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In Vitro Transcription of Two Human Rotaviruses

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The RNA polymerase activities of a cultivatable (Wa) and a noncultivatable (DS-1) strain of human rotavirus were studied. Under optimal conditions, transcription of all of their RNA segments occurred, as evidenced by the hybridization of labeled transcripts to genomic RNA. Cross-hybridization between the two viruses showed that none of their 11 genes were completely homologous. The transcription products could be translated in vitro, yielding proteins with an electrophoretic pattern resembling that obtained with proteins labeled in vivo during infection with the Wa virus.

The ability of several double-stranded RNA (dsRNA) viruses to transcribe in vitro their genomic RNA into single-stranded RNA (ssRNA) has been exploited in a number of ways to study various aspects of their biology. For some of these viruses, a good deal of information has been obtained, not only about the transcription and its regulation (2, 17, 20, 21), but also about the resulting products. Reovirus ssRNA synthesized in vitro has the same polarity as mRNA produced during infection (7) and can direct protein synthesis in cell-free translation systems. These characteristics have permitted study of the viral proteins and the assignment of gene coding functions (15). Furthermore, the abundance of the ssRNAs synthesized in vitro has allowed initial sequencing of their 5′ and 3′ ends (12, 16). Labeled ssRNAs synthesized in vitro can be used as hybridization probes to recognize sequence homologies among different viruses (13). The transcription reaction becomes particularly useful in studying viruses, such as the human rotaviruses, which do not grow in tissue culture or do so to only a limited extent. RNA polymerase activity in calf and human rotaviruses has been described (1, 3, 4, 8, 22). Similar activity in the simian rotavirus SA11 has been used recently to characterize the viral gene products (18).

We have studied the in vitro transcription of two different human rotavirus strains (Wa and DS-1) and have used the transcription products to gain information concerning the protein coding capabilities of their genomes and the genetic relatedness between the two viruses. The Wa strain is a human rotavirus that has been adapted to grow in tissue culture after multiple passages in piglets (24). The DS-1 strain is a noncultivatable human rotavirus. The Wa strain and a DS-1 bovine rotavirus reassortant with the same neutralization specificity as the human virus are distinct in reciprocal plaque neutralization tests with hyperimmune sera (6); in addition, the Wa and DS-1 strains are distinct in enzyme-linked immunosorbent assay and immune adherence hemagglutination assays (10).

MATERIALS AND METHODS

Virus purification. The DS-1 rotavirus was originally obtained from the feces of a child with diarrhea in the Washington, D.C., area. To increase the amount of virus for study, the strain was passaged in gnotobiotic calves, a permissive host for this virus. The gel electrophoretic pattern of RNA from virus grown in calves was identical to that of virus present in the infected child. Virus obtained from the stools of an experimentally infected calf was employed in this study.

Human rotavirus Wa was grown in MA104 cells in the presence of trypsin (0.5 μg/ml) after preincubating the inoculum with 10 μg of trypsin per ml for 1 h and adsorbing it onto the cells for 1 h. Cytopathic effects were initially detected after 48 h, and the cultures were harvested at 72 h postinfection.

The virus was purified after fluorocarbon extraction (Genetron 113; Allied Chemical Corp., Morristown, N.J.) and centrifugation at intermediate speed (10,000 rpm for 10 min in a Sorvall RC4 centrifuge). The virus in the supernatant was then pelleted through a 30% sucrose cushion by centrifugation at 35,000 rpm in a Beckman SW40 rotor for 2 h. The pellets were further purified by centrifugation overnight on a 40 to 55% CsCl gradient. Visible bands corresponding to densities of 1.38 and 1.36 g/ml were collected and pooled; they represented single- and double-shelled particles, respectively. The pooled suspension was treated with 10 mM EDTA (30 min at 37°C) to convert double-shelled particles into single-shelled particles and centrifuged again on a 40 to 55% CsCl gradient. Fractions containing single-shelled particles were pelleted and suspended in 50 mM Tris-hydrochloride (pH 7.0).

