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Mutagenesis of the 3′ Nontranslated Region of Sindbis Virus RNA

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A cDNA clone from which infectious RNA can be transcribed was used to construct 42 site-specific mutations in the 3′ nontranslated region of the Sindbis virus genome. The majority of these mutations were made in the 3′-terminal 19-nucleotide conserved sequence element and consisted of single nucleotide substitutions or of small (1 to 8) nucleotide deletions. An attempt was made to recover mutant viruses after transfection of SP6-transcribed RNA into chicken cells. In most cases, viable virus was recovered, but almost all mutants grew more poorly than wild-type virus when tested under a number of culture conditions. In the case of mutations having only a moderate effect, the virus grew as well as the wild type but was slightly delayed in growth. Mutations having a more severe effect led to lower virus yields. In many cases, virus growth was more severely impaired in mosquito cells than in chicken cells, but the opposite phenotype was also seen, in which the mutant grew as well as or better than the wild type in mosquito cells but more poorly in chicken cells. One substitution mutant, 3NT7C, was temperature sensitive for growth in chicken cells and severely crippled for growth in mosquito cells. Insertion mutations were also constructed which displaced the 19-nucleotide element by a few nucleotides relative to the poly(A) tail. These mutations had little effect on virus growth. Deletion of large regions (31 to 293 nucleotides long) of the 3′ nontranslated region outside of the 19-nucleotide element resulted in viruses which were more severely crippled in mosquito cells than in chicken cells. From these results, the following principles emerge. (i) The entire 3′ nontranslated region is important for efficient virus replication, although there is considerable plasticity in this region in that most nucleotide substitutions or deletions made resulted in viable virus and, in some cases, in virus that grew quite efficiently. Replication competence was particularly sensitive to changes involving the C at position 1, the A at position 7, and a stretch of 9 U residues punctuated by a G at position 14. (ii) The panel of mutants examined collectively deleted the entire 3′ nontranslated region. Only mutants in which 8 nucleotides in the 3′ terminal 19 nucleotides had been deleted or in which the 3′ terminal C was deleted were nonviable. Although the 3′ terminal C was essential for replication, it could be displaced by at least 7 nucleotides from its 3′ terminal position adjacent to the poly(A) tract. (iii) The sizes of the plaques produced by different mutants could not be correlated with the growth rates of the mutants, as determined in one-step growth curves. (iv) The differential host effects observed suggest that host proteins interact with the 3′ nontranslated sequence. (v) The sequence of the 3′ nontranslated region found in virus isolated from nature appears to represent a compromise between sequences that allow the most efficient replication in mosquitoes and those most efficient for replication in vertebrates, which provides the optimal solution for alternating between these two hosts.

The 25 currently recognized alphaviruses form a group of closely related viruses whose proteins share from 30 to 80% sequence similarity, depending upon the viruses or proteins compared (22, 23). The RNA genomes of alphaviruses, which vary from 11.4 to 11.8 kilobases in length, serve as messengers for the four nonstructural proteins, called nsP1, nsP-2, nsP-3, and nsP-4, in order from 5′ to 3′, and are believed to form components of the viral RNA replicase (21). In the current model, nsP4 is the virus polymerase and the other proteins form components of the replicase that allow for discrimination and specificity in the replication of the viral RNA (1, 6, 7). During replication, the viral RNA must be transcribed into a full-length minus strand and the minus strand in turn serves as template for production of genome-length plus-stranded RNA and for transcription of a subgenomic 26S mRNA from an internal start sequence in the minus-strand RNA. The 26S mRNA serves as the messenger for the structural proteins of the virion.

The discovery of conserved nucleotide sequence elements in alphavirus RNAs, 19 to 51 nucleotides in length, led to the hypothesis that these elements act as promoter sequences or replicase-binding sites during replication of the viral RNA and transcription of the subgenomic mRNA (13–16). In this model the replicase binds specifically to these domains in order to initiate transcription of minus strands, of full-length plus strands, or of subgenomic RNAs.

Excluding the poly(A) tail, the 3′ nontranslated region of alphaviruses is 121 to 524 nucleotides in length, depending on the virus (4, 8, 16). At the extreme 3′ terminus adjacent to the poly(A) tail, there is a 19-nucleotide element which is highly conserved among all alphaviruses (14, 16). This sequence element was postulated to play a key role in initiation of minus-strand production from a plus-strand genomic template. Levis et al. (11) showed by deletion mapping that this element was essential for replication and amplification of Sindbis virus defective interfering (DI) RNAs. Upstream of this conserved sequence, the 3′ nontranslated region contains two other features in most alphaviruses: an AU-rich segment near the 3′ end and repeated sequence elements 25 to 72 nucleotides in length. The function of the AU-rich domain and the repeated sequences is unclear. They are not invariably present, and the sequences within the repeated elements are not conserved among different alphaviruses, although short motifs (9 to 12 nucleotides) within the repeats are shared in some instances. Even among geographic variants of the same virus, the

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number of repeated sequence elements has been shown to vary (5).

The development of a full-length clone of Sindbis virus from which infectious RNA can be transcribed in vitro (17) allows us the opportunity to define more precisely the function of the 3′ nontranslated region in general and of the 19-nucleotide conserved sequence element in particular. In this paper we report the construction and characterization of 42 mutations in this region.

MATERIALS AND METHODS

Cells and viruses. All virus propagation was performed on secondary chicken embryo fibroblast (CEF) monolayers maintained in Eagle minimal essential medium supplemented with 3% fetal calf serum. Mosquito cell lines (*Aedes albopictus*) C6/36 and C7/10 were obtained from the American Type Culture Collection and Dennis Brown (The Cell Research Institute, University of Texas at Austin), respectively, and propagated as previously described (3). Site-directed mutagenesis was performed on the full-length cDNA clone pToto50 (17) to insert a unique *Bst*EII restriction site in the region near the stop codon of the structural polyprotein at nucleotide 11380 (nucleotide numbers given for restriction sites will denote the 5′ end of the recognition sequence) (21). The recovered virus, E1-11381G, was indistinguishable from Sindbis HRSP virus, the heat-resistant, small-plaque wild-type virus derived from pToto50, and was designated Toto52. It was used as the wild-type parental virus for all further studies described in this paper. As described in detail below, pToto52 contains the vector sequences of pToto1101.

Nomenclature of virus mutants. We propose a nomenclature for alphavirus mutants which have been generated by site-directed mutagenesis of full-length cDNA clones. The gene product or genome location will be listed first. Examples are: 5NT (5′ nontranslated region), 3NT (3′ nontranslated region), P1 (nonstructural protein 1), E1 (envelope glycoprotein 1), etc. For substitutions, the location of the nucleotide change and the nucleotide present in the mutant will then follow. For deletions, the mutants are designated with an italicized *d* followed by parentheses enclosing the numbers of the nucleotides deleted. For insertions, an italicized *i* is followed by the location of the insertion, a slash (/), and the number of nucleotides inserted. For mutations mapping to the 3′ nontranslated region, numbering will start at the 3′ ultimate nucleotide adjacent to the poly(A) tract and follow 3′ to 5′. Names beginning with a small p refer to cDNA clones, whereas names without the p refer to the virus derived from these clones. For consistency and ease of comprehension, we will also distinguish between Toto clones and virus derived from these clones in the same way. Thus, p3NT7U refers to a full-length plasmid in which a uridine residue has been substituted for the parental nucleotide at position 7 in the 3′ nontranslated region, and 3NT*d*(9-14) is virus rescued from a construct in which nucleotides 9 through 14 of the 3′ nontranslated region have been deleted. Although not relevant for the results presented here, we note that changes resulting in an amino acid substitution can be indicated by the nucleotide substitution effected or the resulting amino acid change (numbering from the NH_2 terminus of the protein) or both.

