0 mM EDTA, pH 8) per well. V8 protease (4 or 20 μg/ml) or chymotrypsin (100 or 1,000 μg/ml) in soaking buffer containing 10% glycerol was then added to the wells along with 0.001% bromophenol blue as a tracking dye. Electrophoresis was performed in the normal manner with the exception that the current was switched off for 30 min when the bromophenol blue dye neared the bottom of the stacking gel.

In vitro transcription and translation of rotavirus mRNA. Preparation of rotavirus mRNA was as described by Mason et al. (17). Translation of the mRNA in rabbit reticulocyte lysates followed the procedure of Pelham and Jackson (21) as modified by Mason et al. (17).

Amino acid analog experiments. MA104 cells were

grown in 35-mm dishes and infected as described for radiolabeling of intracellular polypeptides. Cell monolayers were preincubated for 30 min before the pulse with MEM-AD containing 1/10 the normal concentration of the amino acid(s) substituted by the analog(s). The amino acid analogs (Sigma) used and their normal amino acid counterparts were ethionine (methionine), 2-azetidine carboxylic acid (proline), *p*-fluorophenylalanine (phenylalanine), canavanine sulfate (arginine), and *B*-hydroxynorvaline (threonine). The concentrations of the analog(s) used are indicated in the figure legends. The cells were pulsed for 10 min with [^{35}S]methionine at 4 h p.i. For chase experiments, the cells were incubated in medium containing the respective analog and 400× unlabeled methionine for the indicated times.

Immunoprecipitation of infected and mock-infected cell lysates. Hyperimmune guinea pig antiserum was prepared against purified pooled double-shelled and single-shelled SA11 virus particles as previously described (9). Monospecific antisera were prepared in guinea pigs by inoculation of glycoprotein bands from SDS-polyacrylamide gels. A detailed report of this method will be described elsewhere (H. H. Hanssen, D. Y. Graham, and M. K. Estes, manuscript in preparation). Infected and mock-infected cells in 32-oz (ca. 96-ml) prescription bottles (10^7 cells/bottle) were labeled with [^{35}S]methionine as described for a 10-min pulse. In some experiments, lysates were prepared from cultures that had been treated with tunicamycin (TM; 1 μg/ml; Eli Lilly & Co., Indianapolis, Ind.; a generous gift of R. C. Hamill to B. C. Altenburg) for 4 h before a 3-h pulse from 4 to 7 h p.i. Cell lysates were prepared by treating the labeled cells with 4 ml of ice-cold RIPA buffer and scraping the cell monolayer with a rubber policeman. The lysates were collected with a pipette, and a 100K supernatant was prepared by centrifugation at 35,000 rpm for 30 min in a 50 Ti rotor (Beckman) at 4°C. Portions (0.5 ml) of the supernatant were adsorbed with 50 μl of a 10% Formalin-fixed *S. aureus* A (strain Cowan I) suspension (14) for 15 min at 22°C. A 20-μl portion of antiserum was added to the adsorbed lysates and incubated at 4°C for 1 h. A second portion (50 μl) of bacteria was added to each sample and incubated at 4°C for 15 min. The bacteria with the adsorbed immune complexes were collected by centrifugation and washed sequentially as previously described (17, 20). After washing, the immune complexes were disrupted by boiling the samples in 50 μl of disruption buffer for 2 min with subsequent pelleting of the bacteria by centrifugation. Supernatants were either analyzed immediately by SDS-PAGE or stored at −20°C for future analysis.

TM block of rotavirus polypeptide glycosylation. MEM-AD containing 1 μg of TM per ml (stock solution 200 μg/ml in 0.01 M NaOH, pH 9.0) was added, after a 1-h virus adsorption period at 37°C, to plates to be labeled 4 h p.i., whereas those to be labeled at 7 h p.i. received 1 μg of TM per ml at 4 h p.i. Thirty minutes before labeling, the medium was changed to 0% MEM with AD (5 μg/ml) and TM (1 μg/ml) without methionine, and the infected cells were pulsed with [^{35}S]methionine (40 μCi/ml) for 10 min. The cells were either harvested immediately after the pulse or chased for different lengths of time in 0% MEM containing AD (5 μg/ml), TM (1 μg/ml), and 400× unlabeled methionine, and then harvested and analyzed as before.

RESULTS

Time course of synthesis of viral polypeptides in infected cells. MA104 cell monolayers were infected at a multiplicity of infection of 20 to 30 PFU/cell, labeled with either ^{3}H-amino acids or [^{35}S]methionine for 1-h intervals p.i., and analyzed by SDS-PAGE (Fig. 1A and B) to determine the time course of synthesis of virus-specific polypeptides. By 3 h p.i., polypeptides not seen in uninfected cells, with apparent molecular weights of 125,000, 94,000, 88,000, 53,000, 41,000, 38,000, 35,000, 34,000, 29,000, 28,000, and 26,000 were detected. The entire polypeptide pattern (these bands and an additional 20K band—a total of 12 polypeptides) were clearly visible by 5 h p.i. Comparison of ^{3}H-amino acid (data not shown)- and [^{35}S]methionine-labeled patterns revealed identical polypeptide profiles, demonstrating that all the rotavirus polypeptides contained at least one methionine residue. Later time points did not reveal the appearance of any new viral proteins, indicating that all of the SA11 polypeptides were synthesized by 4 to 5 h p.i.

The polypeptide pattern was not altered by changing conditions of electrophoresis (e.g., running gradient gels from 3 to 9% or 10 to 18% polyacrylamide or by running the gels longer to improve resolution of the high-molecular-weight species). The high-molecular-weight region (125,000 to 88,000) was carefully examined for the presence of four high-molecular-weight polypeptides because of reports of four high-molecular-weight proteins in calf and SA11 rotavirus-infected cells (19, 25). We observed a fourth polypeptide in the region between the 94K and 88K polypeptides at 3 to 4 h p.i. (see arrow, Fig. 1A), but this band disappeared at later times, and it comigrated with a similar band in uninfected cells. Occasionally a fourth band was observed immediately below the 88K band, but this band was not seen when careful precautions were taken to avoid proteolysis (8).

The polypeptide patterns observed after 1-h labeling periods did not change qualitatively throughout the infection. However, scans of the patterns (data not shown) revealed a change in the relative synthesis of individual polypeptides. In particular, the 53K polypeptide decreased in relative amount after 5 h p.i. Whether the differences in the relative amounts of the individual polypeptides reflected regulation of transcription, translation, or post-translational modifications was not determined by these experiments.

