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Rotavirus Genome Segment 7 (NSP3) Is a Determinant of Extraintestinal Spread in the Neonatal Mouse

Eric C. Mossel and Robert F. Ramig*

Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas 77030

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We used the neonatal mouse model of rotavirus infection to study extraintestinal spread following oral inoculation. Five-day-old pups were inoculated with either SA11-Cl3, SA11-Cl4, SA11-4F, RRV, or B223. By using virus detection in the liver as a proxy determination for extraintestinal spread, rotavirus strains capable of extraintestinal spread at high frequency (rhesus rotavirus [RRV]) and very low frequency (SA11-Cl4) were identified. Both strains productively infected the gastrointestinal tract. Oral inoculation of mice with RRV/SA11-Cl4 reassortants and determination of virus titers in the gut and liver revealed that the extraintestinal spread phenotype segregated with RRV genome segment 7 to a high level of significance ($P = 10^{-3}$). RRV segment 7 also segregated with the growth of virus in the gut ($P = 10^{-5}$). Although infection of the gut was clearly required for tropism to the liver, there was no correlation between virus titers in the gut and detection of virus in the liver. Five days after intraperitoneal administration to bypass the gut barrier to virus spread, RRV and SA11-Cl4 both were recovered in the liver. However, only RRV was found in the liver following subcutaneous inoculation, suggesting that this peripheral site presented a similar barrier to virus spread as the gut. Sequence analysis of segment 7 from parental RRV and SA11-Cl4 and selected reassortants showed that (i) amino acid differences were distributed throughout the coding sequences and not concentrated in any particular functional motif and (ii) parental sequence was preserved in reassortants. These data support the hypothesis that NSP3, coded for by genome segment 7, plays a significant role in viral growth in the gut and spread to peripheral sites. The mechanism of NSP3-mediated tropism is under investigation.

Rotaviruses (family *Reoviridae*) are the major cause of viral gastroenteritis among children and animals, accounting annually for 450,000 to 650,000 deaths of children worldwide (U. D. Parashar, E. G. Hummelman, J. S. Bresee, M. A. Miller, and R. I. Glass, Abstr. Vaccines Enteric Dis., p. 76, 2001). The virus particle consists of three concentric protein capsids surrounding a genome of 11 double-stranded RNA (dsRNA) segments encoding six structural proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6) (12). Coinfection of cells with group A rotaviruses allows the reassortment of genome segments, a property that has been extensively used in the study of rotaviruses (32)

Rotavirus transmission is fecal-oral. Virions infect the enterocytes of the small intestine, causing cytolytic lesions on intestinal villi. In large animals, this infection can result in villus blunting, atrophy, or fusion and crypt hyperplasia. Sloughing of the enterocytes may expose the basement membrane at the tip of the villus. In contrast, mice show only mild histopathologic changes (8). Although it occurs rarely in immunocompetent children, a growing body of evidence suggests that group A rotavirus infection may spread beyond the intestine with generally unknown clinical consequences. In children, viral antigen and viral RNA have been observed in the central nervous system, liver, and kidney (14, 23). Several cases of afebrile seizures with concomitant rotavirus infection have been described (9, 18), and one report associated group C rotavirus infection with biliary atresia in humans (34). In orally inoculated mice, rotavirus and/or rotavirus antigen has been recovered from the blood, liver, spleen, kidneys, lungs, and mesenteric lymph nodes (5, 10, 20, 35; R. F. Ramig, unpublished data), and a mouse model of rotavirus-induced hepatitis has been described (40).

Genetic studies in vitro and in vivo demonstrated cosegregation of a specific genome segment(s) with specific viral phenotypes, including host range and virulence. Genome segments 3 (encoding VP3), 4 (VP4), 5 (NSP1), 8 (NSP2), 9 (VP7), and 10 (NSP4) have been associated with the virulent phenotype (1, 3, 4, 19, 24). (Segments are numbered as SA11 functional equivalents for consistency.) However, one of these reports dissociated segments 4 and 9 from virulence and host range in mice (4). The disparity in the data may result from the use of different host species and virus strains in these experiments.

A neonatal mouse model of heterologous rotavirus infection was described, characterized, and used extensively to study rotavirus immunology and pathogenesis (25, 31). Despite relatively low growth of heterologous viruses compared to that of murine strains (17), infection of mice with the heterologous viruses SA11-Cl3, RRV, B223, and WC3 demonstrated that certain rotavirus strains could be detected in the liver following oral inoculation (40; Ramig, unpublished), making this model useful for genetic studies of the viral determinants of extraintestinal spread. Here we examine the strain specificity of rotavirus escape from the gut of the neonatal mouse as measured by virus detection in the liver following oral infection. Reassortants were generated between a spread-competent strain of rhesus rotavirus (RRV) and a spread-incompetent strain, SA11-Cl4. Administration of these reassortants to neonatal mice, characterization of their gut escape phenotype, and identification of the parental origin of their genome segments in-

* Corresponding author. Mailing address: Department of Molecular Virology and Microbiology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-4830. Fax: (713) 798-5025. E-mail: rramig@bcm.tmc.edu.

dicated genome segment 7 (encoding NSP3) is the primary determinant for extraintestinal spread in the neonatal mouse.

MATERIALS AND METHODS

Cells and viruses. MA104 African green monkey kidney epithelial cells (BioWhittaker, Walkersville, Md.) were grown in medium 199 (M199) supplemented with 5% fetal bovine serum (FBS) (Gibco/BRL, Gaithersburg, Md.), 0.15% $NaHCO_3$ (Gibco/BRL), 0.03% L-glutamine (Gibco/BRL), 25 U of penicillin per ml, and 250 μg of streptomycin per ml as described previously (13). Twenty-four hours prior to infection, cell monolayers were switched to serum-free medium.