Transfections and plaque assays. Full-length mutagenized derivatives of pToto52 were prepared for transcription by linearization with the appropriate restriction enzyme, usually *Xho*I. Transcription by SP6 RNA polymerase was carried out essentially as previously described (17). Template DNA was not normally removed after transcription. The transcript RNA was introduced into susceptible secondary CEF monolayers by using DEAE-dextran. A 1% agarose (SeaKem ME; FMC Corp., Marine Colloids Div., Rockland, Maine) overlay containing Eagle minimal essential medium supplemented with 2% fetal calf serum was added to the cells, and they were then incubated for 48 h at 30 or 40°C. Plaques were visualized by staining with neutral red, and mutant virus was normally isolated after 2 to 3 successive rounds of plaque purification. For production of virus stocks, the plaque-purified virus was grown in a T25 flask for 14 to 18 h at 30°C in CEF monolayers and the titer of the resulting stock was determined on CEF monolayers as described previously (20).

Plasmid constructions and site-directed mutagenesis. All mutants in the 3′ nontranslated region of Sindbis virus were constructed in a similar manner. Briefly, a *Stu*I (nucleotide 10770)-*Eco*RI (nucleotide 11743) fragment from the full-length cDNA clone pToto52 was subcloned into the *Sma*I and *Eco*RI restriction sites of an M13mp19 vector. Following growth of the M13mp19 derivative in *Escherichia coli* BW313 (10), site-directed mutagenesis with synthetic oligonucleotides was performed as described previously (24). For the nucleotide substitutions, the synthetic oligonucleotides were degenerate at the appropriate nucleotide. Positive clones were identified by dideoxynucleotide sequencing (19) by using cloned T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, Ohio). After digestion with the restriction enzymes *Sal*I (nucleotide 11087) plus *Eco*RI (nucleotide 11743), the mutagenized fragment was purified by electrophoresis in low-melting-temperature agarose and was recloned into the pToto50 background in a four-fragment ligation. The other fragments used in this ligation were: a *Sac*I (nucleotide 13552)-*Bss*HII (nucleotide 9804) fragment from pToto50; a *Bss*HII (nucleotide 9804)-*Sal*I (nucleotide 11087) fragment from pToto50; and an *Eco*RI (nucleotide 11743)-*Sac*I (nucleotide 13552) fragment from pToto1101. This last fragment from pToto1101 contributes only vector sequences to the new clone and was included because the restriction sites in pToto1101 were preferable to those found in pToto50. The full-length cDNA clone containing the desired mutation was isolated, and the mutation was confirmed by sequencing the double-stranded DNA (2).

The large deletion clones in the 3′ nontranslated region were constructed by using standard procedures (12). In addition to pToto52, which was mentioned above, full-length clones were constructed after oligonucleotide mutagenesis in M13, which inserted new restriction sites in the 3′ nontranslated region. p3NT52G inserted a *Pst*I site at nucleotide 11647; p3NT26T28G inserted an *Hpa*I site at nucleotide 11676; p3NT6A inserted a *Dra*I site at nucleotide 11697; and p3NT*i*1/3 inserted an *Xba*I site at nucleotide 11702. These new restriction sites were then used to construct specific deletions in the 3′ nontranslated region.

To produce 3NT*d*(26-56), p3NT52G was digested with *Pst*I and the 3′ overhang was removed by treatment with T4 DNA polymerase. The *Pst*I site was then joined to the *Hpa*I site of p3NT26T28G in a four-fragment ligation. p3NT*d*(polyA-4) was constructed by a fill in of the *Eco*RI site at nucleotide 11743 with the large fragment of *E. coli* polymerase I (Klenow) to generate a blunt end; this end was then ligated to the blunt end of the *Dra*I site found in p3NT6A at nucleotide 11697. For the construction of p3NT*d*(53-318), the *Bst*EII site of pToto52 (nucleotide 11380) was filled in with Klenow to create a blunt end. The

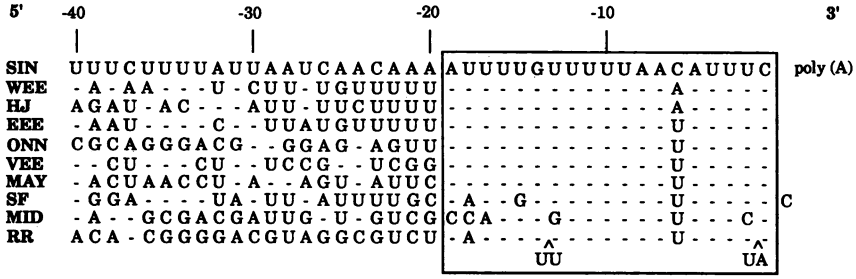


FIG. 1. Conserved sequences at the 3′ terminus in alphavirus RNAs. The genomic RNA is shown 5′ to 3′; nucleotides are numbered 3′ to 5′ from the poly(A). A dash indicates that the nucleotide is the same as that in the top line. The 19-nucleotide conserved element is boxed. Although not shown, all viruses are polyadenylated at their 3′ termini. Virus abbreviations are: SIN, Sindbis; WEE, Western equine encephalitis; HJ, Highlands J; EEE, Eastern equine encephalitis; ONN, O'Nyong-nyong; MAY, Mayaro; VEE, Venezuelan equine encephalitis; SF, Semliki Forest; MID, Middelburg; and RR, Ross River. Sequence data are from Ou et al. (16) for SIN, WEE, HJ, EEE, VEE, SF, and MID; Dalgarno et al. (4) for RR; R. Levinson, J. H. Strauss, and E. G. Strauss (Virology, in press) for ONN; and J. Dalrymple (personal communication) for MAY.

*Pst*I site of p3NT52G (nucleotide 11647) was blunt ended by treatment with T4 DNA polymerase. The two blunt ends were then joined in a two-fragment ligation. In a similar fashion, p3NT*d*(26-318) was generated by ligating the blunt-ended *Bst*EII site from above with the *Hpa*I site of p3NT26T28G. The construction of p3NT*d*(2-318) also used the blunt-ended *Bst*EII site of pToto52. This end was ligated to the *Xba*I site of p3NT*i*1/3 at nucleotide 11702 after the 5′ overhang was filled in with Klenow to produce a blunt end. Finally, p3NT*d*(polyA-318) was constructed in a two-fragment ligation by joining the *Bst*EII site of pToto52 with the *Eco*RI site (nucleotide 11743) of the same plasmid after both had been treated with Klenow to produce blunt ends.

Preparation of viral RNA and nucleotide sequencing. CEF monolayers were infected at a multiplicity of infection of 50 with the different mutants. At 7 h postinfection, the infected monolayers were washed twice in ice cold phosphate-buffered saline, and then phosphate-buffered saline containing 1% Nonidet P-40 was added. The cells were scraped from the plate, and nuclei and unbroken cells were removed by centrifugation at 600 × *g* for 10 min. The supernatant was recovered, and sodium dodecyl sulfate was added to a final concentration of 1%. Following two phenol-chloroform extractions, the RNA was precipitated by the addition of ethyl alcohol. Dideoxynucleotide sequencing with reverse transcriptase was then performed directly on total cytoplasmic RNA by using a $(dT)_{10}dGdA$ primer (5a).

Alternatively, for viral RNAs with mutations lying close to the 3′ end, cDNA was synthesized complementary to the 3′ end of the genomic RNA by using the $(dT)_{10}dGdA$ primer (18). The cDNA was sequenced by the procedure described for double-stranded DNA by using a synthetic primer which binds to the Sindbis virus cDNA at nucleotides 11571 to 11588 (using plus-strand numbering) (2). The annealing step in this procedure (5 min; 85°C; in 0.2 M NaOH) effectively removes the original RNA template.

One-step growth analysis. Differential growth curves were performed by infecting confluent secondary CEF monolayers or C6/36 mosquito cell monolayers (35-mm plate) at a multiplicity of infection of 5 in phosphate-buffered saline containing 1% fetal calf serum. Following 1 h of incubation at room temperature, the inoculum was removed, Eagle minimal essential medium containing 2% fetal calf serum was added, and the cells were incubated at the appropriate temperature. 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 secondary CEF monolayers at 30°C. The results shown are averages of at least two independent growth experiments.

