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Chimeric Sindbis-Ross River Viruses To Study Interactions between Alphavirus Nonstructural and Structural Regions

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Sindbis virus and Ross River virus are alphaviruses whose nonstructural proteins share 64% identity and whose structural proteins share 48% identity. Starting from full-length cDNA clones of both viruses, we have generated two reciprocal Sindbis-Ross River chimeric viruses in which the structural and nonstructural regions have been exchanged. These chimeric viruses replicate readily in several cell lines. Both chimeras grow more poorly than do the parental viruses, with the chimera containing Sindbis virus nonstructural proteins and Ross River virus structural proteins growing considerably better in both mosquito and Vero cell lines than the reciprocal chimera does. The reduction in replicative capacity in comparison with the parental viruses appears to result at least in part from a reduction in RNA synthesis, which suggests that the structural proteins or sequence elements within the structural region interact with the nonstructural proteins or sequence elements within the nonstructural region, that these interactions are required for efficient RNA replication, and that these interactions are suboptimal in the chimeras. The chimeras are able to infect mice, but their growth is attenuated. Western equine encephalitis virus, a virus widely distributed throughout the Americas, has been previously shown to have arisen by natural recombination between two distinct alphaviruses, but other naturally occurring recombinant alphaviruses have not been found. The present results suggest that most nonstructural/structural chimeras that might arise by natural recombination will be viable but that interactions between different regions of the genome, some of which were previously known but some of which remain unknown, limit the ability of such recombinants to become established.

The alphaviruses comprise a group of plus-strand RNA viruses that includes many important pathogens of humans and domestic animals (16, 64). The alphavirus genome of about 11.7 kb is translated into a polyprotein that is cleaved to form four nonstructural proteins, nsP1, nsP2, nsP3, and nsP4, which form components of the viral RNA replicase. A subgenomic mRNA of 4.1 kb is transcribed during infection and is translated into a polyprotein that is cleaved to form the structural proteins, which consist of a basic nucleocapsid protein that assembles with the RNA to form an icosahedral nucleocapsid with T=4 symmetry (5, 51) and two envelope glycoproteins, E1 and E2. During virus assembly, nucleocapsids bud through the cell plasma membrane and acquire a lipid envelope containing the two virus-encoded glycoproteins (reviewed in reference 65). Two other polypeptides are also produced upon cleavage of the precursor structural polyprotein, i.e., a glycoprotein called E3, which remains associated with the virion for some alphaviruses but not others, and a small hydrophobic polypeptide referred to as the 6K protein, which is involved in an unknown way with efficient transport and incorporation of the glycoproteins into the progeny virions (12, 13, 22, 35, 59, 77).

During replication of alphaviruses and assembly of virions, the various virus proteins must interact with one another, with the RNA genome, and with cellular proteins. Many specific interactions between alphavirus proteins or between alphavirus proteins and sequence elements in the RNA are possible, and such interactions have been studied to various extents. These interactions include the following. (i) The nonstructural proteins interact with one another and with host proteins to assemble an RNA replicase complex (3, 31, 62, 64). In particular, interactions between nsP1 and nsP2 (1, 20), between nsP2 and nsP4 (19), and between nsP1 and nsP4 (61) have been postulated, but there certainly must exist interactions among other virus nonstructural proteins. (ii) *cis*-acting regulatory elements in the viral RNA are required for replication of the RNA. These elements are believed to specifically bind virus nonstructural proteins, host proteins, and possibly the capsid protein to control RNA replication (26, 27, 33, 34, 43, 44, 48–50, 75). (iii) The glycoproteins, specifically glycoprotein E2, are involved in some unknown way in RNA replication, possibly through interaction with host major histocompatibility complex antigens (17, 74). (iv) There is believed to be a packaging signal in the viral RNA that binds the nucleocapsid protein to initiate encapsidation of the RNA (4, 15, 58, 75). (v) The glycoproteins E1 and E2 are known to interact with one another to form heterodimers and higher assemblages that are required for efficient transport of the glycoproteins and assembly into progeny virions (2, 7, 56, 69, 70, 78). These interactions are known to involve, at a minimum, specific E1-E2 interactions and E1-E1 interactions. (vi) During virus budding, there is a specific interaction between the cytoplasmic domain of glycoprotein E2 and the nucleocapsid protein that drives virus budding (14, 18, 36, 38, 65).

To study the interactions among virus components and to better define the contributions of different components to virus growth and virulence, we have embarked on a series of experiments involving chimeric alphaviruses. In chimeras, portions of the virus genome are derived from one alphavirus and the remainder of the genome is derived from a second alphavirus.

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If the alphavirus components derived from the different genomes can interact with one another, a functional virus is obtained. If not, the chimera is attenuated or nonviable. Attenuated or nonviable viruses can be used to further define the interacting sites by site-specific mutagenesis to adapt the disparate parts to one another or by selection of viruses that grow better and mapping of the compensating mutations. We have previously reported the construction of chimeras between Ross River virus (RR) and Sindbis virus (SIN) in which the 5′ or 3′ nontranslated regions (NTRs) were interchanged (27). We found that the 3′ NTRs were effectively interchangeable between these two viruses (at least for growth in the cell lines studied). In contrast, exchange of the 5′ NTRs gave rise to attenuated viruses in some cases and exchanges within the 5′ NTRs were not tolerated, probably because structures required for replication were disrupted. We have also reported a study of the interactions of the glycoproteins with the nucleocapsid during virus assembly, using chimeric RR-SIN viruses in which the capsid protein was derived from one virus and the remainder of the genome, including the glycoproteins, was derived from the other virus (36). In the present paper, we report results with chimeric viruses in which the entire nonstructural domain is derived from one of these two viruses and the entire structural domain is derived from the second virus.

