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Attenuation of Sindbis Virus Neurovirulence by Using Defined Mutations in Nontranslated Regions of the Genome RNA

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We examined a panel of Sindbis virus mutants containing defined mutations in the 5′ nontranslated region of the genome RNA, in the 3′ nontranslated region, or in both for their growth in cultured cells and virulence in newborn mice. In cultured cells, these viruses all had defects in RNA synthesis and displayed a wide range of growth rates. The growth properties of the mutants were often very different in mouse cells from those in chicken cells or in mosquito cells. We hypothesize that host factors, presumably proteins, interact with these nontranslated regions to promote viral replication and that the mammalian protein and the chicken or mosquito protein are sufficiently divergent that alterations in the viral RNA sequence can affect the interactions with these different host proteins in different ways. Some of the mutants were temperature sensitive for plaque formation, whereas one mutant was slightly cold sensitive in its growth in chicken cells. Upon inoculation into mice, viruses that grew well in cultured mouse cells retained their virulence, but mice that succumbed usually had extended survival times. One virulent mutant that grew slightly less well in cultured mouse cells than did the parental virus produced eight times as much virus in mouse brain following intracerebral inoculation, suggesting that changes in these regulatory regions may have tissue-specific as well as host-specific effects. Viruses that were severely crippled in their growth in mouse cells in culture were usually, but not always, attenuated in their virulence. In particular, temperature sensitivity was correlated with attenuation. The effect of two mutations was found to be cumulative, and double mutants that contained mutations in both the 5′ and 3′ nontranslated regions were more attenuated than was either single mutant. Three of four double mutants tested were severely crippled for virus production in cultured cells and were avirulent for mice, even when inoculated intracerebrally.

The *Alphavirus* genus of the family *Togaviridae* contains 26 registered members. Many of these viruses are important causative agents of human disease and/or veterinary pathogens, variously causing encephalitis, arthritis, fever, and rash (20). These viruses are transmitted in nature by mosquitoes and replicate alternately in their invertebrate vectors and in their vertebrate hosts (1). Sindbis virus, the prototypic alphavirus, is one of the least virulent of the alphaviruses, but certain Sindbis virus strains are important causative agents of human polyarthritis in northern Europe (17, 23) and of a disease characterized by fever and arthralgia in southern Africa (16). Sindbis virus also causes an age-dependent disease of mice in which young mice contract fatal encephalitis upon inoculation with the virus, whereas older mice survive (6, 9). The virus has been extensively used as a model system for the encephalitic diseases caused by alphaviruses (5).

The development of full-length cDNA clones of RNA viruses from which infectious RNA can be transcribed in vitro makes possible in principle new ways to develop attenuated virus strains for use as vaccines. We have previously characterized a number of mutants of Sindbis virus that have mutations in RNA sequence elements believed to be important in RNA replication (10, 14, 15). These mutants showed various degrees of attenuation when grown in tissue culture cells, and the effects of the mutations were often host dependent, with some mutations having a more dramatic effect upon the replication of the virus in mosquito cells than in chicken cells and some showing the opposite phenotype. We report here our studies on the ability of four deletion mutants in the 5′ nontranslated region (NTR) and of four mutants in the 3′ NTR (three deletions and a point mutation) to replicate in mouse cells in culture and to cause encephalitis in mice. These viruses were chosen to be representative of the phenotypes expressed in tissue culture by the 5′ and 3′ NTR mutants. All are deficient in RNA synthesis and have an extremely low frequency of reversion. We have also characterized a number of double mutants containing mutations in both the 5′ and 3′ NTRs for their ability to replicate in tissue culture cells and to cause disease in mice.

MATERIALS AND METHODS

Construction and isolation of mutant viruses. Plasmid constructions were performed by standard methods (22). In vitro RNA transcription and transfection into permissive cells have been previously described (10, 21). Mutants 5NT*d*(15-25), 5NT*d*(10-14), 5NT*d*(41-55), and 5NT*d*(8,36), in the 5′ NTR, were described by Niesters and Strauss (14). Mutants 3NT*d*(18-25), 3NT*d*(26-318), 3NT*d*(9-14), and 3NT7C, in the 3′ NTR, were described by Kuhn et al. (10). These mutants were constructed in the background of the HRSP (heat-resistant small plaque) strain of Sindbis virus (Toto50). To test the effects of these mutations on the virulence of Sindbis virus for mice, each mutation was moved into a background virus referred to as TE12 (11) in