SA11 clone 3 (SA11-Cl3, P5B[2], G3), SA11 clone 4 (SA11-Cl4, P5B[2], G3) (13), SA11 variant 4F (SA11-4F, P6[1],G3) (27), rhesus rotavirus (RRV, P5B[3],G3) (37), and bovine rotavirus B223 (P8[11],G10) (42) from laboratory stocks were triple plaque purified as described previously (7). Each picked plaque was placed in 1 ml of gelatin-saline (1.7% gelatin, 0.8% NaCl, 0.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM boric acid, 0.13 mM sodium borate), sonicated, and rediluted for additional plaque purification or added to MA104 cell monolayers for passage. Triple-plaque-purified viruses were grown to high titer by passage twice in MA104 cells.

Animals. Untimed pregnant CD-1 mice were obtained from Charles River Laboratories (Wilmington, Mass.). Mice were fed standard laboratory chow and water ad libitum. Two days postbirth, pups were fostered to generate uniform litters of 10 to 12 mice. Four days postbirth, serum was collected from dams by tail bleed. At 5 days of age, pups were inoculated either by peroral insertion of a polyethylene catheter (PE-10; Intramedic, Clay Adams, Becton Dickinson, Franklin Lakes, N.J.) into the stomach, intraperitoneal (i.p.) injection, or subcutaneous (s.c.) injection at the back of the neck. Unless noted otherwise, virus-inoculated pups received 2×10^6 PFU of virus in 50 μl of serum-free M199 with 10% blue food coloring (Kroger Foods, Houston, Tex.) added as a tracer. Mock-inoculated animals received uninfected MA104 lysate in M199 and blue food coloring.

Three and five days postinoculation, pups were sacrificed by cervical dislocation. Livers were excised and placed in 1 ml of cold gelatin-saline solution. Next, intestines (from pyloric sphincter to anus) were excised and were placed in 1 ml of gelatin-saline. Excised organs were kept on ice during the procedure and stored at −20°C until analysis.

Tissue analysis. Tissues were thawed at room temperature, homogenized with a tissue homogenizer (Tekmar, Cincinnati, Ohio), and refrozen at −20°C. Homogenates were thawed at room temperature, sonicated, and assayed for replication-competent rotavirus by plaque formation on confluent MA104 cell monolayers as described above. Plaques were counted 24, 48, and 72 h after addition of neutral red agar. Virus titer was determined by averaging duplicate wells for the lowest dilution in which an accurate count could be obtained and multiplication by the appropriate dilution factor and expressed as PFU per milliliter of tissue homogenate. Due to cytotoxicity of concentrated homogenate, the limit of detection of rotavirus in intestinal homogenates is generally 500 PFU/ml, while in the liver, it is usually 50 PFU/ml.

Contribution of individual genome segments to the designated phenotypes was determined by the parametric Wilcoxon rank-sum test and the nonparametric two-sample *t* test as used previously for similar data (36, 38, 39). For Wilcoxon rank-sum analysis, reassortants were arranged in order of the frequency of detection in each tissue. *P* values for each segment were determined for the rank sum of RRV-derived segments compared to that of SA11-Cl4-derived segments. The *t* test analysis *P* values were determined for each segment by comparing the frequency of detection of reassortants containing RRV-derived segments versus that of reassortants containing SA11-Cl4-derived segments.

Generation, analysis, and purification of reassortants. Two-dram glass flat-bottom vials (Wheaton, Millville, N.J.) were seeded with MA104 cells in 1 ml of M199. Monolayers were coinfected with RRV and SA11-Cl4 at multiplicities of infection (MOI) of 5 and 15, 10 and 10, or 15 and 5 PFU/cell in 0.2 ml of serum-free M199 containing 1 μg of trypsin per ml. After 1 h of adsorption at 37°C, 0.8 ml of serum-free M199 was added. After 2 to 3 days, or when cytopathic effect was confluent, vials were placed at −20°C until plaque isolation as described above.

Reassortants were generated in vivo by peroral coadministration of 2×10^6 PFU (each) of RRV and SA11-Cl4 in 50 μl as described above. Intestines were harvested from infected mice 3 and 5 days postinfection (dpi). Progeny virus was plaque purified from intestinal homogenates as described above.

Plaque-purified viruses were passaged once in MA104 cells. The resulting cell lysates were freeze-thawed once, and the parental origin of the dsRNA genome segments (genotype) was determined by electrophoretic mobility on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (electropherotype) as described previously (15). dsRNA was visualized by autoradiography. Labeled parental virus RNA was included in each gel to serve as markers for genotypic scoring. Useful and interesting reassortants were plaque purified twice and then passaged two to three times to high titer in MA104 cells. Following amplification, the genotype was confirmed as described above. Reassortant designations are derived from the first letter of the parental virus contributing the smaller number of gene segments followed by the segment number(s) contributed by that parental virus.

TABLE 1. Frequency of detection of several rotavirus strains following oral inoculation of neonatal CD-1 mice

Virus	No (%) positive/total in[a]:	
	Intestine	Liver
SA11-Cl3	0/16 (0)	0/16 (0)
SA11-Cl4	18/67 (27)	1/67 (1)
SA11-4F	5/23 (22)	1/23 (4)
RRV	101/110 (92)	29/110 (26)
B223	0/8 (0)	0/8 (0)

[a] Data from 3 and 5 dpi are combined, since no significant difference was observed between data obtained at the two time points.

