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Group A Human Rotavirus Genomics: Evidence that Gene Constellations Are Influenced by Viral Protein Interactions▽†

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Group A human rotaviruses (HRVs) are the major cause of severe viral gastroenteritis in infants and young children. To gain insight into the level of genetic variation among HRVs, we determined the genome sequences for 10 strains belonging to different VP7 serotypes (G types). The HRVs chosen for this study, D, DS-1, P, ST3, IAL28, Se584, 69M, WI61, A64, and L26, were isolated from infected persons and adapted to cell culture to use as serotype references. Our sequencing results revealed that most of the individual proteins from each HRV belong to one of three genotypes (1, 2, or 3) based on their similarities to proteins of genogroup strains (Wa, DS-1, or AU-1, respectively). Strains D, P, ST3, IAL28, and WI61 encode genotype 1 (Wa-like) proteins, whereas strains DS-1 and 69M encode genotype 2 (DS-1-like) proteins. Of the 10 HRVs sequenced, 3 of them (Se584, A64, and L26) encode proteins belonging to more than one genotype, indicating that they are intergenogroup reassortants. We used amino acid sequence alignments to identify residues that distinguish proteins belonging to HRV genotype 1, 2, or 3. These genotype-specific changes cluster in definitive regions within each viral protein, many of which are sites of known protein-protein interactions. For the intermediate viral capsid protein (VP6), the changes map onto the atomic structure at the VP2-VP6, VP4-VP6, and VP7-VP6 interfaces. The results of this study provide evidence that group A HRV gene constellations exist and may be influenced by interactions among viral proteins during replication.

Group A human rotaviruses (HRVs) cause severe gastroenteritis in children and are responsible for up to 600,000 deaths each year worldwide (37). The rotavirus genome consists of 11 segments of double-stranded RNA contained within a triple-layered capsid (39). In general, each gene segment encodes a single polypeptide, allowing rotaviruses to express five or six nonstructural proteins (NSP1 to NSP5 and sometimes NSP6) and six capsid proteins (VP1 to VP4, VP6, and VP7) (12). The inner layer of the virion is composed of the core lattice protein (VP2) and encases the viral RNA-dependent RNA polymerase (VP1), the RNA capping enzyme (VP3), and genomic RNA (39). The intermediate layer is made entirely of VP6, which is the most recognized group- and subgroup (SG)-specific rotavirus antigen used for classification purposes (12, 23). Comprising the outermost layer of the rotavirus particle, the capsid proteins VP7 and VP4 are involved in virus entry into the cell and elicit neutralizing antibodies during infection of a host (12, 23). Serotypes defined by these outer capsid proteins are referred to as G types (VP7) and P types (VP4) and, along with G/P genotypes, are a predominant method of characterizing individual group A rotavirus isolates (23, 30). To date, a total of 14 G types (19 G genotypes) and 14 P types (27 P genotypes) have been identified in nature (23, 30).

Molecular epidemiology studies suggest that HRV G-type strains G1 to G4 are the most prevalent and are often combined with P-type specificities P[4], P[6], and P[8] (13, 23). Nonetheless, other serotypes, some of which bear resemblance to animal rotaviruses, are emerging as important agents of human disease in certain geographical regions (11, 13). For example, recent reports indicate an increase in incidences of HRV serotypes G5 and G8 in developing countries in South America and Africa (25). Unusual HRV serotypes, such as G9 and G12, are being reported at high levels in many countries, including those that are industrialized (13, 43). Of the 19 known G genotypes, 11 of them have been isolated from patients hospitalized for gastroenteritis; genotypes G7 and G13 to G19 have so far been isolated only from animals (25). These epidemiological observations suggest that the circulating rotavirus population is diverse and evolving. However, aside from the genes encoding outer capsid proteins (VP7 and VP4), little is known about the genetic makeup of emerging and endemic HRV strains because very few full-genome sequences have been determined. The limited insight into nucleotide similarities among group A HRVs that we do have is based on the results of qualitative RNA hybridization assays. This approach assigns a viral isolate into one of three distinct genogroups when seven of its gene segments anneal with those of a genogroup prototype strain: Wa (G1P[8]), DS-1 (G2P[4]), or AU-1

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(G3P[8]) (35). Of the three HRV genogroups, Wa and DS-1 are overwhelmingly prominent, whereas the AU-1 genogroup is extremely rare (34). Intragenogroup resemblances are often reflected in the antigenic properties of their VP6 proteins (SG-I versus SG-II) and the electrophoretic migration pattern of viral genome segments (short or long electropherotype) but not necessarily in their serotype specificities (34). For example, G3 HRV isolates have been categorized into each of the three genogroups (Wa, DS-1, and AU-1), suggesting that they are serotypically similar but genetically separate (34). The results of these hybridization studies reveal that the diversity of HRVs in nature is far greater and more complex than that indicated by serotyping alone. Still, genogroup analysis via RNA hybridization does not identify specific amino acid changes that distinguish proteins of one genogroup from another. With the increasing ease of genetic sequencing, studies aimed at comparative genomic analyses of HRV genogroups are warranted.

The factors that influence the emergence of new group A rotavirus serotypes and genogroups within the human population are not completely understood. Reassortment events, in which two distinct rotavirus strains exchange gene segments, are predicted to play a major role in generating diversity (13, 23). In support of this idea, strains with different serotypes and genogroup specificities have been found circulating together during a single rotavirus season, and coinfections of individuals with more than one rotavirus strain (some of animal origin) are reported with frequencies of up to 23% (14). As a result, rotaviruses are hypothesized to exist as a heterogeneous pool of reassortants that is under continuous extrinsic and intrinsic selection pressures (14). Maternal antibodies, previous infection, and vaccination are just some extrinsic, host-derived pressures that could lead to the emergence of novel HRV G/P types. Yet, there is also evidence suggesting that intrinsic selection pressures, such as the capacity of divergent rotavirus proteins to functionally interact during replication, are important in determining the emergence of reassortant strains. For instance, although the genes encoding VP7 and VP4 can segregate independently, certain combinations of G/P types seem preferred among naturally circulating HRVs; G1 is almost always associated with P[8], whereas G2 is commonly seen with P[4] (9). The existence of genogroups is also consistent with the idea that biases toward certain gene constellations among HRVs occur. In fact, independent segregation of genes between different genogroup strains, aside from those encoding VP7 and VP4, is uncommonly reported (49). It is possible that viral protein-protein interactions drive the intrinsic selection of HRV reassortant strains that can replicate better inside an infected cell. In other words, a Wa-like protein might function more effectively with another Wa-like protein than it would with a DS-1-like protein. To test this possibility, we need a more complete understanding of the genetic composition of proteins from group A HRVs belonging to different serotypes and genogroups.