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which the structural glycoproteins E1 and E2 of HRSP have been replaced by the proteins from NSV, a neurovirulent strain of Sindbis virus (6). For this purpose, the *Sac*II (11481)-*Bgl*II (2288) fragment from each mutant clone was joined in a three-piece ligation with the *Stu*I (8569)-*Sac*II (11481) fragment from clone TE12 and the *Bgl*II (2288)-*Stu*I (8569) fragment from clone Toto50 (21). For mutants 3NT*d*(26-318) and 5NT*d*(41-55)/3NT*d*(26-318), the strategy was the same but the restriction sites used for construction were different. The rescued viruses, containing the NSV sequences, will be referred to by their original designations followed by an asterisk; for example, the 3NT7C mutant virus containing NSV envelope proteins will be designated 3NT7C*. A parental virus called Toto50* was also constructed. This virus contains the envelope proteins of NSV, while the remainder of the genome comes from the HRSP strain. Toto50* differs from TE12 in that TE12 contains the envelope proteins of NSV, while the remainder of the genome comes from the cDNA referred to as Toto1101 (11, 21).

One-step growth analysis and RNA synthesis. Differential growth curves were performed as described by Kuhn et al. (10). For determination of RNA synthesis, secondary chicken embryo fibroblast (CEF) monolayers (35-mm plates) were infected with either Toto50 (wild type) or mutant viruses at a multiplicity of 5 PFU per cell at either 30 or 40°C. At the times indicated, total cytoplasmic RNA was isolated and blotted onto nitrocellulose. Viral RNA was determined by the dot hybridization method of White and Bancroft (26), using a ^{32}P-labeled minus-strand RNA probe containing nucleotides 6267 to 4633 transcribed from a subclone of the Toto50 cDNA clone with T7 RNA polymerase. To quantitate specific time points, the dot blots were first autoradiographed, the individual blots were cut out of the nitrocellulose filter, and the radioactivity was quantitated by liquid scintillation counting. The amount of RNA found was corrected for the total amount of RNA applied at each time point, as determined by quantitation of vimentin-specific RNA bound to a portion of the sample.

Animal experiments. One- to two-day-old CD-1 mice (virus antibody free; Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were inoculated either intracerebrally in the right hemisphere or subcutaneously in each hindfoot with 1,000 PFU of virus in 30 μl of Hanks balanced salt solution containing 1% fetal bovine serum. Mice were observed daily for 21 days to determine the percent mortality and the mean day of death. In separate experiments, brains were removed from intracerebrally inoculated mice at various times after infection. The tissues were frozen and thawed and 10% homogenates prepared in Hanks balanced salt solution. Virus content was assayed by plaque formation in BHK-21 cells.

RESULTS

Growth of the single mutants in cultured cells. We have previously determined the ability of a panel of mutants containing changes in the 5′ NTR and of a panel with changes in the 3′ NTR to replicate in cultured cells, either mosquito cells or chicken cells (10, 14). For this project, four mutants from each group, forming a representative sampling of the phenotypes observed in tissue culture, were chosen for analysis of the effects of these mutations in mice. The replication efficiencies of these mutant viruses in CEF and mosquito cells (C6/36) are shown in Table 1. The nomenclature used for the mutants describes the changes that they contain (10). As examples, mutant 5NT*d*(15-25) has nucleotides 15 to 25 of the 5′ NTR deleted, and mutant 3NT*d*(18-25) has nucleotides 18 to 25 of the 3′ NTR deleted (numbering from the 3′ end of the RNA in the latter case). All of the mutations used were deletions, with the exception that in mutant 3NT7C, the A residue at nucleotide 7 from the 3′ end of the viral RNA was replaced with a C.

TABLE 1. Relative rate of virus release at 12 h post infection

Virus[a]	Relative rate of virus release[b]			
	3T3, 37°C	CEF		C6/36, 30°C
		30°C	40°C	
5NT*d*(15-25)	90.9	1.6	ND	25.0
5NT*d*(10-14)	1.1	3.2	ND	0.6
5NT*d*(41-55)	39.0	10.0	ND	320.0
5NT*d*(8,36)	1.2	6.3	0.1	0.1
3NT*d*(18-25)	81.8	5.0	40.0	40.0
3NT*d*(26-318)	3.2	79.0	63.0	1.6
3NT*d*(9-14)	4.0	3.2	4.0	79.0
3NT7C	0.16	13.0	3.2	0.6
5NT*d*(15-25)/3NT*d*(18-25)	50.0	1.6	25.0	2.0
5NT*d*(10-14)/3NT*d*(9-14)	0.03	0.6	0.06	0.003
5NT*d*(41-55)/3NT*d*(26-318)	0.52	1.6	0.2	0.1
5NT*d*(8,36)/3NT7C	0.13	0.2	0.01	0.001

[a] Viruses tested in 3T3 cells had the Toto50* background; viruses tested in CEF and C6/36 cells had the Toto50 background.