MATERIALS AND METHODS

Plasmid construction and virus isolation. Oligonucleotide-directed mutagenesis was used to create a unique *Mlu*I restriction site in both SIN (pToto52) and RR (pRR64) full-length clones (27, 55). Mutagenesis was carried out with an M13mp18 vector as previously described (26, 28). Following isolation of the mutation, a DNA fragment containing the change was isolated by electrophoresis in low-melting-temperature agarose and used to replace the wild-type fragment in either pToto52 or pRR64. The resulting full-length clones, pToto54 and pRR40, respectively, each had a new unique *Mlu*I restriction site within them. The *Mlu*I site was then used to exchange the structural and nonstructural regions of the two genomes. The chimeric plasmids were prepared for transcription by linearization with *Sac*I. Transcription was carried out with SP6 RNA polymerase as previously described (26). Confluent monolayers of BHK-21 cells were transfected with RNA by using DEAE-dextran, and the cells were overlaid with 1% agarose (SeaKem ME; FMC Corp., Marine Colloids Division, Rockland, Maine) in Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum. At 2 to 3 days after transfection, plaques were picked prior to neutral red staining to improve virus viability. Stocks were generated from these plaques by growth on BHK cell monolayers.

Neutralization assays. A solution containing 100 PFU of either RR, SIN, or one of the reciprocal chimeras was mixed with various dilutions of neutralizing antibody made against either RR or SIN and incubated for 60 min at 37°C. The mixture was then applied to BHK cell monolayers (35-mm wells), and the monolayers were incubated for 60 min at room temperature. The inoculum was removed, and the cells were overlaid with 1% agarose in MEM containing 5% fetal bovine serum. Following incubation at 37°C for 48 h, the virus plaques were visualized by staining with neutral red.

Analysis of virus growth. Differential growth curves were generated from growth in Vero cell monolayers, chicken embryo fibroblast monolayers, or C6/36 mosquito cell monolayers, as previously described (27). For these growth curves, the cells were grown to confluency in 35-mm plates and infected at a multiplicity of infection of 5, the medium was changed at intervals after infection, and the titer of the virus released into the medium during each interval was determined. The results shown are means of two independent growth experiments. Cumulative growth curves were generated from growth in mouse embryo fibroblasts prepared from 14-day CD-1 mouse embryos (Charles River Breeding Laboratories, Wilmington, Mass.). The cells were grown to confluency in 12-well plates containing MEM supplemented with 15% fetal bovine serum and infected at a multiplicity of infection of 5, and the medium was changed at 2 h. At various times thereafter, the medium in one of the wells was collected for virus titer determination. All plaque assays were performed on BHK cell monolayers at 37°C.

RNA synthesis. The relative amounts of RNA synthesized were analyzed as described previously (27). The RNA species synthesized were analyzed by using Northern (RNA) blots. Vero or C6/36 monolayers (60-mm plate) were infected at a multiplicity of infection of 5 and harvested at 12 h postinfection. Total cytoplasmic RNA was prepared essentially as described previously (27) and electrophoresed in a 1% agarose–formaldehyde gel. RNA was transferred to a nylon membrane (Hybond-N; Amersham Corp.) and probed with [^{32}P]RNA. Labeled RNA probe was generated by transcription of pGEM3Z plasmids containing sequences from either SIN or RR. For RR, minus-strand RNA corresponding to nucleotides 8235 to 10327 was transcribed. For SIN, minus-strand RNA corresponding to nucleotides 8569 to 10768 was transcribed. Radiolabeled probe bound to the membrane was quantitated with a Fuji BAS 2000 Imaging Plate Scanner.

Animal tests. One-week-old virus-antibody-free CD-1 mice (Charles River) were inoculated intracerebrally in the right cerebral hemisphere or subcutaneously in both hind feet with 1,000 PFU virus in 30 μl of Hanks balanced salt solution supplemented with 1% fetal bovine serum. The mice were observed for 14 days for clinical disease and death. Individual tissues (foot, brain, spinal cord, muscle, and brown fat) were taken from two to four mice at various times from 16 h to 10 days after infection, and homogenates were prepared in Hanks balanced salt solution–1% fetal bovine serum. The virus content was assessed by measuring plaque formation of serial 10-fold dilutions of the tissue homogenates on BHK cells, and geometric means of the titers were determined.

RESULTS

Virulence for mice of RR rescued from cDNA. SIN and RR cause similar diseases in humans (66) but different diseases in mice (16). SIN, including strains called Ockelbo, Pogosta disease, and Karelian fever viruses, causes outbreaks of rash and arthritis in humans in Scandinavia, Russia, and Africa (45, 46, 52). RR causes epidemic polyarthritis in Australia and the South Pacific (57, 67). The diseases caused by SIN and RR are temporarily disabling, but fatal disease is unrecorded and encephalitis has not been reported in humans following infection with either virus. In mice, however, SIN and RR cause different, age-related diseases. Both viruses replicate in muscles and the brain and cause paralysis, but virulent strains of SIN cause death due to encephalitis while virulent strains of RR cause death due to myositis and myocarditis (23, 39, 42, 60).

In mice, the pathology of the disease caused by SIN viruses rescued from cDNA clones is similar or identical to the disease caused by the strain of virus from which the cDNA clone was derived (37, 53, 68). In the case of RR, the cDNA clone pRR64 was derived from RR strain T48 (27), an early isolate of RR that is virulent for mice and has often been used as a standard RR strain in the laboratory. The growth in cell cultures of RR rescued from this clone is identical to that of RR T48 (27), but the disease caused by the rescued RR in mice has not been examined. It was important to examine such virus to show that it had the same virulence in mice as does the well-characterized parental RR T48 strain to rule out the presence of inadvertant changes in the cDNA clone or in virus produced from it that might affect the virulence of the virus. We therefore compared the infection of mice by RR T48 and by RR rescued from clone pRR64. Following subcutaneous inoculation of 1-week-old mice, both T48 and RR64 caused 100% mortality preceded by paralytic illness. The mean day of death was similar for the two viruses, 7.1 days for T48 and 7.6 days for RR64. The pathology of the disease caused by the rescued RR was also similar to that caused by T48 and did not differ from that reported previously for RR (39, 42), although the pathology did differ from that caused by SIN. Thigh muscle from RR64-infected mice showed intense inflammation and necrosis by day 7, which was not observed following infection with either virulent or attenuated strains of SIN. In contrast, brain inflammation in RR64-infected CD-1 mice was less intense than in mice infected with neurovirulent SIN. RR64 infection of mice by intracerebral inoculation caused 100% mortality with a mean time to death of 7.9 days, whereas following inoculation with virulent SIN strains, the mean time to death was 4.3 days. Finally, infection of mice suffering from severe combined immunodeficiency (SCID mice) with RR64 led to 100% mortality, suggesting that direct effects of virus replication were responsible for the disease observed. We conclude that the disease in mice that is caused by virus rescued from the cDNA clone pRR64 is indistinguishable from the disease caused by

