

Nucleotide Sequence of VP4 and VP7 Genes of Human Rotaviruses with Subgroup I Specificity and Long RNA Pattern: Implication for New G Serotype Specificity

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We sequenced the genes coding for the two neutralization proteins, VP4 and VP7, of human rotavirus strains L26 and L27 with subgroup I specificity but the long RNA pattern. The deduced VP7 amino acid sequence of strains L26 and L27 showed a low homology (73.6 to 81.9%) to those of rotavirus strains of the established serotypes. This finding, together with the previous serological characterizations, suggests that the VP7 (G) serotype of the L26 and L27 strains is distinct from those of strains of the previously established serotypes. In contrast, the VP4 sequences of the L26 and L27 strains were quite similar to those of virulent serotype 2 strains (DS-1, S2, and RV-5).

Group A rotaviruses have two neutralization proteins, VP4 and VP7, in the outer layer of the double-shelled particle (15, 27, 31). By cross-neutralization tests with hyperimmune sera, 11 distinct serotypes have been identified in group A rotaviruses so far (2, 5, 16, 24, 28, 35, 36). The serotype specificity of rotaviruses is ascribed mainly to the antigenic specificity of glycoprotein VP7 (5, 20). According to the recent proposal for the binary terminology of rotavirus serotypes, the antigenic specificity of VP7 of the 11 established serotypes can be designated G1 to G11 by using the prefix G for glycoprotein. VP4, a target protein for trypsin activation of infectivity, also has an independent antigenic specificity (15, 27). The proposed designation for the type specificity carried by VP4 is to use the prefix P for protease (P1, P2, and so on).

Recent comparative studies on the nucleotide sequences of VP4 genes from representative human rotavirus strains suggested the presence of at least four P types of VP4 (10, 32). In this article we tentatively designate them as follows: P1, found in virulent strains with G1, G3, or G4 specificity; P2, in virulent strains with G2 specificity; P3, in asymptomatic strains; and P4, in virulent strain K8 with G1 specificity.

Five additional types of VP4 in bovine, simian, and porcine rotaviruses have been suggested by sequence analyses (19, 23, 25, 26). However, systematic antigenic typing of VP4 from human and animal rotavirus strains has not yet been performed. Furthermore, it has been found that several cross-reactive neutralization epitopes exist at least in P1 and P2 VP4s and in P1, P2, and P3 VP4s (31), suggesting ambiguity in the serotyping of VP4 compared with that of VP7.

We recently characterized 20 unusual human rotavirus strains derived from Philippine infants with diarrhea which had subgroup I specificity and the long RNA pattern typical of subgroup II rotaviruses (21). By cross-neutralization tests with the four Philippine strains isolated in cell culture and other established strains, these unusual strains were found

not to be antigenically related to any other established serotypes except for serotypes 7, 10, and 11, antisera for which were not available (S. Urasawa, T. Urasawa, F. Wakasugi, N. Kobayashi, K. Taniguchi, I. C. Lintag, M. C. Saniel, and H. Goto, *Arch. Virol.*, in press).

In this study, we sequenced the genes encoding the two neutralizing proteins VP4 and VP7 of the Philippine strains L26 and L27. A comparison of their sequence with those of established serotypes supported the idea that strains L26 and L27 have a new G serotype on VP7, while their P type on VP4 is similar to that of P2 found in strains with the G2 serotype.

Strains L26 and L27 were grown in MA-104 cells in the presence of trypsin (1 μ g/ml), and single-shelled particles were purified by differential centrifugation, fluorocarbon treatment, and CsCl gradient centrifugation after EDTA treatment. Viral mRNA was synthesized *in vitro* by the endogenous transcriptase present in single-shelled particles. Synthetic oligonucleotide primers were used to sequence mRNA by reverse transcriptase in the presence of dideoxynucleotides as described previously (11). The terminal 100 nucleotides of the RNA were determined by using denatured double-stranded virion RNA.

Figure 1 shows the complete nucleotide sequence and the deduced amino acid sequence of the VP7 gene of strain L26. The fundamental structure of the VP7 gene from strain L26 was similar to those of other rotavirus strains (5, 20). The entire VP7 gene of the L26 strain consisted of 1,062 nucleotides, with two potential open reading frames beginning at an ATG at positions 49 to 51 or 136 to 138 and ending at a TAG at positions 1027 to 1029. The open reading frames had the capacity to code for a VP7 of 297 or 326 amino acids. Two potential glycosylation sites were found at amino acid positions 69 to 71 and 238 to 240. Eight cysteine residues were conserved, as has been found for all strains examined to date. The VP7 sequence of strain L27 was identical to that of strain L26 VP7.

