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Defined Mutations in the 5′ Nontranslated Sequence of Sindbis Virus RNA

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We have constructed 24 deletion mutants which contain deletions of from 1 to 15 nucleotides in the 5′ nontranslated region of Sindbis virus RNA and tested the effect of these mutations on virus replication. The results showed that the first 44 nucleotides, which are capable of forming a hairpin structure, are important for virus replication, as all deletions tested in this region were either lethal or resulted in virus that grew poorly in comparison to the parental virus. Many of these deletions had different effects in mosquito cells than in chicken cells, suggesting that cellular factors, presumably proteins, bind to this region. This domain may function in at least two processes in viral replication. It seems likely that in the minus strand, this sequence element is bound by the viral replicase and promotes RNA replication. In the plus strand, this element may modulate initiation of translation of the nonstructural proteins. The results suggest that the hairpin structure itself is important. All deletions within it had deleterious effects on virus replication, and in particular, deletion of one of the G residues at nucleotide 7 or 8 or of one of the C residues at nucleotide 36 or 37 which are theoretically base-paired with these G's resulted in temperature-sensitive viruses that behaved very similarly. In contrast, large deletions between the 44-nucleotide hairpin and the translation start site at nucleotides 60 to 62 resulted in virus that grew as well as or better than the parental virus in both chicken and mosquito cells. The A residue at position 5 of the HRSP strain used was examined in more detail. Deletion of this A was lethal, whereas substitution by G resulted in a virus that grew poorly, despite the fact that G is present at position 5 in the AR339 parent of HRSP. U at position 5 resulted in a virus that grew less well than the A5 strain but better than the G5 mutant.

Sindbis virus is an enveloped plus-stranded RNA virus, the prototype alphavirus of the family *Togaviridae*. Its entire genome of 11,703 nucleotides has been sequenced (14). Comparative studies of alphaviruses have identified four conserved sequences and/or structures that may serve as promoters for the replication of the viral RNA (for a review, see reference 15), and mutational analyses of Sindbis virus have confirmed their importance in viral replication. A conserved sequence of 24 nucleotides in the junction region between the nonstructural and structural coding regions of the genome which contains the initiation site for the subgenomic 26S RNA (9) has been found to be necessary and sufficient for transcription of a subgenomic RNA (2, 6). A conserved 19-nucleotide sequence adjacent to the poly(A) tail at the 3′ terminus of the RNA was postulated to serve as a promoter sequence for initiation of minus-strand synthesis (11). Mutational analysis of the 3′ nontranslated region has shown that this 19-nucleotide sequence element in particular but also the entire nontranslated region is important for efficient virus replication (5). Near the 5′ terminus of the genomic RNA, a highly conserved sequence, referred to as the 51-nucleotide region, is found that is capable of forming two hairpin loops (10). We previously found that nucleotide substitutions within this domain have dramatic effects on virus replication (8). Finally, there is a conserved stem-and-loop structure at the very 5′ end of the genomic RNA which might serve as a promoter (in the minus-strand RNA) for the initiation of plus-strand synthesis (10). In this article we report the results of a mutational analysis of the 5′ nontranslated region of Sindbis virus. Mutations, either deletions or nucleotide substitutions, were introduced into a full-length clone of Sindbis virus, pToto51, derived from the HRSP strain (5, 8, 12), the newly constructed plasmids were transcribed in vitro with SP6 RNA polymerase to produce infectious transcripts, and the viruses rescued from these constructs studied. With this method, the sequence requirements of the 5′ region could be defined more precisely.

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MATERIALS AND METHODS

Cells and virus strains. Chicken embryo fibroblasts and mosquito C6/36 cells from *Aedes albopictus* were propagated as described previously (3, 5). Monolayers of secondary chicken cells were used for the production of virus stocks and for the titration of viruses. All virus stocks were derived from a full-length cDNA clone of Sindbis virus (pToto51) or its mutagenized derivatives by transfection of chicken cells with RNA synthesized in vitro with SP6 RNA polymerase as described previously (5, 8, 12).

One-step growth curves. Analysis of virus replication was performed by infecting secondary chicken cell or mosquito C6/36 monolayers in 35-mm culture dishes at a multiplicity of 10 in 250 μl of phosphate-buffered saline (PBS). After absorption at room temperature for 60 min, the inoculum was removed, the monolayers were washed once with PBS, and 1 ml of prewarmed medium was added. The plates were incubated at 30, 37, or 40°C as indicated, and the medium was removed every hour and replaced with fresh prewarmed medium. Virus present in the medium was assayed by plaque titration on chicken cells at 30°C.

Site-directed mutagenesis. Oligonucleotide-directed mutagenesis and recovery and characterization of mutant virus were done as described previously (8).