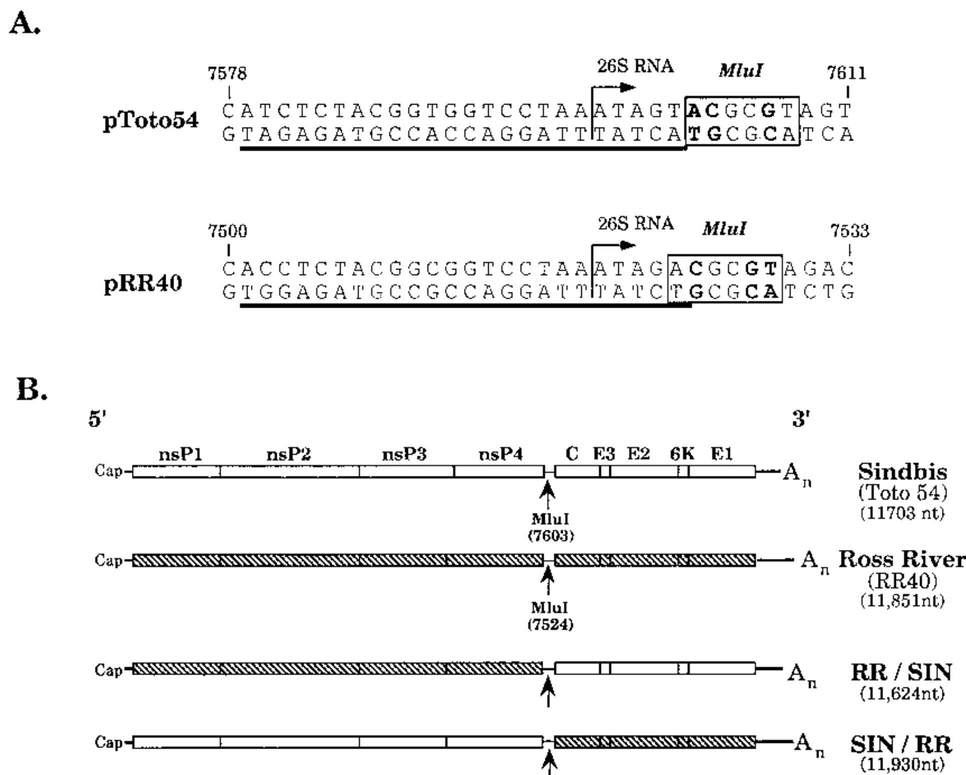

FIG. 1. Introduction of unique *Mlu*I restriction sites into the SIN and RR cDNAs and construction of chimeric clones. (A) Nucleotide sequences surrounding the *Mlu*I restriction sites in pToto54 (SIN) and pRR40 (RR) are shown. Nucleotide numbers starting from the 5′ end of the viral genome are shown above each clone. The start of the 26S subgenomic RNA is indicated by the arrow; the minimal promoter used to transcribe SIN 26S mRNA (33), and by homology assumed to be used for RR, is indicated by the heavy lines under the sequences. The *Mlu*I restriction site is shown as a shaded box with the substituted residues in boldface type. (B) The SIN (Toto54) and RR (RR40) genomes containing the *Mlu*I restriction sites are schematically illustrated, together with the two chimeric viruses constructed by using this new site. SIN coding sequences are indicated by open boxes, and RR coding sequences are indicated by hatched boxes. Virus proteins are indicated above the Toto54 schematic. The unique *Mlu*I restriction site introduced into SIN and RR is indicated by the arrow, with the 5′ nucleotide of the site given in parentheses. The size of the genome is shown in parentheses next to the name of the virus. nt, nucleotides.

the parental T48 strain of RR in all properties examined, and thus this cDNA clone is useful for model studies with RR.

Construction of RR and SIN containing a new restriction site. To construct chimeric viruses in which the structural and nonstructural domains of SIN and RR viruses are interchanged, cDNA clones of these viruses that have a new unique restriction site in the nontranslated region between the structural and nonstructural domains were constructed. The junction regions of the two virus genomes are compared in Fig. 1A, and the nucleotides changed to produce a unique *Mlu*I site in this region in both viruses are indicated.

Virus was rescued from the cDNA clones containing the new *Mlu*I restriction sites, Toto54 for SIN and RR40 for RR. These viruses were compared with the viruses derived from cDNA clones pToto52 and pRR64 by plaque morphology and by virus production as measured by one-step differential growth curves in Vero and mosquito cells (data not shown). Plaque morphology was identical to that of the parental viruses. In mosquito cells, both *Mlu*I-containing viruses grew at levels that were indistinguishable from those of their respective parents. In Vero cells, however, the *Mlu*I-containing viruses were slightly impaired, being somewhat delayed in virus release and releasing virus at rates one-third to about 100% of parental rates at different times after infection.

Isolation of chimeric viruses. The *Mlu*I restriction sites were used to make two chimeric viruses in which the structural and nonstructural domains of SIN and RR viruses were interchanged. These constructs are shown schematically in Fig. 1B. In these chimeras, the 5′ NTR and the entire nonstructural coding region are derived from one virus and the entire structural region and the 3′ NTR are derived from the second virus.

Both chimeric viruses were viable and could be rescued from the cDNA clones. Stocks were prepared, and the neutralization of these viruses with neutralizing antibodies prepared against RR or SIN is shown in Table 1. SIN/RR, which contains the nonstructural proteins of SIN but the structural proteins of RR, was efficiently neutralized by anti-RR neutralizing antibody, as was RR rescued from the parental RR clone, but anti-SIN neutralizing antibody had no effect. Conversely, RR/SIN and SIN, which both contain the structural proteins of SIN, were efficiently neutralized by the anti-SIN antibody whereas the anti-RR antibody had no effect.