RESULTS

The 3′ nontranslated region of Sindbis virus. The high degree of conservation found in the 3′ terminal 19 nucleotides of the alphaviruses suggests that this region may function as a replicase-binding site for the initiation of minus-strand RNA synthesis (16). The sequences of several alphaviruses are compared in Fig. 1. The 3′ terminal 19 nucleotides are invariant in most alphaviruses, with the exception of position 6 [numbering is 3′ → 5′ from the nucleotide preceding the poly(A) tract]. Studies in which DI RNAs were used have shown conclusively that functional DIs require precisely the 19-nucleotide region (11). Deletion of two nucleotides into the 5′ end of the 19 nucleotide region was sufficient to render the DI RNA inactive for replication.

Based on these data, we undertook a molecular genetic approach to investigate the function of this conserved region. Our strategy was to construct a series of nucleotide substitutions in and around the 3′ terminal 19-nucleotide conserved region by using a full-length cDNA clone of Sindbis virus. A fragment of the cDNA encoding the 3′ nontranslated region was subcloned into an M13mp19 vector, and site-directed oligonucleotide mutagenesis was performed. After isolation of the mutant and its subsequent replacement into the full-length Sindbis virus cDNA, RNA transcripts were synthesized from linearized templates. The RNA transcripts were then introduced into susceptible secondary CEF by transfection with DEAE-dextran, and the resulting virus was characterized.

Construction of pToto52. To facilitate the construction and identification of the mutants to be described below, a unique *Bst*EII restriction site was created in the full-length cDNA clone pToto50 (17) by changing the last codon of the structural polyprotein AGA (R) to AGG (R) (mutant pE1-11381G). The vector sequences of pToto1101 were also inserted into the new clone, designated pToto52. Following linearization with *Xho*I, RNA was synthesized by using SP6 polymerase and was transfected into cells. The resulting Toto52 virus was compared with the parental Sindbis HRSP virus derived from Toto50 and found to be identical in plaque morphology and efficiency of plating at 30, 37, and 40°C. The growth kinetics of the two viruses were also identical. The original M13 subclone which contained this *Bst*EII site was

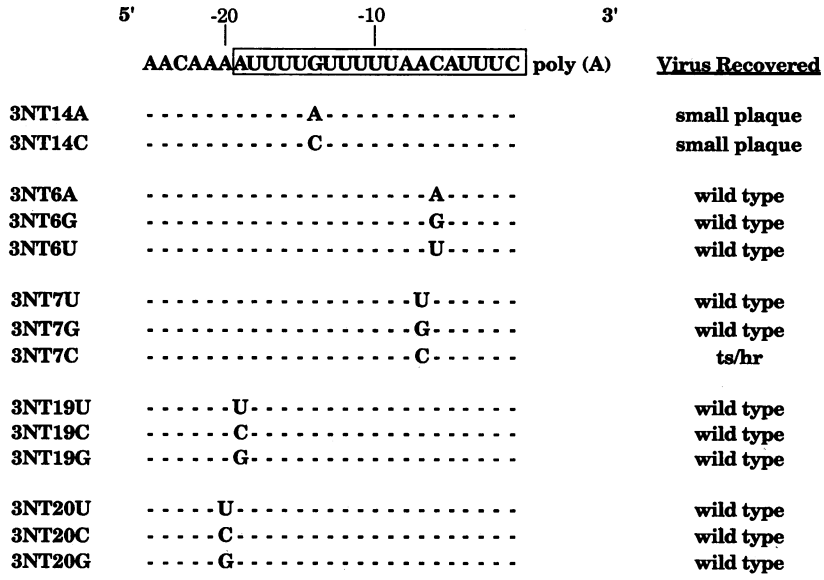


FIG. 2. Nucleotide substitutions in the 3'-terminal 19-nucleotide conserved element. The sequence of the 3' terminal 25 nucleotides of Sindbis virus genomic RNA is shown at the top from 5' to 3'. Numbering is 3' to 5' from the poly(A) tract. A dash indicates no change from the wild-type sequence; substitutions are shown directly below the altered nucleotide. The name of the mutant is shown to the left. The 19-nucleotide conserved sequence element is boxed. The phenotype in CEF of the recovered virus is shown to the right of the mutant. ts, Temperature sensitive; hr, host restricted.

then used as a template to generate all further mutants. The presence or absence of the *Bst*EII restriction site was used to screen for mutants after replacement of the mutagenized construct back into the full-length Sindbis virus cDNA. In addition, the site was used to generate site-specific deletion mutations within the 3' nontranslated region.

Construction of substitution mutants. Since position 6 exhibited variability among the alphaviruses, we predicted that a nucleotide substitution at that position would have little or no effect. The same would be true for nucleotide substitutions outside the conserved region. In contrast, position 19, which was essential for DI RNA activity, would probably be sensitive to nucleotide change. These predictions formed our initial strategy for investigating the region, in which nucleotides at positions 6, 7, 14, 19, and 20 were replaced with the three other possible nucleotides (Fig. 2).

Following isolation of the appropriate mutant clone and in vitro synthesis of RNA by using SP6 RNA polymerase, the RNA was serially diluted in phosphate-buffered saline (minus Mg^{2+} and Ca^{2+}) prior to transfection. Three serial dilutions of RNA were transfected in duplicate into chicken cells, and the efficiency of plaque formation was determined at 30 and 40°C. The results are shown in Fig. 2 for all but construct p3NT14U, which gave anomalous results and will be discussed later. For all of these substitution mutants, infectious virus was recovered. Furthermore, the specific infectivity of mutant RNA transcripts was similar to that of the wild-type control (2×10^4 PFU/μg of RNA, ± a factor of 3), suggesting that the mutations which were made were not lethal for the virus and that selection for revertants or for minor RNA variants in the SP6-transcribed population was not occurring.

The results in Fig. 2 show that substitution at position 6, 19, or 20 gave rise to virus that was wild type by the criteria that the efficiency of plating at 30 and 40°C was identical to that of the wild type and that the virus grew to a high titer in liquid culture. Substitution of A or C for G14 gave rise to virus that formed small plaques. Results from mutants altered at position 7 depended upon the change effected. 3NT7U and 3NT7G were pseudo-wild type, whereas 3NT7C was temperature sensitive in chicken cells and host restricted, in that it grew poorly in mosquito cells (see below).

To confirm the presence of the mutation in the progeny virus, virus stocks were prepared from plaque-purified virus and RNA isolated from virus-infected cells was sequenced through the region containing the change by chain-terminating methods. In the case of all the mutants in Fig. 2, the RNA sequence was as predicted.

Growth curve analysis of substitution mutants. To examine the effects of the mutations more closely, mutants were tested in one-step differential growth experiments for their ability to produce virus compared with a wild-type control. Mutant stocks were prepared and used to infect confluent monolayers of cells (either CEF or mosquito C6/36) at a multiplicity of infection of 5. After a 60-min adsorption period, the cells were washed to remove free virus. They were then incubated at the appropriate temperature with a change of medium every hour. The virus present in the medium was determined by plaque titration in CEF at 30°C. This experiment measures the rate of infectious virus release each hour. One-step growth experiments were performed both in C6/36 mosquito cells at 30°C and in CEF at 40°C (and in some cases 30°C as well) to examine possible differences in the rate of replication in cells from two natural hosts. In each case, the growth curve analysis was done two or more times to ensure the reproducibility of the results and the curves presented represent averages of two or more independent experiments.

