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Sindbis Virus *ts*103 Has a Mutation in Glycoprotein E2 That Leads to Defective Assembly of Virions

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Sindbis virus mutant *ts*103 is aberrant in the assembly of virus particles. During virus budding, proper nucleocapsid-glycoprotein interactions fail to occur such that particles containing many nucleocapsids are formed, and the final yield of virus is low. We have determined that a mutation in the external domain of glycoprotein E2, Ala-344 → Val, is the change that leads to this phenotype. Mapping was done by making recombinant viruses between *ts*103 and a parental strain of the virus, using a full-length cDNA clone of Sindbis virus from which infectious RNA can be transcribed, together with sequence analysis of the region of the genome shown in this way to contain the *ts*103 lesion. A partial revertant of *ts*103, called *ts*103R, was also mapped and sequenced and found to be a second-site revertant in which a change in glycoprotein E1 from lysine to methionine at position 227 partially suppresses the phenotypic effects of the change at E2 position 344. An analysis of revertants from *ts*103 mutants in which the Ala → Val change had been transferred into a defined background showed that pseudorevertants were more likely to arise than were true revertants and that the *ts*103 change itself reverted very infrequently. The assembly defect in *ts*103 appeared to result from weakened interactions between the virus membrane glycoproteins or between these glycoproteins and the nucleocapsid during budding. Both the E2 mutation leading to the defect in virus assembly and the suppressor mutation in glycoprotein E1 are in the domains external to the lipid bilayer and thus in domains that cannot interact directly with the nucleocapsid. This suggests that in *ts*103, either the E1-E2 heterodimers or the trimeric spikes (consisting of three E1-E2 heterodimers) are unstable or have an aberrant configuration, and thus do not interact properly with the nucleocapsid, or cannot assembly correctly to form the proper icosahedral array on the surface of the virus.

Sindbis virus is a small enveloped RNA virus that belongs to the genus *Alphavirus* of the family *Togaviridae*. The nucleocapsid of the virus is an icosahedral structure (T=3) that contains the single-stranded RNA of 11,703 nucleotides complexed with 180 copies of a 30,000-dalton capsid protein (6, 10, 27). During the final stages of virus assembly, the nucleocapsid, assembled in the cytoplasm, buds through the plasma membrane to acquire an envelope consisting of a lipid bilayer containing two virus-encoded integral membrane glycoproteins, E2 and E1. In the final virion structure, 240 copies of E1 and E2 are present in the membrane in an icosahedral array (T=4). Each external spike of the virus consists of a trimer of E1-E2 heterodimers, and these spikes and the nucleocapsid fit together in a precise fashion (6). The precision of virus assembly and rigid exclusion of nonviral proteins from the virus (22) has led us and others to hypothesize that there is a specific interaction between the glycoproteins and the nucleocapsid that furnishes the free energy required to drive virus budding.

Mutant *ts*103 was isolated more than a decade ago after mutagenesis with nitrous acid (25). It is a minute plaque former that grows slowly at any temperature and produces, under optimal conditions, virus yields of 3 to 10% of those of the parental HRSP strain of Sindbis virus (23). After *ts*103 infection, RNA synthesis and protein synthesis as well as nucleocapsid formation are virtually indistinguishable from those following infection by the parental strain of virus, and the very slow rate of *ts*103 virus production appears to be due to a defect in the final stages of virus maturation, the budding of nucleocapsids through the capsid membrane to produce the infectious virus. Electron microscopy of *ts*103-infected cells reveals the presence of large numbers of nucleocapsids apparently in the process of budding. However, the release of mature virus is delayed, and the final yield of virus is reduced. Examination of the released virions by sedimentation velocity centrifugation or by electron microscopy showed that most virus particles contain more than one nucleocapsid in a single envelope (23). Thus, the interactions of the nucleocapsids with the glycoproteins appear to be weak, leading to a slow rate of virus assembly and to the formation of aberrant virions.

Here we report the sequence of the entire structural protein region of *ts*103 and of a partial revertant, *ts*103R, and a comparison of these sequences with that of the parental HRSP strain. In addition, we have mapped the location of the mutation resulting in the *ts*103 phenotype by constructing recombinant viruses between *ts*103 and HRSP, using a cDNA clone of the HR strain of Sindbis virus from which infectious RNA can be transcribed in vitro (18), and in this way shown that the *ts*103 phenotype maps to a change in glycoprotein E2.

MATERIALS AND METHODS

Cells and viruses. Culturing of chicken embryo fibroblast cells, infection with Sindbis virus, and plaque assay were carried out as previously described (16, 25) except that 1 to 1.2% agarose was used for overlay during plaque formation in all experiments (18, 23). Mutant *ts*103 and the revertant *ts*103R were isolated as previously described (23, 25).

