JOURNAL OF VIROLOGY, Jan. 1986, p. 110–116
0022-538X/86/010110-07$02.00/0

Vol. 57, No. 1

Analysis of Reassortment of Genome Segments in Mice Mixedly Infected with Rotaviruses SA11 and RRV

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Seven-day-old CD-1 mice born to seronegative dams were orally inoculated with a mixture of wild-type simian rotavirus SA11 and wild-type rhesus rotavirus RRV. At various times postinfection, progeny clones were randomly isolated from intestinal homogenates by limiting dilution. Analysis of genome RNAs by polyacrylamide gel electrophoresis was used to identify and genotype reassortant progeny. Reassortment of genome segments was observed in 252 of 662 (38%) clones analyzed from in vivo mixed infections. Kinetic studies indicated that reassortment was an early event in the in vivo infectious cycle; more than 25% of the progeny clones were reassortant by 12 h postinfection. The frequency of reassortant progeny increased to 80 to 100% by 72 to 96 h postinfection. A few reassortants with specific constellations of SA11 and RRV genome segments were repeatedly isolated from different litters or different animals within single litters, suggesting that these genotypes were independently and specifically selected in vivo. Analysis of segregation of individual genome segments among the 252 reassortant progeny revealed that, although most segments segregated randomly, segments 3 and 5 nonrandomly segregated from the SA11 parent. The possible selective pressures active during in vivo reassortment of rotavirus genome segments are discussed.

Reassortment of genome segments among the progeny of in vitro mixed infections is well documented for viruses with segmented genomes (2, 16, 20). These mixed infections result in the production of reassortant progeny that each derives a subset of its genome segments from each parent.

The potential for reassortment in vivo must also exist if single cells in the infected host become infected with different strains of virus that can exchange genome segments. Indeed, there is indirect evidence that new pandemic influenza viruses may arise by reassortment between human and animal strains or two human strains in some mixedly infected host (1, 4, 29, 31). Direct evidence for in vivo reassortment of influenza virus genome segments is more limited, but reassortment has been demonstrated in vivo by using defined parental viruses and natural conditions of virus transmission (25–28). For other segmented genome viruses the evidence for in vivo reassortment is also indirect (22, 23), with the exception of reovirus, for which in vivo reassortment under controlled laboratory conditions has been reported (30; D. Rubin, personal communication).

Although the ability of rotaviruses to reassort genome segments in vitro is well established (7, 8, 12), the potential for in vivo reassortment has not been assessed. Cocirculation of different virus strains within animal populations (6, 17) and the demonstration of mixedly infected individual animals (19, 21) have been cited as evidence for potential in vivo reassortment of rotavirus strains (3). However, none of these studies has conclusively demonstrated that in vivo mixed infection leads to reassortment of rotavirus genes.

Here we report an analysis of reassortment of genome segments of two well-characterized rotavirus strains that occurred in infected animals. These experiments document that reassortment does indeed occur in vivo and can occur at very high frequency. In addition, the results indicate that reassortment is an early event in the in vivo infectious cycle and suggest that certain reassortant constellations of genome segments may be selected for or against within the primary infected host.

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MATERIALS AND METHODS

Media and buffers. Medium 199 with and without serum, gelatin-saline, nutrient agar, and neutral red agar were prepared as described previously (14).

Viruses and cells. Wild-type simian rotavirus SA11 was obtained from our laboratory collection and has been previously described (7, 14). Wild-type rhesus rotavirus RRV (MMU18006) was obtained from H. Greenberg, Veterans Administration Hospital, Palo Alto, Calif. Both viruses were plaque purified on MA104 cell monolayers and were used at the third passage level after plaque purification. MA104 cells were maintained as monolayers as previously described (14).

Mice. Multiparous, untimed pregnant CD-1 mice were obtained from the Charles River Breeding Laboratories, Inc., Portage, Mich., colony. Randomly selected dams were screened for existing serum antibody to rotavirus at the time of entry into the laboratory by immune precipitation from ^{3}H-amino acid-labeled lysates of SA11-infected cells, followed by electrophoresis of the immunoprecipitates (11). All mice screened were rotavirus negative by this method. Each dam was placed into a sterile MicroIsolator cage (Lab Products), and the cages were kept in a cage ventilation rack (model VR-1; Lab Products) that provided a unidirectional flow of filtered air over the hooded cages. The dams were kept with their litters throughout the course of the experiments and were given food (mouse chow; Ralston Purina) and water ad libitum.