Genome segment 7 cloning and sequence analysis. RNA was isolated from infected MA104 cell lysates. Five hundred microliters of cell lysate with 1% SDS and 0.1 M sodium acetate (NaOAc) (18.5 μl of 3 M NaOAc [pH = 5.2]) was incubated for 15 min at 37°C. Samples were extracted twice with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]). RNA was precipitated from the aqueous phase by the addition of 1/10 volume of 3 M NaOAc (pH 5.2) and 2 volumes of ethanol and incubation at −80°C overnight.

Oligonucleotides (Gibco/BRL) were prepared complementary to the termini of the SA11-4F genome segment 7 sequence determined by Mattion et al. (21) (GenBank accession no. M87502): SA11-RNA7-For (forward, 5′-CCAGGTAC*CTAATACGACTCACTATA***GGCATTTAATGCTTTTCAGTG**-3′; with *Kpn*I site underlined, T7 promoter italic, and segment 7 nucleotides [nt] 1 to 21 in boldface), SA11-RNA7-Rev2 (reverse, 5′-GGTGTCGAC*CCGCGG***CCACATAACG CCCC**-3′; with the *Xba*I site underlined, the *Sac*II site italic, and segment 7 nt 1092 to 1105 in boldface), and SA11-RNA7-Rev3 (reverse, 5′-GGTTCTAGA*C CGCGG***CCACATAACGCCCC**-3′; with the *Hin*cII site underlined, the *Sac*II site italic, and segment 7 nt 1092 to 1105 in boldface). SA11-Cl4-derived sequences were reverse transcribed from primer SA11-RNA7-Rev3 and PCR amplified with primers SA11-RNA7-For and SA11-RNA7-Rev3. RRV sequences were reverse transcribed from primer SA11-RNA7-For and PCR amplified from primers SA11-RNA7-For and SA11-RNA7-Rev2. PCR products were agarose gel purified, digested with the appropriate restriction enzymes, and cloned into pUC19 (Gibco/BRL). Both strands of insert DNA were sequenced (Seqwright, Houston, Tex.) from purified plasmid with standard M13 and internal primers. Internal primers were prepared based on sequences obtained from the M13 primers: SA11-Cl4 forward 5′-AATGAAAATATGCATTCTC-3′ (nt 632 to 650), SA11-Cl4 reverse 5′-GAATCATCCACTTCAAC-3′ (nt 479 to 495), RRV forward 5′-AATGAAAATATGAATTCTC-3′ (632 to 650), and RRV reverse 5′-GAATCATCAACTTCAAC-3′ (nt 479 to 495). Sequences were aligned with DNAMAN (Lynnon BioSoft, Quebec, Canada).

Nucleotide sequence accession number. The sequences of the RRV and SA11-Cl4 NSP3 genes obtained in this study were submitted to GenBank under the following accession numbers: RRV, AY065842; and SA11-Cl4, AY065843.

RESULTS

RRV and SA11-Cl4 display different intestinal escape phenotypes. To determine the virus pair most suitable for the study of extraintestinal rotavirus spread, triple-plaque-purified SA11-Cl3, SA11-Cl4, SA11-4F, RRV, and B223 were orally administered to 5-day-old mice. On days 3 and 5 postinoculation, intestine and liver were harvested, and titers of infectious virus were determined by plaque assay as described in Materials and Methods. Table 1 lists the frequency with which each virus was found in the intestine and liver. Based on these data, RRV and SA11-Cl4 were selected as the strains capable and

TABLE 2. Fraction of SA11-Cl4-derived genome segments among SA11-Cl4 and RRV reassortant viruses

Condition	n^a	MOI (RRV/SA11)	Fraction in genome segment:										
			1	2	3	4	5	6	7	8	9	10	11
In vitro	9	5:15	0.77	0.66	0.88	0.66	0.77	0.66	0.55	0.55	0.55	0.66	0.77
	10	10:10	0.40	0.40	0.60	0.60	0.60	0.60	1.00	0.60	0.50	0.30	0.80
	12	15:5	0.42	0.42	0.67	0.50	0.50	0.25	0.58	0.50	0.42	0.67	0.83
In vivo	49		0.41	0.31	0.69	0.36	0.86	0.22	0.43	0.20	0.18	0.27	0.18
Gombold in vivo[b]	61		0.57	0.57	0.93	0.33	0.97	0.33	0.41	0.51	0.33	0.13	0.02

[a] *n*, no. of reassortants examined.
[b] Reference 16 (day 3 and 4 postinoculation data only).

incapable of extraintestinal spread, respectively, with virus detection in the liver as the proxy for extraintestinal spread. Although RRV was recovered from the livers of 26% of orally inoculated mice, indicating extraintestinal spread is a relatively weak genetic trait, it was reproducibly isolated from the livers of two or three pups in each litter.