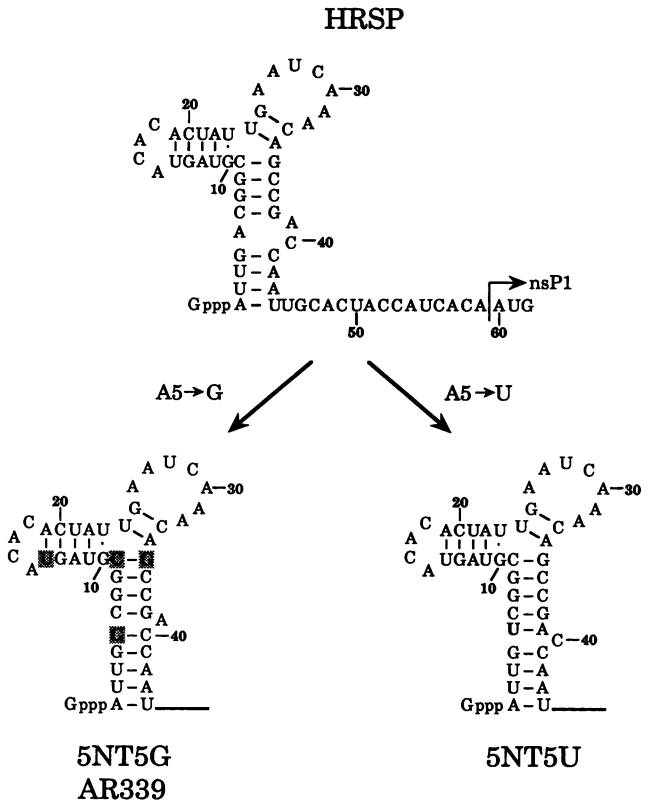

FIG. 1. The 5′ nontranslated sequence of Sindbis virus RNA. The sequence of the 5′ nontranslated region is shown in the form of a terminal hairpin that requires the first 44 nucleotides. Initiation of translation is at the AUG at positions 60 to 62 as shown. Three sequences are shown: HRSP, derived from clone pToto51; mutant 5NT5G, in which the A at position 5 has been changed to G and whose 5′ nontranslated sequence is identical to that in Sindbis strain AR339, which was the parent of HRSP; and mutant 5NT5U, in which the A at position 5 has been changed to U. In the AR339 sequence, four nucleotides that vary in laboratory strains derived from it are shaded. Nucleotide 5 is G in HRSP as shown (10), nucleotide 9 is U in at least some HR strains (13), U14 is C in HR strain *ts*24R1 (13), and G35 is A in the HRLP strain (10).

RESULTS

Construction of mutations in the 5′ noncoding region of Sindbis virus. An overview of the 5′ nontranslated region of Sindbis virus, 59 nucleotides in length, is shown in Fig. 1; a possible stem-and-loop structure which can be formed by the first 44 nucleotides is illustrated for three different strains of virus. Four nucleotides in this region, 5, 9, 14, and 35, which are shaded in the AR339 sequence, have been found to vary in sequenced laboratory strains of Sindbis virus derived from strain AR339. It is of interest that two of these, the C at position 9 (C9) and G35, form a base pair in the hypothetical structure shown.

To assess the importance of the 5′ nontranslated region for virus replication, a large number of mutations were introduced into a full-length cDNA clone of Sindbis virus by in vitro mutagenesis. An overview of these mutations, which consisted for the most part of deletions, is shown in Fig. 2. Mutant RNA transcribed in vitro with SP6 RNA polymerase was transfected into monolayers of secondary chicken cells, and plaques were allowed to develop at 30 or 40°C. In almost all cases viable virus was recovered, and the specific infectivity of the RNA was the same as that for RNA transcribed from the parental clone pToto51, within experimental error (± a factor of 3). Thus, it is unlikely that viable virus arose by selection of same-site or second-site revertants. Deletion of nucleotides 2 to 4 or deletion of nucleotide 5 was lethal. Deletion of nucleotide 7 or 8 (which are equivalent) or of 37 or 38 (equivalent), or of both, gave rise to temperature-sensitive virus that formed plaques at 30°C but not at 40°C. Three mutations, all found downstream of the stem-and-loop structure, gave rise to mixed large and small plaque sizes, as indicated. All other deletions or substitutions tested gave rise to virus that formed plaques indistinguishable from wild-type plaques, even though these deletion mutants grew poorly in comparison to the parental HRSP virus (see below).

