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Processing the Nonstructural Polyproteins of Sindbis Virus: Nonstructural Proteinase Is in the C-Terminal Half of nsP2 and Functions Both in *cis* and in *trans*

W. REEF HARDY AND JAMES H. STRAUSS*

Division of Biology, California Institute of Technology, Pasadena, California 91125

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The processing of the Sindbis virus nonstructural polyprotein translated in vitro has been studied. When Sindbis virus genomic RNA was translated in a reticulocyte lysate, polyprotein P123 was cleaved efficiently to produce nsP1, nsP2, and nsP3. Inhibition of this processing by anti-nsP2 antibodies, but not by antibodies specific for nsP1, nsP3, or nsP4, suggested that the viral proteinase was present in nsP2. To localize the proteolytic activity more precisely, deletions were made in a full-length cDNA clone of Sindbis virus, and RNA was transcribed from these constructs with SP6 RNA polymerase and translated in vitro. Although virtually all of the nsP1, nsP3, and nsP4 sequences could be deleted without affecting processing, deletions in the N-terminal half of nsP2 led to aberrant processing, and deletions in the C-terminal half abolished proteolysis. However, inactive polyproteins containing the nsP2 deletions could be processed by exogenously supplied proteins translated from virion RNA, demonstrating that cleavage was virus specific and not due to a protease present in the reticulocyte lysate and that the deleted polyproteins still served as substrates for the enzyme. From these results and from experiments in which processing was studied at increasingly higher dilution, we have concluded the following: (i) the viral nonstructural proteinase is located in the C-terminal half of nsP2; (ii) in the P123 precursor the cleavage between nsP2 and nsP3 occurs efficiently as a bimolecular reaction (in *trans*) to remove nsP3, while the bond between nsP1 and nsP2 is cleaved inefficiently, but detectably, in *trans*, but no autoproteolysis of P123 was detected; (iii) once nsP3 has been removed, the bond between nsP1 and nsP2 in the P12 precursor is cleaved efficiently by autoproteolysis (in *cis*). This mode of processing leads to a slow rate of cleavage, particularly early in infection, suggesting that the polyproteins might play roles in virus RNA replication distinct from those of the cleaved products. A hypothesis is presented that the proteinase is a thiol protease related to papain.

Many animal virus mRNAs are translated as polyprotein precursors that are cleaved posttranslationally to produce the final protein products (reviewed in reference 26). Some cleavages that occur during the processing of glycoprotein precursors are effected by organelle-bound cellular proteases (reviewed in reference 44), but cleavages that occur in the cytosol of the cell, which include the processing of all nonstructural proteins as well as of many structural protein precursors, are effected by virus-encoded proteinases (38, 47). Proteinases of the picornaviruses and their plant virus counterparts, the comoviruses and nepoviruses, of several retroviruses, and of adenoviruses have been studied in some detail and mapped to specific locations in the virus genome, and proteinases that act both in *cis* and in *trans* have been identified (reviewed in reference 26).

In the case of alphaviruses, two virus-encoded proteinases have been postulated, one required for the processing of the structural polyprotein precursor, which is translated from a subgenomic mRNA of 4.1 kilobases, and the second required for the processing of nonstructural polyproteins, which are translated from the genomic RNA of 11.7 kilobases (reviewed in references 46 and 47). The structural proteinase releases the capsid protein from the nascent structural polyprotein by instantaneous autoproteolysis (2, 41). The location of mutations that render the proteinase temperature sensitive, sequence similarities between the alphavirus capsid proteins and cellular serine proteases, and site-specific mutagenesis of selected residues are consistent with the hypothesis that the capsid autoprotease is a serine protease in which His-141, Asp-147, and Ser-215 form the catalytic triad (4, 17, 32; C. S. Hahn, Ph.D. thesis, California Institute of Technology, Pasadena, 1988).

The nonstructural proteins of Sindbis virus are translated as two large polyprotein precursors (reviewed in reference 45). The smaller precursor (P123, 200 kilodaltons [kDa]) results from the translation of a single open reading frame of 1,896 amino acids that encodes the first three nonstructural proteins, nsP1, nsP2, and nsP3, numbered according to their position in the genome from 5′ to 3′. A fourth nonstructural protein, nsP4, is also produced, albeit in much smaller amounts, by readthrough of an in-frame opal codon to produce a larger precursor (P1234, 250 kDa). Proteolytic processing of these precursors occurs either cotranslationally or posttranslationally to yield the mature nonstructural proteins (21), which constitute the viral RNA-dependent replicase/transcriptase complex.

Recently, two advances have made it easier to examine the processing of these proteins from their precursors, both in vivo and in vitro. First, monospecific antisera to each of the nonstructural proteins have been generated and used to establish the processing kinetics and stability of the mature proteins in vivo (21). Second, a full-length cDNA clone of Sindbis virus has been constructed (37) from which infectious RNA can be transcribed and translated in vitro. Using this clone, Ding and Schlesinger (13) translated a nested set of truncated RNAs in vitro; their results suggested that the proteolytic activity was located within nsP1 or nsP2, and probably within nsP2. In addition, Hahn et al. (19) have

* Corresponding author.

TABLE 1. Deletions in the nonstructural protein region of Toto1101

Name	Location[a]	End modifications	Subclone	Size (aa[b])
Δ1A	*Bal*I (205)-*Bal*I (1101)	None	πnsP12	299
Δ1B	*Sma*I (765)-*Sma*I (944)	None[c]	None	60
Δ1C	*Ban*II (1145)-*Ban*II (1660)	None[d]	πnsP12	172
Δ2A	*Nhe*I (1809)-*Nhe*I (2168)	None	πnsP12	120
Δ2B	*Mst*II (2124)-*Eco*RV (2750)	*Mst*II site filled in with the Klenow fragment	pMT2/3	209
Δ2C	*Cla*I (2714)-*Pvu*II (3103)	*Cla*I site blunt ended with mung bean nuclease	pMT2/3	130
Δ2D	*Eco*RV (2751)-*Nhe*I (3437)	*Nhe*I site filled in with the Klenow fragment	pMT2/3	229
Δ2E	*Pvu*II (3104)-*Ava*I (3550)	*Ava*I site blunt ended with mung bean nuclease	pMT2/3	149
Δ2F	*Xmn*I (3595)-*Tth*111 I (3912)	*Tth*111 I site filled in with the Klenow fragment	pMT2/3	106
Δ2G	*Ban*I (3862)-*Ban*I (4257)	None	pMT2/3	132
Δ2H	*Nhe*I (1809)-*Nhe*I (3437)	None	None	543
Δ34A	*Ssp*I (4130)-*Bam*HI (4638)	*Bam*HI site blunt ended with S1 nuclease[e]	None	—[e]
Δ34B	*Bam*HI (4638)-*Bam*HI (7334)	Both *Bam*HI sites were filled in with the Klenow fragment	None	899
Δ4A	*Hin*dIII (6272)-*Hpa*I (6919)	*Hin*dIII site filled in with the Klenow fragment	None	216

[a] Sindbis virus sequences are numbered according to Strauss et al. (43).
[b] aa, Amino acids.
[c] The clone isolated had three nucleotides deleted from the 5′ *Sma*I site.
[d] The clone isolated had three nucleotides deleted from within the *Ban*II site.
[e] The clone isolated had an extra nucleotide deleted from the *Bam*HI site, causing a frameshift to occur and resulting in termination approximately five amino acids downstream from the *Ssp*I site.

found that three lesions that render the nonstructural proteinase temperature sensitive all map to nsP2.

We have now constructed deletions within the nonstructural region which localize the proteinase activity to a region of ~350 amino acids at the C terminus of nsP2. We have also found that processing was inhibited when virion RNA was translated in the presence of anti-nsP2 immunoglobulin G (IgG). Finally, we have demonstrated that in vitro the proteinase may act both in *cis* and in *trans*.

MATERIALS AND METHODS

Preparation of virion RNA. Primary chicken embryo cells were infected with the HR strain of Sindbis virus (7); virus was purified and RNA was extracted as previously described (34). RNA was stored at −70°C in either 70% ethanol or in diethyl pyrocarbonate-treated water containing 33 U of Inhibit-Ace, an RNase inhibitor (5 Prime-3 Prime, Inc.), per ml. The RNA concentration was determined spectrophotometrically.