Generation of RRV and SA11-Cl4 reassortants. To test the hypothesis that rotavirus spread from the intestine could be mapped to a distinct genome segment or segments, a library of SA11-Cl4 and RRV reassortants was created. Reassortants were generated in vivo by oral coadministration of 2×10^6 PFU of RRV and SA11-Cl4 to two litters of 5-day-old CD-1 mice. Three and 5 dpi, pups were sacrificed, and intestinal homogenates were titrated by plaque assay on MA104 cells. Well-separated plaques were picked and passaged to high titer on MA104 cells. The parental origin of the genome segments was determined by the electrophoretic mobility of ^{32}P-labeled genomic RNA on SDS-PAGE. Of 118 plaques picked for analysis, 15 clones gave no radiolabeled genomic RNA, and two clones contained more than 11 genome segments (indicating mixed plaque picks). Electropherotype assignment of all 11 genome segments was possible for the remaining 101 clones. Of these, 52 clones were of the parental genotype: 35 RRV clones and 17 SA11-Cl4 clones. Forty-nine clones with reassortant genotypes were identified; 40 of them were unique. Four clones were represented twice, while the genotype of SA11-Cl4-derived segment 5, encoding NSP1 (reassortant S5 in Table 3), on a background of RRV was isolated six times.

Reassortant genotypes were analyzed for in vivo selective pressures on reassortment of RRV and SA11-Cl4 (Table 2). The overall strongest selection was for SA11-Cl4-derived genome segment 5; 86% of reassortants contain this segment. The only other favored SA11-Cl4-derived segment was genome segment 3, found in 69% of reassortants. The remaining nine gene segments were derived predominantly (57 to 82%) from the RRV parent, perhaps reflecting the better growth of RRV in the gut (15). The livers of mice coinoculated with RRV and SA11-Cl4 were examined for reassortants with the intestinal escape phenotype. Although virus was recovered from the intestines of each of the 25 coinoculated mice, no virus was recovered from the livers of these animals. Fisher's exact test indicates that extraintestinal spread following inoculation with both RRV and SA11-Cl4 is similar to that in the SA11-Cl4 phenotype ($P = 1$) and different from that in the RRV phenotype ($P = 0.001$).

Reassortants were also generated in vitro by high-multiplicity coinfection of MA104 cell monolayers and subsequent plaque isolation of progeny virus. MA104 cells were infected with RRV and SA11-Cl4 at MOI ratios of 5:15, 10:10, and 15:5. Twenty well-separated plaques were picked from each coinfection, and the parental origin of the genome segments was determined as described above. On genotypic analysis of the 60 plaques picked, 3 were excluded because there was no labeled genomic RNA and 4 were excluded because they contained more than 11 labeled segments. Of the 53 clones for which the genotype was determined, 22 were parental. The remaining 31 had reassortant genotypes.

Despite the relatively low number of in vitro reassortants, several observations were made. With the notable exception of segments 7 and 11, all segments appeared to segregate randomly at equal MOI (Table 2). The strong SA11-Cl4 segment 7 and slight RRV segment 10 biases observed at equal MOI were reduced to near random levels in both unequal MOI infections. However, the SA11-Cl4 segment 11 bias was maintained in all three coinfections. Among the remaining eight segments, there was a trend from RRV bias to SA11-Cl4 bias, because the RRV/SA11-Cl4 MOI ratio changed from 15:5 to 10:10 to 5:15, as would be expected in the absence of selective pressure. Thirty different genotypes were isolated in vitro, and 28 of those were not isolated in vivo.

In addition to the parental virus crosses described above, a number of unique reassortants were created by backcrossing first-generation reassortants to parental viruses or to another reassortant in vitro. In this way, we were able to create different selective pressures in genome segment segregation and selectively generate several single-segment reassortants from double-segment reassortants. For example, reassortant S5,7 was isolated from the intestinal homogenate of a mouse coinoculated with RRV and SA11-Cl4. The single-segment reassortant S7 was generated from the backcross of S5,7 with RRV in MA104 cells.

RRV genome segment 7 (NSP3) is a significant determinant of the intestinal escape phenotype among reassortants. From the panel of reassortants, 16 were selected for oral administration to neonatal mice (Table 3). Eleven of these were single-segment reassortants, including the reciprocal genotype pairs for segments 5, 7, 10, and 11. Four double-segment reassortants were selected for segregation of segments for which there was no single-segment reassortant. A single three-segment reassortant, R4,6,9, was included, because this virus places the RRV-derived genes 4, 6, and 9, which encode a complete RRV middle and outer capsid, on a background of SA11-Cl4. Of

TABLE 3. Frequency of detection in gut and liver of RRV and SA11-Cl4 reassortants following oral inoculation of neonatal mice

Virus (*n*)[a]	% Recovery		Parental origin of genome segments[d] (gene product)										
	Gut[b]	Liver[c]	1 (VP1)	2 (VP2)	3 (VP3)	4 (VP4)	5 (NSP1)	6 (VP6)	7 (NSP3)	8 (NSP2)	9 (VP7)	10 (NSP4)	11 (NSP5)
RRV (110)	92	26	R	R	R	R	R	R	**R**	R	R	R	R
SA11-Cl4 (67)	27	1	S	S	S	S	S	S	**S**	S	S	S	S
S4,5 (35)	100	42	R	R	R	S	S	R	**R**	R	R	R	R
S10 (36)	98	27	R	R	R	R	R	R	**R**	R	R	S	R
R5,7 (69)	78	17	S	S	S	S	R	S	**R**	S	S	S	S
S11 (28)	54	11	R	R	R	R	R	R	**R**	R	R	R	S
R7 (35)	89	9	S	S	S	S	S	S	**R**	S	S	S	S
S3,5 (31)	84	9	R	R	S	R	S	R	**R**	R	R	R	R
S5 (98)	93	6	R	R	R	R	S	R	**R**	R	R	R	R
R5 (55)	45	4	S	S	S	S	R	S	**S**	S	S	S	S
S7 (30)	77	3	R	R	R	R	R	R	**S**	R	R	R	R
R10 (36)	25	2	S	S	S	S	S	S	**S**	S	S	R	S
R11 (32)	28	0	S	S	S	S	S	S	**S**	S	S	S	R
S5,7 (35)	26	0	R	R	R	R	S	R	**S**	R	R	R	R
R4 (30)	23	0	S	S	S	R	S	S	**S**	S	S	S	S
R9 (44)	23	0	S	S	S	S	S	S	**S**	S	R	S	S
R4,6,9 (27)	16	0	S	S	S	R	S	R	**S**	S	R	S	S
R3 (35)	11	0	S	S	R	S	S	S	**S**	S	S	S	S

