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CELL FREE TRANSCRIPTION AND TRANSLATION OF ROTAVIRUS RNA

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SUMMARY

Rotavirus single stranded RNA synthesized in vitro by endogenous RNA transcriptase consists of 11 segments that hybridize with genomic RNA. It displays messenger activity when incubated with rabbit reticulocyte lysate. The products of translation were precipitable by anti-rotavirus serum, but not by normal serum.

INTRODUCTION

Rotavirus RNA polymerase is found within the virion and becomes active when the outer capsid layer is removed from the fully infectious particles by calcium chelation (1,2). Dense (D) particles obtained by this treatment exhibit a highly active polymerase that synthesizes, using the double stranded genome as template, single stranded RNA that hybridizes with viral RNA. By analogy with reovirus (3,4) it can be hypothesized that the in vitro rotavirus enzyme product might serve as messenger. The data presented here characterize the rotavirus polymerase product and demonstrate its ability to stimulate polypeptide synthesis in a rabbit reticulocyte lysate.

MATERIALS AND METHODS

a) Preparation of SS RNA

The isolation and purification of calf rotavirus, as well as the in vitro production of rotavirus SS RNA have been described previously (1). For mass production of SS RNA 500 µg of D particles were suspended in 5 ml of reaction mixture (containing the four nucleoside triphosphates, 2 mM each; $MgCl_2$, 10 mM; phosphoenol pyruvate, 5 mg/ml; pyruvate kinase, 0.5 mg/ml; tris pH 8, 100 mM). The reaction mixture was incubated at 37°C for 6 hours, followed by ultracentrifugation at 35,000 rpm for 60 minutes to remove rotavirus particles. The supernatant was extracted twice with phenol and

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the RNA precipitated with 66% ethanol at -20°C. The dense precipitate was dissolved in sterile distilled water and an equal volume of 4 M LiCl solution was added and SS RNA was allowed to precipitate overnight at -20°C. This procedure was repeated twice in order to remove trace amounts of DS RNA which remained in the supernatant. At this step the yield, estimated by spectrophotometry (O.D. 260) was 1.2 mg of RNA. The transcription product was analyzed by electrophoresis either in 5% polyacrylamide slab gels or using composite agarose-acrylamide (0.6% vs 2%, respectively) slab gels as described by Loening (5). The RNA was stained with ethidium bromide, or in case of isotopically labeled RNA, it was detected by fluorography as described by Laskey and Mills (6).

b) Cell free protein synthesis

Rotavirus SS RNA was translated in a rabbit reticulocyte lysate prepared essentially as described by Pelham and Jackson (7). Incorporation [^{35}S]-L-Methionine into protein was measured by spotting 1 µl aliquots of the 25 µl incubation mixture on Whattman 3 MM paper. After precipitation in cold, followed by hot 5% TCA (containing 5% casamino acids), the papers were washed in ethanol, dried, and the radioactivity counted in a liquid scintillation counter using a toluene based scintillation cocktail. All values were corrected for 0 time control. The translation products were analyzed by electrophoresis in 12.5% polyacrylamide slab gels as described by Laemmli (8) followed by autoradiography. Immunoprecipitation was performed as described by Kerr *et al.* (9).

RESULTS AND DISCUSSION

a) Characterization of the rotavirus polymerase product

When tritiated SS RNA was hybridized with an excess of viral RNA, the gel profile obtained after fluorography (Fig. 1A) was qualitatively identical to the pattern obtained with purified viral DS RNA (Fig. 1B). This indicated that each of the viral genome segments were fully transcribed *in vitro* into SS RNA. However, the relative amounts of each hybrid segment did not correspond to the molar ratio of those observed in the virus particle. For example, as shown in Figure 1A, the dsRNA hybrids of the large size class were reduced in quantity when compared to that present in the virion (Fig. 1B) indicating that the *in vitro* transcription of large size class RNA is less efficient than those of the middle and small size RNA's. When the product of the endogenous polymerase was analyzed by composite agaros-acrylamide gel electrophoresis, eight bands of SS RNA were resolved (Fig. 1C). The slowest band co-migrated with 23S ribosomal RNA as would be expected for the single stranded copy of the largest DS RNA genome segment (Mol. wt. 2.2 x 10^6)(10,11). However, no precise molecular weight