Comparison of the polypeptide patterns from infected cells with those synthesized in vitro in rabbit reticulocyte lysates programmed with SA11-specific mRNA (17) demonstrated the synthesis of the nine primary gene products (125K, 94K, 88K, 53K, 41K, 35K, 34K, 26K,

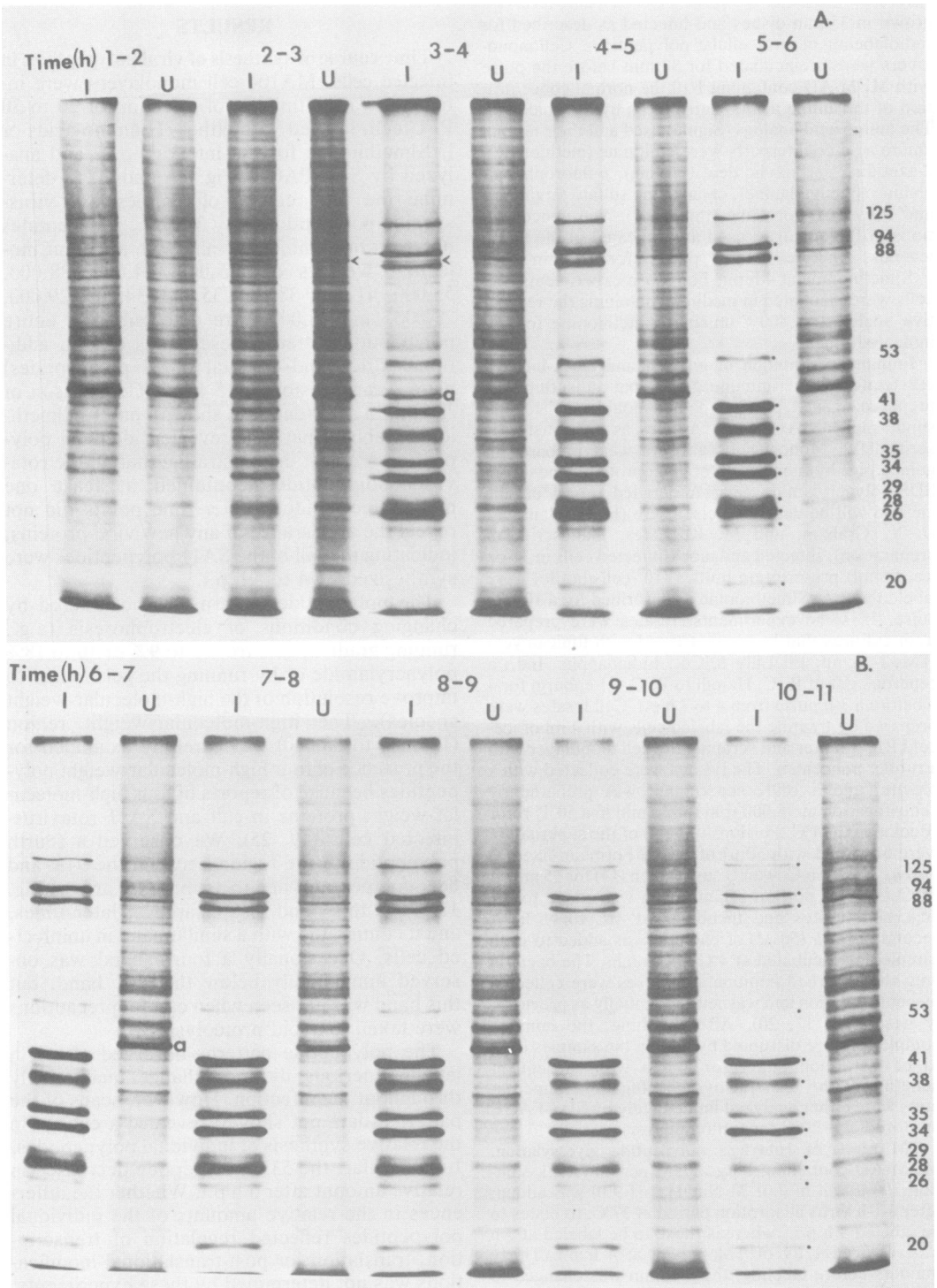

FIG. 1. Time course of synthesis of simian rotavirus SA11-infected cell polypeptides analyzed by SDS-PAGE. Infected (I) and mock-infected (U) MA104 cells were labeled with [^{35}S]methionine as described in Materials and Methods. One-hour pulse intervals from (A) 1 to 6 h and (B) 6 to 11 h p.i. are shown along with the estimated molecular weights ($\times 10^3$) of the viral polypeptides. The virus-specific polypeptides with these molecular weights in this and subsequent figures are indicated by a dot. Arrow denotes an extra high-molecular-weight polypeptide seen only at early time points. The "a" denotes actin.

and 20K) we had previously detected. The remaining three polypeptides (38K, 29K, and 28K) were not detected with in vitro systems, but these polypeptides were glycosylated (see below, Fig. 3) and would not have been modified from the polypeptides synthesized in vitro in the reticulocyte lysate system without the addition of microsomal membranes.

Quantitation of the effect of viral infection on macromolecular synthesis revealed that protein synthesis was maximal at 3 to 5 h p.i. in both viral and mock-infected cells treated with AD, but protein synthesis declined more rapidly in infected cells. At 12 h p.i., an eightfold difference in incorporation of methionine was observed between infected and mock-infected cells, demonstrating that rotavirus infection (in conjunction with AD) inhibited cellular protein synthesis.

Pulse-chase experiments in infected cells. To investigate processing of the viral polypeptides, SA11-infected cells were pulsed with [^{35}S]methionine for 10 min at 5 h p.i. and either harvested immediately or chased with unlabeled methionine (data not shown) or with unlabeled methionine and cycloheximide (50 μg/ml) (Fig. 2). A 10-min pulse revealed a polypeptide pattern similar to that of the 1-h pulse. However, differences were observed in all of the polypeptide bands with molecular weights below 34,000. The 29K and 26K polypeptide bands appeared more intense, whereas the 20K polypeptide appeared less intense. The small amount of label detected in the 20K polypeptide suggested a rapid turnover of this polypeptide. This observation was confirmed by subsequent experiments with TM (see below, Fig. 8), which showed that large amounts of the 20K polypeptide were synthesized during a 10-min pulse in infected cells treated with TM. Processing of the 29K and 26K polypeptides was clearly illustrated in the chase period, when these polypeptides disappeared.