[a] *n*, number of individual pups inoculated with each virus.
[b] Percentage of animals in which virus was recovered from the intestine 3 and 5 days postinoculation.
[c] Percentage of animals in which virus was recovered from the liver 3 and 5 days postinoculation.
[d] RRV-derived genome segment numbers converted to SA11 functional equivalent. R, segment derived from RRV; S, segment derived from SA11-C14.

note, segments 1, 2, and 8 were always derived from the same parent in each reassortant examined.

Table 3 lists the reassortants in descending order of the frequency at which virus was recovered from the liver 3 and 5 days following oral inoculation. No significant difference was observed between the 3- and 5-day time points for any reassortants, and Table 3 combines the two time points. RRV-derived segment 7, encoding NSP3, is present in each of the seven reassortants found most frequently in the liver, while SA11-Cl4-derived segment 7 is present in each of the remaining nine reassortants. RRV genome segment 7 significantly segregated with the difference in virus spread to the liver (*t* test, $P = 0.001$; rank-sum test, $P = 0.003$). No other segments significantly associate with virus spread to the liver (*t* test and rank-sum test, $P > 0.05$) (Table 4). RRV genome segment 7 also significantly segregated with the frequency of virus detection in the gut at 3 and 5 dpi (*t* test, $P = 10^{-5}$; rank-sum test, $P = 0.001$), although, segments 1, 2, 8, and 11 also segregated with virus detection in the gut (*t* test and rank-sum test, $P \leq 0.05$) to a lower level of significance (Table 5).

Among the reassortants, virus titer recovered from the gut followed a generally decreasing trend with the frequency of recovery from the gut (Table 6). However, for each RRV segment 7-containing reassortant, virus titers in the gut of animals in which virus was recovered from the liver were not different from the virus titers in the gut of animals from which virus was not recovered from the liver (*t* test, $P = 0.1$). In addition, the geometric mean titer in the gut of animals administered reassortants with RRV-derived segment 7 was not different from the geometric mean titer in the gut of animals administered reassortants with SA11-Cl4-derived segment 7 (*t* test, $P = 0.07$). Finally, significant overlap was observed in the ranges of gut virus titers of animals in which virus could be recovered from the liver and the ranges of gut virus titers in animals in which virus could not be recovered from the liver. These data suggest that virus detection in the gut following oral inoculation of neonatal mice is most significantly determined by genome segment 7, but also that growth in the gut very likely has multiple genetic determinants. Detectable virus in the gut appears to be necessary for detection in the liver, because only five animals had detectable virus in the liver with no detectable virus in the gut. However, we detected no correlation between virus titer in the gut and virus detection in the liver.

SA11-Cl4 is found in the liver following i.p. inoculation, but not after s.c. administration. To examine the ability of RRV and SA11-Cl4 to transit to the liver when the intestinal barrier to spread is removed, 5×10^5 PFU in a 50-μl inoculum was administered to neonatal mice either i.p. or s.c. at the back of the neck. Intestine and liver were harvested 3 and 5 days postinoculation. No significant difference in the frequency of detection was seen between the time points (data not shown), and the data were combined.

TABLE 4. Statistical analysis of the contribution of single genome segments to virus detection in the liver

Analysis	*P* for genome segment:										
	1	2	3	4	5	6	7	8	9	10	11
Student's *t* test	0.09	0.09	0.16	0.67	0.32	0.19	0.003	0.09	0.33	0.55	0.24
Wilcoxon rank-sum test	0.08	0.08	0.33	0.96	0.07	0.23	0.001	0.08	0.52	0.35	0.48

TABLE 5. Statistical analysis of the contribution of single genome segments to virus detection in the gut

Analysis	*P* for genome segment:										
	1	2	3	4	5	6	7	8	9	10	11
Student's *t* test	0.01	0.01	0.22	0.53	0.19	0.07	10^{-5}	0.01	0.13	0.34	0.05
Wilcoxon rank-sum test	0.02	0.02	0.20	0.60	0.26	0.08	0.001	0.02	0.19	0.40	0.03

RRV was isolated from both the intestines and livers of all 10 animals inoculated i.p. (Table 7) and was recovered from the livers of 4 of 10 animals at higher than input titer. After s.c. inoculation, RRV was recovered from the intestines of 22% (2 of 9) of animals and from the livers of 66% (6 of 9) of animals. SA11-Cl4 was recovered from both the intestine and liver at high frequency following i.p. inoculation (90 and 100%, respectively), but was not recovered from the intestine or liver of any animals following s.c. inoculation. RRV was generally recovered from the liver in higher titers than SA11-Cl4, but this difference was not significant (*t* test, $P = 0.06$). The fact that both viruses were recovered from the livers from all animals examined up to 5 days after i.p. inoculation suggests that both are capable of replication in the liver when the intestinal barrier to spread is removed. That RRV could be recovered from the liver following s.c. inoculation, but SA11-Cl4 could not, indicates that the barrier to spread from the s.c. inoculation site may be similar to the barrier to spread from the gut after oral inoculation.