The results presented in this report are part of a collaborative effort by our laboratory and others that resulted in the establishment of a classification system for all 11 group A rotavirus genome segments (30). Based on phylogenetic analyses of the nucleotide sequences, distinct genotypes were determined for each individual rotavirus segment. The results not only confirmed the G/P types of HRVs by use of appropriate percent identity cutoff values but also increased our understanding of the variation seen in the nine other rotavirus genes. The majority of analyzed HRV gene segments were assigned into genotypes that correlate with the previously described genogroup strains Wa (genotype 1), DS-1 (genotype 2), and AU-1 (genotype 3) (30). In this report, we present the history and detailed sequence characteristics of G-type laboratory reference strains D, DS-1, P, ST3, IAL28, Se584, 69M, WI61, A64, and L26. Our results are consistent with the idea that viral protein-protein interactions influence the extent of group A HRV diversity and the maintenance of preferred gene constellations.

MATERIALS AND METHODS

Cells and rotaviruses. Monkey kidney (MA104) cells were propagated in 199 medium supplemented to contain 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.1% amphotericin B. Human cell culture-adapted rotavirus strains D (G1P[8]), DS-1 (G2P[4]), P (G3P[8]), ST3 (G4P[6]), IAL28 (G5P[8]), Se584 (G6P[9]), 69M (G8P[10]), WI61 (G9P[8]), A64 (G10P[14]), and L26 (G12P[4]) were generously provided by Y. Hoshino (NIH) and were used to infect MA104 cells after pretreatment with 10 μg/ml trypsin, as described previously (16). Infected cells were incubated in serum-free 199 medium for 24 to 72 h until cytopathic effect was visible.

RNA extraction, RT-PCR, and sequencing. Total cytoplasmic RNA was extracted from approximately 2×10^6 rotavirus-infected cells by use of Trizol-LS as described in the manufacturer's protocol (Invitrogen). RNA was denatured by incubation for 10 min at 94°C in a final concentration of 50% dimethyl sulfoxide. Denatured RNA (from approximately 1×10^4 infected cells) was then used as a template for reverse transcription (RT)-PCR to amplify cDNAs corresponding to regions of rotavirus gene 1, 2, 3, or 6 (encoding VP1, VP2, VP3, or VP6, respectively). For genes 5, 6, 9, 10, and 11, RT-PCR was performed using a SuperScript one-step RT-PCR system (Invitrogen). For genes 1 and 2 and genes 3, 4, 7, and 8, respectively, SuperScript II RT and Platinum *Taq* DNA polymerase (Invitrogen) were used according to the manufacturer's protocols. The PCR products were purified using a QIAquick PCR purification kit (Qiagen). In cases when nonspecific PCR bands were present, samples were electrophoresed in a 1% agarose gel, and specific cDNA products were excised and purified using a QIAquick gel extraction kit (Qiagen). The purified PCR products were then sequenced with an ABI Prism BigDye v3.1 terminator cycle sequencing kit (Applied Biosystems Group). The dye terminator was removed using Performa DTR (Edge Biosystems), and sequences were obtained with a 3100 genetic analyzer for genes 5, 8, and 11 or a 3730 DNA analyzer (both Applied Biosystems) for genes 1 to 4, 6, 7, 9, and 10.

For sequencing the 3′ ends of 69M and L26 gene 3 (approximately nucleotides 2155 to 2507) and the 3′ end of ST3 gene 7, the PCR products were ligated into the pGEM-T-Easy vector (Promega) and three independent clones were sequenced. For sequencing the 5′ ends of gene 4 for all of the viruses, PCR products were ligated into the pCR-TOPO-2.1 vector (Invitrogen). Strain A64 genes 10 and 11 were ligated to the pGEM-T-Easy vector and then cut with restriction enzymes BsmBI and MfeI (New England Biolabs), respectively, so that the duplicated genes could be sequenced accurately. Sequence files were analyzed and assembled using Sequencher 4.5 (Gene Codes Corporation).

The nucleotide sequences of the entire open reading frames (ORFs), as well as additional flanking sequences, were determined. Terminal primers were used for RT-PCR amplification, and therefore the extreme 3′ and 5′ termini were not determined de novo.

Amino acid alignments. Amino acid alignments were constructed with MacVector 8.1.2. (Accelrys) using ClustalW, BLOSUM Series, with the defaults set (open gap penalty of 10.0, extended gap penalty of 0.05, and delay divergence of 40%). GenBank accession numbers for the viruses used in this study are presented in Table 1.

RESULTS AND DISCUSSION

The nucleotide sequences of the gene segments for HRV G-type reference strains D, DS-1, P, ST3, IAL28, Se584, 69M, WI61, A64, and L26 were determined by automated sequencing. Table 2 summarizes pertinent features of these strains, as

TABLE 1. GenBank accession numbers

HRV strain	GenBank accession no.										
	VP1	VP2	VP3	VP4	VP6	VP7	NSP1	NSP2	NSP3	NSP4	NSP5/6
Wa	DQ490539	X14942	AY267335	L34161	K02086	M21843	L18943	L04534	X81434	AF093199	AF306494
KU	AB022765	AB022766	AB022767	AB222784	AB022768	D16343	AB022769	AB022770	AB022771	AB022772	AB022773
Dhaka 16-03	DQ492669	DQ492670	DQ492671	DQ492672	DQ492673	DQ492674	DQ492675	DQ492676	DQ492677	DQ492678	DQ492679
D	EF583021	EF583022	EF583023	EF672570	EF583024	EF672574	EF672571	EF672573	EF672572	EF672575	EF672576
DS-1	EF990691	EF990692	EF990693	EF672577	EF990694	EF672581	EF672578	EF672580	EF672579	EF672582	EF672583
TB-Chen	AY787653	AY787652	AY787654	AY787644	AY787645	AY787646	AY787647	AY787648	AY787649	AY787650	AY787651
P	EF583037	EF583038	EF583039	EF672598	EF583040	EF672602	EF672599	EF672601	EF672600	EF672603	EF672604
AU-1	DQ490533	DQ490536	DQ490537	D10970	DQ490538	D86271	D45244	DQ490534	DQ490535	D89873	AB008656
ST3	EF583045	EF583046	EF583047	EF672612	EF583048	EF672616	EF672613	EF672615	EF672614	EF672617	EF672618
IAL28	EF583029	EF583030	EF583031	EF672584	EF583032	EF672588	EF672585	EF672587	EF672586	EF672589	EF672590
Se584	EF583041	EF503842	EF583043	EF672605	EF583044	EF672609	EF672606	EF672608	EF672607	EF672610	EF672611
DRC86	DQ005125	DQ005124	DQ005123	DQ005122	DQ005121	DQ005120	DQ005119	DQ005118	DQ005117	DQ005116	DQ005115
DRC88	DQ005114	DQ005113	DQ005112	DQ005111	DQ005110	DQ005109	DQ005108	DQ005107	DQ005106	DQ005105	DQ005104
69M	EF576937	EF576914	EF576915	EF672556	EF576916	EF672560	EF672557	EF672559	EF672558	EF672561	EF672562
WI61	EF583049	EF583050	EF583051	EF672619	EF583052	EF672623	EF672620	EF672622	EF672621	EF672624	EF672625
A64	EF583017	EF583018	EF503819	EF672563	EF503820	EF672567	EF672564	EF672566	EF672565	EF672568	EF672569
L26	EF583033	EF583034	EF583035	EF672591	EF583036	EF672595	EF672592	EF672594	EF672593	EF672596	EF672597
Dhaka 12-03	DQ146660	DQ146661	DQ146662	DQ146663	DQ146664	DQ146665	DQ146666	DQ146667	DQ146668	DQ146669	DQ146670
Matlab13-03	DQ146671	DQ146672	DQ146673	DQ146674	DQ146675	DQ146676	DQ146677	DQ146678	DQ146679	DQ146680	DQ146681
N26-02	DQ146682	DQ146683	DQ146684	DQ146685	DQ146686	DQ146687	DQ146688	DQ146689	DQ146690	DQ146691	DQ146692
RV176-00	DQ490551	DQ490552	DQ490553	DQ490554	DQ490555	DQ490556	DQ490557	DQ490558	DQ490559	DQ490560	DQ490561
RV161-00	DQ490545	DQ490546	DQ490547	DQ490548	DQ490549	DQ490550	DQ490540	DQ490541	DQ490542	DQ490543	DQ490544
B4633-03	DQ146638	DQ146639	DQ146640	DQ146641	DQ146642	DQ146643	DQ146644	DQ146645	DQ146646	DQ146647	DQ146648
Dhaka 25-02	DQ146649	DQ146650	DQ146651	DQ146652	DQ146653	DQ146654	DQ146655	DQ146656	DQ146657	DQ146658	DQ146659
T152	DQ146699	DQ146700	DQ146701	AB077766	DQ146702	AB071404	AB097459	DQ146703	DQ146704	DQ146705	DQ146706