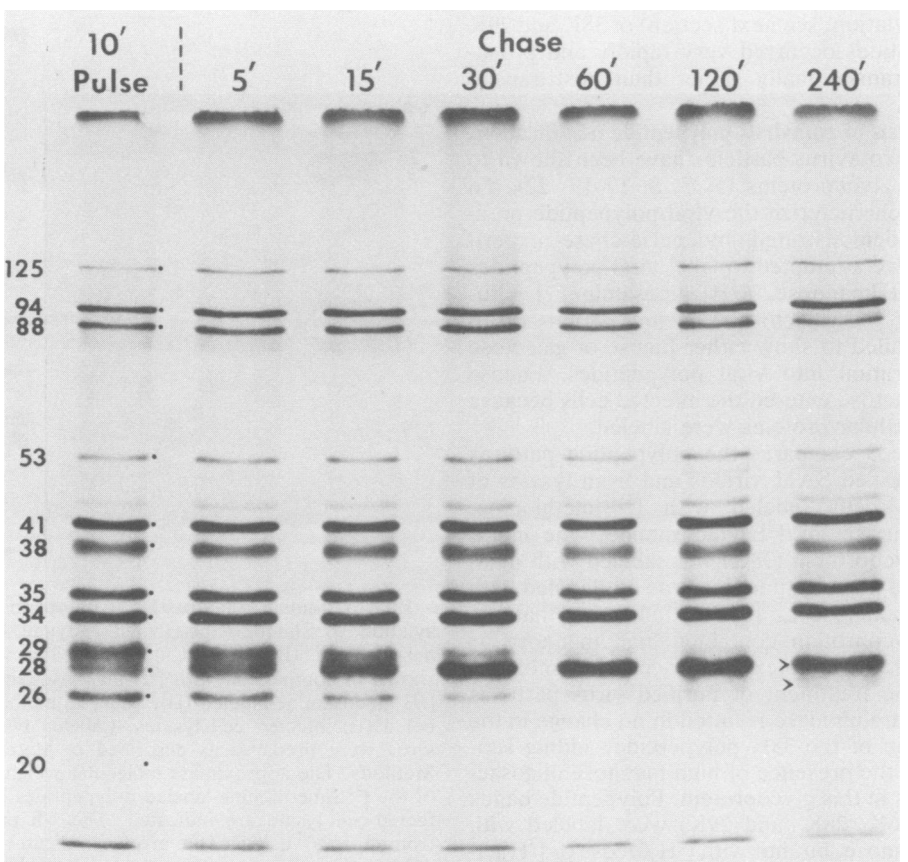

FIG. 2. Kinetics of synthesis of [^{35}S]methionine-labeled SA11 polypeptides in infected cells. MA104 cells were infected, pulsed for 10 min (5 h p.i.) with [^{35}S]methionine as the label, and chased with unlabeled methionine and cycloheximide as described in Materials and Methods. The arrows indicate the 28K and 27K polypeptides in the 4-h (240-min) chase lane.

The chase period also demonstrated a slight decrease in the migration of the 26K polypeptide with time, suggesting that this band might be the precursor to a polypeptide of 27K. A slight increase in the migration of the 38K glycoprotein (minor trimming) was also observed during the chase period.

From in vitro data, we had hypothesized that a 35K polypeptide was the precursor to the 38K glycoprotein (17). No precursor-product relationship was revealed between this major viral glycoprotein (38K) and the 35K band in infected cells. The chase experiments were performed in the presence of cycloheximide to eliminate the possibility that large methionine pools masked the conversion of 35K to 38K (or other precursors into products) by further incorporation of label into the precursor(s) during the chase. "Short" pulse experiments (30 s to 5 min) revealed a polypeptide pattern identical to that seen after a 10-min pulse. These results, in conjunction with the chase experiments with cycloheximide, indicated that modifications (glycosylation; see next section) of 38K and 29K polypeptides occurred very rapidly and probably cotranslationally rather than post-translationally.

Analysis of rotavirus polypeptide modification. Purified rotavirus particles have been shown to contain glycoproteins (5, 7, 8, 17–19, 22). To further characterize the viral polypeptide processing demonstrated by pulse-chase experiments, we attempted to label viral polypeptides with [^{3}H]mannose, [^{3}H]glucosamine, [^{3}H]fucose, or [^{3}H]galactose. Labeling periods of up to 8 h failed to show either fucose or galactose incorporation into viral polypeptides. Fucose and galactose entered the infected cells because many cellular proteins were labeled.

Figure 3 compares the polypeptide patterns from purified SA11 virions and from lysates of infected cells labeled with [^{35}S]methionine, [^{3}H]mannose, or [^{3}H]glucosamine. The major viral glycoprotein (38K) was labeled with mannose and glucosamine, but was not labeled with either galactose or fucose, indicating that the 38K glycoprotein contained high mannose as opposed to complex oligosaccharide moieties (15). The treatment of purified virus particles with neuraminidase resulted in no change in the migration of the 38K polypeptide, adding support for the presence of high-mannose oligosaccharides in this glycoprotein. Polypeptide bands (23K, 26K, 28K, and 29K) were labeled with [^{3}H]mannose, but not with [^{3}H]fucose or [^{3}H]galactose, suggesting that they also contained high-mannose oligosaccharides. In addition, polypeptide bands (23K, 26K, and 28K) were labeled with [^{3}H]glucosamine.

The 41K and 94K bands from purified virions and infected cells incorporated label from [^{3}H]mannose and [^{3}H]glucosamine to varying degrees with long-term (8 h or longer) labeling periods, but to a much less extent with 1-h pulses, suggesting interconversion of label into protein. The 94K band was also labeled with [2-^{3}H]mannose in a 15-min chase period after a 10-min pulse. This observation warrants further study, since this sugar precursor reportedly does not readily undergo interconversion.

The polypeptide patterns of infected cells were also analyzed after labeling with $^{32}PO_4$ or $^{35}SO_4$. Neither phosphorylation nor sulfation was detected. Other possible polypeptide modifications (such as acetylation or methylation) have not been investigated.

Processing of SA11 glycoproteins: identification of intermediates. Since labeling with [^{3}H]mannose and [^{3}H]glucosamine revealed that most of the polypeptides were glycosylated, we performed a pulse-chase experiment with [^{3}H]man-

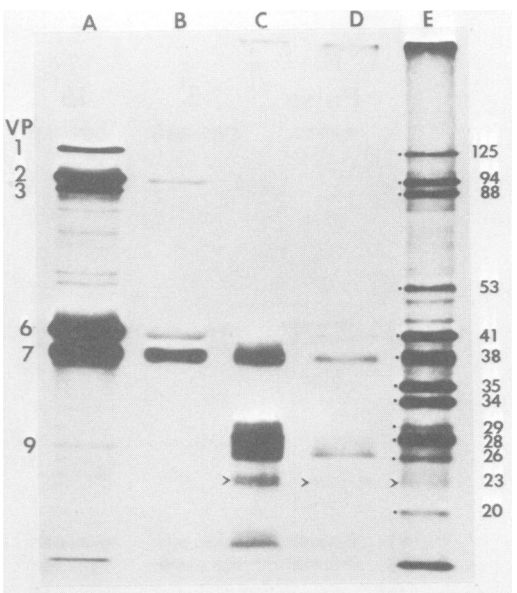

FIG. 3. Simian rotavirus SA11 polypeptide glycosylation. [^{35}S]methionine-labeled (A) and [^{3}H]mannose-labeled (B) purified double-shelled virus are shown in comparison with [^{3}H]mannose-labeled (C), [^{3}H]glucosamine-labeled (D), and [^{35}S]methionine-labeled (E) infected cell lysates. Labeled lysates and virus were prepared as described in Materials and Methods. The approximate molecular weights ($\times 10^3$) of the [^{35}S]methionine-labeled polypeptides in the infected-cell lysate are indicated. The 1-h pulses are from 5 to 6 h p.i. The arrows indicate the 23K polypeptide. The viral structural polypeptides are designated as VP1 through VP9 as previously described (8). The extra bands detected in this virus preparation were assumed to represent proteolytic degradation products (8). They were not labeled in this figure, as their exact origin in this preparation was not proven.

nose (Fig. 4) to follow the processing of only the glycoproteins. Mannose label was used because it is rapidly incorporated into glycoproteins (15). The chase was again performed in the presence of cycloheximide.

