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Conservation of the Fourth Gene among Rotaviruses Recovered from Asymptomatic Newborn Infants and Its Possible Role in Attenuation

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RNA-RNA hybridization was performed to assess the extent of genetic relatedness among human rotaviruses isolated from children with gastroenteritis and from asymptomatic newborn infants. ^{32}P-labeled single-stranded RNAs produced by in vitro transcription from viral cores of the different strains tested were used as probes in two different hybridization assays: (i) undenatured genomic RNAs were resolved by polyacrylamide gel electrophoresis, denatured in situ, electrophoretically transferred to diazobenzyloxymethyl-paper (Northern blots), and then hybridized to the probes under two different conditions of stringency; and (ii) denatured genomic double-stranded RNAs were hybridized to the probes in solution and the hybrids which formed were identified by polyacrylamide gel electrophoresis. When analyzed by Northern blot hybridization at a low level of stringency, all genes from the strains tested cross-hybridized, providing evidence for some sequence homology in each of the corresponding genes. However, when hybridization stringency was increased, a difference in gene 4 sequence was detected between strains recovered from asymptomatic newborn infants ("nursery strains") and strains recovered from infants and young children with diarrhea. Although the nursery strains exhibited serotypic diversity (i.e., each of the four strains tested belonged to a different serotype), the fourth gene appeared to be highly conserved. Similarly, each of the virulent strains tested belonged to a different serotype; nonetheless, there was significant conservation of sequence among the fourth genes of three of these viruses. Significantly, the conserved fourth genes of the nursery strains were distinct from the fourth gene of each of the virulent viruses. These results were confirmed and extended during experiments in which the RNA-RNA hybridization was carried out in solution and the resulting hybrids were analyzed by polyacrylamide gel electrophoresis. Under these conditions, the fourth genes of the nursery strains were closely related to each other but not to the fourth genes of the virulent viruses. Full-length hybrids did not form between the fourth genes from the nursery strains and the corresponding genes from the strains recovered from symptomatic infants and young children.

The shedding of rotavirus by newborn infants has been documented in several studies (3, 15, 16). Such shedding is rarely associated with any symptoms; however, this type of infection appears to confer protection against subsequent rotavirus illness (1). What makes the newborn baby refractory to rotavirus illness is an important question which has yet to be answered. Rotavirus antibodies acquired from colostrum or by transplacental transfer may play a role in this phenomenon. It is also possible that the intestinal enzymes responsible for activation of the virus have not yet developed at this early age. It is known that activation of rotavirus infectivity in vitro requires the action of trypsin. It is also possible that the rotavirus strains which produce silent infection in the nursery differ from conventional rotaviruses and hence are attenuated. This was suggested by the work of Rodger et al. (16) and Perez-Schael et al. (15), who observed a constancy of the RNA electropherotype of strains isolated from the same nursery over a prolonged interval while there were considerable differences between the electropherotype of these strains and those of strains circulating outside of the nursery in ill infants and children during the same interval. Of interest, in a recent study, Hoshino et al. (12) demonstrated antigenic diversity among nursery strains; each of the four strains tested belonged to a different human rotavirus serotype.

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In this study, RNA-RNA hybridization was used to assess the genetic relatedness of different rotavirus strains isolated from asymptomatic newborn infants in hospital nurseries and to compare these strains with rotaviruses isolated from symptomatic infants and young children. We observed that there was conservation of the fourth gene among the "nursery strains." Also, the conserved fourth genes of these strains differed from the corresponding genes of virulent rotavirus strains.

MATERIALS AND METHODS

Strains. The following strains isolated from asymptomatic newborn infants were studied: M37, a subgroup 2, serotype 1 strain from Venezuela (15); 1076, a subgroup 1, serotype 2 strain from Sweden; McN, a subgroup 2, serotype 3 strain from Australia (1); ST3, a subgroup 2, serotype 4 strain from England (3).

Four well-characterized laboratory strains, originally isolated from ill infants or children, were also studied: Wa, subgroup 2, serotype 1 (20); DS1, subgroup 1, serotype 2 (21); P, subgroup 2, serotype 3 (21); and VA70, subgroup 2, serotype 4 (9). The first three of these strains (Wa, DS1, and P) were isolated in Washington, D.C.; VA70 was isolated from a child in Italy.

Each of the rotavirus strains studied was grown in MA-104 cells in the presence of trypsin (0.5 μg/ml), and virions were purified as previously described (6).

RNA extraction and preparation of probes. Single-shelled particles were extracted twice with phenol and once with chloroform to obtain genomic double-stranded (ds) RNAs. Single-stranded (ss) RNA probes (mRNAs) were prepared by in vitro transcription of rotavirus single-shelled particles and purified by lithium chloride precipitation (6). [α-^{32}P]GTP was included during the transcription reaction. The specific activity was 5×10^4 cpm/μg of RNA for those probes tested in liquid hybridization and 5×10^4 to 5×10^5 cpm/μg for those used in Northern blot hybridization. The integrity of all 11 RNA segments in each probe was assessed by electrophoresis in 1.4% low-melting-temperature agarose gels containing 0.1% sodium dodecyl sulfate (SDS).

Northern blot hybridization. Genomic dsRNAs from each of the strains were blotted onto diazobenzyloxymethyl (DBM)-paper in a manner similar to that described by Street et al. (17). The dsRNAs were initially run in 6% discontinuous polyacrylamide gels with a 3% stacking gel. The gels were stained with ethidium bromide, photographed, and then immersed in 0.1 M NaOH for 20 min to denature and partially break up the RNA and thus facilitate transfer. The NaOH was neutralized and the gels were then transferred by electrophoresis onto freshly prepared DBM membranes (Schleicher & Schuell, Inc., Keene, N.H.) during 4 h at 2 A in a transblot unit (Bio-Rad Laboratories, Richmond, Calif.). The membranes were then baked for 2 h at 80°C and stored at −70°C.

