# The Characterization and Processing of the Nonstructural Proteins of Sindbis Virus

Thesis by

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*In Partial Fulfillment of the Requirements*

*for the Degree of*

*Doctor of Philosophy*

California Institute of Technology

Pasadena, California

1990

(Submitted September 19, 1989)

## DEDICATION

This thesis is dedicated to my mother and grandmother, without whose love, support and encouragement this day never would have come. Should I stand to receive my degree there shall be two more that stand with me.

## ACKNOWLEDGEMENTS

During my graduate study, so many others have intervened when I was ready to quit and walk away that I feel the degree belongs to them as much as it does to me. My gratitude is long overdue, especially when I have spent such a large part of my time here at Caltech being ungrateful and bitter, sometimes flaunting my dissatisfaction and venting my anger on those who have been most helpful to me. Therefore, it is both an apology and a thank-you that I now write in acknowledgement of those who have been loyal and have "hoped against hope" in anticipation of my completion.

First, I would like to thank Sabine, my fiancée, whose love, patience, and reassurance inspired me to finish early in spite of my doubts. Many thanks to Warren, Caren, Art and JR; you were there whenever I had a problem, even when it seemed redundant. Also, my appreciation to Dr. Leighton King and Dr. Bruce Mattson for giving me a head start in science, and to Betty for all of her prayers and support.

I wish to thank especially Jim and Ellen for all their time and help, and for giving me the hope I needed to believe I could finish. Charlie, thank you for your friendship, guidance and pep-talks. Also, many thanks to Chang, Young, Ricardo, Richard, Randy, Kang-sheng, Nathalie, Raoul, Frank, Edith, Yukio and Hong for your help and friendship. Finally, I would like to thank my other advisor, Dr. John Richards, for his hospitality, support and encouragement despite my lack of participation in the group, as well as the other members of my committee, Drs. Judith Campbell, Scott Emr and John D. Baldeschwieler; especially J.D.B. for his time and kind encouragement.

Above all thanks be to God, the only wise God, without whose grace and tender mercy I would be lost. Although mentioned last, all praise and

thanks belong to Him first. Jesus said of himself, "I am the way, the truth, and the life. No one comes to the Father (God) except through me" (John 14:6). This is a timeless reality.

## SHORT ABSTRACT

The nonstructural proteins of Sindbis virus, the type alphavirus of the family Togaviridae, are produced by proteolytic cleavage of two polyprotein precursors. One precursor (P123) is 1,896 amino acids in length and contains the sequences of nsP1, nsP2, and nsP3, while the second (P1234) of 2,513 amino acids is produced by readthrough of an opal termination codon and contains a fourth nonstructural protein, nsP4.

In order to study the kinetics of processing of these polyproteins, monospecific antisera were produced in rabbits to fusion protein antigens containing the N-terminal two-thirds of the *trpE* protein of *E. coli* fused to a large part of the sequence within each of the nonstructural proteins of Sindbis virus. Using these antisera the following details of processing were elucidated: i) most nonstructural proteins arise from the processing of the completed precursor P123 and its cleavage product, P12; ii) in the P123 precursor, the primary cleavage occurs in *trans*, between nsP2 and nsP3 to generate P12 and nsP3 with a half-life of ~19 min. *in vivo*, followed by processing of P12 to produce nsP1 and nsP2, either intramolecularly or in *trans*, at a rate which exceeds that of the first cleavage; iii) processing of the 3-4 site is complex; and iv) nsP3 was found to be phosphorylated during posttranslational modification.

*In vitro*, processing could be inhibited with antibodies to nsP2, but not with antisera to the other three nonstructural proteins, suggesting that the nonstructural proteinase is in nsP2. Deletion-mapping studies were performed which further localized the proteinase to a region of approximately 334 amino acids at the C terminus of nsP2. From a comparison of the deduced sequences of several alphaviruses in this part of nsP2 with the sequences of cellular proteinases, a hypothesis was presented

that the proteinase is a thiol protease related to papain. Finally, the examination of several temperature-sensitive mutants of Sindbis virus has confirmed the importance of this region as only mutants that possessed a mutation in the C-terminal domain of nsP2 produced aberrant processing patterns at the nonpermissive temperature.

## ABSTRACT

In Sindbis virus, the type alphavirus of the family Togaviridae, the nonstructural proteins involved in viral replication are produced by proteolytic cleavage of two polyprotein precursors. One precursor (P123) is 1896 amino acids in length and contains the sequences of nsP1, nsP2, and nsP3; while the second (P1234) of 2513 amino acids is produced by readthrough of an opal termination codon and also contains the sequences of a fourth nonstructural protein, nsP4.

In order to study the processing of these proteins, monospecific antisera were produced in rabbits to fusion protein antigens containing the N-terminal two-thirds of the *trp E* protein of *E. coli* fused to a large part of the sequence within each of the nonstructural proteins of Sindbis virus. Each antiserum was specific for the corresponding nonstructural protein and its precursors, and was used in immunoprecipitations to examine the kinetics of polyprotein processing both *in vivo* and *in vitro*. While some processing can occur during elongation of the polyprotein, most nonstructural proteins arise from the processing of the completed precursor P123 and its cleavage product, P12. In the P123 precursor, the primary cleavage occurs in *trans*, between nsP2 and nsP3, to generate P12 and nsP3. This cleavage had a half-life of ~19 min *in vivo*. Processing of P12 to produce nsP1 and nsP2 can then proceed, either intramolecularly or in *trans*, at a rate which exceeds that of the first cleavage. The half-life of this precursor *in vivo* is 9 min or less. Processing in which a cleavage in *trans* must precede a *cis* cleavage is unique among virus proteinases and results in the slow kinetics of processing observed *in vivo*. Processing of the 3-4 site is more complex since very little mature nsP4 is detected. *In vitro*, nsP4 appears to be cleaved efficiently from polyprotein P1234 but not from precursor P34.

However, nsP4 does not appear to accumulate significantly throughout pulse-chase experiments *in vivo*. It is possible that the concentration of nsP4 is regulated by some means other than opal suppression of translation and processing.

When nonstructural polyproteins were translated from virion RNA *in vitro*, processing could be inhibited with antibodies to nsP2, but not with antisera to the other three nonstructural proteins, suggesting that the nonstructural proteinase is 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 polymerase and translated *in vitro*. Nearly all of the sequences external to nsP2 could be deleted without affecting processing. However, deletions in the N-terminal half of nsP2 led to aberrant processing, and deletions in the C-terminal half abolished processing. These results localized the proteinase to a region of approximately 334 amino acids at the C terminus of nsP2. When the deduced amino acid sequences of several alphaviruses in this part of nsP2 were compared with the sequences of cellular proteinases, regions surrounding one cysteine and two histidine residues in nsP2 showed limited sequence similarity with sequences in the vicinity of the active site residues of the papain superfamily of cysteine proteinases. If nsP2 is evolutionarily related to the papainlike proteinases, it would represent the first example of such a proteinase in a virus.

Large numbers of temperature-sensitive RNA-minus mutants of Sindbis virus that are defective in viral RNA synthesis at the nonpermissive temperature have been isolated and assigned by complementation to four groups (A, B, F and G). Recently, the *ts* lesions of representative mutants from each group have been mapped to specific amino acid changes in the

nonstructural proteins. We have examined the processing of nonstructural polyproteins by several of these mutants and have found that only mutants possessing mutations in the C-terminal domain of nsP2 produce aberrant processing patterns at the nonpermissive temperature. These results are consistent with our deletion-mapping studies that show that the C-terminal domain of nsP2 contains the proteinase.

The stability of the nonstructural proteins was also examined by immunoprecipitation of proteins labeled in pulse-chase experiments *in vivo*. Although all four proteins appeared to be stable, nsP3 exhibited anomalous behavior. Following its synthesis and processing, nsP3 was chased from a species of ~76 kDa to various high molecular-weight forms. In parallel experiments, these forms could be labeled with $^{32}$P-inorganic phosphate, suggesting that nsP3 is phosphorylated. We have investigated the phosphorylation of nsP3 in chick and mosquito cells infected with Sindbis and in chick cells infected with Middelburg virus, a closely related alphavirus. Our results suggest that the phosphorylation of nsP3 is a general feature in infection among the alphaviruses.

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# INTRODUCTION

Within the last 15 years recombinant DNA technology has revolutionized the field of virology by providing a new conceptual and experimental framework for studying the replication and pathogenesis of viruses. The cloning and sequencing of viral genes have uncovered many details about the organization and structure of viral genomes, and the expression of these genes has led to the development of tools, such as immuno-specific reagents, for exploring the properties of viral proteins further. Finally, cDNA clones of many plus-strand RNA viruses including Sindbis virus (74) have been constructed, from which infectious virus may be resurrected. These clones represent an important vehicle with which to assess the functional effects of site-specific alterations in viral proteins.

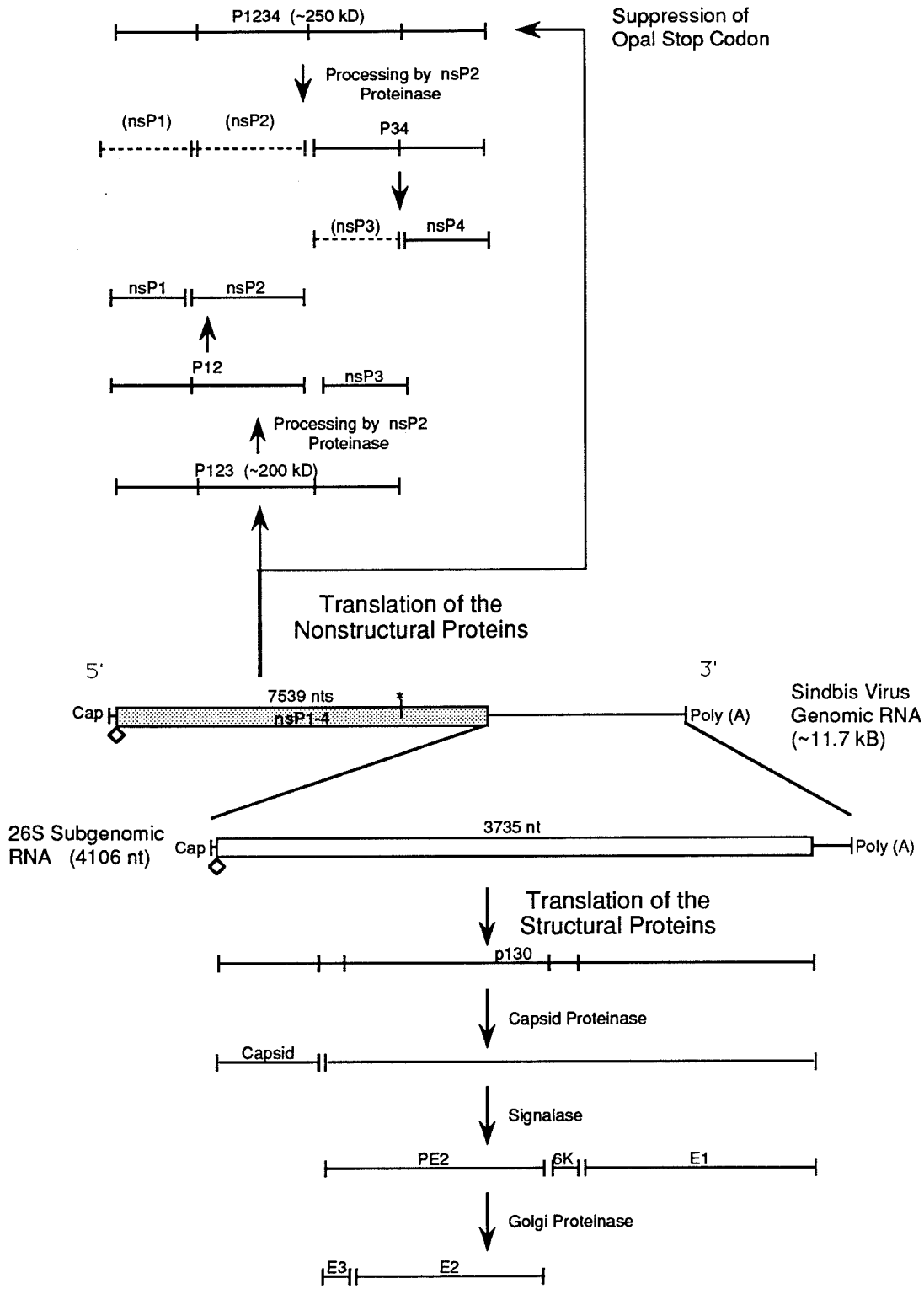
There are over 25 members in the alphavirus genus of the family Togaviridae, many of which are pathogenic for man and domestic animals. While alphaviruses infect a wide variety of cultured cells, in nature they primarily infect birds and mammals and are transmitted by mosquitoes (13). Research has focused mainly on Semliki Forest virus and Sindbis virus, the type alphavirus, both of which have low virulence in man (22). The genome of Sindbis virus consists of a plus-strand RNA molecule of 11,703 nucleotides, which is capped and methylated at the 5' terminus and polyadenylated at the 3' terminus (87). In the virion, a single molecule of RNA is enclosed by multiple copies of the basic capsid protein (C) to form a nucleocapsid of icosahedral symmetry (89). This nucleocapsid is in turn surrounded by a lipid envelope, which is derived from the host plasma membrane and contains two transmembrane glycoproteins, E1 and E2, encoded by the virus.

Experiments with Semliki Forest virus have shown that virions enter the cell through clathrin-coated pits and vesicles by receptor-mediated endocytosis (30, 55). Following endocytosis, the virions are transported to early endosomes where under mild acidic conditions membrane fusion and penetration of virion RNA occur (42, 43, 99). This RNA then serves as a message for the synthesis of the nonstructural proteins of the virus required to replicate the virus RNA (82). Replication of the incoming genomic RNA begins with the transcription of full-length minus strand RNA, which in turn acts as a template for the synthesis of both 26S subgenomic mRNA and additional 49S genome-length RNA (reviewed in 91 and 36).

Both the structural and nonstructural proteins of Sindbis virus are translated as polyproteins which must subsequently undergo proteolytic processing (Fig. 1). The subgenomic mRNA serves as a message for the structural polyprotein precursor, which contains the capsid protein, the two envelope glycoproteins E1 and E2, and two small peptides that do not appear in the virion (75, 76). The capsid protein is an autoproteinase that cleaves itself from this precursor (1, 83), while the remaining nascent polyprotein is believed to be processed by cellular proteinases within the rough endoplasmic reticulum and Golgi apparatus (75, 92).

The nonstructural proteins of Sindbis virus are translated as two polyprotein precursors from the genomic RNA. Translation of a single open reading frame of 1,896 amino acids produces the smaller precursor, which contains the first three nonstructural proteins nsP1, nsP2, and

**Fig. 1. Translation and processing strategy of Sindbis virus polyproteins.** Both the structural and nonstructural polyproteins are initiated at the first AUG codon (designated by an open diamond) in their respective mRNAs. In the region encoding the nonstructural proteins, translation either terminates at an in-frame opal codon (designated by an asterisk) to produce P123, or continues for another 616 amino acids to produce P1234. Nontranslated sequences are indicated by bold lines extending from the open boxes (open reading frames) in each mRNA.


P1234 (~250 kD)
Suppression of
Opal Stop Codon
Processing by nsP2
Proteinase
(nsP1)
(nsP2)
P34
(nsP3)
nsP4
nsP1
nsP2
P12
nsP3
Processing by nsP2
Proteinase
P123 (~200 kD)
Translation of the
Nonstructural Proteins
5'
3'
7539 nts
Cap
nsP1-4
Poly (A)
Sindbis Virus
Genomic RNA
(~11.7 kB)
26S Subgenomic
RNA (4106 nt)
3735 nt
Cap
Poly (A)
Translation of the
Structural Proteins
p130
Capsid Proteinase
Capsid
Signalase
PE2
6K
E1
Golgi Proteinase
E3
E2

nsP3, numbered according to their order 5' to 3' in the genome (82, 87). However, at some lower frequency, readthrough of an in-frame opal termination codon at the 3' end of nsP3 occurs, leading to the production of a larger precursor that contains the forth nonstructural protein, nsP4 (29, 53, 86). Proteolytic processing of these precursors occurs at discrete sites, either cotranslationally or posttranslationally, to yield the mature nonstructural proteins, and is catalyzed by a viral-encoded proteinase [Chapter 1].

Cleavage sites between the nonstructural proteins have been located by partial amino acid sequencing of the N terminus of nsP2 (37), nsP3, and nsP4 (94) of Semliki Forest virus and have been assigned to homologous sequences in the deduced amino acid sequences of the other alphaviruses (87, 92). In every case, cleavage occurs after an amino acid with a short side chain, such as glycine, alanine or cysteine, which is preceded by a glycine residue. The amino acid following the cleavage site is normally glycine or alanine, although in the site between 3-4 there is a tyrosine residue. Since this consensus triplet is present at other locations throughout the nonstructrual proteins in the deduced amino acid sequence of other alphaviruses, it is clear that other structural determinants are important as well.

**Viral Proteinases**

Throughout their development, viruses have evolved different strategies to contend with the problem of encoding a sufficient number of functions for efficient replication, packaging and delivery, while at the same time limiting their genome size because of packaging constraints and the moderate error frequency, and lack of proofreading functions, of most viral

polymerases. One strategy that is prevalent among the plus strand RNA viruses, retroviruses and adenoviruses is to encode these functions in the form of polyproteins that must undergo regulated and specific proteolysis to activate or express these functions. In many instances, the structural proteins that comprise the virion, particularly the glycoproteins, are processed on their way to the plasma membrane by organelle-bound cellular proteinases located in the rough endoplasmic reticulum and the Golgi apparatus. However, in every case where it is known, the nonstructural proteins used in replication, as well as the capsid protein(s) which encapsidate the virus in the cytosol, are processed by a viral-encoded proteinase(s) of high specificity.

The study of these viral proteinases has many obvious implications both for viral chemotherapy (46), and for dissecting the evolutionary relationships that exist within a superfamily of viral proteinases and between superfamilies of viral and cellular proteinases (5, 6, 19). Currently, the viral proteinases of the picornaviruses and their plant virus counterparts, the comoviruses and the nepoviruses, as well as several retroviruses and members of the adenoviruses, have been studied in some detail, and some have been mapped to specific locations in the viral genome (Reviewed in 46).