Reagents and general methods. Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs, Inc., Boehringer Mannheim Biochemicals, or Promega Biotec. *Escherichia coli* DNA polymerase (large fragment), S1 nuclease, and mung bean nuclease used to modify restriction sites before cloning were purchased from Bethesda Research Laboratories, P-L Biochemicals, Inc., and New England BioLabs, respectively. The restriction fragments generated were purified from low-melting agarose (FMC Corp.) after electrophoresis (51). Recombinant plasmids were transformed into MC1061. Dideoxy sequencing was performed using the Sequenase system (U.S. Biochemicals). A full-length cDNA clone of Sindbis virus from which full-length infectious RNA can be transcribed by SP6 RNA polymerase (Toto1101) was kindly provided by C. M. Rice (37), as was Toto1000.S, a derivative of full-length clone Toto1000 in which the opal codon between nsP3 and nsP4 has been replaced by a serine codon (27).

Construction of deletion mutants. Deletions were engineered into clone Toto1101 throughout the region encoding the nonstructural proteins, taking advantage of convenient restriction sites. Details of these constructions are given in Table 1. Since many of the restriction sites used occur several times in Toto1101, two shuttle vectors were used, πnsP12 and pMT2/3. πnsP12 (18) contains the *Sac*I (nucleotide [nt] 13552, upstream from the SP6 promoter in Toto1101)-to-*Eco*RV (nt 2750) fragment from Toto1101 cloned into πAN7 (29). pMT2/3 was constructed by cloning the *Eco*RI (nt 1921)-to-*Asu*II (nt 4705) fragment filled in with the Klenow fragment into the *Sma*I site of pMT21. Deletions were named Δ, followed by 1, 2, 3, or 4 for the nonstructural protein containing the deletion and then, A, B, etc., in order 5′ to 3′ within that protein. Deletions Δ1A, Δ1C, and Δ2A were constructed by treating shuttle vector πnsP12 with *Bal*I, *Ban*II, and *Nhe*I, respectively, followed by ligation. Then, in each case, the *Sac*I (nt 13552)-*Bgl*II (nt 2288) fragment containing the deletion was cloned back into Toto1101. Deletions Δ1B and Δ2H were made directly in Toto1101 by digestion with *Sma*I and *Nhe*I, respectively, followed by ligation.

All other deletions within nsP2 were engineered in shuttle vector pMT2/3 and later transferred to Toto1101. When deletions required the use of dissimilar restriction enzymes and produced incompatible ends, modifications were made to preserve the reading frame across the junction (Table 1). Deletions Δ2B (*Mst*II-*Eco*RV) and Δ2D (*Eco*RV-*Nhe*I) were transferred by ligating the *Dra*III (nt 1933)-*Avr*II (nt 4280) fragment of pMT2/3 into Toto1101 partially digested with *Dra*III and cut with *Avr*II. For deletions Δ2C (*Cla*I-*Pvu*II), Δ2E (*Pvu*II-*Ava*I), Δ2F (*Xmn*I-*Tth*111I), and Δ2G (the *Ban*I deletion which spans the nsP2/nsP3 boundary), the *Bgl*II (nt 2289)-*Avr*II (nt 4280) fragment was ligated into Toto1101 digested with *Avr*II and *Bgl*II.

The deletions in nsP3 and nsP4 were constructed directly in Toto1101. All three deletions, Δ34B (*Bam*HI), Δ34A (*Ssp*I-*Bam*HI), and Δ4A (*Hin*dIII-*Hpa*I) required modification of the termini before ligation. The Δ34B deletion was straightforward. After cutting with *Bam*HI, the cohesive ends were filled in with the Klenow fragment and the plasmid was reclosed with T4 ligase. Deletion Δ4A was constructed by a two-piece ligation of the *Bgl*II (nt 2289)-*Hin*dIII (nt 6272, filled in with the Klenow fragment) and *Hpa*I (nt 6919)-*Bgl*II (nt 2288) fragments. Deletion Δ34A (*Ssp*I-*Bam*HI) was generated by a three-piece ligation of the

*Bam*HI (nt 4638, blunt ended with S1 nuclease)-*Spe*I (nt 5262), *Spe*I (nt 5263)-*Bgl*II (nt 2288), and *Bgl*II (nt 2289)-*Ssp*I (nt 4130) fragments.

Deletions made in the shuttle vectors were screened by restriction analysis before they were transferred to Toto 1101. Transformants were checked by sequencing across the junctions created by the deletions, using either chain termination methods (48) or chemical methods (31). The sequence at the juncture was identical to that predicted from the cloning strategy in all but three cases, as noted in Table 1, footnotes *c* to *e*. First, deletion Δ1B was missing an additional three nucleotides (one amino acid) from the 5′ *Sma*I site. Second, in Δ1C three nucleotides were missing from the *Ban*II site, thereby destroying the site and deleting an additional amino acid. Finally, in Δ34A an extra nucleotide was removed from the 3′ *Bam*HI site, probably by S1 nuclease treatment, resulting in a shift in the reading frame. This change causes termination to occur five amino acids downstream, effectively deleting all nsP3 and nsP4 sequences.

In vitro transcription and translation of deleted clones. Small preparations of DNA for transcription were made by alkaline lysis (30). After digestion with either *Xho*I (nt 11749), which cuts downstream from the poly(A) tract so that a full-length polyadenylated RNA transcript is produced, or *Bss*HII (nt 9804), which cuts at the end of the E2 sequence, the DNA was incubated with 100 μg of proteinase K per ml at 37°C for 30 min, extracted twice with phenol-chloroform (1:1), and ethanol precipitated in the presence of 0.2 M sodium acetate. SP6 transcriptions were performed as previously described (37) with the following modifications: 10 mM dithiothreitol and 0.5 mM $m^7G(5')ppp(5')G$ were used in all of the reactions, and Inhibit-Ace at 33 U/ml was used in place of human placental RNase inhibitor. The quantity and integrity of the transcripts were checked on nondenaturing agarose gels before translation in vitro.

Nuclease-treated, methionine-depleted, rabbit reticulocyte lysate (Promega Biotec) was supplemented with 1 mCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham Corp.) per ml and 20 μM unlabeled amino acids lacking methionine, according to the instructions of the manufacturer. Inhibit-Ace was included at 33 U/ml to inhibit RNase activity. Unless otherwise noted, in vitro translations were carried out at 30°C for 60 min, using 13 to 17 μg of virion RNA per ml or 5 to 10 μg of SP6-transcribed RNA per ml in a total volume of 10 to 20 μl. For experiments which examined the posttranslational processing of labeled precursors over time, unlabeled methionine and cycloheximide were added to each reaction to a final concentration of 1 mM and 0.6 mg/ml, respectively, to prevent further protein synthesis. Incorporation was monitored by precipitation with trichloroacetic acid. The translation products were either examined immediately by discontinuous polyacrylamide gel electrophoresis in sodium dodecyl sulfate or diluted (1:1) with a solution containing 62 mM Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate, and 1% 2-mercaptoethanol for immunoprecipitation.

Immunoinhibition of processing. To an in vitro translation mixture containing virion RNA prepared as described above was added 0.1 volume of preimmune serum or antisera to each of the nonstructural proteins of Sindbis virus. The RNA was then translated for 60 min at 30°C in the presence of the various sera, or with 0.1 volume of water as a control, and the protein products were examined by polyacrylamide gel electrophoresis.

Translation of virion RNA was also performed in the presence of various amounts of anti-nsP2 IgG for 60 min at 30°C, and the protein products were examined as before. For these experiments the IgG was purified from polyclonal antiserum to nsP2 obtained from rabbits (21) by protein A affinity chromatography, using the monoclonal antibody purification system (Bio-Rad Laboratories), and had been tested for its ability to immunoprecipitate nsP2 and its precursors from Sindbis virus-infected cell lysates. The protein concentration of the purified IgG was determined using the dye-binding assay of Bradford (5).