The 38K, 29K, 28K, 26K, and 23K polypeptide species were labeled with mannose in a 15-min pulse (Fig. 4). The 29K polypeptide was processed during the 1st hour of chase as previously seen in the [^{35}S]methionine labeling experiment (Fig. 2). The 29K band chased into a band with an apparent molecular weight of 28,000 which did not change further during longer chase periods. The 26K polypeptide increased in molecular weight during the chase, apparently becoming a polypeptide of approximately 27,000. The 23K polypeptide disappeared with chase, and it was not clear whether it was further modified or degraded. The [^{3}H]mannose-labeled 38K structural glycoprotein was trimmed to a minor extent as was seen with [^{35}S]methionine labeling. Mannose-labeled polypeptides with molecular weights less than that of the 20K virus-specific polypeptide were also detected in mock-infected cells and were presumably cellular proteins. These results characterized some of the modifications seen in the pulse-chase experiments as resulting from glycosylation.

Designation of polypeptides as structural or nonstructural. Immunoprecipitation experiments with antisera prepared against double-shelled virus particles were used to determine the structural or nonstructural status of the polypeptides in infected cells (Fig. 5). The four major virus structural proteins (94K, 88K, 41K, and 38K) were immunoprecipitated, whereas the 125K protein, a component of the inner capsid, was not. We previously reported difficulty in immunoprecipitating the 125K polypeptide (17). The 125K protein was identified as virus specific by partial proteolysis mapping (data not shown).

Several of the polypeptides (53K, 35K, 34K, 29K, 28K, or 20K) were not immunoprecipitated in any experiment, suggesting that they were nonstructural. The 35K polypeptide, in agreement with the results of pulse-chase experiments, was not immunoprecipitated from infected-cell lysates. In infected cells, the 35K polypeptide was a nonstructural polypeptide, and the precursor to the 38K glycoprotein (detected only in TM-treated infected cells) migrated very close to 35K (35.5K, see below). Occasionally, a protein in the 26K to 28K polypeptide region was immunoprecipitated from infected cells, but this precipitation was not quantitative (data not shown) as we reported for immunoprecipitation of the 26K polypeptide synthesized in vitro (17). Since preparations of purified virus contain a polypeptide of approximately 27K (VP9), which is a minor viral component (8, 25), we hypothesize that the 26K polypeptide may be the precursor to this structural component of the SA11 virion. However, the specificity of our present antisera and the small amounts of this polypeptide in virions preclude definitive proof of this hypothesis.

Proof of viral specificity of the 53K and 34K

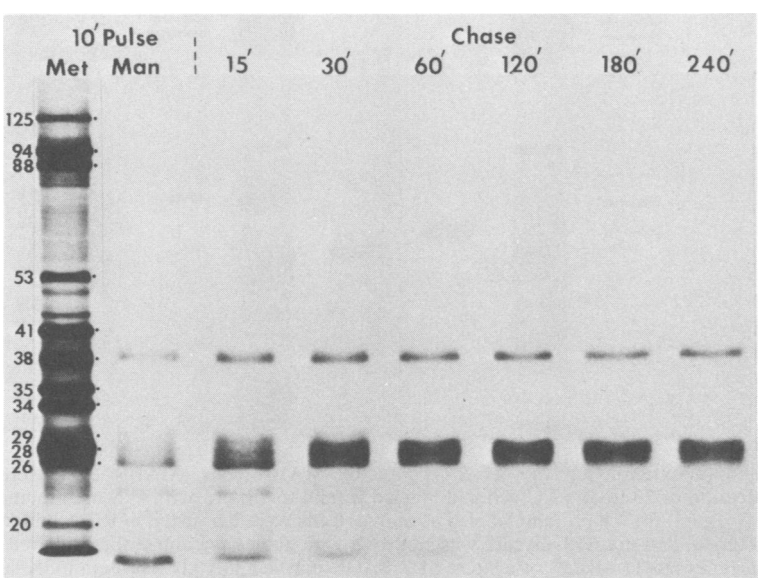

FIG. 4. Kinetics of synthesis of SA11 glycoproteins in infected cells. MA104 cells were infected, pulsed for 10 min (5 h p.i.) with [2-^{3}H]mannose, and chased with excess unlabeled mannose and cycloheximide.

polypeptides was examined by comparing V8 protease peptide maps of these polypeptides synthesized in infected cells with their counterparts synthesized in vitro from RNA transcripts (Fig. 6). The maps of these proteins were identical, proving that they were not host proteins, but were coded by the virus. The 53K and 34K proteins were nonstructural, since neither protein was recognized by hyperimmune sera or detected in purified virions.

Identification of the precursors to the rotavirus glycoproteins. Treatment of infected cells with TM (1 μg/ml), which inhibits de novo synthesis of high-mannose oligosaccharides (15), resulted in the disappearance of the 38K glycoprotein and the appearance of a 35.5K polypeptide (Fig. 5). This new band was presumably the precursor to the 38K glycoprotein. A second, previously undetected polypeptide of 37K was also observed. This polypeptide was not detected in infected cells without TM or mock-infected cells with TM, suggesting that it was either a viral polypeptide or a cellular polypeptide stimulated by both viral infection and TM treatment. Although, in the absence of TM, the 37K polypeptide could have been masked by the 38K glycoprotein, other data suggested this was not the explanation. Different stocks of plaque-purified SA11, each with a glycoprotein with a different molecular weight (37.5K, 37K, or 36K), or with an unglycosylated 35.5K polypeptide, also induced a new band in infected cells treated with TM. These TM-induced bands did not comigrate with the structural glycoprotein in these viruses and therefore eliminated the possibility of masking in the absence of TM (Estes et al., submitted for publication).

To evaluate the relatedness of the 35.5K band to the 38K structural glycoprotein (Fig. 5), immunoprecipitation of lysates of infected and TM-treated infected cells was done with antiserum directed against whole virus particles (total antiserum) and with antiserum specific for the 38K glycoprotein. Immunoprecipitation of infected cell lysates not treated with TM precipitated the 94K, 88K, 41K, and 38K proteins with the total antiserum and the 38K glycoprotein with the 38K glycoprotein antiserum. The total antiserum directed against the TM-treated infected cell lysates precipitated not only the 94K, 88K, and 41K proteins, but also the 37K and the 35.5K