In vitro transcription. Five to ten micrograms of virus was utilized for a 500-μl transcription reaction

that contained 2.5 mM ATP, GTP, UTP, and CTP; 0.5 mM *S*-adenosylmethionine; 0.1% bentonite; 12 mM $MgCl_2$; 100 mM Tris-acetate (pH 8.0), and a nucleoside triphosphate regenerating system consisting of 8 mM phosphoenolpyruvate and 50 μg of pyruvate kinase per ml. In several instances the regenerating system was omitted, and the concentration of ATP was increased to 10 mM. When labeled RNA was synthesized, either [^{32}P]GTP or [^{32}P]ATP (60 μCi per reaction) was used, and the concentration of the corresponding unlabeled nucleotide was lowered to 0.625 mM.

Optimal transcription occurred when the reaction mixture was incubated for 6 h at 42°C. Although the reaction was still linear beyond 6 h, the larger RNA segments synthesized became less abundant after longer incubations. After 6 h of incubation, viral particles and bentonite were pelleted, the supernatant was extracted with phenol, and RNA was precipitated with 3 volumes of ethanol after overnight incubation at −20°C. In some instances, ssRNAs synthesized were separated from the remaining genomic dsRNA by precipitation with 2 M LiCl (overnight incubation at 4°C).

Hybridization of ssRNA to genomic RNA. To assess whether all of the RNA segments were synthesized in vitro and to determine segment homologies between the two viral strains, preparations of labeled ssRNAs were hybridized to dsRNA obtained by phenol extraction of purified virus. For this purpose, dsRNAs were denatured and allowed to hybridize to homologous or heterologous ^{32}P-labeled ssRNAs. About 0.2 μg of dsRNAs was incubated for 30 min at 40°C in 90% dimethyl sulfoxide, followed by the addition of 10 μg of wheat germ tRNA (added as a carrier), 0.5 volume of 0.9 M NaCl, 3 volumes of ethanol, and 20,000 to 30,000 cpm of ssRNAs. The mixture was left overnight at −20°C, after which the RNAs were precipitated and taken up in hybridization buffer (100 mM NaCl, 50 mM Tris-hydrochloride [pH 8.0], 0.1% sodium dodecyl sulfate) and incubated at 72°C for 6 h to allow hybridization to occur. RNAs were then precipitated with ethanol and taken up in a mixture containing 100 U of S1 nuclease (Bethesda Research Laboratories, Bethesda, Md.) in 50 mM NaCl–1 mM $ZnSO_4$–5% glycerol–30 mM sodium acetate (pH 4.6). This mixture was incubated for 30 min at 37°C, diluted in sample buffer, and analyzed electrophoretically on a 7.5% discontinuous gel by the method of Laemmli (14), except for the omission of sodium dodecyl sulfate. After overnight electrophoresis at 10 to 15 mA, the gel was stained with ethidium bromide, photographed under UV light, dried, and autoradiographed on Kodak XA R film.

Agarose gel electrophoresis of RNA. RNA produced by in vitro transcription was electrophoresed in 1.5% low-melting-temperature agarose gels (Sea Plaque; Marine Colloids, Rockland, Maine) in Tris-acetate-EDTA buffer (40 mM Tris, 5 mM sodium acetate, 1 mM EDTA [pH 7.8]). Flat-bed gels (0.5 by 20 by 25 cm) were run overnight at a constant current of 75 mA and then stained with ethidium bromide, photographed, and autoradiographed.

Translation of ssRNA. Unlabeled transcription products prepared as described above were used to direct protein synthesis in a rabbit reticulocyte in vitro translation system (Bethesda Research Laboratories) which included 2 μCi of [^{35}S]methionine per reaction. The ssRNAs were incubated in this system for 90 min at 30°C. The mixture was then treated with pancreatic ribonuclease (30 min at 37°C), and aliquots of the product were studied by electrophoresis in 7.5 to 12% Laemmli gels (14) which were fixed in 10% acetic acid–30% methanol, dried, and autoradiographed.