Immunoprecipitation. Before immunoprecipitation, the diluted translation products were heated for 3 min at 90°C, followed by centrifugation at 16,000 × *g* for 8 min to remove high-molecular-weight aggregates. A 2- to 6-μl volume of the supernatant (i.e., 1 to 3 μl of the in vitro translation mix) was added to ~200 μl of RIPA buffer (23), and 4 μl of preimmune serum or antiserum monospecific for each of the nonstructural proteins of Sindbis virus (21) was added to each tube. After 45 min at room temperature, 20 μl of a 10% (wt/vol) suspension of *Staphylococcus aureus* cells (Calbiochem-Behring) was added to each reaction and incubation was continued for an additional 30 min at room temperature. The cells were pelleted by centrifugation for 2 min at 4,000 × *g* and washed three times with 500 μl of RIPA buffer.

The immunoprecipitated products were suspended in loading buffer (62.5 mM Tris hydrochloride [pH 6.8], 2.3% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10% glycerol), denatured by heating to 90°C for 3 min, and centrifuged for 8 min at 16,000 × *g* to remove the cells. These samples were then analyzed by polyacrylamide gel electrophoresis as previously described (21).

RESULTS

Kinetics of nonstructural polyprotein processing in vitro. A schematic diagram of the nonstructural region of Sindbis virus is shown in Fig. 1, together with the processing pathway for polyprotein P123 deduced from in vivo studies (21). Readthrough of the in-frame opal codon, indicated by the asterisk, also results in the production of a larger polyprotein, P1234, which is processed to give at least two additional products, P34 and nsP4.

To study the synthesis and processing of Sindbis virus nonstructural proteins in vitro, virion RNA was translated at 30°C in the presence of 1 mCi of [^{35}S]methionine per ml for various lengths of time. The translation products were then examined, either directly or after immunoprecipitation, by polyacrylamide gel electrophoresis (Fig. 2). Diffuse bands which migrate at the positions of nsP1 and nsP2 are visible as early as 14 min after the start of translation, and a sharp band of nsP1 is readily apparent after 20 min (Fig. 2A). Immunoprecipitation of the translation products confirms these assignments (Fig. 2B). The appearance of these products before nsP3 sequences have been completely translated (polyprotein P123 is not visible until 23 min of translation) suggests that the processing of the polyprotein precursor can occur nascently. Furthermore, precursor P12 can be detected before P123, as early as 17 min, also suggesting that cleavage at the 2/3 site can occur in the nascent polyprotein. P123 is readily detectable at 23 min, giving a calculated elongation rate of about 85 amino acids per min and demonstrating that cleavage does not always occur while the polyprotein is nascent.

While not readily apparent in Fig. 2, readthrough product P1234 can be detected by 32 min. Shortly thereafter, at 35 min, a band identified as P34 is detectable. This product

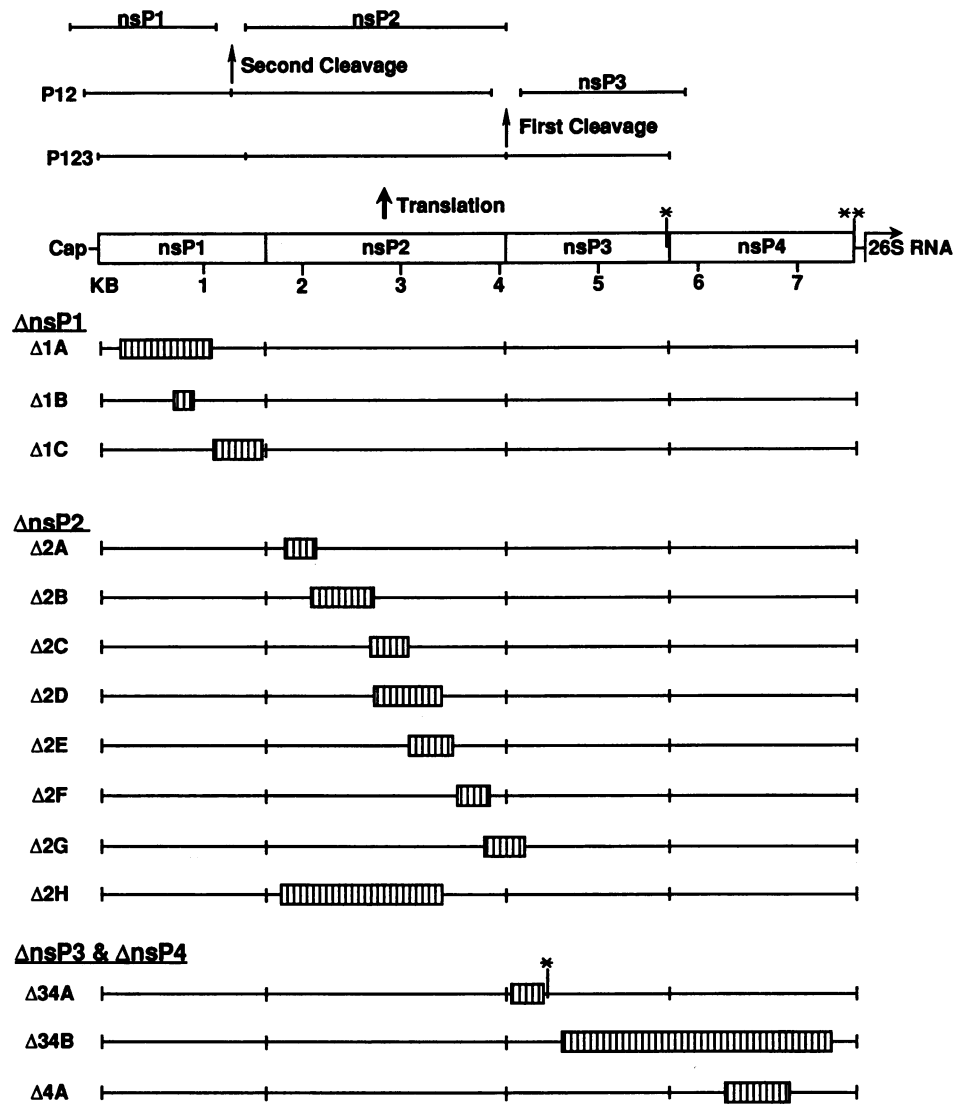

FIG. 1. Processing scheme of the nonstructural polyproteins of Sindbis virus and schematic representation of the deletion constructions. The mature nonstructural proteins of Sindbis virus, nsP1, nsP2, nsP3, and nsP4, result from the processing of two polyprotein precursors, P123, illustrated above a schematic of the nonstructural region of the Sindbis virus genome, and P1234 (not shown), which is produced by readthrough of an in-frame opal codon (asterisk). In vivo the bond between nsP2 and nsP3 in polyprotein P123 must be processed first to produce P12 and nsP3 before the bond between nsP1 and nsP2 can be cleaved, even though the latter cleavage is more rapid (21). Below is a schematic representation of the deletion constructs tested (deletions are illustrated as the hatched boxes; more details are presented in Table 1). The asterisks indicate locations of termination codons.

accumulates with time such that by 60 min it is readily seen in Fig. 2A (see also Fig. 2B).

In all of the gel patterns shown, there is a heterogeneous population of diffuse bands between P12 and nsP2 that is specifically immunoprecipitated by antisera to both nsP1 and nsP2 (Fig. 2B) and that probably results from premature termination. Also, a sharply defined precursor band at 155 kDa, which appears to possess nsP1, nsP2, and nsP3 sequences (Fig. 2B), accumulates with time from 20 to 35 min and then disappears by 60 min (Fig. 2A). The origin of this band is unclear.