well as those of other group A HRVs whose genome sequences have been reported to date. With a few exceptions, the individual genes/proteins for the 10 reference strains were assigned to genotypes that correlate with previously described genogroup strains Wa (genotype 1), DS-1 (genotype 2), and AU-1 (genotype 3) (30) (Fig. 1). This classification system afforded the opportunity to identify HRVs that are either (i) pure genogroup strains, with all of their proteins classified into a single genotype, or (ii) intergenogroup reassortants, with proteins of more than one genotype.

TABLE 2. Group A human rotaviruses analyzed

HRV strain	G type	P type	Yr of isolation	Country of isolation
Wa	G1	P8	1974	United States
KU	G1	P8	1974	United States
Dhaka 16-03	G1	P8	2003	Bangladesh
D	G1	P8	1974	United States
DS-1	G2	P4	1976	United States
TB-Chen	G2	P4	1991	China
P	G3	P8	1974	United States
AU-1	G3	P9	1981–1983	Japan
ST3	G4	P6	1975	United Kingdom
IAL28	G5	P8	1992	Brazil
Se584	G6	P9	1998	United States
DRC86	G8	P6	2003	Democratic Republic of the Congo
DRC88	G8	P8	2003	Democratic Republic of the Congo
69M	G8	P10	1980	Indonesia
WI61	G9	P8	1983	United States
A64	G10	P14	1987	United Kingdom
L26	G12	P4	1987	Philippines
Dhaka 12-03	G12	P6	2003	Bangladesh
Matlab13-03	G12	P6	2003	Bangladesh
N26-02	G12	P6	2002	Bangladesh
RV176-00	G12	P6	2000	Bangladesh
RV161-00	G12	P6	2000	Bangladesh
B4633-03	G12	P8	2003	Belgium
Dhaka 25-02	G12	P8	2003	Bangladesh
T152	G12	P9	1998–1999	Thailand

Wa genogroup (genotype 1) strains D, P, ST3, IAL28, and WI61. The Wa strain is one of the best-studied HRVs, in large part because it was the first to be grown successfully in culture and because it causes acute diarrhea in gnotobiotic piglets (51). The virus was isolated in the United States in 1974, and as the prototype for the Wa genogroup, the virus exhibits a long electropherotype and VP6 SG-II and VP2 SG-II specificities (50) (Fig. 1 and Table 2). Using the aforementioned classification system, we found that 5 of the 10 viruses (D, P, ST3, IAL28, and WI61) are genetically more similar to Wa than to either the DS-1 or the AU-1 strain (30) (Fig. 1). With the exception of VP4 and VP7, each of the proteins encoded by these five viruses can be classified as genotype 1. The observation that these HRVs do not encode proteins similar to the DS-1 and AU-1 genogroups (genotypes 2 and 3, respectively) suggests that they are not intergenogroup reassortants but instead can be viewed as pure Wa genogroup strains. The Wa-like strains D, P, and ST3 have been characterized over the years because they represent three of the four most common HRV G types (G1, G3, and G4, respectively). The VP7-encoding genes from these strains (in addition to that of the G2 strain DS-1) have been incorporated into the live-attenuated, multivalent rotavirus vaccines RotaTeq and RotaShield (37). The D and P strains were originally isolated in 1974 in the United States (Children's Hospital, Washington, DC) from infants suffering from gastroenteritis (24, 50). The ST3 strain was isolated in 1975 from an asymptomatic newborn in England (St. Thomas Hospital, London) (6, 50). Despite the importance of the D, P, and ST3 strains, their genome sequences have not been determined previously.

While the D, P, and ST3 strains represent more commonly circulating HRV serotypes, IAL28 and WI61 reflect globally emerging serotypes G5 and G9, respectively (Table 2). Strain IAL28 was isolated in 1992 from a child with diarrhea in Brazil