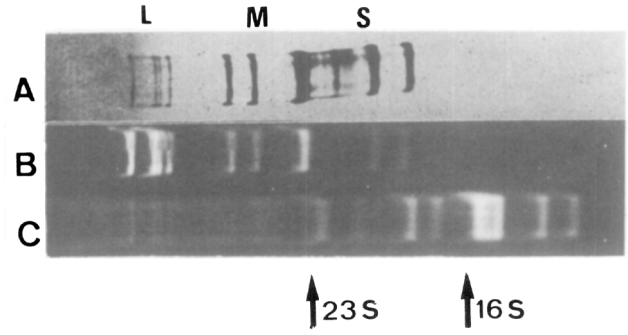

Figure 1: Electrophoretic pattern of in vitro transcription product
Acrylamide gel electrophoresis in 5% gel of ^{3}H-uridine labeled transcription product (SS RNA) annealed with excess unlabeled, denatured virion DS RNA (line A). Approximately 5 µg of purified DS RNA was annealed with 15,000 cpm of transcriptase product as described previously (1). The preparation was treated with RNase A (10 µg/ml in 0.3 M NaCl) precipitated with 66% ethanol and resuspended in Loening's buffer. After electrophoresis, the gel was processed for fluorography. On line B and C the genome template and the transcriptase product are analyzed in composite agarose - 2% acrylamide gels and stained with ethidium bromide. E. coli 23S and 16S rRNA's were run in parallel wells to serve as molecular weight markers. L, M, and S indicates the location of large, medium, and small size class virion dsRNA's in lines A and B. Migration was from left to right.

determination could be done as electrophoresis was not performed under denaturing conditions. Traces of DS RNA are visible in Fig. 1C where the in vitro transcription product was analyzed before LiCl precipitation. When used in protein synthesis, DS RNA was always removed by LiCl precipitation since DS RNA is known to inhibit cell free protein synthesis (12).

b) Translation of rotavirus SS RNA in rabbit reticulocyte lysates

Addition of in vitro produced rotavirus SS RNA to rabbit reticulocyte lysates resulted in the incorporation of [^{35}S]-L-Methionine into large molecular weight, acid precipitable product. This incorporation continued at a linear rate for 30 minutes at 37^{o}C and for 2 hours at 22^{o}C (Fig. 2). At the lower temperature, the extent of amino acid incorporation was increased by the added rotavirus RNA about 15 fold above the control. At 37^{o}C, the initial rate of protein synthesis was higher than at 22^{o}C, however, it stopped completely after 20 minutes of incubation. The

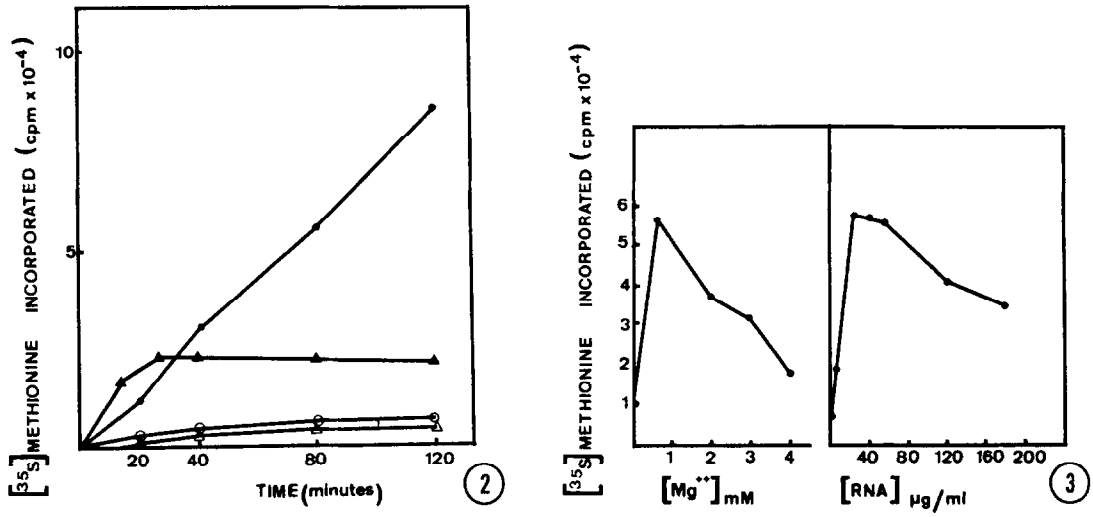

Figure 2: Time course of protein synthesis

Control lysates (open symbols) and reticulocyte lysates supplemented with 24 µg/ml of rotavirus SS RNA (filled symbols) were incubated at 37°C and 22°C. [^{35}S]-L-Methionine incorporation was assayed by taking 1 µl aliquots at various times as shown. Control lysate 22°C (O) and 37°C (Δ); and lysate with 24 µg/ml of SS RNA at 22°C (●) and 37°C (▲).