To confirm that the rescued viruses contained the expected chimeric genomes, cytoplasmic RNA was isolated from BHK cells infected with chimeric or parental viruses, and reverse transcription-PCR was performed with primers chosen to lie on either side of the chimeric junction. Analysis of the amplified DNA products by both size and digestion with restriction enzymes confirmed the identity of the chimeras (data not shown).

Virus production in several cell lines. The growth of the chimeric viruses was compared with that of parental viruses (derived from cDNA clones) following infection of mammalian

TABLE 1. Relative titers of RR, SIN, and the chimeric viruses after treatment with anti-RR and anti-SIN neutralizing antibodies

Anti-E2 antibody	Virus	Plaque titer[a] at antibody dilution of:			
		None	1:10	1:100	1:1,000
Anti-RR	RR	<1	6	56	94
	SIN/RR	<1	2	32	76
	SIN	85	91	100	101
	RR/SIN	50	82	102	106
Anti-SIN	RR	88	117	108	100
	SIN/RR	88	83	82	92
	SIN	<1	3	7	15
	RR/SIN	<1	<1[b]	<1S[b]	<1[b]

[a] Plaque titers were determined in duplicate. Each titer is shown as the percentage of the titer for the same virus whose titer was determined without antibody.

[b] The apparently greater effect of the antiserum against RR/SIN than against SIN probably arises because the plaques formed by RR/SIN are very tiny, and small amounts of residual antiserum in the overlay may cause them to become invisible.

TABLE 2. Plaque-forming efficiencies on different cells

Virus	No. of plaques[a] on cell line:		
	BHK	Vero	C6/36
SIN	1	0.5	0.004
SIN/RR	1	1.0	2.1
RR/SIN	1	1.3	0
RR	1	5.6	62

[a] The table shows the number of plaques formed on the different cells lines normalized to the number of plaques formed on BHK cells; values are the mean of three independent experiments except for SIN on C6/36 cells, for which the values are from a single experiment. Zero indicates that no detectable plaques were formed.

cells (Vero or primary mouse embryo fibroblast), of mosquito cells (*Aedes albopictus* C6/36), or of chicken cells (primary chicken embryo fibroblasts). The growth curves shown in Fig. 2 for Vero, chicken, and C6/36 cells are differential curves in which the medium over the cells was changed at each time point so that the rate of virus release was measured as a function of time rather than the cumulative yield of the virus at that time. Such growth curves provide a very sensitive assay for virus replication.

In Vero cells, SIN and RR grew similarly, with SIN producing virus at a two- to threefold-higher rate than RR late in infection. The two chimeric viruses grew less well, with SIN/RR producing more virus than RR/SIN. The rate of SIN/RR release was about 10-fold lower than that of SIN at late times, whereas that of RR/SIN was about 10^{-2} that of SIN. Thus, the chimeric viruses are attenuated in this cell line, growing less well than the parental viruses; RR/SIN grows particularly poorly.

In mosquito cells, RR grew distinctly better than did SIN, producing virus at a rate about 2 orders of magnitude greater than SIN. SIN/RR grew almost as well as did RR and distinctly better than SIN, whereas RR/SIN grew very poorly, producing 4 orders of magnitude less virus than did RR and 2 orders of magnitude less virus than did SIN. Since SIN/RR grows almost as well as RR and much better than SIN, the increased growth rate of RR in mosquito cells relative to SIN appears to be determined in large part by the RR structural proteins rather than by the RNA replicase.

All infectivity titers used to determine input multiplicities were obtained by plaque assay on BHK cells. We examined the relative abilities of the different viruses to form plaques on Vero and C6/36 cells (Table 2). Plaque-forming efficiencies on Vero cells were very similar to those on BHK cells, with the exception that RR forms plaques more efficiently on Vero cells than on BHK cells. Thus, the differences in the growth curves on Vero cells do not result from different effective multiplicities of infection. On mosquito cells, however, SIN formed plaques poorly and RR formed plaques very efficiently; therefore, the differences in growth rates between SIN and RR could result from differences in effective multiplicities of infection, but it is also possible that the differences in plaque-forming efficiencies result from the difference in growth rates. The plaques formed by SIN were indistinct and difficult to quantitate. Second, RR/SIN is unable to form a plaque on C6/36 cells, probably because it grows very poorly, but some virus is produced, showing that cells are infected even if no plaque is formed. It is possible that RR structural proteins in

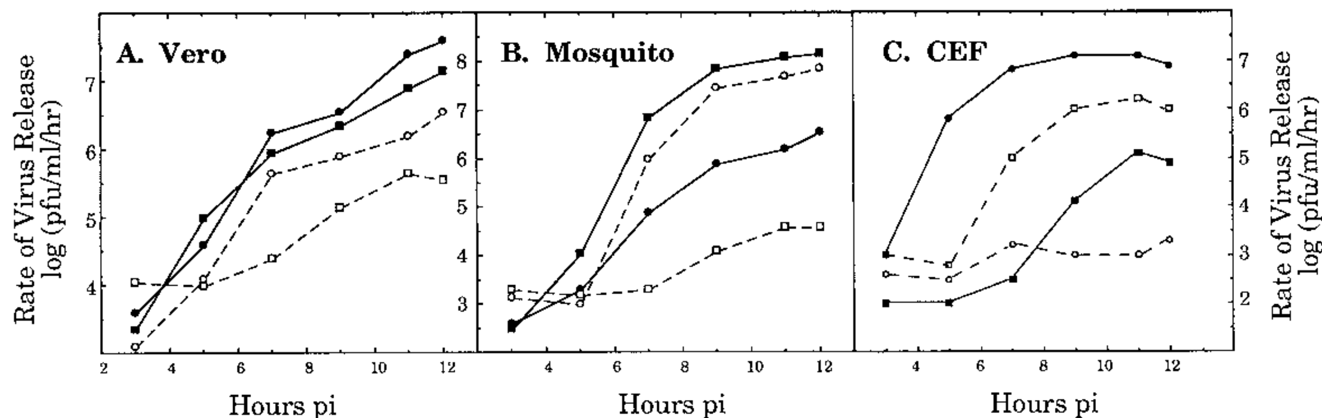

FIG. 2. One-step differential growth curves for the chimeric viruses and the parental viruses in Vero, mosquito, and chicken cells. Cells were infected at a multiplicity of infection of 5, and the medium was changed at intervals. Virus released into the medium during each interval was determined by plaque assay on BHK cells at 37°C. The growth curves of viruses in Vero cells (A) and secondary chicken embryo fibroblasts (CEF) (C) were generated at 37°C, and those of viruses in C6/36 mosquito cells (B) were generated at 30°C. SIN refers to the wild-type SIN virus rescued from cells transfected with pToto52 RNA (Vero or mosquito cells) or pToto54 RNA (chicken cells); RR refers to the wild-type T48 strain of RR rescued from cells transfected with pRR64 RNA (Vero or mosquito cells) or pRR40 RNA (chicken cells). Symbols: ●, SIN; ■, RR; ○, SIN/RR; □, RR/SIN. Results are expressed as log PFU released per milliliter per hour and are the results of two independent growth curve experiments. pi, postinfection.