For the Picornaviridae, a family of small, nonenveloped animal viruses, a single polyprotein is produced from their plus strand RNA genomes, and this appears to be processed exclusively by viral-encoded proteinases (reviewed in 61, 65, 46). In entero- and rhinoviruses, the primary cleavage to separate the structural and nonstructural protein domains occurs rapidly while the polyprotein is still nascent and is mediated by viral polypeptide 2A (96). Secondary processing of the polyprotein, which

includes most of the remaining cleavages, is then effected by viral proteinase 3C, most elegantly shown for poliovirus by the inhibition of processing using antiserum to 3C (27).

The action of the 3C proteinase has been deduced from several kinetic studies. From the results of pulse-chase experiments in which a precursor of the 3C proteinase was produced in E. coli, Hanecak et al. (28) suggested that proteinase 3C was capable of autocatalytic excision from its precursor and showed a distinct preference for cleavage first at its amino-terminus. Investigations on the cardiovirus, encephalomyocarditis virus, which show that processing of 3C from its precursors *in vitro* is dilution-insensitive, have led to similar conclusions (64). Nevertheless, both proteinases, in their mature form and also as precursors, have been shown to act also in *trans*, suggesting that a dual activity is possible and maybe even common for these enzymes (35, 66, 67, 102). The finding that polyproteins containing the 3C proteinase possess a cleavage specificity and processing activity that are distinct from that of the mature proteinase may also indicate that these processing intermediates possess unique functions in viral replication or assembly.

Studies using thiol-specific proteinase inhibitors indicate that the 2A proteinase of poliovirus (45) and several picornaviral 3C proteinases (12, 21, 69) are cysteine-type proteinases. Furthermore, an examination of amino acid sequence similarities conserved among the 3C proteinases of picornaviruses has implicated Cys-147 and His-161 as the active-site residues (2) and subsequent site-directed mutagenesis experiments support this conjecture (34). If these amino acids are the catalytic residues, the 3C proteinases of the picornaviruses will comprise a new family of cysteine proteinases which may be evolutionarily related to the trypsinlike family of

serine-proteinases (5, 19). According to preliminary studies, the 2A proteinase and the 24 kDa proteinase of comoviruses are closely related to 3C and should also belong to this family (2, 45, 46).

Polyproteins of the Retroviridae family are encoded by mRNA that is synthesized by cellular RNA polymerase II from the integrated proviral DNA (reviewed in 98). In every case, the proteinase is located between the structural (*gag*) and nonstructural (*pol*) genes in the virus and is synthesized either as part of a *gag* or *gag-pol* polyprotein precursor. In the latter case, this is achieved by suppression of a termination codon at the end of the *gag* gene or by ribosomal frameshifting at, or near, the end of the *gag* gene (56). From a comparison of the deduced amino acid sequences of several retroviral proteinases with those of known cellular proteinases, it appears that the retroviral proteinases possess a highly conserved region that shows marked similarity to the active site region of the aspartic proteinases (68, 72, 95). X-ray structural determination has now established that the proteinases of Rous sarcoma virus and human immunodeficiency virus type I, and quite probably all retroviral proteinases, belong to the family of aspartyl pepsinlike proteases (59, 60, 100).

In adenoviruses, proteolytic processing of structural protein precursors is required for the production of infectious virus and occurs late in infection during virus maturation (46). However, unlike the plus strand RNA and reteroviruses, adenoviruses do not encode polyproteins, but rather produce precursor proteins that require the processing of small terminal peptides for activation (14, 33). Recently, the proteinase has been localized by sequence analysis of a temperature-sensitive processing mutant and several of its revertants (51, 97). From a comparison of the deduced amino

acid sequences of three adenovirus proteinases with many cellular serine proteinases, it has been suggested that residues His-54, Asp-102 and Ser-187 form the catalytic triad (31).

Much less is known about the proteinase(s) that process the polyprotein of flaviviruses, a family of arthropod-borne viruses which, like the picornaviruses, encode all their proteins in one long, open reading frame (reviewed in 77, 93). Although it seems clear that the four structural proteins at the N terminus of the polyprotein are processed by cellular enzymes (73, 92), the proteinase(s) responsible for processing the nonstructural proteins in the cytosol is not known. Sequence analysis and structural modeling of the deduced amino acid sequence of NS3 in several flaviviruses has revealed a conserved N-terminal domain of ~180 amino acids, which is homologous to the cellular trypsinlike proteases (6). Downstream of this proteinase domain and separated by a spacer element of about 11 amino acids is another conserved segment, which is predicted to encode a nucleoside-triphosphate binding (NTPase) or helicase activity (6, 20). This model reinforces the concept that a virus may encode multiple functions in a single protein as separate and possibly independent domains to satisfy the requirements of viral economy; however, support for this model awaits further testing.

For the alphaviruses, which belong to the family Togaviridae, both structural and nonstructural polyproteins are translated from separate mRNAs whose relative populations are regulated temporally and quantitatively during viral RNA synthesis (reviewed in 90, 93). In addition, several, but not all, alphaviruses have an opal stop codon at the 3' end of nsP3 that acts to decrease the amount of nsP4 produced, and consequently, the amount of the readthrough polyprotein P1234 present (85, 86).

Processing of these polyproteins is carried out by at least two viral-encoded enzymes, the capsid proteinase (23) and a proteinase within nsP2 [Chapter 2].

Currently, somewhat more is known about the capsid autoproteinase than about the nonstructural proteinase. On the basis of the mapping of mutations that render the capsid proteinase temperature-sensitive, the discovery of sequence similarities between the capsid protein and cellular serine proteases, and the effects of site-directed mutagenesis of residues believed to be important in catalysis, it has been postulated that the capsid proteinase is a serine proteinase in which His-141, Asp-147 and Serine-215 form the catalytic triad (7, 23, 57) [C. S. Hahn, Ph.D. thesis, California Institute of Technology, Pasadena, 1988].

Since the translation of the nonstructural proteins is a very early event in virus replication and occurs amidst a large background of host cell protein synthesis, early studies to characterize the processing of these proteins *in vivo* relied upon special labeling techniques (9, 41, 48, 82) and mutants of the virus that are temperature-sensitive in processing (8, 40). Later, processing was examined with the aid of monospecific antisera produced against either synthetic peptides (53) or fusion protein antigens (70) [Chapter 1] containing sequences within the nonstructural proteins.

In general, the kinetics of processing the polyprotein precursors of Sindbis virus *in vivo* (9, 82) [Chapter 1] and *in vitro* (16) [Chapter 2] is very similar. While some processing at the 1-2 and 2-3 cleavage sites can occur during elongation of the polypeptide chain, most of the nonstructural proteins arise from the processing of completed precursor P123 and its cleavage product P12. Following the completion of the P123 precursor, the bond between nsP2 and nsP3 must be cleaved first and in *trans*, generating

P12 and nsP3. Pulse-chase experiments *in vivo* show that this cleavage occurs with a half-life of ~19 min, but other results indicate that this rate will vary with proteinase concentration. Processing of P12 to produce mature nsP1 and nsP2 can then occur, either intramolecularly or in *trans*, at a rate that appears to exceed that of the 2-3 cleavage. This processing cascade leads to a slow rate of cleavage and to the accumulation of several long-lived processing intermediates, suggesting that these polyproteins may play a role in viral replication which is distinct from those of the cleaved products.

The processing of nsP4 appears to be more enigmatic. At least two alphaviruses, Semliki Forest virus (94) and O'nyong-nyong virus (85), do not possess an opal stop codon at the end nsP3 and thus produce a single polyprotein containing all four nonstructural proteins. A significant amount of nsP4 can be detected in cells infected with these viruses, suggesting that it is efficiently processed from its precursors (41, 48, 70). However, in cells infected with wild-type Sindbis virus (53, 82), or with site-specific mutants that replace the opal codon with a sense codon (50), very little mature nsP4 can be detected, even though significant quantities of precursors containing nsP4 sequences are present. Since it was reported that nsP4 is less stable than the other three nonstructural proteins in Semliki Forest virus-infected cells (41), it is possible that it is also less stable or somehow regulated in Sindbis virus-infected cells. Another possibility is that nsP4 is lost in preparation.

Recently, the nonstructural proteinase has been localized to the C terminus of nsP2 in several studies that utilized Toto 1101, a full-length cDNA clone of Sindbis virus from which infectious RNA can be transcribed *in vitro* (74). Using the infectious clone, Ding and Schlesinger examined

proteolytic processing of the nonstructural proteins *in vitro* by transcribing and translating a nested set of truncated RNA's (17). They concluded that the proteinase is within nsP1 or nsP2, probably in nsP2. This was supported by the mapping of lesions in four temperature-sensitive mutants of Sindbis virus which are defective in processing their nonstructural polyproteins at the nonpermissive temperature (26) [Chapter 3]. In Chapter 3, deletion mapping studies are described, which localized the protease activity to a region of approximately 334 amino acids at the C terminus of nsP2 [Chapter 2].

Closer scrutiny of the deduced amino acid sequences of nsP2 in five alphaviruses suggested that the polypeptide has at least two functional domains, a replicase region homologous to a protein encoded by several Sindbis-like plant viruses and a proteinase domain that possesses some similarity to the papain superfamily of cysteine proteinases. If the nsP2 proteinase is related to the papain family of thiol proteinases, it will represent the first example of such a proteinase in a virus.

**Temperature-sensitive processing mutants**

Large numbers of temperature-sensitive mutants that are defective either in the production of structural proteins ($RNA^+$) or in the replication of viral RNA ($RNA^-$) have been isolated, characterized and assigned by complementation to three $RNA^+$ groups (C, D, E) and four $RNA^-$ groups (A, B, F, G) (10, 11, 84, 88). Sequence analysis of representative mutant-revertant pairs of $RNA^+$ mutants has been used to locate the lesion causing the *ts* phenotype (3, 23, 52).

A different approach was employed for the $RNA^-$ mutants (25, 26). The construction of Toto1101, a full-length cDNA clone of Sindbis virus from

which infectious RNA may be transcribed *in vitro*, has greatly facilitated our understanding of the molecular genetics of the virus by allowing strain-specific phenotypes to be mapped to particular regions of the viral genome (54, 74). To map the mutations responsible for the *ts* phenotypes of several RNA$^-$ mutants, large segments of RNA sequence from within the genome of these *ts* mutants were reverse-transcribed and cloned, at convenient restriction sites, into the corresponding region of Toto1101. These hybrid clones were then transcribed with SP6 polymerase to produce infectious RNA and the phenotypes of the recombinant viruses were assayed. In this way, the portion(s) of the genome of the *ts* mutant, which confers temperature sensitivity, can be identified and the mutation within that segment can be located by nucleotide sequencing. This technique also has the advantage of rescuing the individual mutations into a uniform genetic background so as to isolate the lesion from the effects of unknown alterations in other portions of the genome.

From these mapping results (25, 26) and from previous work, which characterized the functional defects of some of these RNA$^-$ mutants (4, 40, 78, 79, 80, 81), some of the activities of the nonstructural proteins were deduced. The only B group mutant, *ts*11, has a mutation in nsPl, implicating this protein in the initiation of minus strand synthesis (26, 79). This protein has also been implicated in methylation of the 5' terminal cap structure on virion RNAs (58). Three group F mutants have been mapped to nsP4 (25). The best characterized member, *ts*6, fails to synthesize any RNA upon shift to the nonpermissive temperature (4, 40, 79) and this, together with the presence of the GDD motif found in a number of viral polymerases (39), has led to the hypothesis that nsP4 is the major RNA polymerase of Sindbis virus.

Members of group G (*ts*18 and *ts*7) and group A (*ts*17, *ts*21 and *ts*24) have all been found to have *ts* lesions in nsP2 (26), and thus the previous complementation found between these groups was intracistronic. In addition, *ts*7 was found to possess a second change in nsP3, which renders the virus temperature sensitive and is also responsible for the RNA$^-$ phenotype. From the phenotypes of these various mutants, nsP2 has been implicated in the initiation of 26S mRNA synthesis, the regulation of minus-strand synthesis and the proteolytic processing of nonstructural polyproteins (26, 40, 78, 79, 81).

In Chapter two of this thesis, results on the synthesis and processing of nonstructural polyproteins by several temperature-sensitive mutants, representing the four RNA$^-$ complementation groups, are reported and show that only mutants that possess a lesion in the C-terminal half of nsP2 exhibit aberrant processing patterns at the nonpermissive temperature. For those mutants most defective in processing, hybrid viruses [in which the original mutation from the parental virus was rescued into a uniform genetic background (Toto1101)] were tested and gave virtually identical results. These results support the hypothesis that the nonstructural proteinase is located within the C-terminal domain of nsP2.

**Phosphorylation of nsP3**

In eucaryotic organisms, protein phosphorylation performs a diverse role in cellular regulation, affecting protein synthesis (63) and cell division (62), and modulating the activity of many enzymes involved in central metabolism (15, 47). In addition, phosphorylation is known to affect the interaction and assembly, or disassembly, of components in macromolecular structures such as chromatin (101) and the nuclear

envelope (18). Recent work with viral oncogenes, some of which encode protein kinases, and with their cellular homologs, many of which are growth-factor receptors and therefore tyrosine kinases, has also highlighted the involvement of phosphorylation in the transduction of extracellular signals (32).

Besides retroviruses, a wide variety of animal and insect viruses (49), as well as at least one plant virus (24), are known to possess phosphoproteins. Most of these are structural components of the virion, although a few of these, such as the NS protein of vesicular stomatitis virus (44) and the NP protein of influenza virus (38), also participate in viral replication. Nonstructural phosphoproteins are less common but have been reported for herpes simplex virus type 1 (71). In the few cases where the functional significance of phosphorylation has been established, it appears to affect the interaction of the phosphoprotein with nucleic acid.

In Sindbis virus, the posttranslational modification of nonstructural protein nsP3 was first observed in pulse-chase experiments in which labeled proteins were examined by immunoprecipitation with monospecific antisera to the nonstructural proteins [Chapter 1]. Following its synthesis and processing, nsP3 was chased from a species of ~76 kDa to various high molecular weight forms which in parallel experiments were labeled with $^{32}$P-inorganic phosphate.

In similar experiments with Semliki Forest virus, phosphorylation of nsP3 was also observed although no change in its migration behavior on SDS-polyacrylamide gels was detected (70). Phosphoamino acid analysis revealed the presence of phosphoserine and phosphothreonine residues, but no phosphotyrosine was found. Moreover, Peränen et al. (70) have found from cell fractionation studies that the nsP3 associated with the viral

polymerase fraction in infected cell lysates is more heavily phosphorylated than that which is not, suggesting that phosphorylation plays some role in viral replication.

In Chapter 4, results are presented on the phosphorylation of nsP3 in chick and mosquito cells infected with Sindbis virus and in chick cells infected with Middelburg virus, an alphavirus closely related to Sindbis virus and Semliki Forest virus. These results indicate that the phosphorylation of nsP3 is a general feature of alphavirus infection.

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# Chapter 1

## Processing the Nonstructural Polyproteins of Sindbis Virus: Study of the Kinetics *In Vivo* by Using Monospecific Antibodies

This chapter was published in the *Journal of Virology*.

# Processing the Nonstructural Polyproteins of Sindbis Virus: Study of the Kinetics *In Vivo* by Using Monospecific Antibodies

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Received 21 September 1987/Accepted 24 November 1987

## ABSTRACT

Plasmids were constructed that contained a large portion of each of the four nonstructural genes of Sindbis virus fused to the N-terminal two-thirds of the *trpE* gene of *Escherichia coli*. The large quantity of fusion protein induced from cells containing these plasmids was subsequently used as an antigen to generate polyclonal antisera in rabbits. Each antiserum was specific for the corresponding nonstructural protein and allowed ready identification of each nonstructural protein and of precursors containing the sequences of two or more nonstructural proteins. These antisera were used to determine the stability of the mature nonstructural proteins and to examine the kinetics of processing of the nonstructural proteins from their respective precursors *in vivo*. Pulse-chase experiments showed that the precursor P123 is cleaved with a half-life of ~19 min to produce P12 and nsP3; P12 is then cleaved with a half-life of ~9 min to produce nsPl and nsP2. Thus, although the rate of cleavage between nsPl and nsP2 is faster than that between nsP2 and nsP3, the latter cleavage must occur first and is therefore the rate-limiting step. The rate at which P34 is chased suggests that the cleavage between nsP3 and nsP4 is the last to occur; however, the regulation of nsP4 function in Sindbis virus-infected cells may be even more complex than was previously thought. The products nsPl and nsP2 (and nsP4) are relatively stable; nsP3, however, is unstable, with a half-life of about 1 hour, and appears to be modified to produce heterodisperse, higher-molecular-mass forms. In general, the processing schemes used by Sindbis virus and Semliki Forest virus appear very similar, the major difference being that most nsP3 in Sindbis virus results from termination at an opal codon, whereas in Semliki Forest virus cleavage of the P34 precursor is required.

## INTRODUCTION

The nonstructural proteins of Sindbis virus are translated as two large polyprotein precursors from the 5'-terminal two-thirds of the genomic 49S RNA (for a review, see Reference 25). For both precursors initiation begins at the first methionine codon 59 nucleotides downstream from the 5'-terminal cap (17). Translation continues either for 1,896 amino acids until an opal stop codon is reached (resulting in a polyprotein of 200 kilodaltons [kDa]), or at a much lower frequency, readthrough of the opal codon can occur so that translation may continue for another 616 amino acids to produce a larger 250-kDa precursor (14, 22, 23). Processing of the 200-kDa precursor leads to the appearance of mature nsPl, nsP2, and nsP3, and it is believed that the 250-kDa precursor is processed to yield the first three mature nonstructural proteins and a fourth, nsP4 (2, 3, 14, 20).

The use of opal suppression in regulating the amount of a gene product is a novel form of control worthy of study; unfortunately, specific immunochemical regents have not been available until recently to allow the separation and characterization of these nonstructural proteins amidst the large background of host cell protein synthesis. One approach to the production of specific immunological reagents has been to construct synthetic peptides that possess sequences within the protein that seem most likely to reside on the protein surface, e.g., sequences rich in hydrophilic residues. These oligopeptides are then linked to larger carrier proteins and used as antigens to generate high-titer antisera (13). Such an approach has been used to produce an antiserum specific for nsP4 of Sindbis virus (14).

Another method of generating specific antisera has been to construct hybrid gene fusions in which a eucaryotic gene or portion of a gene is placed adjacent to a well-defined procaryotic gene which is under the

control of a strong procaryotic promoter (6). Upon induction, the fusion protein product accumulates in the periplasmic space as a secreted protein or in the cytoplasm, depending on the type of fusion.

