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Genetic Relatedness Among Human Rotaviruses as Determined by RNA Hybridization

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Viral RNAs from human rotaviruses were compared by gel electrophoresis and by hybridization to probes prepared by in vitro transcription of two well-characterized laboratory strains (Wa and DS-1). Also, the viral RNAs were compared by hybridization to probes prepared from three of the test viruses. Thirteen specimens (diarrheal stools) were obtained from infants and children 5 to 21 months old on a single day at the emergency ward of the Caracas Children's Hospital, and an additional specimen was obtained from the same hospital 6 months before. When the electrophoresed viral RNAs were stained with ethidium bromide and examined by UV light, five different migration patterns (electropherotypes) were distinguished on the basis of differences in mobility of the RNA segments. The hybridization technique that was employed permitted only qualitative comparisons of corresponding genes of different human rotaviruses. Ten of the specimens contained enough virus to yield sufficient RNA for hybridization studies. Eight of the viruses studied by hybridization contained 4 to 11 genes that reacted specifically with the Wa probe to yield double-stranded RNA segments with a mobility similar to that of Wa viral RNA or test virus RNA. The other two viruses contained 11 genes that reacted specifically with the DS-1 hybridization probe to yield double-stranded RNA segments with a mobility similar to DS-1 viral RNA or test virus RNA. A more complex picture emerged when hybridization probes were prepared from three of the test viruses and used to compare the different electropherotypes. Corresponding genes that exhibited similar migration did not necessarily exhibit homology when studied by hybridization. Also, some corresponding genes that exhibited homology did not have the same mobility by gel electrophoresis.

The recognized importance of rotaviruses as a major cause of gastroenteritis has prompted many efforts directed toward the identification of biologically different types (27, 28). Until recently, the failure of most human rotaviruses to grow well in cell culture has made this task a difficult one since it has been impossible to differentiate virus isolates by classical neutralization tests (26). Although a fluorescent focus neutralization assay has been described (6), its applicability has been limited, and the nature of the viral antigen(s) that is(are) measured by this test is uncertain. Recently, an indication of the existence of at least two serotypes of human rotavirus has been obtained by a plaque reduction neutralization assay. The evidence is based on the ability of the human rotavirus to reassort with a cultivatable rotavirus yielding cultivatable progeny with the human neutralization phenotype (7). In addition, two subgroups of human rotavirus have recently been identified by an immune adherence hemagglutination assay (IAHA) (14). On the other hand, the relative ease of studying the rotavirus genomic double-stranded (ds) RNA has given rise to a series of epidemiological studies based on RNA analysis (4, 5, 21, 22) similar to those reported for influenza virus (19) or for reovirus (8, 20). Although the differences in RNA migration patterns between rotavirus strains were originally thought of as a useful means of identifying the species derivation of a rotavirus (9), subsequent reports have demonstrated the existence of polymorphism within strains derived from humans (12, 17) or from animals (24). Therefore, attempts to classify the members of this family of Reoviridae into individual electropherotypes, far from clarifying the origin and distribution of given strains, have led to the conclusion (not very helpful for purposes of classification) that a high degree of

genetic variability exists even among strains circulating simultaneously (23). Electrophoretic migration is obviously not a very sensitive way of analyzing the genomic relatedness among viral strains, and the many electropherotypes detected for human rotavirus may misrepresent the actual differences among them. In this report we have used molecular cross-hybridization of rotavirus RNA as a more specific way of looking into genomic relationships among several rotavirus strains of human origin and their relation to two prototype strains from our laboratory. We have taken advantage of the transcriptase activity present in the rotavirus virion that allows the synthesis in vitro of complete replicas of the plus-strand from the viral ds RNA (1). Inclusion of labeled nucleotides during the reaction gives rise to RNAs that can be used as hybridization probes to determine the existence of homologous segments among different strains.

MATERIALS AND METHODS

Clinical specimens. Stool specimens were obtained on a single day (17 July 1980) from children hospitalized in the emergency ward of the Hospital de Niños, J. M. de Los Rios (Caracas Children's Hospital). The children (5 to 21 months old) had all been admitted with acute diarrhea with various degrees of dehydration. Samples were collected only from children who had been in the hospital for 48 h or less. Stool suspensions (5%) were prepared and tested for rotavirus by an enzyme-linked immunosorbent assay (ELISA) (15). Thirteen specimens obtained from 30 children were positive by this test.