Mutations at position A5. Deletion of A5 was lethal; no virus could be recovered from two independent constructs. In other Sindbis virus strains sequenced, the nucleotide at position 5 is G, including the AR339 strain from which HRSP was derived by passage in cultured cells. This change results in a difference in the hypothetical 5′-terminal hairpin structure, as illustrated in Fig. 1. To test the importance of this change, substitution mutant 5NT5G (see Fig. 2 legend for explanation of designations) was constructed. We also changed A5 to U, so that A39 would be drawn into the stem of the structure and result in an extrahelical C40 rather than A39 (Fig. 1), because extrahelical A residues have been found to be important in the interaction of at least some proteins with RNA (17). Both 5NT5G and 5NT5U were viable and formed plaques indistinguishable from wild-type plaques. The effects of these mutations were examined more closely by determining differential growth curves in both chicken and mosquito cells (Fig. 3). The medium was changed every hour, and the amount of virus present in the medium was assayed by plaque assay at 30°C in chicken cells. Thus, each time point represents the rate of virus release at that time. Surprisingly, 5NT5G (whose 5′ nontranslated sequence is identical to that of the AR339 strain) was significantly impaired in both cell lines, producing virus at rates less than 10% of the rate of the parental HRSP virus derived from pToto51 throughout most of the time period examined. We assume that HRSP must have compensating mutations elsewhere in the genome that allow more efficient replication with A5 and that cause the virus to replicate poorly with G5. Although side-by-side comparisons have not been performed, we have consistently found that AR339 grows to higher titers in chicken cells than does HRSP. 5NT5U was less affected. In mosquito cells it was essentially indistinguishable from HRSP. In chicken cells it had a longer latent period than did HRSP but eventually produced virus at rates greater than HRSP. Thus, the extrahelical A39 is not essential for replication.

Temperature-sensitive deletions in the stem structure. Mutants 5NT*d*(7), which is the same as 5NT*d*(8), and 5NT*d*(36), which is the same as 5NT*d*(37), produced small plaques at 30°C and failed to produce plaques at 40°C. (Independent M13 constructs were used to isolate independent mutants in the case of both nucleotides 7 and 8 and 36 and 37 and are called by the different nucleotides to distinguish them.) In the hypothetical structure illustrated in Fig. 1, G7 pairs with C37 and G8 pairs with C36. Deletion of either the G or the C in the base pair leads to temperature sensitivity, a phenotype not seen with any of the other deletions, suggesting that this structure does in fact exist in solution and that the G · C base pair is recognized by a viral or cellular protein during replication. Double mutants constructed to remove both