Since we possess a cDNA copy of the 49S RNA genome of Sindbis virus, we set out to produce fusion proteins of the Sindbis virus nonstructural proteins by using the *trpE* system. In general, many *trpE* fusion proteins have the property of being insoluble and resistant to proteolytic degradation in *Escherichia coli* (10, 21). This facilitates purification since the protein can usually be pelleted under conditions in which most *E. coli* proteins are soluble. Large quantities of the fusion protein can be produced in this fashion for use as an immunogen to generate antisera against a wide repertoire of antigenic determinants. Our hybrid gene fusions consisted of the leader and the N-terminal two-thirds of the *E. coli trpE* protein fused to a large region of sequence from within each of the genes encoding the nonstructural proteins of Sindbis virus. The resulting fusion proteins were used to generate antisera which were effective in immunoprecipitating virus-specific proteins from lysates of infected cells. These immunoprecipitated proteins were of the proper size based on the genomic sequence and previous observations in experiments that examined the nonstructural proteins of Sindbis virus. These antisera were used as probes to elucidate the kinetics of processing of the nonstructural polyprotein precursors to form mature proteins and to determine the relative stability of these mature products.

## MATERIALS AND METHODS

**Cells and virus strains.**

Virus stocks were prepared in primary monolayers of chicken embryo fibroblasts (18). The heat-resistant, small-plaque strain of Sindbis virus (Sindbis virus HRSP) used in these experiments has been described previously (22, 23). BHK-21 cells (American Type Culture Collection) were maintained at 37°C under 5% $C0_2$ in Eagle minimal essential medium (5) supplemented with 10% fetal calf serum. *E. coli* MC1061 ($RecA^-$) was used in all cloning steps .

**Labeling of infected cells and preparation of lysates.**

Confluent monolayers of chicken embryo fibroblasts or BHK cells grown in either 100- or 60-mm plastic petri plates were washed once with the room-temperature, phosphate-buffered saline (PBS) of Dulbecco and Vogt (4), lacking divalent cations and then infected at a high multiplicity (50 to 100 PFU per cell) with Sindbis virus in PBS containing $Ca^{2+}$, $Mg^{2+}$, 1 μg of actinomycin D per ml, antibiotics, and 1% dialyzed fetal calf serum. Virus absorption was allowed to occur at 37°C for 70 min, and the inoculum was removed. The plates were then washed once with room-temperature PBS to remove unabsorbed virus, and the incubation was continued in Eagle medium containing 10% dialyzed fetal calf serum, 1 μg of actinomycin D per ml, antibiotics, and 1/20 the normal concentration of methionine.

The time postinfection at which the cells were labeled is described in the figure legends. In most instances, labeling was carried out for 30 min in prewarmed Eagle medium containing 40 μCi of [$^{35}$S]methionine per ml (>600 Ci/mmol; Amersham Corp.). In some experiments the monolayers were incubated in hypertonic medium containing an excess of 220 mM

NaCl for 30 min before the cells were labeled to synchronize initiation of translation (19)

After the labeling period the monolayers were placed on ice, washed three times with ice-cold PBS, and lysed with either 480 μl (60-mm plates) or 800 μl (100-mm plates) of lysis solution (0.5% sodium dodecyl sulfate [SDS], 2 mM EDTA, 170 μg of phenylmethylsulfonyl fluoride [PMSF] per ml). The monolayers were then scraped from the plates with a rubber policeman, and the lysates were stored at -70°C.

**SDS-PAGE analysis.**

Slab gels containing 10% (wt/vol) acrylamide (acrylamide-bisacrylamide ratio, 30:0.4 [wt/wt]) were used for discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) (11). Electrophoresis was carried out at 150 V (constant voltage), after which the gels were fixed overnight in 50% methanol-12% acetic acid. The gels were stained briefly in Coomassie brilliant blue and then destained to highlight adjacent molecular mass standards (SDS-high-molecular-mass standards [Bio-Rad Laboratories] and various bands from chicken gizzard extract [250, 200, 130, 100, and 55 kDa] provided by H. Hinssen). The gels were soaked for 30 min in distilled water and 30 min in Fluoro-Hance (Research Products International) before being dried. Radioactive ink was used to mark the molecular mass standards before fluorography at -70°C using prefogged X-Omat R film (Eastman Kodak Co.) (12).

**Construction of *trpE* hybrid gene fusions.**

The plasmids used for the construction of *trpE* hybrid gene fusions were provided by T. J. Koerner. The pATH vectors (plasmids amenable for

making *trp* hybrids) contain the *trp* operator and promoter, *trp*L leader and attenuation sequences, and the N-terminal two-thirds of the *trpE* gene from *E. coli* (21). A polylinker juxtaposed to this gene allows construction of open reading frame gene fusions which, when induced with ß-indoleacrylic acid, yield large quantities of a *trpE* fusion protein product (10).

Using these vectors and pSVC2N.10 (provided by C. M. Rice), a plasmid containing the genes that encode the nonstructural proteins of Sindbis virus HRSP, we constructed four gene fusions, each of which contains most of a gene encoding one of the nonstructural proteins of Sindbis virus (the fusion construct for nsP4 contains some sequences from a preliminary fusion protein vector we tested, pORF 2 [provided by J. Kobori]). Standard procedures (15) were used to isolate these fusion constructs. Restriction enzymes and T4 DNA ligase were from New England BioLabs, Inc., T4 DNA polymerase was from New England Nuclear Corp., and calf intestinal alkaline phosphatase was from Boehringer Mannheim Biochemicals. Restriction fragments used in cloning were isolated by electrophoresis in low-melting- point agarose. Transformants judged to be promising on the basis of restriction analysis and, for the nsP3 and nsP4 constructs sequencing across the 5' junction, were induced with ß-indoleacrylic acid, and the protein pattern of the whole-cell lysate was examined by using discontinuous SDS-PAGE.

**Expression and purification of *trpE* fusion proteins.**

The expression and purification protocol was a modification of that used by Kleid et al. (10). Cultures of MC1061 cells containing the hybrid gene fusion constructions were grown overnight in M9CA (15) containing 100 µg of ampicillin per ml and 20 µg of tryptophan per ml. These cultures were

diluted 1:10 in M9CA plus ampicillin, and the mixture was incubated at 37°C with shaking. One hour later ß-indoleacrylic acid was added to a final concentration of 5 μg/ml, and the culture was incubated at 37°C for an additional 2 h with shaking. The cells were then cooled on ice for 10 min and pelleted in a GSA rotor for 12 min at 10,000 x g at 4°C in a Sorvall centrifuge. The pelleted cells were suspended in 22 ml of TEN buffer (50 mM Tris hydrochloride [pH 7.5], 0.5 mM EDTA, 0.3 M NaCl) containing 2 mg of lysozyme per ml and left at 12°C for 20 min. (For the *trpE*-nsP3 fusion product, PMSF was included throughout the purification at a final concentration of 20 μg/ml. This was done to prevent the occurrence of smaller discrete bands that appeared if PMSF was absent.) These suspensions were first frozen on dry ice and then thawed at 37°C. Nonidet P-40 was added to a final concentration of 0.1%, and the solutions were vortexed and left for 15 min at 12°C. Then, 16.5 ml of a solution of 1.5 M NaCl and 12 mM $MgCl_2$ containing 3 μg of DNase I per ml were added, and the solutions were left on ice for 1 h with periodic vortexing every 10 min. The insoluble fusion protein was then pelleted by centrifugation in an SS34 rotor at 12,000 x g and 12°C for 3 min, and the pellet was washed three times by suspension in TEN buffer containing 0.1% Nonidet P-40, followed by centrifugation. The final pellet was then suspended in 700 μl of cracking buffer (0.01 M sodium phosphate [pH 7.2], 1% ß-mercaptoethanol, 1% SDS, 6 M urea), and the mixture was heated at 37°C for 30 min to solubilize the pellet. The yield of fusion protein determined by discontinuous SDS-PAGE using Bio-Rad molecular mass standards for comparison was about 1.2 to 1.4 mg of protein per 200 ml of culture.

**Immunizations.**

Serum was collected from each rabbit before immunization to serve as a control. Approximately 200 to 300 μg of each of the semipurified fusion proteins were gel-purified on 7.5% discontinuous SDS-polyacrylamide gels. The gels were stained briefly with Coomassie blue, and the fusion protein bands were excised and washed for 30 min in distilled water. The gel strip was then homogenized in an equal volume of PBS-0.1% SDS, and the resultant slurry was emulsified with an equal volume of Freund's complete adjuvant. This mixture was injected intradermally along the back and intramuscularly near the lymph nodes in the hind legs of 4- to 5-kg New Zealand White rabbits. The animals were boosted every 4 weeks with 100 to 150 μg of fusion protein in incomplete Freund's adjuvant. They were bled from the ear vein 5 weeks after the first injection and then 2 consecutive weeks after each booster. The blood was allowed to clot at room temperature for 1 h before centrifugation to separate serum. The serum was subsequently frozen at -20°C until ready for use.

**Immunoprecipitations.**

The whole-cell lysates were thawed at room temperature and vortexed vigorously. The lysates were then sonicated at room temperature for 1 min in a Bransonic 12 (Branson Cleaning Equipment Co.) sonicator bath and vortexed. This was repeated five times before the samples were spun for 15 min in an Eppendorf centrifuge to pellet insoluble cell debris. At this point the high viscosity that was due to cellular DNA was significantly reduced and the resulting lysate was homogeneous.

Preliminary experiments were performed to determine the optimum conditions and amounts of antiserum and protein A-bearing

*Staphylococcus aureus* (Cowan 1 strain; Calbiochem-Behring) (9) required for near quantitative immunoprecipitation of the nonstructural proteins from whole-cell lysates. For the immunoprecipitation of nsPl, nsP2, and nsP3, 80 μ1 of whole-cell lysate (1/10 of a 100-mm petri plate) was diluted 1:5 with immunoprecipitation buffer (binding buffer) (50 mM Tris hydrochloride [pH 7.4], 0.3 M NaCl, 4 mM EDTA, 0.5% Triton X-100, 200 μg of bovine serum albumin per ml, 20 μg of PMSF per ml) and 20 μl of antisera were added. Because nsP4 is produced in much smaller amounts, twice the amount of whole-cell lysate (i.e., 160 μl) was diluted 1:4 with binding buffer and 20 μl of antisera were added. After the reaction mixtures were mixed end-over-end for 90 min at room temperature, approximately 100 μl of a 10% (wt/vol) solution of *S. aureus* cells (representing a binding capacity of 215 μg of human immunoglobulin G per reaction), which had been equilibrated in binding buffer, were added to each mixture. Shaking was continued at room temperature for 2 h. The antigen-antibody-*S. aureus* complexes were layered over a solution of 20% sucrose in binding buffer and pelleted at 1,500 x g and 20°C for 10 min in an International centrifuge (International Equipment Co.). The pellet was resuspended in 0.5 ml of wash buffer (50 mM Tris hydrochloride [pH 7.4], 0.6 M NaCl, 4 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 200 μg of bovine serum albumin per ml, 20 μg of PMSF per ml) and vortexed vigorously, after which the cells were pelleted at 1,000 x g and 20°C for 10 min in the International centrifuge. This was performed three times before the pellets were suspended in 100 μl of sample loading buffer (62.5 mM Tris hydrochloride [pH 6.8], 2.3% SDS, 5% ß-mercaptoethanol, 10% glycerol). The immunoprecipitates were incubated at 65°C for 15 min, and the *S. aureus* cells were removed by spinning for 3 min in an Eppendorf

centrifuge. The radioactivity in 10 µl of the supernatant was counted in a scintillation cocktail, and normally, 20 µl were loaded per lane for discontinuous SDS-PAGE analysis.

## RESULTS

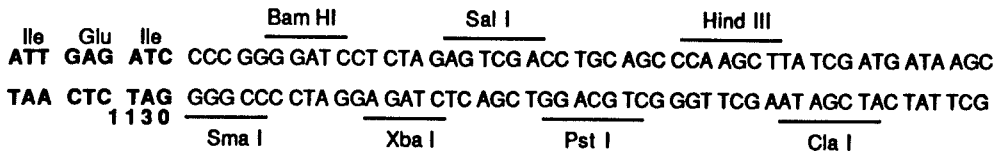
### Expression of *trpE* fusion proteins.

Using the *trpE* expression system in *E. coli* and the cDNA clone of Sindbis virus, we generated fusion proteins from constructs containing most of the sequence of each nonstructural protein gene fused to the N-terminal two-thirds of the *trpE* gene of *E. coli*. In each instance, the Sindbis virus insert was chosen to contain the maximal amount of cDNA encoding each nonstructural protein, consistent with the distribution of convenient restriction sites in cloned Sindbis virus cDNA. These open reading frame inserts were cloned into the polylinker of a plasmid expression vector, pATH 1 or pATH 2, obtained from T. J. Koerner. Details concerning the subcloning and the junctional regions of these constructs are shown in Fig. 1. In each instance the constructs contained between 75 and 96% of each nonstructural protein gene fused to about 75% of the *trpE* gene (Table 1).

Clones were screened by restriction fragment analysis, SDS-PAGE analysis of inducible fusion protein products, and in some instances nucleotide sequencing (16). Induction of the selected fusion constructs with ß-indoleacrylic acid yielded large amounts of fusion protein, which were subsequently purified by the procedure given in Materials and Methods. Samples of the bacterial whole-cell lysate and purified material were run on a 10% discontinuous SDS-polyacrylamide gel alongside molecular mass standards of known concentration to estimate the amount of fusion protein produced and to monitor the purification steps and protein integrity (Fig. 2).

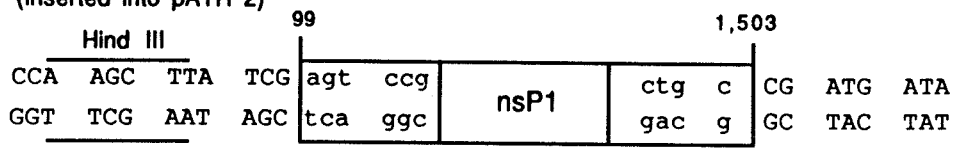
**Fig. 1.** The polylinker of pATH 2 and junctional sequences for constructs. The nucleotides in boldface correspond to sequences from the *trpE* gene of *E. coli* and are numbered in accordance with Yanofsky et al. (27). The lightface capital letters denote sequences within the polylinker of the pATH vector, whereas the small letters are Sindbis virus-specific sequences numbered in accordance with Strauss et al. (23). The clone containing sequences from nsP1 was constructed from the *Hin*fI-*Pst*I fragment (nucleotides 99 to 1503) of Sindbis virus cDNA. This fragment was treated with the Klenow fragment to fill in the *Hin*fI site and T4 DNA polymerase to blunt-end the *Pst*I site, and inserted into the pATH 2 *Cla*I site which had been blunt-ended with the Klenow fragment. The nsP2 fusion hybrid was constructed by ligating the *Eco*RI-*Pst*I fragment (nucleotides 1921 to 3953) into the *Eco*RI and *Pst*I sites in the polylinker of pATH 1 (not shown). In addition, a small deletion was made in the polylinker by removing a *Sac*I site upstream of the *Eco*RI site to adjust the reading frame with respect to the *trpE* gene. For the nsP3 construct, the *Bst*XI-*Ban*II fragment (nucleotides 4176 to 5415) was blunt-ended by using T4 DNA polymerase and ligated into the *Cla*I site, which had been filled in with the Klenow fragment, in the polylinker of pATH 2. The nsP4 insert was initially derived by ligating the blunt-end products from a *Bal* 31 digestion of the *Bgl*I-*Nar*I fragment (nucleotides 5518 to 7872) into a shuttle vector. After preliminary screening of these clones to select one that would give an open reading frame, the insert was excised with *Bam*HI and ligated into the *Bam*HI site of pATH 2.

The Polylinker of pATH 2

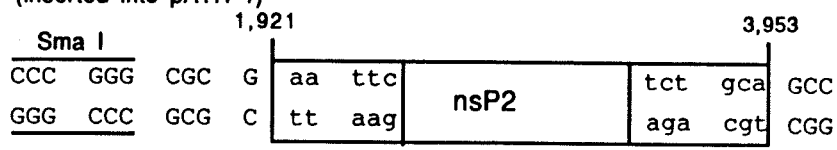



Junctional Sequences For Constructs

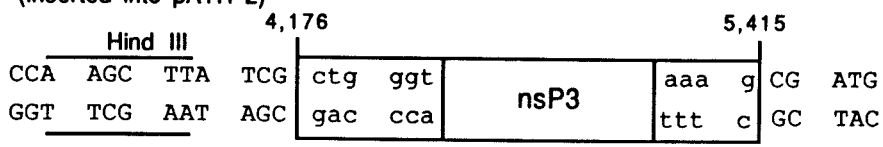
**P1** (inserted into pATH 2)




**P2** (inserted into pATH 1)




**P3** (inserted into pATH 2)




**P4** (inserted into pATH 2)

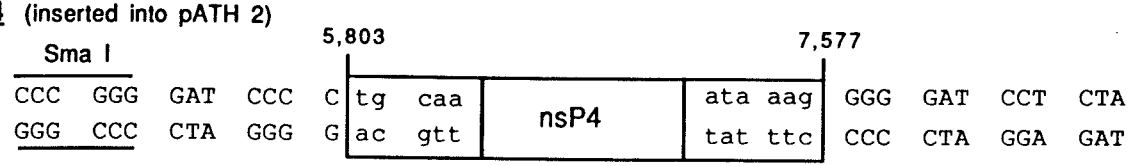


TABLE 1.