The ^{32}P-labeled ssRNA probes (0.5×10^6 to 1×10^6 cpm) were hybridized to the blotted RNAs following the procedure of Thomas (18). Initially the blotted membranes were incubated for 2 h in a solution containing 2.5 to 5× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM $NaPO_4$ buffer (pH 6.5), 0.1% SDS, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll (Pharmacia, Inc., Piscataway, N.J.), and 50% formamide. During a second 2-h prehybridization period, the blots were incubated in a similar solution also containing 100 μg of sheared and denatured salmon sperm DNA per ml. The salmon sperm DNA as well as the RNA probes were boiled for 5 min and quenched on ice for 5 min before being used. Next, hybridization with the RNA probes was carried out for 20 to 30 h in the latter solution to which dextran sulfate (Pharmacia) was added to a concentration of 10%. Following hybridization, the membranes were washed four times at room temperature in 1× SSC–0.1% SDS, twice at the temperature used for hybridization (at either 42 or 52°C) in 2× SSC–0.1% SDS, and twice in 1× SSC at the temperature used for hybridization. The membranes could be reused by removing the probes during a 30-min wash at 80°C in 50% formamide–0.1× SSC–0.1% SDS.

Two different stringencies were used for hybridization in the analysis of corresponding genes of the eight strains under study. Under conditions of low stringency, hybridization was performed at 42°C with 50% formamide in 5× SSC (0.75 M Na^+), while under conditions of high stringency hybridization was performed at 52°C with 50% formamide and 2.5× SSC (0.375 M Na^+). Under these conditions the effective hybridization temperatures were T_m (RNA) − 44°C and T_m (RNA) − 29°C, respectively.

Hybridization in solution. Total (about 0.2 μg) or individually isolated genomic dsRNA segments (approximately 50 ng) were mixed with the ^{32}P-labeled transcription probes (30 to 60 ng or 1,500 to 3,000 cpm) and denatured by boiling for 2 min, followed by quenching on ice for 5 min. Hybridization was then allowed to occur during overnight incubation at 65°C in a buffer containing 50 mM Tris acetate (pH 8.0), 250 mM NaCl, and 0.2% SDS. Following hybridization the RNAs were precipitated with ethanol and electrophoresed in discontinuous polyacrylamide gels. The gels were visualized by ethidium bromide and then dried and autoradiographed. Two nursery strains, 1076 and McN, grew poorly in tissue culture, and it was not possible to produce sufficient labeled single-strand probes for all of the desired liquid hybridization experiments.

RESULTS

Relationships among illness and asymptomatic infection rotavirus strains studied by Northern blot hybridization. Genomic dsRNAs from the eight strains tested, four from newborn infants who underwent silent infection (nursery strains) and four from infants and young children with diarrhea, were resolved by polyacrylamide gel electrophoresis (PAGE) and blotted onto DBM membranes. Transcription probes from Wa, DS1, P, VA-70, M37, and ST3 were first hybridized to these blots at low stringency (42°C, 50% formamide, 5× SSC). Homologous hybridizations were performed in each instance to confirm the integrity of the transcription probes. Under these conditions of hybridization, the eight strains could not be differentiated (data not shown). In tests with every strain, each segment which could be resolved by PAGE gave a positive signal with the corresponding ssRNA of each of the probes. In some cases it was not possible to resolve segments 2 and 3 or 7, 8, and 9 because the segments migrated very closely together.

Hybridization at a higher stringency (52°C, 50% formamide, 2.5× SSC) was also performed with probes from each of the eight strains. All of the pairwise comparisons were performed by Northern blot at higher stringency in both directions. Again, homologous hybridizations were included to confirm the integrity of the transcription probes (shown in lane 1 under Wa probe, lane 3 under DS1 probe); strains obtained from symptomatic infants and young children (Wa, DS1, P, and VA70) clearly hybridized with every segment that was resolved from the eight strains which were blotted, with the exception of the fourth segment from the strains which were recovered from asymptomatic newborn infants, i.e., M37, 1076, McN, and ST3. The fourth segment of the latter viruses failed to produce a signal or produced a very faint signal compared with that produced by the other gene segments. Examples of these patterns with ssRNA probes from Wa and DS1 are shown in Fig. 1.

Similarly, probes from the nursery strains, M37, 1076, McN, and ST3, failed to hybridize (or hybridized faintly) to the fourth segment of strains recovered from symptomatic infants and young children (Wa, DS1, P, and VA70), while these probes reacted with each of the other gene segments of these strains which could be clearly resolved by PAGE. The results obtained with probes from M37 and ST3 are shown in Fig. 2; probes from 1076 and McN yielded similar results. In every instance probes prepared from each of the four nursery strains hybridized strongly to the fourth gene of the other three nursery strains. The nursery strain probes also hybridized to all of the other heterotypic nursery strain genes which could be resolved by PAGE.

Probes prepared from Wa, P, VA70, M37, McN, and ST3 (members of the Wa family of human rotaviruses [7]) hybridized less strongly to the first three genes of DS1 and 1076 (members of the DS1 family of human rotaviruses [7]) than to the corresponding genes of viruses in the former group (shown in Fig. 1 and 2, lanes 3 and 4, for Wa, M37, and ST3;

similar results were obtained with McN and VA70). Conversely, probes prepared from DS1 and 1076 hybridized less strongly to the first three genes of Wa family viruses than to 1076 or DS1 (lanes 1 and 2 and 6 through 8 at the bottom of Fig. 1 for DS1 probe; similar results were observed with the 1076 probe).

Thus, we observed a hierarchy of hybridization reactions. Probes for genes 5 through 11 hybridized strongly with the corresponding genes of all other strains. Probes from Wa or DS1 family viruses also hybridized strongly with the first three genes of other viruses in their family group, while reactions were less intense with the first three genes of viruses belonging to the other family. Finally, the fourth genes of illness strains hybridized strongly among themselves but not with the corresponding genes of nursery strains and vice versa. The degree of hybridization observed between the fourth genes of illness and nursery strains was clearly less than that seen between the first three genes of Wa and DS1 family viruses. Thus, the fourth genes of the nursery strains appear to be highly related and distinct from the corresponding genes of illness strains. The latter genes also appear to be highly related when assayed by Northern blot hybridization. The observed conservation of the fourth gene among nursery strains and among illness strains is independent of serotype or Wa/DS1 family relationship.