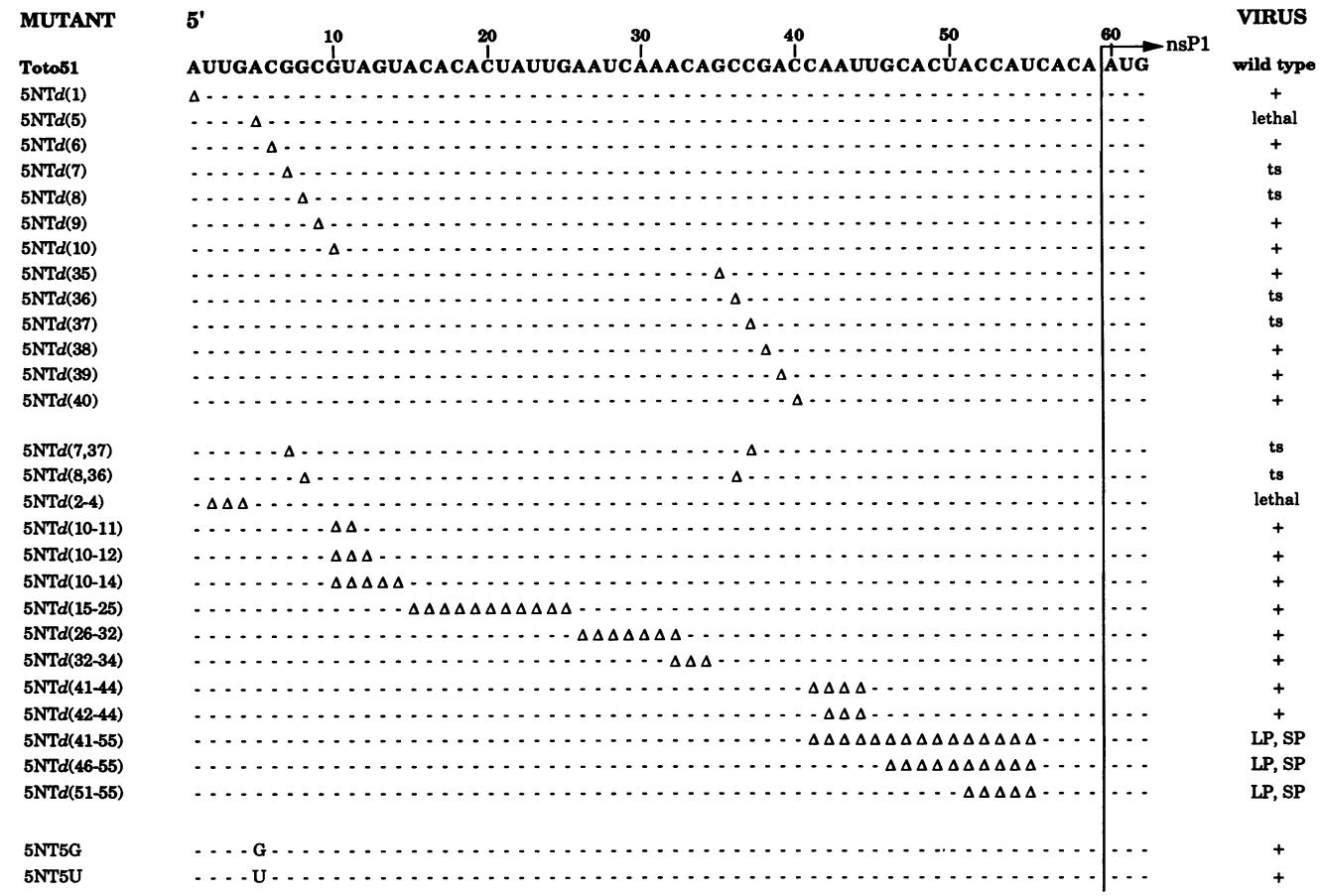

FIG. 2. Mutations in the 5′ nontranslated region of Sindbis virus. The sequence of the 5′ nontranslated region is shown in the top line, and the changes effected in the various mutants are shown below (–, nucleotide identical to that in the top line; Δ, nucleotide deleted; G and U, nucleotide 5 substituted with the indicated nucleotide). The name of the mutant is shown at the left in the nomenclature of Kuhn et al. (5). 5NT signifies that the mutation is in the 5′ nontranslated region; *d* signifies a deletion, with the nucleotide(s) deleted following in parentheses; 5G and 5U signify that nucleotide 5 has been substituted by G or U, respectively; p signifies the full-length plasmid construct, whereas the name without the p refers to the virus recovered from the cDNA clone. Virus recovered by plaque assay on chicken cells is shown at the right, where + signifies that the mutant is viable and forms plaques indistinguishable from those produced by RNA transcripts from the parental clone pToto51; ts signifies that plaques are formed at 30°C but not at 40°C; LP and SP signify that a mixture of both large and small plaques was present in the primary transfection assay; lethal signifies that no virus could be recovered from RNA transcripts of the mutant clone.

members of the base pair G7 · C37 (equals G8 · C36) (again independent constructs were made) also formed small plaques at 30°C and were temperature sensitive. RNAs isolated from 5NT*d*(7) and from the double mutant were sequenced and found to have the predicted deletions and no other changes over the area sequenced.

Growth curves of these mutants in both chicken and mosquito cells are shown in Fig. 4. At 30°C the two single mutants were indistinguishable from one another; in both cell types they produced virus at rates of about 10^{-1} to 10^{-2} that of the parental virus. The double mutant behaved quite differently. At 30°C in chicken cells it had a very long latent period, some 4 h or so greater than HRSP, but by 10 h produced virus at rates greater than either of the single mutants and about 25% of the HRSP rate. In mosquito cells the double mutation was almost lethal; virus was produced at only 10^{-3} to 10^{-4} of the parental rate, and the mutant is effectively host restricted. The differential effects in the two cell types suggest that host cell factors interact with the 5′ nontranslated region, possibly with the structure shown in Fig. 1, during replication.

The temperature sensitivity of 5NT*d*(8,36) in chicken cells

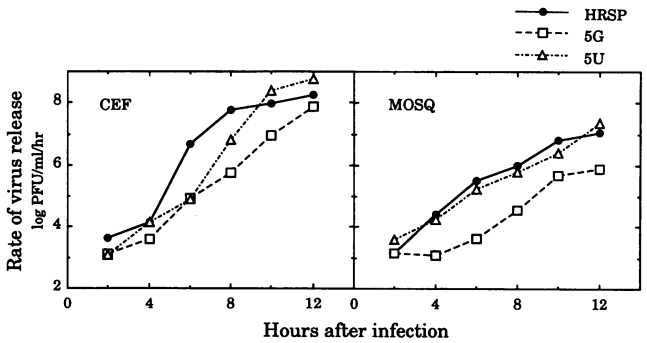

FIG. 3. Growth curves of virus 5NT5G (5G) and 5NT5U (5U) compared with HRSP recovered from clone pToto51. Chicken cells (CEF) or mosquito cells (MOSQ) were infected at a multiplicity of 10 at 30°C, and the medium was changed each hour. Titers in selected samples were determined on chicken cells at 30°C. The results thus represent the rate of virus release (PFU per hour) at the different times after infection. These and all other growth curves are the average of two independent experiments to ensure the reproducibility of the results.