[b] Expressed as percentage of the wild-type virus (Toto50 or Toto50*) rate. Data for CEF and C6/36 cells for the single mutants in the 3′ NTR are from Kuhn et al. (10); data for the single mutants in the 5′ NTR are from Niesters and Strauss (14). ND, not determined.

These mutants were constructed in the background of the HRSP strain of Sindbis virus, which is of low virulence for mice. To test the effects of these mutations on the virulence of Sindbis virus for mice, each mutation was moved into a background virus in which the structural glycoproteins E1 and E2 of HRSP have been replaced by the proteins from NSV, a neurovirulent strain of Sindbis virus (11). The mutant viruses containing NSV E1 and E2 were then tested for their growth properties in cultured mouse cells and for their virulence in suckling mice. The growth properties of the viruses in 3T3 mouse cells at 37°C are shown in Table 1. We have previously noted that the growth rates of different mutants relative to those of their parents were often quite different in chicken and mosquito cells (10, 14, 15). As seen in Table 1, the relative growth properties of different mutants in mouse cells in this panel were often quite different from those in chicken cells. 5NT*d*(15-25) replicated very poorly in chicken cells but almost normally in mouse cells. Similarly, 5NT*d*(41-55) and 3NT*d*(18-25) replicated much better in mouse cells than in chicken cells. Conversely, 3NT*d*(26-318), as well as 3NT7C, replicated much better in chicken cells than in mouse cells. Mutants 5NT*d*(8,36) and 3NT7C are temperature sensitive in their ability to form a plaque, forming plaques at 30°C but not at 40°C (10, 14). In contrast, 3NT*d*(18-25) is slightly cold sensitive in chicken cells. A spectrum of growth properties different from those in mouse cells or in chicken cells was seen in mosquito cells (Table 1). (It should be noted that the viruses tested in 3T3 cells had the envelope proteins of NSV and thus differ at four amino acids from the virus tested in chicken or mosquito cells; however, in each case the mutant containing a deletion or substitution in the NTR was compared with a parental virus identical to

TABLE 2. Neurovirulence of Sindbis virus mutants in suckling mice[a]

Virus	Intracerebral			Subcutaneous		
	No. tested	% Mortality	MDOD[b]	No. tested	% Mortality	MDOD
Toto50*	30	100	2.4	27	100	3.6
5NT*d*(15-25)*	31	100	2.7	27	100	4.1
5NT*d*(10-14)*	30	100	3.3	28	64	6.0
5NT*d*(41-55)*	31	100	4.0	20	50	11.0
5NT*d*(8,36)*	54	74	7.9	52	12	12.3
3NT*d*(18-25)*	19	100	2.9	20	100	3.4
3NT*d*(26-318)*	24	100	3.9	31	81	8.2
3NT*d*(9-14)*	37	81	6.6	41	71	9.0
3NT7C*	30	17	4.8	32	13	10.5
5NT*d*(15-25)/3NT*d*(18-25)*	18	100	2.9	20	100	4.6
5NT*d*(10-14)/3NT*d*(9-14)*	42	7	14.0	38	3	16.0
5NT*d*(41-55)/3NT*d*(26-318)*	50	2	3.0	53	2	9.0
5NT*d*(8,36)/3NT7C*	21	0		21	0	

[a] Mice were inoculated with 1,000 PFU of virus at 1 to 2 days of age by the intracerebral or subcutaneous route.
[b] MDOD, mean day of death after virus inoculation.

it except for the change in the NTR, and it seems unlikely that the relative effects of the mutations would be influenced by changes in E1 or E2.)