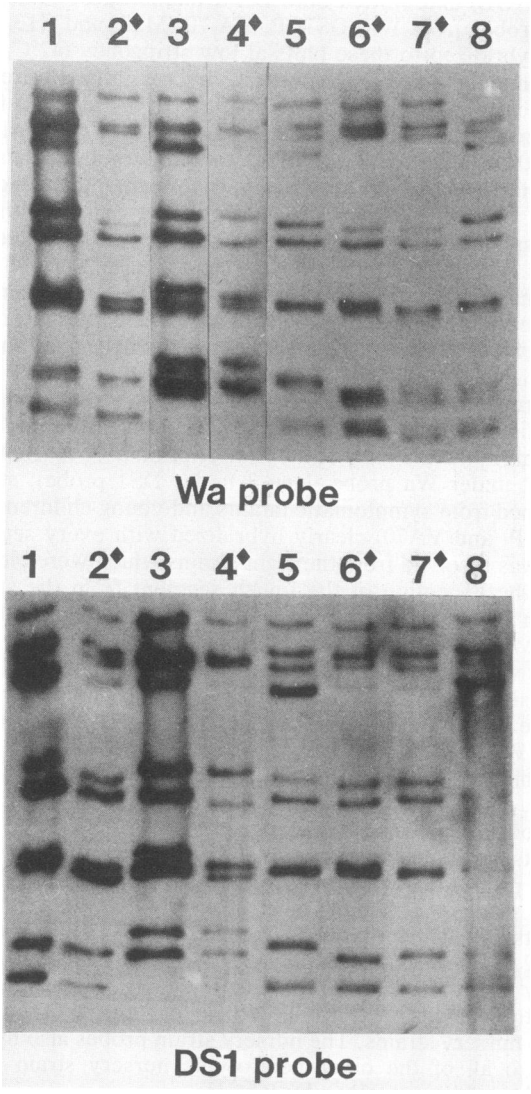

FIG. 1. Autoradiogram of Northern blot hybridization of ^{32}P-labeled Wa (top) and DS1 (bottom) ssRNA probes to denatured genomic RNAs from the following rotavirus strains immobilized on DBM membranes after PAGE: lane 1, Wa; lane 2, M37; lane 3, DS1; lane 4, 1076; lane 5, P; lane 6, McN; lane 7, ST3; lane 8, VA70. ◆ identifies strains recovered from asymptomatic newborn infants. Lanes 3 and 4 (top) were exposed for a longer period of time.

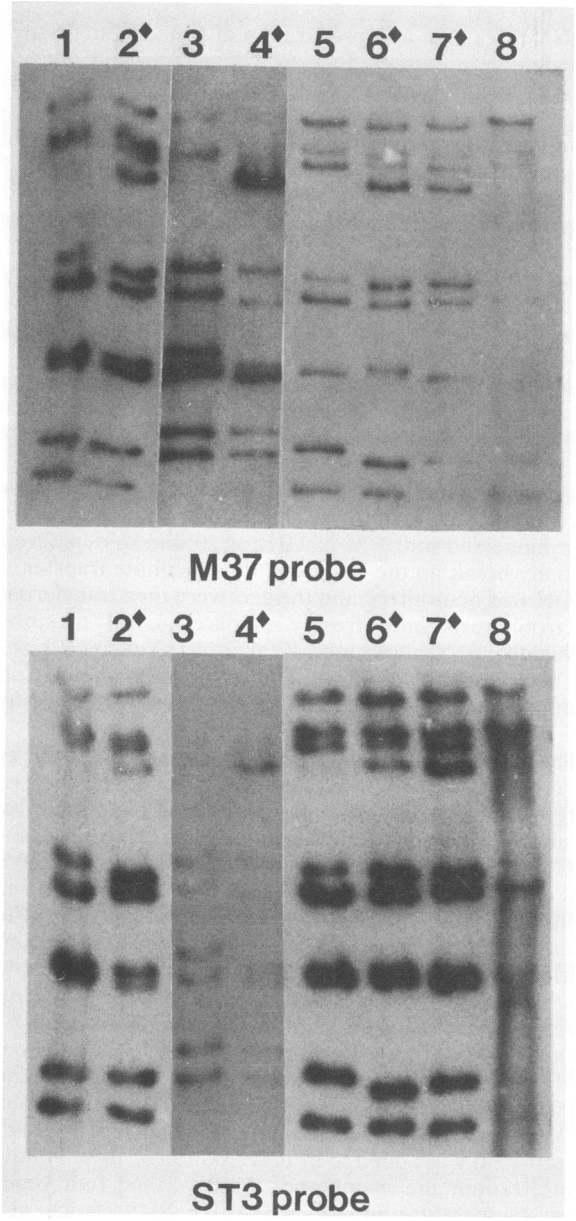

FIG. 2. Autoradiogram of Northern blot hybridization of ^{32}P-labeled M37 (top) and ST3 (bottom) ssRNA probes to denatured genomic RNAs from the following rotavirus strains immobilized on DBM membranes after PAGE: lane 1, Wa; lane 2, M37; lane 3, DS1; lane 4, 1076; lane 5, P; lane 6, McN; lane 7, ST3; lane 8, VA70. ◆ identifies strains recovered from asymptomatic newborn infants. Lanes 3 and 4 were exposed for a longer period of time.