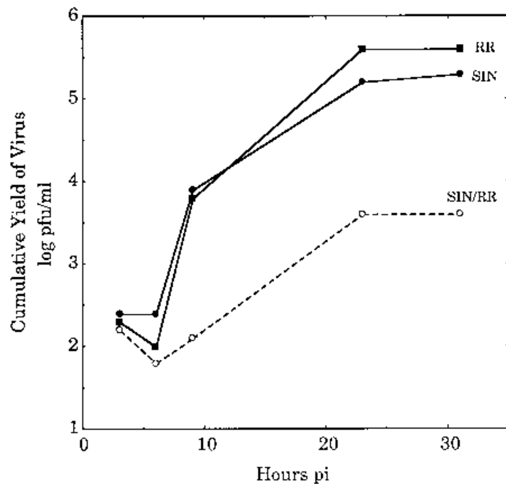

FIG. 3. Cumulative growth curves in mouse embryo fibroblasts. Mouse embryo fibroblasts were infected at 37°C at a multiplicity of infection of 5, and the medium in different wells was harvested at the times shown. The cumulative yield of virus released into the medium at each time point was determined by plaque assay on BHK cells. pi, postinfection.

RR and SIN/RR lead to better growth in the growth curves and higher plaque-forming efficiency on mosquito cells because they lead to more efficient entry mediated by interactions with (different) high-affinity receptors, as is the case for SIN envelope proteins in chicken cells.

In chicken cells, SIN grew decidedly better than did RR. RR is maintained in nature primarily in small mammals whereas SIN, although capable of replicating in mammals, has birds for its primary vertebrate reservoir (24, 45). The T48 strain of RR infects only about 2% of primary or secondary chicken embryo fibroblasts in culture (determined by immunofluorescence assay of infected cells with anti-envelope protein antibodies), apparently because most of the cells lack high-affinity receptors for the virus (74), resulting in the poor growth seen in Fig. 2C and an inability of the virus to form plaques on chicken cells. SIN/RR grows even more poorly in chicken cells. The structural proteins of this chimera are derived from RR, and, as is the case for RR, the chimera is able to infect only a fraction of chicken cells; the infected cells produce almost no virus, however, and the chimera is thus attenuated relative to RR. In contrast, SIN infects and replicates in chicken cells very efficiently, and the plaque-forming efficiency of the virus on chicken cells is about the same as on BHK cells. Chimera RR/SIN, whose structural proteins are derived from SIN, is able, like SIN, to infect most or all chicken cells in culture, as shown by the ability of this chimera to form small plaques on chicken cell monolayers. However, the chimera produces only low yields of virus in chicken cells, as well as in other cells, apparently because of incompatibilities between SIN structural proteins and RR sequence elements.

Growth of virus in cultured mouse cells is often a good predictor of virus virulence in mice (25). For this reason, we also examined the cumulative yield of SIN/RR grown in primary mouse embryo fibroblasts for different times in comparison with the growth of the parental viruses (Fig. 3). Under the conditions of this experiment, SIN and RR produced yields of $>10^5$ after 23 h whereas SIN/RR produced a yield that was reduced by 2 orders of magnitude. Because of technical difficulties, the growth of RR/SIN could not be examined in this experiment, but as shown below, this chimera replicates very poorly in mice.

Thus, in each case the chimeras grew less well than did at least one of the parental viruses. RR/SIN grew poorly relative to SIN/RR in all cell lines and was almost nonviable in mosquito cells. The simplest interpretation is that there are incompatibilities between the nonstructural and structural protein domains, with incompatibilities between the SIN structural proteins and the RR nonstructural domain being more pronounced.

RNA synthesis. Synthesis of viral RNA in Vero cells and mosquito cells infected with the two chimeras was examined and compared with that in cells infected with the parental viruses, also rescued from cDNA clones (Fig. 4). Also exam-