Virulence of the single mutants for mice. The effects of these different mutations on the virulence of the virus in mice are shown in Table 2. The parental virus Toto50*, which is essentially Sindbis virus HRSP containing the E1 and E2 of the neurovirulent NSV and similar to TE12 characterized by Lustig et al. (11), leads to 100% mortality in 1- to 2-day-old mice inoculated either intracerebrally or subcutaneously (Table 2). Intracerebral inoculation leads to a slightly accelerated infection of the central nervous system, and the mice die approximately 1 day earlier. The four mutations in the 5′ NTR tested for their virulence in mice had effects in mice which were correlated with their attenuation for growth in cultured mouse cells, but the correlation was not precise (Table 3). Mutant 5NT*d*(15-25) grew well in cultured mouse cells and was as virulent as the parental virus in mice (note that it grew poorly in chicken cells and moderately well in mosquito cells, however, as shown in Table 1). Mutant 5NT*d*(41-55) grew somewhat less well in mouse (and mosquito) cells and was attenuated in mice, showing only 50% mortality when inoculated subcutaneously and leading to a much longer survival time in the mice that did ultimately succumb. Mutant 5NT*d*(8,36) is temperature sensitive for growth in cultured cells, growing poorly at 40°C and failing to form plaques at this temperature. It also grew poorly in mouse cells at 37°C. This virus was more attenuated in its virulence for suckling mice when inoculated subcutaneously, leading to only 12% mortality and with a survival time more than four times as long as that for the wild-type virus. When inoculated intracerebrally, the majority of mice still died (74%), but the survival time was extended. Finally, mutant 5NT*d*(10-14) also grew poorly in mouse cells in culture, but it was only slightly attenuated relative to the wild type.

TABLE 3. Correlation of virus replication in 3T3 cells with virulence for mice inoculated subcutaneously

Virus	% Virus replication in 3T3 cells[a]	% Mortality in mice[a]
5NT*d*(15-25)*	90.9	100
3NT*d*(18-25)*	81.8	100
5NT*d*(15-25)/3NT*d*(18-25)*	50.0	100
5NT*d*(41-55)*	39.0	50
3NT*d*(9-14)*	4.0	71
3NT*d*(26-318)*	3.2	81
5NT*d*(8,36)*	1.2	12
5NT*d*(10-14)*	1.1	64
5NT*d*(41-55)/3NT*d*(26-318)*	0.52	2
3NT7C*	0.16	13
5NT*d*(8,36)/3NT7C*	0.13	0
5NT*d*(10-14)/3NT*d*(9-14)*	0.03	3

[a] Expressed as percentage of the level of the wild-type virus (Toto50*).

The effects of mutations in the 3′ NTR on virulence in mice were better correlated with the effects in tissue culture than were those of the 5′ mutants (Table 3). Mutant 3NT*d*(18-25) was as virulent as the parental virus in mice, and the mutant grew well in 3T3 cells (note that it is slightly cold sensitive in chicken cells). Mutant 3NT*d*(26-318), which grew poorly in 3T3 cells but well in chicken cells, was slightly attenuated in mice, with longer survival times when inoculated subcutaneously and causing only 80% mortality. Mutant 3NT*d*(9-14) was somewhat more attenuated in mice. In cultured mouse cells, this virus grew poorly although about as well as 3NT*d*(26-318). Finally, 3NT7C, which is temperature sensitive for plaque formation in chicken cells in culture and grows quite poorly in mosquito cells at 30°C, grew very poorly in mouse cells at 37°C. It was attenuated in mice when inoculated subcutaneously or intracerebrally. Only 13 to 17% of the mice succumbed, and the survival time was extended for those mice that did die.

We conclude that temperature sensitivity of a mutant leads to a strong attenuation in its virulence for mice. However, virus that is attenuated for growth in cultured cells, producing as little as 1 to 4% as much virus as does the wild type, may still be quite virulent in this mouse system, and attenuation of growth in cultured cells is not necessarily correlated with attenuation in virulence for mice. Not surprisingly, attenuation of growth in mouse cells is a better predictor of attenuation of virulence in mice than is attenuation in chicken or mosquito cells.

Replication of virus mutants in mouse brain tissue. To further characterize the virulence of the mutants in mice, the amounts of virus present in the brains of mice at two