Except for serotype 7 strains, sequence data for which are not yet available, the nucleotide and amino acid sequences of strains representing the established serotypes, including non-serotype 6 bovine strain 61A (possible serotype 10)

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GGC	TTT	AAA	AGA	GAG	AAT	TTC	CGT	TTG	GCT	AGC	GGT	TAG	CTC	CTT	TTA	ATG	TAT	GGT	ATT	60
																M	Y	G	I	4
GAA	TAT	ACC	ACA	ATT	CTA	ACC	ATT	TTG	ATA	TCA	ATC	GTT	CTA	CTA	AAT	TAT	ATA	TTA	AAA	120
E	Y	T	T	I	L	T	I	L	I	S	I	V	L	L	N	Y	I	L	K	24
TCG	ATA	ACT	AGT	ATG	ATG	GAC	TTT	ATT	ATA	TAT	AGA	TTC	TTA	CTA	GTT	TTT	GTT	ATC	GTA	180
S	I	T	S	M	M	D	F	I	I	Y	R	F	L	L	V	F	V	L	V	44
CTG	CCA	TTT	ATT	AAA	GCT	CAA	AAC	TAT	GGA	ATA	AAT	CTT	CCA	ATC	ACA	GGT	TCT	ATG	GAT	240
L	P	F	I	K	A	Q	N	Y	G	I	N	L	P	I	T	G	S	M	D	64
ACT	GCA	TAT	GTA	AAC	TCT	ACG	CAA	CAA	GAA	AGT	TTT	ATG	ACT	TCC	ACT	TTA	TGC	TTG	TAT	300
T	A	Y	V	N	S	T	Q	Q	E	S	F	Y	A	S	T	L	C	L	Y	84
TAT	CCG	AAT	TCA	GTT	ACG	ACT	GAA	ATA	ACT	GAC	CCC	GAT	TGG	ACG	CAT	ACA	CTA	TCA	CAA	360
Y	P	N	S	V	T	T	E	I	T	D	P	D	W	T	H	T	L	S	Q	104
CTA	TTT	CTG	ACT	AAA	GGA	TGG	CCA	ACA	AAT	TCT	GTT	TAC	TTC	AAG	AGT	TAT	GCT	GAC	ATA	420
L	F	L	T	K	G	W	P	T	N	S	V	Y	F	K	S	Y	A	D	I	124
GCG	TCC	TTC	TCT	GTA	AAT	CCA	CAG	TTA	TAC	TGT	GAT	TAC	AAT	ATC	GTG	TTA	GTA	CAA	TAT	480
A	S	F	S	V	N	P	Q	L	Y	C	D	Y	N	I	V	L	V	Q	Y	144
CAA	AAT	TCA	TTA	GCG	TTA	GAT	GTT	TCG	GAA	CTC	GCT	GAT	TTA	ATT	TTA	AAT	GAA	TGG	TTA	540
Q	N	S	L	A	L	D	V	S	E	L	A	D	L	I	L	N	E	W	L	164
TGT	AAT	CCG	ATG	GAC	GTA	ACG	TTA	TAT	TAT	TAT	CAA	CAG	ACT	GAC	GAA	GCC	AAT	AAA	TGG	600
C	N	P	M	D	V	T	L	Y	Y	Y	Q	Q	T	D	E	A	N	K	W	184
ATA	TCA	ATG	GGA	GAT	TCA	TGT	ACA	GTT	AAA	GTA	TGT	CCT	TTA	AAT	ATG	CAA	ACG	TTA	GGA	660
I	S	M	G	D	S	C	T	V	K	V	C	P	L	N	M	Q	T	L	G	204
ATT	GGA	TGT	ACA	ACA	ACC	GAC	GTC	GCA	ACA	TTT	GAA	GAA	GTA	GCA	AAC	GCG	GAA	AAG	TTA	720
I	G	C	T	T	T	D	V	A	T	F	E	E	V	A	N	A	E	K	L	224
GTA	ATT	ACT	GAT	GTT	GTA	GAC	GGA	GTC	AAT	CAT	AAG	ATC	AAT	ATT	ACA	TTG	AAT	ACA	TGC	780
V	I	T	D	V	V	D	G	V	N	H	K	I	N	I	T	L	N	T	C	244
ACT	ATA	CAA	AAT	TGT	AAA	AAA	TTG	GGA	CCT	AGA	GAA	AAC	GTA	GCA	ATT	ATA	CAA	GTA	GGT	840
T	I	Q	N	C	K	K	L	G	P	R	E	N	V	A	I	I	Q	V	G	264
GGT	TCT	GAC	ATC	ATA	GAT	ATA	ACA	GCA	GAT	CCA	ACA	ACA	ATT	CCA	CAA	ACT	GAA	AGA	ATA	900
G	S	D	I	I	D	I	T	A	D	P	T	T	I	P	Q	T	E	R	I	284
ATG	CGA	ATA	AAT	TGG	AAA	AAA	TGG	TGG	CAA	GTG	TTT	TAT	ACC	GTA	GTA	GAT	TAC	ATA	AAT	960
M	R	I	N	W	K	K	W	W	Q	V	F	Y	T	V	V	D	Y	I	N	304
CAA	ATA	GTT	CAG	GTA	ATG	TCT	AAA	CGA	TCT	AGA	TCA	CTA	AAT	TCA	GCT	GCA	TTT	TAT	TAC	1020
Q	I	V	Q	V	M	S	K	R	S	R	S	L	N	S	A	A	F	Y	Y	324
AGA	ATT	TAG	ATA	TAG	CTT	AGG	TTA	GAG	TTG	GTC	GAT	GTG	ACC							1062
R	I																			326