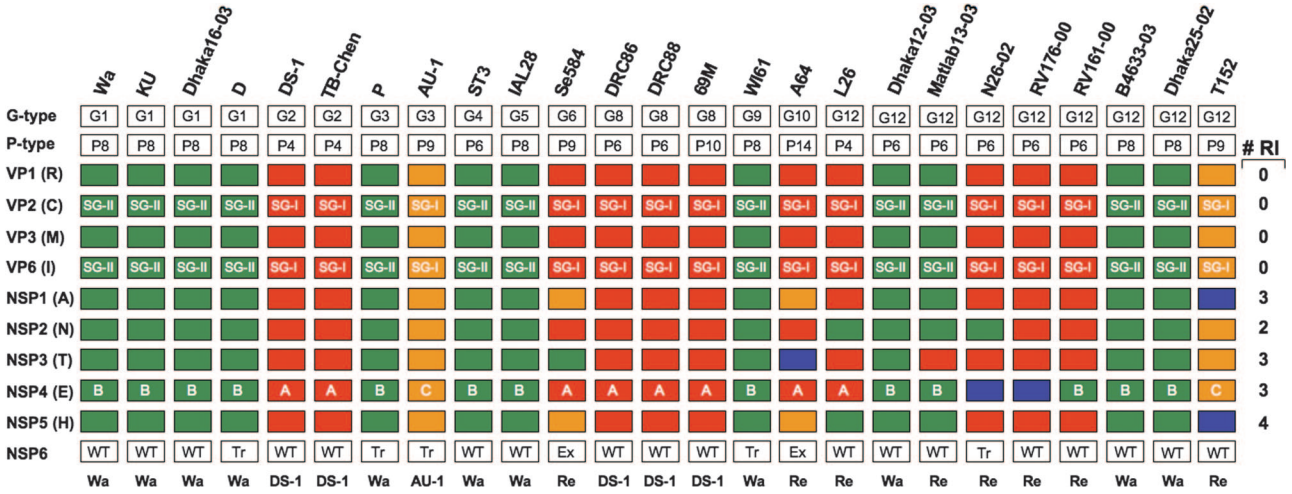

FIG. 1. Summary of HRV protein genotypes, genogroups, and reassortment incidences. Individual HRV strains analyzed in this study are listed at the top, with their corresponding genes/proteins aligned underneath. The VP7 and VP4 genotypes (G/P types) of the strains are listed. Colors indicate the genotype of each gene/protein, as described previously (30): green for Wa-like genotype 1, red for DS-1-like genotype 2, orange for AU-1-like genotype 3, and blue for animal RV-like genotypes. The single-letter abbreviation for each protein is indicated in parentheses (30). VP6 and VP2 SG specificities and NSP4 genotypes (A, B, and C) are classified according to previous studies (17, 26, 32). The NSP6 ORFs were found to be identical to that of the Wa strain (WT), truncated (Tr), or extended (Ex) (also see Fig. S3 in the supplemental material). Below each genome, the HRVs are classified as pure Wa genogroup strains (Wa), pure DS-1 genogroup strains (DS-1), a pure AU-1 strain (AU-1), or intergenogroup/interspecies reassortants (Re). The numbers of reassortment incidences (# RI) detected in this study for genes encoding VP1 to -3, VP6, and NSP1 to -5 are listed on the right.

and was initially shown to have dual G5-G11 antigenic determinants; this virus was later classified as a G5 strain based on sequence analysis (30, 48). Because G5 and G11 serotypes are commonly seen in pigs, IAL28 might represent an interspecies reassortant, with its VP7 gene coming from a porcine virus and its 10 other genes coming from a Wa genogroup HRV (48). Strain WI61 was recovered in 1983 from a child with gastroenteritis in the United States (Children's Hospital, Philadelphia, PA) (7). At the time of its isolation, only G1 to G4 serotypes had been identified in humans, and therefore WI61 represented the fifth G type to be discovered. With the recognition that the incidence of G9 HRV infections is steadily increasing, WI61 becomes a particularly important reference strain (44). In addition to those of D, P, ST3, IAL28, and WI61, the full-genome sequences of other Wa genogroup HRVs have been determined, including two G1P[8] strains (KU and Dhaka 16-03) and several G12 isolates: Dhaka 12-03 (G12P[6]), B4633-03 (G12P[8]), and Dhaka 25-02 (G12P[8]) (Table 1) (43, 50). Our analyses suggest that despite the differences in serotype specificities (i.e., G1, G3, G4, G5, G9, and G12), these HRVs are genetically similar viruses (Fig. 1). Thus, pure Wa genogroup viruses can bear a large number of VP7 serotypes.

DS-1 genogroup (genotype 2) strains DS-1 and 69M. Strain DS-1 is the well-known prototype for its genogroup and was isolated in 1976 in the United States (Children's Hospital, Washington, DC) from an infant with diarrhea (45, 50) (Table 2). Like many other strains belonging to the DS-1 genogroup, the virus has VP6 SG-I and VP2 SG-I specificities and a short electropherotype, the latter due to an extended gene 11 3′ untranslated region (UTR) (28). In addition to Wa-like strains D, P, and ST3, the VP7 gene of DS-1 represents one of the most predominant HRV serotypes (G2) and is included as a component of the tetravalent rotavirus vaccines. This is the first report of the full-genome sequence for this important vaccine-related strain.

Of the HRVs sequenced in this study, 69M is the only strain besides DS-1 that is classified as a pure member of the DS-1 genogroup (Fig. 1). 69M was isolated in Indonesia in 1980 from a child with diarrhea and is unusual in that, instead of the short electropherotype of most DS-1-like strains, 69M has a super-short electropherotype (19, 28, 29). The super-short migration pattern of the 69M genome is due to its gene 11 3′ UTR being twice the length of the already extended DS-1 gene 11 3′ UTR (28). Since 69M is a pure member of the DS-1 genogroup, all of the individual proteins encoded by 69M, with the exception of VP4 and VP7, can be classified as genotype 2. Other DS-1-like HRVs of which genome sequences are known include the Chinese isolate TB-Chen (G2P[4]) and the DS-1-like isolates DRC86 (G8P[6]) and DRC87 (G8P[8]), which are from the Democratic Republic of the Congo (5, 31). These results indicate that DS-1 genogroup HRVs can display VP7 proteins from diverse G types (e.g., G2 and G8), although seemingly not as diverse as the G types that are associated with Wa genogroup strains.

Intergenogroup reassortant strains Se584, A64, and L26. We found that three of the reference strains we sequenced, namely, Se584, A64, and L26, show proteins originating from more than one genotype. These three viruses encode mostly genotype 2 (DS-1-like) proteins but have a few proteins that can be classified as either genotype 1 or genotype 3 (Wa-like or AU-1-like, respectively). Strain AU-1 was isolated in Japan during the years 1981 to 1983, and based on the lack of RNA hybridization with the Wa or DS-1 probe, AU-1 became the prototype strain for a third HRV genogroup (34, 36). However, this genogroup is very rare, and with the exception of the

prototype strain, no pure AU-1 HRVs have been isolated to date. The only other AU-1 genogroup strain recovered from a human is strain T152 (G12P[9]), which was discovered in Thailand and encodes only seven genotype 3 proteins (Fig. 1) (30, 42, 43). Interestingly, T152 expresses two proteins (NSP1 and NSP5) more closely related to animal rotaviruses, suggesting that this AU-1-like virus may represent an interspecies reassortant (30, 43). For HRVs of which full-genome sequences are known, we find that genotype 3 proteins tend to appear in reassortant strains with predominantly genotype 2 (DS-1-like) backgrounds (Fig. 1).

Of the 10 HRV reference strains, only strains Se584 and A64 encode proteins that can be classified as genotype 3 (30). Strain Se584 was isolated in 1998 from a 7-year-old child as part of a rotavirus surveillance project in the United States (18). The occurrence of this G6 serotype strain, especially in an older child, suggests that Se584 is an unusual isolate. Previous RNA hybridization studies of this virus showed that the AU-1 genogroup probes annealed to three Se584 gene segments and that no gene segments were recognized by the Wa or DS-1 probe (18). Our sequencing results contradict these RNA hybridization data; we found that Se584 encodes six genotype 2 proteins (VP1, VP2, VP3, VP6, NSP2, and NSP4), one genotype 1 protein (NSP3), and two genotype 3 proteins (NSP1 and NSP5) (Fig. 1). The reason for the lack of detectable hybridization of Se584 gene segments with the Wa or DS-1 probe in the previous study is unknown.