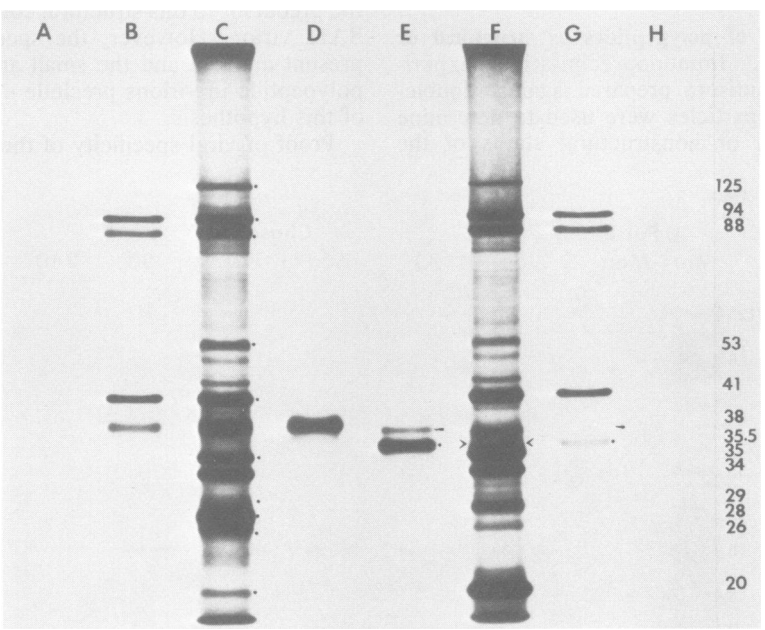

FIG. 5. Immunoprecipitation analysis of SA11-infected MA104 cells. MA104 cells were infected at a multiplicity of infection of 20 to 30 PFU/cell and labeled from 4 to 7 h p.i. with [^{35}S]methionine (20 μCi/ml) or treated with TM (1 μg/ml) for 4 h p.i. and labeled as above. Cells were harvested in RIPA buffer as described in Materials and Methods, and the RIPA lysate was analyzed with guinea pig antiserum prepared against purified double-shelled particles (SA11 serum) or against SDS-PAGE-purified 38K glycoprotein (38K serum). Infected-cell lysates (A–D) or TM-treated infected-cell lysates (E–H) were precipitated with (A, H) preimmune serum, (B, G) SA11 serum, (C, F) no serum added, or (D, E) 38K serum. In lanes E and G, the short line points to the 37K; dots represent the 35.5K polypeptide; in lane F, the arrows show the 35.5K polypeptide.

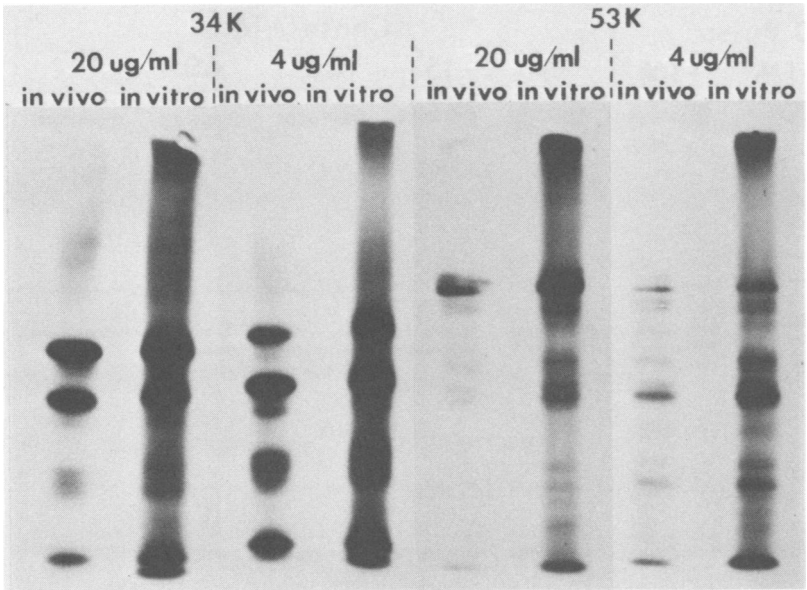

FIG. 6. Peptide mapping analysis of the 34K and 53K polypeptides. V8 protease was used as described in Materials and Methods at 20 and 4 μg/ml. Comparison of the maps of the 34K or 53K polypeptides synthesized in vivo with the maps of their respective counterparts synthesized in vitro from total SA11 transcripts (17).

polypeptides. The 38K antiserum also immunoprecipitated these latter two proteins, strongly suggesting they were directly related to the 38K polypeptide.

Partial proteolytic peptide mapping provided additional evidence that the 35.5K polypeptide was the precursor to the 38K glycoprotein (Fig. 7). The maps were identical except for one peptide that has been shown to contain the oligosaccharide moiety (B. B. Mason, D. Y. Graham, and M. K. Estes, manuscript in preparation). The partial protease map of the 35K polypeptide was sufficiently different from those of the 35.5K and the 38K polypeptides to support our designation of the 35K band as a nonstructural protein.

The 29K glycoprotein was also absent from TM-treated infected cells, and a concomitant build-up of the 20K polypeptide was observed, suggesting a relationship between the 29K glycoprotein and the 20K polypeptide. The bands of 23K, 26K, and 27K previously shown to incorporate mannose (Fig. 4) were also not found in TM-treated cells, suggesting that they were glycosylation intermediates of the 29K glycoprotein.

Unexpectedly, a 28K polypeptide remained in the presence of TM (Fig. 5), even though the previous pulse-chase experiments (Fig. 2 and 4) had shown that a band migrating as 28K was labeled with mannose. The same pattern was observed in infected cells treated with 0.05, 1, 5, or 10 μg of TM per ml. This glycosylated 28K band apparently had comigrated with the unglycosylated 28K band detected in TM-treated infected cells. To further investigate the origin of the unglycosylated 28K protein, a pulse-chase experiment was performed in the presence of

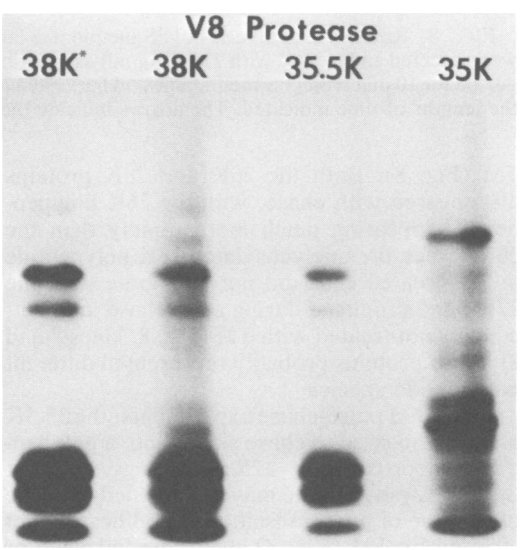

FIG. 7. Characterization of the SA11 glycoprotein and its precursor by peptide mapping. V8 protease peptide mapping (4 μg/ml) was used to compare the relationship of the glycoprotein from purified virus (38K*), the glycoprotein from infected cells (38K), the postulated precursor to this glycoprotein detected in TM-treated infected cells (35.5K), and the postulated nonstructural polypeptide (35K).