The substitutions at position 6, tested because this position is variable in alphaviruses, produced virus with growth characteristics similar to those of the wild type in both cell lines, in that the mutants grew well and produced high yields of virus (Fig. 3A and B; mutants 3NT6A, 3NT6G, and 3NT6U). However, all three mutants showed some impairment in growth. 3NT6A and 6U were delayed by about 1 h in both cell lines; the rate of virus production eventually

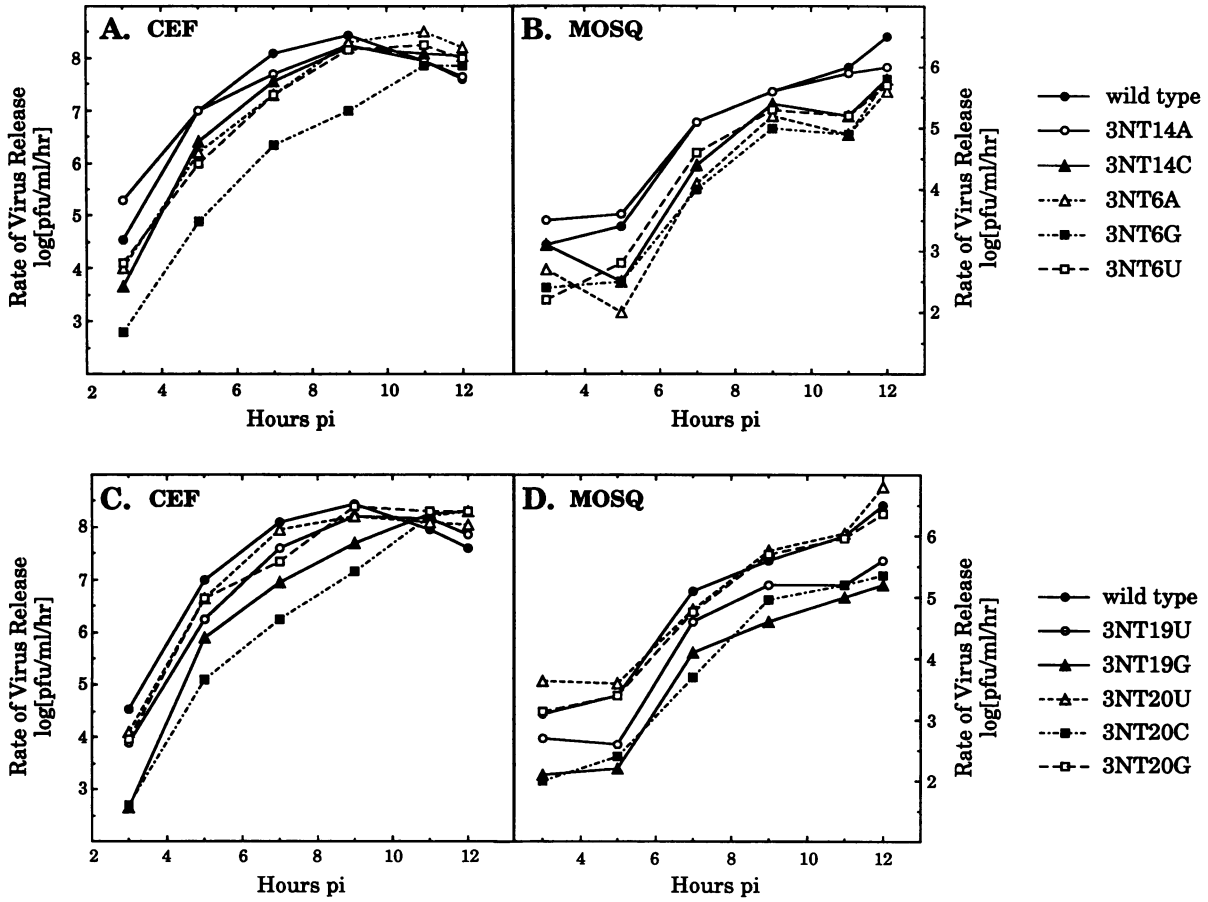


FIG. 3. One-step differential growth curves for the 3′-terminal 19-nucleotide substitution mutants. Growth curve experiments were performed as described in Materials and Methods. Wild type refers to virus rescued from the pToto52 construction. Substitution mutants were tested in CEF at 40°C (A and C) and in C6/36 mosquito cells at 30°C (B and D). Symbols for the viruses are shown in the corresponding legends to the right of the graphs. Results are expressed as log (PFU per milliliter per hour) released as measured by plaque assays on CEF monolayers at 30°C.

reached wild-type levels (at 8 h) in chicken cells but never exceeded 10% of wild-type levels in mosquito cells. Mutant 3NT6G grew less well in chicken cells than 6A or 6U, being delayed by 3 to 4 h and growing to a lower yield, although in mosquito cells it grew about as well as the other two mutants.

Nucleotide substitution at position 14 was performed to test the importance of the G residue in the middle of the conserved oligo(U) tract. 3NT14A was indistinguishable from the wild type, whereas 3NT14C exhibited slightly reduced growth kinetics in chicken and mosquito cells (Fig. 3A and B). The virus rescued from the p3NT14U mutant produced small plaques in chicken cells and reduced growth kinetics in mosquito cells. Following sequencing of the virus-specific RNA, it was apparent that this RNA had undergone a deletion of four nucleotides (ΔU_4). The growth properties of this virus, 3NT*d*(11-14), will be described in greater detail below. Since the full-length cDNA p3NT14U had the correct sequence, the deletion must have been generated at a later step. Sequencing of the SP6 RNA transcripts from p3NT14U revealed that the majority of the transcripts had deletions around position 14 and indicated that the deletion event was an SP6 RNA polymerase artifact and, also, that the stretch of 10 U residues generated by the insertion of a U for a G at position 14 was not copied faithfully. Transcription of these sequences with T7 RNA polymerase resulted in the same deletion (data not shown).

Mutations at position 19, at the 5′ border of the 19-nucleotide region, produced virus similar to the position 6 mutants (Fig. 3C and D; mutants 3NT19U and 3NT19G). The viruses grew well but were delayed in chicken cells and grew to lower titer in mosquito cells.

The substitutions at position 20 were made because this is outside the 3′ terminal conserved element. 3NT20U and 3NT20G were essentially indistinguishable from the wild type in both cell lines, but 3NT20C was significantly impaired in both cell lines (Fig. 3C and D). In chicken cells, 3NT20C was delayed by 3 to 4 h but it did ultimately produce virus at wild-type rates; in mosquito cells, not only was virus production delayed but the maximal rate of virus production was an order of magnitude less than that in wild-type infected cells.

Mutations changing the A at position 7 to either a U or a G had minor effects on the resulting virus (Fig. 4A, B, and C; mutants 3NT7U and 3NT7G), with the viruses again being somewhat delayed in growth in chicken cells and growing to a lower titer in mosquito cells. However, if the A was substituted with a C in position 7, to give mutant 3NT7C, the recovered virus was temperature sensitive for plaque formation, forming plaques at 30°C but not at 40°C. In one-step

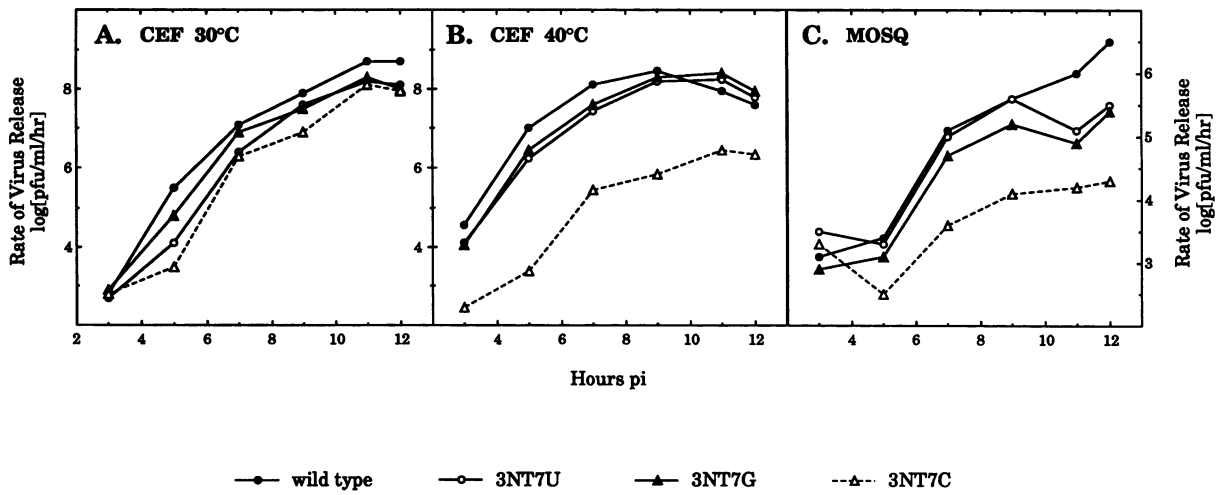


FIG. 4. One-step differential growth curves for the substitution mutants at position 7. Growth curve experiments were performed as described in Materials and Methods. Wild type refers to virus rescued from the pToto52 construction. Substitution mutants were tested in CEF at 30°C (A) and 40°C (B) and in C6/36 mosquito cells at 30°C (C).

growth curves, 3NT7C grew fairly well in chicken cells at 30°C, but at 40°C the production of virus was decreased by more than 2 orders of magnitude (see Fig. 4A and B). In C6/36 mosquito cells at 30°C, its growth was also severely crippled (Fig. 4C); in these cells, the virus grew slowly and never reached the level of virus released in the wild-type control. The ability of the 3NT7C virus to form plaques in mosquito cells (C7/10) was also restricted (data not shown).

