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Genetics

Phenotypes of rotavirus reassortants depend upon the recipient genetic background

(segregation analysis/phenotypic expression)

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ABSTRACT **We have previously characterized the biological and immunological properties of a simian rotavirus SA11 variant (4F) with an altered genome segment 4. The SA11-4F variant formed large plaques in the presence of protease, formed small clear plaques in the absence of protease, and grew to high titer in the presence of protease when compared to our standard wild type (SA11 clone 3). To determine the genome segment of the rotavirus SA11 variant 4F that encoded the unique protease-associated phenotypes of the variant, reassortants were generated that segregated the outer capsid genes of 4F onto a genetic background derived from either the bovine rotavirus B223 or our standard SA11 wild type (clone 3), both of which have contrasting protease-associated phenotypes. The parental and reassortant viruses were examined to determine which genes from the 4F variant encoded the ability (*i*) to form large plaques in the presence of protease, (*ii*) to form small clear plaques in the absence of exogenous protease, and (*iii*) to grow to significantly higher titer in the presence of protease. These phenotypes could be transferred to a clone 3 genetic background by a single genome segment from the 4F variant segment 4. However, in the 4F/B223 reassortants a different and unexpected situation was found. On a B223 genetic background the same phenotypes segregated with a combination of a minimum of two 4F genome segments, segments 4 and 9. These results indicate that the recipient genetic background onto which the genes of a donor rotavirus are reassorted can affect the phenotypes conferred by the presence of the donor segments. Thus, the results of segregation mapping experiments using reassortant viruses should be interpreted with caution.**

Rotaviruses are recognized as a leading cause of infantile gastroenteritis infecting most avian and mammalian species (1). Because of the significance and prevalence of rotavirus infection, considerable effort is being directed toward the development of a vaccine (2).

Rotaviruses are members of the *Reoviridae* and contain a genome of 11 segments of double-stranded RNA enclosed in a nonenveloped icosahedron with two concentric protein coats. The outer capsid of the serotype 3 prototype virus SA11 contains two structural proteins: VP4 and VP7, the products of genome segments 4 and 9, respectively. The viral hemagglutinin, VP4, is an 88-kDa protein that is associated with a number of properties including neutralization (3), protease-cleavage enhancement of viral infectivity (4), and virulence (5). The outer capsid glycoprotein, VP7, has a molecular mass of ≈37 kDa and is associated with neutralization and hemagglutination inhibition (6) and viral attachment to the host cell (7) and is synthesized in two forms in the infected cell (8). An understanding of the functions of these outer capsid proteins at the molecular level is clearly important.

An interesting naturally occurring variant of SA11, called 4F, was isolated by Pereira *et al.* (9) and is characterized most prominently by a rapid electrophoretic migration of genome segment 4. We reported (10) a phenotypic characterization of the 4F variant and found that it differs from our standard laboratory wild-type strain of SA11 (clone 3) in a large number of phenotypes. Among the phenotypes of the 4F variant that differed significantly from clone 3 were (*i*) formation of very large plaques in the presence of protease, (*ii*) the ability to form small clear plaques in the absence of exogenous protease, and (*iii*) the ability to grow to significantly higher titer in the presence of protease. The prior association of protease effects with the cleavage of VP4, the product of genome segment 4 (4), suggested that these unique properties of the 4F variant were a consequence of its unique genome segment 4.

We generated reassortants between the 4F variant and second parents that differed with respect to the protease phenotypes to confirm that genome segment 4 was indeed responsible for the unique properties of 4F. Here, we report the results of these reassortment studies and the finding that the genetic background onto which the genes of the 4F variant were reassorted affected the phenotypes conferred by the presence of the 4F genome segments.

MATERIALS AND METHODS

Viruses and Cells. Our standard laboratory wild-type SA11 (SA11 clone 3) was isolated as described (11) from an SA11 stock obtained from H. Malherbe (Gull Laboratories, Salt Lake City). The SA11 variant 4F (SA11-4F) was isolated by Pereira *et al.* (9) from an SA11 preparation originally obtained from G. W. Gary (Centers for Disease Control, Atlanta). The bovine rotavirus B223 was isolated and adapted to culture by Woode *et al.* (12). All viruses were plaque-purified three times in Houston and used for these studies within three passages of final plaque purification. MA104 fetal monkey kidney cells (M.A. Bioproducts), grown in medium 199, were used for all plaque assays and for propagating virus as described (13).