Inoculation of suckling mice. Seven-day-old suckling mice were orally inoculated by the method of Rubin and Fields (18). Briefly, mice were intubated with polyethylene tubing (PE-10; Becton Dickinson Labware, Oxnard, Calif.), and 0.05 ml of medium 199 without serum and containing 1.85×10^{6} PFU each of SA11 and RRV and 10% blue food coloring (Kroger Co., Cincinnati, Ohio) was instilled directly into the stomach. The nonadsorbable food coloring served as a marker for the site of inoculation and passage of the

inoculum through the intestine. Single infections were performed similarly with 3.70×10^6 PFU of virus. Viruses were not trypsin activated before inoculation. At various times postinfection, mice were observed for signs of disease and sacrificed. The combined small and large intestine of each animal was removed into 1.0 ml of gelatin-saline and frozen. When all intestinal samples had been collected, they were thawed and homogenized at 4°C for 10 s with a microprobe homogenizer (Tissuemizer; Tekmar, Cincinnati, Ohio). The intestinal homogenates were then sonicated, and serial dilutions were prepared and titrated on MA104 cell monolayers as previously described (14), except that incubation was at 37°C and neutral red agar was added on day 6 postinfection.

Isolation and characterization of progeny virus clones. Intestinal homogenates were plated on MA104 cell monolayers at dilutions appropriate to yield well-isolated plaques and incubated at 37°C. On day 7 postinfection individual plaques were picked without regard to plaque morphology and passaged to high titer on MA104 cell monolayers. The genotype of each resulting progeny virus clone was determined essentially as described previously (7). Briefly, 0.45 ml of each virus was activated with 7.5 μg of trypsin per ml at 37°C for 30 min and then inoculated onto MA104 cell monolayers in 35-mm-diameter dishes. RNA was labeled by the addition of 1.0 ml of medium 199 without serum and containing 7.5 μg of actinomycin D and 75 μCi of [^{32}P]orthophosphoric acid. After 2 days of incubation at 37°C, the cells were collected and pelleted, the pellet was lysed in 0.5% Nonidet P-40, the lysate was phenol extracted once, and the RNA was ethanol precipitated. The resulting ^{32}P-labeled double-stranded RNAs were subjected to electrophoresis in 8% Laemmli gels (9) in slabs 45 cm long and 0.75 mm thick. The gels were run in a "Poker Face" (Hoefer) apparatus for 24 to 26 h at 350 V. After electrophoresis, gels were dried and exposed to X-ray film, and the origin of each genome segment of each progeny clone was determined by comparison with parental markers run in the same gel.

RESULTS

Viral growth kinetics and disease in vivo. Multiple litters of 7-day-old CD-1 mice, containing 8 to 12 pups per litter, were orally inoculated with SA11, RRV, or a mixture of SA11 and RRV. At various times postinfection, the pups were examined for external signs of diarrhea and overt dehydration and then sacrificed. After dissection of the intestine, the consistency of the feces in the colon, distension of the colon, and passage of the marker dye were noted. Infection with either SA11 or RRV resulted in the onset of diarrhea 24 to 36 h postinfection (hpi) (Fig. 1A and B). Semisolid feces were noted as early as 24 hpi, when the dissected intestine was examined. External signs of diarrhea were typically first noted at 36 to 48 hpi and usually subsided by 72 hpi. However, watery feces were often seen as late as 96 hpi. The onset, severity, and duration of disease in mice infected with the SA11-RRV virus mixture were not significantly different from those in singly infected mice (Fig. 1C).

After dissection and examination, combined individual small and large intestines were homogenized, sonicated, and titrated to determine the content of infectious virus. Both SA11 and RRV exhibited similar growth kinetics in animals. An initial loss of nearly 2 logs in titer during the first 8 h was followed by a slight increase in titer during the subsequent 16 h, albeit not to the level originally used for infection (Fig. 1A and B). SA11 appeared to replicate to higher titer in vivo than did RRV. Titers of both SA11 and RRV gradually decayed to undetectable levels over the following 3 to 5 days. The slight increase in titer seen in RRV-infected mice on days 4 and 5 was observed in only one of four litters of mice examined. The reason for this increase and its significance are unknown.

The kinetics of growth of virus in mice infected with the SA11-RRV mixture was intermediate to that of either SA11 or RRV alone (Fig. 1C). As in the single-virus infections, an initial loss of titer was observed within 8 hpi. However, the increase in titer normally seen by 12 hpi was absent. The decay of virus from the intestine of mixedly infected mice was, in general, similar to that seen in single-virus infections. However, in one litter (L139) the rate of decay was slower and resulted in the presence of appreciable numbers of virus at 96 hpi.