Cleavage between nsP3 and nsP4. To examine the processing of P34 in vitro under our conditions, we translated RNA transcribed from Toto1000.S, in which the opal stop codon between nsP3 and nsP4 has been replaced with a serine codon (27). Samples were removed at 20, 30, 40, 50, and 60 min and analyzed by polyacrylamide gel electrophoresis (Fig. 3). At 60 min after the start of translation, unlabeled methionine and cycloheximide were added, and samples were removed after various times of further incubation to examine the stability of the polypeptides present at 60 min (Fig. 3). The overall pattern of polyprotein synthesis and processing was similar to that from virion RNA. Both nsP1 and P12 were just visible 20 min after initiation, and nsP2 was apparent by 30 min. P123, identified from its mobility and immunoreactivity, appeared at 30 min and reached a maximum concentration at about 40 to 50 min, at about the same time that P12 and P34 achieve their maxima. Readthrough products, as evidenced by the heterodisperse bands above 200 kDa, were visible by 30 min, and a discrete

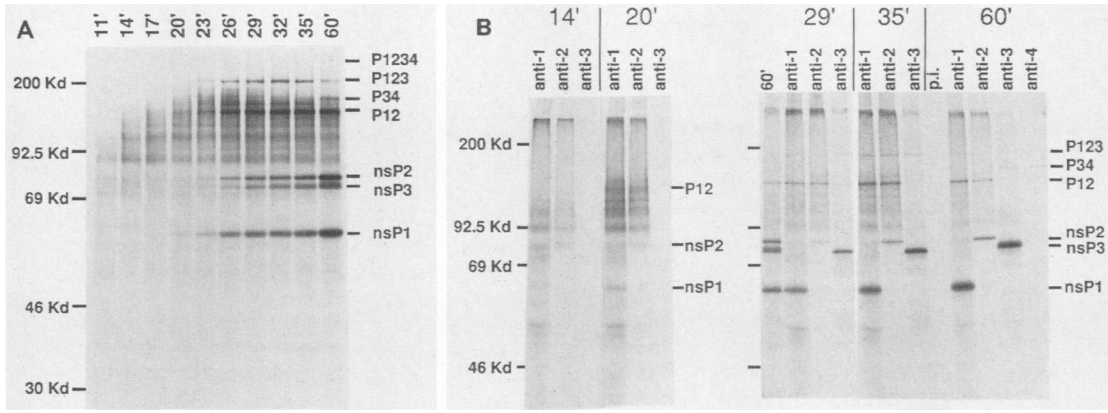

FIG. 2. Synthesis and processing of Sindbis virus polyproteins in vitro. (A) Virion RNA was translated at 30°C for the times indicated and then analyzed on a 10% polyacrylamide gel. The molecular masses of ^{14}C-standards (Amersham) are shown at the left and are as follows (in kilodaltons): myosin, 200; phosphorylase *b*, 92.5; bovine serum albumin, 69; ovalbumin, 46; and carbonic anhydrase, 30. The protein products are indicated on the right. (B) Labeled products from the 14-, 20-, 29-, 35-, and 60-min time points in panel A were immunoprecipitated with monospecific antisera to nsP1, nsP2, or nsP3, respectively; in the case of the 60-min time point, anti-nsP4 and preimmune serum (p.i.) were also used. These samples were then analyzed on 10% sodium dodecyl sulfate–polyacrylamide gels alongside the unfractionated translation products from the 60-min time point (60′) to aid in the identification of the precursors and mature nonstructural proteins visible in panel A.

band of the proper size for the P1234 precursor was visible by 40 min.

The appearance of only small amounts of nsP3, the absence of detectable nsP4 (examined more carefully by immunoprecipitation of the 30-, 80-, and 130-min samples [data not shown]), and the stability of P34 suggest that very little processing of P34 occurs under our conditions.

Inhibition of proteolysis by anti-nsP2 antibodies. In preliminary experiments to locate the nonstructural proteinase, virion RNA was translated in the presence of 10% preimmune serum or of one of the four nonstructural protein antisera for 60 min at 30°C. The incorporation of [^{35}S]methionine into protein was assayed by precipitation with trichloroacetic acid, and the translation products were analyzed by polyacrylamide gel electrophoresis (Fig. 4A). All five sera, including the preimmune serum, led to a decrease in the incorporation of [^{35}S]methionine into acid-precipitable material and to some inhibition of processing as compared with the control. The reaction containing anti-nsP2 showed a dramatic effect on processing, however, with only minute quantities of nsP1, nsP2, or nsP3 being produced.

To separate the antibodies from inhibitors which might be present in the serum, IgG was purified from the anti-nsP2 antiserum by protein A affinity chromatography. Virion RNA was then translated in the presence of various concentrations of anti-nsP2 IgG to examine the sensitivity of processing to the IgG concentration (Fig. 4B). At very high concentrations, 1.1 mg of IgG per ml or higher, elongation was inhibited (data not shown). In the presence of 850 μg of IgG per ml, elongation occurred but processing was virtually undetectable. In addition, a clear band of P1234 was present.

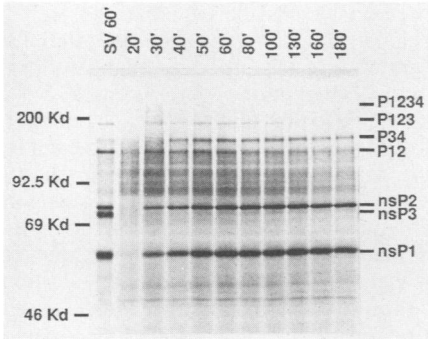

FIG. 3. Synthesis and processing of precursors translated in vitro from Toto1000.S. RNA transcribed from Toto1000.S by SP6 polymerase was translated in vitro at 30°C for the times indicated. Excess unlabeled methionine was added at 60 min to prevent further incorporation of label, and thus samples after this constitute a chase. Samples were analyzed by polyacrylamide gel electrophoresis. Virion RNA which had been translated for 60 min at 30°C (SV 60′) was included for comparison. Standards and the position of Sindbis virus precursors and nonstructural proteins are the same as in Fig. 2.

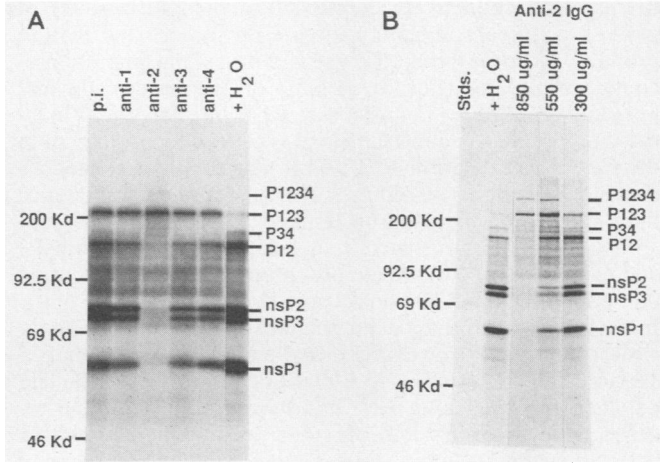

FIG. 4. Inhibition of processing by antisera to the nonstructural proteins of Sindbis virus. (A) A 9-μl volume of rabbit reticulocyte lysate primed with virion RNA was incubated at 30°C for 60 min in the presence of 1 μl of preimmune serum or of antiserum specific for one of the four nonstructural proteins of Sindbis virus. Water was used in place of serum in the control. The samples were then analyzed by gel electrophoresis as in Fig. 2. (B) Virion RNA was translated in the presence of nsP2-specific IgG. The final concentration of IgG in the translation mix is shown.