Construction and Characterization of Reassortants. Reassortants were constructed using the SA11-4F variant and the bovine B223 virus as parents. Crosses were performed as described (13), and the yields of the crosses were plated at limiting dilution. Plaques were randomly picked and grown to high titer, the genome RNA was labeled with ^{32}P, and the genotypes of the progeny clones were determined by electrophoresis as described (14). Reassortants with desired genotypes were additionally plaque-purified three times and grown to high titer, and the genotypes were confirmed by electrophoresis prior to use in these experiments. In these studies, we concentrated on reassortants that contained the

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outer capsid genes 4 and 9 of the 4F variant on a genetic background of segments derived from B223. Multiple, independent isolations of each reassortant genotype were made from independent crosses.

A second reassortant genotype was constructed using the R-144 reassortant derived from the cross of the 4F variant and B223 as one parent and SA11 clone 3 as the second parent. This cross allowed us to move genome segment 4 of the 4F variant onto a genetic background derived from our standard SA11 wild-type clone 3. These reassortants were constructed as above except that the yield of the cross was treated with the anti-VP4 monoclonal antibody 9F6 (15) that fails to neutralize the 4F variant but does neutralize clone 3 (unpublished data). Multiple, independent isolations of this reassortant genotype were also made.

Plaque Assays and One-Step Growth Curves. These assays were performed as described (10, 13).

RESULTS

Construction and Characterization of Reassortants. To determine if the unique phenotypes identified for the 4F variant were encoded in genome segment 4, we undertook the reassortment of the genome segments of 4F onto the genetic background of a second parent with contrasting phenotypes. The virus chosen as the second parent was the bovine strain B223 that, in contrast to the 4F variant, forms small plaques in the presence of protease, does not form plaques in the absence of exogenous protease, and grows to a lower relative titer in the presence of protease. We concentrated on reassorting the segments of 4F encoding the outer capsid proteins (segments 4 and 9) onto the foreign genetic background since the outer capsid protein VP4 had been associated with protease effects of SA11 (4). The quantitative phenotypes of the 4F variant and B223 parental viruses are shown in Table 1 and Figs. 1 and 2.

So that the effect of the unique segment 4 of the 4F variant could be examined on a familiar genetic background, a second reassortant genotype was constructed by crossing the R-144 reassortant with SA11 clone 3 (our standard, well-characterized wild-type clone). Reassortants containing segment 4 from the 4F variant and the remaining segments from clone 3 were selected after treatment of the yield of the cross with a monoclonal anti-VP4 antibody (9F6) that neutralized clone 3 but not the 4F variant (data not shown). A two-step reassortment procedure was necessary to isolate this reassortant genotype because only segment 4 of the 4F variant and clone 3 were sufficiently different in gels to allow reliable determination of parental origin. As clone 3 resembles B223 in its protease-related phenotypes (Table 1 and Figs. 1 and 2), this reassortant genotype (which we call "reconstructed 4F") would provide independent confirmation of any phenotype seen to segregate with segment 4.

The "electropherotypes" of the 4F variant, B223, clone 3, and reassortants derived from these viruses are shown in Fig. 1. The genotypes of the reassortants, determined from the electropherotypes, are summarized in Table 1.

Protease-Dependent Plaque Size. The parental viruses and the reassortants were plated at dilutions yielding well-isolated plaques in the presence of the normal concentration of pancreatin (0.136 international trypsin units/ml). After development of the plaques, the plates were overlaid with neutral red, and plaque size was measured. In each case the plaque sizes of the parental viruses were distinct, and the ranges of the two plaque sizes did not overlap (Table 1). Among the 4F/B223 reassortants, only those reassortants containing both segments 4 and 9 from the 4F variant made large plaques like the 4F variant parent (Table 1). 4F/B223 reassortants that contained only segment 4 or segment 9 from the 4F variant parent made plaques slightly larger than the B223 parent, but the range of sizes overlapped with that of B223 and did not overlap with the size range of the 4F parent. The small number of plaques analyzed has not allowed a definitive demonstration of an intermediate plaque size for these reassortants. Although the "reconstructed 4F" reassortants were derived from two small-plaque parents (R-144 and clone 3), the plaques made by those reassortants were even larger than those produced by the 4F variant (Table 1).