Isolation and screening of progeny virus clones from mixedly infected mice. Intestinal homogenates from mice mixedly infected with SA11 and RRV were inoculated onto MA104 cell monolayers at dilutions to give well-isolated plaques. The plaques picked were passaged to high titer on

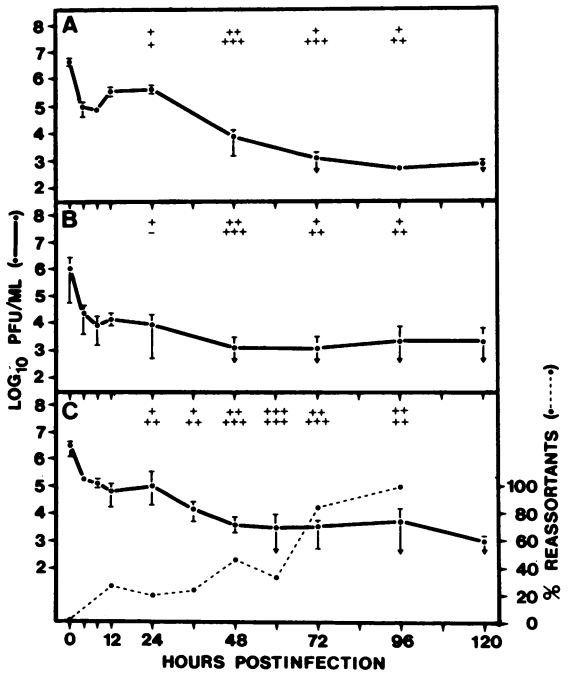

FIG. 1. Kinetics of growth and disease in mice orally inoculated with SA11 (A), RRV (B), or a mixture of SA11 and RRV (C). Seven-day-old mice were inoculated as described in Materials and Methods. At the indicated times postinfection, mice were examined for signs of disease and sacrificed. The range of disease observed is indicated across the top of each panel (+, semisolid feces in the colon; ++, liquid feces in the colon; +++, liquid feces with significant distension of the colon [these animals expelled liquid feces when the abdomen was palpated]; no indication, no visible disease). Homogenates of individual intestines were plated on MA104 cell monolayers to determine the infectious virus contained in combined small and large intestine at each time. Plotted (solid lines) are the mean and range for two (A) or four (B and C) litters of infected mice. Downward arrowheads indicate values below the limit of detection (5×10^2 PFU/ml) in the assays performed. Virus was not detectable in samples taken at 144 and 168 hpi in all litters, and these points are not shown in the figure. The broken line in panel C indicates the mean percentage of reassortant progeny in the yield of mixedly infected mice.