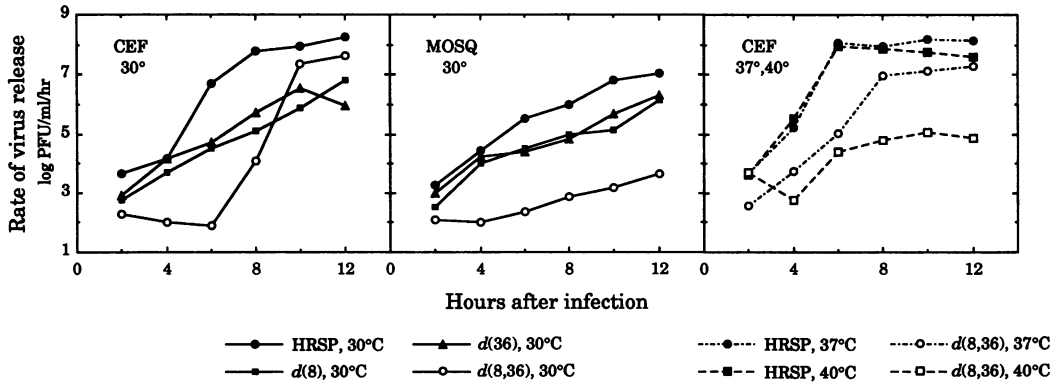

FIG. 4. Growth curves of mutants 5NT*d*(8), 5NT*d*(36), and 5NT*d*(8,36) in comparison to HRSP. Conditions were as described in the legend to Fig. 3 except that for the right panel growth was at 37 or 40°C as indicated, and virus titer was assayed by plaque formation at 30°C in chicken cells.

is shown in the third panel of Fig. 4. At 37°C the virus behaved similarly to the case at 30°C, but with a shorter latent period (as is also the case for the wild-type virus). At 40°C, however, the virus had a longer latent period and produced virus at only about 10^{-3} of the wild-type rate.

Other deletions in the proposed 5' hairpin structure. Of 21 different deletions of 1 to 11 nucleotides in the 5'-terminal 44 nucleotides, 16 deletions gave rise to virus that formed plaques indistinguishable from those formed by HRSP. When examined more closely, however, all 16 mutants were impaired in their growth. Differential growth curves in both chicken and mosquito cells are shown in Fig. 5 for nine of these mutants to illustrate this. All demonstrated a longer latent period and produced virus at rates less than the wild-type rate late in infection. 5NT*d*(6) was particularly impaired, producing virus at $<10^{-3}$ of the wild-type rate in both cell types. 5NT*d*(1) was also significantly impaired. 5NT*d*(32-34) was more impaired in chicken cells (rate of virus production, $\sim 10^{-3}$ of HRSP rate) than in mosquito cells (rate of virus production, ~5% of HRSP rate). 5NT*d*(40) and 5NT*d*(9) were less impaired, producing virus at 10^{-1} of the wild-type rate late in infection, although 5NT*d*(9) had an extended latent period.

Mutants 5NT*d*(41-44), 5NT*d*(26-32), 5NT*d*(15-25), and 5NT*d*(10-14) behaved similarly to one another in chicken cells, having a latent period 2 h longer than the wild-type period but by 12 h producing virus at 5 to 40% of the wild-type rate (Fig. 5). In mosquito cells, however, 5NT*d*(10-14) grew very poorly and produced virus at about 10^{-3} of the wild-type rate. This deletion effectively removes the second stem in the hypothetical folded RNA structure. It is of interest that 5NT*d*(32-34) was relatively more severely affected for growth in chicken cells, whereas 5NT*d*(10-14) was more severely affected in mosquito cells, suggesting once again that host factors interact with these sequences.

It is particularly noteworthy that 5NT*d*(15-25) was viable and grew moderately well (rate of virus production, ~5% of the rate for HRSP). This deletion was lethal for a defective interfering RNA (16). To confirm the presence of the deletion, RNA from the mutant was sequenced and found to

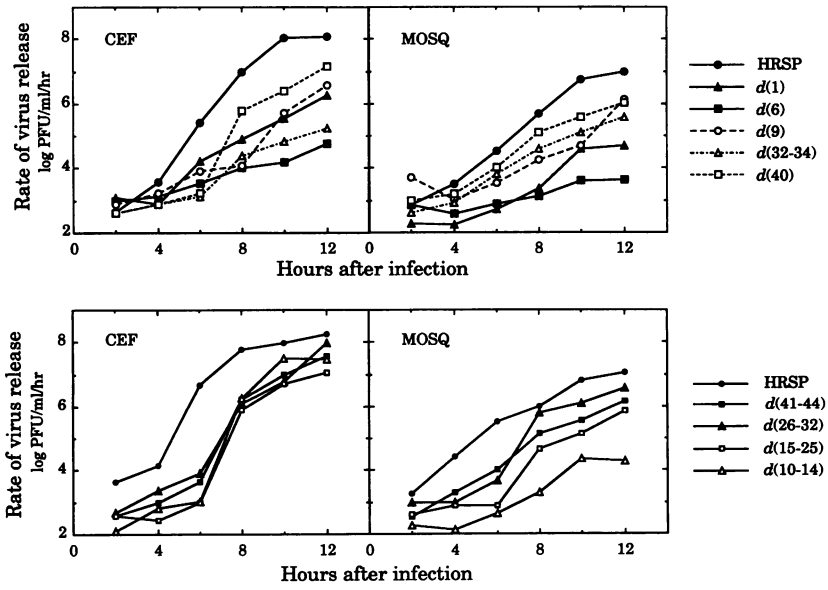

FIG. 5. Growth curves of mutants with deletions in the 5'-terminal 44 nucleotides. Conditions were as described in the legend to Fig. 3. The mutants are indicated at the right.