Protease-Dependent Plaque Formation. Serial dilutions of stocks of the parental and reassortant viruses were made and plated either in the presence of normal protease concentrations or in the absence of exogenous protease. After development of the plaques (5 days in the presence and 9 days in the absence of protease), the titers in the respective conditions were determined (Table 1). The relative growth of each virus was measured as the efficiency of plating (EOP = titer

Table 1. Genotypes of reassortant clones and segregation of protease-dependent plaque size and protease-dependent plaque formation

Virus	Parental origin of segment											Protease-dependent plaque size		Protease-dependent plaque formation, titer		
	1	2	3	4	5	6	7	8	9	10	11	No. measured	Plaque size, mm	− protease	+ protease	EOP (−/+)
SA11-4F	F	F	F	F	F	F	F	F	F	F	F	16	4.7 ± 1.1	7.3×10^5	1.4×10^8	2.7×10^{-3}
B223	B	B	B	B	B	B	B	B	B	B	B	16	1.8 ± 0.4	$<1.0 \times 10^3$	8.5×10^7	$<1.2 \times 10^{-5}$
R-141	B	B	B	F	B	B	B	B	F	B	B	16	4.9 ± 0.7	8.0×10^5	1.4×10^8	5.7×10^{-3}
R-21	B	B	B	F	B	B	B	B	F	B	B	16	4.4 ± 1.0	1.8×10^5	2.5×10^7	7.2×10^{-3}
R-144	B	B	B	F	B	B	B	B	B	B	B	16	2.8 ± 0.8	$<1.0 \times 10^3$	2.0×10^8	$<5.0 \times 10^{-6}$
R-197	B	B	B	F	B	B	B	B	B	B	B	16	2.3 ± 0.5	$<1.0 \times 10^3$	1.3×10^8	$<8.0 \times 10^{-6}$
R-284	B	B	B	F	B	B	B	B	B	B	B	16	2.1 ± 0.5	$<1.0 \times 10^3$	2.9×10^7	$<3.5 \times 10^{-5}$
R-76	B	B	B	B	B	B	B	B	F	B	B	16	2.2 ± 0.5	$<1.0 \times 10^3$	4.0×10^7	$<2.5 \times 10^{-5}$
R-198	B	B	B	B	B	B	B	B	F	B	B	16	2.1 ± 0.4	$<1.0 \times 10^3$	1.2×10^7	$<8.7 \times 10^{-5}$
SA11-cl3	S	S	S	S	S	S	S	S	S	S	S	16	1.6 ± 0.4	$<1.0 \times 10^3$	1.6×10^8	$<6.3 \times 10^{-6}$
R-23(A^+)	S	S	S	F	S	S	S	S	S	S	S	16	5.2 ± 1.0	2.7×10^5	$1.9 \times 10^{7*}$	1.4×10^{-2}
R-29(A^+)	S	S	S	F	S	S	S	S	S	S	S	16	5.1 ± 1.0	2.0×10^5	1.3×10^7	1.5×10^{-2}
R-33(A^+)	S	S	S	F	S	S	S	S	S	S	S	5	5.7 ± 1.4	8.0×10^5	5.6×10^7	1.4×10^{-2}

Each reassortant virus listed was isolated from an independent cross. The parental origin of reassortant genome segments was determined by electrophoresis. F, B, and S indicate origin from the SA11-4F variant, B223, and SA11 clone 3 wild type (SA11-cl3), respectively. For plaque size determination, plaques were formed in the presence of a normal concentration of protease, stained with neutral red, measured 5 days after infection, and reported as mean ± SD. To determine protease-dependent plaque formation, plaques were formed either in the absence of exogenous protease (−) or in the presence of a normal concentration of protease (+) and counted 5 days (for +) or 9 days (for −) after infection. EOP is the efficiency of plaque formation (EOP = titer without protease/titer with protease).