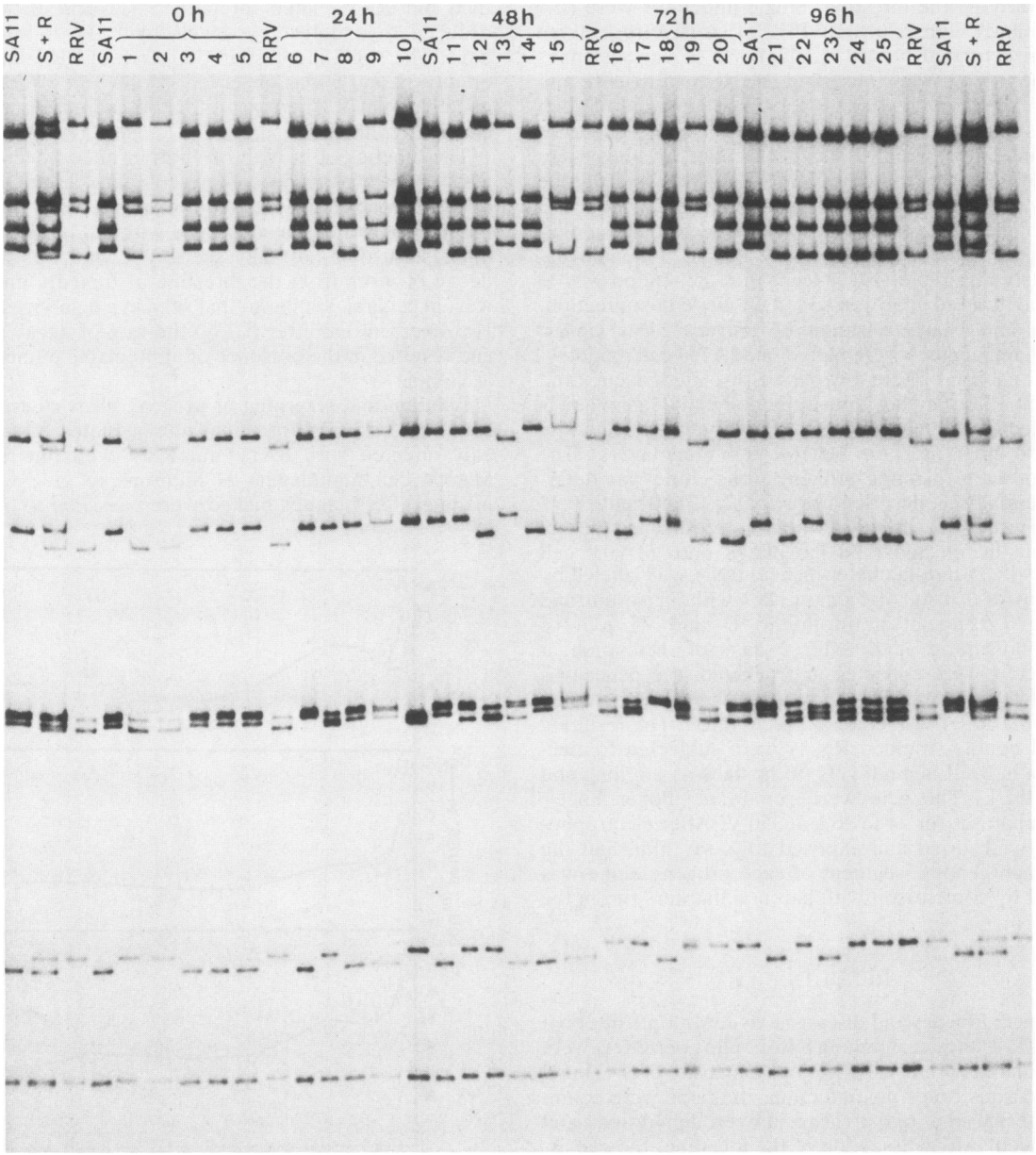

FIG. 2. Electrophoresis of ^{32}P-labeled genome RNAs. The genome RNAs of 25 reassortants and the parental viruses were subjected to electrophoresis as described in Materials and Methods. At each side of the figure, SA11 and RRV were coelectrophoresed to illustrate the magnitude of the mobility differences between the genome segments of the two viruses. The mobility differences for segments 2 and 11 were small but highly reproducible. The numbered lanes contain clones isolated from in vivo mixed infections at the times indicated. Clones 1 through 5 were isolated immediately after inoculation and were parental. Clones 6 through 25 were reassortant, as determined by comparison of segment mobility to the mobility of parental markers. The genome segments of SA11 and RRV correspond in order of electrophoretic mobility except in the region of segments 7 through 9. The seventh segment in the RRV profile is the functional equivalent of SA11 segment 9 as revealed by reassortants 6 and 10. The eighth and ninth RRV segments functionally correspond to SA11 segments 7 and 8, respectively. In this communication, functional equivalents have been used, so that the RRV segment refered to as RRV 9 is actually the seventh segment in the RRV genome profile. Likewise, the eighth and ninth segments of the RRV profile are refered to as RRV 7 and RRV 8, respectively. In the original gel the distance between RRV segments 1 and 11 was 23.5 cm.

MA104 cell monolayers, and the resulting progeny were identified as being of parental or reassortant genotype by polyacrylamide gel electrophoresis of double-stranded RNA (Fig. 2). Of 769 plaques originally picked for analysis, 27 clones yielded mixed genotypes that contained more than 11 segments. These clones were not subcloned and included in the analysis, because segregation of genome segments during subcloning would have occurred in vitro. Another 22 clones did not yield labeled genome segments on screening gels, indicating that they contained either no virus or virus in insufficient titer to yield detectable genotypes. These clones were also excluded from the analysis. Table 1 summarizes the number of parental and reassortant genotypes of the 720 pure clones isolated at various times postinfection from four

TABLE 1. Summary of genotypes of clones derived in vivo from animals mixedly infected with SA11 and RRV