In summary, all but one of the substitution mutants in the 19-nucleotide conserved element grew at reduced rates compared with the wild-type virus and these defects in growth were usually more pronounced in mosquito cells than in chicken cells. These results suggest that the host range of the virus may play a role in the conservation of this element. It also appears that the size of the plaques formed by various mutants does not correlate with the kinetics of virus growth. In particular, 3NT14A is essentially indistinguishable from the wild type in growth curves but forms small plaques, whereas other mutants form wild-type plaques but grow more slowly (see also below). We have reached similar conclusions from studies of mutants in the 5′ end of Sindbis virus (12a). Why this should be is not understood, but it is clear that simply screening mutants by plaque assay can be misleading.

Construction of deletion mutants in the 19-nucleotide domain. The data provided by the nucleotide substitutions suggested a greater plasticity in tissue culture cells than had been predicted for the 3′-terminal 19-nucleotide conserved region. To investigate this more thoroughly, a panel of nucleotide deletions was constructed within the 19-nucleotide conserved region.

Figure 5 shows the deletion mutants which were made and the phenotypes of the recovered viruses. Surprisingly, deletions of up to 6 nucleotides within the 19-nucleotide region can be made and virus can be recovered. As with the substitution mutants, the specific infectivities for most of the deletion mutant RNAs were within threefold of the wild type; the exceptions will be discussed below. Moreover, none of these deletion mutants were temperature sensitive, as measured by the efficiency of plating at 30 and 40°C on chicken cells. Deletions constructed at the 5′ border of the conserved sequence region [3NT*d*(20-25), *d*(18-25), and *d*(18-19)], as well as single point deletions at position 6 or 7, produced normal-size plaques on CEF monolayers. Deletions within the uridine-rich region (nucleotides 9 through 18) resulted in viruses producing small plaques, as did simultaneous deletion of the two A residues at nucleotides 7 and 8. This latter deletion was combined with 3NT*d*(9-14) to generate p3NT*d*(7-14). Two independent clones of p3NT*d*(7-14) have failed to produce virus.

The final set of deletion mutants constructed in the 19-nucleotide conserved region were at the very 3′ end of the genome, directly adjacent to the poly(A) tract. Virus was not recovered after DEAE-dextran transfection of the transcript RNA into chicken cells from any mutant in which the 3′ terminal C was deleted.

Growth curve analysis of deletion mutants. The ability of these deletion mutants to grow, as measured by one-step differential growth experiments, differed markedly depending on the host cell tested. Some viruses grew poorly in both chicken cells and mosquito cells, whereas others showed a preference for one cell type over another. Virus 3NT*d*(20-25) grew well in chicken cells at 40°C, exhibiting only a slight delay compared with the wild type (Fig. 6A). In mosquito cells, it lagged considerably behind the wild type and at the end of 12 h was producing virus at only one-third the level of the wild type (Fig. 6B). Extending the deletion by two nucleotides into the 19-nucleotide conserved region produced a virus, 3NT*d*(18-25), which grew substantially more slowly and to a lower yield in chicken cells, although its growth in mosquito cells was nearly identical to that of 3NT*d*(20-25) (Fig. 6A and B). Virus 3NT*d*(18-19), in which only nucleotides 18 and 19 were deleted, grew significantly less well than 3NT*d*(18-25) in mosquito cells and chicken cells, even though 3NT*d*(18-25) also had those two nucleotides deleted (Fig. 6A and B). Deletion of an additional two nucleotides within the 19-nucleotide conserved region produced a virus, 3NT*d*(16-19), which grew better than 3NT*d*(18-19) and 3NT*d*(18-25), but still not as well as the wild type in chicken cells at 40°C (Fig. 6A). Surprisingly, 3NT*d*(16-19) grew better than the wild-type virus in C6/36 mosquito cells (Fig. 6B). If 6 nucleotides starting from the 5′ boundary of the 19-nucleotide region were deleted, the rescued virus, 3NT*d*(14-19), grew slightly slower than 3NT*d*(16-19) in chicken cells (Fig. 6A). In mosquito cells, 3NT*d*(14-19) grew at a rate similar to that of the wild-type

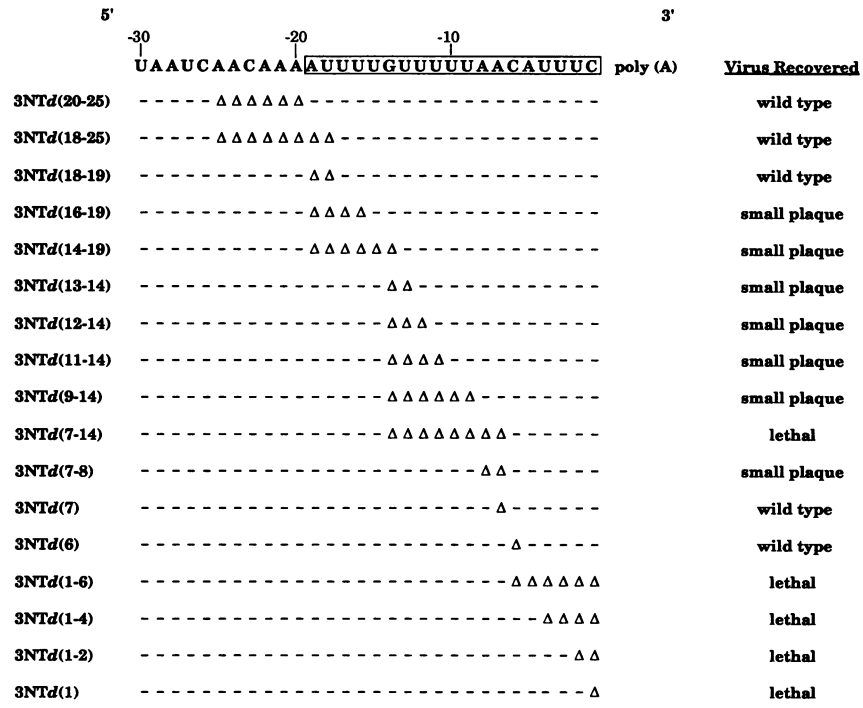


FIG. 5. Nucleotide deletion mutants in the 3′ terminal region of Sindbis virus RNA. The sequence of the 3′ terminal 30 nucleotides of Sindbis virus genomic RNA is shown at the top from 5′ to 3′. Numbering is 3′ to 5′ from the poly(A) tract. A dash indicates no change from the wild-type sequence. A Δ indicates the deletion of the nucleotide by mutagenesis. The name of the mutant is shown to the left. The 19-nucleotide conserved element is boxed. The phenotype of the recovered virus in CEF is shown to the right of the mutant. Lethal indicates a failure to rescue infectious virus after DEAE-dextran transfection.

virus (Fig. 6B). Thus, it appears that the overall structure of the 3′ region is important and that the effect upon context induced by the various deletions is unpredictable at present. It also appears that the nucleotide sequence present in the 3′ sequence element is a compromise, since at least some changes enable the virus to grow better than the wild type in mosquito cells.