*Properties of Fusion Proteins*

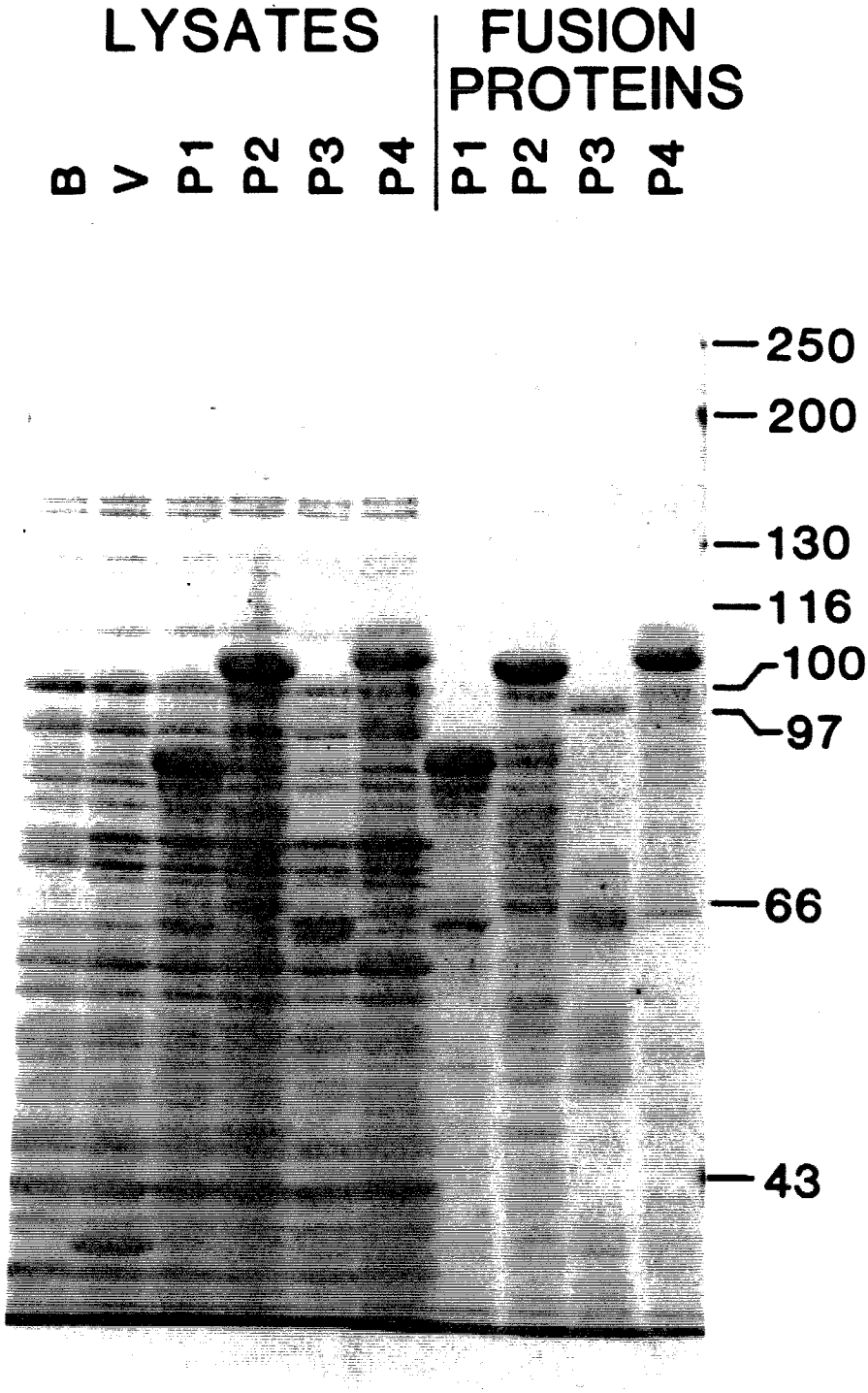
| Nonstructural Protein | Length of gene NT/AA[a)] | Location of fusion protein insert[b)] | Predicted % of protein represented | Apparent size of fusion protein (Kd) | size from SDS-PAGE (Kd) |
|---|---|---|---|---|---|
| nsP1 | 1,620/540 | 99 - 1,503 | ~87% | 90 | 83 |
| nsP2 | 2,421/807 | 1,921 - 3,953 | ~84% | 113 | 105 |
| nsP3 | 1,647/549 | 4,176 - 5,415 | ~75% | 84 | 98 |
| nsP4 | ~1,848/~616 | 5,803 - 7,577 | ~96% | 105 | 110 |

a) NT = number of nucleotides encoding the protein

AA = number of amino acids

b) Nucleotide numbers in the Sindbis virus genome according to Strauss *et al.* (24).

**Fig. 2.** Cultures of *E. coli* containing the hybrid gene fusions were induced with ß-indoleacrylic acid. To obtain whole-cell lysates, the cells from 1 ml of culture were pelleted, resuspended in 50 µl of cracking solution (0.01 M sodium phosphate [pH 7.2], 1% ß-mercaptoethanol, 1% SDS, 6 M urea), and heated for 1 h at 37°C. Purified fusion proteins were prepared as described in the text. Samples were electrophoresed on a 10% discontinuous SDS-polyacrylamide gel alongside Bio-Rad high-molecular-mass standards and chicken gizzard proteins; the locations and molecular masses (in kilodaltons) of the standards are indicated on the right. Lanes: B, lysate of induced bacteria lacking plasmid pATH 2; V, lysate of cells containing vector (pATH 2) only; Pl, P2, P3, and P4, lysates or semipurified fusion proteins from cells containing the plasmids described in the legend to Fig. 1.


LYSATES
FUSION PROTEINS
B
V
P1
P2
P3
P4
P1
P2
P3
P4
250
200
130
116
100
97
66
43

We consistently obtained yields of between 5 and 10 mg of fusion protein per liter of culture.

The predicted sizes of the fusion proteins are given in Table 1 with the apparent molecular masses obtained from SDS-PAGE analysis. Only for the nsP3 construct did the apparent molecular mass differ markedly (approximately 14 kDa greater) from the predicted value. This difference was probably due to the Sindbis virus sequences present, since nsP3 precipitated from infected cells migrates anomalously, corresponding to a mass approximately 16 kDa larger than predicted (3, 14).

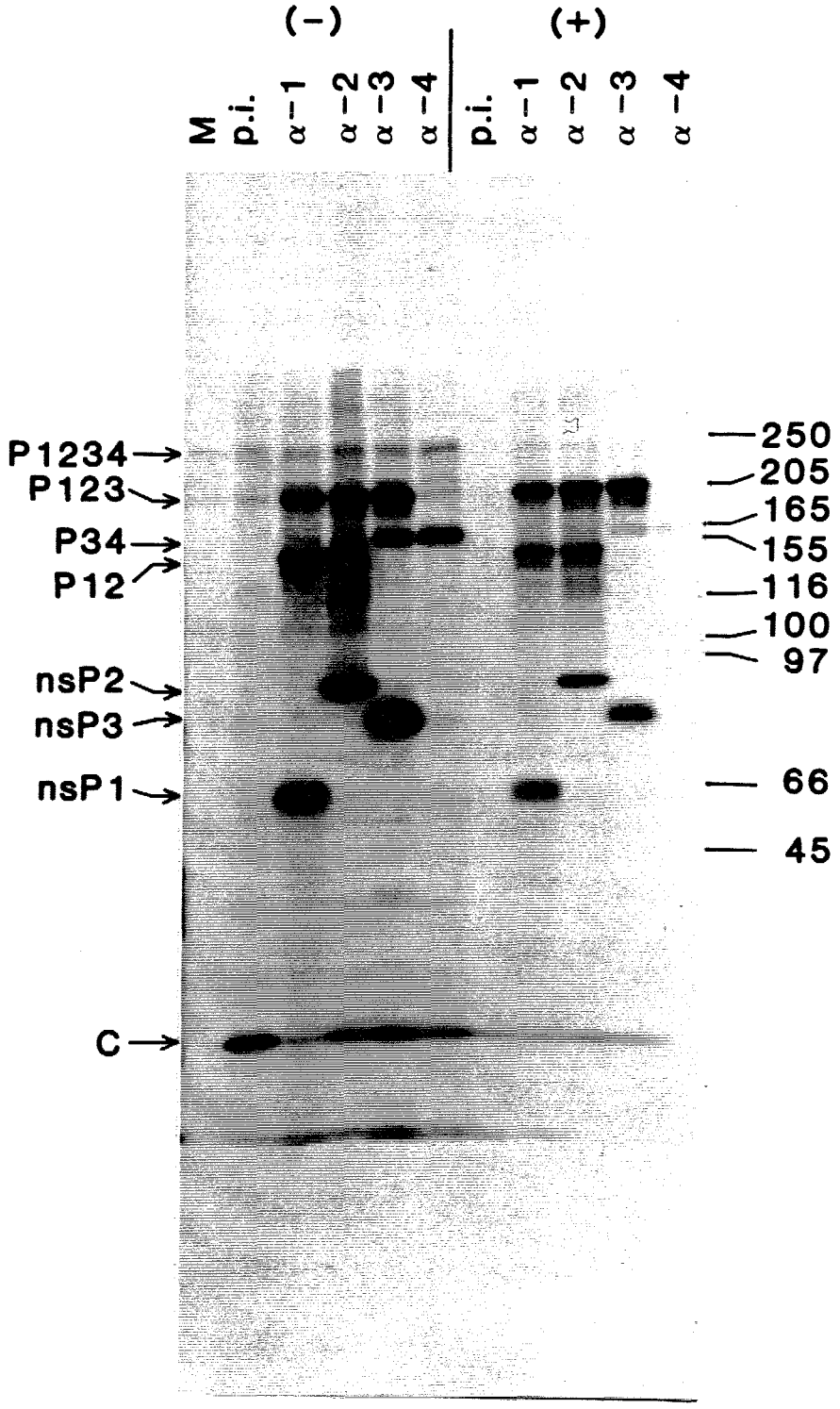
**Production of antibodies to the fusion proteins.**

The various fusion proteins were purified by SDS-PAGE and injected into rabbits. The antisera obtained were screened by immunoprecipitation of the fusion proteins. In each instance the antiserum recognized and immunoprecipitated the fusion protein antigen used to generate it (data not shown). The specificity of these antibodies is demonstrated in the following sections.

**Analysis of the nonstructural proteins of Sindbis virus in infected cells.**

BHK monolayers were infected with Sindbis virus HRSP and labeled for 30 min beginning at 4 h postinfection. The cells were then lysed, and the lysate was immunoprecipitated with the anti-fusion protein antibodies (Fig. 3). Bands corresponding to each of the nonstructural proteins, nsPl (60 kDa), nsP2 (89 kDa), and nsP3 (76 kDa), specifically immunoprecipitated by the corresponding antibody can be readily seen. However, nsP4, which is reported to migrate at 73 kDa, is not visible. In addition, a number of proteins were precipitated by specific combinations of antisera, and these

**Fig. 3.** Fluorogram of immunoprecipitated virus-specific nonstructural proteins of Sindbis virus. Duplicate monolayers of BHK cells were infected as described in the text. At 3.5 h postinfection, the media over one set of monolayers were replaced with hypertonic media. At 4 h postinfection, both sets of monolayers were labeled for 30 min in isotonic media containing [$^{35}$S]methionine, harvested, and immunoprecipitated as described in the text. The resuspended immunoprecipitates were analyzed on a 10% discontinuous SDS-polyacrylamide gel. Lanes: M, mock-infected cells immunoprecipitated with a mixture of the four immune antisera; p.i., infected cells immunoprecipitated with preimmune serum; α-1, α-2, α-3, and α-4, infected cells immunoprecipitated with antisera made to fusion proteins containing sequences from nsPl, nsP2, nsP3, and nsP4, respectively; (+) and (-), cells with and without hypertonic treatment, respectively. Molecular mass standards are indicated as described in the legend to Fig. 2. C, Capsid protein.


(−)
(+)
M
p.i.
α−1
α−2
α−3
α−4
p.i.
α−1
α−2
α−3
α−4
P1234
P123
P34
P12
nsP2
nsP3
nsP1
C
250
205
165
155
116
100
97
66
45

proteins are putative precursors to the mature proteins. They include P1234 (~250 kDa), P123 (~200 kDa), P12 (~135 kDa), and P34 (~150 kDa). Similar- size proteins have been observed previously and identified by molecular mass. There are also faint bands visible, at approximately 200, 220, and 250 kDa, in the mock-infected samples and infected samples precipitated with preimmune serum, which appear to have been immunoprecipitated nonspecifically; these nonspecific bands interfere with the analysis of P1234 and, to a much lesser extent, of P123. Some capsid protein (C) was also precipitated nonspecifically.

Total incorporation of [$^{35}$S]methionine label in Sindbis virus-infected cells was monitored by trichloroacetic acid precipitation of infected-cell lysates. Typically, we found that 1 to 2% of the total labeled protein in the infected cell was immunoprecipitated in each reaction by α-nsPl, α-nsP2, or α-nsP3 (data not shown). For nsP4 the signal was too small to be significant.

To examine the kinetics of processing of the viral precursors, infected cells were subjected to hypertonic conditions for 30 min before being labeled in isotonic medium to synchronize translation initiation (19). There was a generalized reduction in protein synthesis after such a block, as shown by trichloroacetic acid precipitation to quantitate total protein synthesis (data not shown) or by examination of virus-specific proteins after immunoprecipitation (Fig. 3). However, host protein synthesis was reduced more than viral protein synthesis. After immunoprecipitation with α- nsPl, α-nsP2, or α-nsP3, approximately 6 to 7% of the total labeled protein was precipitated (data not shown), in contrast to the 1 to 2% found in the absence of salt synchronization, and a greater proportion of

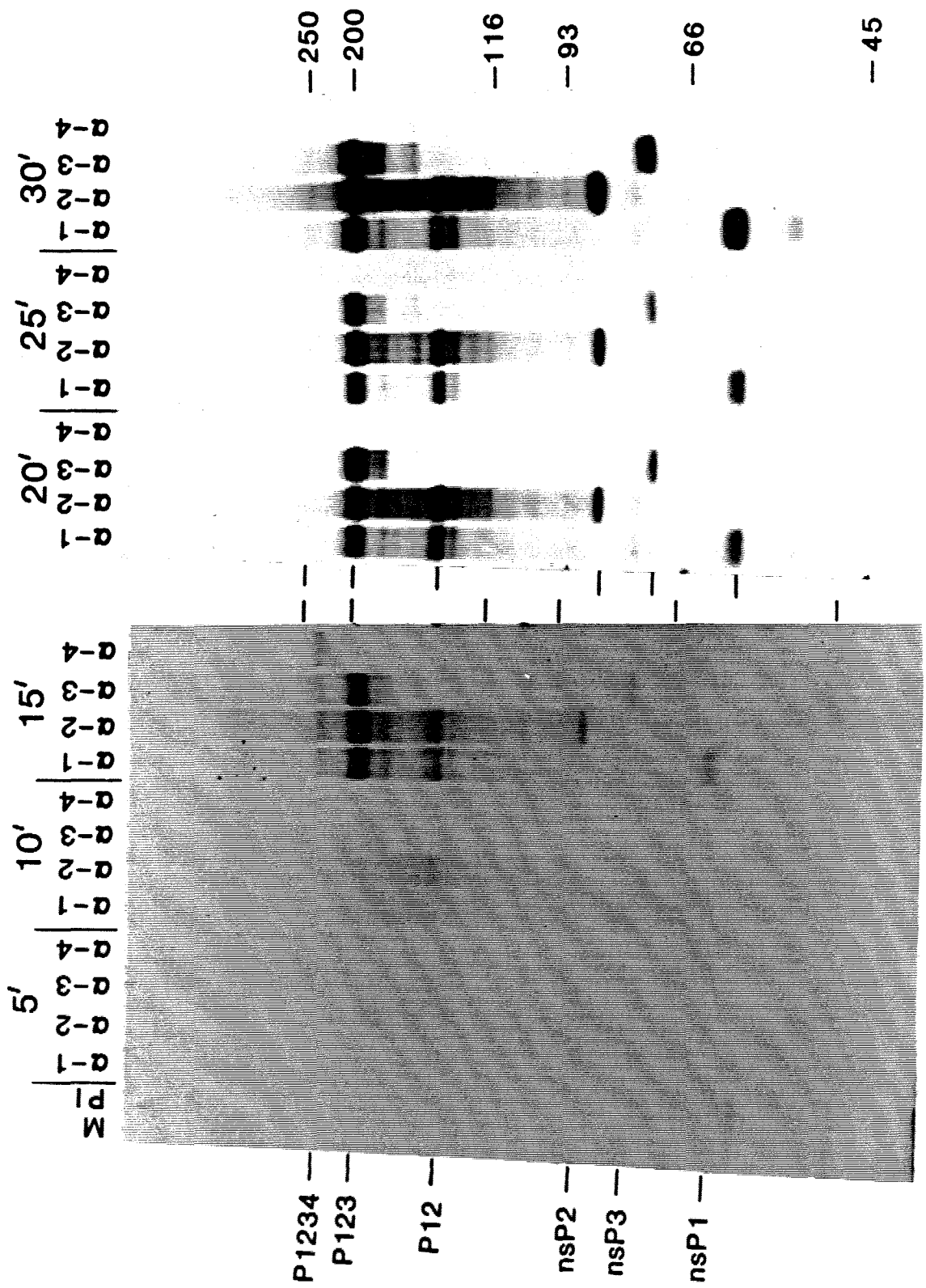
incorporated label was thus found in virus-specific proteins. The percentage of radioactivity precipitated by α-nsP4 was less than 0.5%.

**Accumulation of precursors and mature proteins.**

After a hypertonic block to synchronize initiation of translation, Sindbis virus-infected cells were labeled for various periods in isotonic media to determine the time required to translate the precursors and to examine the kinetics of the initial processing events. After a 5-min pulse only faint heterodisperse bands were present (Fig. 4). After 10 min of labeling the P12 precursor was visible, but in this experiment no mature nsP3 or P123 was seen (Fig. 4). This suggests that the cleavage that separates P12 and nsP3 can occur before the completion of nsP3, while the polyprotein is nascent (but see below). In a similar experiment, Brzeski and Kennedy (2) also observed a band of approximately 150 kDa 10 min after synchronous initiation, in the absence of other nonstructural polypeptides or their precursors.

By 15 min, P123 was present in large amounts, indicating that under these conditions it takes between 10 and 15 min to synthesize this polyprotein. The final products, nsPl, nsP2 and nsP3 were also seen, suggesting that both internal cleavages can occur within 15 min. However, the amount of P123 present relative to the quantities of the mature proteins, as well as to the amount of P12, indicates that the P123 precursor moiety is usually completed before processing occurs. In addition, small amounts of the readthrough products P1234 and P34 were first visualized at this time, although they could not be easily seen in this experiment after synchronization with high salt treatment.