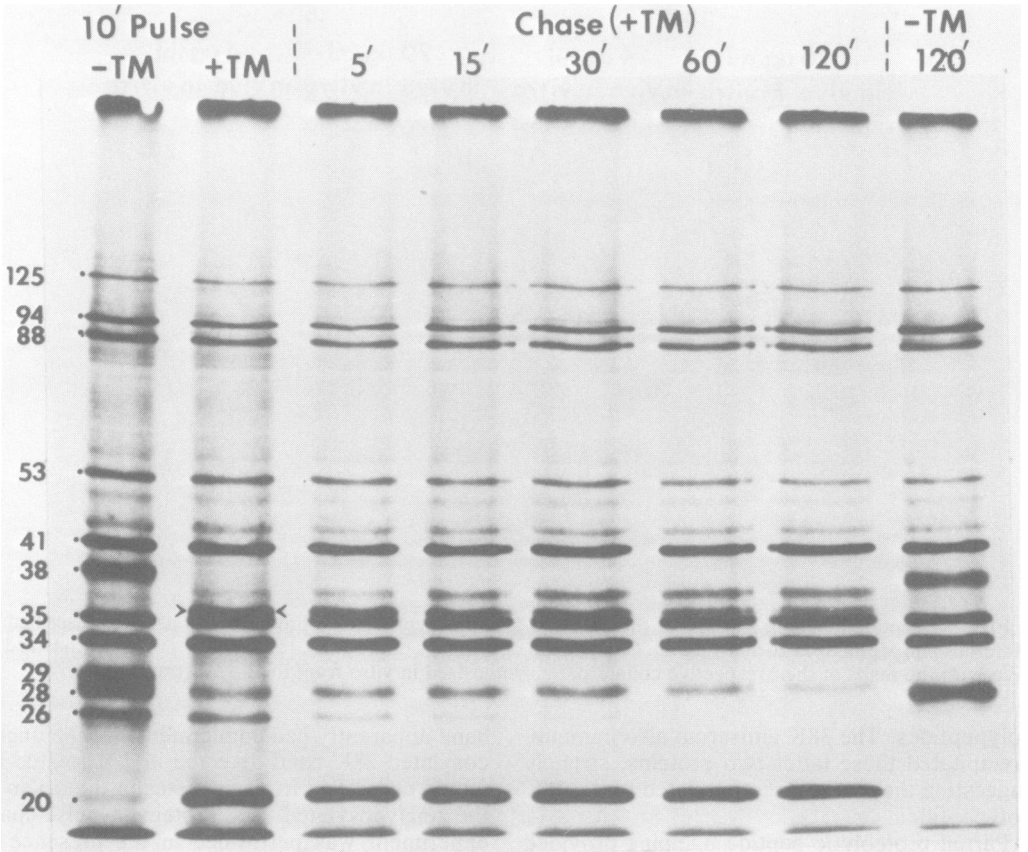

FIG. 8. Kinetics of synthesis of [^{35}S]methionine-labeled SA11 polypeptides in TM-treated cells. MA104 cells were infected and treated with TM (1 μg/ml) as described in Materials and Methods. The infected cells were then pulsed for 10 min with [^{35}S]methionine and harvested immediately or chased with 400× unlabeled methionine for the lengths of time indicated. The arrows indicate the 35.5K polypeptide.

TM (Fig. 8). Both the 26K and 28K proteins disappeared with chase, with the 26K polypeptide disappearing much more rapidly than the 28K. Since the unglycosylated 28K polypeptide in TM-treated cells did not comigrate with the 27K band produced during a 2-h chase in infected cells not treated with TM (Fig. 8, lanes 7 and 8), these proteins probably represented different polypeptide species.

In the TM pulse-chase experiments, the 35.5K protein appeared to chase slowly into a polypeptide of approximately 37K. This suggested that the 35.5K polypeptide may be modified by some other type of post-translational modification not affected by TM (e.g., *O*-glycosidic linkages) or by aberrant processing induced by TM (15). The exact modification of the 37K polypeptide remains unclear, but it did not incorporate [^{3}H]mannose, [^{3}H]galactose, or [^{3}H]fucose when labeling was attempted in the presence of TM (data not shown). The relationship of the 37K polypeptide to the 35.5K and 38K polypeptides, however, was confirmed by peptide mapping (data not shown).

28K and 26K are related polypeptides. We further investigated the origin of the unglycosylated 28K polypeptide by comparing the peptide maps of the 28K and 26K polypeptides from TM-treated infected cells with each other and with the 26K polypeptide synthesized in vitro from mRNA (Fig. 9). The results of these experiments suggested that the 26K viral polypeptide in infected cells was related to the 26K polypeptide synthesized in vitro. Furthermore, the 26K and 28K polypeptides from the TM-treated infected cells also appeared to be closely related; their protease map patterns were identical in that every peptide produced from the 26K polypeptide was present in the 28K polypeptide map. However, the 28K polypeptide map was "staggered," and the relative mobilities of the larger peptides were slower than those of similar bands in the protease map of the 26K polypeptide. This may indicate that the two proteins differ at either

their carboxy or their amino terminus. The mechanism by which these two polypeptides arise is of interest because these two bands appear to be encoded by one gene segment: the peptide maps are similar and the two polypeptides are detected simultaneously in short pulses, yet no post-translational modifications have been detected to indicate a precursor-product relationship between them.

Effects of amino acid analogs on SA11 rotavirus polypeptide synthesis. Amino acid analogs inhibit proteolytic processing and glycosylation of protein precursors (1, 10, 11, 13, 28, 29). Since this and our previous studies demonstrated that both processes were involved in the maturation of SA11 polypeptides or in the activation of viral infectivity, we attempted to better characterize these events by attempting to alter processing with amino acid analogs. Analogs for proline (2-azetidine carboxylic acid), phenylalanine (*p*-fluorophenylalanine), methionine (ethionine), and threonine (*B*-hydroxynorvaline) had no effect on polypeptide synthesis or processing (data not shown) when tested alone and in combination at concentrations ranging from 2 to 8 mM.

In contrast, with canavanine sulfate (an arginine analog), a new band migrating slightly slower than the 125K polypeptide was observed (Fig. 10). The origin of this band is unknown, but it could have represented the fourth high-molecular-weight gene product that we failed to detect either in vitro or in infected cells. More likely, this band represented one of the other high-molecular-weight proteins in which the substitution of canavanine for arginine altered the migration of the polypeptide in polyacrylamide gels. The 41K polypeptide also was altered; it migrated as a broader band when canavanine was used. This effect presumably represents incorporation of several canavanine residues into the 41K polypeptide and indicates that the 41K polypeptide may be a basic protein.

Canavanine had an effect on processing of the 29K polypeptide; chase experiments, with unlabeled methionine in the presence of 4 mM canavanine, showed that the efficiency of the processing of the 29K polypeptide to the 28K polypeptide was reduced compared with that of the sample without canavanine. Canavanine interferes with trypsin or trypsin-like enzymatic activity (13), suggesting that proteolytic processing and not oligosaccharide trimming was responsible for the observed apparent change in molecular weight. Incorporation of canavanine into rotavirus polypeptides did not noticeably affect glycosylation of rotavirus polypeptides.

This experiment also clarified the processing of the 26K polypeptide, as it appeared to increase in molecular weight during the chase, supporting our hypothesis that the 26K polypeptide gives rise to a 27K band.