Immunoprecipitation of the proteins translated in vitro was performed by incubating them with specific antirotavirus antisera. Antiserum against DS-1 was prepared with a partially purified preparation of the virus extracted from stools. Antiserum against Wa was prepared with virus sedimented at high speed from infected-culture lysates. Both antisera were obtained by inoculating guinea pigs with the respective viral preparations, following the immunization protocol described by Kapikian et al. (11). The labeled protein-antibody complex was precipitated with staphylococcal protein A and analyzed by sodium dodecyl sulfate gel electrophoresis.

Labeling of viral proteins during infection. Labeling of rotavirus proteins during infection of MA104 cells was done by the following method. Wa virus at a multiplicity of infection of ≥10 was adsorbed for 1 h onto cell monolayers (in six-well plates). The wells were then washed, and after 15 h of infection, the medium (minimal essential medium with Eagle salts) was replaced with methionine-free medium. [^{35}S]Methionine was added to the wells 2 h later at a final activity of 200 μCi/ml, and the cells were washed with buffered saline and lysed and harvested with sodium dodecyl sulfate sample buffer 1 h later. Electrophoresis of the resulting material was performed as described for the translation products. Mock-infected cell monolayers were used as controls.

RESULTS

Transcription. Incorporation of ^{32}P into trichloroacetic acid-precipitable material was used to monitor transcription. Although single-shelled particles showed full activity without being treated with EDTA, double-shelled particles (complete virions) had to be pretreated with EDTA to activate transcription. Such pretreatment resulted in a shift of density from 1.36 to 1.38 g/ml due to the removal of the outer capsids. When the incubation took place at 42°C, the reaction was linear for up to 10 h (longest time studied); however, prolonged incubation resulted in a high degree of RNA fragmentation. The products of Wa transcription are shown in Fig. 1A. There were 10 bands of ssRNA resolved, and they appeared to represent the 11 virion RNA segments in approximately equimolar amounts. Figure 1B is an autoradiograph corresponding to the same preparation depicted in Fig. 1A and shows the presence of radioactivity in all of the RNA segments. The pattern is reproducible; however, it is not known whether the order of mobility corresponds to that of the virion dsRNAs. Figure 1C represents the migration pattern of the RNAs transcribed in vitro by the DS-1 virus. Nine bands of high intensity which represent the newly synthesized ssRNAs

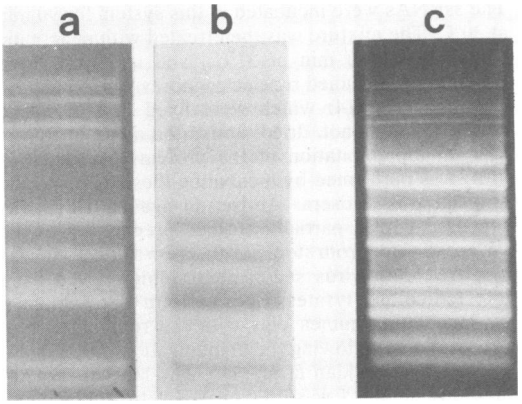

FIG. 1. (a and b) ^{32}P-labeled ssRNA synthesized in vitro by Wa single-shelled particles. The RNA segments were resolved by electrophoresis in low-melting-temperature agarose (Sea Plaque) and analyzed by staining with ethidium bromide (a) or by autoradiography (b). (c) DS-1 transcription products as seen under UV light after ethidium bromide staining. The thinner bands represent virion dsRNA segments.

were visualized (although in some experiments the second band was resolved into two). The thinner bands seen at the top of Fig. 1C represent genomic dsRNA segments which were not completely removed from this preparation. Treatment with RNase before electrophoresis resulted in complete digestion of the major bands, whereas the thinner bands were unaffected (data not shown).