**Fig. 4.** Monolayers of BHK cells were infected with Sindbis virus HRSP as described in the text. At 3 h postinfection, 5 M NaCl was added to the medium in each petri plate to give a final concentration of 220 mM NaCl in excess of that in the normal medium; 30 min later the hypertonic medium was removed and isotonic medium containing 40 μCi of [$^{35}$S]methionine per ml was added to each plate for various periods before the cells were lysed. The lysates were processed, immunoprecipitated, and electrophoresed. The lanes are defined in the legend to Fig. 3. Molecular mass standards are indicated as described in the legend to Fig. 2. The time of labeling is shown in minutes ('). The autoradiogram depicting the 5-, 10-, and 15-min time points was exposed 2.5 times as long as the one showing the 20-, 25-, and 30-min time points.


M
PI
5′
10′
15′
20′
25′
30′
α-1
α-2
α-3
α-4
P1234
P123
P12
nsP2
nsP3
nsP1
250
200
116
93
66
45

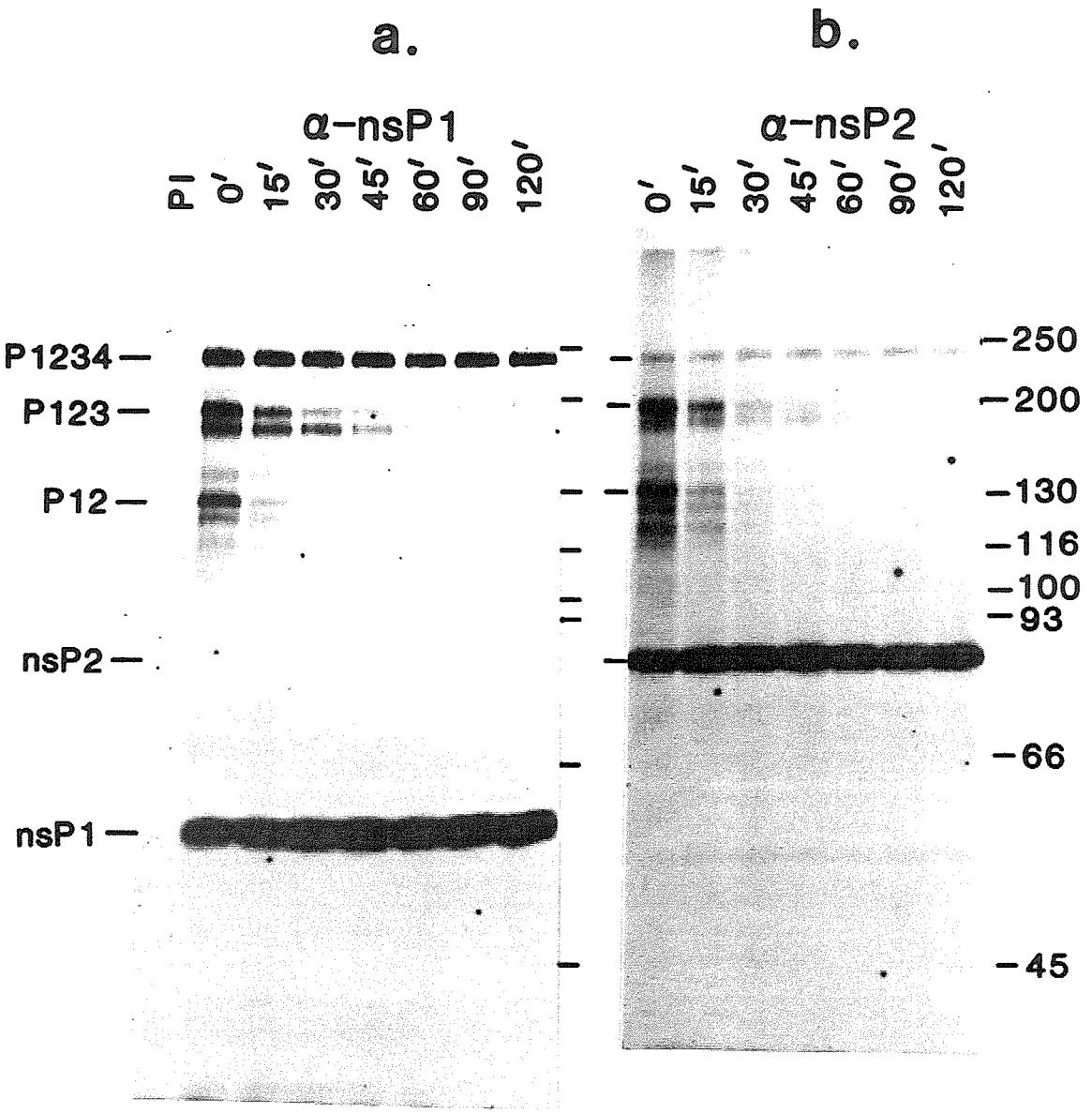
At 20 to 30 min after initiation, all of the precursors and products were present (note that the length of exposure for these time points differs from that for the 5- to 15-min points). Bands that appear to represent degradation products were also present but do not obscure the major conclusions. On the basis of the relative intensities of the mature products and precursors after various labeling periods, we obtained a preliminary estimate of 15 to 20 min to complete processing of P123 after synthesis. On the basis of these results a 30-min labeling period was chosen for further experiments.

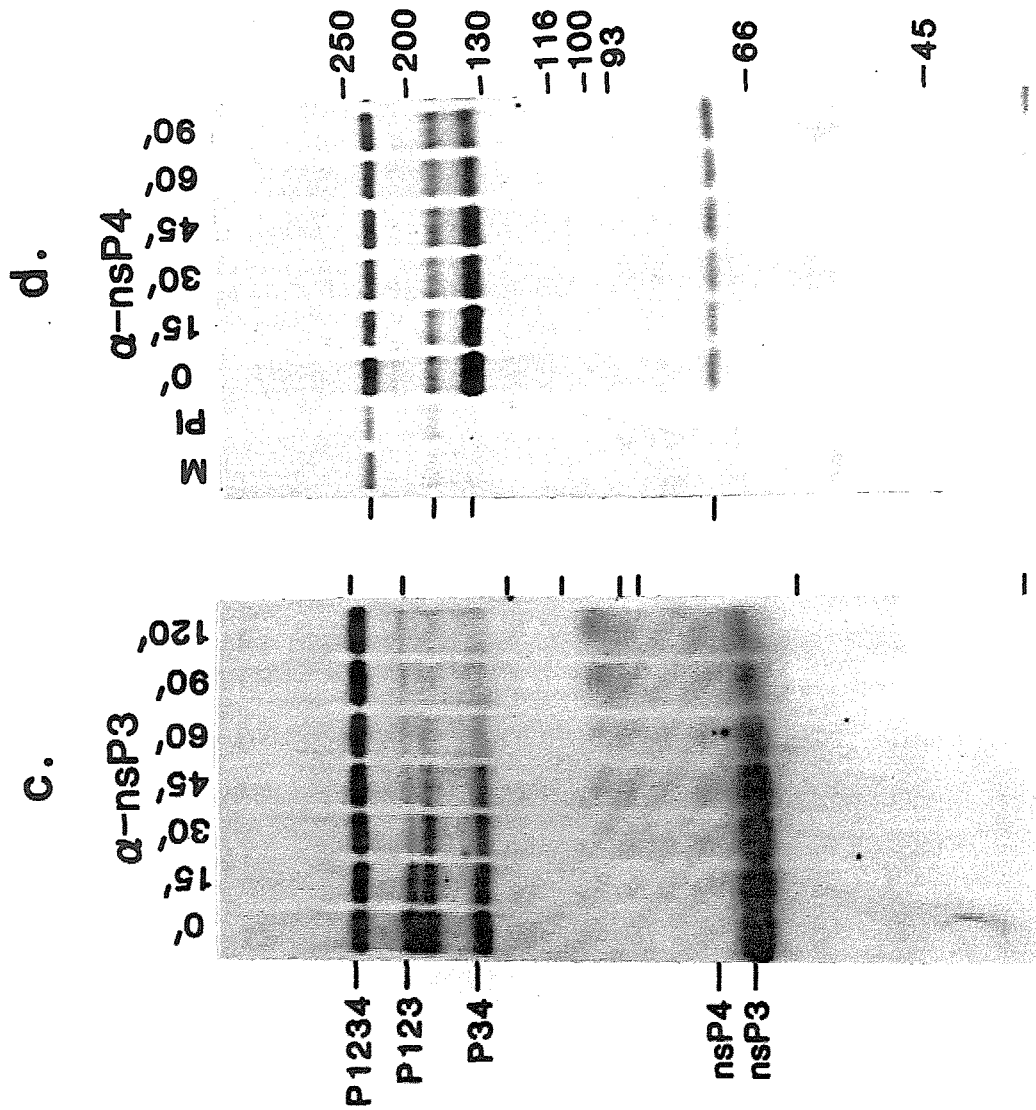
**Pulse-chase analysis of the stability of nonstructural polypeptides.**

Monolayers of infected chicken embryo fibroblasts or BHK cells were pulsed for 30 min with [$^{35}$S]methionine and then chased for various times in media containing an excess of unlabeled methionine. The concentration of PMSF was raised to 1 mM to inhibit protein breakdown. Cell lysates were immunoprecipitated to compare the relative stabilities of the nonstructural proteins and their precursors. The results are shown for chicken embryo fibroblasts (Fig. 5) because they expressed more of the readthrough product, P1234, than did BHK cells; otherwise the results were very similar.

Immediately after the 30-min pulse, all of the major precursor bands were present. Two additional bands were also present. One just below P123, at approximately 180 kDa, was immunoprecipitated by α-nsPl, α-nsP2, and α-nsP3 and was chased more slowly than P123. The second appeared only in the nsPl and nsP2 immunoprecipitates and migrated slightly below P12. It is unclear whether these two bands were degradation products or were produced by aberrant processing or host modification; these products were much more prominent in chick cells than in BHK cells.

**Fig. 5.** Monolayers of chick cells were infected with Sindbis virus HRSP and labeled beginning at 3 h postinfection for 30 min in medium containing 40 μCi of [$^{35}$S]methionine per ml. The cells were then chased for 0, 15, 30, 45, 60, 90, and 120 min (') in medium containing a 20-fold excess of nonradioactive methionine. The cells were lysed, the lysates were immunoprecipitated, and the immunoprecipitates were analyzed by SDS-PAGE. The samples in panels a, b, c and d were immunoprecipitated with α-nsPl, α-nsP2, α-nsP3, and α-nsP4, respectively. The time of chase is indicated above each lane. Molecular mass standards (masses in kilodaltons) are indicated on the right, and nonstructural proteins are indicated on the left. Note that twice as much lysate was used for the immunoprecipitations with α-nsP4 (d) as for the other antibodies.


a.
b.
α-nsP1
α-nsP2
PI
0'
15'
30'
45'
60'
90'
120'
P1234
P123
P12
nsP2
nsP1
250
200
130
116
100
93
66
45

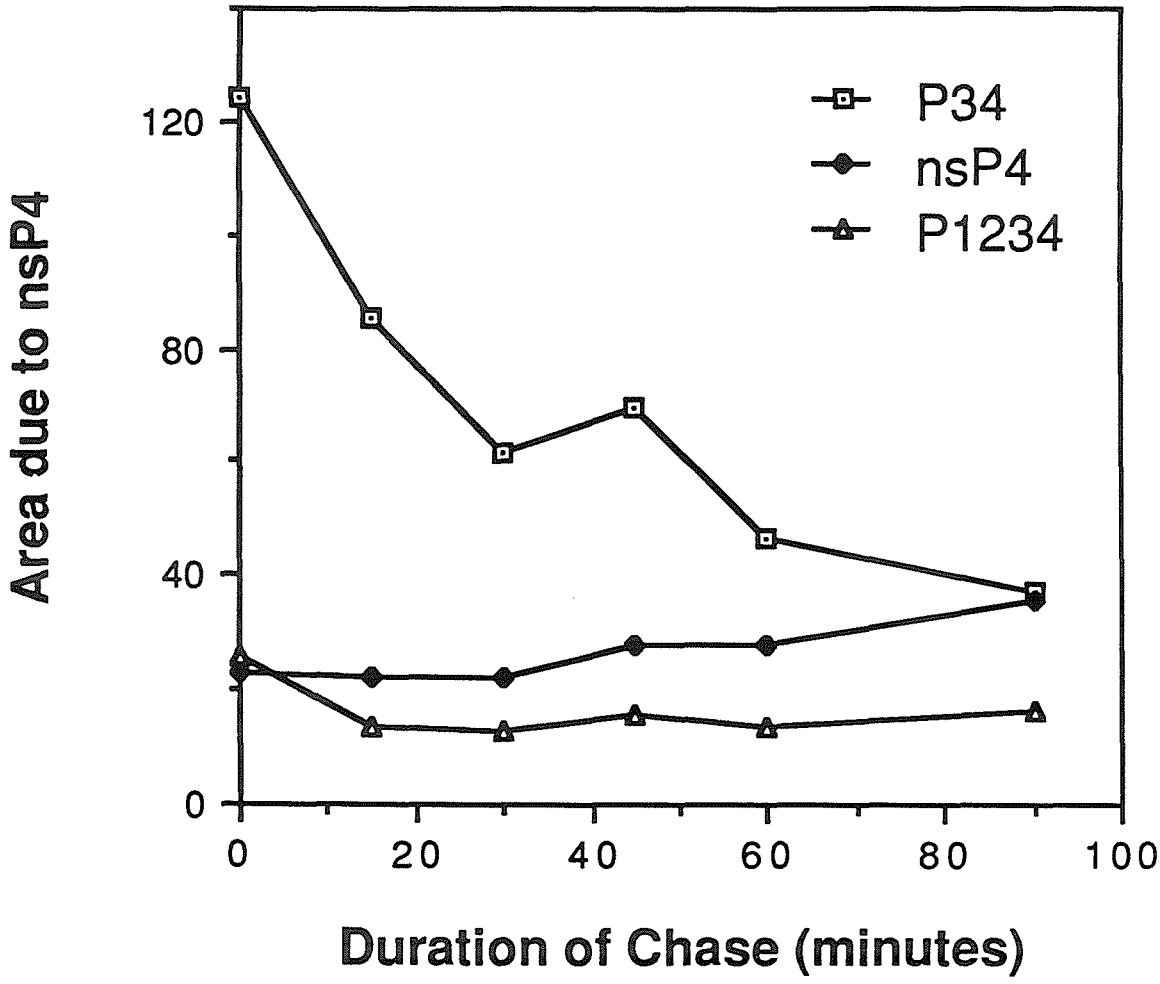

d.
α-nsP4
M
Pi
0'
15'
30'
45'
60'
90'
250
200
130
116
100
93
66
45
c.
α-nsP3
0'
15'
30'
45'
60'
90'
120'
P1234
P123
P34
nsP4
nsP3

Of the precursors, P123, P12, and P34 clearly turn over fairly rapidly, whereas P1234 does not. The kinetics of this turnover is explored in greater detail in the next section.

Of the four mature proteins produced during infection, only nsP3 seemed appreciably labile under the conditions used in this experiment. This protein disappeared with a half-life of 40 to 60 min (Fig. 5c). Coincident with the disappearance of nsP3, a heterogeneous population of polypeptides with apparent molecular masses of 79 to 113 kDa appeared. These polypeptides were precipitated only with α-nsP3 serum (Fig. 5), and it seems likely that they arose from a modification of the nsP3 that disappeared during the chase. However, we cannot rule out the possibility that they arose from P123 or P34 by a different type of processing or that they might contain non-nsP3 sequence which was not detected by the nsP2 or nsP4 antibodies. Assuming they represent a modified form of nsP3, the nature of the modification is obscure. It has been observed that nsP3 is phosphorylated (G. Li and C. M. Rice, personal communication). Based on parallel pulse-chase studies using [$^{35}$S]methionine and $^{32}P_i$, nsP3 appears to be chased to two predominant phosphorylated forms with apparent molecular masses, as determined by gel electrophoresis, of approximately 79 and 113 kDa (data not shown).

By using more lysate and longer exposure times, we could also monitor the processing of nsP4 from its precursors (Fig. 5d). The amount of material in nsP4 and its precursors was quantitated by densitometry of the autoradiogram shown in Fig. 5d. These data were normalized for the number of methionines in the nsP4 region of each precursor and plotted (Fig. 6). The data demonstrate that nsP4 accumulated during the chase at a remarkably slow rate, much more slowly than the disappearance of P34,

**Fig. 6.** The autoradiogram of the α-nsP4 immunoprecipitation (Fig. 5d) was subjected to densitometry. The y axis is in arbitrary units of area corresponding to the quantity of nsP4 either in nsP4 itself or in the nsP4 region of a precursor. For example, P34 has 27 methionine residues, of which only 16 are in nsP4; therefore, the areas for P34 were multiplied by 16/27 to arrive at the area due to nsP4 alone. The x axis gives the time of chase.


P34
nsP4
P1234
120
80
40
0
0
20
40
60
80
100
Area due to nsP4
Duration of Chase (minutes)

which possesses a half-life of approximately 30 min. This indicates either that some P34 was being degraded rather than being processed to produce mature nsP3 and nsP4 or that nsP4 was unstable and was turning over at a rate slightly less than the rate at which P34 was being processed. The interpretation of the processing of the P1234 precursor is obscured by the presence of a band at 250 kDa which was immunoprecipitated nonspecifically, as can be seen in the mock-infected and preimmune control samples. It is unclear whether P1234 is processed to mature products. Based on the abundance of P34 relative to that of P1234 (Fig. 5), P34 is probably the predominant species from which nsP4 is derived, and any processing of P1234 probably makes a negligible contribution to the pool of nsP4.