Virus preparation. The rotavirus-positive specimens were extracted with Genetron 113, and the virus in the aqueous phase was sedimented through a 30% sucrose cushion by centrifugation for 2 h in a Beckman SW27 rotor at 25,000 rpm; the pellets were fractionated in a 40 to 55% cesium chloride gradient by overnight centrifugation at 35,000 rpm in a Beckman SW40 rotor. Fractions with densities of 1.38 to 1.36 g/ml were pooled and fractionated again in similar gradients. The resulting fractions containing the virus were pelleted and taken up in phosphate-buffered saline.

In addition to the clinical specimens, two laboratory strains of human rotavirus were used in this study: the Wa strain, recently adapted to growth in tissue culture (25), and the DS-1 strain, a non-cultivatable virus that had been amplified by passage in gnotobiotic calves. The Wa virus was grown in MA 104 cells, harvested after 3 days, and purified as described for the clinical isolates. The DS-1 virus employed in this work originated from the stool of an infected calf.

RNA extraction and electrophoresis. Genomic ds RNA was obtained from the purified virus by phenol extraction and ethanol precipitation as described previously (12). After being resuspended in 50 mM Tris, pH 8.0, the RNA was analyzed by polyacrylamide gel electrophoresis in 7.5 or 12% discontinuous gels run overnight at 12 to 15 mA of constant current. The system employed was the same as that described by Laemmli (16) for proteins except that sodium dodecyl sulfate was omitted from all of the solutions.

After electrophoresis, the gels were stained for 2 h in ethidium bromide and photographed under UV light. Only 9 of the 13 rotavirus-positive specimens had enough RNA to be studied in this manner.

Transcription. Purified virus particles from the two laboratory strains (Wa and DS-1) and from some of the Venezuelan specimens were preincubated in 5 mM EDTA to activate transcriptase (2). The final composition of the transcription mixture was as follows: 30 to 50 μg of virus per ml; 1 mM ATP (including 50 μCi of [^{32}P]ATP; Amersham Corp.); 2.5 mM GTP, UTP, and CTP; 0.5 mM *S*-adenosylmethionine; 0.1% bentonite; 12 mM $MgCl_2$; 8 mM phosphoenolpyruvate; 50 μg of pyruvate kinase per ml; 0.5 mM EDTA and 100 mM Tris-acetate, pH 8.0. Total volume of this mixture was 250 μl. After 6 h of incubation at 42°C, virus was removed from the reaction mixture by centrifugation, and the supernatant containing the labeled single-stranded (ss) RNA was extracted with phenol and precipitated with ethanol.

Hybridization. The labeled, ss RNA produced by in vitro transcription was hybridized to genomic ds RNA. Denaturation of the ds RNA was accomplished by incubation for 30 min at 42°C in 90% dimethyl sulfoxide. Immediately after this incubation, 0.5 volume of 0.9 M NaCl, 3 volumes of ethanol, 5 μg of tRNA (added as carrier), and about 10,000 cpm of ss RNA (probe) were added. The RNA was precipitated overnight and then taken up in hybridization buffer (100 mM NaCl, 50 mM Tris-chloride [pH 8.0], 0.1% sodium dodecyl sulfate) and incubated for 6 h at 72°C. A second precipitation with ethanol was carried out before the RNA was analyzed by polyacrylamide gel electrophoresis as described above. The gels were dried after UV light visualization and autoradiographed on Kodak XAR film.