TABLE 4. Replication of Sindbis virus mutants in mouse brain cells

Virus	Replication of virus in mouse brain cells			
	18 h postinfection		48 h postinfection	
	PFU/g of mouse brain	% of wild-type level	PFU/g of mouse brain	% of wild-type level
Toto50* (wild type)	1.1×10^9	100.0	2.5×10^{10}	100.0
5NT*d*(15-25)*	1.7×10^9	155.0	2.7×10^{10}	108.0
5NT*d*(10-14)*	1.9×10^6	0.2	4.0×10^{10}	160.0
5NT*d*(41-55)*	2.5×10^8	22.7	4.8×10^9	19.0
5NT*d*(8,36)*	$<5 \times 10^2$	<0.001	1.6×10^6	0.006
3NT*d*(18-25)*	9.3×10^9	845.0	Dead	
3NT*d*(26-318)*	5.0×10^5	0.05	1.3×10^8	0.5
3NT*d*(9-14)*	1.2×10^7	1.1	1.9×10^7	0.08
3NT7C*	$<5 \times 10^2$	<0.001	5.0×10^2	<0.001
5NT*d*(15-25)/3NT*d*(18-25)*	1.2×10^9	109.1	8.5×10^9	34.0
5NT*d*(10-14)/3NT*d*(9-14)*	$<5 \times 10^2$	<0.001	2.5×10^3	<0.001
5NT*d*(41-55)/3NT*d*(26-318)*	$<5 \times 10^2$	<0.001	2.8×10^3	<0.001
5NT*d*(8,36)/3NT7C*	4.5×10^3	<0.001	5.3×10^4	<0.001

different times after intracerebral inoculation with the different mutants were determined (Table 4). Growth of the virus in mouse brain cells was correlated reasonably well, but not perfectly, with growth in 3T3 cells in culture and with virulence. Note that mutant 5NT*d*(10-14) is delayed in its growth in brain cells but ultimately reaches greater than wild-type levels and is virulent. Note also that mutant 3NT*d*(18-25), which is virulent, reaches virus titers almost 10-fold greater than wild-type levels at 18 h but grows somewhat less well than the wild type in 3T3 cells. Finally, several mutants that are somewhat attenuated, such as 5NT*d*(8,36), 3NT*d*(9-14), and 3NT7C, produced only small amounts of virus, 1% or less of the amount produced by the parental virus, in the brain cells after 48 h, although these viruses did kill a significant percentage of inoculated animals.

The result for 3NT*d*(18-25), in which growth in mouse brain cells was greatly increased whereas growth in 3T3 cells was depressed, suggests that deletions in regulatory regions can have tissue-specific as well as host-specific effects and that the putative host proteins that bind to these elements are differently expressed in different tissues. It seems unlikely that the increased rate of growth in mouse brain cells was due to a secondary mutation in the genome because the virus titer was determined after only 18 h of growth, and such a high-yielding variant was not seen with any other strain studied. Virus isolated from the brains of mice infected with 3NT*d*(18-25) had the same plaque phenotype as did the virus used to infect the mice.

Construction of double mutants. We wished to follow up these studies by constructing a number of double mutants in which mutations in the 5′ NTR were combined with mutations in the 3′ NTR. Four double mutants were first constructed in the background of Sindbis virus HRSP. Growth curves of these mutants in chicken cells at 30 and 40°C and in mosquito C6/36 cells at 30°C were determined in order to compare their properties with those previously obtained for the single mutants. These growth curves (Fig. 1) can be compared with published curves for the single mutants (10, 14). The growth rates at 12 h after infection are summarized in Table 1. At 30°C in chicken cells, all four mutants tested were indistinguishable from one another and all produced virus at about 1% of the rate of the parent. At 40°C, the results varied among the mutants, although all mutants were attenuated for growth relative to the parental viruses. Mutant 5NT*d*(15-25)/3NT*d*(18-25) was delayed in its growth but ultimately produced virus at about 25% of the rate of the parental virus. Mutants 5NT*d*(41-55)/3NT*d*(25-318) and 5NT*d*(10-14)/3NT*d*(9-14) produced virus at a rate of 10^{-2} to 10^{-3} of the parent. Finally, mutant 5NT*d*(8,36)/3NT7C was delayed by about 7 h in its growth and produced virus at only about 10^{-4} of the parental virus rate. In mosquito cells, these last two mutants were totally unable to replicate, whereas the first two mutants exhibited long delays in their growth curves and produced small amounts of virus in comparison with the parental virus.

The effect of two mutations is thus cumulative. In each case, the double mutants were more severely affected for virus growth than was either of the single mutants containing only one of the two mutations. The combination of mutations sometimes had unpredicted effects upon growth properties of the viruses. For example, mutant 3NT*d*(9-14) grows at a wild-type rate in mosquito cells. When combined with mutation 5NT*d*(10-14), which grows poorly but detectably in mosquito cells, the double mutant is unable to replicate in mosquito cells.