DISCUSSION

This study of the biosynthesis of the simian rotavirus polypeptides in infected cells is part of our continuing efforts to understand the molecu-

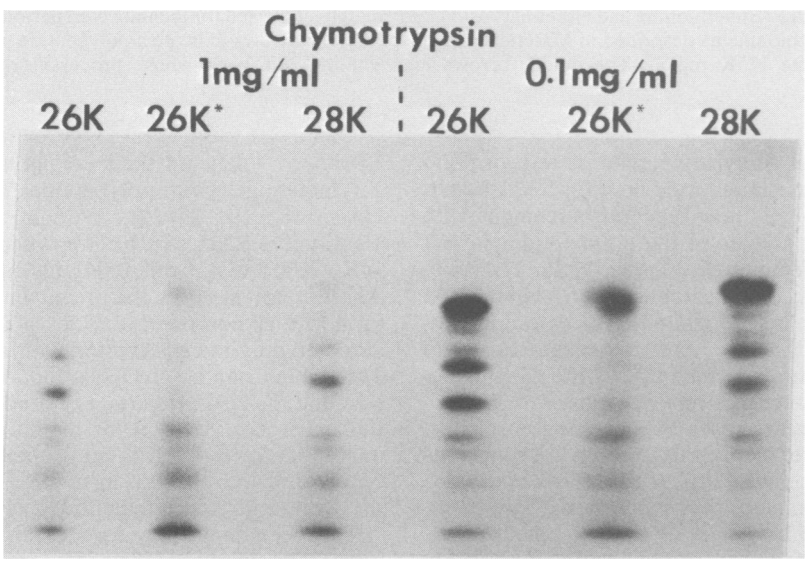

FIG. 9. Comparison by peptide mapping of the 26K and 28K polypeptides from TM-treated cells with the 26K polypeptide synthesized in vitro. Chymotryptic peptide mapping was used to compare the relationship of the 26K polypeptide synthesized in vitro (26K*) with the 26K and 28K polypeptides detected in TM-treated cells. Note the apparent "staggering" of the 28K peptide map in relation to the 26K peptide maps.

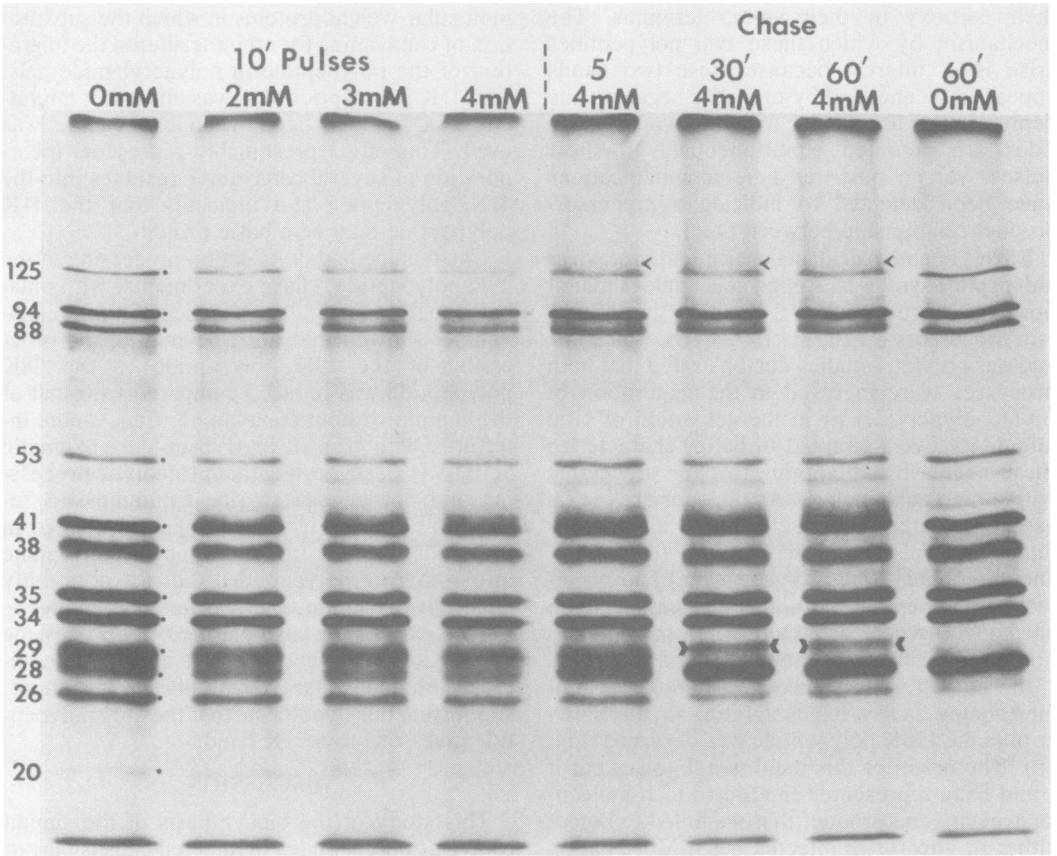

FIG. 10. Effects of canavanine sulfate on SA11 rotavirus polypeptide synthesis. MA104 cells were infected and treated with various concentrations of canavanine sulfate as indicated. At 5 h p.i., the cultures were pulsed for 10 min with [^{35}S]methionine and either harvested immediately or chased for the indicated periods of time with unlabeled methionine as described in Materials and Methods. The arrows indicate a polypeptide previously not seen above the 125K region. The double arrows highlight the 29K band whose processing is blocked by canavanine.

lar biology of rotaviruses. The pattern of polypeptide synthesis reported here for SA11-infected monkey cells is in general agreement with other recent studies of the polypeptides in calf rotavirus-infected cells (3, 18, 19, 26, 27). As in those studies, we have found that SA11 infection of MA104 cells (at high multiplicity and in conjunction with AD) greatly decreases host cell protein synthesis, which allows the easy detection of virus-specific polypeptides. This pattern shows that rotaviruses exhibit virus-host interactions more similar to those of orbiviruses (blue-tongue virus [12]) than reoviruses.

We used immunoprecipitation, partial proteolytic peptide mapping, or both to establish the structural or nonstructural status of the viral polypeptides detected in the infected cells. This approach and our results differ from previous studies which designated infected-cell polypeptides as structural (ranging in number from 6 to 15) based solely on their comigration in polyacrylamide gels with polypeptides from purified virus (18, 19, 25–27). We can definitively identify five SA11 structural polypeptides (125K, 94K, 88K, 41K, and 38K) in infected cells. Another polypeptide, the precursor to the structural 38K glycoprotein (a 35.5K polypeptide), is detected only in cells treated with the antibiotic TM, which blocks glycosylation. We hypothesize that the 26K primary gene product polypeptide is processed (type of modification as yet unidentified) to a ~27K polypeptide that is incorporated into the virion in small amounts as the viral structural polypeptide, VP9.

The remaining polypeptides that were not immunoprecipitated (53K, 35K, 34K, 29K, 28K, and 20K) represented either nonstructural polypeptides or precursors to the structural polypeptides. Our accumulated data suggest that there are four primary nonstructural polypeptides: the

53K and 34K polypeptides, which were shown to be virus-specific by their identity with their in vitro-synthesized counterparts; the 29K to 28K glycoprotein synthesized from the 20K precursor; and the 35K polypeptide. The 35K polypeptide was distinguished from the 35.5K precursor to the structural glycoprotein. The 35K polypeptide from infected cells has not yet been proven identical to its in vitro-synthesized counterpart, because proteolytic cleavage which occurs in the in vitro system has made it difficult to obtain purified 35K polypeptide synthesized in vitro.