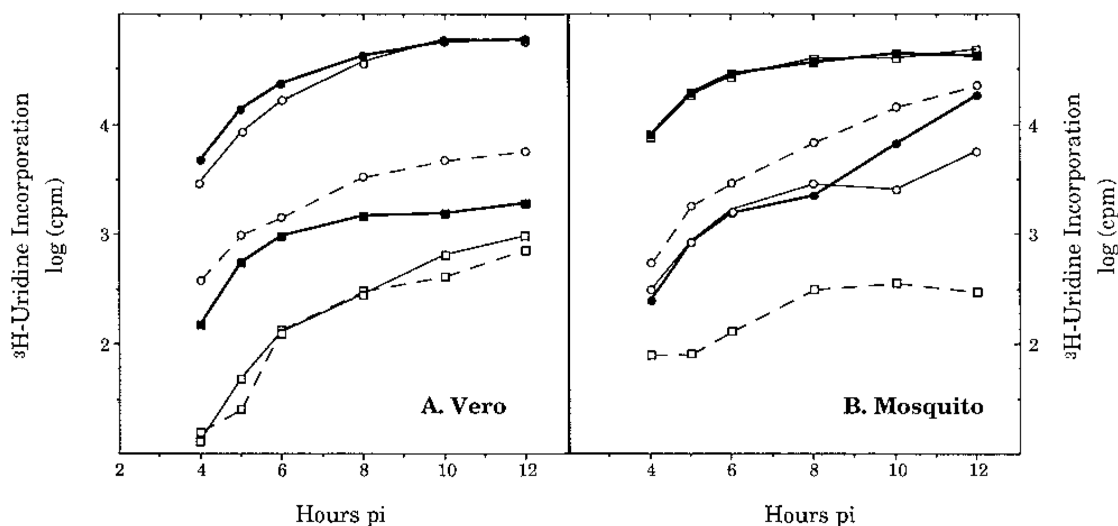

FIG. 4. RNA synthesis following infection of Vero cells and mosquito cells. RNA synthesis experiments were conducted in Vero cells at 37°C (A) and in C6/36 mosquito cells at 30°C (B). Symbols: —●—, SIN; —■—, RR; —○—, SIN/3′RR; —□—, RR/3′SIN; ---○---, SIN/RR; --□--, RR/SIN. Cells were labeled beginning at 2 h postinfection (pi) and harvested at the indicated times. Results are expressed as log cpm of [^{3}H]uridine incorporated, as assayed by liquid scintillation counting, and represent the radioactivity incorporated into 3.5×10^3 cells. The chimeras SIN/3′RR and RR/3′SIN have exchanged only the 3′ NTRs and are described in reference 27.

TABLE 3. Molar ratios of subgenomic to genomic RNA

Cell line	Virus	26S/49S RNA ratio
Vero	SIN	2.6
	RR	3.0
	SIN/RR	1.5
	RR/SIN	2.5
C6/36	SIN	2.8
	RR	1.4
	SIN/RR	1.0
	RR/SIN	2.7

ined, for reference, were chimeras in which only the 3′ NTRs were exchanged. As described previously (27), exchange of the 3′ NTRs leads to no difference in virus growth rates, but there are detectable differences in RNA synthesis.

In Vero cells, in which SIN grew slightly better than RR, SIN produced 50-fold more RNA than did RR; note that SIN/3′RR produced RNA at an identical rate to SIN. It is interesting that this large difference in RNA synthesis led to only a twofold difference in the rates of virus release (Fig. 2). SIN/RR produced less RNA than did SIN but more RNA than did RR (although producing less virus than RR). RR/SIN produced the least RNA but produced an amount equivalent to that produced by RR/3′SIN. We interpret these results to mean that viruses with the SIN replicase make more RNA than do viruses with the RR replicase and that mismatched structural proteins in the case of SIN/RR lead to a reduced rate of RNA synthesis. For RR/SIN, the decrease in RNA synthesis can be attributed to the effects of the wrong 3′ NTR.

In mosquito cells, in which RR grew better than did SIN, RR produced approximately 10-fold more RNA than did SIN and RR/3′SIN produced the same amount of RNA as did RR. Thus, the mismatch in the 3′ NTR affects RNA synthesis in Vero cells but not in mosquito cells. SIN/3′RR and SIN produced the same amount of RNA early, but SIN produced more later (conceivably because of reinfection). SIN/RR produced more RNA than did SIN but less than did RR, whereas RR/SIN produced very little RNA. In mosquito cells, viruses with the RR structural proteins made more RNA than did viruses with the SIN structural proteins and yielded more virus; as described above, we cannot rule out the possibility that these differences arise from more efficient infection by viruses containing RR envelope proteins.

The relative amounts of genomic RNA and of subgenomic RNA produced after infection by these viruses was examined by Northern blotting, and the results are shown in Table 3. In no case were dramatic differences in the ratio of subgenomic to genomic RNA observed, but detectable differences were present for some chimeras. In Vero cells, the molar ratio of subgenomic RNA to genomic RNA was 2.5 to 3 for both RR and SIN as well as for RR/SIN but slightly less (1.5) for SIN/RR. In mosquito cells, the molar ratio of 26S to 49S RNA was 2.7 to 2.8 for SIN and RR/SIN but less (1.0 to 1.4) for RR and for SIN/RR. It is interesting that in mosquito cells, viruses with RR structural proteins make less 26S RNA than 49S RNA but produce more RNA and more virus than do the viruses with SIN structural proteins. It is unclear whether the small differences in the ratios of subgenomic RNA to genomic RNA observed are significant in terms of the differences in RNA synthesis rates and rates of virus production observed.

Biological properties of the chimeric viruses. One-week-old mice were infected with the two chimeric viruses and with the parental viruses rescued from cDNA clones pToto52 (SIN HRSP) or pRR64 (RR T48), and the mortality caused by the viruses was determined after intracerebral and subcutaneous inoculation (Table 4). RR64 is virulent in mice and led to 100% mortality whether inoculated peripherally or intracranially. The HRSP strain of SIN is relatively avirulent in mice, and virus rescued from pToto clones derived from HRSP cause 61% mortality, due to encephalitis, in 1-day-old mice (37) but no mortality in 1-week-old mice (Table 4). The two chimeric viruses were also attenuated. No deaths were recorded in mice inoculated subcutaneously with either chimera. In mice inoculated intracranially, SIN/RR caused no mortality and RR/SIN led to only 6% mortality.

We examined virus growth in several tissues after infection by RR64, SIN Toto52, SIN/RR, and RR/SIN. Tissues assayed included the foot (the site of subcutaneous inoculation), blood, brain, spinal cord, brown fat, and muscle near to (thigh) and distant from (pectoral or triceps) the site of subcutaneous inoculation. After subcutaneous inoculation, no virus was detected in mice inoculated with RR/SIN, but other viruses replicated at the site of inoculation. All three viruses induced a viremia, but that present after RR64 infection was higher (titer, $10^{7.5}$) and more sustained (6 days) than that induced by SIN Toto52 or SIN/RR (3 days; maximum titer, $10^{3.2}$ for SIN and 10^{5} for SIN/RR). All three viruses also grew in muscle, brain, spinal cord, and brown fat, with RR64 producing greater amounts of virus than did SIN Toto52 or SIN/RR. The chimera SIN/RR grew less well than RR in any tissue tested but did replicate to somewhat higher titer than SIN in spinal cord and

TABLE 4. Biological properties of the nonstructural/structural chimeric viruses

Inoculation route	Virus	% Mortality (n)[a]	Maximum titer (log PFU/g) in:					
			Brain	Spinal cord	Muscle	Brown fat	Blood	Foot
Subcutaneous	SIN (HRSP)	0 (9)	3.6	4.1	4.5	4.6	3.2	6.4
	RR(T48)	100 (28)	6.8	6.9	8.6	6.8	7.5	8.1
	SIN/RR	0 (28)	3.5	5.1	3.2	4.3	5.0	5.6
	RR/SIN	0 (47)	ND[b]	ND	ND	ND	<2.7	<2.7
Intracranial	SIN (HRSP)	0 (9)	6.5	7.1	ND	4.2	3.0	ND
	RR(T48)	100 (23)	6.6	7.1	ND	7.0	7.0	ND
	SIN/RR	0 (22)	5.1	5.6	ND	4.3	5.0	ND
	RR/SIN	6 (34)	3.2	ND	ND	ND	4.0	ND

[a] n, number of 1-week-old mice inoculated for the mortality assay.
[b] ND, not done.

produced a higher viremia. In view of the growth of SIN/RR in mice in comparison with SIN Toto52, the lower titers of this virus produced in cultured mouse cells are of interest (Fig. 4).