Hybridization. When labeled ssRNAs were hybridized to the homologous dsRNAs and analyzed by acrylamide gel electrophoresis and autoradiography, the resulting pattern was identical to that obtained with UV light visualization of ethidium bromide-stained dsRNAs. However, when heterologous hybridizations were carried out (i.e., dsRNAs from Wa and ssRNAs from DS-1 and vice versa), none of the 11 segments seen under UV light incorporated any radioactivity. The results are shown in Fig. 2.

Translation. The ability of the ssRNAs from the two viruses to direct protein synthesis in a rabbit reticulocyte in vitro translation system is shown in Fig. 3A. When DS-1 transcripts were translated in vitro, at least nine protein bands ranging in molecular weight from 32,000 to 118,000 were observed. A similar but not identical protein pattern resulted when Wa transcripts were translated. Immunoprecipitation with homologous sera of the translation products of each virus was carried out to ascertain the viral nature of the proteins translated in vitro. With both Wa and DS-1, all of the proteins synthesized were precipitated, except for a protein with an approximate molecular weight of 48,000, which was present in the control translation mixture to which no exogenous mRNA was added (Fig. 4).

The migration pattern of the polypeptides labeled during Wa infection of MA104 cells is shown in Fig. 3B. Under the conditions used, Wa virus was not able to totally suppress cell protein synthesis; however, new protein bands were easily detected after 18 h postinfection. Shorter infection times did not allow clear distinction between proteins synthesized in the presence of the virus and those from uninfected control cells. The migration patterns and molecular weights of these proteins are compared with those of the translation products in Fig. 3B and Table 1.

DISCUSSION

Studies of the pathogenesis of human rotavirus infection have been hampered by the difficulty in obtaining a suitable cell line or finding the appropriate conditions under which the virus can multiply efficiently or both. Although a cultivatable mutant of the Wa virus had been

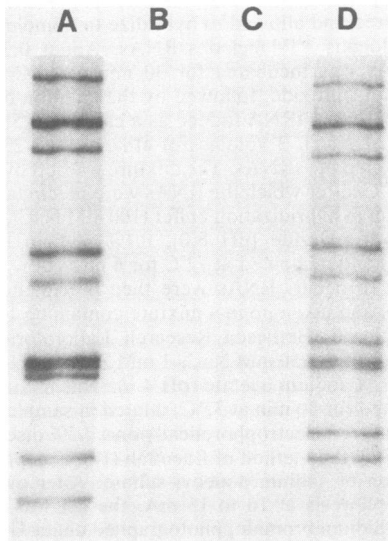

FIG. 2. Cross-hybridization of Wa and DS-1 RNAs. Virion dsRNA (200 ng) from Wa or DS-1 was hybridized to labeled ssRNA (25 ng) obtained by in vitro transcription from the same virus. In lane A, dsRNA from the Wa virus was hybridized to a homologous probe (^{32}P-labeled ssRNA from Wa); in lane D, dsRNA from DS-1 was hybridized to ssRNA from DS-1. In lane B, dsRNA from Wa was hybridized to a DS-1 probe; in lane C, dsRNA from DS-1 was hybridized to a Wa probe. The hybrids were electrophoresed on a 7.5% acrylamide gel, dried, and autoradiographed. The electrophoretic patterns in lanes A and D are identical to those observed for the undenatured dsRNAs from the corresponding viruses.