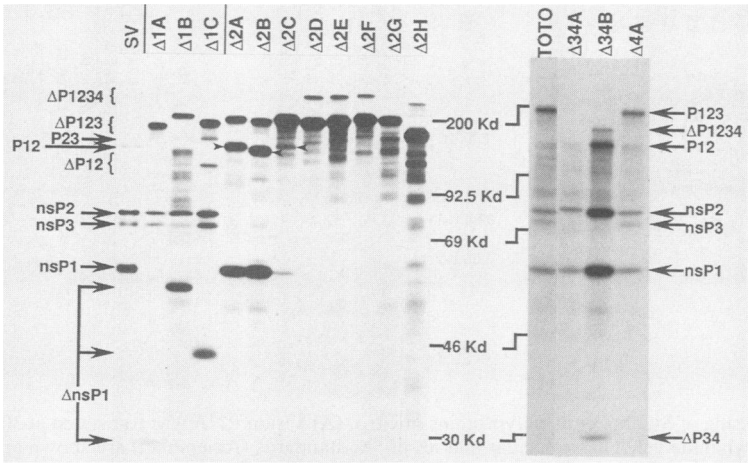

FIG. 5. In vitro translations of deletion clone RNAs. Virion RNA (SV) or RNA transcribed with SP6 polymerase from Toto1101 (TOTO) and from each of the deletion clones was translated for 60 min at 30°C in a rabbit reticulocyte lysate. The translation products were then analyzed by polyacrylamide gel electrophoresis. Precursors with reduced electrophoretic mobility because of internal deletions are indicated (ΔP1234, ΔP123, and ΔP12), as are deleted forms of nsP1 (ΔnsP1). The identity of these proteins was determined by immunoprecipitation (not shown). The arrowheads in lanes Δ2A, Δ2B, and Δ2C point to polypeptide ΔP23, containing the sequence of deleted nsP2 plus nsP3. The clones indicated at the top are described in detail in Table 1 and are shown in Fig. 1.

With 550 μg of IgG per ml some processing occurred, whereas at 300 μg/ml processing was extensive but still less than that in the control. These data are consistent with the hypothesis that nsP2 is the nonstructural proteinase.

Deletion mapping. To map the location of the proteinase activity responsible for processing the nonstructural polyproteins, a number of deletions were engineered into a full-length cDNA clone of Sindbis virus, Toto1101 (37). These deletions are illustrated schematically in Fig. 1. RNA was transcribed with SP6 RNA polymerase from each deletion construct and translated for 60 min in a reticulocyte lysate system. The individual reactions were then examined directly (Fig. 5) or after immunoprecipitation with antisera to each of the four nonstructural proteins (not shown).

Translation products of the three deletion constructs in nsP1, which collectively delete 470 of the 540 residues of nsP1, processed completely to give precursors and mature products with the predicted gel mobilities and immunoprecipitation patterns (Fig. 5). A band of the correct size and immunoreactivity to be precursor P23 was also found in all three nsP1 deletion constructs and is especially prominent in the C-terminal deletion, Δ1C. This suggests that sequences in nsP1, although not required for function, may influence the context in which the proteinase interacts with its substrate. Similarly, constructs that deleted sequences in nsP3 and nsP4 also gave complete processing, with the exception that construct Δ34A did not cleave between nsP2 and nsP3. In this case a product accumulated that migrated slightly slower than nsP2, consisting of nsP2 plus the N-terminal 15 amino acids from nsP3. Note that even in this case the site between the nsP1 and nsP2 bond was cleaved. Taken together, these results indicate that only sequences within nsP2, plus at most a few amino acids from the C terminus of nsP1 and the N terminus of nsP3, are required for proteolytic processing.

In contrast, clones with deletions in nsP2 showed aberrant processing. Deletions in the N-terminal half of nsP2 (Δ2A, Δ2B, and Δ2C) failed to cleave between nsP2 and nsP3, giving deleted forms of P23, as indicated with arrowheads, and at least some mature nsP1 (markedly less in Δ2C than in Δ2A or Δ2B). On the other hand, clones with deletions that are downstream from the *Pvu*II site (nt 3103), or amino acid 475 of nsP2, namely, deletions Δ2D, Δ2E, Δ2F, Δ2G, and Δ2H, showed no evidence of processing. It is also of note that these deletions, especially Δ2D, Δ2E, and Δ2F, lead to the accumulation of deleted forms of P1234, analogous to the accumulation of P1234 when RNA was translated in the presence of anti-nsP2 IgG to inhibit processing (Fig. 4B). From these results we conclude that the proteinase-active site, assuming that a single proteinase is responsible for the two or three cleavages required to process the polyproteins, must be encoded between the *Pvu*II (nt 3103) and *Ssp*I (nt 4130) sites, that is, between amino acids 475 and 807 in the C-terminal half of nsP2.

***trans* processing of uncleaved precursors from deletion clones.** To determine whether the Sindbis virus nonstructural proteinase could function intermolecularly (in *trans*), we took advantage of several deletion clones which do not process one or more of the precursors. These were transcribed and translated in the presence of [^{35}S]methionine as described previously. Parallel incubations were also performed in which virion RNA was translated in the absence of radioactive amino acids to produce unlabeled nonstructural proteins. Control incubations were identical but contained no added RNA. After 60 min at 30°C, cycloheximide and excess unlabeled methionine were added to each reaction. A sample of the control reticulocyte lysate or the lysate containing the unlabeled nonstructural proteins was then mixed with an equal volume of the reactions containing the labeled, uncleaved precursors from the deletion clone constructs. The samples were incubated at 30°C for an additional 90 min and prepared for analysis by polyacrylamide gel electrophoresis (Fig. 6). For all seven deletion clones tested, at least some additional processing was seen when Sindbis virus nonstructural proteins from the translation of virion RNA were added (compare lanes labeled with the name of the deletion clone with those marked +SV), but not when a control lysate incubated without added RNA was supplied (lanes marked +BL). The processing was most extensive in clones Δ2A, Δ2B, and Δ2C, which contain nsP2 deletions 5′ of the *Pvu*II site at position 3103 (Fig. 6A) and in which some processing occurred even in the absence of added translation

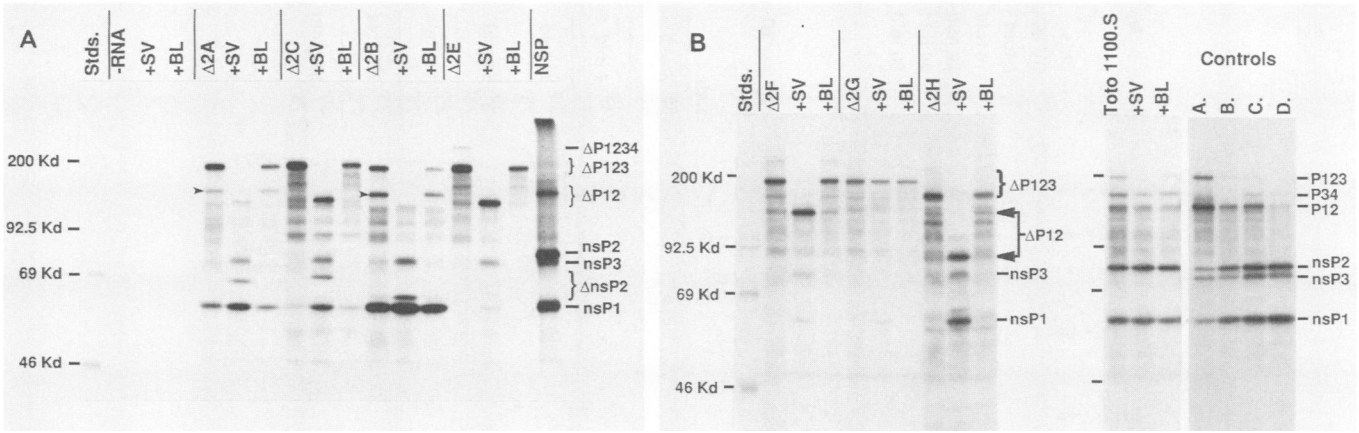

FIG. 6. *trans* processing by the Sindbis virus nonstructural proteinase. RNAs from seven of the deletion clones were translated for 60 min at 30°C to produce labeled precursors (lanes labeled according to the RNA translated). A control lacking RNA (−RNA) was included to ensure that no additional incorporation occurred after the cycloheximide and unlabeled methionine were added. To each translation reaction an equal volume of unlabeled nonstructural proteins translated from Sindbis virion RNA (+SV) in a mix supplemented with 20 μM methionine and the other amino acids at 40 μM, or of blank rabbit reticulocyte lysate (+BL), was then combined with the labeled precursors, and the mixture was incubated for an additional 90 min at 30°C in the presence of cycloheximide and unlabeled methionine to prevent further incorporation. Arrowheads in lanes Δ2A and Δ2B indicate deletion polypeptide ΔP23. Labeled nonstructural proteins (NSP) produced by translating virion RNA in vitro were included as markers in panel A. Panel B at the right also shows the results when labeled translation products from RNA transcribed from clone Toto1000.S were incubated with unlabeled translation products from Sindbis virus RNA, as well as a number of controls that examined both the synthesis and processing of nonstructural proteins during translation of virion RNA. Virion RNA was translated for either 30 min (control A) or 60 min (control C) at 30°C, and to portions of both translation mixtures were then added cycloheximide and unlabeled methionine. Control A was then incubated for an additional 30 min (control B) and control C was incubated for an additional 90 min (control D), at 30°C. Electrophoresis was as described in the legend to Fig. 2. Lane Toto1100.S, Toto1000.S, as defined above.