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Virus purification and isolation of RNA. *ts*103 and *ts*103R were grown in primary chicken embryo fibroblast cells in hypotonic saline for 10 to 20 h, and virus was harvested in hypertonic saline as described elsewhere (16, 23). Virus was purified and RNA was extracted as previously described (19).

Isolation of cDNA clones. Clones containing the 3′-terminal 5,438 nucleotides [plus a variable length of poly(A)] of the genomes of *ts*103 and of *ts*103R were obtained by using a T-tailed plasmid vector for first-strand synthesis as previously described (7, 11). A library of cDNA clones representing the rest of the genome was obtained by using calf thymus random primers for first-strand synthesis and *Eco*RI linkers to clone the double-stranded cDNA into a plasmid vector essentially as described previously (15, 17). To identify clones containing inserts representing the 5′ ends of the genomes, colonies were probed with a radiolabeled RNA consisting of the 5′-terminal 500 nucleotides of Sindbis virus HRSP, derived by transcribing RNA in vitro from clone Toto 1101 (18).

Sequence analysis. Sequences of *ts*103 and of *ts*103R were obtained by the chemical sequencing methods of Maxam and Gilbert (14) as modified by Smith and Calvo (20). Most of the sequences of the structural regions of *ts*103 and *ts*103R were obtained from sequencing of single-stranded cDNA restriction fragments produced by *Hae*III digestion (1, 19). This method has the advantage that a consensus sequence is immediately obtained. In addition, the cDNA clones that were used to construct the hybrids containing the structural regions of *ts*103 and *ts*103R were sequenced throughout this region by Maxam-Gilbert chemical sequencing as previously described (26). To confirm the nature of recombinant viruses produced from construction of hybrid genomes, certain regions of the recombinant virus genomes were sequenced by direct RNA sequencing, using reverse transcriptase, synthetic primers, and RNA templates as described elsewhere (31; C. S. Hahn, E. G. Strauss, and J. H. Strauss, Methods Enzymol., in press).

General recombinant DNA techniques. Restriction endonucleases and DNA-modifying enzymes were purchased from commercial sources and used essentially as recommended by the manufacturers. Plasmids were grown, purified, and analyzed by standard methods, with minor modifications (13).

Construction of recombinant viruses. Hybrid genomes were constructed by replacing restriction fragments in Sindbis virus cDNA clone Toto 1101 (18) with the corresponding regions from cDNA clones derived from either *ts*103 or *ts*103R. Details of the restriction sites used are given in the figure legends. Virus was rescued from these recombinant clones essentially as described previously (12), and the virus was tested for its biological properties.

Isolation of revertants from rescued *ts*103 mutants. A hybrid virus stock, called *ts*103D, was obtained which consisted of Toto 1101 (18) in which the sequence from *Hpa*I (nucleotide 6919) to *Bss*HII (nucleotide 9804) was derived from *ts*103. This virus stock was plaqued in 100-mm-diameter petri plates (~1,000 plaques per plate), and larger plaques were picked and replaqued to obtain (pseudo)revertants.

Analysis of revertants by oligonucleotide hybridization. An oligonucleotide TCTCCTGGTACTGACTCTT, complementary to the *ts*103 sequence from nucleotides 9652 to 9670, was synthesized (the underlined A is specific to the *ts*103 sequence), ^{32}P labeled with T4 kinase, and used as a probe in slot blots with *ts*103 stocks and their revertants, using a modification of the method of Suggs et al. (29). Hybridization was in 5× SSPE–5× Denhardt solution–0.1% sodium dodecyl sulfate–100 μg of denatured salmon sperm DNA per ml, followed by washing in 2× SSPE containing 0.1% sodium dodecyl sulfate (13). The filters were subjected to increasing temperature of washing, and a temperature was found at which the probe remained bound if perfectly base paired to the RNA (i.e., the sequence was that of *ts*103) but washed off if there was a single mismatch in the RNA (e.g., the RNA had the parental sequence) (this temperature was found to correspond closely to that predicted by the formulas of Davis et al. [4]; see also references 2 and 13).

Analysis of virus stocks for multicored particles. Viral RNA was labeled with [^{3}H]uridine, and 1 to 4 ml of the virus-containing culture fluid from a 100-mm-diameter tissue culture plate was layered onto a 10-ml sucrose gradient (15 to 30% [wt/vol] sucrose in 200 mM sodium chloride, 50 mM Tris [pH 7.4], 1 mM EDTA). Centrifugation was at 5°C for 80 to 90 min at 27,000 rpm or 50 to 60 min at 36,000 rpm in an SW40 or SW41 rotor (Beckman Instruments, Inc.). The gradients were fractionated and assayed for radioactivity in a liquid scintillation counter. For some experiments, the virus was concentrated by precipitation with polyethylene glycol as previously described (16) before analysis by sucrose gradient sedimentation.