FIG. 1. Complete nucleotide sequence and deduced amino acid sequence of the VP7 gene of strain L26. Two potential glycosylation sites are shown in boxes.

recently isolated in Thailand (32a), were compared with those of strain L26. The nucleotide and amino acid sequences determined were only 72.9 to 77.4% and 73.6 to 81.9% homologous, respectively (Table 1). These low homology values contrast strongly with the 91 to 100% homology found among the strains of a given serotype (14).

VP7 has six serotype-specific regions, designated A to F (amino acids 39 to 50, 87 to 101, 120 to 130, 143 to 152, 208 to 221, and 233 to 242, respectively) (14). Amino acids in these regions are well conserved among strains of the same serotype but differ considerably among strains belonging to different serotypes. Three regions in particular, B, D, and E, are considered the major antigenic sites, since mutants resistant to anti-VP7 serotype-specific neutralizing monoclonal antibodies had amino acid changes only in these three regions (3, 5, 22, 29). A comparison of amino acid sequences in these regions between strain L26 and strains with serotype

1, 2, 3, 4, 5, 6, 8, 9, 11, or possibly 10 specificity revealed a great difference (Fig. 2). The amino acid sequence homologies in the three regions were only 34 to 51%. In our previous studies (21; Urasawa et al., in press), neutralizing monoclonal antibodies specific for serotype 1, 2, 3, or 4 could not recognize strains L26 and L27 either in an enzyme-linked immunoassay or a neutralization test. Furthermore, two-way cross-neutralization tests with hyperimmune sera showed no serological relationship of the two strains to other serotypes except for serotypes 7, 10, and 11, for which antisera were not available. Thus, the present sequence data strongly support the notion that these strains represent a new G (VP7) serotype.

The complete nucleotide sequences of VP4 genes from strains L26 and L27 were also determined. The VP4 nucleotide sequence of the two strains was 2,359 bases long and contained a single long open reading frame beginning with

Strain	G Serotype	B region		D region		E region	
		87	101	143	152	208	221
L26	12	NSVTTEITDPDWTHT		QYQNSLALDV		TTTDVATPEEVANA	
KU	1	TEAS-Q-A-G--KD-		K--Q--E--M		Q--N--DS--MI-EN	
Wa	1	TEAS-Q-A-G--KD-		K--Q--E--M		Q--N--DS--MI-EN	
S2	2	AEAKN--S-DE-EN-		R-D-TSE---		KI---D---I--SS	
DS-1	2	AEAKN--S-DE-EN-		R-D-TSE--A		K---N---I--SS	
P	3	TEAA---N-NS-KD-		K-DAT-Q--M		L---TN-----T-	
SA11	3	TEAA---N-NS-KD-		K-DAT-Q--M		L---AT-----T-	
ST-3	4	SEAP-Q-S-TE-KD-		RPVSGEE--I		Q--NT---T--DS	
VA70	4	SEAP-Q-S-TE-KD-		KPASGEE--I		Q--N-----M--DS	
OSU	5	-EAA---A-TK-KE-		K-DGN-Q--M		S---INS--T----	
NCDV	6	VEASN--A-TE-KD-		K-DSTQE--M		LI-NPD---T--TM	
UK	6	VEASN--A-TE-KD-		K-DSTQE--M		LI-NPD---T--TT	
69M	8	VEAE---A-SS-KDH		K-NANSE--M		L---TT-----T-	
B37	8	VEAE---A-SS-KDH		K-NANSE--M		L---TT-----T-	
WI61	9	AEAS-Q-G-TE-KD-		K-DST-E--M		---NT-----AS	
F45	9	AEAS-Q-G-TE-KD-		K-DST-E--M		---NT-----AS	
61A	10?	TEAR---N-NE----		R-NS--E--M		Q--NTR-----T-	
YM	11	HEAA-Q-A-DK-KD-		K-DGNSQ--M		L---PT-----S-	