Animal[a]	Time (hpi)	No. of clones examined	No. (%) of clones with genotype:		
			SA11	RRV	Reassortant
L139-A1	0	20	14 (70)	6 (30)	0 (0)
L167-A1	0	20	18 (90)	2 (10)	0 (0)
L177-A1	0	18	17 (94)	1 (6)	0 (0)
L121-A1	12	10	6 (60)	2 (20)	2 (20)
L121-A2	12	10	6 (60)	0 (0)	4 (40)
L139-A2	12	13	3 (23)	2 (15)	8 (62)
L167-A4	12	20	14 (70)	1 (5)	5 (25)
L177-A4	12	18	16 (89)	2 (11)	0 (0)
L121-A3	24	20	13 (65)	3 (15)	4 (20)
L121-A4	24	18	14 (78)	0 (0)	4 (22)
L139-A3	24	8	7 (88)	0 (0)	1 (12)
L139-A4	24	10	9 (90)	0 (0)	1 (10)
L167-A5	24	19	13 (68)	0 (0)	6 (32)
L177-A5	24	19	16 (84)	0 (0)	3 (16)
L121-A5	36	74	51 (69)	2 (3)	21 (28)
L121-A6	36	83	65 (78)	0 (0)	18 (22)
L139-A5	36	9	7 (78)	1 (11)	1 (11)
L139-A6	36	10	8 (80)	0 (0)	2 (20)
L121-A7	48	80	31 (39)	8 (10)	41 (51)
L121-A8	48	83	19 (23)	23 (28)	41 (49)
L139-A7	48	10	3 (30)	1 (10)	6 (60)
L139-A8	48	10	9 (90)	1 (10)	0 (0)
L167-A6	48	16	7 (44)	1 (6)	8 (50)
L177-A6	48	20	16 (80)	0 (0)	4 (20)
L121-A10	60	15	10 (67)	4 (27)	1 (7)
L139-A9	60	9	0 (0)	7 (78)	2 (22)
L139-A10	60	9	0 (0)	1 (11)	8 (89)
L121-A11	72	10	0 (0)	2 (20)	8 (80)
L167-A7	72	19	0 (0)	2 (11)	17 (89)
L177-A7	72	20	0 (0)	4 (20)	16 (80)
L139-A12	96	20	0 (0)	0 (0)	20 (100)
Total[b]	12–96	662	343 (52)	67 (10)	252 (38)

[a] Designation is a combination of the litter number (e.g., L139) and the animal number (e.g., Al).

[b] Clones from 0 hpi were omitted from the total, since reassortment could not yet have occurred.

different litters of mixedly infected mice. Of the 662 clones derived at times when reassortment could have occurred (12 to 96 hpi), 252 reassortants (38%) were identified.

Two observations support the premise that reassortment occurred in the infected animal and not in vitro during plaque formation. First, all progeny isolated from animals sacrificed immediately after inoculation had parental genotypes (0 hpi; Table 1). Second, reconstruction experiments were performed by mixing SA11- and RRV-containing infected intestinal homogenates and using the mixture to pick plaques. Of the 20 clones examined, 8 (40%) were SA11 genotype, 11 (55%) were RRV genotype, and 1 (5%) contained 22 genome segments, indicating that it originated from an SA11-RRV mixed plaque.

The distribution of progeny clones between the two parental genotypes was decidedly unequal (12 to 96 hpi; Table 1); the SA11 genotype represented 52% of the total examined. The bias toward the SA11 parental genotype was most pronounced at early times (12 to 48 hpi) and paralleled the times when SA11 appeared to replicate more than RRV in the singly infected animals (Fig. 1A and B). The RRV parental genotype was more prominent at late times (60 to 72 hpi), when RRV showed a slight rise in titer in single-virus infection. All clones were reassortant at the latest time examined. However, only one animal yielded detectable virus at 96 hpi, making the significance of this observation uncertain.

Significant variation in the isolation rates of parental and reassortant progeny was noted among litters and among animals within single litters (Table 1). For example, animal L139-A2 yielded significantly fewer clones with SA11 parental genotype and significantly more clones with reassortant genotype than did animals from other litters sampled at the same time (12 hpi). Furthermore, animals L139-A7 and L139-A8, both from the same litter and sampled at 48 hpi, yielded quite different virus populations; reassortants predominated in the former, while no reassortants were isolated from the latter.

Kinetics of reassortment in vivo. Although no reassortants could be detected before virus had an opportunity to replicate (0 hpi), by 12 hpi reassortants represented more than 25% of the yield (Table 1; Fig. 1C). At later times the proportion of reassortants varied, but the trend was toward a higher frequency of reassortants with increasing time. These data indicate that reassortment occurred at high frequency early in the in vivo infectious cycle.

Characterization of the reassortant population derived in vivo. The 252 reassortants obtained represented 122 of the 2,048 (2^{11}) genotypes possible for reassortment of two rotaviruses. The majority of the genome segment constellations observed were isolated only once (85 genotypes) or twice (21 genotypes; data not shown). The 16 genome segment constellations isolated three or more times are listed in Table 2. Five of these constellations were isolated from only a single animal. It is impossible to determine whether these large groups of identical genotypes resulted from some selective advantage conferred by the constellation of genome segments or whether sisters of an early reassortment event were simply picked. In contrast, some genotypes were isolated from more than one animal from one to three different litters (e.g., the 4-, 6-, 12-, and 23-member groups). In these cases, it appears that a particular constellation of genome segments may have conferred a selective advantage, since the same genotype was selected independently several times. In general, the favored genotypes were isolated late in the in vivo infectious cycle, suggesting that selection had played a role in the enrichment of those genotypes.