*Lower titers reflect the use of first passage reassortants as compared to second passage parental strains.

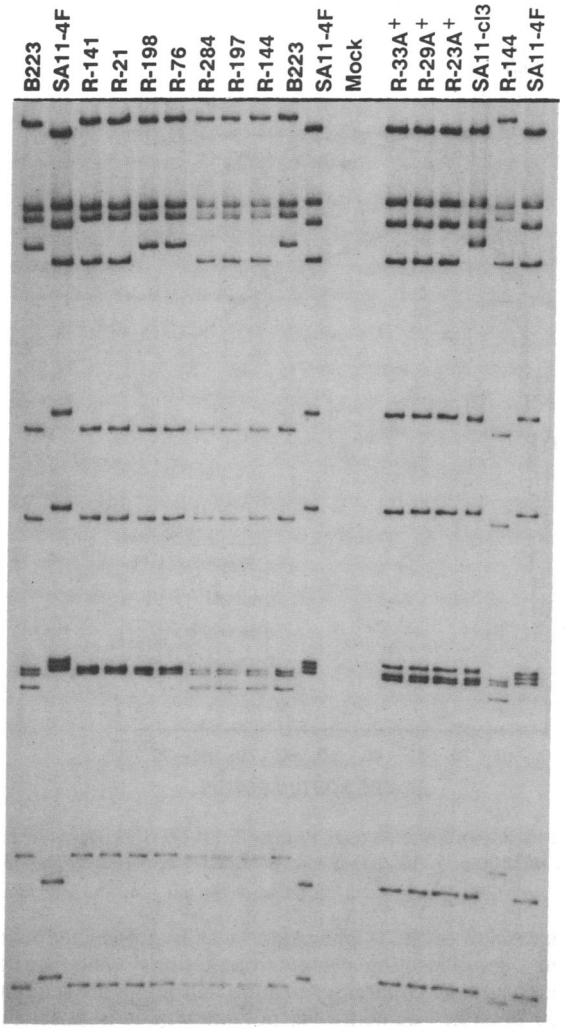

FIG. 1. Electrophoresis of ^{32}P-labeled genome RNAs of SA11-4F, B223, SA11 clone 3, and reassortants derived from these viruses. The parental origin of the genome segments of reassortants was determined by comparison to the cognate segments of the parental viruses. The genome segments of SA11-4F, B223, and SA11 clone 3 correspond in order of electrophoretic mobility. The migrational difference observed between regions 7–9 of SA11-4F and SA11 clone 3 is due to a differential migration of segment 8 (unpublished data). In the original gel the distance between B223 segments 1 and 11 was 20.4 cm. Lane labels identify strains from which RNA was purified.

without protease/titer with protease). The 4F variant was capable of forming small clear plaques in the absence of protease, although with an efficiency of only 10^{-3} the efficiency as in the presence of protease, whereas for B223 the efficiency was much lower, $<10^{-5}$. Among the 4F/B223 reassortants, only those with both segments 4 and 9 from the 4F variant parent were capable of forming plaques in the absence of protease at an efficiency like that of the 4F parent (10^{-3}). Neither the 4F segment 4 or 9 alone was capable of rescuing a B223 genetic background to relative protease independence for plaque formation. The "reconstructed 4F" reassortant was derived from parental viruses incapable of forming plaques in the absence of protease. However, the reconstructed 4F was able to form plaques in the absence of protease with an efficiency (10^{-2}) greater than that of the 4F parent (10^{-3}; Table 1).

Protease-Dependent Growth. One-step growth curves at high multiplicity of infection (moi = 10) were determined for each of the parental viruses and reassortants representative of each reassortant genotype. In these experiments, a standard concentration of trypsin (1.0 μg/ml) was present in the medium to facilitate virus growth. As shown in Fig. 2*A*, the 4F variant grew reproducibly to a titer 0.5–1.0 orders of magnitude higher than did B223, although the kinetics of growth appeared to be identical. Among the reassortants, only R-141, which contained both segments 4 and 9 from the 4F parent, was able to grow as well as the B223 parent, and reassortants containing 4F segment 4 alone (R-144) or 9 alone (R-76) grew less well than the B223 parent. Thus none of the outer capsid genes, in any combination, was capable of rescuing the growth of a B223 genetic background to a level similar to that of the 4F parent. A contrasting situation was found when the "reconstructed 4F" was examined (Fig. 2*B*). Both parental viruses (R-144 and clone 3) grew to similar peak titer with similar kinetics. However, the reconstructed 4F reassortants (R-23, R-29) grew to similar peak titer, and with similar kinetics, to the 4F variant parent from which only segment 4 of the reassortants had been derived.