A single point deletion at nucleotide 14 (G) was predicted to have an effect similar to the substitution of the residue with a U (p3NT14U). The G residue interrupts a stretch of U residues. Substitution or deletion of the G creates a sequence of at least nine contiguous U residues, which might be expected to perturb the function of this region. The deletion mutant p3NT*d*(14) was constructed, and a virus was recovered following transfection of the mutant RNA. Analysis of the nucleotide sequence of the rescued viral RNA showed the deletion of the G residue, along with an additional deletion of three U residues. This virus, 3NT*d*(11-14), had the same deletion which was present in the substitution mutant derived from p3NT14U described previously. Sequencing of the SP6 RNA transcript confirmed that the deletion occurred during in vitro transcription. The specific infectivity of the RNA transcript was similar to that of the wild type, suggesting, along with the sequence analysis, that the majority of RNA transcripts produced by the SP6 polymerase contained the exact deletion found in the viral RNA and that selection for minor RNA variants following transfection did not occur. The precision of the observed deletion suggests that the phenomenon studied here differs from that observed by Konarska and Sharp (9), who reported slippage of SP6 RNA polymerase during in vitro transcription of a polypyrimidine stretch which resulted in a heterogeneous length of oligo(U) synthesized.

Growth curves from four deletion mutants having a 5′ boundary at the G at position 14 and proceeding 3′ are shown in Fig. 6C and D. All four viruses, 3NT*d*(13-14), 3NT*d*(12-14), 3NT*d*(11-14), and 3NT*d*(9-14), released virus in chicken cells at rates significantly slower than the wild-type virus at both 30 (data not shown) and 40°C (Fig. 6C). In C6/36 mosquito cells, 3NT*d*(13-14), 3NT*d*(12-14), and 3NT*d*(11-14) were also substantially reduced in growth, while 3NT*d*(9-14) behaved similarly to the wild type (Fig. 6D). These four viruses, together with 3NT*d*(14-19), form a series in which G14 has been removed and the resulting U tract varies from 4 to 8 residues. Mutant 3NT*d*(14-19) also has A19 deleted, but since nucleotides 20, 21, and 22 are all A's, the deletion of A19 per se should have little effect on the resulting virus. Within this series, the general rule is that the shorter the U tract, the better the virus grows [U_4 in 3NT*d*(9-14) > U_5 in 3NT*d*(14-19) > U_6 in 3NT*d*(11-14) > U_7 in 3NT*d*(12-14) > U_8 in 3NT*d*(13-14)], although the exact order is slightly different in the two different cell lines. In fact, 3NT*d*(13-14) grew very poorly, producing some 3 orders of magnitude less virus and lagging by about 4 h. As noted above, viruses with 9 or 10 U's could not be produced because of the inability of RNA polymerases to transcribe this region.

Interestingly, deletion of the A residue at position 7 produced a virus, 3NT*d*(7), which was similar to the wild-type virus in growth properties (data not shown). Thus substitution of A7 by C produced a temperature-sensitive virus (3NT7C), as described above, but deletion had very little effect. It should be pointed out, however, that deletion of position 7 cannot be distinguished from a deletion at position 8, since both nucleotides are A residues. If the A residues at both positions 7 and 8 were removed, the virus that was rescued, 3NT*d*(7-8), grew well at 30°C in both CEF

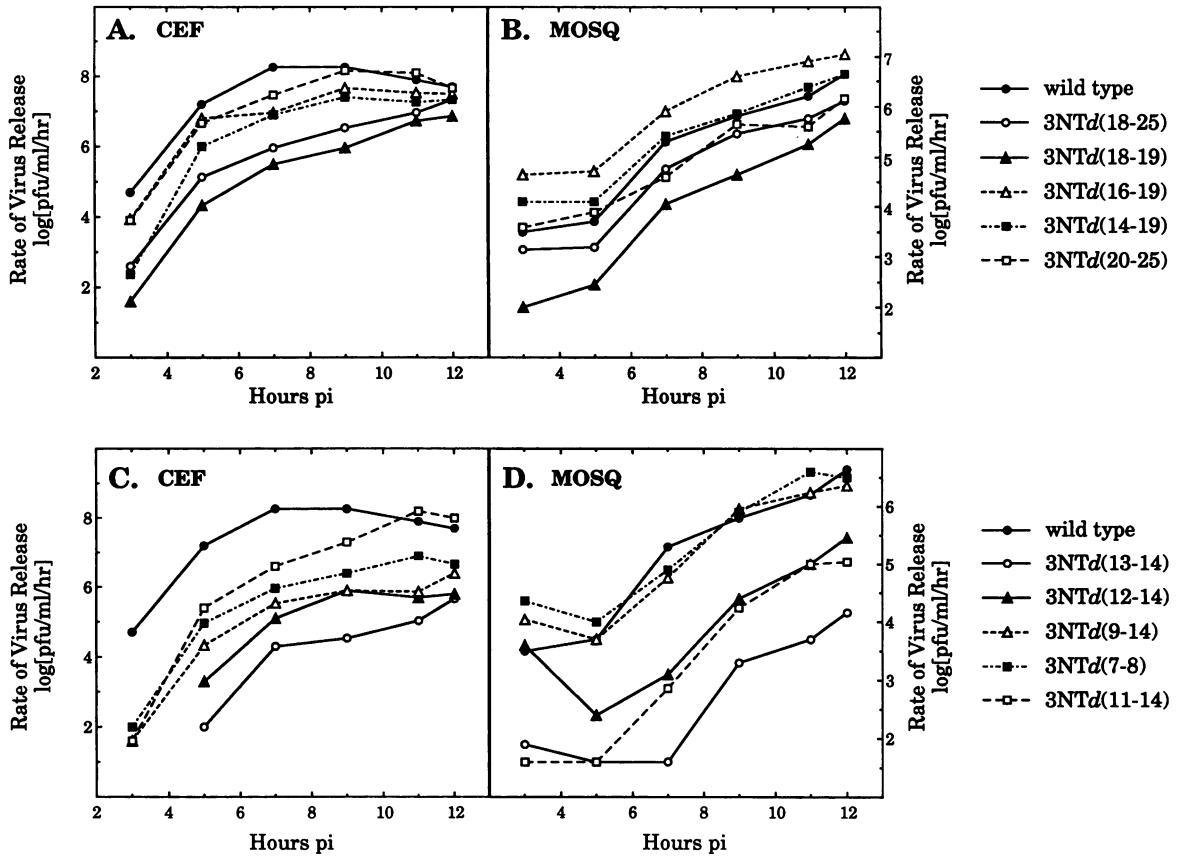


FIG. 6. One-step differential growth curves for deletion mutants in the 3'-terminal 19-nucleotide sequence element. Growth curve experiments were performed as described in Materials and Methods. Wild type refers to virus rescued from the pToto52 construction. Deletion mutants were tested in CEF at 40°C (A and C) and in C6/36 mosquito cells at 30°C (B and D). Symbols for the viruses are shown in the corresponding legends to the right of the graphs.

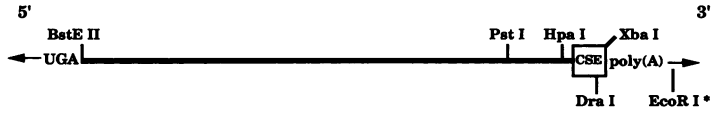
(not shown) and C6/36 cells (Fig. 6D) but grew poorly in CEF at 40°C (Fig. 6C). Position 6, which shows variation in the alphaviruses sequenced, can be deleted with no significant effect on virus growth [3NT*d*(6); data not shown].

Construction and isolation of insertion mutants. Because the 19-nucleotide conserved sequence element is followed by a 3' terminal poly(A) tract in all alphaviruses, it is possible that the poly(A) tract is a component of the recognition sequence. To investigate whether the sequences within the 19-nucleotide conserved sequence element had to be closely coupled to the poly(A) tract, nucleotides were inserted between them. We first inserted three nucleotides, UAG, immediately before the poly(A) tract, which resulted in the creation of a unique *Xba*I restriction site, while leaving the sequence of the 19-nucleotide element intact. The virus rescued from this clone, 3NT*i*1/3, grew nearly as well as the wild-type virus (data not shown). This unique restriction site at the junction between the poly(A) tract and the 19-nucleotide conserved sequence was then used to insert additional nucleotides. p3NT*i*1/3 was linearized at the *Xba*I restriction site, and the 5' overhang was filled in with the large fragment of *E. coli* polymerase I (Klenow) and ligated, resulting in the insertion of an additional four nucleotides. The resulting virus, 3NT*i*1/7, produced slightly smaller plaques but produced virus at a rate comparable to that of the wild type. These results show that the distance of the poly(A) from the 19-nucleotide conserved element can be varied within the limits tested with little effect on virus replication. This result is of particular interest in view of the finding that deletion of the 3' terminal C was lethal (Fig. 5).