RESULTS

Sequence analysis of the structural proteins of *ts*103 and *ts*103R. Since *ts*103 has a defect in the assembly of virions, it seemed probable that the mutation would lie in one of the structural proteins. To start, the entire structural protein regions of the genomes of *ts*103 and *ts*103R were sequenced and compared with the sequence of the parental HRSP strain. Most of the sequence was obtained by direct sequencing of first-strand cDNA after digestion with *Hae*III (19), which gives the consensus sequence in the RNA population. These sequencing results were confirmed and extended by sequencing cDNA clones of *ts*103 and *ts*103R (Fig. 1).

There were no changes in the 5′ or 3′ nontranslated regions flanking the structural protein region, and there were no coding differences in the capsid proteins of the viruses (although there was a silent change in the second codon of the capsid protein, AAU → AAC, in *ts*103 and *ts*103R). In the remainder of the structural region, there was only a single change between HRSP and *ts*103, a C-to-U change at position 9661 leading to the substitution of Ala-344 in the E2 glycoprotein in HRSP by Val in *ts*103 (GCC → GUC). *ts*103R was found to contain Val-344, as did *ts*103, but contained one additional change in E2 and two differences in the E1 region. These were changes of nucleotide 9330 from A to U (leading to the substitution of Thr-234 in E2 by Ser), of 10744 from A to U (leading to the substitution of Lys-227 in E1 by Met), and of 10848 from G to U (leading to the substitution of Ala-262 in E1 by Ser). These data indicated that if, in fact, the assembly defect of *ts*103 was due to a change within the structural protein region, that change must have been the Ala-to-Val change at position 344 of E2 and that the partial revertant isolated was a second-site revertant, presumably involving one or more of the changes in glycoprotein E2 or E1.

Localization of the mutation responsible for the *ts*103 phenotype. To map the location of the change responsible for the *ts*103 phenotype, recombinant viruses were constructed. Sindbis virus cDNA clone Toto 1101 contains a complete cDNA copy of Sindbis virus inserted downstream from an

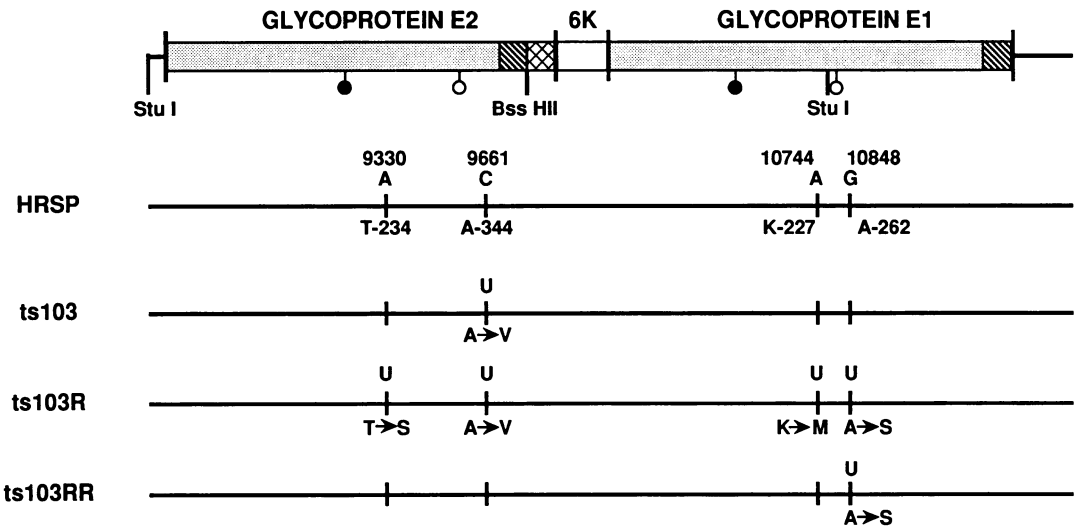

FIG. 1. Sequence differences between HRSP, *ts*103, *ts*103R, and *ts*103RR in the glycoprotein region and diagram of the proteins. Symbols: □, external domains; ▨, transmembrane domains; ▩, putative cytoplasmic domain of E2. Also shown are sites of carbohydrate attachment (complex [●] and simple [·] carbohydrate chains). Three restriction sites used in the constructions are indicated. Where differences occur, nucleotides are shown above the line together with the nucleotide coordinate, and the encoded amino acids are shown below the line (in single-letter code) together with the amino acid position numbered from the amino terminus of glycoprotein E2 or E1. Where no nucleotide or amino acid is indicated, there is no difference from the HRSP sequence.