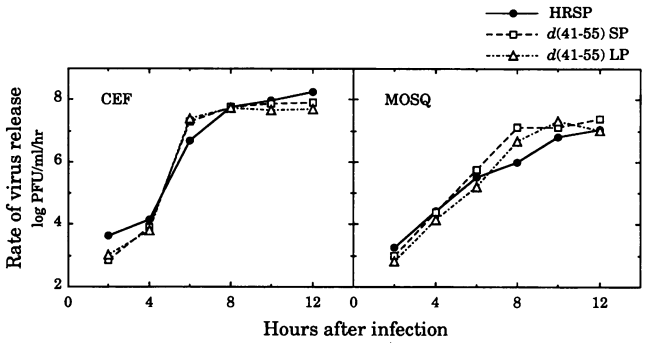

FIG. 6. Growth curves of mutant 5NT*d*(41-55). Large-plaque (LP) and small-plaque (SP) strains of this mutant were prepared and used to infect chicken or mosquito cells at a multiplicity of 10, and growth curves were performed as described in the legend to Fig. 3.

contain the predicted deletion and no other changes in the region sequenced.

It is also of note that 5NT*d*(1) was viable. In the cDNA constructs there is an additional G at the 5′ end of the RNA transcript, necessary to obtain specific initiation by SP6 RNA polymerase. In wild-type constructs, this G is removed during replication (12). Attempts to sequence the 5′ end of RNA from 5NT*d*(1) were inconclusive, but it is possible that the extra G is not removed and the effect is to substitute G for the first A.

Deletions between the 5′ structure and the initiating AUG codon. To examine the importance of the nucleotides between the 5′-terminal structure and the initiating AUG codon of nsP1, three deletions were made which removed part or all of this sequence. The largest deletion removed nucleotides 41 to 55, which begin in the stem structure and include all downstream nontranslated nucleotides except for the CACA preceding the AUG start codon (Fig. 1). This construct retains the so-called Kozak sequences optimal for protein synthesis (4). Transfection of RNA transcribed from p5NT*d*(41-55) into chicken cells yielded two different sizes of plaques. Isolation of the different plaques resulted in two stable virus strains, a large-plaque-forming virus (plaques ~6 mm) and a small-plaque-forming virus (plaques ~1 mm), which are indicated by LP and SP, respectively (Toto51 virus and HRSP produced plaques of ~2 mm). Sequencing the viral genome in the mutated region showed that both 5NT*d*(41-55)LP and 5NT*d*(41-55)SP contained the deletion introduced and did not contain any other change in the 5′ region sequenced (data not shown). Two other constructs, which removed nucleotides 46 to 55 and 51 to 55, respectively, gave similar results in that large and small plaques were both present in the original transfection plaque assay; the large plaques were largest for the largest deletion.

Growth curves of the large- and small-plaque strains of 5NT*d*(41-55) are shown in Fig. 6. Both variants grew quite well in both chicken and mosquito cells, and if anything produced virus at faster rates than did the parental HRSP strain derived from the clone pToto51, particularly in mosquito cells. The origin of the small-plaque and large-plaque variants is thus a mystery. Neither has a selective advantage, both are present in the original transfection assay, and the specific infectivity of the RNA is the same as that transcribed from pToto51, so that selection of second-site mutants appears unlikely. The SP variant was present at a 50- to 100-fold excess in the primary transfection assay and is presumably the deletion mutant in the Toto51 background, and the LP variant presumably has a second mutation somewhere in the genome that leads to the large plaque size. Because both the SP and LP strains are stable once plaque purified, the change does not appear to occur during virus replication. It appears that there must be a change introduced by SP6 RNA polymerase during transcription in vitro and that this change occurs at a fairly high frequency.

DISCUSSION

Sequence requirements in the 5′ nontranslated region. We report here the results of studies of 26 different mutations in the 5′ noncoding region of Sindbis virus. Most of these mutants were deletion mutants to minimize the occurrence of same-site revertants and facilitate the search for second-site revertants in proteins that bind to these sequence elements. The results clearly establish the importance of the 5′-terminal sequence for virus replication. Deletion of nucleotides 2 to 4 was lethal, as was deletion of nucleotide 5. Deletion of nucleotide 6 was almost lethal, giving a virus that replicated very poorly. Deletion of nucleotide 7 or 8 gave a temperature-sensitive virus that was impaired even at the permissive temperature (30°C). Deletion of nucleotide 9 gave a virus that had an extended latent period and which produced virus at only about 1% of the parental rate late in infection. Deletion of nucleotides 10 to 14 led to a virus that had a long latent period and in mosquito cells was almost nonviable, producing virus at 0.1% of the wild-type rate. In general, deletions further removed from the 5′ terminus were less affected for growth, although only the mutants with deletions beyond position 40 showed near wild-type growth patterns, and deletion of positions 32 to 34 gave a virus that grew quite poorly in chicken cells. It would be of interest to make more comparisons of the effects of nucleotide substitutions with deletions, to determine how much of the effect is due to the displacement of sequence elements or secondary structures from one another and how much is the result of an absolute requirement for a particular nucleotide at a particular position. At position 5, deletion of the A was lethal, whereas substitution by U gave a virus that was only modestly affected for growth and substitution by G had a more pronounced effect. At position 8, deletion of the G gave rise to temperature-sensitive virus. Preliminary results suggested that substitution by A or C also gave rise to temperature-sensitive virus (unpublished data), implying that the G · C base pair at this position is required for efficient replication.