Pulse-labeling experiments and labeling with sugar precursors suggest that the rotavirus 38K and 29K glycoproteins are glycosylated cotranslationally, as was also recently suggested for VP7 of the calf rotavirus (19). This is consistent with these glycoproteins containing N-linked oligosaccharides (15). Additional modification of both the 38K structural glycoprotein and the 29K nonstructural glycoprotein was observed during the chase experiments, as was also reported for the calf rotavirus (19, 27). Since the use of cycloheximide in the chase periods did not alter this processing, both of these events apparently occur post-translationally. It remains to be proven whether these final polypeptide modifications result from proteolytic cleavages or carbohydrate trimming, but blockage of the 29K trimming by the analog canavanine supports proteolytic cleavages for the processing of the 29K polypeptide.

Previous reports (17–19) had shown that rotavirus glycoproteins incorporate [^{3}H]glucosamine into the oligosaccharide moieties. Our experiments showed that [^{3}H]mannose was also incorporated to a very large extent into several polypeptides (38K, 29K, 28K, 27K, 26K, and 23K) along with glucosamine, whereas fucose and galactose were not incorporated. This suggests that all the rotavirus glycoproteins contain at least one high-mannose oligosaccharide moiety (15). The fact that neuraminidase treatment also did not alter the migration of the 38K glycoprotein and the digestion of the 38K glycoprotein by endo-β-acetylglycosaminidase H confirms the presence of high-mannose oligosaccharides (data not shown).

We have further extended these results by examining the polypeptide(s) synthesized in MA104 cells infected with calf (Lincoln strain) and porcine (OSU strain) rotaviruses in the presence and absence of TM (data not shown). Our results with SA11 offer a general model for all these viruses, except that the molecular weights for the individual polypeptides differ in the different virus strains, in agreement with previous reports (22, 26). In addition, the relative migration of the precursor to the structural glycoprotein can vary between virus strains. In some cases, the glycoprotein precursor migrates more slowly than the 35K nonstructural protein, and with other virus strains the precursor migrates between the 35K and 34K nonstructural proteins. The different migration of these precursors may explain the report that the hypothesized precursor to the calf virus glycoprotein (UK strain) migrates more rapidly than the calf virus proteins designated VP8 and VP9 by McCrae and Faulkner-Valle (19).

Amino acid analogs have been used to inhibit proteolytic processing or glycosylation of viral proteins (or both) (1, 10, 11, 13, 28, 29), but their effectiveness as probes of rotavirus polypeptide modification was varied. *B*-Hydroxynorvaline can block glycosylation when the glycosylation site has the sequence of Asn-X-Thr (10). The failure of this analog to block glycosylation of any of the rotavirus glycoproteins might suggest

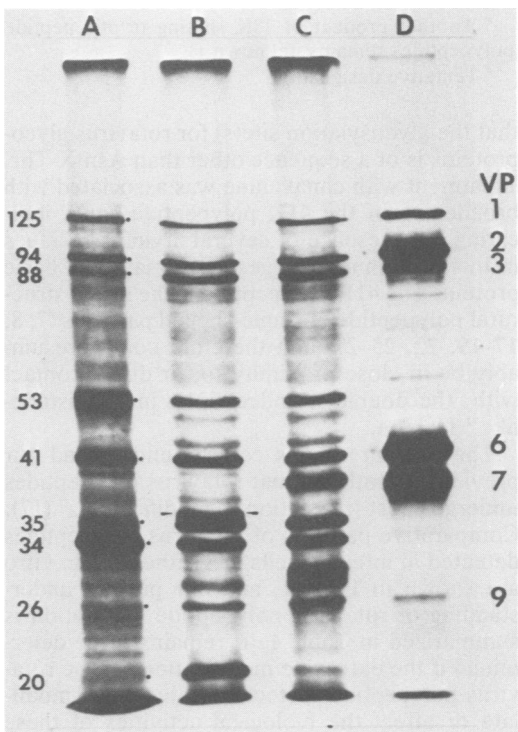

FIG. 11. Comparison of SA11 polypeptides. SA11 polypeptides synthesized from mRNA in a rabbit reticulocyte lysate in vitro translation system (A). The dots represent the nine previously identified primary gene products (17). The polypeptide pattern, in a 10-min pulse, at 4 h p.i. in infected cells with (B) or without (C) TM treatment. (D) Purified double-shelled SA11 virus polypeptides. The viral proteins are labeled as VP1 through VP9 as previously described (8). All of the above polypeptide patterns have been labeled with [^{35}S]methionine.

TABLE 1. Summary of SA11 polypeptides and their modifications

Primary product	Designation	Modification	Modified product
125K	VP1 (inner[a])	None	None
94K	VP2 (inner)	Cleaved	VP3*, VP4*[b] (88K, 84K)
88K	VP3 (outer)	Cleaved	VP5*, VP8* (60K, 28K)
53K	Nonstructural	None	None
41K	VP6 (inner)	None	None
35.5K	VP7 (outer precursor)	Glycosylated and trimmed[c]	VP7 (38K)[d]
35K	Nonstructural	None	None
34K	Nonstructural	None	None
26K[e]	VP9 (outer precursor)[f]	Yes, type unknown	~27K
20K	Nonstructural[f] (precursor)	Glycosylated and trimmed[c]	28K, 29K

[a] Location in inner or outer capsid of the virus.
[b] *, Cleavage product from nomenclature in Estes et al. (8).
[c] Type of trimming unknown.
[d] The molecular weight of the structural glycoprotein can vary from 36,000 to 38,000, depending on the plaque isolate of virus analyzed.
[e] Another product of 28K sharing tryptic peptides is also detectable. The origin of both of these related polypeptides remains unknown.
[f] Tentative designation.

that the glycosylation site(s) for rotavirus glycoproteins is of a sequence other than Asn-X-Thr. Treatment with canavanine was associated with broadening of the 41K polypeptide band, indicating the presence of several arginine residues in this protein and suggesting it may be a basic protein. The 41K polypeptide is the major structural polypeptide of single-shelled particles (7, 8, 17–19, 22, 25–27) and therefore could presumably be in close proximity to, or direct contact with, the double-stranded RNA in a "histone-like" fashion.

The present studies confirm and extend our previous hypothesis that rotavirus polypeptides undergo post-translational modifications (17). Comparative patterns of rotavirus polypeptides detected in infected cells or synthesized in vitro are shown in Fig. 11, and our present understanding of rotavirus polypeptide maturation is summarized in Table 1. It remains to be determined if the extensive modifications of the rotavirus polypeptides detected in this study modulate or affect the biological activities of these proteins. It is apparent that some of the differences in the amounts of the polypeptides synthesized in vivo or in vitro which have suggested gene regulation can be explained on the basis of post-translation modifications.

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