Viral RNA synthesis in chicken cells infected with the various double mutants was also examined (Fig. 2). At 30°C, the production of RNA in cells infected by any of the mutants was delayed relative to RNA synthesis in cells infected with the parental virus. At 12 h postinfection, the rate of RNA production by the mutants was 25 to 40% of the parental virus rate. Thus, RNA synthesis was not affected as drastically as production of progeny virus (see Fig. 1). At 40°C, mutant 5NT*d*(8,36)/3NT7C was the most reduced in RNA synthesis, producing only about 10% as much RNA as did the parental virus. Mutant 5NT*d*(15-25)/3NT*d*(18-25) produced almost 40% as much RNA as did the parental virus, and the other two double mutants produced intermediate amounts at 40°C. RNA synthesis thus correlates with the production of virus but is not as severely affected.

Growth of double mutants in mouse cells. The double mutants were recloned into the NSV envelope background (Toto50*) and tested for growth in 3T3 cells in culture. Rates of virus release at 12 h postinfection are shown in Table 1 and compared with those of the mutants in chicken and

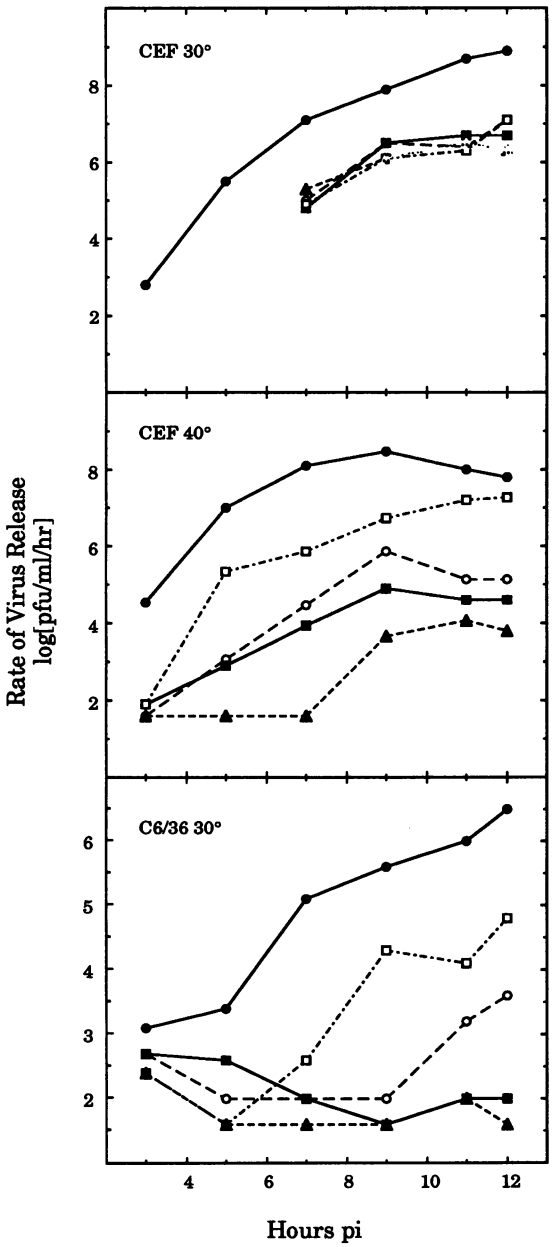

FIG. 1. One-step differential growth curves for the double mutants. Monolayers of CEF or of mosquito cells (C6/36) were infected with virus at a multiplicity of infection of 5. Virus was allowed to adsorb for 1 h, at which time the inoculum was removed and Eagle minimal essential medium containing 2% fetal calf serum was added. The culture fluid was removed, and fresh medium was added every 30 min for the first 2 h and every hour after that. Culture fluid samples were then assayed for plaque-forming virus by titration on monolayers of secondary CEF at 30°C. Symbols: ●, Toto50 (wild type); ○, 5NT*d*(41-55)/3NT*d*(26-318); ▲, 5NT*d*(8,36)/3NT7C; ■, 5NT*d*(10-14)/3NT*d*(9-14); □, 5NT*d*(15-25)/3NT*d*(18-25). pi, postinfection.