SP6 RNA polymerase promoter, and infectious RNA can be transcribed from these clones in vitro (18). Hybrid cDNA clones were constructed by replacing restriction fragments within this clone with the corresponding sequences from cDNA clones of *ts*103 or *ts*103R. RNA was transcribed from the recombinant clones and transfected onto secondary chicken embryo fibroblast cells. Monolayers were incubated at 30°C, and virus was harvested when the cells showed a full cytopathic effect. The parental HR strain or phenotypically wild-type recombinants required about 72 h for this, whereas *ts*103 took up to 6 days. Monolayers were also incubated at 30 or 40°C under 1 to 1.2% agarose for plaque assay. The constructs tested are diagrammed in Fig. 2B, which also includes the coordinates of the restriction sites used to make the hybrid genomes. In each construct, most of the sequence was derived from clone Toto 1101, except for a single contiguous region derived from *ts*103. For simplicity, these various constructs and the viruses rescued from them will be referred to as 103A through 103F (Fig. 2B).

The phenotypes of each of the six recombinants illustrated in Fig. 2B are shown in Table 1. The plaque sizes of 103A at 30°C and of 103B and 103C at both 30 and 40°C were indistinguishable from that of Toto 1101. Recombinants 103D, 103E, and 103F, in contrast, formed minute plaques at both 30 and 40°C, as did *ts*103. Furthermore, the sizes of the virus plaques correlated with the kinetics of virus release and with the formation of multicored particles. Virus from 103A, 103B, and 103C had the same growth rate as did that from Toto 1101, whereas *ts*103 and recombinants 103D, 103E, and 103F exhibited very slow kinetics of virus release. Similarly, tests for the production of multicored particles by sucrose gradient sedimentation (Fig. 3) showed that virus from Toto 1101 or from 103A, 103B, and 103C gave a single sharp virus peak sedimenting at 280S. In contrast, *ts*103 and recombinant 103D showed multiple peaks of virus sedimenting from 280S to >700S (recombinants 103E and 103F were not tested). Since recombinants 103D, 103E, and 103F share only the E2 region of *ts*103, we conclude that glycoprotein E2 from *ts*103 is necessary and sufficient to obtain the *ts*103 phenotype and that the plaque size, growth pattern, and production of multicored particles are all due to the same mutation, namely, the substitution of Ala-344 in E2 by Val (Fig. 1).

The plaques formed by 103A at 40°C were smaller than those formed by Toto 1101 but considerably larger than those formed by *ts*103 (Table 1), suggesting that there is a change in the A region of *ts*103 that leads to small plaques at 40°C. Because this change appeared to be unrelated to the *ts*103 phenotype, as discussed above, it was not further investigated.

Second-site reversion in *ts*103R. The partial revertant *ts*103R was found to be intermediate in phenotype between *ts*103 and HR with respect to virus release and plaque morphology (Table 2). Multicored particles were produced by this virus (Fig. 3 and Table 2), but larger amounts of virus were released at a faster rate. Since *ts*103R possesses Val-344 in E2, as does *ts*103, the altered phenotype must be due to partial suppression of this mutation by a second-site mutation. To confirm this, a recombinant virus between Toto 1101 and *ts*103R analogous to construct 103D (Fig. 2B) was made. This recombinant virus, 103RD (Fig. 2C), was tested for its biological properties (Table 2) and found to possesses the *ts*103 phenotype, showing that the original *ts*103 mutation was in fact still present and that the suppressing mutation must lie outside the region defined by this construct, that is, outside the coordinates 6919 to 9804 in the viral RNA (Fig. 2C).

The location of the suppressor mutation was defined by two other recombinant viruses tested, 103RE and 103RF (Fig. 2C). Recombinants 103RE and 103RF had the same phenotype as did *ts*103R (Table 2); i.e., they formed small plaques (as opposed to minute plaques made by *ts*103) at 40 and 30°C, released virus more rapidly than did *ts*103 (but still more slowly than did HR), and made multicored particles. These results show that the suppressor mutation must lie between coordinates 9804 and 10770 (Fig. 2C) and therefore that the suppressor mutation must be the Lys-227 → Met change in glycoprotein E1 (Fig. 1). Note, however, that