The 5′ nontranslated sequence may be important for at least two processes in virus replication. Ribosomes bind to the 5′ end of the plus strand to initiate translation, and replicase must bind to the 3′ end of the minus strand to initiate transcription of plus strands. Two features of our results suggest that the stem-and-loop structure at the 5′ end is a key feature in one or both processes, rather than the linear sequence of RNA per se. The fact that deletion of either G7 (or G8) or its base-paired partner in the structure (C37 or C36) gave rise to virus that behaved very similarly, being temperature sensitive and exhibiting similar growth patterns, suggests that this base pair is an important feature in replication. The fact that any deletion within the first 40 to 44 nucleotides had a deleterious effect on growth, whereas deletions downstream of this had only modest effects, if anything promoting virus growth, suggests that some factor interacts with this 5′-terminal structure in order to promote RNA replication or translation. Even if the stem structure is a key element, however, the first five nucleotides appear to

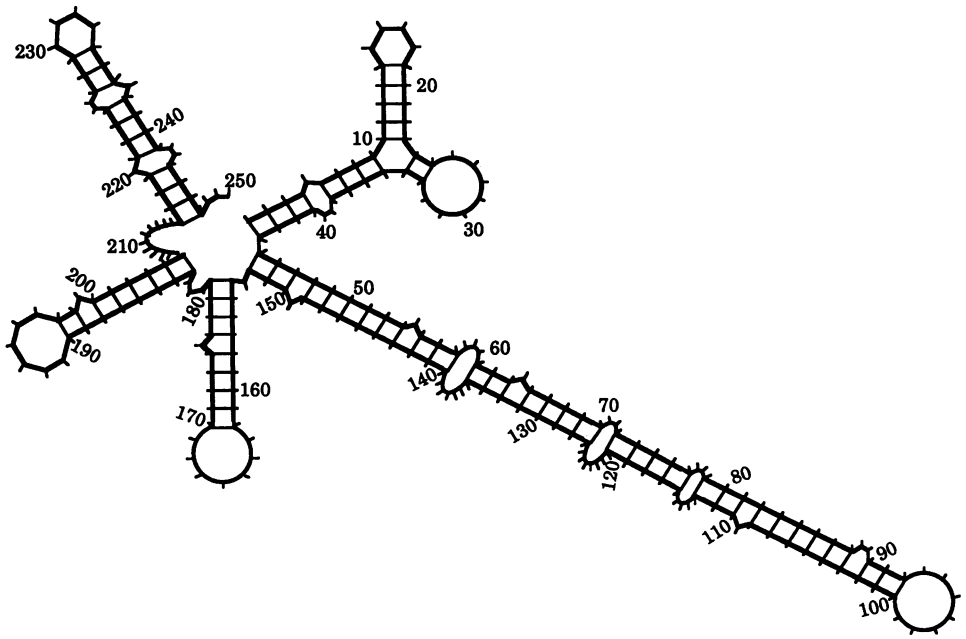

FIG. 7. Predicted structure formed by the 5′-terminal 250 nucleotides of Sindbis virus RNA. The software package from the University of Wisconsin Genetics Computer Group, which uses the Zucker folding program (1, 18), was used to construct the figure.

have an importance in viral replication greater than simply participating in this structure. Deletion of nucleotides 2 to 4 was lethal, as was deletion of nucleotide 5, whereas deletion of nucleotides 41 to 44 or of nucleotide 40, which are theoretically base-paired with nucleotides 2 to 4 and nucleotide 5, respectively, led to viable virus that grew at a rate about 10% of the wild-type rate late in infection.