Relationships among the fourth genes of illness and asymptomatic infection rotavirus strains studied by hybridization in solution. Total genomic RNAs from all strains were denatured and hybridized to transcription probes in solution. The resulting hybrids were analyzed by PAGE, fluorography,

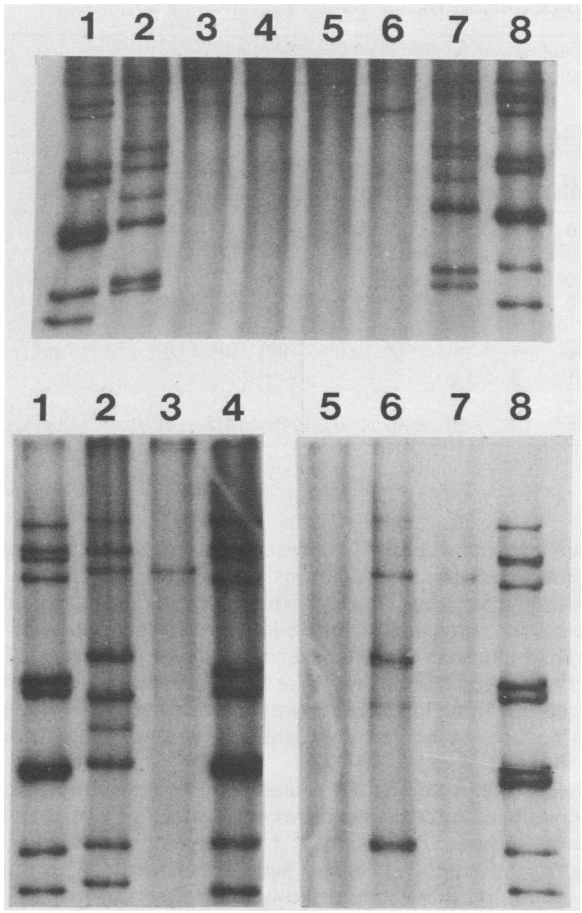

FIG. 3. Hybridization of denatured genomic RNAs from several rotavirus strains to ^{32}P-labeled ssRNA probes. Hybridizations were carried out in solution (overnight incubation at 65°C), and the RNAs were electrophoresed in 10% acrylamide gels, dried, and autoradiographed. Number on top of each lane identifies pairwise hybridization (dsRNA-ssRNA probe, in that order) as follows: (top)—(1) Wa/Wa; (2) P/Wa; (3) isolated gene 4 from P/Wa; (4) isolated gene 4 from P/P; (5) isolated gene 4 from Wa/P; (6) isolated gene 4 from Wa/Wa; (7) Wa/P; (8) P/P; (bottom)—(1) ST3/ST3; (2) McN/ST3; (3) isolated gene 4 from McN/ST3; (4) P/P; (5) isolated gene 4 from McN/P; (6) McN/M37; (7) isolated gene 4 from McN/M37; (8) M37/M37.

and autoradiography. Homologous hybridizations were included in each experiment to establish the integrity of the probes. In many cases, certain of the hybrids which formed between ssRNA probes and denatured genomic dsRNAs of other strains could not be identified because their migration pattern on PAGE differed from that of the corresponding genes of both viruses being compared. Thus, to extend our analysis of relatedness among the fourth genes of the strains under study, it was neccesary in certain instances to isolate and extract gene 4 by preparative gel electrophoresis. The isolated gene was then denatured and hybridized to transcription probes. Examples of this procedure are shown in Fig. 3 and 4, and a summary of the results obtained by this approach is presented in Table 1.

The top portion of Fig. 3 shows the patterns observed when the total genomic dsRNAs or only the fourth gene from Wa and P were cross-hybridized to probes from these strains. A hybrid formed between the fourth genes of Wa and P which had an electrophoretic mobility different from the fourth gene of either strain. The fourth gene of Wa also hybridized under these conditions to the fourth gene of VA70 (Fig. 4), but it did not hybridize to the fourth gene of DS1 or either of the nursery strains tested, M37 or ST3 (not shown).

The bottom of Fig. 3 shows the hybrids which formed between denatured dsRNAs of McN and probes from ST3 and M37. The fourth gene of the nursery strain McN formed hybrid bands with the corresponding gene from nursery strains ST3 and M37, and these hybrids exhibited the same pattern of migration as that of McN. However, the fourth gene of McN did not form a hybrid with P, a strain recovered from an ill child.

Figure 4 (top) shows the hybrids which formed between denatured RNAs from the nursery strain 1076 and probes from M37 and DS1. A homologous DS1/DS1 or M37/M37 hybridization established the integrity of the DS1 and M37 probes. Total genomic RNAs from 1076 formed a series of hybrid bands with a DS1 probe (lane 4); however, none of the hybrids corresponded to the fourth gene since the isolated fourth gene of 1076 did not form a hybrid segment with the DS1 probe (lane 3). In contrast, the fourth gene of strain 1076 formed a hybrid band with the M37 probe (lane 2). Pairwise comparison of three of the nursery strains (M37, 1076, and ST3) was of particular interest because only the fourth genes of 1076 and ST3 appeared to be closely related, while only two genes of M37 and 1076 appeared to be closely related, the fourth and seventh genes.

The bottom of Fig. 4 shows the hybrids which formed between VA70 and ST3 (both serotype 4 strains) and be-