Our sequencing results show that strain A64 is similar to Se584 and encodes six genotype 2 proteins (VP1, VP2, VP3, VP6, NSP2, and NSP4) and two genotype 3 proteins (NSP1 and NSP5) (Fig. 1). However, while Se584 possesses a genotype 1 NSP3, the A64 NSP3 protein is not phylogenetically related to any HRV but instead seems related to an animal rotavirus (i.e., the bovine-like genotype 6) (30) (Fig. 1). Strain A64 is an unusual HRV in that it was isolated from an immunocompromised child (20) and therefore grew under conditions with atypical selective pressures. In addition to being a reassortant, this virus contains rearrangements in genes 7, 10, and 11 that stem from head-to-tail sequence duplications (2, 20). As shown in Fig. 2, all three sequence duplications initiate downstream of the ORF, thereby leaving genes 7, 10, and 11 capable of encoding full-length proteins (NSP3, NSP4, and NSP5/6, respectively). The sequence duplication in gene 11 introduces a second ORF for NSP6 in the viral RNA; yet, given its downstream position, it is not clear whether this ORF is capable of being translated (Fig. 2). Our description of the duplication of the A64 gene 10 is in agreement with that of a previous report (20). For A64 genes 7 and 11, this is the first description of the sequence duplications.

Strain L26 also represents an intergenogroup reassortant, in this case formed by genetic exchange of genes from viruses belonging to the Wa and DS-1 genogroups. The L26 strain is identified as a member of the DS-1 genogroup, since seven of its genes encode genotype 2 proteins, with two encoding genotype 1 proteins (NSP2 and NSP5) (Fig. 1). Originally isolated from a sick infant in the Philippines in 1987, L26 is a representative of the globally emerging G12 serotype (46). This strain has a long electropherotype but shows VP2 and VP6 SG-I specificities, an unusual combination for a DS-1 genogroup strain.

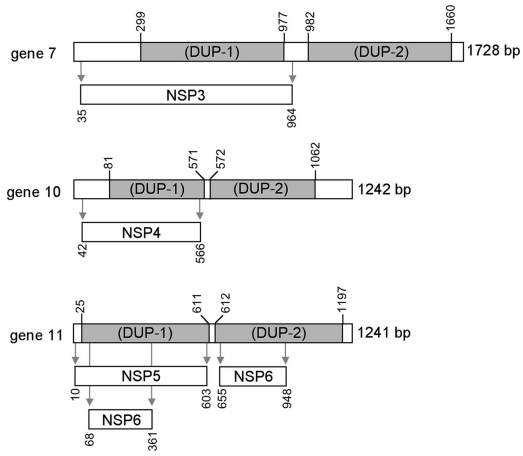

FIG. 2. Schematic of intragenic sequence rearrangements for A64 genes 7, 10, and 11. Each aberrant RNA segment contains a single head-to-tail sequence duplication (DUP-1 or DUP-2). The duplication initiates downstream of the ORF for each gene, thereby allowing A64 to express full-length NSP3, NSP4, and NSP5/6 proteins, respectively. The gene 11 rearrangement introduces a second NSP6 ORF, one that is positioned downstream of the NSP5 ORF.

Other HRVs that are known intergenogroup reassortants include several G12P[6] strains: Matlab13-03, N26-02, RV176-00, and RV161-00 (43). Matlab13-03 is predicted to belong to the Wa genogroup but encodes a genotype 2 NSP3 (Fig. 1). Strains N26-02, RV176-00, and RV161-00 can be described as DS-1-like, because the majority of their proteins are related to this genogroup (Fig. 1). N26-02 contains a genotype 1 NSP2 and an NSP4 from an animal rotavirus (genotype 6) (30) (Fig. 1). RV176-00 and RV161-00 encode NSP4 proteins that are genotype 1 and animal-like (genotype 6), respectively (30) (Fig. 1).

The fact that the HRV reassortants described above are viable demonstrates that the proteins encoded by the reassorted genes functionally replace the proteins encoded by the parental genes. Nonetheless, it is interesting that certain combinations of genes seem to exist, even among the reassortant strains. For example, the genes encoding VP1, VP2, VP3, and VP6 for each individual strain invariably belong to the same genotype. Additionally, we found that most of the reassortment events were associated with serotypes that are rarely seen in humans or that otherwise represented atypical infections (e.g., immunocompromised child or gastroenteritis in a 7-year-old) and that the predominant HRV serotypes (G1 to G4) showed no evidence of genetic exchange. Thus, these observations raise several important questions regarding possible intrinsic selection pressures influencing rotavirus diversity. Do rotaviruses have preferred gene constellations? Can proteins of different genotypes functionally substitute for each other in all cases? What are the precise amino acid changes that determine whether a protein belongs to genotype 1, 2, or 3? Do these changes cluster at sites of viral protein-protein interactions?

Correlating genotype-specific changes with protein interaction sites. To characterize the level of variation among HRV proteins belonging to genotype 1, 2, or 3, we first calculated the average intergenotype and intragenotype percent identities. In

TABLE 3. Intragenotype and intergenotype percent identities

Protein	% Identity (avg ± SD)[a] for genotypes:					
	1 vs 1	2 vs 2	3 vs 3	1 vs 2	1 vs 3	2 vs 3
VP1	98.2 ± 1.1	97.9 ± 0.9	98.3*	89.9 ± 0.4	91.3 ± 0.3	94.8 ± 0.3
VP2	97.1 ± 1.0	97.7 ± 1.8	98.8*	89.1 ± 0.8	91.5 ± 0.6	93.8 ± 0.4
VP3	96.6 ± 1.3	94.4 ± 3.6	93.2*	81.3 ± 0.6	83.4 ± 0.6	83.7 ± 0.4
VP6	98.7 ± 0.9	98.7 ± 0.8	97.5*	92.1 ± 0.4	91.8 ± 0.3	94.9 ± 1.0
NSP1	88.1 ± 5.8	96.6 ± 2.9	89.2 ± 5.1	68.9 ± 0.8	57.6 ± 1.2	58.5 ± 0.7
NSP2	95.9 ± 2.1	95.6 ± 1.7	95.0*	89.8 ± 0.8	89.3 ± 0.8	89.0 ± 0.8
NSP3	95.4 ± 2.7	97.5 ± 1.7	95.5*	82.0 ± 0.9	83.1 ± 0.8	88.8 ± 0.5
NSP4	95.9 ± 1.8	93.7 ± 1.9	94.3*	84.1 ± 1.2	84.1 ± 0.9	84.0 ± 1.2
NSP5	96.2 ± 2.3	96.1 ± 3.1	95.8 ± 1.1	83.0 ± 1.4	90.0 ± 0.9	86.4 ± 1.7

[a] *, single value; no standard deviation given.

this analysis, we utilized amino acid sequence information for the 25 HRVs for which full-genome sequences are available. We note that, due to the lack of availability of genotype 3 sequences, we are more confident regarding changes differentiating genotype 1 and 2 proteins. As shown in Table 3, proteins belonging to the same genotype (excluding NSP1) have a high degree of sequence identity (93.2 to 98.8%). In contrast, the sequence identity of proteins belonging to different genotypes is at least 5% lower. Thus, on average, proteins of different genotypes will differ by 5 or more amino acids per 100 from proteins of the same genotype, suggesting a greater evolutionary distance between genotypes. While the amino acid identities for NSP1 proteins of the same genotype ranged from 88.1 to 96.6%, the identities for those of different genotypes were substantially lower (57.6 to 68.9%) (Table 3). These data indicate that the evolutionary distance between NSP1 proteins of different genotypes vastly exceeds that of the other viral proteins analyzed (see below). To identify the precise amino acid residues that distinguish proteins from genotypes 1, 2, and 3, amino acid alignments were constructed (see Fig. S1 and Table S1 in the supplemental material). Residues in the alignments that differed depending on the genotype of the protein (vis-à-vis genotype-specific differences) were identified and mapped to defined regions or known structural or functional domains (Fig. 3). We found that for some of the proteins, genotype-specific changes often clustered in sites of known viral protein-protein interactions.