Construction of large deletions in the 3' nontranslated region. To investigate the importance of the different domains in the 3' nontranslated region for virus growth, a series of viruses containing large deletions were constructed. For this, we inserted a number of restriction sites within the 3' nontranslated region, some of which have been described earlier (Fig. 7). In all cases, mutants containing the engineered restriction site alone were tested for their growth kinetics. When pToto52 was digested with *Bst*EII and the 5' overhang was filled in with Klenow, the UGA stop codon was retained and was followed by a single C. Blunt-end ligation allowed deletion of the downstream 3' nontranslated sequence without disruption of the C-terminal arginine residue of glycoprotein E1 or of the UGA stop codon. Two other restriction sites were created, which inserted a *Pst*I site at nucleotide 11647 (mutant p3NT52G) and an *Hpa*I site at nucleotide 11676 (mutant p3NT26T28G). The creation of a *Dra*I site within the 19-nucleotide conserved region at nucleotide 11697 occurred in nucleotide substitution clone p3NT6A (the growth properties of this mutant were described above). Finally, creation of an *Xba*I site at nucleotide 11702, which separates the 19-nucleotide region from the poly(A) tract, was described above.

The *Pst*I site at nucleotide 11647 lies at the 5' boundary of the AU-rich region. Deletion of the sequences between the *Pst*I site and *Hpa*I site (nucleotide 11676) removed most of

3' Nontranslated Region Deletion Mutants



Name	Deletion	Size (nt)	Viable
3NT*d*(26-56)	*Pst*I (11647) - *Hpa*I (11676)	31	yes
3NT*d*(polyA-4)	*Dra*I (11697) - *Eco*RI (11743)	44◆	no
3NT*d*(53-318)	*Bst*EII (11380) - *Pst*I (11647)	266	yes
3NT*d*(26-318)	*Bst*EII (11380) - *Hpa*I (11676)	293	yes
3NT*d*(2-318)	*Bst*EII (11380) - *Xba*I (11702)	317	no
3NT*d*(polyA-318)	*Bst*EII (11380) - *Eco*RI (11743)	358◆	no

FIG. 7. Deletion mutants in the 3' nontranslated region of Sindbis virus genomic RNA. The schematic at the top shows the 3' nontranslated region as found in the pToto50 cDNA clone. The UGA stop codon at the 5' end punctuates the end of the structural polyprotein. The 19-nucleotide conserved sequence element (CSE) is represented by the box. Newly engineered restriction sites are indicated. The nucleotide numbers given for restriction sites denote the 5' end of the recognition sequence. The asterisk (*) indicates the preexisting *Eco*RI restriction site. This site is found immediately 3' of the poly(A) tract at nucleotide 11743. The diamond (◆) indicates that the size of the deletion includes 37 A residues present in the poly(A) tract of the cDNA clone. The table underneath lists the deletions which were made and whether infectious virus was recovered. Details of the constructions are given in Materials and Methods.

the AU-rich sequence, with the exception of the 19-nucleotide conserved sequence and 6 adjacent nucleotides (see Fig. 7). The rescued virus 3NT*d*(26-56) produced slightly smaller plaques than the wild type in chicken cells, and its growth was reduced in C6/36 mosquito cells (Fig. 8B). Deletion of the nontranslated sequence upstream of the AU-rich region to produce mutant 3NT*d*(53-318) had a greater effect on virus growth. This deletion removed about 84% of the sequences in the 3' nontranslated region of Sindbis virus and covered the region between the *Bst*EII site and the *Pst*I site. Virus produced from this deletion clone produced smaller plaques at 30 and 40°C in chicken cells. Its growth was impaired at both temperatures, but a reduction in growth rate was most clearly seen in mosquito cells (Fig. 8B). A larger viable deletion which removed an additional sequence in the 3' nontranslated region was also generated. This mutant, 3NT*d*(26-318), had 92% of the sequences in the 3' nontranslated region removed. It possessed a 3' nontranslated region of only 26 nucleotides: 25 nucleotides from the 3' terminus of the genome and 1 nucleotide 3' of the UGA termination codon of the structural polyprotein. Mutant 3NT*d*(26-318) produced smaller plaques and grew more slowly than either 3NT*d*(53-318) or 3NT*d*(26-56). As seen in Fig. 8, although this virus grows moderately well in chicken cells at 40°C, it is severely restricted in mosquito cells.

Two very large deletions from which viable virus could not be recovered were also constructed. By using the *Xba*I restriction site of p3NT*i*1/3 and the *Bst*EII site, a deletion was generated which removed all but two of the original 3' nontranslated nucleotides but left the poly(A) intact (be-

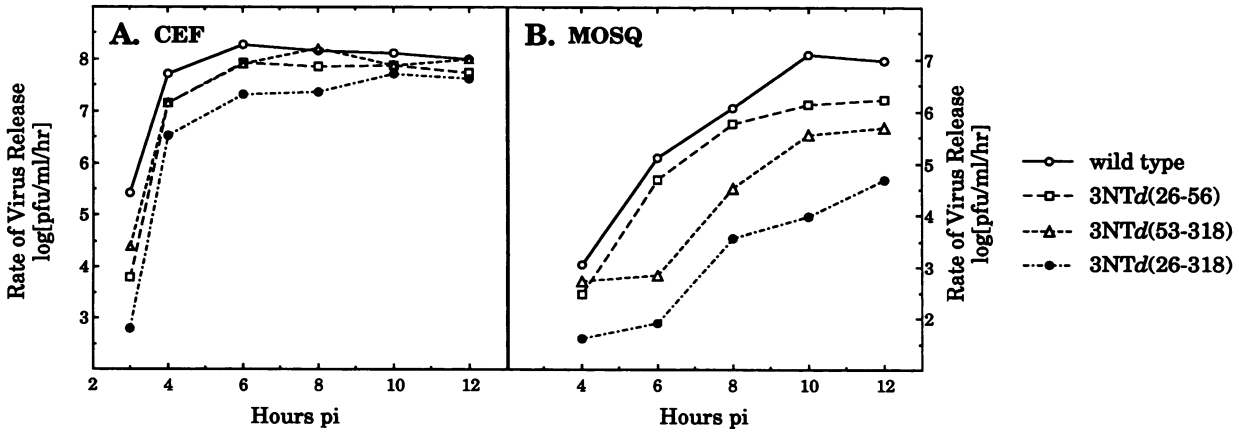


FIG. 8. One-step differential growth curves for the 3' nontranslated region deletion mutants. Growth curve experiments were performed as described in Materials and Methods. Wild type refers to virus rescued from the pToto52 construction. Deletion mutants were tested in CEF at 40°C (A) and in C6/36 mosquito cells at 30°C (B).

cause of the *Xba*I site, an additional three nucleotides were also inserted). RNA was transcribed with SP6 RNA polymerase, but despite numerous attempts with two independent clones, transfection of the RNA failed to yield infectious virus. Thus the 3′ 25 nucleotides adjacent to the poly(A) contain sequences that are absolutely required for virus replication. A further deletion clone which removed all viral sequences 3′ of the UGA stop codon was generated. This clone was made by deleting a *Bst*EII (nucleotide 11380) to *Eco*RI (nucleotide 11743) fragment. This clone also failed to yield virus.

In summary, although the 3′ terminal 25 nucleotides are required for virus replication, the remaining 293 nucleotides are not essential and can be deleted. The fact that all three viable deletion viruses grew more poorly in mosquito cells at 30°C than in chicken cells at 40°C suggests that some factor in the arthropod host reacts specifically with these sequences.