We previously showed that B223 was undetectable in the gut and liver by 3 days following oral inoculation (Table 1), and we suspected it would not replicate well following parenteral inoculation. Therefore, it was included as a negative control to ensure that we were not measuring residual inoculum. B223 was recovered from the intestines of two of five animals inoculated i.p. and sacrificed 3 days postinoculation, but from none of the five animals sacrificed 5 days postinoculation. As expected, B223 was not found in the liver by either route of administration at either time point. Because B223 was not recovered from the liver, B223 recovery from the intestine at low frequency is likely due to a low level of virus replication that is cleared by 5 dpi. B223 has been shown to be less stable than SA11-Cl3 and SA11-4F during in vitro manipulation (6). This instability may also contribute to the lack of detection in this experiment.

Analysis of RRV and SA11-Cl4 NSP3 sequences. To determine if differences in the spread phenotype of RRV and SA11-Cl4 could be associated with any identified NSP3 functional domain, the open reading frames of RRV and SA11-Cl4 NSP3 were sequenced. NSP3 from the RRV biological clone used in these experiments was found to differ from the RRV NSP3 sequence published by Rao et al. (33) (GenBank accession no. X81426) by 5 of 312 residues. Our SA11-Cl4 clone differed from the SA11-4F NSP3 sequence published by Mattion et al. (21) (GenBank accession no. M87502) at 1 of 315 residues and from the SA11 NSP3 sequence published by Both et al. (2) (GenBank accession no. X00355) at 3 of 315 residues (Fig. 1). Overall, our RRV NSP3 and SA11-Cl4 NSP3 were found to be 82.5% (260 of 315 residues) identical and 90.8% (286 of 315 residues) similar. Deletion mutants have been used to map several functional and structural domains on NSP3 (28). The RNA-binding domain (amino acids 4 to 149) was 85.5% iden-

TABLE 6. Virus titers in the gut and liver of orally inoculated neonatal mice

Virus	Gut titer (PFU/ml of tissue homogenate) in:				Liver titer (PFU/ml of tissue homogenate)		*P*[c]
	Gut-positive animals		Liver-positive animals				
	Mean[a]	Range[b]	Mean[a]	Range[b]	Mean[a]	Range[b]	
RRV	5,430	500–290,000	9,670	0–285,000	609	5–410,000	0.08
SA11-Cl4	1,121	100–13,000	0	NA[d]	50	NA	NA
S4,5	9,112	500–70,000	10,574	2,500–32,500	599	50–49500	0.58
S10	7,508	1,000–115,000	5,064	0–15,000	674	10–100,000	0.10
R5,7	17,197	300–400,000	77,306	25,000–400,000	530	50–2,000,000	0.22
S11	1,907	500–11,500	1,581	1,000–2500	663	50–21,500	0.44
R7	6,986	1,000–21,500	9,655	7,500–16,000	224	100–750	0.65
S3,5	2,012	500–10,500	1,414	500–4000	71	50–100	0.77
S5	3,147	500–25,500	2,759	500–12000	161	50–500	0.81
R5	12,098	500–10,000	NA	0–70,000	1,936	150–25,000	0.55
S7	2,404	500–17,500	17,500	NA	100	NA	NA
R10	1,162	300–4500	4,500	NA	48,000	NA	NA
R11	1,035	500–4500	NA	NA	NA	NA	NA
S5,7	1,649	1,000–4500	NA	NA	NA	NA	NA
R4	1,140	500–2500	NA	NA	NA	NA	NA
R9	595	200–1500	NA	NA	NA	NA	NA
R4,6,9	500	500–500	NA	NA	NA	NA	NA
R3	783	100–500	NA	NA	NA	NA	NA

[a] Geometric mean titer of virus-positive tissues.
[b] Virus-positive tissues only.
[c] Two-sample *t*-test comparing gut virus titers of animals with virus-positive livers versus gut virus titers of animals with virus-negative livers.
[d] NA, not applicable.

TABLE 7. Rotavirus detection in the gut and liver following parenteral inoculation of neonatal mice

Virus	Route	No. (%) positive/total in:		Mean titer (range) in[a]:	
		Gut	Liver	Gut	Liver
RRV	i.p.	10/10 (100)	10/10 (100)	4.8 ± 0.8 (3.3–5.6)	5.3 ± 1.0 (3.0–6.6)
	s.c.	2/9 (22)	6/9 (66)	2.7 ± 0.0 (2.7–2.7)	2.4 ± 0.5 (1.7–3.1)
SA11-Cl4	i.p.	9/10 (90)	10/10 (100)	3.6 ± 0.5 (2.7–4.2)	3.2 ± 0.6 (1.7–3.9)
	s.c.	0/10	0/10		
B223	i.p.	2/10 (20)	0/10	3.0 ± 0.0 (3.0–3.0)	
	s.c.	0/10	0/10		

[a] Gut and liver virus titers expressed as geometric mean of the log PFU per milliliter of tissue homogenate ± standard deviation calculated from only tissues with a measurable virus titer.

tical and 91.0% similar. The eIF4GI-binding domain (amino acids 206 to 315) was 74.1% identical and 88.0% similar. The proposed dimerization domain (amino acids 150 to 206) and coiled-coiled motif (amino acids 160 to 250) were slightly better conserved, with 91.2 and 89.0% identity and 94.7 and 92.3% similarity, respectively. Thus, sequence comparison of RRV and SA11-Cl4 NSP3 did not reveal differences clustering in defined functional domains, although identity was somewhat lower in the eIF4GI-binding domain.