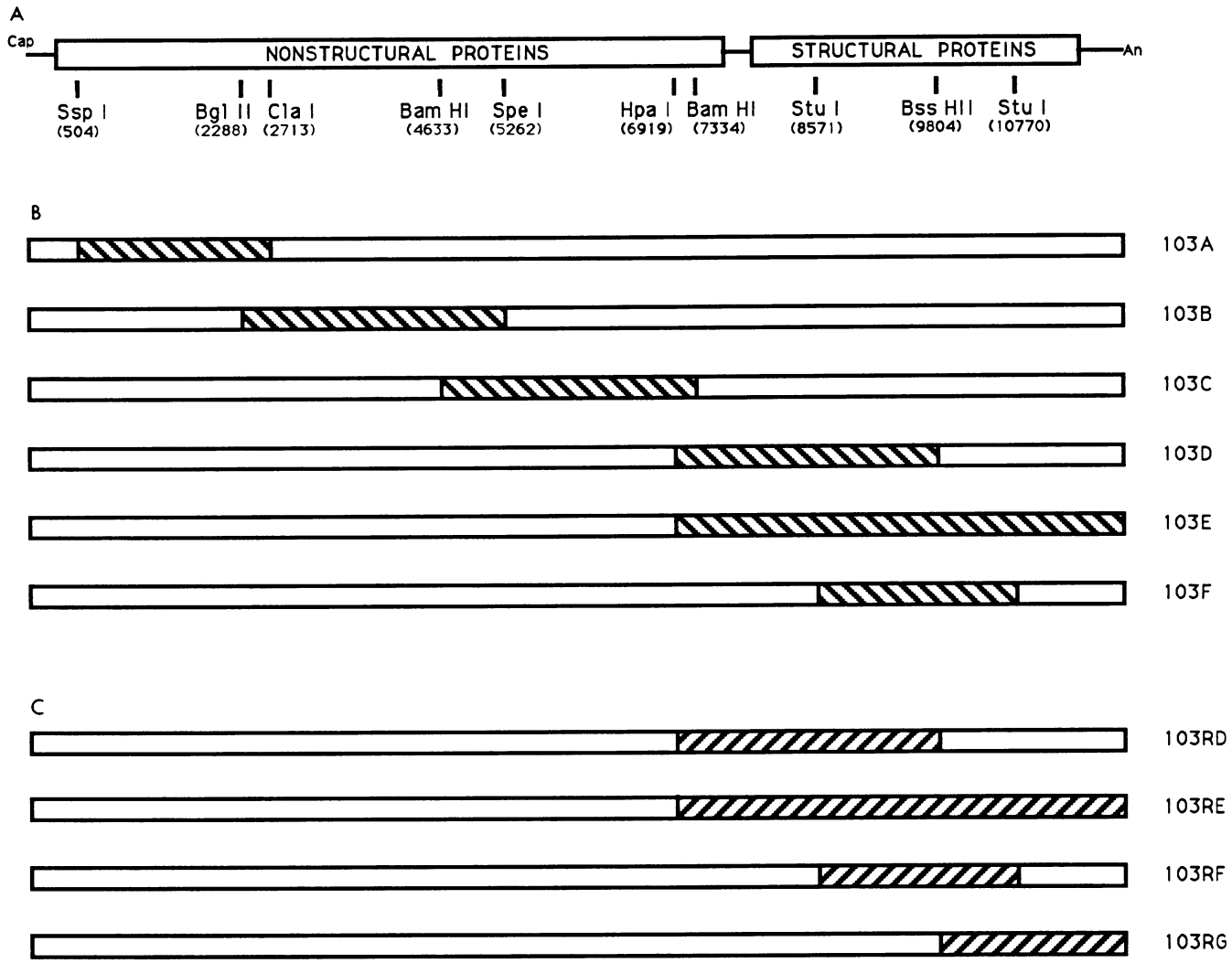

FIG. 2. Construction of recombinant genomes. (A) Schematic of the Sindbis virus genome. Symbols: □, open reading frames encoding the nonstructural and structural proteins; —, nontranslated regions. The restriction sites used and their coordinates in the viral genome are indicated for the constructs, which were named 103A, 103B, etc., in the approximate order, from 5′ to 3′ in the genome of the sequences derived from *ts*103. (B) Constructs for mutant *ts*103. Restriction fragments in Sindbis virus cDNA clone Toto 1101 (18) were replaced with the corresponding fragments from cDNA clones of *ts*103 (▨). (C) Constructs for revertant *ts*103R. Restriction fragments in clone Toto 1101 were replaced with the corresponding fragments from clones of *ts*103R. Note that in construct 103RD, the same restriction fragment of Toto 1101 was replaced with *ts*103R cDNA as was replaced with cDNA from *ts*103 in construct 103D. The same is true for constructs 103RE and 103E and constructs 103RF and 103F.

103RF contains Ser at residue 234 of E2; although this change alone will not suppress the *ts*103 mutation (construct 103D), we cannot rule out an effect of this change on suppression, acting in concert with Met-227 in E1.