FIG. 2. Comparison of the VP7 amino acid sequence in three antigenic regions (B, D, and E) of strain L26 with those of human rotavirus strains with different G serotypes. The entire VP7 amino acid sequences of strains other than L26 have been reported previously (4, 5, 8, 9, 12-14, 17, 28, 32a). NCDV, Nebraska calf diarrhea virus.

ATG at positions 10 to 12 and terminating with TAG at positions 2335 to 2337. The entire deduced amino acid sequences of strains L26 and L27 are shown in Fig. 3. Four nucleotide sequences, three of which caused amino acid changes, were different between the two strains: AGA (codon 51), AAC (codon 324), GAA (codon 392), and TTT (codon 405) in strain L26 were GGA, AAT, AAA, and TGT, respectively, in strain L27.

The nucleotide and amino acid sequences of L26 and L27 were compared with those of human rotavirus strains recovered from symptomatic and asymptomatic patients. The VP4 nucleotide sequence of both strains showed higher homology (95%) to those of strains (DS-1 and RV-5) with P2 specificity than to those of strains with P1, P3, and P4 specificities (87, 74, and 67%, respectively).

Figure 4 shows the comparison of the amino acid sequences in a selected region (amino acids 361 to 430) where P2-specific amino acid sequences are found. In our previous study (30), we showed that amino acid residue 392 is crucial to neutralization epitope specific to P1 and P2 by using KU

TABLE 1. Nucleotide and amino acid sequence homology of strain L26 VP7 with VP7 from other rotavirus strains with different G serotype specificities

Strain (G serotype)	% Homology with VP7 of strain L26	
	Nucleotide sequence	Amino acid sequence
KU (1)	74.7	76.9
DS-1 (2)	73.3	76.1
SA11 (3)	77.4	81.3
Rhesus rotavirus (3)	76.1	81.9
VA70 (4)	73.1	74.2
Gottfried (4)	72.9	73.6
OSU (5)	75.5	80.7
Nebraska calf diarrhea virus (6)	76.0	78.2
69M (8)	74.2	79.2
WI61 (9)	76.3	80.9
61A (10?)	73.8	77.5
YM (11)	74.3	80.8

(P1) and DS-1 (P2) antigenic mutants resistant to each of the anti-VP4 neutralizing monoclonal antibodies specific to P1 or P2. L27 VP4 had a different amino acid from L26 at position 392, Glu in L26 and Lys in L27. Interestingly, the DS-1 mutant resistant to the P2-specific neutralizing monoclonal antibody (S2-2F2) had an amino acid substitution at this residue (Lys-392 to Glu). Indeed, S2-2F2 antibody, which was reactive with strain L27, did not recognize strain L26 in a neutralization test (21). Strain L26 may be a naturally occurring variant which acquired resistance to the antibody directed to the epitope involving amino acid residue 392 during an infection process in an individual who had pre-existing antibodies directed to that epitope.

In general, there was a cosegregation between VP4 and VP7 genes of human rotaviruses detected in nature; strains with G1, G3, or G4 have P1 specificity, while strains with G2 have P2 specificity (10, 30, 31). An exception is found in strains recovered from asymptomatic infections in neonates, which have P3 specificity and either G1, G2, G3, or G4 specificity (6, 10, 11). In our recent study (32), we reported another exception, strain K8 with G1 and unique VP4 (P4). However, strains like L26 and L27 having the P2 type on VP4 and a G serotype other than G2 on VP7 have not been described. The L26 and L27 strains have subgroup I specificity on VP6, which is generally found in serotype 2 strains