After intracerebral inoculation, a small amount of virus was detected in the blood and brain of RR/SIN-infected mice. SIN Toto52 and RR64 replicated to similar titers in the brain and spinal cord, but the viremia was higher for RR. SIN/RR replicated less well than either parent in nervous system tissue; however, after subcutaneous inoculation, the maximal viremia was greater than for SIN.

As a control, the virus recovered from infected mice was tested by reverse transcription-PCR with primers on either side of the chimeric junction. In each case, the recovered virus arose from the parent or chimera injected. We cannot rule out the possibility, however, that point mutations leading to better virus growth in mice arose during the infection.

We conclude that both chimeras are attenuated relative to RR. It is noteworthy that RR/SIN is highly attenuated and replicates very poorly in mice, consistent with its very poor growth in cultured cells. The growth of SIN/RR in spinal cord and the height of viremia are greater than for SIN, suggesting that the structural proteins contribute to the more efficient growth observed for RR in infected mice.

DISCUSSION

Structural protein effects on RNA replication. All alphaviruses are closely related in their genome organization and share a minimum of 40% amino acid sequence identity in the structural proteins and 60% identity in the nonstructural proteins (32, 64). SIN and RR are among the more distantly related of the alphaviruses: their nonstructural proteins share 64% identity and the structural proteins share 48% sequence identity (8, 63). Despite these sequence divergences, both reciprocal chimeric viruses that exchange the nonstructural and structural domains are viable, making it possible to study interactions between the structural domain and the nonstructural domain during the replication and assembly of alphaviruses. We have previously shown that the 3′ NTRs of RR and SIN can be interchanged without apparent effect on the growth rate of the virus in either Vero cells or mosquito cells, although RNA synthesis is depressed in Vero cells in the case of RR/3′SIN (Fig. 4) (27). Studies of defective interfering RNAs have suggested that there are no *cis*-active sequence elements required for RNA replication or encapsidation in the structural region of alphavirus RNAs other than the 3′ NTR, at least for SIN (34, 40, 41) and Semliki Forest virus (29, 30). Thus, the chimeras constructed for this study are useful for studying the effects of the glycoproteins upon RNA replication and for studying interactions between the capsid protein and *cis*-active elements present in the nonstructural domain of the viral RNA required for RNA synthesis or encapsidation during replication of nondefective viruses.

Interpretation of the results is complicated because more than one factor appears to influence replication. However, for at least some chimeras, the results indicate that there are incompatibilities between the structural proteins and either the nonstructural proteins or RNA sequence elements within the nonstructural domain that lead to reduced rates of RNA replication and reduced levels of virus production. One possible hypothesis to explain such an effect is that there are incompatibilities in the binding of the capsid protein to sequence elements present in the nonstructural domain. Weiss et al. (75) have shown that there is a domain in the nonstructural region of the SIN genome, between nucleotides 746 and 1226, that binds the SIN capsid protein and that is required both for efficient RNA replication and for efficient RNA encapsidation. Thus, it is possible that the capsid protein must bind to this region to stimulate or regulate RNA replication as well as, during assembly of nucleocapsids, to initiate encapsidation of the viral RNA. A role for a viral coat protein in RNA replication has been demonstrated for alfalfa mosaic virus and the ilarviruses, in which binding of the capsid protein to the 3′ end of the RNA is required for RNA synthesis (reviewed in reference 21), and for turnip crinkle virus, in which the coat protein binds to an RNA stem structure thought to be involved in readthrough translation of the viral polymerase (73). The location of the RR domain corresponding to the capsid-binding domain in SIN is not known but is also presumed to lie in the nonstructural region. If binding of alphavirus capsid protein is required for efficient RNA synthesis, a defective interaction of SIN capsid protein with the RR sequence element could lead to depressed RNA synthesis, whereas a better but still defective interaction of the RR capsid protein with the SIN element could lead to more extensive RNA synthesis than for the reciprocal chimera.

Although this model is attractive, it probably cannot account for the full extent of the depression observed. Frolov and Schlesinger (10) have found that a chimeric virus that contains the entire RR genome, derived from pRR64, except for the capsid protein, which was derived from SIN pToto54 (described in reference 36), synthesized the same amount of RNA in BHK cells as did RR derived from pRR64. However, a virus consisting of SIN Toto54 with the RR capsid gene synthesized only 30% as much RNA in BHK cells. In both chimeras, less 26S subgenomic RNA was made, resulting in decreased production of structural proteins. Thus, chimeras containing only a mismatched capsid protein gene have altered RNA synthesis patterns but are not as affected as chimeras in which the capsid gene, the glycoprotein genes, and the 3′ NTR are mismatched. Mismatched 3′ NTRs can account for the differences in RNA synthesis seen in some chimeras in some cell lines but cannot account for all the differences observed for the chimeras in this study. It is conceivable that an incompatibility in the 3′ NTRs could have a synergistic effect with a capsid protein-RNA incompatibility that leads to depressed RNA synthesis.