RESULTS

Analysis of the RNA by polyacrylamide gel electrophoresis. The migration pattern of the genomic ds RNA of the viruses purified from stool specimens is shown in Fig. 1. Five different patterns (electropherotypes) can be distinguished. For purposes of comparison, they will be referred to as: A (viruses no. 1, 5, 10, and 13), B (no. 4), C (no. 11 and 19), D (no. 21), and E (no. 22). Rotavirus no. 4 is the most distinctive of the group exhibiting slow migration of genes 10 and 11. The migration of genes 1, 3, 4, 6, 10, and 11 is very similar among the other electropherotypes. Differences in migration of segments 7, 8, and 9 account for the major dissimilarities among electropherotypes A, C, D, and E, although smaller differences in the migration of genes 2 and 5 are also seen. Patterns of viruses A, C, D, and E correspond to electropherotype 2L and subgroup 2 according to the migration of their segments 10 and 11, while virus B corresponds to the 2S pattern and subgroup 1 (10).

Hybridization of genomic RNA to ss RNA probes of DS-1 and Wa. Figure 2 shows the patterns obtained when ds RNA from A (no. 1),

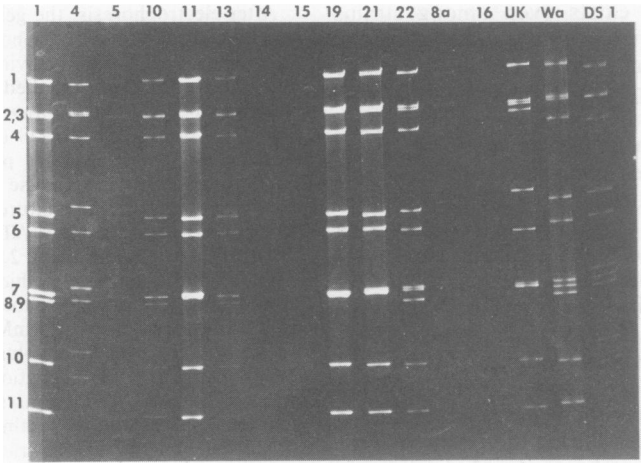

FIG. 1. Polyacrylamide gel electrophoresis of ds RNA from one animal (UK) and several human rotavirus strains as visualized by UV light after ethidium bromide staining. Electrophoresis was carried out overnight at 12.5 mA in a 7.5% gel. Numbers on the left of the figure represent the RNA segment order used throughout the paper.

B (no. 4), C (no. 11 and 19), and D (no. 21) was hybridized to a probe prepared from the DS-1 strain. Only ds RNA of virus no. 4 showed homology with the DS-1 virus; the migration pattern of the resulting hybrids resembles that of virus no. 4 genomic RNA. RNA from viruses representing the other electropherotypes failed to hybridize sufficiently to DS-1 to yield discernible bands similar in migration pattern to their genomic ds RNA. When no. 4 virus was hybridized to a Wa probe, a similar result was observed; i.e., no discernible hybrid bands were apparent in the gel (Fig. 3). Given the unique quality of this pattern B virus, RNA from a virus

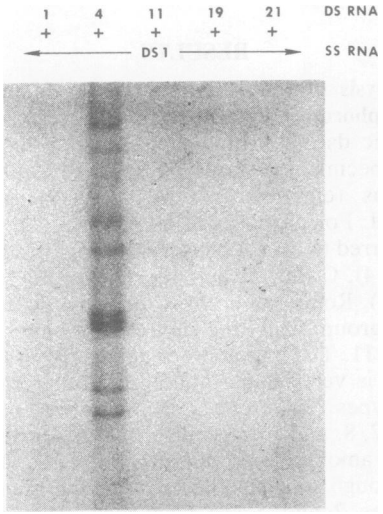

FIG. 2. Autoradiography showing the hybridization of ds RNA from rotavirus no. 1, no. 4, no. 11, no. 19, and no. 21 to a ss RNA labeled probe prepared by in vitro transcription of DS-1. Electrophoresis was carried out as described. Although the ds RNA in the lanes for viruses 1, 11, 19, and 21 was visualized by UV light (not shown), the bands observed in those lanes were free of radioactivity. Segment 1 of virus no. 4 was more clearly visualized in other X-rays.