A fourth recombinant virus, 103RG, was also constructed and tested (Table 2). This virus contains the E1 region from *ts*103R with the rest of the genome derived from Toto 1101 (Fig. 2C). This recombinant virus formed very large plaques (much larger than the plaques formed by Toto 1101), grew somewhat faster than Sindbis virus HR, and did not lead to the formation of multicored particles (Fig. 3). Thus, the suppressor mutation, when separated from the *ts*103 mutation, leads to the formation of large plaques and may be a generalized suppressor that increases the efficiency of virus assembly and release in some way.

Revertant *ts*103RR. At the time of the isolation of *ts*103R, since it seemed to be only a partial revertant, we attempted to isolate a complete revertant from the *ts*103 population. This required multiple rounds of passage to amplify revertants in the population after they arose, as previously described (23). One presumptive revertant made large (rather than intermediate) plaques and produced no multicored particles; this was called *ts*103RR. Sequence analysis of *ts*103RR showed that it had Ala-344 at E2 (like HR) and therefore was a true (same-site) revertant (Fig. 1). This revertant also contained Ser-262 in E1 rather than Ala, the significance of which is unclear.

Reversion frequency of *ts*103. During the isolation of revertants from *ts*103, the reversion frequency was found to be less than 10^{-7} (23). This reversion frequency is quite low in view of the fact that the *ts*103 phenotype is obtained with a single nucleotide change in the E2 coding region. These experiments relied in part on the amplification of revertants once they arose in the population. Such amplification might

TABLE 1. Properties of Toto 1101, *ts*103, and recombinant viruses from *ts*103

Virus[a]	Plaque size, 40°C/30°C	Growth rate	Multicored particles[b]
Toto 1101	Normal/normal	Normal	No
*ts*103	Minute/minute	Very slow	Yes
103A	Small/normal	Normal	No
103B	Normal/normal	Normal	No
103C	Normal/normal	Normal	No
103D	Minute/minute	Very slow	Yes
103E	Minute/minute	Very slow	NT
103F	Minute/minute	Very slow	NT

[a] Constructs are shown in Fig. 2.
[b] Assayed by sucrose gradient sedimentation. NT, Not tested.

be adversely affected by the presence of even a few mutant spikes on virions arising from mixedly infected cells (negative complementation). In addition, *ts*103 was originally isolated after nitrous acid mutagenesis of HRSP, making it possible that other unmapped changes in the genome might have affected the detection of revertants. Therefore, we reexamined the reversion frequency of *ts*103 by direct screening of plaques for the presence of revertants. Recombinant 103D, which differs from Toto 1101 by only one coding difference, the change in position 344 of glycoprotein E2, was plaque purified once and passed at low multiplicity in chicken cells. This stock was plaqued at 40°C. More than 40,000 plaques were screened, and three large plaques were obtained (reversion frequency of 7×10^{-5}). These three new revertant viruses, however, all differed in plaque size and titer produced from virus from Toto 1101, suggesting that they may all be second-site revertants and that in each case the suppressor mutation is different.

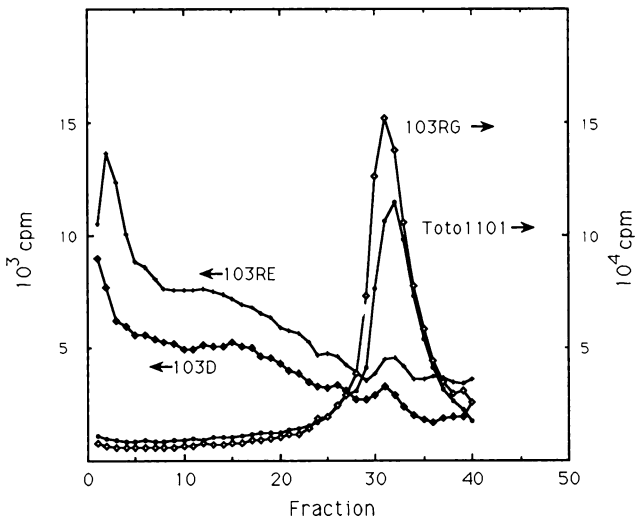

FIG. 3. Sedimentation profiles of Sindbis virus. Chicken cells were infected with various hybrid viruses in the presence of [^{3}H]uridine. The culture fluid was harvested, virus was precipitated with polyethylene glycol, and resuspended virions were layered over 15 to 30% sucrose gradients. Fractions were collected and assayed for radioactivity. Sedimentation is from right to left. The top of the gradient is not shown. Viruses examined were Toto 1101 (●), 103RG (◇), 103D (◆), and 103RE (+). The constructs used to obtain these viruses are illustrated in Fig. 2. Scales for Toto 1101 and 103RG are 10-fold higher than those for 103D and 103RE. The peak of virus in Toto 1101 sediments at 280S.