Overall, the segregation of individual genome segments among the reassortants was strikingly random, with 54.5 and 45.5% of the segments being derived from SA11 and RRV, respectively (Table 3). However, significant variation among litters and among animals within litters was noted in the parental derivation of genome segments. For example, reassortants from animal L121-A7 had a preponderance of SA11 segments, while reassortants from animal L121-A8 contained mostly RRV segments. The RRV parental genotype was isolated from animal L121-A8 three times more frequently than from animal L121-A7 (Table 1), which may explain the difference in parental origin of segments among reassortants isolated from these animals.

Although the segregation of many genome segments among the reassortant progeny was nearly random, segments 3 and 5 nonrandomly segregated from SA11 in 85 and 92% of the reassortants, respectively, and segments 9, 10, and 11 segregated preferentially from RRV (Table 3). The preference for SA11 segments 3 and 5 was seen in reassortants isolated at every time and in every animal examined. When segments 3 and 5 were examined as a pair, SA11-SA11

TABLE 2. Genotypes of SA11-RRV reassortants isolated more than twice[a]

No. of times genotype isolated	Parental origin of segment[b]											No. of different isolations from:		Time of isolation (hpi)[e]
	1	2	3	4	5	6	7	8	9	10	11	Litters[c]	Animals[d]	
3	S	S	S	R	S	S	S	S	S	S	S	1	2	24,48
	S	S	S	S	S	S	S	R	S	S	S	2	2	36
	R	R	S	S	S	S	S	S	S	S	S	2	3	12,24,36
	S	S	S	R	S	S	R	S	R	S	S	1	1	60
4	S	S	S	S	S	S	R	S	S	S	S	1	3	24,36
	S	S	S	S	S	S	S	S	S	R	S	2	3	24,36
	R	R	R	R	S	R	R	R	R	R	S	2	2	12,48
	S	S	S	S	S	S	S	S	S	S	R	2	2	24,36
6	S	R	S	S	S	S	S	S	S	S	S	2	3	36,48
7	S	R	S	S	S	S	R	S	R	R	R	1	1	48
10	R	R	S	R	S	R	R	S	R	R	R	1	1	72
12	R	R	R	R	S	R	R	R	R	R	R	2	3	36,48,72
	S	S	S	S	S	S	S	S	R	S	S	3	5	24,36,48
13	S	S	S	R	S	R	S	R	S	R	R	1	1	96
14	S	S	S	S	S	S	R	S	R	R	R	1	1	72
23	R	R	S	R	S	R	R	R	R	R	R	1	2	48,72

[a] In addition to the geneotypes shown here, 85 different genotypes were isolated once each and 21 different genotypes were isolated twice each.
[b] Symbols: S, SA11; R, RRV. RRV genome segments are numbered according to their SA11 cognate segment.
[c] Number of different litters from which genotype was isolated.
[d] Number of different animals from which genotype was isolated.
[e] Times of infection from which indicated genotype was isolated.

and RRV-SA11 origin was seen in 80 and 12% of the reassortants, respectively. Only 8% of the reassortants derived segment 5 from RRV, regardless of the origin of segment 3. This observation suggests that genome segment constellations containing RRV segments 3 and 5 were selected against in vivo. It is interesting to note that, among the favored genotypes (Table 2), segments 3 and 5 of RRV were underrepresented and not represented, respectively, whereas the remaining nine segments were of relatively random parental origin. The basis of this nonrandom segregation, and possible selection, of genome segments in vivo is unknown.

DISCUSSION

This examination of in vivo reassortment extends our ongoing studies of the genetic interactions between rotaviruses. The results presented here clearly demonstrate (i) that in vivo mixed infection with two distinct rotaviruses results in a high frequency of reassortment of genome segments, and (ii) that reassortment occurs early in the in vivo infectious cycle. In addition, some genome segments were found to reassort in a distinctly nonrandom manner in the population of in vivo reassortants examined, and certain constellations of genome segments were repeatedly isolated from independently infected animals. However, owing to small sample size and the unknown nature of any selective pressures active in vivo, any conclusions about selection must be speculative.

Both SA11 and RRV replicate in the infant mouse host, with no significant differences noted in the kinetics of replication or the disease induced. The peak of replication observed at 12 to 24 hpi was consistently lower than the input virus dose. However, this is a feature of the high virus doses that were used to promote mixed infection of enterocytes. When SA11 was administered to 7-day-old mice at doses of 10^2 to 10^3 PFU, replication to titers above the input level was noted in the intestine at 12 to 36 hpi (R. F. Ramig, unpublished data). The kinetics and disease observed with SA11 and RRV were similar to those previously reported for SA11 in suckling mice (13).