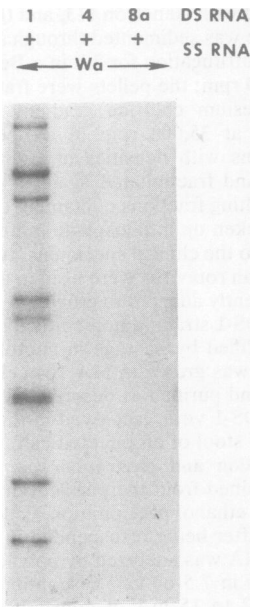

FIG. 3. Autoradiography showing the hybridization of specimens 1, 4, and 8a to a labeled ss RNA probe prepared from Wa.

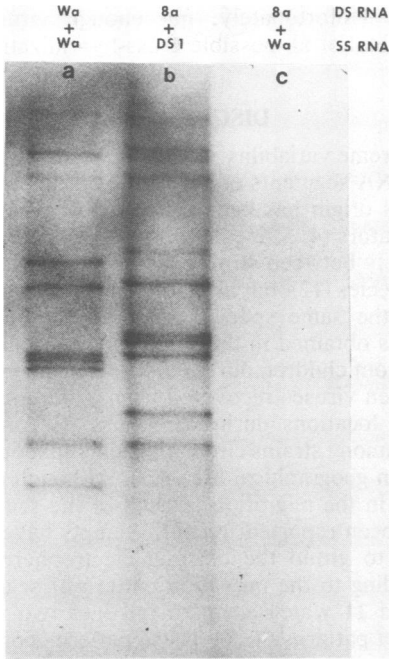

FIG. 4. Autoradiography showing the hybridization of ds RNA from Wa (lane a) and 8a rotavirus (lanes b and c) to ss RNA probes prepared from Wa (lanes a and c) and DS-1 (lane b).

previously obtained from the same hospital (virus 8a, January 1980) which had a migration pattern of segments 10 and 11 similar to that of B (2S) was hybridized to probes prepared from Wa and DS-1. Its hybridization pattern with DS-1 was very similar to that of virus no. 4 (Fig. 4, lane b); again virus 8a did not yield discernible bands with the probe made from Wa (Fig. 4, lane c) that did hybridize in a homologous reaction (Fig. 4, lane a). A schematic representation of the pattern obtained by hybridizing RNA from viruses 4 and 8a to DS-1 can be seen in Fig. 5.

When ds RNA from an A virus (no. 1) was denatured and hybridized to probes prepared from Wa, nine bands were observed (Fig. 3; the second band appears to be double and the sixth may be double or triple). Similar results were obtained when other viruses of electropherotype A (no. 5 and 10) were hybridized to Wa probes (not shown).

The patterns resulting from the hybridization of viruses of electropherotypes C, D, or E with Wa are presented schematically in Fig. 5, and an example is given in Fig. 6. Various patterns of hybridization between those viruses and Wa were apparent; i.e., gene 1, the double region 2-3, and gene 11 produced hybrids that comigrated with the corresponding segments of Wa in every case. In addition, genes 5 and 10 from viruses 11 and 19 (C) and genes 6 and 10 and one or more genes of the triplet 7-8-9 from virus no. 22 (E) hybridized to Wa, maintaining their expected mobility. It is interesting that the Wa probe produced two hybrid bands with virus no. 11 and virus no. 19 (C) and one with virus no. 21 (D) that were not equivalent in mobility to the corresponding genomic ds RNA of Wa or either of those viruses. The fourth gene of the former viruses (no. 11 and 19) did not form a hybrid of equivalent mobility to gene 4 of Wa when hybridized to a Wa probe.

Cross-hybridization between viruses A, C, D, and E. Figures 7 and 8 represent the results obtained when ds RNAs from the Venezuelan viruses were hybridized to probes prepared from themselves. Hybridization of virus no. 19 (C) to a probe from virus no. 1 resulted in a pattern almost identical to that of virus no. 1, except for gene 5 which did not produce a hybrid molecule