DISCUSSION

The experiments described here yielded surprising results. We expected that the unique protease-associated properties of the SA11-4F variant would segregate with its unique genome segment 4 (and VP4) since the segment 4 product of SA11 has been shown to be protease-sensitive (4). If we consider only the "reconstructed 4F" reassortants, this expectation was met. The reconstructed 4F contained only segment 4 from the 4F variant and the remaining genome segments from SA11 clone 3, our standard wild type. On the genetic background of clone 3, segment 4 alone of the 4F variant was capable of conferring the phenotypic properties that we had identified as unique to 4F: formation of large plaques in the presence of protease, formation of small clear plaques in the absence of protease, and the ability to grow to relatively higher titer in the presence of protease. However, in the 4F/B223 reassortants a different and unexpected situation was encountered. When genome segments of the 4F variant were moved onto a B223 genetic background, the combination of 4F segments 4 and 9 was required to rescue the ability of reassortants to form large plaques in the presence of protease or to form plaques in the absence of protease. Although segments 4 or 9 alone resulted in slightly increased plaque size, the basis for the slight increase is not understood. In addition, on a B223 genetic background, the outer capsid proteins of the 4F variant were not able to rescue growth to relatively higher titer, either singly or in combination, suggesting that growth to high titer may be a more complex phenotype. We have isolated the reciprocal genotype to each of the reassortants reported here. Preliminary results indicate that all reciprocal reassortants express phenotypes consistent with the results reported here (unpublished data).

These results indicate that, in reassortment experiments with rotaviruses, the genetic background onto which donor segments are moved can affect the phenotypic expression of those segments. Thus, on one background (SA11 clone 3) the phenotypes examined segregated with a single genome segment from the 4F parent, whereas on a different genetic background (B223) the same phenotypes segregated with a combination of two or more genome segments. This result may have implications for the use of reassortants in a large number of studies to map phenotypes to specific constellations of genome segments.

Segregation analysis, of the type used here, has been used to map a number of phenotypes in rotaviruses and other segmented genome viruses. For example, reassortment has been used to identify VP4 as the rotavirus hemagglutinin and determinant of protease-enhanced plaque formation (16), rotavirus VP6 as the subgroup antigen (17), rotavirus proteins VP4 and VP7 as neutralization antigens (3, 18), genome