DISCUSSION

We have constructed and examined 42 different mutants in the 3′ nontranslated region of the Sindbis virus genome. These include 14 nucleotide substitutions in the 3′ terminal 20 nucleotides; 17 deletion mutants in which from 1 to 8 nucleotides in the 3′ terminal 25 nucleotides are deleted; 6 large deletion mutants; 4 nucleotide substitutions constructed to produce new restriction sites in the 3′ nontranslated region; and 2 insertion mutants, one of which results in the introduction of a unique restriction site between the 3′ terminal nucleotide sequence element and the poly(A) tract. Virtually all of these mutants were viable, and the majority of the mutations had only modest effects upon the ability of the virus to replicate. We have tried to ensure that the phenotype observed resulted from the changes introduced by measuring the specific infectivity of each mutant RNA, by sequencing RNA recovered from mutant viruses, and by working with low-passage stocks. In particular, the specific infectivity of the RNA of each viable mutant was the same as that of the wild type, within experimental error, and there is no reason to believe that (pseudo) revertants were selected and examined in these experiments. Furthermore, the phenotypes of the mutants were stable upon passage. Finally, the overall conclusions of this study are based on the results from 42 independently constructed mutants.

The plasticity of the 3′ nontranslated region of the genome was somewhat unexpected, in view of the high degree of organization shown by alphavirus genomes. The 3′ terminal sequence element of 19 nucleotides is highly conserved among all of the viruses, and the upstream domains contain a number of repeated sequence elements in most of the alphaviruses, as well as regions of high A+U content. Yet Sindbis virus is able to accommodate a large number of changes throughout the 3′ nontranslated region, including deletion of most of this region. In fact, the catalog of mutants constructed collectively deleted the entire 3′ nontranslated region. Deletion of the 3′ terminal C was lethal, as was deletion of 8 nucleotides within the 3′ terminal 19 nucleotides, but all other deletions gave rise to viable virus.

Even though the sequence of the 3′ nontranslated region can accommodate a large number of changes, almost every mutation in the terminal 19-nucleotide sequence element did in fact have a deleterious effect upon virus replication when examined carefully by growth curve analysis. In the case of mutations having only a moderate effect, this showed up as a delay in the growth of the virus by 1 to 2 h, with the virus ultimately producing yields comparable to those of the wild type. Mutations having more severe effects resulted in lower virus yields and, in extreme cases, in temperature sensitivity or in severe restrictions for growth in one of the two host cells examined. Thus, it is clear that the 19-nucleotide sequence element at the 3′ end is important for virus replication. The remainder of the nontranslated region is also important in some way, since large deletions in this region resulted in a virus that grew poorly in mosquito cells.

Because in nature alphaviruses alternate between mosquito hosts and vertebrate hosts, it seemed of interest to explore the replication ability of the viruses both in vertebrate cell lines, such as the chicken cells used here, and in mosquito cell lines. The different mutations had strikingly different effects upon the growth rate of the virus in these two cell lines. In the majority of cases, growth in the mosquito cells was affected more than growth in chicken cells. Many of the mutations which had very modest effects upon growth rate in chicken cells had more severe effects in mosquito cells and led to virus yields 1/10 or less that of wild-type levels. However, the opposite phenotype was also seen. At least two of the mutations studied here resulted in virus that grew as well as or even better than wild-type virus in mosquito cells but which grew more poorly than the wild type in chicken cells. The most straightforward interpretation of this result is that host factors are involved in the replication of the viral RNA and that the nucleotide sequence of the viral genome represents a compromise between that sequence which best adapts it for growth in mosquito cells and that sequence that best adapts it for growth in vertebrate cells. The requirement for alternation between two such different host species in order to maintain transmission in nature may place constraints on the nucleotide and protein sequences of alphaviruses which are greater than those for viruses with a more narrow host range. It is also clear that the fitness of an alphavirus for replication in tissue culture cells is a different test from the fitness of an alphavirus for continued passage in nature. The virus must be transmitted to a susceptible mosquito when the mosquito takes a blood meal from an infected vertebrate and must pass from the midgut to the salivary glands so that it can be transmitted with the injection of small amounts of saliva when the mosquito takes its next blood meal. The resulting infection in the vertebrate must then be severe enough to result in a viremia of sufficient intensity that the virus can again be transmitted to a mosquito. Viremia in the vertebrate host is moderated by the immune response and is often of fairly limited duration. Thus, it does not take much of an attenuation of the virus growth rate, whether in the mosquito or the vertebrate host, to interrupt the cycle of transmission in nature.

The results here make clear that the overall organization of the entire 322-nucleotide 3′ nontranslated region, and not just the 3′-terminal 19-nucleotide conserved sequence element, is important in some way for virus replication. This is in contrast to previous results using deletion mapping of Sindbis virus DI RNAs (11), in which it was found that deletion of the entire nontranslated region except for the 3′ terminal 19 nucleotides had no effect on the DI RNA, whereas deletion into this sequence element by two nucleotides was lethal. Either DI RNA is not as sensitive to deletion in this region for function as is the entire genome or these results are due to the differential effect of such deletions on replication in vertebrate cells versus mosquito cells, since we found that mutants with large deletions upstream of the 3′ terminal sequence element were more impaired for

growth in mosquito cells than in chicken cells. It is also unclear why DI RNA should be inactivated by deletion two nucleotides into the 19-nucleotide sequence element. The deletion mutations we have studied at this boundary, for example, 3NT*d*(18-19), which do not otherwise remove the remainder of the 3′ nontranslated region, are viable and, although somewhat impaired, are no more so than large deletion mutants which remove most of the 3′ nontranslated region and which had no effect on DI function. It appears that the complete sequence of the 3′ nontranslated region is important for virus replication and that deletion mapping studies of DI RNA do not give a precise definition of the sequence elements necessary for efficient replication of infectious virus.

The results here also make clear that a gradient of phenotypes can result from changes in the 3′ nontranslated region, from changes that have very modest effects on virus growth to changes that severely impair virus replication to the extent that no virus can be recovered. At the current time, the severity of the defect resulting from any mutation cannot be predicted. The changes at nucleotide 7 are particularly interesting. Change of the A in the wild-type virus to U or G or even deletion of this nucleotide has only a modest effect upon virus replication. However, change of this nucleotide to C has a profound effect, resulting in a virus which is temperature sensitive for growth in chicken cells and severely restricted for growth in mosquito cells. Although we do not understand why some changes are better tolerated than others, it seems clear that the 3′ nontranslated region, and in particular the 3′-terminal 19-nucleotide element, must interact in a precise way with viral and cellular proteins for efficient replication. The results are compatible with the hypothesis that the viral replicase, possibly in combination with cellular proteins as cofactors, binds specifically to this region to initiate transcription of minus-strand RNA. Further studies will be required to assay the effects of these mutations on RNA replication.

The importance of the G residue that punctuates a string of U's in the 3′-terminal sequence element is shown by the inability of SP6 RNA polymerase to faithfully transcribe DNA in which this G has been changed to U or has been deleted. In either case, four nucleotides from the wild-type sequence have been deleted from the resultant RNA transcripts. Change of the G to U results in a stretch of 10 U's, whereas deletion of this G results in a stretch of 9 U's. In either case, the principal polymerase product contains only six U's. The precision of the deletion suggest that it is not simply a matter of the polymerase stuttering when trying to copy this stretch but that the structure of the RNA product or, alternatively, the DNA template affects the transcription of the RNA. If this sequence element does possess a secondary structure, as yet not understood, that affects its interaction with SP6 and T7 RNA polymerase, this structure could also be important for the interaction of the RNA with the viral replicase. The finding that mutants with shorter U tracts grew better than ones with longer U tracts also bears on this. It has not been possible to generate a secondary structure for the RNA in this region which would explain the results obtained with the mutants.

The overall organization and structure of this region, which is thought to be involved in the initiation of minus-strand RNA synthesis, also may have effects upon the fidelity of replication in this region. Mutant 3NT7C, which is temperature sensitive, has a very low reversion frequency, less than 10^{-8}, despite the fact that change of this nucleotide to any of the other three nucleotides or deletion of this nucleotide would give a virus which is not temperature sensitive. The majority of the revertants of 3NT7C that do arise appear to be second-site revertants, which are currently being further characterized.

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