Figure 3: Mg^{2+} concentration optimum and RNA saturation curve for the translation of rotavirus SS RNA

In the rabbit reticulocyte system, translation assays were performed at 22°C in a total volume of 25 µl. Samples of 1 µl were removed at 20, 40, 80, and 120 minutes, and processed to measure the incorporation of [^{35}S]-L-Methionine into TCA precipitable material. The Mg^{++} concentration optimum was determined using 80 mM K^+ and 24 µg/ml of SS RNA. The RNA saturation curve was measured using a K^+ of 80 mM and a Mg^{++} of 0.7 mM.

optimal magnesium ion concentration was 1 mM and the optimal SS RNA concentration was 0.6 µg for a 25 µl assay (Fig. 3). Residual traces of DS RNA could be responsible for inhibition of protein synthesis at high concentrations of exogenous RNA. In the limited range tested (from 50 to 100 mM) potassium ion concentration had little influence on protein synthesis at a Mg^{++} concentration of 1 mM (data not shown).

A comparison of the electrophoretic pattern on SDS polyacrylamide slab gels of protein synthesized in the cell free system in the presence or absence of added messenger RNA after a two hour incubation period at 22°C is shown in Figure 4.

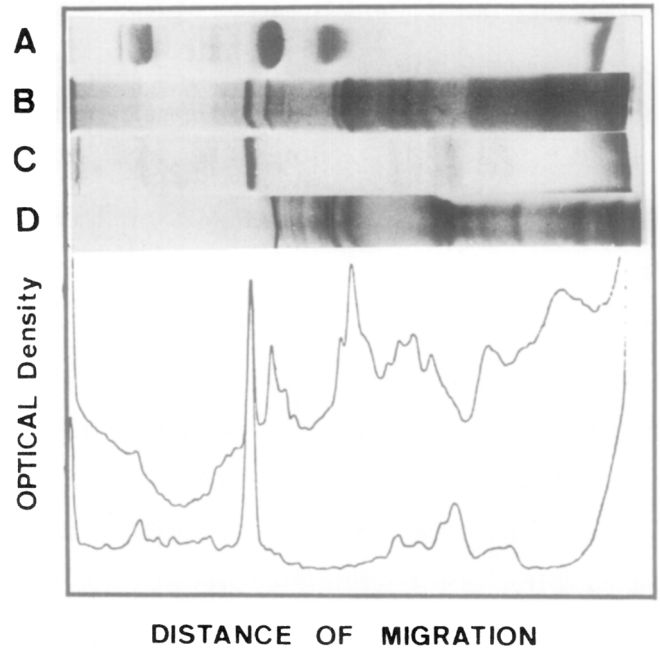

Figure 4: Analysis of the protein products synthesized in rabbit reticulocyte lysate

Purified rotavirus and in vitro translation products analyzed in 12.5% polyacrylamide slab gels. Track A shows rotavirus structural proteins stained with coomassie blue. Track B, C, and D are autoradiograms of labeled products made in vitro ($22^{o}C$, 2 hr incubation) and analyzed in the same gel. Track B and C respectively represents the polypeptides produced with or without rotavirus SS RNA. Track D represents the immune precipitate of the material analyzed in Track B. Curves below are densitometric tracings of Track B (upper) and Track C (lower). Direction of migration is from left to right.

Without added SS RNA, little protein is synthesized in nuclease incubated reticulocyte lysate (Fig. 4C). Analysis of the rotavirus SS RNA directed products shows several proteins in the molecular weight range from 44,000 to 12,000. One of them has the same mobility as the major rotavirus capsid protein. This polypeptide is precipitated by anti-rota-hyperimmune rabbit serum (Fig. 4D). Two heavy bands migrate a little faster than the doublet of glycoprotein present in the outer capsid layer of rotavirus (apparent molecular weight for the glycosylated peptides, 33,000 and 31,000). These two bands are also precipitated by the hyperimmune serum, and could correspond to the unglycosylated form of the structural polypeptides (Fig.

4D). It is noteworthy that the single polypeptide present as an endogenous reticulocyte product in Track C (also present in Track B) is not present in the immunoprecipitated product (Track D). In the immune precipitate eight smaller polypeptides are visible; they could correspond to premature termination products or products due to degraded SS RNA of the large size class, which under the experimental conditions used could not be translated into high molecular weight polypeptides. No labeled proteins were detectable in the immune precipitate control. Unfortunately, tryptic peptide maps of the labeled polypeptides produced in the cell free system could not be compared to those of labeled virion proteins because rotavirus replicates very poorly in tissue culture, thus the growth and purification of [^{35}S]-L-Methionine labeled rotavirus is not feasible at present. For this reason fidelity of translation was established using anti-rotavirus immune serum. The above results provide evidence that rotavirus SS RNA obtained *in vitro* exhibits messenger activity as it has been shown previously for reovirus (3,4). Some of the polypeptides synthesized seem to be virus specific as they are precipitated by a specific immune serum. The results will be of use in further studies on virus directed protein synthesis in the infected cell.

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