The finding of a significant frequency of reassortant progeny (>25%) early in the in vivo infectious cycle was similar to the early reassortment noted for mixed infection of rotavirus temperature-sensitive mutants in vitro. However, two differences between in vivo and in vitro reassortment were noted. (i) Significant numbers of reassortants were detected at 12 hpi in vivo whereas the first significant reassortants were detected at 16 hpi in vitro (15). This slight difference in kinetics may simply reflect the adaptation of rotaviruses for replication in enterocytes compared with their more restricted growth in cultured cells, or the lower temperature (31°C) at which the in vitro kinetic experiments were performed. (ii) The frequency of reassortant progeny in vivo increased throughout the infectious cycle, whereas maximal frequencies of reassortants were noted at the earliest time in standard in vitro crosses performed at high multiplicity of infection (15). This observation suggests that either multiple cycles of replication and mating occur in vivo or that certain genotypes have selective advantage in vivo, or both. In any event, the early kinetics of reassortment in vivo would allow sufficient time for postulated selective forces to act on the pool of reassortant progeny, resulting in selection of favored genotypes.

The frequency of reassortant genotypes was high (38%) among the progeny of mixed infection in vivo. This frequency of reassortants observed was probably a low estimate of the overall reassortment frequency, since the majority of reassortants were isolated at early times, when the frequency tended to be low. However, this frequency was significantly higher than the 10% reported for mice mixedly infected with reovirus types 1 and 3 (30). The high frequency of reassortment in vivo with rotaviruses suggests that in vivo mixed infection may yield more efficient reassortment than in vitro mixed infection of two wild-type viruses. Thus, in vivo mixed infections may be a useful tool when collections of rotavirus reassortants are generated for use in other studies.

The 252 reassortant progeny isolated from in vivo mixed infection represented 122 of the 2,048 constellations of genome segments possible from mixed infection of two

TABLE 3. Segregation ratios of SA11/RRV reassortant clones derived in vivo[a]

Animal	Time (hpi)	Segregation ratio of indicated segment (SA11/RRV)										
		1	2	3	4	5	6	7	8	9	10	11
L121-A1	12	1/1	2/0	2/0	1/1	2/0	2/0	1/1	1/1	1/1	2/0	0/2
L121-A2	12	1/3	3/1	3/1	2/2	2/2	2/2	1/3	3/1	2/2	2/2	2/2
L139-A2	12	2/6	1/7	4/4	1/7	6/2	2/6	3/5	2/6	4/4	2/6	5/3
L167-A4	12	3/2	4/1	5/0	3/2	4/1	4/1	4/1	4/1	0/5	3/2	4/1
L121-A3	24	4/0	3/1	4/0	4/0	3/1	3/1	1/3	4/0	3/1	3/1	3/1
L121-A4	24	4/0	4/0	4/0	2/2	4/0	4/0	4/0	4/0	2/2	4/0	4/0
L139-A3	24	1/0	1/0	1/0	1/0	1/0	1/0	1/0	0/1	1/0	1/0	1/0
L139-A4	24	1/0	1/0	1/0	1/0	1/0	0/1	1/0	1/0	1/0	0/1	1/0
L167-A5	24	4/2	3/3	5/1	4/2	5/1	4/2	6/0	4/2	6/0	4/2	4/2
L177-A5	24	2/1	2/1	3/0	1/2	3/0	3/0	2/1	2/1	3/0	1/2	2/1
L121-A5	36	21/0	17/4	19/2	16/5	19/2	18/3	11/10	15/6	12/9	15/6	13/8
L121-A6	36	12/6	13/5	16/2	15/3	18/0	15/3	14/4	11/7	13/5	11/7	12/6
L139-A5	36	0/1	1/0	1/0	0/1	0/1	0/1	0/1	0/1	0/1	1/0	0/1
L139-A6	36	2/0	2/0	2/0	2/0	2/0	2/0	1/1	1/1	1/1	1/1	0/2
L121-A7	48	28/13	21/20	36/5	31/10	36/5	27/14	26/15	26/15	12/29	23/18	26/15
L121-A8	48	3/38	3/38	30/11	4/37	40/1	4/37	0/41	1/40	1/40	2/39	9/32
L139-A7	48	5/1	3/3	5/1	4/2	6/0	4/2	4/2	4/2	3/3	3/3	5/1
L167-A6	48	5/3	4/4	7/1	6/2	7/1	7/1	6/2	7/1	3/5	6/2	5/3
L177-A6	48	0/4	0/4	1/3	0/4	4/0	0/4	3/1	3/1	3/1	1/3	2/2
L121-A10	60	0/1	0/1	1/0	1/0	0/1	1/0	0/1	0/1	0/1	0/1	0/1
L139-A9	60	0/2	1/1	0/2	0/2	2/0	2/0	2/0	0/2	0/2	0/2	2/0
L139-A10	60	5/3	4/4	7/1	1/7	7/1	7/1	2/6	5/3	2/6	5/3	5/3
L121-A11	72	1/7	3/5	8/0	6/2	7/1	3/5	3/5	3/5	3/5	3/5	1/7
L167-A7	72	14/3	14/3	14/3	14/3	17/0	14/3	0/17	14/3	0/17	0/17	0/17
L177-A7	72	0/16	0/16	15/1	0/16	15/1	0/16	4/12	12/4	2/14	3/13	0/16
L139-A12	96	20/0	18/2	20/0	0/20	20/0	3/17	18/2	2/18	15/5	2/18	0/20
Total fraction SA11		0.55	0.51	0.85	0.48	0.92	0.52	0.47	0.52	0.37	0.39	0.42