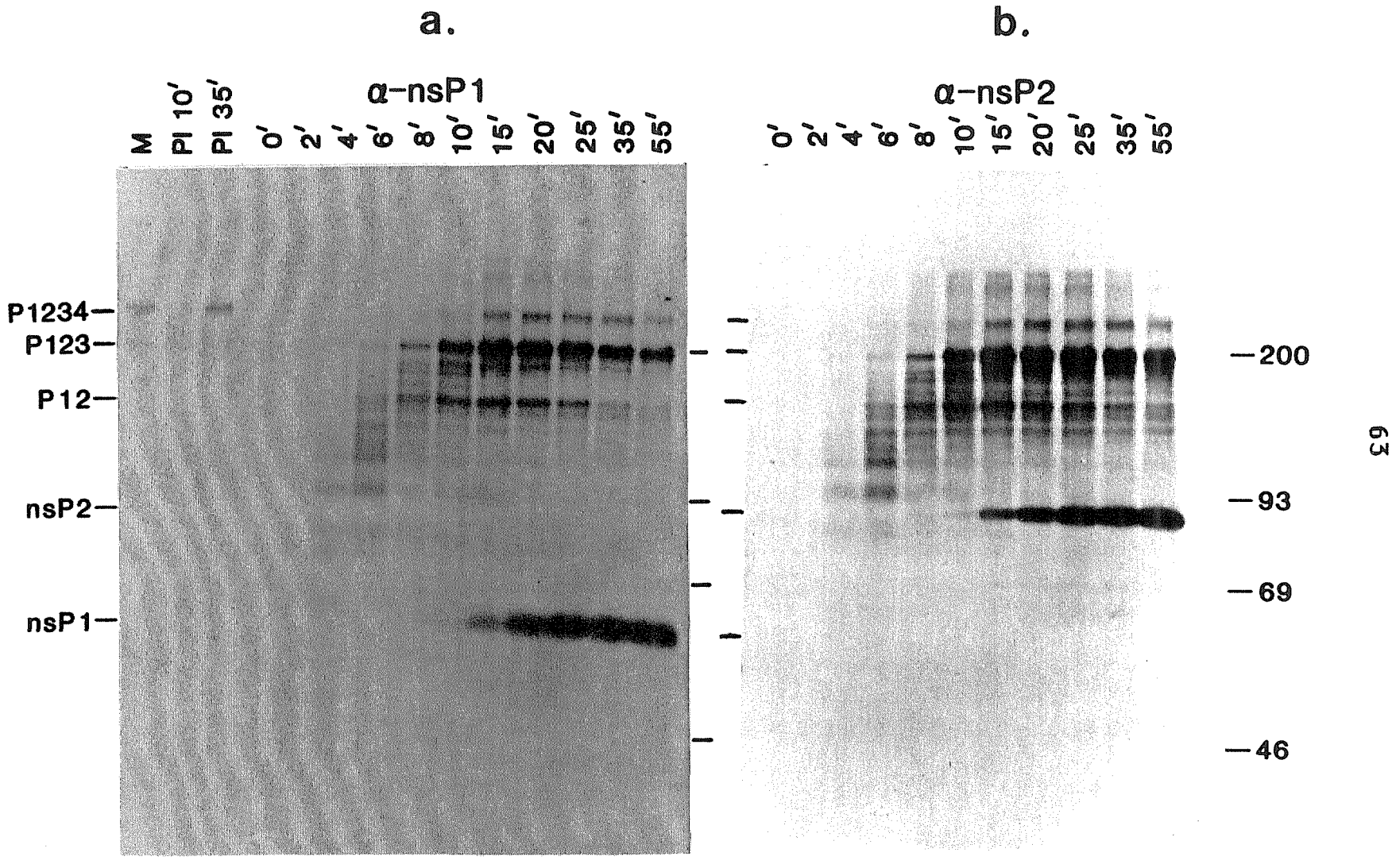
**Kinetics of polyprotein processing.**

We performed short pulse-chase experiments to measure the kinetics of processing, but it was difficult to disentangle the effects of polypeptide chain elongation from processing. The time required to synthesize a complete polypeptide chain is not dissimilar to that required for processing, and the processing kinetics depends upon the stage of completion of the polypeptide. We therefore devised a modified protocol for a pulse-chase experiment in which we monitored elongation and processing of polypeptide chains initiated in a 2-min time period. To accomplish this, infected cells at 3 h postinfection were synchronized by treatment with 220 mM excess NaCl for 30 min as before and released from the hypertonic block in the presence of 50 μCi of [$^{35}$S]methionine per ml. Two min later pactamycin was added to 1 μM to block further translation initiation. Protein synthesis (and processing) were allowed to continue in the presence of label for various

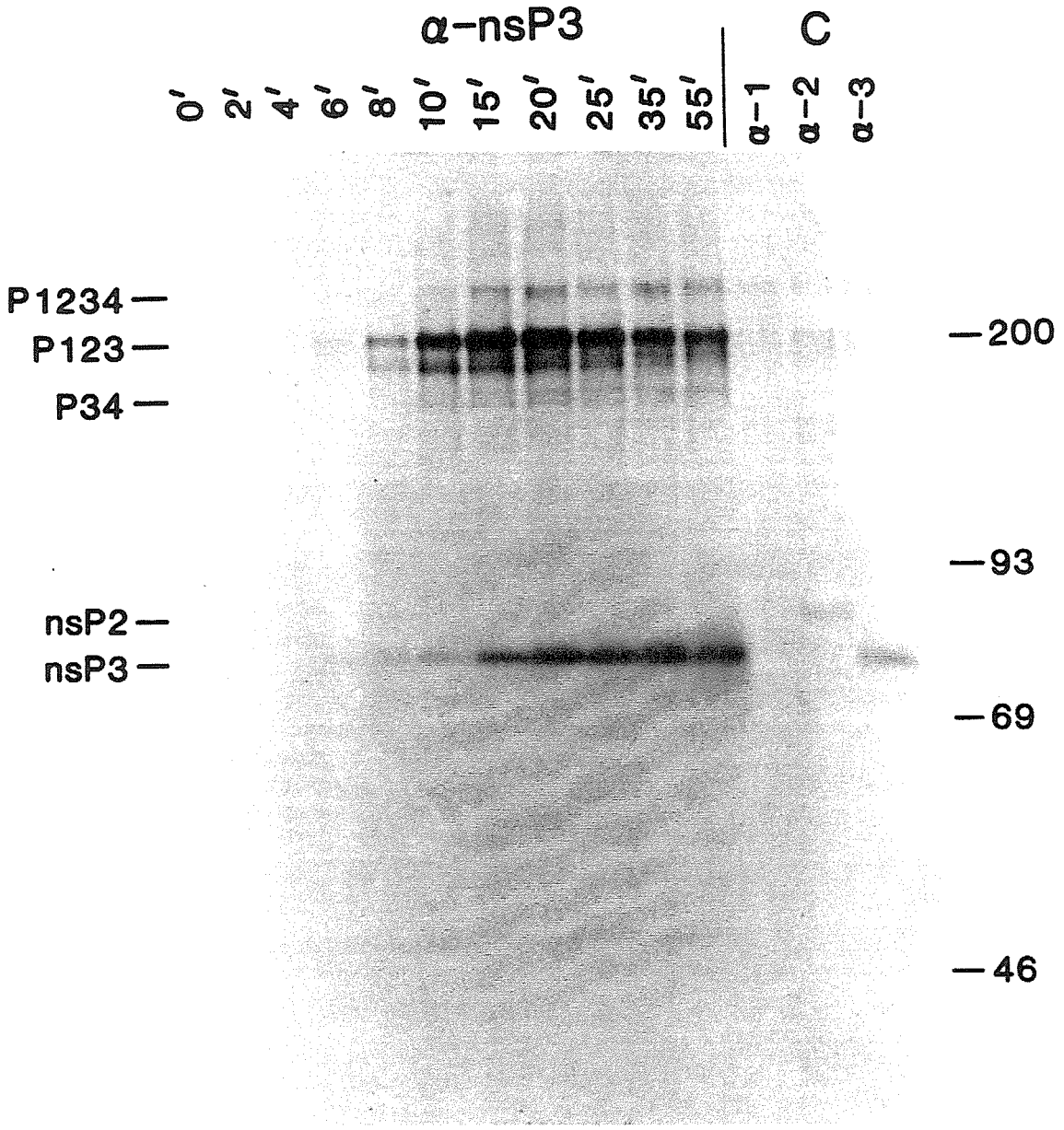
periods, and the cells were then lysed and prepared for immunoprecipitation (Fig. 7). Because the purpose of the experiment was to determine cleavage kinetics accurately, the temperature of incubation was carefully controlled, and the synthesis kinetics appeared to be slightly faster than in the experiment shown in Fig. 4. We determined that 1 μM pactamycin was sufficient to inhibit initiation of new polypeptide chains while still allowing elongation of previously initiated chains by the following experiment. At 3.5 h postinfection without hypertonic treatment, 1 μM pactamycin was added to cells. After 5 min, [$^{35}$S]methionine was added, and incubation was continued for an additional 25 min in the presence of pactamycin. Immunoprecipitation of these cell lysates (Fig. 7c, control lanes) showed that nsP3 was labeled predominantly; a small amount of label was also found in nsP2, and no label was observed in nsPl. Because the order of proteins in the polyprotein precursor is $NH_2$-nsPl-nsP2-nsP3-COOH, this was the expected result if the pactamycin inhibition was effective.

In cells synchronized by a hypertonic block and allowed to initiate polypeptide chains for 2 min before the addition of pactamycin, the incorporation of [$^{35}$S]methionine into elongating strands plateaued between 15 and 20 min after the addition of the drug, as determined by hot trichloroacetic acid precipitation of the whole-cell lysates, again demonstrating the effectivenss of pactamycin treatment. Completed chains of P123 were first detectable 6 min after the addition of the drug, that is, 8 min after synchronous initiation of translation (Fig. 7). This would require a maximum elongation rate of approximately 240 amino acids per minute, which is somewhat faster than the rate estimated for translation of the poliovirus genome (19). Although nsP3, P12, and P123 appeared at 6 min

**Fig. 7.** Monolayers of chick cells were infected at high multiplicity with Sindbis virus HRSP. At 3 h postinfection, NaCl was added to inhibit initiation of new polypeptide chains; 30 min later the hypertonic media were removed and Eagle medium containing 50 μCi of [$^{35}$S]methionine per ml was added. After 2 min, pactamycin was added to a final concentration of 1 μM. The monolayers were then lysed at various times after the addition of the drug and immunoprecipitated. The antisera used were α-nsPl (a), α-nsP2 (b), α-nsP3 (c). The numbers above the lanes are minutes after the addition of pactamycin. The results of a control experiment (C) are also shown in panel c, in which infected cells were labeled with [$^{35}$S]methionine beginning 5 min after the addition of pactamycin, and the cells were lysed 25 min later. The cell lysates were immunoprecipitated with α-nsPl, α-nsP2, or α-nsP3 as indicated. M and PI are defined in the legend to Fig. 3.


a.
b.
α-nsP1
α-nsP2
M
PI 10'
PI 35'
0'
2'
4'
6'
8'
10'
15'
20'
25'
35'
55'
P1234
P123
P12
nsP2
nsP1
200
93
69
46

c.


α-nsP3
C
0'
2'
4'
6'
8'
10'
15'
20'
25'
35'
55'
α-1
α-2
α-3
P1234
P123
P34
nsP2
nsP3
200
93
69
46

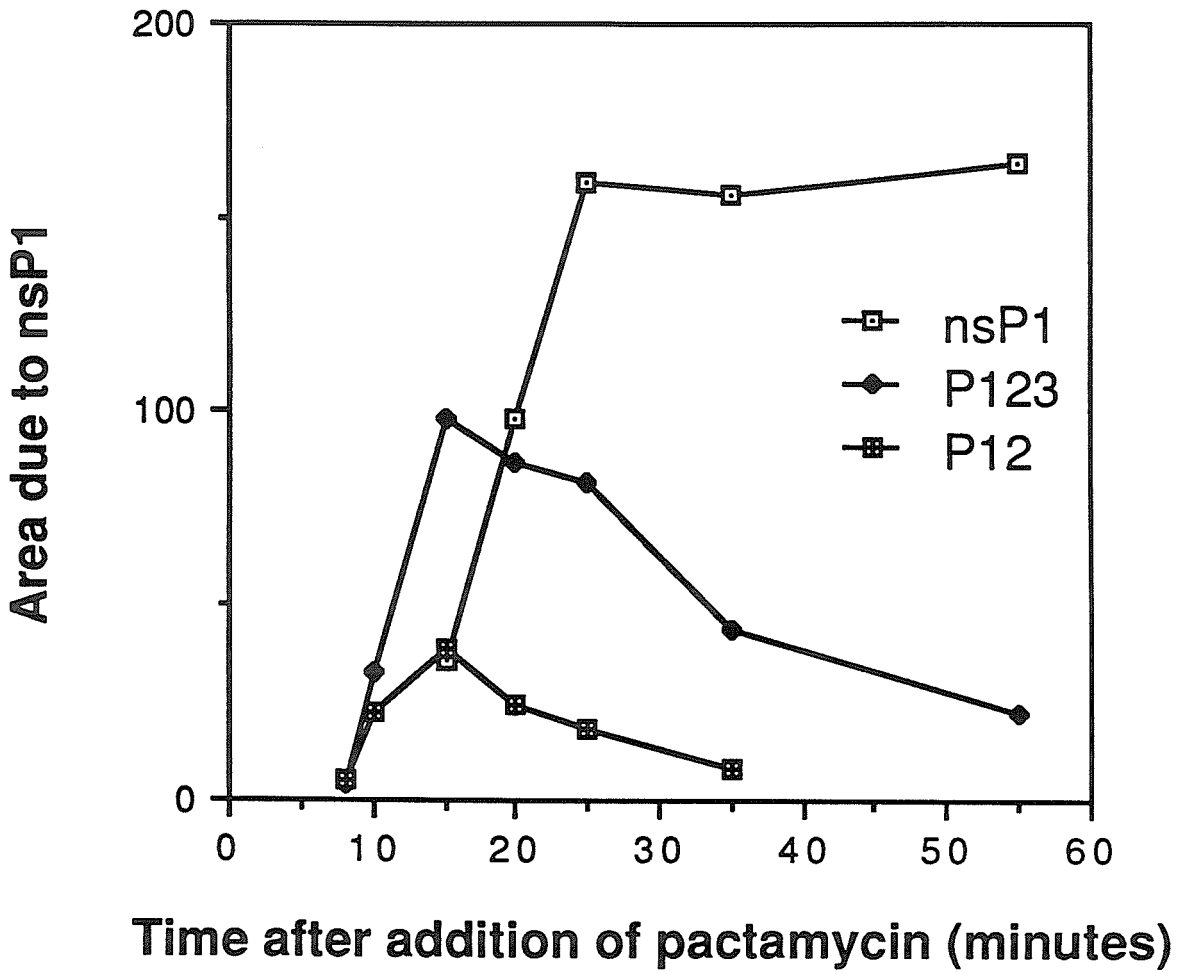
after the addition of pactamycin, mature nsPl and nsP2 were not detectable before 10 min (Fig. 7). Whether P1234 was synthesized or processed under these conditions cannot be determined because of the presence of a nonspecific band at ~250 kDa.

To quantitate these data, the autoradiograms were subjected to densitometry; a graph of the densitometry data for anti-nsPl immunoprecipitation is shown in Fig. 8. It can be seen that the accumulation of P123 peaked between 10 and 15 min after the addition of the drug, signifying the completion of elongation. P123 was then cleaved with a half-life of approximately 19 min to produce P12 and nsP3. The amount of P12 was also maximal at about 10 to 15 min, but it was cleaved with a half-life of about 9 min and was almost absent by 55 min. Thus, the cleavage between nsPl and nsP2 occurred more rapidly than that between nsP2 and nsP3. However, since there was no P23 visible in any of the pulse-chase experiments, it appears that nsP3 must be cleaved from P123 before nsPl can be cleaved from nsP2. The densitometry data for nsP2 and nsP3 (not shown) lead to similar conclusions.

## DISCUSSION

A brief labeling of Sindbis virus-infected cells at 3 to 4 h after infection, followed by immunoprecipitation with antibodies specific for the four nonstructural proteins, clearly showed the following virus-specific nonstructural polypeptides: P1234 (~250 kDa), P123 (200 kDa), P34 (150 kDa), P12 (135 kDa), nsP2 (89 kDa), nsP3 (76 kDa), and nsPl (60 kDa). The 72 kDa protein (nsP4) could be visualized by using special procedures and longer exposures. These molecular masses are consistent with the predicted molecular masses of the nonstructural proteins deduced from RNA

**Fig. 8.** The autoradiogram of immunoprecipitation by α-nsPl (Fig. 7a) was subjected to densitometry to quantitate the amount of nsPl and its precursors present at various times after pactamycin addition. Areas have been normalized as described in the legend to Fig. 6.

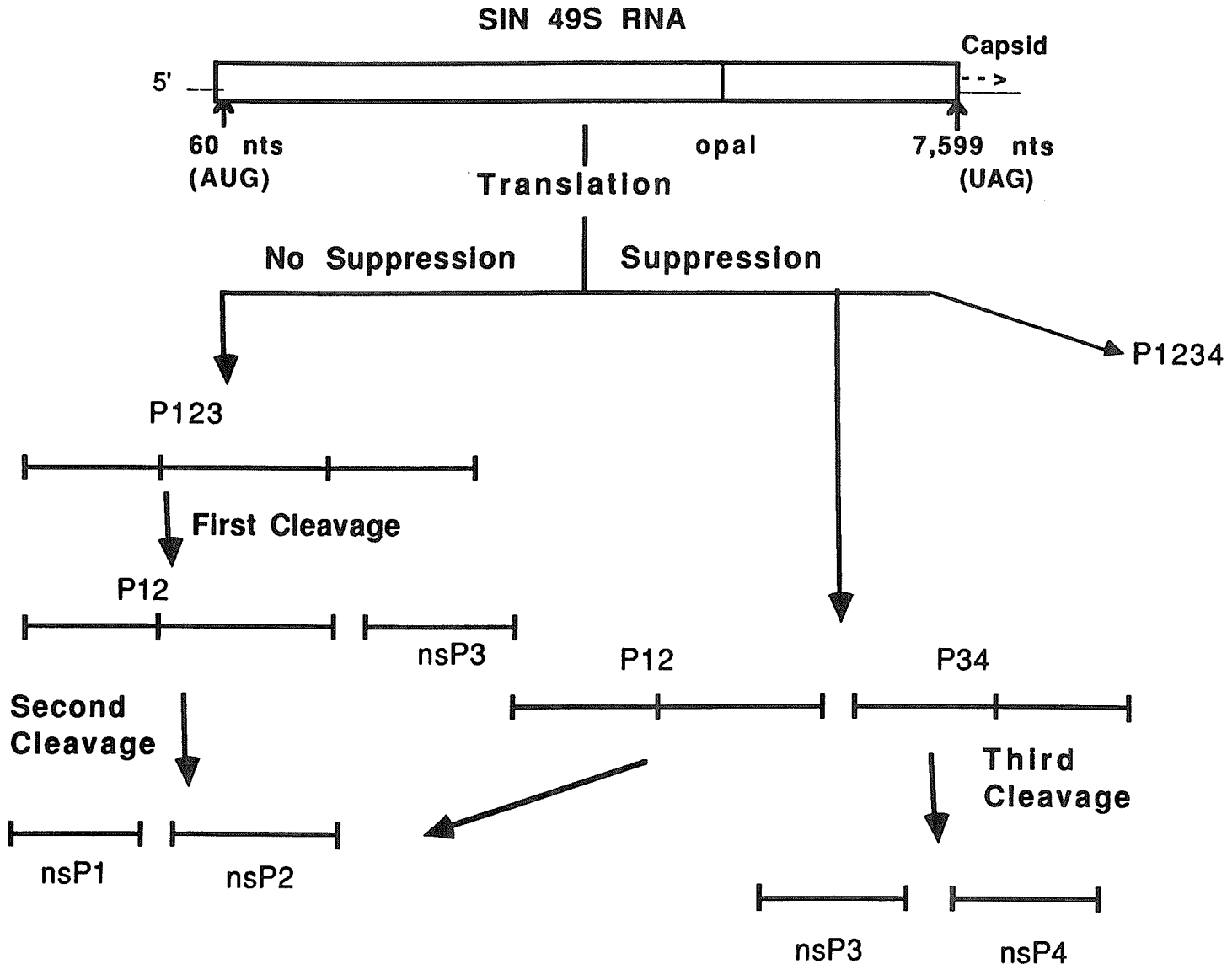

200
100
0
0
10
20
30
40
50
60
Area due to nsP1
Time after addition of pactamycin (minutes)
nsP1
P123
P12

sequence data (23), with the exception that nsP3 and precursors containing nsP3 migrate anomalously. These molecular masses are also in excellent agreement with the sizes of the polypeptides found in previous studies (1-3, 7, 14). Using α-nsP4 peptide antibody, Lopez et al. (14) also immunoprecipitated a band corresponding to a molecular mass of approximately 220 kDa which they classified as the P234 precursor but which we did not detect in our experiments. In chick cells we also found additional bands that migrated between 200 and 100 kDa, especially in the pulse-chase experiments. The label in these bands appeared to chase, and they may represent alternative or aberrant processing after the hypertonic block. The concentration of these precursors was reduced in infected BHK cells, and their participation in the overall cleavage scheme is unclear.