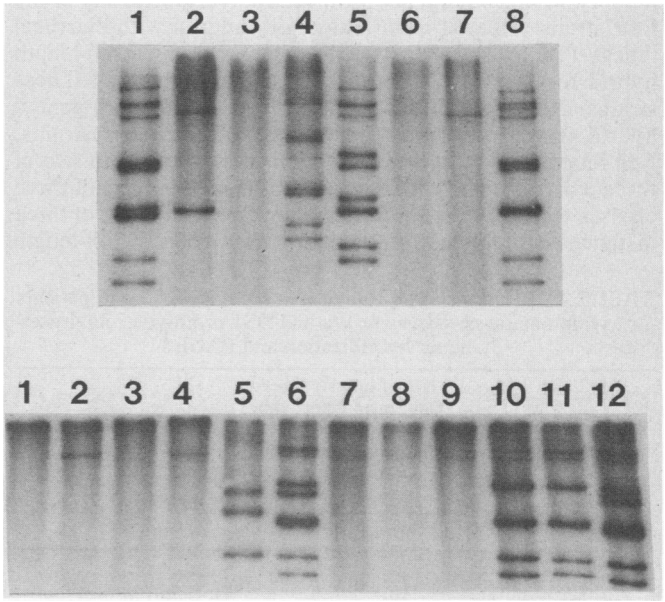

FIG. 4. Hybridization of denatured genomic RNAs from several rotavirus strains to ^{32}P-labeled ssRNA probes. Hybridizations were carried out in solution (overnight incubation at 65°C), and the RNAs were electrophoresed in 10% acrylamide gels, dried, and autoradiographed. Number on top of each lane identifies pairwise hybridization (dsRNA/ssRNA probes, in that order) as follows: (top)—(1) M37/M37; (2) 1076/M37; (3) isolated gene 4 from 1076/DS1; (4) 1076/DS1; (5) DS1/DS1; (6) isolated gene 4 from 1076/ST3; (7) 1076/ST3; (8) ST3/ST3; (bottom)—(1) isolated gene 4 from VA70/ST3; (2) isolated gene 4 from VA70/VA70; (3) isolated gene 4 from ST3/VA70; (4)isolated gene 4 from ST3/ST3; (5) ST3/VA70; (6) VA70/VA70; (7) isolated gene 4 from VA70/Wa; (8) isolated gene 4 from Wa/VA70; (9) isolated gene 4 from Wa/Wa; (10) VA70/Wa; (11) Wa/VA70; (12) Wa/Wa.

TABLE 1. Relationship of fourth genes of various rotaviruses as studied by Northern blot and hybridization in solution

Recovery from:	Strain from which ^{32}P-labeled ssRNA transcription probe was prepared		Hybrid formation when indicated denatured ds genomic RNA(s) incubated with labeled transcription probe															
			Northern blot (higher stringency)								Hybridization in soln and analysis by PAGE							
	Designation	Serotype	Wa	DS1	P	VA70	M37	1076	McN	ST3	Wa	DS1	P	VA70	M37	1076	McN	ST3
Ill infants	Wa	1	⊞[a]	+	+	+	0	0	0	0	⊞[b]	0	+[b]	+[b]	0[b]	0	0	0
	DS1	2	+	⊞	+	+	±	0	0	0	0	⊞	0	0	0	0[b]	0	0
	P	3	+	+	⊞	+	0	0	0	0	+[b]	0	⊞[b]	0[b]	0	0	0[b]	0[b]
	VA70	4	+	+	+	⊞	0	0	0	0	+[b]	0		⊞[b]	0	0	0	0[b]
Asymptomatic newborn infants	M37	1	0	0	0	0	⊞	+	+	+	0[b]	0[b]	0[b]	0[b]	⊞[b]	+[b]	+[b]	+[b]
	1076	2	0	0	±	0	+	⊞	+	+								
	McN	3	0	0	0	0	+	+	⊞	+								
	ST3	4	0	0	0	0	+	+	+	⊞	0[b]	0	0	0[b]	+[b]	+[b]	+[b]	⊞[b]

[a] Homologous reaction boxed.
[b] Hybridization also performed with denatured genomic RNA of isolated gene 4.

tween Wa and VA70. Although ST3 and VA70 formed at least seven hybrid bands, their fourth genes did not hybridize. The fourth gene of VA70, on the other hand, did form a hybrid with the fourth gene of Wa, and this hybrid exhibited the same mobility as the Wa fourth gene.

A summary of the liquid hybridization analysis of the fourth genes of the eight strains tested is presented in Table 1, which contains observations not included in Fig. 3 and 4. Gene 4 of nursery strain M37 or ST3 formed a full-length hybrid with the corresponding gene of each of the other three nursery strains but did not form such a hybrid with any of the four strains isolated from infants or children with diarrheal illness (Table 1). A less consistent pattern of full-length hybrid formation was observed in pairwise tests of illness strains. No illness strain probe formed a full-length gene 4 hybrid with every one of the three other illness strains. Full-length gene 4 hybrid formation was observed in two of three pairwise tests with the Wa strain probe, in one of three crosses with the P or VA70 strain probe, and in none of three instances with the DS1 strain probe. Significantly, full-length gene 4 hybrids were not observed in any of the pairwise tests of illness versus nursery strains.

It should be noted that hybridization in solution proved to be a more stringent test for homology because seven of the pairwise comparisons that were positive by Northern blot (high-stringency) analysis were not positive by the former procedure. In no instance, however, was a pairwise comparison positive by liquid hybridization and negative by Northern blot.

Relationships among the remaining 10 genes of illness and asymptomatic infection rotavirus strains studied by hybridization in solution. Previously it had been observed that the majority of genes of most human rotaviruses produced full-length hybrids when tested with labeled transcripts from the Wa strain but not when tested with transcripts from the DS1 strain or vice versa (7). This suggested that most human rotaviruses belonged to the Wa or DS1 family of viruses. The relationship of the genes of each of the illness and nursery strains, other than gene 4, to the corresponding genes of the Wa and DS1 strains is summarized in Table 2. In all but one instance, hybridization was observed with only one of the two probes tested, Wa or DS1. The exception was the M37 strain, which produced seven hybrids with the Wa probe and one with the DS1 probe. Three of the illness strains and three of the nursery strains belonged to the Wa family, while a single illness strain and a single nursery strain belonged to the DS1 family. Of interest, both of the DS1 family viruses shared the same subgroup and serotype, while the six Wa family viruses shared another subgroup and represented three different serotypes. Thus, conservation of gene 4 among nursery strains was observed independent of Wa or DS1 family relationship as well as of subgroup and serotype.