The NSP3 coding sequences of several reassortants (S4, S5, S11, R3, and R11) were also determined. NSP3 sequence analysis confirmed the parental origins of segment 7 in these reassortants and showed that they were identical to the parental virus from which they were derived. The single exception was reassortant R11, which contained a single nucleotide mutation in NSP3 that resulted in a leucine-to-arginine change at position 297. Finally, the NSP3 sequence of RRV 1010-5L, a clone isolated from the liver of an orally inoculated neonatal mouse, was determined to be identical to that of the RRV inoculum. These results indicate that NSP3 mutants were not selected during reassortant construction and characterization.

DISCUSSION

The present studies confirmed that in the neonatal mouse model of rotavirus infection, there are differences in the ability of RRV and SA11-Cl4 to spread to the liver following oral inoculation. However, when the intestinal barrier to spread was removed by i.p. inoculation, both viruses were recovered from the liver at high frequency. By inoculating mice with

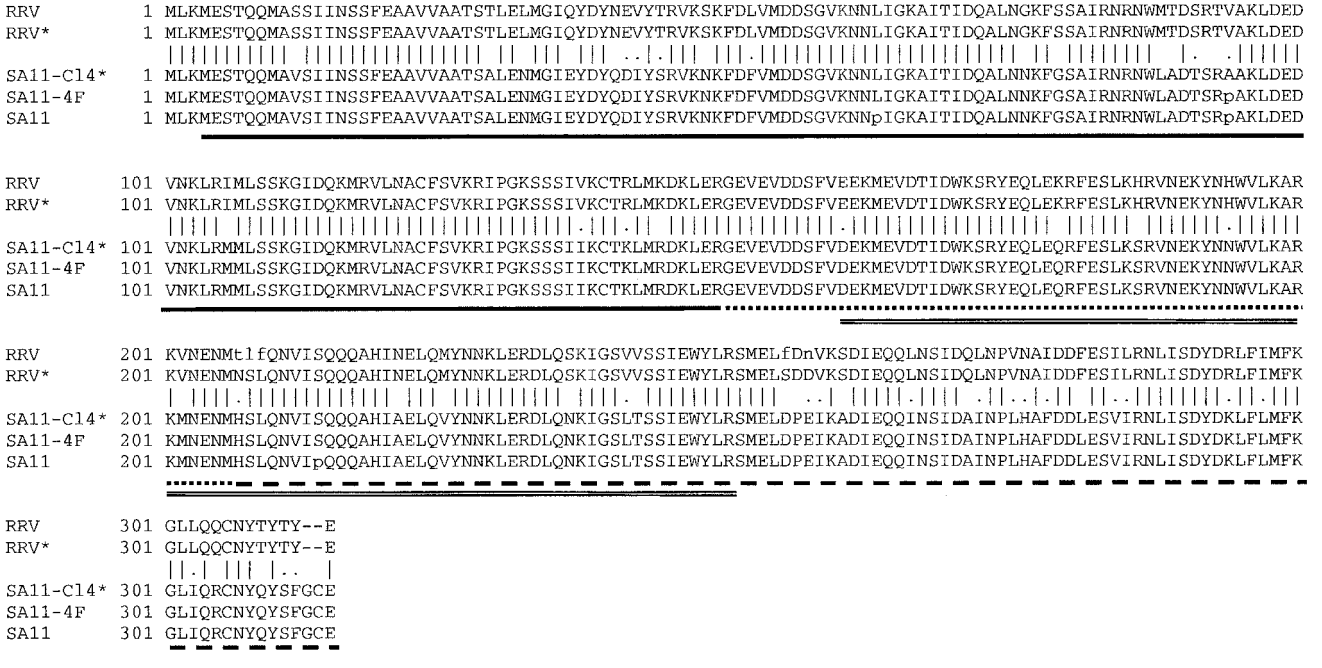

FIG. 1. Alignment of the predicted amino acid sequences for RRV and SA11-Cl4 NSP3 (GenBank accession no. AY065842 and AY065843, respectively). Amino acid identity is noted by vertical lines, and similarity is noted by dots. Dashes indicate gaps in the sequence. Lines beneath the alignment denote NSP3 functional and structural domains: solid line (amino acids [aa] 4 to 149), RNA binding; dotted line (aa 150 to 206), dimerization; dashed line (aa 206 to 315), eIF4G1 binding; and double line (aa 160 to 250), coiled-coil domain. Previously published NSP3 sequences are shown for comparison (GenBank accession numbers: RRV, X81426 [33]; SA11-4F, M87502 [21]; SA11, X00355 [2]). Residues in lowercase differ from those in the sequences observed in this study. NSP3 sequences determined in this study are indicated by asterisks.

RRV and SA11-Cl4 reassortants and screening for virus spread to the liver, we mapped the phenotype to genome segment 7, which encodes NSP3.

It was previously reported that oral inoculation of 1- to 3-day-old SCID and BALB/c pups with 10^5 focus-forming units (FFU) of RRV resulted in virus being recovered from 91 and 71% of the livers examined, respectively (40). The substantially lower percentage reported here might be due to differences in the strain or age of the mice used in this study or differences in the passage history of the biological clone of RRV carried in this laboratory and used in these experiments. In addition, among RRV segment 7-containing reassortants, virus detection in the liver ranged from 6 to 42%. We hypothesize that the reassortants exhibit a frequency range rather than a consistent detection frequency due to differences in the overall fitness of reassortants with different genome constellations relative to each other and to the parental viruses.