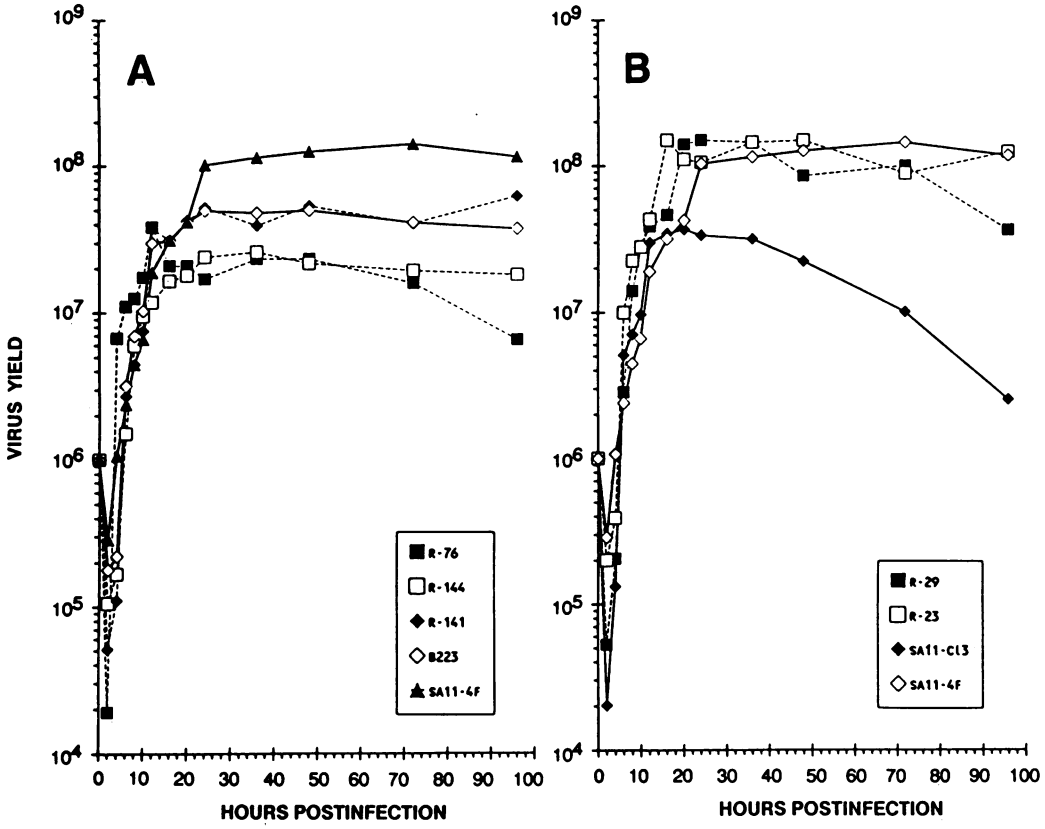

FIG. 2. One-step growth curves of SA11-4F, B223, SA11 clone 3, and selected reassortants. At each time point, titers of progeny virus were determined by plaque assay. Mean growth curves were generated for total infectious yield. (*A*) SA11-4F/B223 reassortants (n = 3). (*B*) "Reconstructed 4F" reassortants (n = 2).

segment 4 as encoding the ability to rescue noncultivatable rotaviruses (19), and VP4 as the determinant of rotavirus virulence (5). Likewise, in other members of the *Reoviridae*, reassortment has been used to identify numerous reovirus genes as determinants of specific phenotypes (for a review, see ref. 20) and to identify neutralization specificity (21) and the hemagglutinin (22) in bluetongue virus. In each of these studies the phenotype under study appeared to segregate with a single genome segment in reassortants. However, in none of these studies were reassortment experiments carried out using different virus pairs as the parental viruses. Our results suggest that it is possible, although not necessary, that different results may have been obtained if different parental virus pairs had been used for the production of reassortants.

How then might the recipient genetic background affect phenotypes expressed by reassortants? We currently favor the hypothesis that the "fit" or noncovalent interaction of proteins derived from divergent parental viruses may play a role in this process. Thus, in the case of our data, VP4 from the 4F variant has a good fit with other capsid proteins derived from clone 3 but has a poorer fit with other capsid proteins from B223. On a genetic background of B223, a second outer capsid protein (VP7) from the 4F variant is required to give a fit good enough to allow the phenotypes to be expressed. Such a hypothesis might predict that reassortants with poorly "fitting" combinations of capsid proteins may be unstable. We have preliminary evidence that, for at least some of the reassortants between 4F and B223, this prediction is realized (unpublished data). One example of the interaction of proteins playing a role in the phenotype of a virus comes from the mapping of extragenic suppressor mutations with a temperature-sensitive (ts) phenotype in reovirus (23). In this case, the ts phenotype of a mutant protein could be suppressed by a mutation that mapped in a second protein with which the ts protein interacted. The suppression of the ts phenotype was hypothesized to result from "compensating protein interactions" between the ts protein and the suppressor protein. Although the sequence of B223 genome segment 9 is not known, it is possible that differential glycosylation of, or different numbers of carbohydrate side chains on, VP7 could play a role in fit of the outer capsid proteins. Of course, other hypotheses could explain the genetic background effects we observed here.

Finally, our results demonstrate that the genetic background upon to which rotavirus genome segments are moved can affect the expression of phenotype in reassortants, at least for the virus pairs used in this study. Pending confirmation of our results, the assignment of phenotypes to specific rotavirus genome segments must be approached with caution. These results may also be relevant to the use of rotavirus reassortants as attenuated vaccines.

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