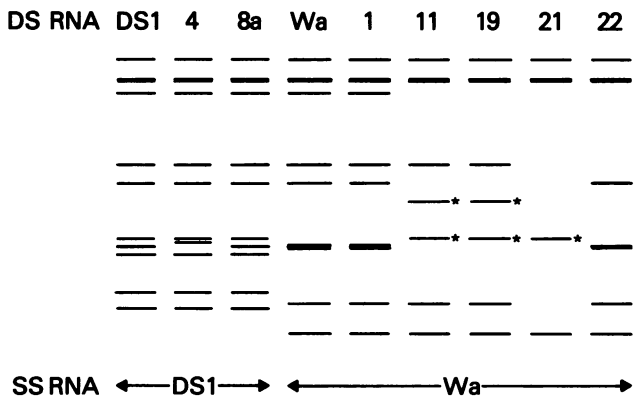

FIG. 5. Schematic summary of the cross-hybridization patterns obtained when ds RNA from the virus in the specimens was hybridized to ss RNA probes prepared from DS-1 or Wa virus. Asterisks beside the bands indicate the lack of comigration between them and the ds RNA from either the specimen or the probe virus.

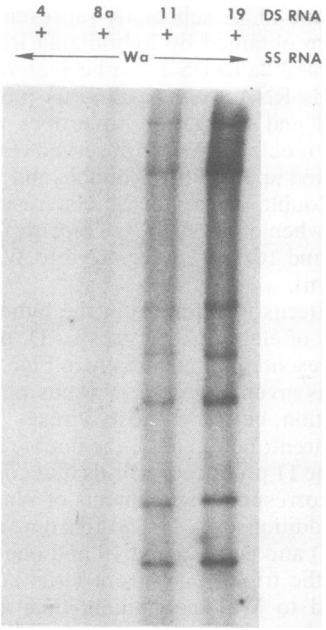

FIG. 6. Autoradiography showing the electrophoretic pattern obtained by hybridizing ds RNA from viruses 4, 8a, 11, and 19 (from left to right in that order) to a labeled ss RNA probe prepared from Wa.

of similar migration (Fig. 7a). Virus no. 21 (D) was less related to virus no. 1 (A) since segments 1, 5, and 10 and possibly part of the 7-8-9 complex did not produce hybrids of equivalent mobility; a number of extra bands were present in this hybridization (not seen in the corresponding UV light photograph, Fig. 1) that may represent complete or fragmented hybrid segments of partial homology (Fig. 7a). Hybridization of viruses 11 and 19 (C), 21 (D), and 22 (E) to a probe prepared from virus no. 11 is shown in Fig. 7c. Hybrids between virus no. 19 and a probe obtained from virus no. 11 resembled the migration pattern of those viruses except for their fifth genes which produced a band with slower mobility. All of the genes of virus no. 21 produced hybrid bands with a virus no. 11 probe of similar migration to the ds RNA of virus no. 11 except for its gene 10 and possibly one or more genes of the 7-8-9 complex; virus no. 22 differs from no. 11 in segments 6 and 7-8-9 (Fig. 7c). Finally, Fig. 7b represents the pattern obtained by hybridizing virus no. 19 (C) and virus no. 21 (D) to a probe prepared from virus no. 22. Both no. 19 and no. 21 differ from no. 22 in genes 6, 7-8-9, and 10 (although a less intense segment 10 is seen with virus no. 19); in addition, segment 5 of virus no. 19 does not comigrate with segment 5 of either no. 19 or no. 22.

Figure 8 presents these relationships schematically. Unfortunately, not enough virus was available for all possible cross-hybridizations.

DISCUSSION

Extreme variability in the migration pattern of the RNA segments of rotaviruses of human and animal origin has been reported by several investigators (4, 5, 21, 22). Variations were found not only between strains recovered from different species (12), but also among those recovered from the same species (12, 17, 24), between viruses obtained in the same geographical location from children during different seasons (21), between viruses recovered from children at different locations during the same season, and even among strains circulating simultaneously in a given geographical area (23). Although differences in the migration of each of the segments have been reported, recent attempts have been made to group the existing electropherotypes according to the migration pattern of segments 10 and 11 which seem to fall into two rather distinct patterns (4, 5). These patterns correlate with antigenic specificities detected by the IAHA or ELISA (10).