[a] For definitions, see Table 1 footnotes.

rotaviruses. Although this sample seemed too small for meaningful statistical analysis, certain points regarding the segregation of genome segments were evident. (i) A number of genotypes were repeatedly isolated from independently infected animals (Table 2). Furthermore, these genotypes tended to be isolated at late times in the in vivo replicative cycle. These observations suggest that some form of selection was taking place in vivo. (ii) The segregation of individual genome segments in vivo showed a decidedly nonrandom segregation of segments 3 and 5 from the SA11 parent (Table 3). This bias against RRV segments 3 and 5 was not due to the presence of reassortants containing only SA11 segments 3 and 5 as the largest class of repeatedly isolated genotypes (Table 2), since similar bias was seen when this large group was omitted from the analysis (data not shown). A similar nonrandom segregation of segments 3 and 5 has been observed in in vitro crosses of SA11 and RRV temperature-sensitive mutants in which segments 3 and 5 were not subject to selection (unpublished data). Since nonrandom segregation of segments 3 and 5 has been observed both in vivo and in vitro, this segregation cannot reliably be ascribed to events occurring during in vivo infection. (iii) The segregation of genome segments 9, 10, and 11 showed a slight preference for RRV as the parent of origin. However, owing to small sample size the significance of this bias was not clear.

Similar nonrandom segregation of genome segments in vivo has been observed by others. Wenske et al. (30) reported that 121 reassortants isolated from mice mixedly infected with reovirus types 1 and 3 fell into only five different genotypes, with two genotypes predominating. Rubin (personal communication) reported similar results. The randomness of segregation in the more extensive in vivo studies of reassortment in influenza viruses is difficult to assess. Most of these studies have made use of parental viruses with widely different capacity for growth in the model host or used immune hosts or in vitro immune selection of the progeny of the in vivo mixed infection (25–28). One in vitro study of influenza reassortment has shown nonrandom segregation (10).

It is important to note that in this study we have not assumed that reassortant viruses were derived from a single infectious particle and not an aggregate of viruses. Controls isolated from animals immediately after mixed infection, or reconstructions of mixed intestinal homogenates from heterologously infected animals, failed to yield reassortant progeny. This indicates that reassortants were not derived by segregation from viral aggregates during in vitro plaque formation.

We have speculated that selection is responsible for the nonrandom segregation of genome segments observed in vivo. The basis for this selection is unknown. It seems unlikely that immune selection was operative, since these experiments were performed in pups born to seronegative dams. It is possible that our method of screening immune status was insensitive. However, any immunity to rotavi-

ruses should have been active on both SA11 and RRV, since both are serotype 3, subgroup 1 viruses (5). Furthermore, the genome segments strongly selected for or against (segments 3 and 5) are not known to encode antigens involved in important immune reactions (5). An alternative to immune selection is selection for genome constellations more favorable than the parental constellations for replication in vivo. This selection would be analogous to the selection of segment 4 from the cultivatable parent during in vitro rescue of noncultivatable rotaviruses (8). Given the immune status of the mice, this alternative seems more likely.

Finally, it is significant to note that demonstration of efficient reassortment in vivo may affect strategies for rotavirus immunization. Live, attenuated vaccines such as those currently in trial (24) presumably have the ability to reassort with field strains of the virus, possibly leading to rescue of virulence. In addition, homologous or heterologous immunity, or both, provided by vaccination may impose important selective pressures in an individual infected with two different strains of field virus. Studies of the effects of homologous and heterologous immunity on in vivo reassortment of rotavirus genome segments are in progress.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 16687 from the National Institutes of Health. J.L.G. was supported by National Research Service Award CA 09197, and R.F.R. is the recipient of National Institutes of Health Research Career Development Award AI 00433.

We thank Kathryn Langly for technical assistance and B. N. Fields and D. H. Rubin for making available their results before publication.

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