TABLE 2. Properties of *ts*103R and recombinant viruses derived therefrom

Virus[a]	Plaque size, 40°C/30°C	Growth rate	Multicored particles[b]
*ts*103R	Small/small	Slow	Yes
103RD	Minute/minute	Very slow	NT
103RE	Small/small	Slow	Yes
103RF	Small/small	Slow	Yes
103RG	Large/large	Normal	NT

[a] Constructs are shown in Fig. 4.
[b] Assayed by sucrose gradient sedimentation. NT, Not tested.

Two of these pseudorevertants were examined in more detail. A synthetic oligonucleotide complementary to the *ts*103 sequence between nucleotides 9652 and 9670, which spans the Ala → Val change in E2, was used as a probe in slot blot hybridization under conditions in which a single-nucleotide mismatch could be detected. One revertant, 103DR1, was found to retain the *ts*103 sequence through this region and thus was clearly a pseudorevertant. Sucrose gradient analysis of the virus produced by this strain confirmed that multicored particles were produced (Fig. 4). The second revertant, 103DR2, had a mismatch with the probe and thus could possibly have reverted to the parental sequence. Sucrose gradient analysis demonstrated that multicored particles were produced (Fig. 4), however, making it unlikely that 103DR2 had reverted to the wild-type sequence. It is unknown whether the change from the *ts*103 sequence occurred in the codon for Val-344 or elsewhere within the 19-nucleotide sequence or even whether this change was responsible for the larger plaque phenotype of the variant. It does seem clear, however, from these results and from those of Strauss et al. (23) that the *ts*103 mutation is very stable and that reversion to the wild-type nucleotide occurs at very low frequency.

DISCUSSION

We have shown here that the *ts*103 phenotype is due to a C → U change at position 9661 of Sindbis virus RNA, leading to the substitution of alanine by valine at position 344 of glycoprotein E2. A C → U transition is consistent with the action of nitrous acid, in which C in the viral RNA is deaminated to produce U. A substitution of Ala by Val is often considered to be conservative, but in this case it leads to dramatic effects on the function of glycoprotein E2 during virus assembly.

We had originally postulated (23) that the defect in *ts*103 might lie in the nucleocapsid protein because it was found that many nucleocapsids isolated from *ts*103-infected cells or from multicored virions sedimented more slowly that did those isolated from wild-type virions or from wild-type-infected cells. More recent results of Coombs et al. (3), however, have shown that there is an immature form of the nucleocapsid that sediments more slowly, which upon maturation becomes a more rapidly sedimenting form. Thus, the finding here that the nucleocapsid protein of *ts*103 is identical to that of wild-type virus suggests that the nucleocapsids in *ts*103-infected cells or many of the nucleocapsids in multicored particles are, in fact, immature forms that are not triggered to assume the mature form because of deficiencies in virus assembly.

It is believed that during virus assembly the cytoplasmic domains of the glycoprotein interact with the nucleocapsid to drive virus budding and to produce the precisely assem-