Immunoprecipitation with these antisera also revealed a previously unrecognized form of nsP3. Over a period of 2 h after synthesis, nsP3 appeared to shift from a 76-kDa band (when newly synthesized) to a heterodisperse series of bands between 79 and 113 kDa. The molecular mechanisms of this apparent posttranslational modification are unclear but may be due in part to phosphorylation.

On the basis of the kinetic data presented above, we formulated a model for the synthesis and processing of Sindbis virus nonstructural proteins (Fig. 9). Translation apparently begins at only a single point in the genome, the AUG codon (arrow), 60 nucleotides from the 5' terminus of the 49S genomic RNA. After translation through nsP3 the nascent polypeptide chains follow one of two courses. Translation either terminates at the opal codon, producing P123, or reads through the opal codon to produce P1234. Frequently, the cleavage between nsP2 and nsP3 occurs before the completion of P1234, leading to large amounts of P34 relative to P1234. If the

**Fig. 9.** Proposed model showing the processing of the polyprotein precursors of Sindbis virus. After the translation of nsP3, either termination or readthrough occurs. Termination produces most of the nonstructural proteins. The major precursor is P123, which is first cleaved to P12 and nsP3. Subsequently, P12 is cleaved to nsPl and nsP2. SIN, Sindbis virus; nts, nucleotides.


SIN 49S RNA
Capsid
5'
- - >
60 nts
(AUG)
opal
7,599 nts
(UAG)
Translation
No Suppression
Suppression
P1234
P123
First Cleavage
P12
nsP3
P12
P34
Second
Cleavage
Third
Cleavage
nsP1
nsP2
nsP3
nsP4

precursor P34 had come from the posttranslational processing of P1234, we would have expected a much higher ratio of P1234 to P34 following a short pulse of label. At a much lower frequency, nascent P123 can be cleaved between nsP2 and nsP3 before nsP3 has been completely translated, such that significant amounts of P12 and nsP3 appear simultaneously with P123 (Fig. 7).

We have previously postulated that the proteolytic activity responsible for cleaving the nonstructural polyprotein resides within the nonstructural proteins themselves and is at least in part autoproteolytic (23). *In vitro* translation results (3; our unpublished observations) are consistent with this hypothesis. Several results from the present study are of particular interest. (i) Processing occurs sequentially; i.e., the cleavage between nsP2 and nsP3 must necessarily precede the cleavage between nsPl and nsP2. (ii) The kinetics of processing is relatively slow, especially if the activity is autoproteolytic. (Keranen and Ruohonen [8], however, have reported that the processing of the nonstructural proteins of Semliki Forest virus (SFV) is much more rapid.) (iii) The second cleavage (to separate nsPl and nsP2) is more rapid than the first.

Some alphaviruses, particularly Sindbis virus (22), Middelburg virus (22), and Ross River virus (unpublished data) possess an opal codon between moieties nsP3 and nsP4, whereas SFV (26) and O'Nyong-nyong virus (E. G. Strauss, personal communication) do not. Aside from the differences necessitated by the lack of an opal codon in the genome at the end of nsP3, the processing scheme for the nonstructural polypeptides of SFV appears to be very similar to that described above for Sindbis virus. It was proposed several years ago that the first cleavage of the SFV nonstructural polyprotein separated P12 (or the 155-kDa precursor) from

P34, then called the 135-kDa precursor (for a review, see Reference 20). After this, the cleavage to produce mature nsPl and nsP2 appears to be virtually identical with that for Sindbis virus. The differences in the processing schemes are in the treatment of the P34 polypeptide. It appears clear that effective replication of either alphavirus requires that significant quantities of nsP3 be produced. In Sindbis virus, most of this nsP3 is the result of termination at the opal codon and not cleavage of the P34 polypeptide. However, for SFV, the production of large amounts of nsP3 requires rapid and efficient cleavage of P34 since termination cannot occur; little P34 accumulates under any conditions, and significant amounts of nsP4 are produced. In Sindbis virus infection, on the other hand, P34 appears to be the moiety that accumulates during infection and little or no free nsP4 is ever seen. These two modes of replication can be brought together if we assume (i) that large amounts of nsP3 are required for successful alphavirus replication and (ii) that little or no nsP4 as nsP4 is required. It is possible that the active form of nsP4 for both Sindbis virus and SFV is the P34 polypeptide and that only small amounts of it are needed. Only small quantities of this polypeptide are made by readthrough in Sindbis virus, and perhaps only small quantities escape processing in SFV.

## ACKNOWLEDGMENTS

We thank E. G. Strauss, C. M. Rice, and M. J. Schlesinger for helpful discussions and assistance in preparation of the manuscript.

This work was supported by Public Health Service grants A120612 and A110793 from the National Institutes of Health; W.R.H. was supported in

part by Public Health Service training grant GM00086 from the National Institutes of Health.

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# Chapter 2

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

This chapter is in press in the *Journal of Virology*.

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

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Received 26 May 1989/Accepted 24 July 1989

## ABSTRACT

The processing of the Sindbis virus nonstructural polyprotein translated *in vitro* has been studied. When Sindbis 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 nsPl, 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 is 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.

## INTRODUCTION

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 proteinases (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 that 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, nsPl, 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 nsPl or nsP2, and probably within nsP2. In addition, Hahn et al. (19) have 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 per ml of Inhibit-Ace, an RNase inhibitor (5 Prime-3 Prime, Inc.). 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 πnAN7 (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 in the Nonstructural Protein Region of Toto 1101

| Name | Location[a] | End Modifications | Subclone | Size (aa) |
|---|---|---|---|---|
| Δ1A | *Bal* I (205)-*Bal* I (1,101) | None | πnsP12 | 299 |
| Δ1B | *Sma* I (765)-*Sma* I (944) | None[b] | none | 60 |
| Δ1C | *Ban* II (1,145)-*Ban* II(1,660) | None[c] | πnsP12 | 172 |
| Δ2A | *Nhe* I (1,809)-*Nhe* I (2,168) | None | πnsP12 | 120 |
| Δ2B | *Mst* II (2,124)-*Eco* RV (2,750) | *Mst* II site filled-in with Klenow. | pMT2/3 | 209 |
| Δ2C | *Cla* I (2,714)-*Pvu* II (3,103) | *Cla* I site blunt-ended with Mung bean nuclease. | pMT 2/3 | 130 |
| Δ2D | *Eco* RV (2,751)-*Nhe* I (3,437) | *Nhe* I site filled-in with Klenow. | pMT 2/3 | 229 |
| Δ2E | *Pvu* II (3,104)-*Ava* I (3,550) | *Ava* I site blunt-ended with Mung bean nuclease. | pMT 2/3 | 149 |
| Δ2F | *Xmn* I (3,595)-*Tth*111 I(3,912) | *Tth*111 I site filled-in with Klenow. | pMT 2/3 | 106 |
| Δ2G | *Ban* I (3,862)-*Ban* I (4,257) | None | pMT 2/3 | 132 |
| Δ2H | *Nhe* I (1,809)-*Nhe* I (3,437) | None | none | 543 |
| Δ34A | *Ssp* I (4,130)-*Bam* HI (4,638) | *Bam*HI site blunt-ended with S1 nuclease.[d] | none | —[d] |
| Δ34B | *Bam* HI (4,638)-*Bam* HI (7,334) | Both *Bam* HI sites were filled-in with Klenow. | none | 899 |
| Δ4A | *Hind* III (6,272)-*Hpa* I (6,919) | *Hind* III site filled-in with Klenow. | none | 216 |

Footnotes:

[a] Sindbis sequences are numbered according to Strauss et al. (1984).

[b] The clone isolated had 3 nucleotides deleted from the 5' *Sma* I site.

[c] The clone isolated had 3 nucleotides deleted from within the *Ban*II site.

[d] The clone isolated had an extra nucleotide deleted from the *Bam* HI site, causing a frameshift to occur, and results in termination approximately 5 amino acids downstream of the *Ssp*I site.

(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 that 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 Klenow) 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 Toto1101. 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 *b* to *d*. 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 caused 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 $m^7$G(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 [$^{35}$S]methionine (>1000 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 that 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 SDS or diluted (1:1) with a solution containing 62 mM Tris chloride (pH 6.8), 2% SDS, and 1% 2-mercaptoethanol for immunoprecipitation.

**Immunoinhibition of processing.**

To an *in vitro* translation mixture containing virion RNA prepared as described above was added 1/10 volume of preimmune sera 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 1/10 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 (MAPS) (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 x 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) were added to each tube. After 45 min at room temperature, 20 μl of a 10% (wt/vol) suspension of *Staphylococcal 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 4000 x g and washed three times with 500 μl of RIPA buffer.

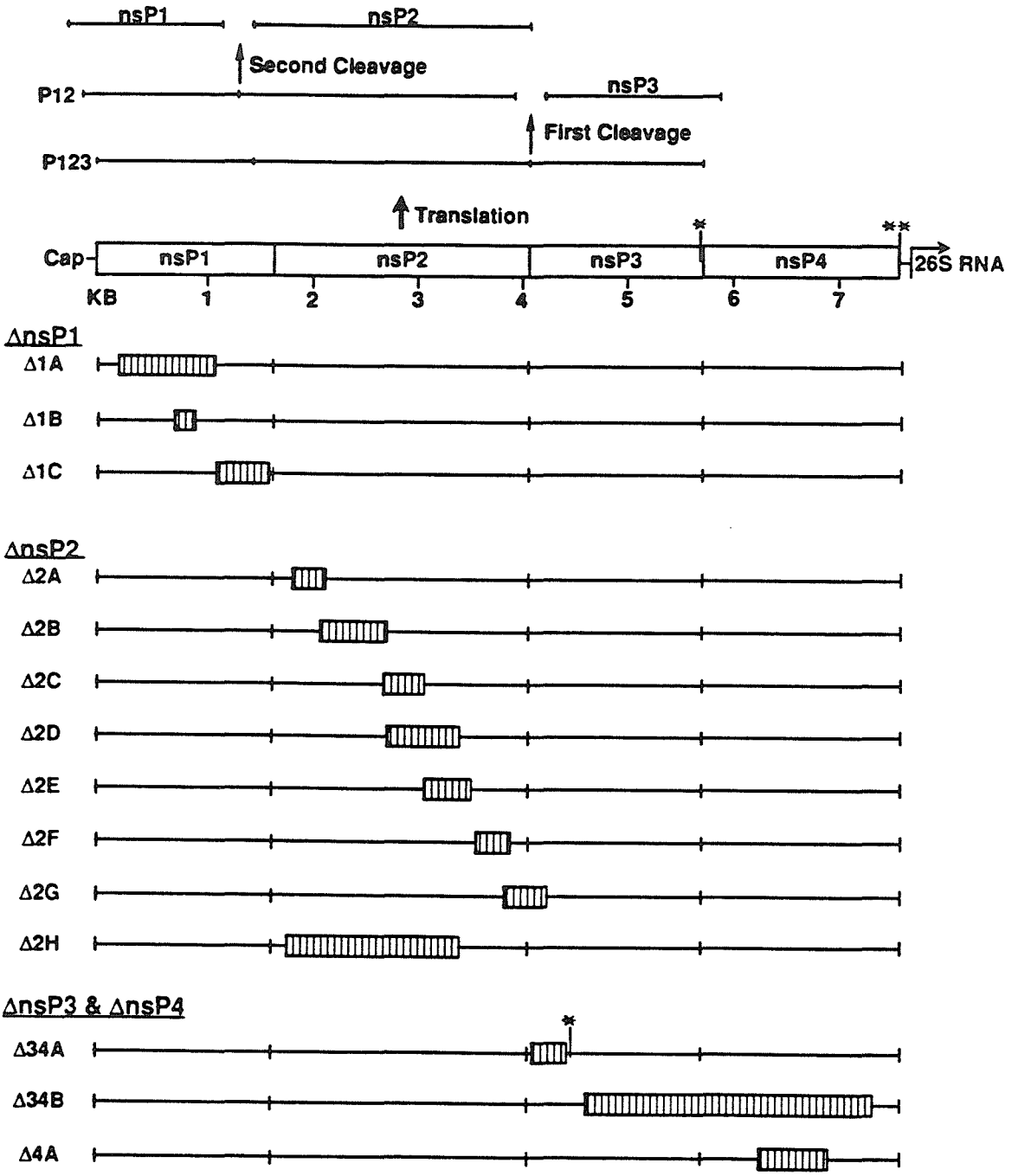
The immunoprecipitated products were resuspended in loading buffer (62.5 mM Tris chloride (pH 6.8), 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol), denatured by heating to 90°C for 3 min, and centrifuged for 8 min at 16,000 x g to remove the cells. These samples were then analyzed by polyacrylamide gel 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.

**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 nsPl 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.


nsP1
nsP2
Second Cleavage
P12
nsP3
First Cleavage
P123
Translation
Cap
nsP1
nsP2
nsP3
nsP4
26S RNA
KB
1
2
3
4
5
6
7
ΔnsP1
Δ1A
Δ1B
Δ1C
ΔnsP2
Δ2A
Δ2B
Δ2C
Δ2D
Δ2E
Δ2F
Δ2G
Δ2H
ΔnsP3 & ΔnsP4
Δ34A
Δ34B
Δ4A

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 [$^{35}$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 that migrate at the positions of nsPl and nsP2 are visible as early as 14 min after the start of translation, and a sharp band of nsPl 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 P 1234 can be detected by 32 min. Shortly thereafter, at 35 min, a band identified as P34 is detectable. This product 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 nsPl 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),

**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% SDS 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.

Fig. 2A

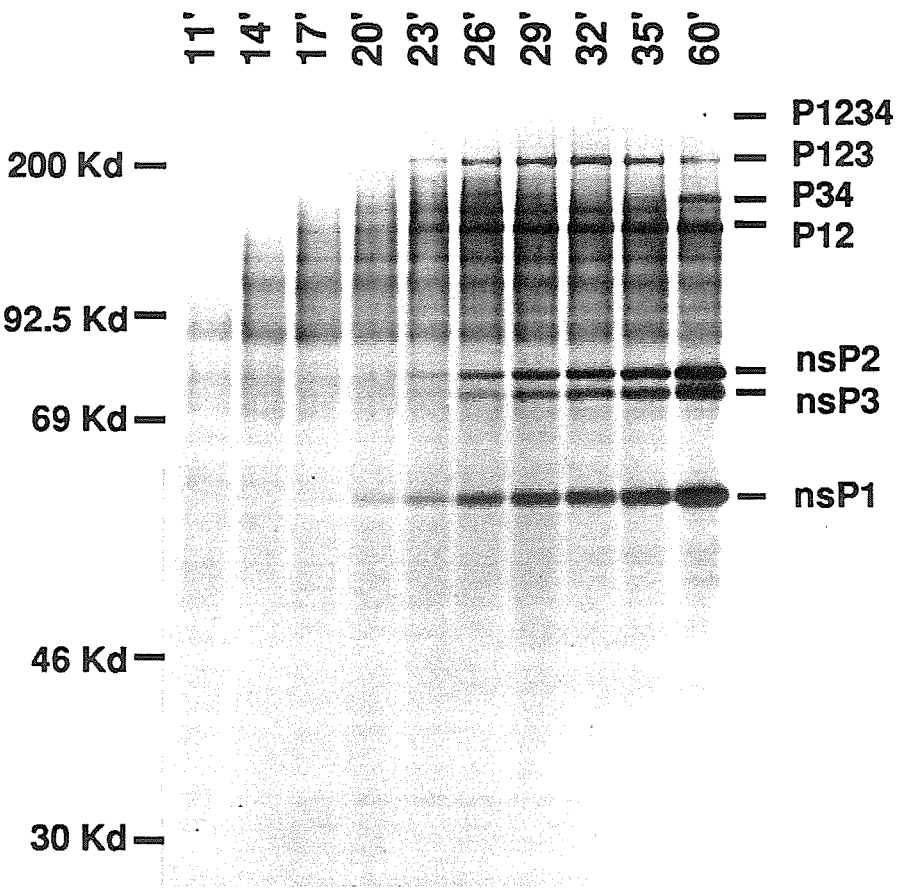
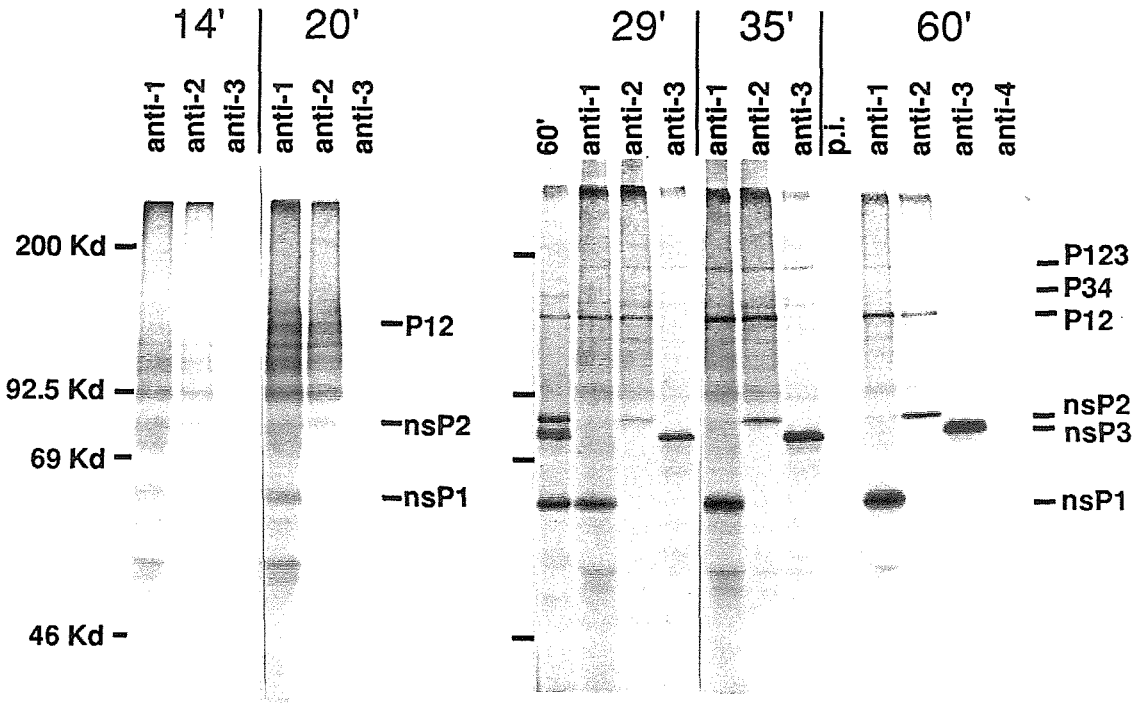