Relationships among the remaining 10 genes of the asymptomatic infection strains studied by hybridization in solution. As expected, the 10 remaining genes of the three nursery strains that belonged to the Wa family and subgroup 2 exhibited a closer relationship to each other than to the corresponding genes of the DS1-like, subgroup 1 rotavirus, strain 1076 (Table 3). Again, these relationships among the non-gene 4 RNA segments were observed independent of the conservation of gene 4 by the four nursery strains.

TABLE 2. Relationship of rotaviruses recovered from ill patients or asymptomatic newborns to Wa and DS1 prototype rotaviruses by liquid hybridization and PAGE[a]

Rotavirus	No. of hybrids other than gene 4 detected by liquid hybridization and PAGE with indicated labeled ssRNA transcripts from:		Comment
	Wa	DS1	
Ill patients			
Wa (serotype 1)	10	0	Wa family prototype
DS1 (serotype 2)	0	10	DS1 family prototype
P (serotype 3)	9	0	Predominantly Wa-like
VA70 (serotype 4)	7	0	Predominantly Wa-like
Asymptomatic newborn infants			
M37 (serotype 1)	7	1	Predominantly Wa-like
1076 (serotype 2)	0	8 (9?)	Predominantly DS1-like
McN (serotype 3)	8	0	Predominantly Wa-like
ST3 (serotype 4)	7	0	Predominantly Wa-like

[a] Because comigration of genes 2 and 3 and genes 7, 8, and 9 often occurs, the number of full-length hybrids listed may represent a minimal estimate of relatedness between viruses except in instances in which hybrids were not detected.

DISCUSSION

Rotavirus infection in newborn babies rarely results in illness. This observation has been documented in studies

TABLE 3. Liquid cross-hybridization among rotaviruses from asymptomatic newborn infants

Rotavirus strain	Full-length hybrids other than gene 4 detected by liquid hybridization and PAGE of indicated melted ds genomic RNAs and labeled ssRNA transcripts[a]		
	1076	McN	ST3
M37	1 (7th gene)	4	6
1076		ND[b]	0
McN			9

[a] Because comigration of genes 2 and 3 and genes 7, 8, and 9 often occurs, the number of full-length hybrids listed may represent a minimal estimate of relatedness between viruses except in instances in which hybrids were not detected.
[b] ND, Not done.

performed in different countries (see reference 12 for review) and may have some significance for developing a strategy for immunoprophylaxis of rotavirus disease. Bishop et al. (1) demonstrated that asymptomatic rotavirus infection during the neonatal period induced resistance to subsequent rotavirus gastroenteritis. Attenuation of rotavirus infection in neonates may depend on host factors such as the presence of rotavirus-neutralizing antibodies, antitrypsin activity, or nonimmunoglobulin antiviral activity in colostrum or milk; however, a correlation between these factors and protection against disease has not been confirmed (19). The roles that transplacentally acquired antibodies and the maturational status of intestinal enzymes, which may be important for the activation of infectivity, play in this phenomenon have not been explored.

On the other hand, it is possible that the asymptomatic nature of some neonatal rotavirus infections may be due to attenuation of certain rotavirus strains that infect newborn infants in the nursery. As a consequence, these viruses would be allowed to persist in the nursery because they do not cause disease. In three separate studies it was observed that the RNA segments of rotaviruses recovered from the same nursery over a period of several years exhibited a similar or identical electrophoretic migration. Rodger et al., in Australia (16), observed only two electrophoretic patterns (almost identical) among a large number of isolates obtained over a 5-year period. Perez-Schael et al. (15) also observed a single RNA pattern among nursery strains from a hospital in Venezuela as analyzed by electrophoresis and RNA-RNA hybridization. Hoshino et al. (12) have shown that three strains recovered in England over a period of 12 months by J. E. Banatvala were identical by RNA electrophoresis and RNA-RNA hybridization. In addition, it was observed that five strains from Sweden, recovered over a 1-year period, had the same electrophoretype. In the study of Rodger et al. (16), 116 strains examined from ill infants and children in the community were grouped into 17 different electrophoretypes; however, none of these patterns was similar to the two closely related patterns observed for 72 strains recovered over a 5-year period from a nursery in the same city. Similarly, the homogeneous group of nursery strains recovered over a 12-month period from a single nursery in Venezuela differed electrophoretically and in hybridization pattern from more than 60 strains obtained during the same period of time from ill infants and children in the community (8, 15).

Initially we adapted the nursery strains from the studies of Bishop et al. (1), Chrystie et al. (3), Perez-Schael et al. (15), and strain 1076 obtained from B. Tufvesson to tissue culture, and this allowed us to study these viruses serologically and genetically. The four known human rotavirus serotypes and the two different rotavirus subgroups were represented among the nursery strains (12). Initial analysis of their electrophoretic migration pattern revealed that the four prototype strains differed significantly from each other. In fact, the 1076 strain exhibited a "short" RNA pattern typical of serotype 2 rotaviruses (12), and this pattern was markedly different from that of the other three nursery strains.

In the present study, relationships among nursery and illness rotavirus strains were investigated by utilizing labeled ssRNA transcription probes that were employed in two different hybridization assays, Northern blot hybridization (with probes from each of the eight strains) and hybridization in solution (with probes from six of the strains). The percent mismatch allowed under the hybridization stringencies utilized can be calculated from the equation T_m (RNA) = 79.8 + 18.5(log M) + 58.4 H_G + 11.8(H_G)^2 − 0.35 (% formamide) (2), where M is the Na^+ molarity and H_G is the guanine-plus-cytosine content as a fraction; a value of 0.43 was used in our calculations. According to these calculations up to 32 and 21% mismatch was allowed under low and high hybridization stringences, respectively, in the Northern blots and 18% during hybridization in solution. This predicted gradient of specificity was observed when comparisons were made by using the three conditions of hybridization; the low-stringency Northern blot technique was least specific, while liquid hybridization was most specific. However, it should be noted that recent sequence analysis of the fourth gene from the strains used here suggested that >92% homology between two VP3 genes is required for formation of a stable hybrid under the conditions used for liquid hybridization (9a). This is because a competition is established for the minus strand of the genomic RNA between the labeled ssRNA (+) probe and the more abundant, fully homologous genomic (+) strand which acts to limit full labeled hybrids from forming.