Interactions among VP1, VP2, VP3, and VP6. Proteins constituting the inner layers of the rotavirus virion (VP1, VP2, VP3, and VP6) segregate with a single genotype for each of the HRVs, suggesting that they are genetically linked (Fig. 1). Because these proteins are known to interact with each other, we hypothesize that the linkage is a reflection of amino acid changes driving more favorable intragenotype interactions. The core lattice protein (VP2) engages both the viral RNA-dependent RNA polymerase (VP1) and the RNA capping enzyme (VP3) via its internally located N-terminal domain (52). The interaction between the VP2 N terminus and VP1/VP3 is thought to be critical during rotavirus RNA synthesis (viral genome replication and transcription) (38). Additionally, the outer surface of the VP2 shell interacts with VP6 during the early stages of virion morphogenesis (12). The VP2-VP6 interaction is important for the formation of double-layered particles (DLPs) and also influences the transcriptional activity of the particle (12). The outer surface of the VP2 shell domain, which is accessible to interactions with VP6, exhibits 27 amino acid differences between genotypes 1 and 2 (32) (Fig. 3). However, we found that most of the genotype-specific differences in VP2 map to the N terminus of the protein and include dramatic sequence variations and insertions/deletions, which are predicted to influence VP1/VP3 binding (32) (see Fig. S2 in the supplemental material). For the viral polymerase VP1, we identified 66 amino acids that differ between genotypes 1 and 2 (Fig. 3). Although the changes are distributed throughout the enzyme, the bulk of them are surface exposed and concentrate at the N- and C-terminal domains and the finger subdomain; in contrast, the predicted catalytic domain of the VP1 is quite conserved (26a) (Fig. 3). To our surprise, VP3, the RNA methyltransferase/guanylyltransferase, shows more genotype-specific amino acid differences than any other inner capsid protein. We found 71 changes between genotypes 1 and 2 and 22 changes among genotypes 1, 2, and 3, many of which clustered in the central region and C terminus of the protein (Fig. 3). Based on what is seen for VP1, we predict that the currently undefined VP3 catalytic sites might be located in the more conserved regions of the protein. The regions of VP1 and VP3 that mediate interactions with each other and with the VP2 N terminus are unknown. It is possible that the identified genotype-specific changes could be sites of VP1/VP2/VP3 interactions.

Interactions among VP2, VP6, VP7, and VP4. Our sequence alignments indicate that VP6 contains relatively few genotype-specific amino acid differences (see Fig. S1 in the supplemental material). Those that are present tend to cluster toward the termini of the protein, with many in or close to known VP2 interactive domains (4) (Fig. 3). Mapping these differences onto the atomic structure of VP6 indicates that the majority of them (18 out of 23 amino acids) are surface exposed and that many are situated in domain B at the base of the VP6 trimer and near the VP2 contact site (Fig. 4). The placement of these residues raises the possibility that genotype-specific changes in VP6 could influence (i) the assembly of the VP6 protein layer onto VP2, (ii) the stability of the DLP, or (iii) the transcriptional activity of VP1 (4). VP6 also engages the outer capsid proteins VP7 and VP4 during formation of infectious triple-layered particles (12). The sites on VP6 where VP7 and VP4 bind have been described previously and can be visualized on the atomic structure of the trimer (27) (Fig. 4). VP6 contains several genotype-specific changes near the VP7-binding site. Interestingly, several of these residues comprise the VP6 SG-