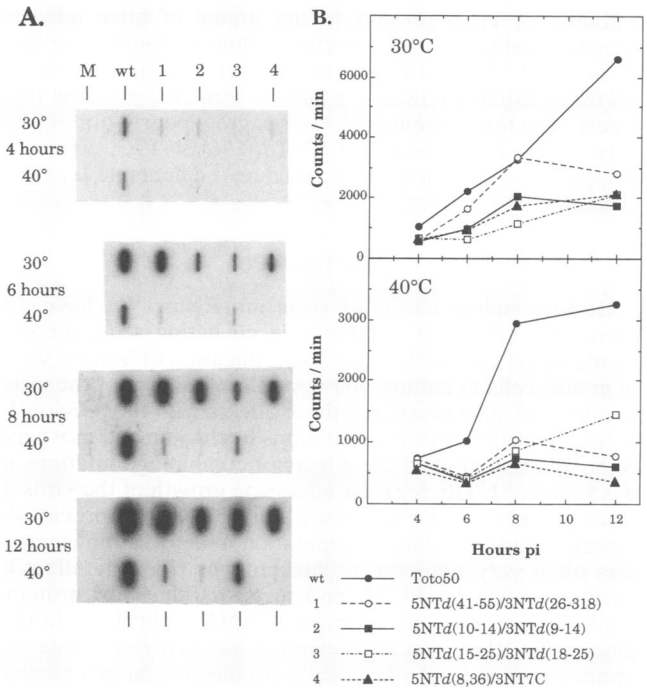

FIG. 2. RNA synthesis by the double mutants. Secondary CEF monolayers were infected with either wild-type (wt) virus (Toto50) or the double mutants or were mock infected (M) as described in Materials and Methods. At the times indicated, cytoplasmic RNA was isolated and treated as described in the text. (A) Autoradiograph of the slot blot hybridization. Numbers at the top refer to the double mutants and are indicated in the legend below panel B. (B) Quantitation of RNA, performed as described in Materials and Methods. Symbols for the various viruses are shown in the legend. pi, postinfection.

mosquito cells. Mutant 5NT*d*(15-25)/3NT*d*(18-25) grew well in mouse cells. In chicken cells, it was cold sensitive like the single mutant 3NT*d*(18-25). The other three double mutants grew poorly in all cell lines tested.

Virulence of the double mutants. The virulence of the double mutants was tested in suckling mice following either intracerebral or subcutaneous inoculation (Table 2). Double mutant 5NT*d*(15-25)/3NT*d*(18-25)* was as virulent as the parental virus, leading to 100% mortality by either route of inoculation, consistent with its vigorous growth in 3T3 cells in culture. The other three double mutants were highly attenuated. After infection with mutants 5NT*d*(10-14)/3NT*d*(9-14)* and 5NT*d*(41-55)/3NT*d*(26-318)*, 93 to 98% of mice survived (in three of four cases, only a single mouse died). Furthermore, the mice that succumbed lived much longer after infection. Thus, even though the individual mutations that make up these two double mutants had only modest effects on virulence, the double mutants were much more severely attenuated. Finally, mutant 5NT*d*(8,36)/3NT7C*, which contained two mutations, each of which alone caused significant attenuation, was completely avirulent; all mice survived infection with this double mutant. It is worth noting that both individual mutations rendered the virus temperature sensitive and that, as for two other double mutants, the combination of the two mutations is much more effective in attenuating the virus in mice than is either mutation separately.

The correlation between growth in mouse 3T3 cells and virulence in young mice is shown in Table 3. It is clear that attenuation for growth in cultured mouse cells is an important predictor of attenuation in mice, but the correlation is not perfect and thus other factors are also important.

Growth of the double mutants in mouse brains. The

amounts of virus present in the brains of mice infected intracerebrally with the various double mutants were also determined at two different times after infection (Table 4). 5NT*d*(15-25)/3NT*d*(18-25), which is virulent, grew to high levels. The three attenuated viruses grew poorly but detectably. It is interesting that 5NT*d*(8,36)/3NT7C, which is completely avirulent in mice, produced detectable levels of virus.

DISCUSSION

Host-dependent effects of virus mutations. We have explored the effects of mutations in *cis*-acting sequence elements in the viral RNA upon the replication of Sindbis virus in mouse cells in culture and upon the virulence of the virus in mice and have compared these effects with the previously determined effects of the mutations in chicken and mosquito cells. We also studied the effects of combining mutations in the 5′ and 3′ NTRs for their effects on growth of the virus in tissue culture and for virulence in mice. We had previously reported that the effect of a mutation in a *cis*-acting element was often very different in chicken cells from the effect in mosquito cells (10, 14, 15) and suggested that host proteins bind to these elements to promote RNA replication. In this model, the RNA sequence element has evolved to interact optimally, or at least efficiently, with the host factors present in all of the natural hosts of the virus, and changes in the element may upset interaction with the protein factor in some of the viral hosts but not in others. The fact that in some instances mutations led to increased growth rates in one of the hosts but reduced growth rates in others suggested that it is possible to refine the interactions of the virus with any particular host but at the expense of interactions with other hosts and thus with the persistence of the virus in nature. Direct evidence for binding of host proteins to some of these elements has recently appeared (12, 13, 19).