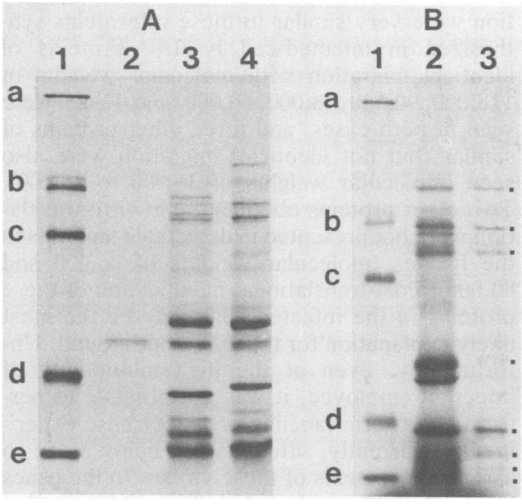

FIG. 3. (A) Electrophoretic analysis of proteins synthesized by the in vitro translation of ssRNA obtained from transcription of the Wa (lane 3) and DS-1 (lane 4) viruses. (Lane 2) Background translation of the system when no exogenous RNA was added to it. The following methylated markers (and their corresponding molecular weights) were run in lane 1: (a) myosin, 200,000; (b) phorphorylase b, 92,500; (c) bovine serum albumin, 69,000; (d) ovalbumin, 46,000; and (e) carbonic anhydrase, 30,000. (B) Electrophoretic analysis of proteins synthesized during infection of MA104 cells with Wa virus as described in the text (lane 3). Bands marked with a ■ were not present in the control mock-infected lysates. (Lane 2) Migration of Wa in vitro translation products; (lane 3) the same molecular weight markers as were run in Fig. 3A, lane 3.

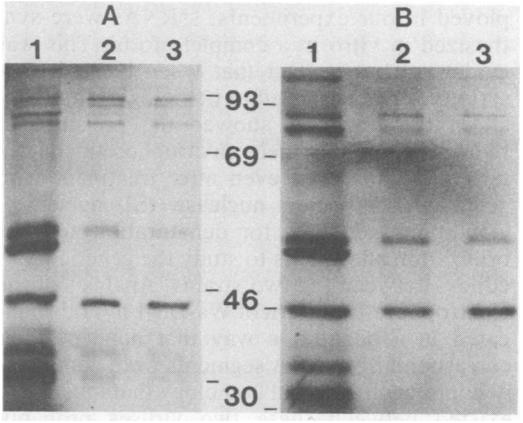

FIG. 4. Immunoprecipitation of proteins translated in vitro from ssRNA synthesized by the Wa (A) and DS-1 (B) viruses. (Lanes A1 and B1), translation products before immunoprecipitation; (lanes A2, B2, A3, and B3, proteins precipitated by incubation with Wa (A) or DS-1 (B) antisera diluted in phosphate-buffered saline at 1:50 and 1:100 dilutions, respectively, followed by the addition of staphylococcal protein A. A major protein, seen in lanes A1 and B1, which was not immunoprecipitated corresponds with the background protein described in the legend to Fig. 3. Numbers in the middle represent molecular weight markers ($\times 10^3$).

recovered after serial passages in gnotobiotic piglets, it is not totally representative of the many different viruses that infect humans in nature. More recently, a procedure to grow and propagate human rotaviruses (19, 23) was described. The efficiency of such a procedure has not yet been established. Fortunately, the transcriptase activity present in virions that can be purified from stools or from cultures offers an opportunity to study several aspects of their biology.

On the one hand, transcripts formed in vitro are likely to be faithful copies of the plus strands of the genomic RNA, as demonstrated for reovirus (16), and thus can be used as hybridization probes to analyze nucleotide sequence relatedness between different viruses. On the other hand, since transcripts formed in vitro may function as mRNA by programming the translation of specific proteins in a cell-free extract, an opportunity is available to identify the primary gene products of rotaviruses and possibly make comparisons between different strains. The transcriptase activity of human rotavirus has been studied to only a limited extent (1, 8, 22). The activation of the enzyme by chelation with EDTA is similar to that described for other rotaviruses (4, 18). Under the conditions em-

TABLE 1. Comparison of molecular weights of polypeptides synthesized by in vitro translation of DS-1 and Wa RNAs obtained by in vitro transcription and polypeptides labeled during Wa infection of MA104 cells

Mol wt ($\times 10^3$) of polypeptides from:		
In vitro-translated RNA		Wa-infected cell lysates
DS-1	Wa	
118	118	118
	90.5	90.5
86	86	
79	80	
78	78	78
50	50	50
44	42	42
37	36	34.5
35	34	32
32	31	31
		24.5
20[a]	23[a]	22.5
17[a]	19[a]	19