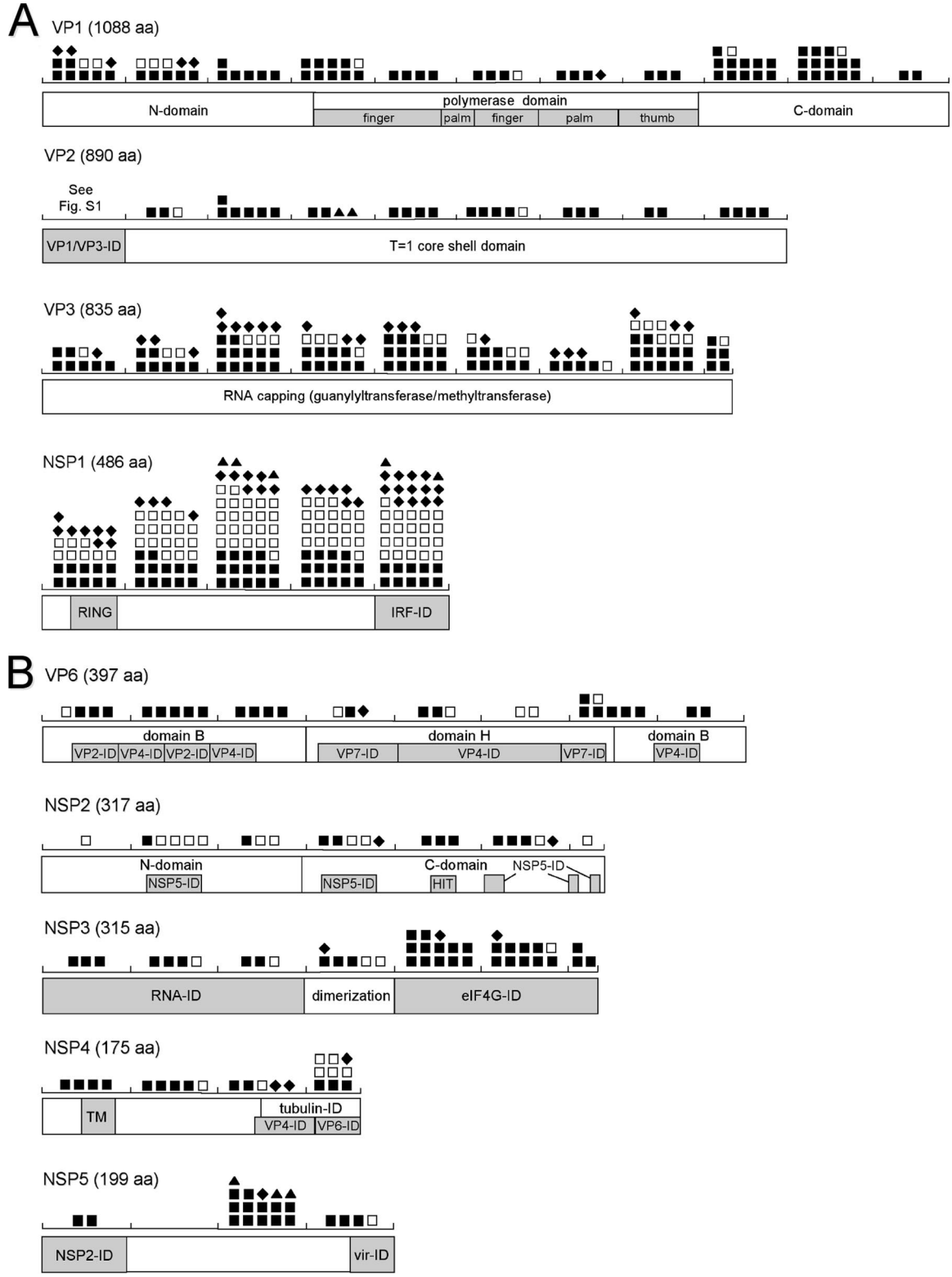

FIG. 3. Locations of genotype-specific changes in rotavirus proteins. Amino acid alignments of proteins encoded by HRVs with fully sequenced genomes were produced using MacVector 8.1.2 (see Fig. S1 in the supplemental material). From these alignments, residues that distinguished each of the three genotypes were identified. Such genotype-specific changes are indicated using symbols. Residues that are conserved among but differ between genotypes 1 and 2 are shown as black squares. Residues unique to genotype 3 proteins and not seen in genotype 1 or 2 are indicated as white squares. Residues that showed specific changes among genotypes 1, 2, and 3 are illustrated as black diamonds. Residues present in one genotype but not others (i.e., deletions) are indicated as black triangles. The amino acids identified in this analysis are presented in Table S1 in the supplemental material. For mapping purposes, the residues were then grouped into intervals of either (A) 100 amino acids (aa) (for VP1, VP2,

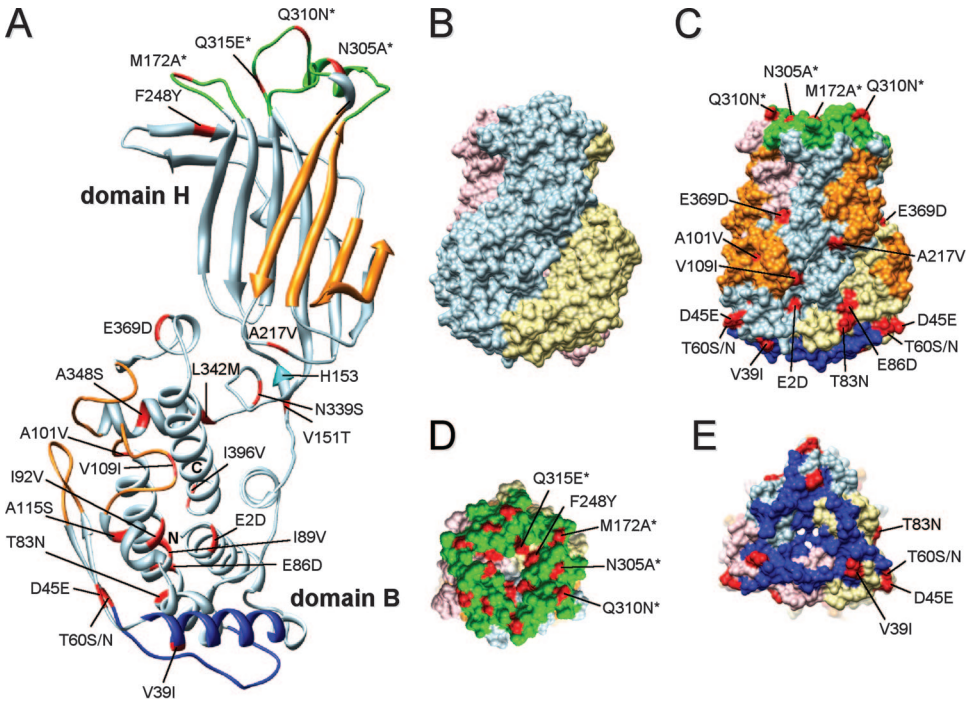

FIG. 4. Mapping genotype-specific differences on the atomic structure of VP6. (A) Ribbon diagram illustrating domain H (residues 151 to 331) and domain B (residues 1 to 150 and 331 to 397) of VP6 (bovine strain RF) (27). Regions of VP6 interfacing with VP2 (blue), VP4 (gold), and VP7 (green) are indicated, as are the locations of the N and C termini and the zinc-coordinating residue (cyan, H153). Amino acids differing between genotype 1 and 2 VP6 proteins are shown in red and are labeled. The genotype-specific residues in the VP7 interface region that define VP6 SG specificity are labeled with asterisks: SG-I (A172 and A305) and SG-II (M172, N305, Q310, and Q315) (17). (B) Surface side-view representation of the VP6 trimer, with monomers colored in light blue, pink, and yellow. (C) VP6 trimer as shown in panel B, with VP2, VP4, and VP7 interface regions and residues differing between genotypes 1 and 2 colored as described for panel A. (D and E) Top and bottom views, respectively, of the trimer shown in panel C.

specific epitopes (Fig. 4). In contrast, the site on VP6 where VP4 binds is quite conserved, and the β-roll of domain H does not show any differences between genotypes 1 and 2. Although genes encoding VP6, VP7, and VP4 of HRVs are suggested to reassort freely and independently in nature, some types of outer capsid proteins tend to appear together more frequently. For instance, genotype 2 VP6 proteins tend to associate most frequently with G2 VP7 and P[4] VP4 proteins. Preferential binding among these capsid proteins could influence (i) assembly of infectious virions, (ii) particle stability in nature, or (iii) efficiency of disassembly following entry into a host cell. Therefore, interactions between the outermost capsid proteins might be subjected to intrinsic selection pressures related to favorable interactions as well.

Interactions between VP6 and NSP4. In addition to binding to rotavirus structural proteins, VP6 engages the transmembrane glycoprotein (NSP4) during virion assembly. This interaction recruits newly formed DLPs into the endoplasmic reticulum, where, through a budding process, the outer capsid shell of the virion is formed (12). A previous study has provided evidence of a genetic linkage between VP6 and NSP4 (21). Specifically, HRVs exhibiting VP6 SG-II specificities (genotype 1) have proteins classified as NSP4B (genotype 1), whereas those bearing VP6 SG-I epitopes (genotype 2) encode NSP4A or NSP4C (genotype 2 or 3, respectively) (21). The results presented in this report are in agreement with this finding, as only three HRVs (N26-02, RV176-00, and RV161-00) for which sequences are available have VP6 and NSP4 proteins belonging to different genotypes (Fig. 1). Furthermore, while NSP4 contains very few genotype-specific amino acid differences, the changes cluster in the C terminus of the protein (Fig. 3). This observation is intriguing, as the C-terminal region of NSP4 is involved in interactions with VP6 (1). Thus, the genotype-specific amino acid variations may be connected to the genetic linkage noted for the VP6 and NSP4 proteins. Unfortunately, no information on the location of the NSP4-binding site on the VP6 trimer exists, but we expect the distal portion of the trimer to be the most likely site (Fig. 4).