In this investigation, we have extended the study of host dependence to show that mutations in the 5′ and 3′ NTRs often have effects in mouse cells very different from those in chicken (or mosquito) cells. Thus, the effects of any mutation must be considered separately in mammalian, avian, and mosquito cells. In nature, Sindbis virus replicates in a mosquito vector, and the main vertebrate reservoir is thought to be different species of birds. The virus also replicates in and causes disease in mammals, including humans. Thus, all three of these cell lines can be considered to be derived from natural hosts of the virus. It would be of interest to further explore whether such mutations in *cis*-acting elements might also have different effects in cells from different mammals or in cells from different tissues. Mutant 3NT*d*(18-25) produced eightfold more virus in mouse brain after 18 h than did the wild-type virus but produced slightly less virus in cultured mouse 3T3 cells, suggesting that these mutations may have tissue-specific effects. Identification of the protein factors involved in these interactions and comparison of the proteins from different animals and tissues would also be of considerable interest.

Attenuation of virulence for mice. We have tested the virulence of a number of mutations in *cis*-acting regulatory elements for their virulence in mice in order to explore whether this might be a feasible approach to vaccine development. The mutations chosen for examination were all deletion mutations with one exception, 3NT7C. Deletion mutations revert at very low frequencies, making them ideal candidates for vaccines, and the nucleotide substitution mutant studied here, 3NT7C, also reverts at a very low frequency (9a). The combination of several deletion mutations within one virus renders the probability of wild-type reversion very low indeed. Our experience has been that most revertants of deletion mutants are second-site revertants that allow the virus, for instance, to form a plaque at 40°C in the case of temperature sensitivity but do not completely restore the wild-type phenotype. The revertant virus is still attenuated for growth in tissue culture and would be expected to be attenuated in an animal host as well.

The mutations studied here are all in conserved sequence elements that we believe are important for viral RNA replication. Consistent with this view is the fact that the mutants are all defective in RNA synthesis. Surprisingly, the reduction in RNA synthesis is, in general, not as great as the decrease in virus production. In the double mutant 5NT*d*(8,36)/3NT7C, RNA synthesis is reduced 10-fold, whereas production of progeny virus is reduced 2 orders of magnitude. This difference may reflect differences in the concentration of viral RNA that is required for packaging versus the concentration required for transcription and translation.

For the single mutations, the effects seen in tissue culture correlated only imperfectly with the effects observed in mice. The most consistent predictor of attenuation in mice was the inability of a virus to form a plaque at the elevated temperature (40°C) in tissue culture cells, in that all of the temperature-sensitive mutants examined had greatly reduced virulence. The significance of this observation may be related to the body temperature of young mice, which is known to be 39°C. Moreover, the greater the attenuation in tissue culture, the higher the probability of attenuation in mice. Finally, three of four double mutants tested were greatly attenuated.

Live attenuated virus vaccines have been produced classically by passing the virus in an unnatural host in order to select avirulent variants. The development of full-length cDNA clones from many RNA viruses has made it possible to compare the nucleotide sequences of virulent viruses and the vaccine strains derived from them (3, 7, 24) and to determine, by constructing chimeric viruses, which of the changes are responsible for or contribute to the attenuation of the vaccine virus (2, 18, 25). Many of the attenuating mutations cause amino acid substitutions in the structural proteins of the virus and probably modulate virus entry into particular populations of host cells, thereby changing tissue tropism, but some attenuating mutations that map to control elements in nontranslated domains have been found (4, 18). The next generation of vaccines may well consist of viruses containing attenuating mutations selected to change the tissue tropism of the virus coupled to deletion mutations such as the ones studied here to further down-regulate the rate of virus replication (8). It is noteworthy that many of these viruses are severely restricted for growth in mosquito cells, and in particular the double mutants 5NT*d*(10-14)/3NT*d*(9-14) and 5NT*d*(8,36)/3NT7C seem to be totally unable to replicate in mosquito cells. Use of such a mutant as a live alphavirus vaccine would ensure that the obligate mosquito-vertebrate-mosquito cycle of natural transmission was interrupted, and thus virus replication would be restricted to the initial vaccine.

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