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MASLIYRQLLTNSYSVDLHDEIEIQIGSEKTQNVTTINPGPPAQTRYAPVNRHGEINDSTTVEPVLDGPIQPTTFKPPNDY 80
G
WLLISSNTDGVVYESTNNSDFWTAIVAVEPRVSQTRNQYILFGENKQFNIENNSDKWKFPEMFKGSSQSNFNRRTLTSN 160
NRLVGMKLYGCRVWTFHGETPRATTDSSNTADLNNISIVIHSEFYIIPRSQESKNEYINGLPPIQNTNRNVVPLSLSSR 240
SIQYRRAQVNEEDITISKTSLWKEMQYNRDIIRFKFPGNSVIKLGGLGYKWSEISYKAANYQYSYSRDGEQVTAHTTCSVN 320
GVNNFSYNGGSLPTDFSISRVEVIKENSYYIDYWDKSAFRNMVYVRSLAANLNSVKCAGGSYNFRLPVGEWPIMNGGA 400
K
VSLHFAGVTLSTQFTNFVSLNSLRFRLVDEPSFSIIRTRTVNLYGLPAANPNNGNEYEMSCRFLSISLVPTNDDYQ 480
C
TPIMNSVTVRQDLERQLSDLREEFNLSLQEIAMSQLIDLALLPLDMFSMFGSIKSTIDLTKSMATSVMKFRKSKLATS 560
SEMTNSLSDAASSASRSASVRSNLSVISNWDASKSTSNTIDLVDVSTQTSTISKLRKEMITQTEGMSFDDISAAVL 640
KTKIDMSTQIGKNTLPDIVTEASEKFKPKRSYRVLKDNEVMEINTEGKFFAYKVDLNEIPFDINKFAELVTDSPVISAI 720
IDFKTLKNLNDNYGITRMEALNLIKSNPNVLRNFINQNNPIARNRIEQLILQCKL 775
    
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FIG. 3. Complete amino acid sequences of VP4 from strains L26 and L27. Only the amino acids of strain L27 which are different from those of strain L26 are shown below the L26 amino acid sequence.

Strain	P type	361 (362)	392 (393)	430 (431)
KU	P1	FRNMVYVRS LAANLNSVKCTGGSYDFSIPVGAWPVMNGGAVSLHFGV TQFTDFVSL		
P	P1	-----		
RV-5	P2	-----	-----RL--G--I-----	
DS-1	P2	-----	-----N-RL--K--I-----	
V-S2-2F2 (DS-1)	P2	-----	-----N-RL--E--I-----	
L26	(P2)	-----	-----N-RL--E--I-----	
L27	(P2)	-----	-----N-RL--K--I-----	
M37	P3	-----	-----S--N--N--QL-----S-----	
1076	P3	-----	-----S--N--N--QM-----S-----	
K8	P4	-----	-----S--S--AL--NH--S--T--TS--LS--QYTDY	

FIG. 4. Comparison of amino acid sequences of a selected region (amino acids 361 to 430) on VP4 of strains L26 and L27 with those of VP4 from human rotaviruses of different P types. The numbers above the sequence refer to amino acid positions. The numbers in parentheses show the amino acid positions in strain K8, which has an insertion of one amino acid after residue 135 (32). The entire VP4 amino acid sequences of strains other than L26 and L27 have been reported previously (10, 18, 23, 29, 32).

having P2 on VP4 and G2 on VP7. Therefore, these strains, which still reserve the cosegregation between P2 specificity and subgroup I specificity, might be naturally occurring reassortants between a serotype 2 human rotavirus and a strain with novel serotype specificity (G12) on VP7. Despite the frequent occurrence of human rotavirus reassortment *in vitro* (7, 34), the appearance of such reassortants in nature seems to be rare. There may be some constraints, which have not been elucidated, on the occurrence of human rotavirus reassortants in nature.

Although the L26 and L27 strains have P2 specificity on their VP4, they showed little relationship with reference strains having VP4 of P2 specificity in cross-neutralization tests (Urasawa et al., *in press*), indicating low immunogenicity of VP4 of these strains. This finding is in accord with the general evidence that antibody reactivity in hyperimmune sera is directed largely against VP7 in most wild strains. In contrast, the reassortants prepared *in vitro* by coinfection with two different rotavirus strains exhibited high immunogenicity of VP4 (16, 27, 34). The surface configuration formed by different combinations of VP4 and VP7 might affect their relative contribution to immunogenicity, as suggested from reassortment studies in which the recipient genetic background affected phenotypic properties such as plaque size (1). Characterization of more naturally occurring unusual strains, most of which may be reassortants, would facilitate understanding of this issue. The prevalence in human and animals of strains with the G12 serotype, like the L26 and L27 strains, is being examined by the use of G12-specific neutralizing monoclonal antibodies and by RNA-RNA hybridization.

Nucleotide sequence accession numbers. The L26 VP7 and L27 VP4 gene sequences have been given GenBank accession numbers M36396 and M36397, respectively.

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