[a] Not always synthesized efficiently by the translation system.

ployed in our experiments, ssRNAs were synthesized in vitro in a complete form. This was evidenced by the fact that when labeled transcripts were hybridized to homologous dsRNA, all of the segments showed the presence of radioactivity, and their electrophoretic migration was conserved even after treatment with single-strand-specific nuclease (S1 nuclease). Conditions adequate for denaturation and hybridization allowed us to study the genetic relatedness between the two strains. Analysis of the electrophoretic pattern of Wa-DS-1 hybrids indicated in a qualitative way that none of their corresponding dsRNA segments were completely homologous. Partial homology that must have existed between these two viruses probably yielded molecules with single-stranded regions susceptible to S1 nuclease digestion, leading to the disappearance of labeled RNA hybrids with electrophoretic mobility equivalent to that of the corresponding virion dsRNAs. This experimental approach offers an excellent way of examining genetic homologies between different rotaviruses. In that respect, we have recently examined the relatedness between other human rotaviruses and the Wa and DS-1 strains. Those studies show various degrees of homology between each of several rotaviruses purified from the stools of children with diarrhea and either of the two strains studied in this report (J. Flores, I. Perez, L. White, M. Perez, A. Kalica, R. Marquina, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock, Infect. Immun., in press)

The primary gene products of both viruses were studied by translating the ssRNA product of in vitro transcription in a rabbit reticulocyte lysate system. With Wa virus, 10 proteins were clearly detected, ranging in molecular weight from 31,000 to 118,000, with an overall protein pattern similar to that described for the SA11 virus (18). The strain Wa protein pattern was similar to the one obtained by labeling viral polypeptides during infection of cells in culture. Precipitation by virus-specific antisera of the proteins translated in vitro further supports their viral origin. In addition, two other proteins with molecular weights of approximately 19,000 and 23,000 (for Wa) and 17,000 and 20,000 (for DS-1) were sometimes synthesized by the ssRNA transcribed in vitro. Proteins of similar molecular weights have been reported by Mason et al. (18) and Dyall-Smith and Holmes (5) for SA11 and Wa viruses. In preliminary experiments in which RNA was extracted from the individual bands that were resolved by agarose gel electrophoresis, it was observed that RNA extracted from the two fastest-moving RNA bands was able to translate proteins of those sizes (19,000 to 23,000).

The migration patterns and molecular weights of the proteins synthesized by in vitro translation were very similar to those of proteins synthesized in infected-cell lysates. Proteins of identical migration with molecular weights of 118,000, 90,500, 78,000, 50,000, and 42,000 were seen in both cases, and three other proteins of similar (but not identical) migration were also seen (molecular weights of 31,000 to 36,000). Two other proteins obtained by in vitro translation were not presented in detectable amounts in the lysates (molecular weights of 86,000 and 80,000). Post-translational modification of these proteins in the infected cells may be the most likely explanation for the differences found. Unfortunately, even at the high multiplicity of infection employed, it was not possible to perform short-term labeling or pulse-chase experiments. Currently, attempts are being made to assign the proteins of these viruses to the genes from which they are coded. It must be emphasized that coupling in vitro transcription and translation could make it possible to study proteins of noncultivatable viruses, such as DS-1, and their antigenic interrelationships.

The two strains utilized in this study, Wa and DS-1, have been shown to be distinct by a number of criteria: their RNAs have different migration patterns (segments 10 and 11 of DS-1 have a slower mobility [9]), and they belong to different subgroups (10). The two viruses also differ in their neutralization antigen, as shown by plaque reduction neutralization assays with the cultivatable mutant of Wa and a reassortant of DS-1 rescued during coinfection with a cultivatable bovine rotavirus (6). The data presented in this paper on the cross-hybridization between the two viruses and on the pattern of their primary gene products substantiates to a great extent the distinctiveness of the two strains.

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