products. For these clones the cleavage of ΔP123 was complete in the presence of added viral proteins, and nsP1, ΔnsP2, and nsP3 were produced. The processing of clones with deletions 3′ of the *Pvu*II site was also apparent (Fig. 6A and B). In clones Δ2E, Δ2F, and Δ2H, the processing of the ΔP123 precursor was complete, but the major cleavage products were ΔP12 and nsP3, with only very small amounts of nsP1 visible in the first two cases and larger amounts present in the last case. Deletion Δ2G, in which the cleavage site between nsP2 and nsP3 had been deleted, was inefficiently processed, but did produce some nsP1 and a faint band of ΔP23 of about 140 kDa. From these results it is clear that the Sindbis virus nonstructural proteinase can act in *trans* to process both cleavage sites in P123, but the cleavage between nsP2 and nsP3 is more efficient, at least in the constructs tested. These results also show that the processing of the nonstructural polyprotein is performed by a Sindbis virus protein and not by a proteinase present in the reticulocyte lysate.

In a further experiment, Toto1000.S was translated and mixed with unlabeled nonstructural proteins (+SV) or blank lysate (+BL) to determine whether supplying the translation mixture with additional nsP2 would lead to more processing of P34. The protein patterns were identical (Fig. 6B, right panel), indicating that the availability of proteinase was not the rate-limiting step in the processing of P34. The right panel of Fig. 6B also shows a series of controls that were performed to examine the nonstructural proteins translated from Sindbis virion RNA and to demonstrate that the methionine and cycloheximide added after 60 min had no deleterious effect on subsequent processing. A minus-RNA control was also included to ensure that no additional [^{35}S]methionine was incorporated after the addition of lysate.

Effects of dilution on proteinase function. The demonstration of *trans* cleavage by the Sindbis virus nonstructural proteinase offered the opportunity to determine whether the protease could also act in *cis*. Dilution experiments were performed to determine whether a dilution could be achieved at which *trans* activity could no longer be detected but at

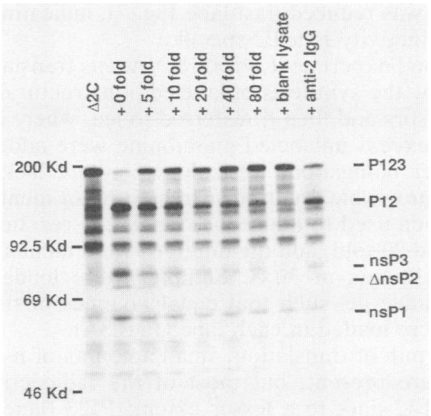

FIG. 7. Dilution of the *trans*-processing activity of the nonstructural proteinase. Labeled precursors were generated by translating RNA from clone Δ2C for 60 min at 30°C. A sample of this material was then combined either with an equal volume of unlabeled nonstructural proteins synthesized from virion RNA as in Fig. 6 that had been diluted 0-, 5-, 10-, 20-, 40-, and 80-fold in blank lysate or with an equal volume of blank lysate. In the last lane the unlabeled nonstructural proteins were preincubated on ice for 5 min with anti-nsP2 IgG before being combined with the Δ2C translation products. To all of the reactions were added unlabeled methionine and cycloheximide as in Fig. 6 to prevent additional elongation, and the mixtures were incubated at 30°C for 30 min to allow processing to occur.

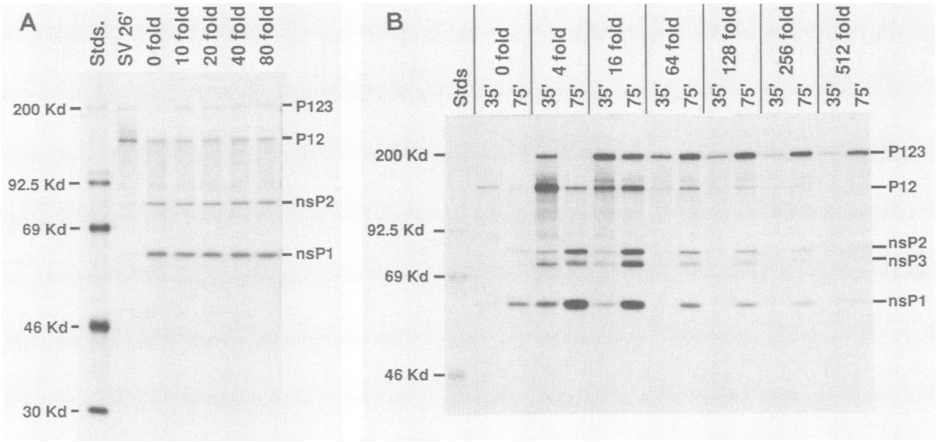

FIG. 8. Effect of dilution upon processing of Sindbis virus polyprotein precursors. (A) Virion RNA was translated at 30°C to allow the synthesis of labeled precursors. After 26 min (SV 26′), translation was terminated by adding excess unlabeled methionine and cycloheximide, and the sample was diluted 0-, 10-, 20-, 40-, and 80-fold with blank rabbit reticulocyte lysate, treated similarly to inhibit elongation. The dilutions were returned to 30°C for another 30 min and then analyzed by polyacrylamide gel electrophoresis. (B) Serial dilutions of virion RNA were translated at 30°C for either 35 or 75 min and then analyzed by polyacrylamide gel electrophoresis. The highest concentration of RNA used was 43 μg/ml.

which *cis* processing still occurred. First, processing in *trans* was examined by incubating a labeled, uncleaved precursor(s) from clone Δ2C with an equal volume of unlabeled nonstructural proteins prepared as above but diluted 0-, 5-, 10-, 20-, 40-, or 80-fold (Fig. 7). The results demonstrate that the *trans*-acting activity of the proteinase is sensitive to dilution. Even a fivefold dilution had a pronounced effect upon the extent of processing, and at the highest dilution processing is greatly reduced, but still detectable, when compared with incubation with blank lysate. As noted above, the *trans* activity is directed primarily at the site between nsP2 and nsP3. Moreover, when anti-nsP2 IgG was incubated with the undiluted nonradioactive nonstructural protein mix before it was added to the labeled precursors, processing was reduced (last lane Fig. 7), indicating that the proteolytic activity is nsP2 specific.

In a second experiment, virion RNA was translated for 26 min to allow the synthesis of labeled nonstructural polyprotein precursors and then transferred to ice, where cycloheximide and excess unlabeled methionine were added to prevent further elongation. Reticulocyte lysate lacking RNA (blank lysate), which had been treated in an identical fashion, was then used to dilute the translation reaction 0-, 10-, 20-, 40-, and 80-fold, and the dilutions were incubated for an additional 30 min at 30°C. Samples were loaded onto a polyacrylamide gel such that equal volumes of the labeled material were loaded in each lane (Fig. 8A).

After 26 min of translation, small amounts of nsP1, nsP2, and nsP3 are present, but most of the radioactivity is in precursor P12 and, to a lesser extent, P123 (lane SV 26′). Dilution appeared to have no effect on the processing of P12 to mature nsP1 and nsP2 over the next 30 min, suggesting that cleavage of the bond between nsP1 and nsP2 was autoproteolytic, while the processing of the P123 precursor was sensitive to dilution, and P123 was completely processed only in the undiluted sample. Mature nsP3 was difficult to detect in this experiment because after 26 min of translation the majority of label is in nsP1 and nsP2 sequences (see the 26-min time point in Fig. 2).