The deletions made after nucleotide 40 offer some insight into the sequence requirements. 5NT*d*(41-44) was attenuated for growth, demonstrating the importance of these nucleotides. 5NT*d*(41-55) grew very well; inspection of the RNA sequence (Fig. 1) shows that in this mutant nucleotides 41 and 42 are CA, as in the wild type, but nucleotides 43 and 44 are different (i.e., the mutation is equivalent to deleting nucleotides 43 to 57). This suggests that it is the first 42 nucleotides that are important and that nucleotides 43 to 55 have only a modest role in replication, if anything leading to a slight attenuation in growth. It would be of interest to explore whether this modest attenuation could be related to the prediction from RNA-folding programs that nucleotides 45 to 57 form a base-paired stem structure with nucleotides 140 to 152. In Fig. 7 is shown the structure predicted to be formed by the first 250 nucleotides of Sindbis virus RNA. The 5′-terminal structure formed from the first 44 nucleotides is followed by a long stem-and-loop structure formed from nucleotides 45 to 152. This is followed almost immediately by the double hairpin structure of the 51-nucleotide conserved sequence element (8). These hairpins begin with nucleotide 154 in Sindbis virus, but the conserved element is formed by nucleotides 155 to 205 (10). Nucleotides 206 to 215 are unpaired, and 216 to 247 can form another hairpin. It is perhaps of interest that the putative hairpin formed by nucleotides 45 to 152 brings the 51-nucleotide element into proximity to the 5′-terminal element.

Mutational analysis of conserved sequence elements in alphavirus genomes. This is the third report in a series of mutational analyses to describe the importance for virus replication of conserved sequence elements in alphavirus genomes. Previously we reported analyses of the 3′ nontranslated region (5) and of the 51-nucleotide sequence element found in the coding domain of nsP1 (8). Although three disparate regions of the genome are involved, and these three elements presumably perform different functions in virus replication, certain themes are common to the results of all three studies. For one, almost all mutations introduced led to viable virus, although most mutants grew poorly compared with the parental virus. Thus, the genome is surprisingly plastic in that most deletions and sequence changes are tolerated in otherwise invariant domains, although the poor growth of most of the mutants would lead to selection in nature against these changes.

A second theme common to all three studies is that many or most of the mutations have strikingly different effects in mosquito cells and chicken cells. In nature, Sindbis virus alternates between vertebrate hosts, often birds, and invertebrate hosts, mosquitoes in the case of Sindbis virus and most, but not all, alphaviruses, and thus the virus has evolved to replicate efficiently in both hosts. The fact that the different mutations have such host-dependent effects suggests that host factors, presumably proteins, interact with all three virus sequence elements examined in these studies to promote replication and translation of the RNA and that the sequence of the virus represents a compromise between that which best adapts the virus to mosquitoes and that which best adapts it to vertebrate hosts. Presumably, different host factors are involved in the recognition of the three different sequence elements.

In all three studies we found that plaque morphology is not correlated with growth rate. The large-plaque and small-plaque variants of 5NT*d*(41-55) grew equally well but produced plaques that differed by sixfold in size. Moreover, many mutants studied here produced plaques that were indistinguishable from wild-type plaques despite producing virus at rates of only 0.1 to 10% of the wild-type rate. On the other hand, 5NT*d*(8,36) failed to produce plaques at 40°C, although at this temperature it produced virus at a rate

greater than did 5NT*d*(6) at 30°C, under which conditions 5NT*d*(6) formed normal plaques.

Fourth, in all three studies it has been difficult to correlate the effects of mutations in the viral genome upon replication of the virus with the effects of the same mutations in defective interfering (DI) RNAs. The sequence requirements for the function of Sindbis virus DI RNAs have been defined by Schlesinger and colleagues (7, 16). In the current study, the deletion 5NT*d*(15-25), which is equivalent to deleting nucleotides 16 to 26, had relatively modest effects upon virus growth but was lethal for a DI that had the Sindbis virus 5′ terminus. Although it could be argued that the effect of the deletion upon virus growth might be sufficient to disable a DI which must be amplified relative to the virus, we have found other deletions that are tolerated by DI RNAs to have even more profound effects upon virus replication (5, 8). Thus, the sequence requirements for DI RNA replication and packaging are related to but are not identical to the requirements of the viral genome for replication and packaging, and the source of these differences remains obscure.

Finally, we found in all three studies that SP6 polymerase may introduce changes during RNA transcription. In the present study the deletions downstream of the stem-and-loop structure [5NT*d*(41-55), 5NT*d*(46-55), and 5NT*d*(51-55)] gave rise to both large and small plaques in the initial transfection which appeared to result from SP6-introduced changes. The large plaques were only about 1% of the total, and thus the rate of error by the polymerase was fairly low. In an earlier study we found that two substitution mutations in the 51-nucleotide sequence element gave rise to mixed plaques and, as in the present case, concluded that they arose from errors during SP6 transcription (8). In that case, however, the frequency of large-plaque variants was considerably greater, about 20%. It is intriguing that in both instances the mutants grew very well, as well as or better than the wild type in cultured cells. Two distinct plaque sizes have not been observed after transcription of the parental pToto51 or of many other mutant clones, and why they arose in only these three instances is unclear. In a third study, we found that SP6 transcription of two mutant clones in the 3′ nontranslated region led to the deletion of four residues with a very high frequency (i.e., the deleted RNA was the majority product) (5). Caution must thus be used in the interpretation of results following SP6 transcription.

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