VP3, and NSP1) or (B) 50 aa (for VP6, NSP2, NSP3, NSP4, and NSP5), spanning the length of the protein. The number of residues in each classification group per interval is plotted above the protein. Locations of key functional and structural domains are approximated. The genotype-specific changes for the VP2 N terminus are presented in Fig. S2 in the supplemental material. The NSP5 interactive domain (ID) on NSP2 was determined based on reference 22 and overlaps the RNA ID. IRF, interferon regulatory factor; HIT, histidine triad; TM, transmembrane domain; vir, viroplasm.

Nonetheless, NSP4 is a multifunctional protein that interacts not only with VP6 but also with VP4 and host cell membranes and proteins (e.g., tubulin) during viral replication. Aside from promoting rotavirus maturation, NSP4 functions as a viral enterotoxin that induces diarrhea through fluid loss triggered by intracellular calcium mobilization and chloride secretion (12). Thus, the pressures influencing the genetic diversity of NSP4 are not merely limited to viral protein interactions.

NSP2-NSP5 interactions and analysis of the NSP6 ORF. During replication inside a host cell, the nonstructural proteins NSP2 and NSP5 interact with each other to mediate the formation of viroplasms, which serve as sites of viral RNA synthesis and early virion morphogenesis (47). The RNA-binding protein NSP2 exists as a doughnut-shaped octamer that is formed by the interaction of two tetramers stacked tail to tail (22). Extending diagonally across the NSP2 octamer are four highly basic grooves; these represent the sites of interaction with NSP5 and single-stranded RNA (22). In addition to binding NSP5 and RNA, NSP2 is suggested to interact with one or more core proteins (e.g., VP1, VP2); yet, the binding sites for these proteins on NSP2 have not been determined (47). NSP2 and NSP5 are remarkably conserved among genotypes 1, 2, and 3, and nearly all of the genotype-specific changes for both of these proteins lie just outside of known interaction sites (Fig. 3). This observation is interesting in light of the fact that the NSP2- and NSP5-encoding genes seem to reassort more freely in the HRVs than do genes encoding the inner capsid proteins (Fig. 1). The conserved nature of the NSP2 and NSP5 interaction sites might allow these two proteins to function in a genotype-independent manner. In other words, NSP2 and NSP5 proteins from different genotypes (or even from different species) can functionally substitute for each other quite easily. For the phosphoprotein NSP5, the majority of the genotype-specific changes were noted to occur in the C-terminal half of the protein; the importance of this region is not known, but it might represent the binding site for cellular protein kinases (Fig. 3).

We should point out that, for many HRVs, the NSP5-coding gene (gene 11) has an alternative ORF that encodes a putative nonstructural protein (NSP6) (Fig. 1; also see Fig. S3 in the supplemental material). The existence of NSP6 in cells infected with HRVs has not been fully demonstrated. However, in studies using the simian strain SA11, NSP6 was detected using specific antisera and was shown to colocalize with NSP2 and NSP5 in viroplasms (33). The lengths of the NSP6 ORF sequences vary quite dramatically for the 25 HRVs analyzed in this study; yet, such variation has been noted for many animal rotavirus strains (33). Five HRVs (D, P, WI61, AU-1, and N26-02) show NSP6 ORFs that are truncated compared with that of SA11, while two of them (Se584 and A64) show extended NSP6 ORFs (Fig. 1; also see Fig. S3 in the supplemental material). We found no correlation between NSP6 ORF length and genotype/genogroup specificity. Future studies are needed to determine whether these HRVs truly express the proposed NSP6 proteins.

NSP1 and NSP3 interactions with host cells. The rotavirus nonstructural proteins NSP1 and NSP3 are unique in that they interact extensively with host cell factors but are not known to bind to any other viral proteins and are nonessential for replication in cell culture. As such, one might expect that NSP1 and NSP3 are subject to extrinsic selection pressures and should show patterns of evolution that differ from those of the other viral proteins. Our sequencing analysis shows that HRV NSP1 and NSP3 proteins can still be assigned into genotype 1, 2, or 3 but that the genes encoding these proteins tend to reassort more freely than those encoding the inner capsid proteins (Fig. 1). Additionally, the percent identity between genotypes 1 and 2 for these proteins is lower than that for any of the others, being 68.9% and 82.0% for NSP1 and NSP3, respectively, suggesting that these proteins are quite divergent (Table 3). NSP1 plays an important role in antagonizing the host cell innate immune response by inducing the proteosome-mediated degradation of transcription factors involved in interferon production (interferon regulatory factors) (3). Extensive genotype-specific changes for NSP1 are seen throughout the entire protein but are even more exaggerated in the C-terminal half (Fig. 3). A region within the C terminus of NSP1 is thought to be important for binding to interferon regulatory factor proteins (15). In contrast, the putative RING domain of NSP1, which contains a motif recognized in many E3 ubiquitin ligases, is quite conserved (Fig. 3). NSP3 is involved in abrogating host cell protein synthesis during infection and is known to interact with viral RNA and components of the cellular translation machinery (10, 40, 41). NSP3 is much more conserved than NSP1 but has genotype-specific changes clustering in its C terminus, a region important for interactions with eIF4G (eukaryotic initiation factor 4G) (Fig. 3). The reason for genotype-specific variation in NSP1 and NSP3 is not known, but it might reveal that these proteins participate in as-yet-unidentified interactions with other viral proteins.

In summary, the results presented in this report provide detailed information regarding the full-genome sequences of 10 commonly used HRV strains (D, DS-1, P, ST3, IAL28, Se584, 69M, WI61, A64, and L26). By utilizing and expanding upon the classification system of Matthijnssens et al. (30), we present evidence for the existence of preferred HRV gene constellations. Several previous reports have provided data to suggest that not all HRV genes reassort randomly; the existence of HRV genogroups is the strongest evidence in favor of this argument (9, 21, 34, 35, 49). Still, this study is the first to combine sequence analyses with structural and functional data to (i) gain insight into the normal variation within and between HRV genogroup proteins (i.e., genotypes) and (ii) provide a molecular explanation for why preferred gene constellations are maintained. Our results are consistent with the idea that genetic linkages are, at least in part, a result of intrinsic selection pressures on viral proteins. Nonetheless, we cannot rule out the possibility that interactions among RNA segments during gene assortment and packaging influence the constellations. It is important to note that intragenogroup reassortant viruses that break seemingly tight genetic linkages (for example, between VP2 and VP6) can be created in the laboratory, demonstrating that the preferences for intragenotypic protein interactions are subtle (data not shown). However, we predict that such reassortants, albeit viable, have decreased evolutionary fitness compared to that of parental strains and would be selected against in the context of infection in the human population. Without a doubt, further epidemiologic studies are required to examine if and how identified genotype-specific differences correlate with biological functions. We expect the

results presented here to provide a scientific platform for such studies that will reveal the true dynamics of group A rotavirus evolution and diversity.

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