In a similar experiment to examine the processing of P12, deletion construct Δ34A (which when translated gives essentially P12) was translated for 26 min, then diluted up to 80-fold, and incubated for 30 min more. The results appeared to be identical to those obtained in Fig. 8A, and no effect of dilution upon the processing of P12 could be detected (data not shown).

To further examine the sensitivity of the cleavage between nsP2 and nsP3 to dilution and to determine the order of processing, we translated serial dilutions of virion RNA for 35 or 75 min at 30°C. Since the concentration of nonstructural proteins present is a function of the concentration of virion RNA translated, this procedure effectively tested for *trans* cleavage as a function of the protein concentration. Translations were terminated by adding 1 volume of sample buffer, and the samples were adjusted such that each lane contained translation products of equivalent amounts of input RNA.

At the highest RNA concentration used, 43 μg/ml, which was threefold higher than that used in the previous experiments, the reticulocyte translation components were limiting; incorporation was reduced and primarily N-terminal products were synthesized (Fig. 8B). At lower concentrations of virion RNA, P123 and its cleaved products were produced in good yield (see especially the 4- and 16-fold dilutions). With increasing dilution, however, precursor P123 accumulated and cleaved products P12, nsP1, nsP2, and nsP3 decreased. At the highest dilution used, very little cleavage occurred. While we cannot rule out limited *cis* cleavage of the bond between nsP2 and nsP3, it appears that this cleavage occurs primarily in *trans* and that this bond must be cleaved to facilitate further processing of P12.

In summary, cleavage of the bond between nsP2 and nsP3 is sensitive to dilution and can be readily demonstrated to occur in *trans*, suggesting that cleavage is normally a *trans* event. Cleavage between nsP1 and nsP2 is insensitive to dilution (at least in the P12 precursor) and is difficult to demonstrate in *trans*, suggesting that mature nsP1 and nsP2 are normally produced by autoproteolysis of the P12 precursor.

DISCUSSION

Location of the proteinase. Antibodies to localize the activity of a viral proteinase have been previously used by

Hanecak et al. (21) and Carrington and Dougherty (9). In our system the use of purified anti-nsP2 IgG was particularly successful in inhibiting processing. From the immunoinhibition results and, more important, from the processing of translation products from deletion constructs, we have localized the nonstructural proteinase to the C-terminal half of nsP2, in agreement with previous results from the mapping of temperature-sensitive mutants in the nonstructural proteinase (19) and from the translation of truncated RNAs (13).

Attempting to localize a proteinase activity by examining the processing of deletion constructs can be complicated by the possibility that changes in conformation induced by the deletions, rather than deletion of the active site per se, may render the proteinase inactive or the sites of cleavage inaccessible (10). The results here with a large number of deletion constructs were remarkably consistent, however, and indicated that the C-terminal domain of nsP2 between amino acids 475 and 807 contained the active site of the enzyme. The results with construct Δ2C, in which the proteinase activity is greatly reduced in comparison with Δ2A and Δ2B, but not abolished, suggests that deletion to the *Pvu*II site at amino acid 474 of nsP2 invaded the proteinase domain but did not eliminate the active site and thus that the N terminus of the proteinase domain is found between the *Eco*RV site used for construct Δ2B (amino acid 356 of nsP2) and the *Pvu*II site (amino acid 474).

It has been found previously that three large domains in the nonstructural proteins of Sindbis virus share sequence homology with domains in the nonstructural proteins of a number of plant viruses, including tobacco mosaic virus (1, 22). In particular, amino acids 30 to 459 of Sindbis virus nsP2 are homologous to a nonstructural protein in these plant viruses. However, while the plant virus proteins terminate at this point, Sindbis virus nsP2 continues for 348 residues. These C-terminal 348 residues of Sindbis virus nsP2 have no plant virus counterpart, and it is precisely within these residues that we have found the proteinase, consistent with the fact that these plant viruses evidently lack a proteinase. Thus, nsP2 appears to possess two distinct domains, an N-terminal domain of about 460 residues required for RNA replication and presumably performing the same functions in RNA replication as the homologous plant virus proteins and a C-terminal domain of about 350 residues that is a proteinase. The two domains appear to function independently. The 10 or so amino acids that form the extreme C terminus of the replicase domain and the N terminus of the proteinase domain are not well conserved among alphaviruses and contain a large proportion of charged residues and a number of proline residues, suggesting that this region might function as a linker that possesses limited secondary structure.

Nature of the proteinase. Recently, Ding and Schlesinger (13) proposed that nsP2 may be a metalloproteinase. We suggest, however, that it may be a thiol proteinase. First, Zn^{2+} inhibits the processing of alphavirus precursors (6), which is a characteristic of proteins with a cysteine residue at the active site. A similar result has been obtained for picornavirus proteinase 3C (8, 35), which has an active-site cysteine residue. Second, the presence of a reducing agent, such as 2-mercaptoethanol, during in vitro translation is also required for efficient processing, suggesting that nsP2 may be a cysteine proteinase. As a note of general interest, it has also been found that general serine protease inhibitors, such as phenylmethylsulfonyl fluoride and Nα-tosyl-L-lysine chloromethy ketone, have no effect on the Sindbis virus nonstructural proteinase (11).

An examination of the deduced amino acid sequence of several alphaviruses reveals the conservation of 11 cysteine residues and 11 histidine residues in nsP2, of which only 2 cysteine residues and 5 histidine residues occur in the C-terminal domain defined here as the proteinase. One cysteine residue in particular is in a region that shows limited sequence similarity to the active-site residue of the cysteine proteases of the papain family (Fig. 9). As shown, Cys-481 is in a domain that is similar to that containing the active-site cysteine residue of papain proteases, and its position with respect to the N terminus of the nsP2 proteinase domain coincides almost precisely with the position of active-site Cys-25 from the N terminus of papain (28, 36). The importance of this region for proteinase function has been shown by the mapping of three mutations of Sindbis virus which result in temperature-sensitive processing of the nonstructural proteins (19). Two of these mutations are shown in Fig. 9 and are located just downstream from Cys-481 (the third change occurred at position 736).

Four conserved histidine residues in the proteinase domain of nsP2 are also shown in Fig. 9. Two of these, His-619 and His-709, are in regions that show similarities in sequence to active-site His-159 of papain. The first, His-619, is spaced by 138 amino acids from Cys-481, almost identical to the spacing of 134 amino acids between the active-site residues of papain. The second, His-709, although spaced by 228 residues from Cys-481, is near the temperature-sensitive lesion of *ts*24 at residue 736. It is impossible to predict which of the histidine residues might be involved in proteolysis on the basis of current information; the adjacent histidine residues at positions 701 and 702 seem unlikely to be a component of the active site.

If our hypothesis is correct that the nonstructural proteinase of alphaviruses belongs to the papain family of thiol proteases, this would represent a new family of proteinases in viruses. Proteinases previously studied have been postulated to belong to the trypsin/chymotrypsin family of serine proteases (even in the case of picornaviruses, where the active-site serine has been replaced by a cysteine residue) (3, 15) or to the aspartate family of proteases, of which pepsin is the cellular model, in the case of retroviruses (reviewed in reference 26).

***cis* and *trans* cleavage.** From the data presented here, it appears that the kinetics of processing of the polyprotein precursors of Sindbis virus in vivo and in vitro are similar, but the early appearance of nsP1, nsP2, and P12, before nsP3 or P123 is detected, indicates that there is more nascent processing in vitro than in vivo. This increase could be due to the reduced rate of elongation in vitro at 30°C, 85 amino acids per min as compared with ~240 amino acids per min in vivo at 37°C (21). In this regard, Collins et al. (11), who obtained a similar elongation rate in vitro, observed the same relative order of appearance for the nonstructural proteins.