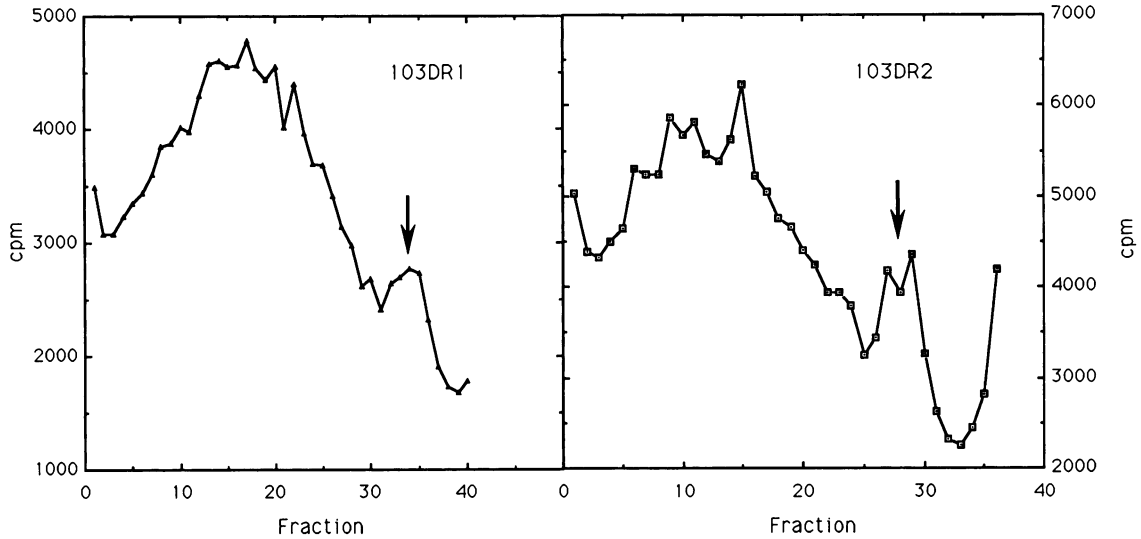

FIG. 4. Sedimentation profiles of *ts*103 pseudorevertants 103RD1 and 103DR2. Analysis was performed as for Fig. 3. ↓, Position of virus particles containing a single nucleocapsid.

bled virion. The fact that the *ts*103 mutation is in a domain of glycoprotein E2 external to the lipid bilayer (Fig. 1) means that the weakened interactions between the glycoproteins and the nucleocapsid proteins are not the results of changes in the domains of these two proteins that interact directly. Instead, it suggests that the E1-E2 heterodimer forms improperly in *ts*103, due either to a change in the conformation of E2 itself induced by the amino acid substitution at position 344 or to steric hindrance, if this altered amino acid is a contact residue essential for heterodimerization. Alternatively or in addition, the amino acid substitution could affect assembly of the spikes (each made up of three dimers). In any event, the mutant spike unit has a suboptimal geometry and fails to interact properly with the nucleocapsid, leading to defective assembly of virions.

A number of formal mechanisms exist which could explain the failure of correct nucleocapsid-glycoprotein interactions. For one, the assembled spike itself could be unstable and dissociate at the cell surface, so that the effective concentration of assembled spikes available for budding is low. A second possibility is that improperly assembled spikes fail to bind properly to the nucleocapsid because of changes in the conformation of the cytoplasmic binding domain induced by improper folding or assembly of the glycoprotein spikes. A third possibility is that the aberrant spikes fail to interact properly with one another or are unable to assume the correct T=4 icosahedral spacing, possibly because of steric hindrance. We note in this regard that a much larger proportion of the *ts*103 virus yield consists of (apparently) perfectly formed virions when virus is made in hypotonic medium and released in hypertonic medium than when virus is formed and released in isotonic medium (22, 23; compare also with Fig. 3 and 4). In hypotonic medium, the final stages of virus assembly and release are inhibited (16, 24, 30), and virus assembly thus occurs over a very long period of time. Thus, it appears that under such conditions the assembly defect of *ts*103 can be overcome at least in part and that the spikes can in fact assume the correct icosahedral architecture, suggesting that the concentration of components involved in budding is low or that suboptimal interactions among the proteins are involved in the assembly defect.

We have also shown that the mutation in *ts*103 can be partially suppressed by a change of Lys-227 to Met in glycoprotein E1. In some way, this change in E1 can compensate in part for the change in E2. One possibility is that Ala-344 of E2 and Lys-227 of E1 are contact residues in E1-E2 dimer formation and that the two changes partially compensate for one another. A second possibility is that the altered conformation of E2 induced by Val-344 is partially compensated for by an altered conformation of E1 induced by the Met-227. A third possibility is that the change in E1 increases the stability of glycoprotein E1-E2 interactions or the affinity of the spike for the nucleocapsid during budding. We favor the third possibility because of the finding that the change in E1, when separated from the *ts*103 mutation, leads to an increase in plaque size and growth kinetics of the virus, suggesting that the change in E1 has a generalized effect rather than being specific for the *ts*103 mutation. Moreover, the *ts*103R mutation only moderates the *ts*103 phenotype rather than specifically suppressing it; multicored particles continue to be produced but in greater yield, suggesting again a general effect of the E1 change upon virus assembly kinetics.

The results reported here and the previous results of Strauss et al. (23) suggest that the true reversion frequency of *ts*103 to the wild-type sequence is on the order of 10^{-7}. In general, RNA viruses have been found to undergo mutation at much higher frequencies, on the order of 10^{-4} (21, 28), and many nucleotide substitutions in Sindbis virus have been found to revert at frequencies of 10^{-3} to 10^{-5} (8, 9, 25). There are now three examples of single nucleotide substitutions in Sindbis virus RNA that revert at much lower frequencies, however. In addition to the *ts*103 mutation described here, these are the *ts*24 mutation (reversion frequency of $<10^{-8}$ [9]) and a mutant described by Durbin and Stollar (5) that reverts at a frequency of $<5 \times 10^{-7}$. Thus, it seems clear that the context in which a nucleotide is found is an important determinant of its mutation rate.

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