We generated SA11-Cl4 and RRV reassortants in vivo and in vitro. Gombold and Ramig (16) previously published an extensive analysis of in vivo RRV and SA11 reassortment. Because our goal was to generate reassortants useful in our pathogenic studies, we did not isolate reassortants in numbers sufficient to draw meaningful statistical conclusions regarding genome segment segregation. However, it is worth noting that our observed in vivo genome segment segregation generally agreed with that of Gombold and Ramig (16) (Table 2), with the possible exception of segments 3 and 8, which may have less SA11 bias than previously observed. In addition, we found it interesting that SA11-Cl4-derived segment 11 was highly selected among in vitro reassortants, while RRV-derived segment 11 was highly selected in vivo. We expected that reassortants capable of intestinal escape might be isolated from the livers of infected animals. Surprisingly, no virus was isolated from the livers of infected animals, suggesting that some feature of the coinfection suppressed extraintestinal spread by a mechanism that is not understood.

The mechanism of virus spread to the liver is not clear. In general, the barriers to extraintestinal spread are the virus crossing the gut barrier, virus transit to the target tissue (including replication in any intermediate tissues or cell types), and virus replication in the target tissue. Inoculation of RRV and SA11-Cl4 i.p. demonstrated that when the gut barrier is removed, both viruses could be recovered from the liver at least 2 days after a control virus was no longer detectable, suggesting that replication in the target tissue is not the limiting step. Despite RRV being recovered from the liver at titers greater than those administered, we do not have direct evidence for virus replication in the liver, and it is possible that replication is not occurring in the liver, but instead that it is a depot for virus produced elsewhere in the mouse. These data also suggest that though these experiments examined only virus spread to the liver, the study of virus spread to the liver represents a proxy for the study of extraintestinal spread. Following s.c. inoculation, RRV was recovered from the liver at high frequency, while SA11-Cl4 was not. Based on this, we postulate a mechanism of spread from the distal s.c. site involving strain-specific transit via the lymphatic system and further hypothesize that this may be similar to the mechanism of strain-specific spread from the intestine.

Data from several reports support a role for the lymphatic system in rotavirus extraintestinal spread. Murine rotavirus antigen was observed 2 to 7 days postinoculation of 14-day-old Swiss-Webster mice in the macrophages of the Peyer's patches and mesenteric and inguinal lymph nodes (5). RRV antigen was not observed extraintestinally in this previous study. In a subsequent report, RRV was found in the Peyer's patches and mesenteric lymph nodes 1 to 10 days after inoculation of adult BALB/c mice with the RRV-based Rotashield vaccine preparation (22). Finally, extraintestinal spread of the closely related reovirus has been shown to occur via transport though the M cells of the intestine to the macrophage-rich subepithelial space. Cell-associated and free reovirus may then spread to the periphery via the lymphatics (26).

The molecular mechanism of the strain specificity of extraintestinal spread is not clear. NSP3 functions in rotavirus replication in a manner analogous to poly(A)-binding protein in eukaryotic cells. NSP3 specifically binds a short sequence (4 to 5 nt) at the 3′ terminus of rotavirus mRNA as well as the eukaryotic translation initiation factor eIF4GI to selectively enhance the translation of rotavirus mRNAs during virus replication (29, 30, 41). In the absence of a reverse genetics system for rotavirus, sequences of RRV-derived NSP3 and SA11-Cl4-derived NSP3 from the parental viruses and several reassortants were examined. Based on published NSP3 sequences, we expected and found that the parental viruses differed significantly. Since segment 7 NSP3 is the only viral determinant of spread to the liver found to be statistically significant in this model, and the efficiency of detection in the liver of reassortants containing RRV-derived segment 7 ranged from 6% to 42%, we hypothesized that the NSP3 sequences of the reassortants isolated might show changes that could account for the increased or decreased efficiency of spread relative to the parental RRV. However, the RRV-derived NSP3 sequences in the reassortants were all identical to that of the parent. The amino acid sequence of our RRV clone differs from the previously published sequence by five residues. The changes at positions 208 and 209 to Ser and Leu render our RRV clone identical at those positions to our SA11-Cl4 clone and to the previously published SA11 NSP3 sequence. Amino acids 208 and 209 are at the 5′ end of the eIF4GI-binding domain of NSP3. It is interesting to speculate whether this additional SA11-like character of RRV NSP3 contributes to the decreased extraintestinal spread of RRV in our model relative to the previously published study of RRV extraintestinal spread.

The scope and significance of the extraintestinal spread of rotavirus infection in humans are not yet clear. Rotavirus antigen has been detected in the livers and kidneys of immunocompromised children (14), and one report associates group C rotaviruses with extrahepatic biliary atresia in infants (34). Case reports have associated rotavirus intestinal infection with neurological disease, and studies also report the detection of rotavirus antigen or RNA in the cerebrospinal fluid of children with seizures or convulsions (9, 18, 23). However, proof of a causal relationship between rotavirus and neurological dysfunction has not been established.

Uhnoo et al. previously recognized the potential for extraintestinal spread of live rotavirus vaccines based on RRV and suggested that special attention be paid to the liver function of vaccinees (40). Although there are no reports of extraintestinal spread to the liver among rotavirus vaccinees, unexplained

intestinal sequelae have been noted (11). Our work focuses on the potential for extraintestinal spread to RNA segment 7 of RRV in a mouse model and reiterates the potential for extraintestinal spread by live rotavirus vaccines. Our work further suggests that extraintestinal spread by live virus vaccines could be attenuated by the inclusion of a genome segment 7 from a virus less capable of extraintestinal spread.

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