While some processing to nsP1 and nsP2 can occur during elongation of the polypeptide chain, most nonstructural proteins arise from the processing of completed precursor P123 and its cleavage product, P12 (Fig. 1). After completion of the P123 precursor, the bond between nsP2 and nsP3 must first be cleaved in *trans* to generate P12 and nsP3. Once P12 is produced, the processing of the bond between nsP1 and nsP2 can occur either autoproteolytically or in *trans* to produce mature nsP1 and nsP2, with autoproteolytic cleavage apparently favored kinetically. The protein species responsible for the *trans* cleavage is not known. We presume that nsP2, once formed, can act in *trans* as a proteinase, although there is no direct evidence for this; but there still appears to be a requirement for a *trans* proteinase that acts

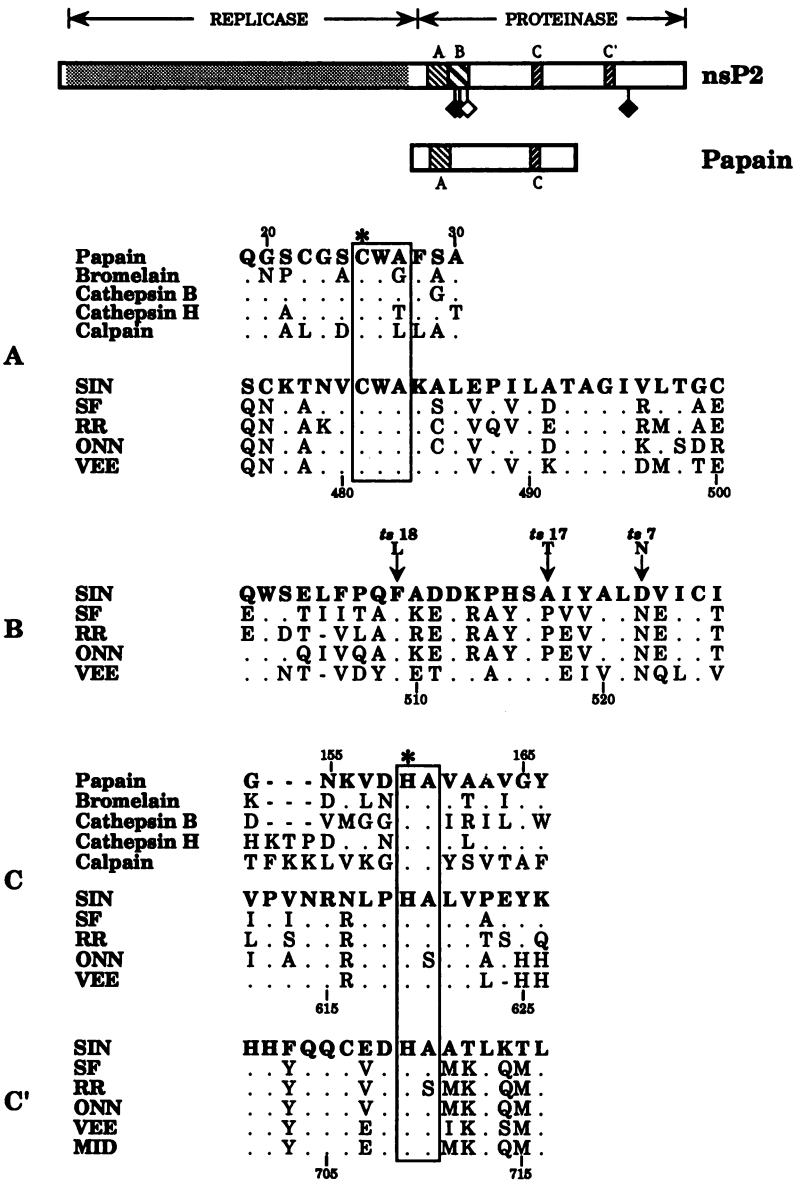

FIG. 9. Protein sequence comparison between several alphaviruses and five members of the papain protease superfamily. A schematic of nsP2 is shown, divided into a replicase region and a proteinase region. Sequences of five or six alphavirus nsP2s in the regions marked A, B, C, and C′ are shown below, aligned in regions A, C, and C′ with regions in papain (A and C as indicated in the schematic) and the corresponding regions of four other papain family proteases. The boxed amino acids indicate possible active-site residues of the alphavirus proteinase, aligned with the catalytic-site cysteine and histidine residues (asterisks) of papain and the four other papainlike cysteine proteases. The location of three mutations (*ts*18, *ts*17, and *ts*24) that render the Sindbis virus proteinase inactive upon a shift from a permissive to a nonpermissive temperature are indicated by the solid diamonds, and sequences for two of these are indicated below in sequence block B (19). The open diamond indicates the position of *ts*7, which is RNA$^-$ but whose proteinase remains functional upon shift. Amino acids which are identical to the sequence in boldface type on the first line of each cluster are indicated by dots, while gaps introduced for alignment have been designated by dashes. The numbering which appears above the papainlike proteases is that of papain (12), while that for the alphavirus sequences is that of Sindbis virus (43). The data are from Dayhoff (12) for papain, Goto et al. (16) for stem bromelain, Ritonja et al. (39) for human cathepsin B, Takio et al. (49) for rat cathepsin H, Ohno et al. (33) for chicken calpain II, Strauss et al. (43) for Sindbis virus (SIN), Takkinen (51) for Semliki Forest virus (SF), Faragher et al. (14) for Ross River virus (RR), E. G. Strauss (unpublished data) for O'nyong-nyong virus (ONN) and Middelburg virus (MID), and Kinney et al. (25) for Venezuelan equine encephalitis virus (VEE).

at the precursor stage, before any nsP2 has been formed, as the results of translation of RNA at a very high dilution suggest that very little processing of P123 occurs except in *trans*. It thus seems likely that one or more of the precursor polyproteins that contain nsP2, such as P123 itself, must possess proteinase activity capable of cleaving P123 in *trans*, although it is possible that the small amount of processing detected while the polyprotein is nascent might be responsible for the initial formation of the *trans*-acting proteinase.

The requirement for a *trans* cleavage followed by a *cis* cleavage is unique among virus proteinases studied to date and gives rise to the slow processing kinetics observed in

vivo (21). The unusual nature of the cleavage kinetics and the relatively long half-lives of the intermediates suggest that the polyprotein intermediates might have functions distinct from those of the processed products. It also seems possible that early in infection the cleavage kinetics might differ and that precursors such as P123 might be involved in minus-strand RNA synthesis. It is also provocative that the plant virus proteins that are homologous to the Sindbis virus proteins function as what is in essence a polyprotein. Perhaps the acquisition of a proteinase by the alphaviruses enabled them to differentially regulate replication and transcription more precisely. Further studies on the capabilities of proteins individually expressed to function as proteinases and of Sindbis virus mutants that are unable to cleave the polyprotein will be instructive.

We have found that when proteinase function is inhibited by antibodies or by deletion of the active site, the amount of readthrough polyprotein detected on gels increases greatly, suggesting that the extent of readthrough is greater than previously thought and that rapid cleavage, possibly while nascent, occurs in a significant fraction of the readthrough polyprotein. Such cleavage could account for the production of P123 in the opal codon mutant, Toto1000.S (Fig. 3). We have detected very little nsP4, however, whether in vitro (this paper) or in vivo (21). Similarly, Li and Rice (27) detected little nsP4 in vivo with the opal codon mutant, although nsP3 was produced. It is unclear at present whether nsP4 escapes detection because it is selectively lost during analysis or whether it is rapidly degraded, possibly by the viral proteinase. Experiments are under way to determine whether the concentration of nsP4 is regulated after its synthesis in Sindbis virus-infected cells; but it is interesting that at least two alphaviruses, Semliki Forest virus (50) and O'Nyong-nyong virus (42), do not possess the opal codon between nsP3 and nsP4 and produce only the equivalent of Sindbis virus readthrough polyprotein P1234. The major cleavage pathway appears to be similar to that in Sindbis virus in that the first cleavage occurs between nsP2 and nsP3 to produce P12 and P34 (21, 40). P34 must be cleaved to produce nsP3, which occurs rapidly, and large amounts of nsP4 are readily found in infected cells (24), in contrast to the situation with the Sindbis virus opal codon mutant (27). Why such closely related viruses differ in what would appear to be a fundamental aspect of the regulation of the amounts of a replicase component is a mystery, and whether different concentrations of nsP4 are required or simply tolerated for efficient replication of some alphaviruses but not others is unknown.

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