Although a rather limited number of specimens was examined in the present report, it is striking that among the nine samples examined, there were five different electropherotypes and six different hybridization patterns, despite the fact that the viruses were obtained on the same

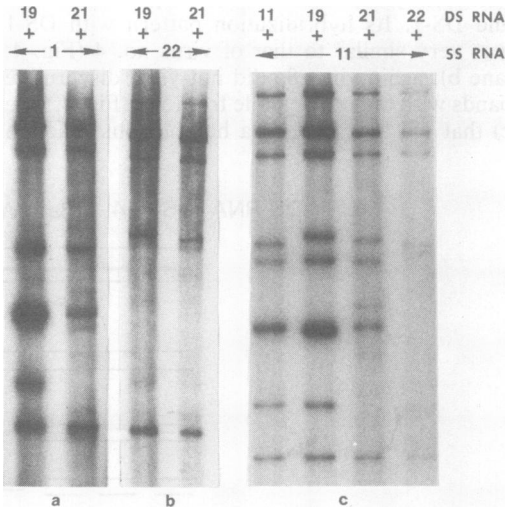

FIG. 7. Autoradiography showing the electrophoretic pattern obtained after hybridization of (a) ds RNA from viruses 19 and 21 to probes from virus 1, (b) ds RNA from viruses 19 and 21 to probes from virus 22, and (c) ds RNA from viruses 11, 19, 21, and 22 to probes from virus 11.

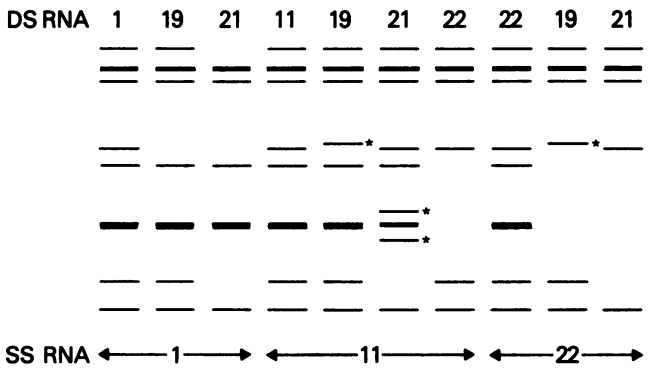

FIG. 8. Schematic representation of the hybridization pattern observed when rotaviruses from the specimens studied were hybridized among themselves. The asterisk beside any band indicates its lack of comigration with the ds RNA from either virus from which the hybrid was obtained.

day in one hospital that serves the metropolitan area of Caracas, Venezuela.

Only one of the samples exhibited a pattern corresponding to that described as 2S (10). For this reason we included in our study a rotavirus with the 2S pattern obtained during a previous study. Both of these 2S-like viruses (no. 4 and 8a) were previously subgrouped by IAHA (10) and shown to belong to subgroup 1, of which DS-1 is the prototype. This is noteworthy in light of the results that show a hybridization pattern of the two viruses and the DS-1 strain that reproduces the migration pattern of DS-1, despite the fact that the migration pattern of the RNA segments of the two viruses, apart from segments 10 and 11, was different. Similarly, it was interesting that none of the other viruses of the group classified previously as antigenic subgroup 2 (10) produced hybrid bands of migration similar to those of the DS-1 virus when hybridized to a probe made with DS-1. These results suggest that although viruses 4 and 8a have a slightly different electropherotype, they are members of a common family that includes DS-1. Furthermore, in recent experiments, some of the genes from virus no. 4 have been rescued by reassortment with the bovine rotavirus UK, in the manner described by Greenberg et al. (7). The reassortants obtained that exhibited the human neutralization phenotype were indistinguishable from DS-1 by neutralization and distinct from Wa (7a).

Several interesting observations emerged from the experiments in which the other viruses were employed. When viruses 1, 5, and 10 of the electropherotype A were hybridized to probes prepared from the Wa virus, the hybrids produced yielded similar migration patterns. Although total identity with Wa did not exist (since these viruses had a slighly different electropherotype from that of Wa), it is possible that the regions of nonhomology between their segments represent a small proportion of the total sequence and thus do not produce detectable alterations of the migration pattern.