Our RNA-RNA hybridization analysis revealed a major difference between the nursery strains and rotavirus strains isolated from infants and children with gastroenteritis. Namely, the fourth gene of the nursery strains failed to hybridize under conditions of high stringency (Northern blot or liquid hybridization) to the corresponding gene of strains recovered from ill infants and children. This difference was independent of the serotype specificity of these viruses. Thus, each of the four nursery strains, which represented four separate serotypes, failed to form a gene 4 hybrid with a rotavirus strain of the same serotype recovered from an ill individual. In contrast, the fourth gene of the four strains from asymptomatic newborns was highly conserved despite serotype polymorphism.

When studied by Northern blot analysis, under conditions of high stringency, the fourth genes of the illness strains constituted a conserved group in which hybridization occurred in each pairwise test, while hybridization was not detected with the fourth gene of any of the nursery strains. The fourth genes of the nursery strains also constituted a conserved group in which cross-hybridization occurred, while hybridization with the fourth gene of illness strains was not detected. In tests in which liquid hybridization, a more stringent technique, was used with two nursery strain probes, M37 and ST3, and the four illness strain probes, the fourth gene of the nursery strains was also observed to be highly conserved. Full-length gene 4 hybrids were detected in each instance when the nursery strains were tested with the former probes but not with the latter probes. The fourth gene of each of the illness strains did not form full-length

hybrids with the two nursery strain probes. The nursery strain probes, however, did form full-length gene 4 hybrids with each of the heterotypic nursery strains. Significantly, the fourth genes of the illness strains exhibited less conservation when studied by liquid hybridization in pairwise tests than was evident by the less stringent Northern blot analysis. Thus, the fourth genes of the nursery strains appeared to be a more closely related group than the fourth genes of the illness strains.

Our findings suggest that the difference in virulence of the nursery and illness strains may result, in part, from a difference in the fourth gene product of these two groups of rotaviruses. The fourth rotavirus gene encodes VP3, an 88,000-dalton outer capsid rotavirus protein, which must be cleaved by trypsin or another peptidase for virus to become infectious in vitro or in vivo (4). Genetic studies also indicate that the fourth rotavirus gene product is important in determining host range of rotaviruses in tissue culture. Initially, we observed that noncultivatable human rotavirus strains could be rescued by gene reassortment with an animal rotavirus that grew well in vitro. These studies established that the fourth gene of human rotaviruses restricted growth in vitro and that its replacement with the corresponding gene of a cultivatable bovine rotavirus, strain UK, made it possible for human rotavirus reassortants to grow efficiently in vitro (5, 13). Presumably restriction of host range in vitro is related to a deficiency of the appropriate proteolytic enzyme(s) in tissue culture cells and the ease with which cleavage of VP3 occurs in vitro. This is an attractive explanation since cultivation of human rotaviruses has now been achieved successfully by including trypsin in the culture medium.

Processing of VP3 by those proteolytic enzymes which are most abundant during the neonatal stage of intestinal maturation may allow nursery rotaviruses to replicate to a moderate level without causing disease. On the other hand, neonatal intestinal enzymes may not activate strains which are able to infect and produce illness in older infants and children. Also, nursery strains may not cause diarrhea in older infants and children because cleavage of VP3 by "mature" proteolytic enzymes may not be efficient. However, it is unlikely that these strains only infect the newborn. Unfortunately, there have been no attempts to recover rotavirus strains from asymptomatic older infants and children.

A better understanding of the fourth gene in avirulence of nursery strains awaits sequence analysis, especially in the cleavage region, and comparison of this sequence with that of the corresponding gene of rotavirus strains recovered from ill children. Such experiments are now in progress (9a).

The role of the fourth gene product in the neutralization of rotaviruses has been the subject of several recent studies. Greenberg et al. (10) generated neutralizing monoclonal antibodies that reacted with VP3. Hoshino et al. (11) discovered that certain rotaviruses, for example, the nursery strain M37 analyzed in this study, exhibited two distinct serotypes. Thus, antibodies against strain M37 neutralize serotype 1 strains, such as Wa, as well as nursery strain ST3, a serotype 4 rotavirus. This intertypic neutralization phenomenon was mapped to the outer capsid VP7 protein of M37 and Wa and to another outer capsid protein, VP3, of M37 and ST3. The latter relationship is of interest because the M37 VP3 gene cross-hybridizes with the VP3 gene of ST3 under conditions of high stringency. Of interest, intertypic neutralization, such as that observed for M37 and ST3, was not detected with the other nursery strains included in this study (1076, McN), although all of the nursery strains share a high level of homology in their fourth gene. In a similar manner, the fourth gene of Wa exhibited a high degree of homology with the corresponding gene of strains P and VA70, which belong to different serotypes and do not exhibit intertypic neutralization with Wa or with each other. Given that the fourth gene product is capable of inducing neutralizing antibodies, it is likely that the epitopes responsible for inducing neutralizing antibodies represent a small portion of the total VP3 and hence may not exert a significiant influence on hybridization of gene 4 RNAs.