A second possible model for the depressed RNA synthesis in the chimeras is an incompatibility between the glycoproteins and the RNA machinery. It has been found that deletions in glycoprotein E2 of RR or SIN (a 7-amino-acid deletion centered on residue 55) lead to altered rates of RNA synthesis in some but not all cell lines (71, 74). It has also been found that substitutions at position 55 of E2 of SIN lead to a difference in the rate of RNA synthesis in mouse cells; the effect of the E2 change is different in mouse cells that differ in their major histocompatibility antigens (17). The mechanisms by which E2 could affect the rate of RNA synthesis are not known, but it is possible that the E2s in the chimeras interact with the RNA replication machinery in an altered fashion. As suggested above, the effect of mismatched glycoproteins could be synergistic with that of mismatched capsid genes or 3′ NTRs.

A third possible model is that the efficiency of the subgenomic promoter is altered in the chimeras, an effect possibly enhanced by the mutations necessary to introduce the *Mlu*I site. The subgenomic promoter of SIN has been well studied, and its activity depends only upon the 24 nucleotides indicated in Fig. 1 and upon upstream elements, not upon any downstream elements (33, 54); however, not all possible sequences have been tested. Furthermore, it is known that there is a translation-enhancing element in the coding sequence for the capsid protein (9, 11), but the activities of the SIN and RR elements have not been compared.

Another, less likely, possibility to explain the results obtained here is that an incompatibility between the capsid protein and encapsidation sequences in the nonstructural region of the viral RNA results in an altered virus that has a lower rate of attachment, penetration, or uncoating, leading to a delay in viral replication and thus to lower levels of replication at early times. However, an encapsidation sequence does not appear to be absolutely required for virus assembly, and there is no evidence that virus assembled in the absence of an encapsidation signal differs in its properties (4). Furthermore, RNA synthesis following infection by the chimeric viruses does not catch up to parental levels with time, which might be expected if the depressed synthesis resulted simply from a delay in the infection process. In an attempt to rule out this model, we used RNA, transcribed in vitro from the cDNA clones, rather than virus to infect cells. Although these experiments were performed several times by a variety of approaches, technical problems with the experiments prevented us from obtaining a clear-cut answer. Thus, at present, although we believe it to be unlikely that defects in the early stages of infection by the chimeras are responsible for the poor growth observed, at least in Vero cells, we cannot rule out this possibility.

Since the RR-SIN chimeras are viable but replicate poorly, it should be possible to further delineate the structural domain and nonstructural domains that interact by obtaining variants of the chimeras that replicate more efficiently by serial passage of the virus. Mapping of compensating mutations that arise during passage of the chimeras would highlight areas that interact. Alternatively, site-specific mutagenesis or fragment exchange experiments can be used in an attempt to obtain viruses that replicate more efficiently. As noted above, the SIN domain between nucleotides 746 and 1226 is implicated in capsid protein binding, and additional studies have suggested that the binding site on the capsid protein is located between amino acids 76 and 107 (15, 47). Other studies suggest that a conserved sequence element surrounding residue 106 of the capsid protein is responsible for binding the capsid protein to ribosomes (76). Additional chimeric viruses could focus upon these regions and provide a molecular genetic approach toward elucidating possible interactions between the capsid protein and RNA sequence elements.

Attenuation of chimeric viruses. It is of interest to compare the artificial chimeras studied in this paper with a similar type of chimeric virus that was generated in nature by a double recombination event between a SIN-like virus and Eastern equine encephalitis (EEE) virus. Sequencing of this recombinant virus, Western equine encephalitis (WEE) virus, identified two sites of recombination (18, 72). The WEE virus 5′ NTR, nonstructural proteins, capsid protein, and part of E3 are derived from EEE virus, whereas the structural glycoproteins E2 and E1 are derived from a SIN-like virus. A second recombination event in the 3′ NTR resulted in the 3′-terminal 80 nucleotides being derived from EEE virus. The initial recombinant appears to have exhibited a number of incompatibilities. The fact that the 3′-terminal 80 nucleotides are derived from the EEE virus parent, requiring an inherently unlikely double recombination event, suggests that the EEE nonstructural proteins interact with this domain and that the second recombination event was required to obtain a that could persist in nature. Second, in WEE virus, the EEE capsid protein interacts with the SIN glycoproteins and incompatibilities appear to have been present in the original recombinant. Amino acid substitutions in the WEE capsid protein and in E2 of WEE virus appear to have adapted the EEE capsid protein to SIN E2 and identify amino acids potentially involved in capsid-E2 interactions that occur during virus budding (6, 18, 65). We have recently characterized RR-SIN chimeras in which only the capsid protein is derived from the second virus, rather than the entire structural protein region, as a way to further study the interactions between E2 and capsid protein during virus assembly (36). Third, it is unknown if there were incompatibilities between the glycoproteins and the RNA replication machinery of the type suggested by the data presented in this paper. There are 134 changes from the SIN sequence in WEE virus E2, of which 28 are to the EEE sequence, and there are 103 changes from the SIN sequence in WEE E1, of which 17 are to the EEE sequence. Most of the changes from the SIN amino acid to the EEE amino acid are scattered in the ectodomains of the proteins, but there is a pronounced clustering of such changes in the transmembrane domains and in the E2 cytoplasmic domain. The four changes in the E2 cytoplasmic domain from the SIN sequence to the EEE sequence are postulated to adapt the SIN E2 domain to the EEE nucleocapsid. There are five changes in the E2 transmembrane domain from the SIN sequence to the EEE sequence and four such changes in the E1 transmembrane domain. These transmembrane domains show little conservation among alphaviruses, and the occurrence of such a cluster of changes from SIN to EEE is curious. These changes could represent further adaptation of the SIN envelope proteins to the EEE nucleocapsid, or they could represent adaptation of the SIN envelope proteins to EEE nonstructural proteins and RNA. Further study of chimeric viruses and identification of suppressor mutations that suppress incompatibilities in chimeras will be useful in sorting out the interactions that occur during alphavirus replication.

It is of interest that WEE virus is attenuated relative to the EEE virus parent. WEE virus can cause encephalitis in humans, but the incidence of encephalitis following infection by the virus is much lower than that following EEE virus infection, and the encephalitis resulting from WEE virus infection is usually less severe than resulting from EEE virus infection (16, 52). The chimeric SIN/RR and RR/SIN viruses are also attenuated relative to the parental strains when examined for growth in tissue culture cells or for virulence in mice. This suggests that it might be possible to attenuate viruses for potential live virus vaccines by judicious construction of chimeric viruses.

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