Certain genes of the viruses from the other groups (C, D, and E) formed hybrids of mobility similar to Wa when hybridized to Wa probes even though some of these genes had a mobility that differed from the corresponding genes of Wa. In no instance was a gene 4 hybrid detected at the expected position in the gel. However, gene 4 of virus A hybridized with the corresponding gene of Wa and also with gene 4 of viruses C, D, and E. This indicated that the fourth gene of viruses C, D, and E has homology to gene 4 of Wa. These findings suggest that genes that are related but not completely homologous can form hybrids that may or may not comigrate with their parental genes. Regions of secondary structure in hybrid segments alter their electrophoretic mobility as compared with that of the parental strands. This seemed to be the case when viruses of electropherotype C or D were hybridized to the Wa probe. Partial hybridization of the segments in the 7-8-9 complex may have yielded bands that did not comigrate with any of the ds virion RNA segments. These unusual bands are marked with an asterisk in Fig. 5 and 8. The lack of identity of the segments of the 7-8-9 complex from virus C or D to the corresponding segments of Wa is relevant in light of the recent observations that gene 9 of Wa codes for the specific neutralization antigen (11). However, when these viruses were hybridized to a probe from A, an observation similar to that described for gene 4 was made; i.e., the 7-8-9 complex hybridized with A, maintaining the same mobility as the parental genes, and in turn, segments 7-8-9 of electropherotype A hybridized to Wa, also maintaining the same mobility as their parents.

It has recently been reported (11) that gene 6 of the human rotavirus codes for the subgroup

antigen measured by the ELISA and IAHA (14) that is independent of the antigen involved in neutralization of virus by antibody. Electropherotypes A, C, D, and E react in the ELISA and IAHA in the same way as Wa, i.e., subgroup 2 (10). As schematized in Fig. 5 and 8, by cross-hybridization, gene 6 of these viruses exhibits a strong relationship among the four groups and with gene 6 of Wa. The exceptions seen in some of the crosses may possibly be explained as discussed above for genes 4 and 7-8-9. When virus 10 was hybridized to a probe from virus 11 (both grouped as C), an interesting observation was made. Although these viruses had an identical electropherotype, the mobility of the hybrid corresponding to gene 5 was different from that of either virus, which further supports the notion that comigration does not necessarily reflect identity between genes of rotaviruses. Although, in general, for each of the segments of the viruses studied there was an analogous gene in one cross-hybridization or another, an exception must be noted. Gene 10 of virus 21 did not have a homologous counterpart in any of the other viruses studied. The significance of this observation is not understood at this time.

The high degree of polymorphism detected among rotavirus strains by electrophoresis of their ds RNA and of the hybrid molecules obtained by cross-hybridization to ss RNA as reported here reflects the extent of genetic variability of rotaviruses as they exist in nature. While the electropherotype of cultivatable rotaviruses does not seem to change as they are passaged in cell culture (13), there is evidence from other viruses that various degrees of genetic variability compatible with viability may occur and be detected by highly sensitive techniques capable of detecting changes in a few bases (RNA fingerprinting) (3). For poliovirus, such genetic variability has recently been explored by following the changes taking place in the oligonucleotide pattern of a vaccine strain of poliovirus RNA during transmission from person to person. This analysis revealed a high mutation rate that might potentially lead to biologically relevant differences from the original strain (18). The known ability of segmented RNA viruses to reassort may provide one more way for the rotavirus genome to change.

Insufficient understanding of the epidemiology and pathophysiology of the rotavirus infection and of the structure and replication of the virus does not allow a thorough interpretation of the findings described here. Quantitative determinations of the actual degrees of homology between the different viruses studied could not be made because of the limited amount of material available. The qualitative observations reported permit, however, the clarification of some points. Genetic variability of human rotaviruses, as seen by electropherotype determination and hybridization, is common. Comigration between equivalent segments of different viruses does not guarantee their identity, and conversely, lack of comigration does not always presage significant sequence variation. Families of rotaviruses seem to exist and to correspond to the reported patterns of genes 10 and 11 and to the subgroup specificity. The viruses studied here by genomic cross-hybridization seem to belong to one of two "families." Members of one (DS-1-like) seem to share homology among themselves but not with the Wa-like "family" that seems to be more heterogeneous. The existence of other "families" remains to be elucidated.

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