When performing RNA-RNA hybridizations in solution, a high degree of homology among many genes other than gene 4 was detected in various crosses. The hybridization conditions used in this assay were rather stringent, since RNA-RNA annealing was allowed to occur at a high temperature which destabilizes partial hybrids with >18% mismatch. The actual degree of homology required to form a stable hybrid was demonstrated by sequence analysis to be even greater, ≥92% (9a). In addition, the migration pattern of partial hybrids was altered with respect to that of the genes from which they originated. Nonetheless, the hybridization patterns observed were consistent with a previous observation that most human rotaviruses studied by liquid hybridization can be classified in one of two major families, one with relatively high homology to Wa and the other with significant homology to DS1 (7, 8). In the present study this dichotomy was maintained independent of the origin of the strains, whether from asymptomatic newborns or symptomatic infants and children. This suggests that illness and nursery strain genes, other than gene 4, may have a common origin and may be derived from one of two distinct ancestral sources, a Wa-like virus or a DS1-like virus. Particularly instructive in this regard was the analysis of three nursery strains, M37, 1076, and ST3. Strain 1076 shared a high degree of homology with only gene 4 of ST3 and genes 4 and 7 of M37. On the other hand, 1076 exhibited significant homology with at least eight or nine genes of DS1, the prototype of the DS1 family, while at least seven of the genes of M37 and ST3 appeared to be highly related to the corresponding genes of the Wa strain. These observations suggest that strain 1076 may have arisen through gene reassortment involving a virus bearing a nursery strain gene 4 and a virus with predominantly DS1-like genes. A similar origin can be suggested for the M37 and ST3 viruses, but in these instances gene reassortment probably occurred with a virus which was Wa-like.

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LITERATURE CITED

1. **Bishop, R. F., G. L. Barnes, E. Cipriani, and J. S. Lund.** 1983. Clinical immunity after neonatal rotavirus infection. A prospective longitudinal study in young children. N. Engl. J. Med. **309**:72–76.
2. **Bodkin, D. K., and D. L. Knudson.** 1985. Sequence relatedness of Palyam virus genes to cognates of the Palyam serogroup viruses by RNA-RNA blot hybridization. Virology **143**:55–62.
3. **Chrystie, I. L., B. Totterdell, M. J. Baker, J. W. Scopes, and J. E. Banatvala.** 1975. Rotavirus infections in a maternity unit.

Lancet ii:79.
4. **Estes, M. K., D. Y. Graham, and B. B. Mason.** 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J. Virol. **39**:879–888.
5. **Flores, J., H. B. Greenberg, J. Myslinski, A. R. Kalica, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1982. Use of transcription probes for genotyping rotavirus reassortants. Virology **121**:288–295.
6. **Flores, J., J. Myslinski, A. R. Kalica, H. B. Greenberg, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1982. In vitro transcription of two human rotaviruses. J. Virol. **43**:1032–1037.
7. **Flores, J., I. Perez, L. White, M. Perez, A. R. Kalica, R. Marquina, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1982. Genetic relatedness among human rotaviruses as determined by RNA hybridization. Infect. Immun. **37**:648–655.
8. **Flores, J., I. Perez-Schael, E. Boeggeman, L. White, M. Perez, R. Purcell, Y. Hoshino, K. Midthun, R. M. Chanock, and A. Z. Kapikian.** 1985. Genetic relatedness among human rotaviruses. J. Med. Virol. **17**:135–143.
9. **Gerna, G., N. Passarani, M. Battaglia, and E. Percivalle.** 1984. Rapid serotyping of human rotavirus strains by solid-phase immune electron microscopy. J. Clin. Microbiol. **19**:273–278.
9a. **Gorziglia, M., Y. Hoshino, A. Buckler-White, R. Glass, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1986. Conservation of amino acid sequence of VP8 and cleavage region of 84-kDa outer capsid protein among rotaviruses recovered from asymptomatic neonatal infection. Proc. Natl. Acad. Sci. USA **83**:7039–7043.
10. **Greenberg, H. B., J. Valdesuso, K. van Wyke, K. Midthun, M. Walsh, V. McAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47**:267–275.
11. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82**:8701–8704.
12. **Hoshino, Y., R. G. Wyatt, J. Flores, K. Midthun, and A. Z. Kapikian.** 1985. Serotypic characterization of rotaviruses derived from asymptomatic human neonatal infection. J. Clin. Microbiol. **21**:425–430.
13. **Kalica, A. R., H. B. Greenberg, R. G. Wyatt, J. Flores, M. M. Sereno, A. Z. Kapikian, and R. M. Chanock.** 1981. Genes of human (strain Wa) and bovine (strain UK) rotaviruses that code for neutralization and subgroup antigens. Virology **112**:385–390.
14. **McLean, B. S., and I. H. Holmes.** 1981. Effects of antibodies, trypsin, and trypsin inhibitors on susceptibility of neonates to rotavirus infection. J. Clin. Microbiol. **13**:22–29.
15. **Perez-Schael, I., G. Daoud, L. White, G. Urbina, N. Daoud, M. Perez, and J. Flores.** 1984. Rotavirus shedding by newborn children. J. Med. Virol. **14**:127–136.
16. **Rodger, S. M., R. F. Bishop, C. Birch, B. McLean, and I. H. Holmes.** 1981. Molecular epidemiology of human rotaviruses in Melbourne, Australia, from 1973 to 1979, as determined by electrophoresis of genome ribonucleic acid. J. Clin. Microbiol. **13**:272–278.
17. **Street, J. E., M. C. Croxson, W. F. Chadderton, and A. R. Bellamy.** 1982. Sequence diversity of human rotavirus strains investigated by Northern blot hybridization analysis. J. Virol. **43**:369–378.
18. **Thomas, P. S.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
19. **Totterdell, B. M., K. G. Nicholson, J. MacLeod, I. L. Chrystie, and J. E. Banatvala.** 1982. Neonatal rotavirus infection: role of lacteal neutralising alpha 1-anti-trypsin and nonimmunoglobulin antiviral activity in protection. J. Med. Virol. **10**:37–44.
20. **Wyatt, R. G., W. D. James, E. H. Bohl, K. W. Theil, L. W. Saif, A. R. Kalica, H. B. Greenberg, A. Z. Kapikian, and R. M. Chanock.** 1980. Human rotavirus type 2: cultivation in vitro. Science **207**:189–191.
21. **Wyatt, R. G., H. D. James, Jr., A. L. Pittman, Y. Hoshino, H. B. Greenberg, A. R. Kalica, J. Flores, and A. Z. Kapikian.** 1983. Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes. J. Clin. Microbiol. **18**:310–317.