11'
14'
17'
20'
23'
26'
29'
32'
35'
60'
200 Kd
92.5 Kd
69 Kd
46 Kd
30 Kd
P1234
P123
P34
P12
nsP2
nsP3
nsP1

Fig. 2B


14'
20'
29'
35'
60'
anti-1
anti-2
anti-3
60'
p.i.
anti-4
200 Kd
92.5 Kd
69 Kd
46 Kd
P12
nsP2
nsP1
P123
P34
P12
nsP2
nsP3
nsP1

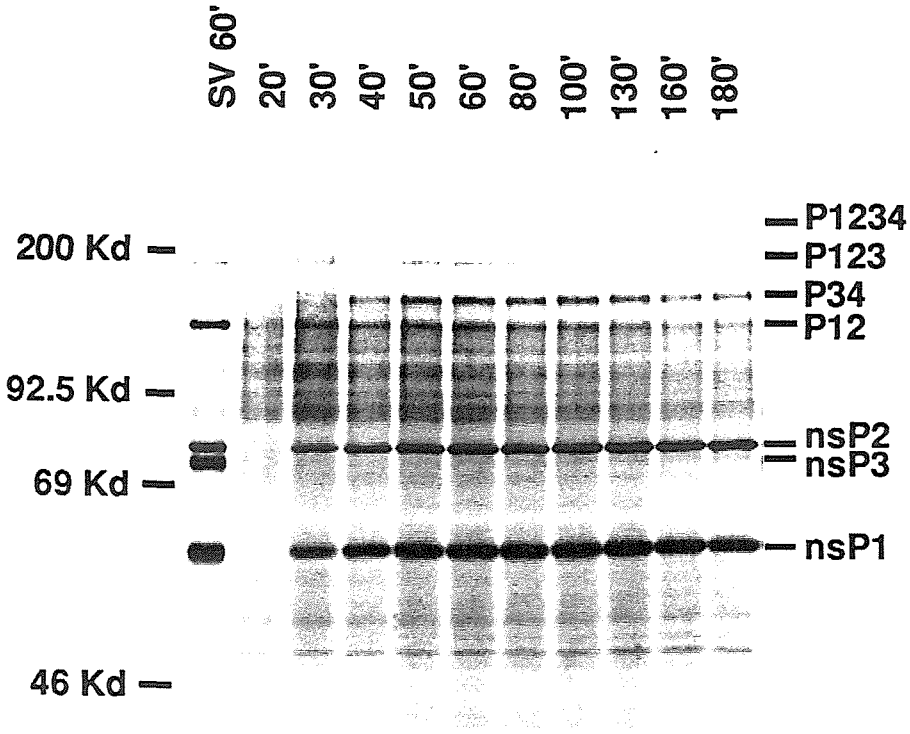
accumulates with time from 20 to 35 min, and then disappears by 60 min (Fig. 2A). The origin of this band is unclear.

**Cleavage between nsP3 and nsP4.**

In order 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 had 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 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.

**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 constituted 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 precursors and nonstructural proteins are the same as in Fig. 2.


SV 60'
20'
30'
40'
50'
60'
80'
100'
130'
160'
180'
200 Kd
92.5 Kd
69 Kd
46 Kd
P1234
P123
P34
P12
nsP2
nsP3
nsP1

**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 that 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 IgG concentration (Fig. 4B). At very high concentrations, 1.1 mg IgG per ml or higher, elongation was inhibited (data not shown). In the presence of 850 μg IgG per ml, elongation occurred, but processing was virtually undetectable. In addition, a clear band of P1234 was present. With 550 μg of IgG per ml some processing occurred, whereas at 300 μg/ml processing was extensive but still less than than that in the control. These data are consistent with the hypothesis that nsP2 is the nonstructural proteinase.

**Fig. 4.** Inhibition of processing by antisera to the nonstructural proteins of Sindbis. (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 lgG. The final concentration of IgG in the translation mix is shown.

Fig. 4A

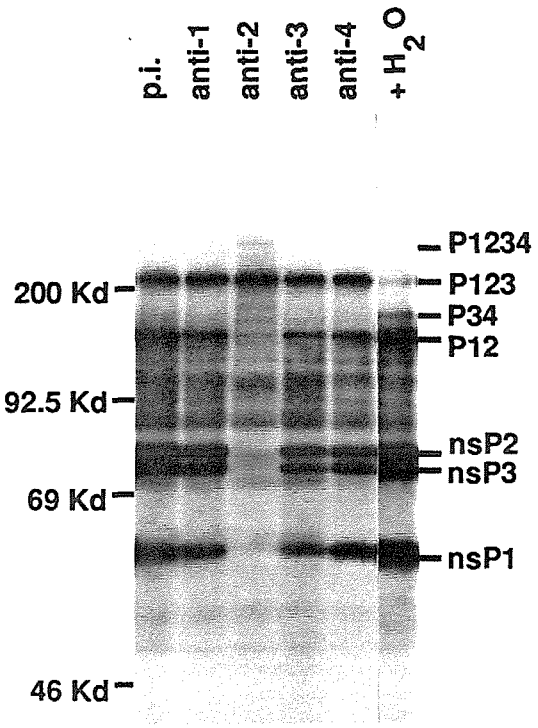
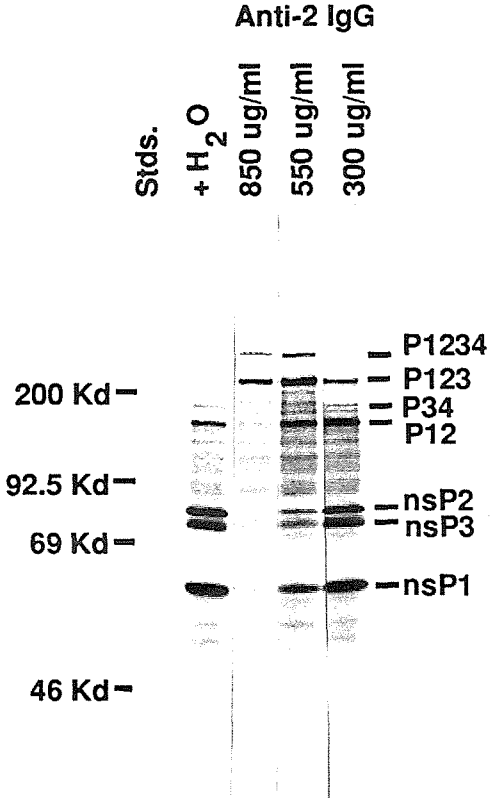

p.i.
anti-1
anti-2
anti-3
anti-4
+ $H_2O$
200 Kd
92.5 Kd
69 Kd
46 Kd
P1234
P123
P34
P12
nsP2
nsP3
nsP1

Fig. 4B

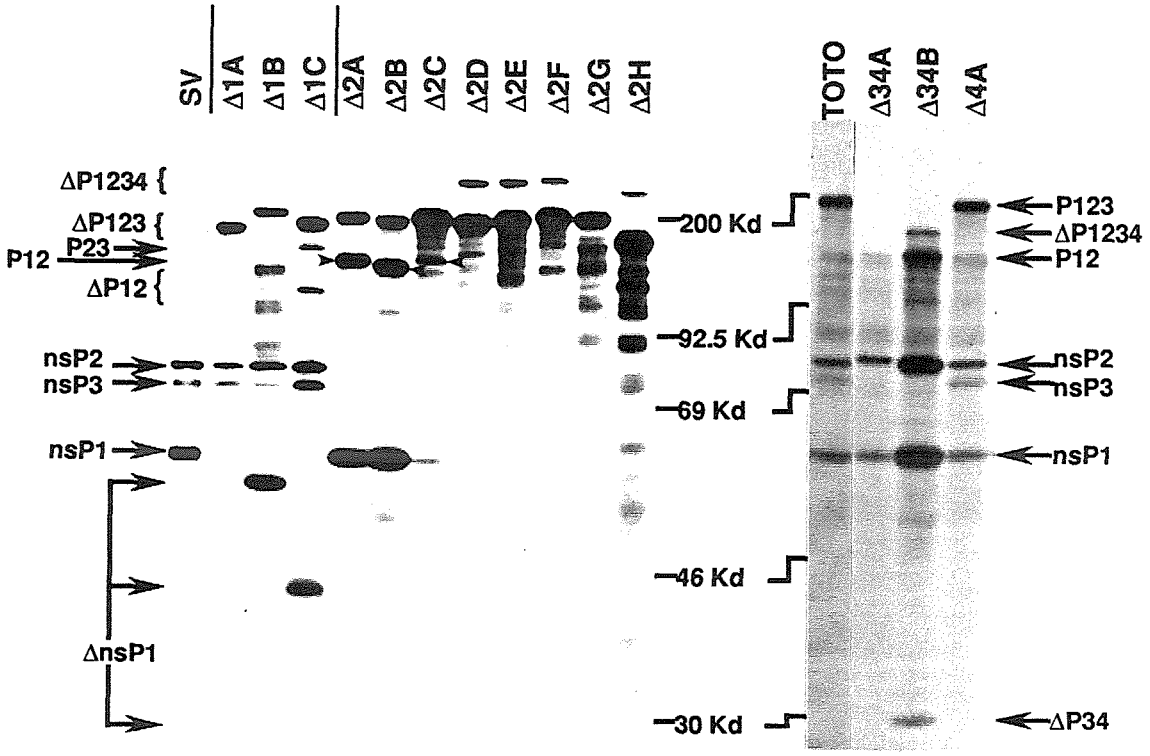

Anti-2 IgG
Stds.
+ $H_2O$
850 ug/ml
550 ug/ml
300 ug/ml
200 Kd
92.5 Kd
69 Kd
46 Kd
P1234
P123
P34
P12
nsP2
nsP3
nsP1

**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 nsPl, were 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 nsPl deletion constructs and was 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 more slowly than nsP2, consisting of nsP2 plus the N-terminal 15 amino acids from nsP3. Note that even in this case the site between the nsPl 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 nsPl and the N terminus of nsP3, are required for proteolytic processing.

**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, 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 Fig. 1.


SV
Δ1A
Δ1B
Δ1C
Δ2A
Δ2B
Δ2C
Δ2D
Δ2E
Δ2F
Δ2G
Δ2H
TOTO
Δ34A
Δ34B
Δ4A
ΔP1234
ΔP123
P12
P23
ΔP12
nsP2
nsP3
nsP1
ΔnsP1
200 Kd
92.5 Kd
69 Kd
46 Kd
30 Kd
P123
ΔP1234
P12
nsP2
nsP3
nsP1
ΔP34

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 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, led 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 that 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, that contained 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 products. For these clones the cleavage of ΔP123 was complete in the presence of added viral proteins and nsP1, ΔnsP2, and nsP3 were produced. 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, not by a proteinase present in the reticulocyte lysate.

**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. A was then incubated for an additional 30 min (control B), and C was incubated for an additional 90 min (control D), at 30°C. Electrophoresis was as in Fig. 2.

Fig. 6A

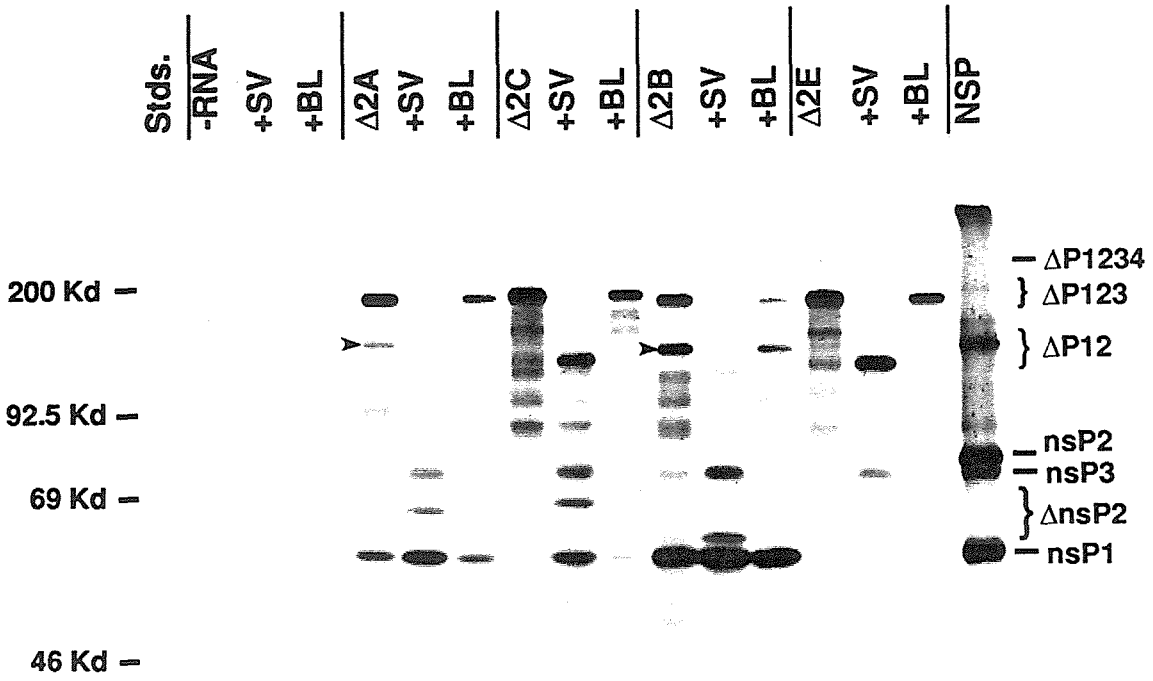
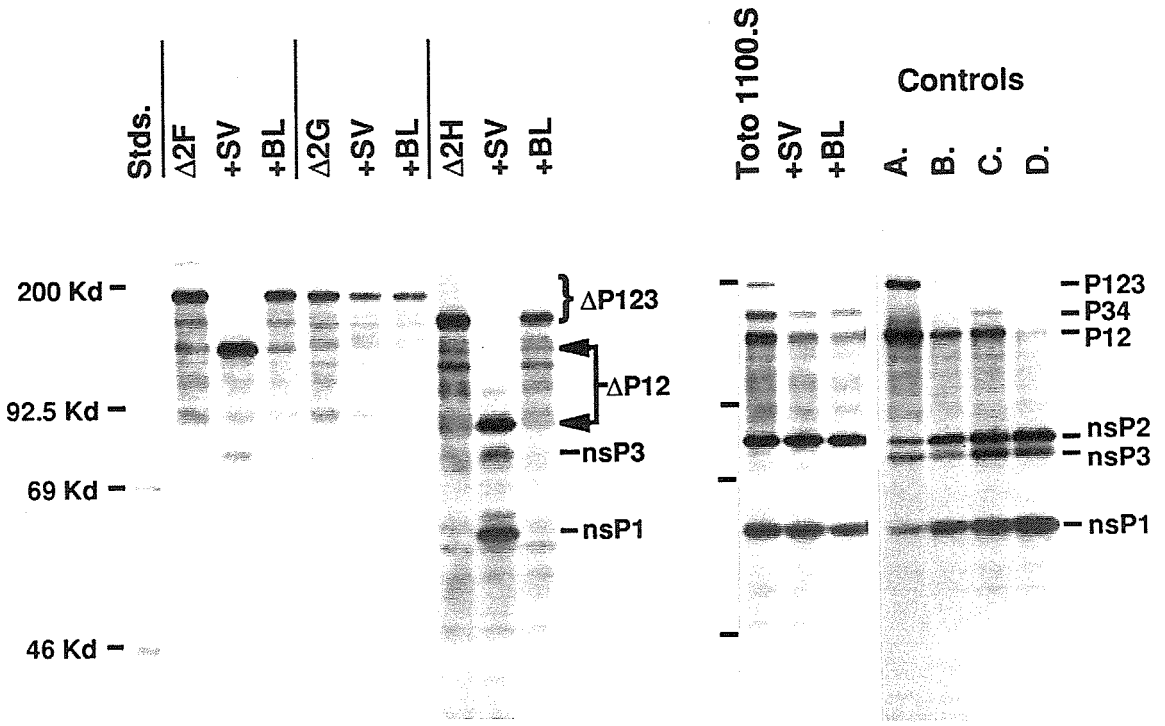

Stds.
-RNA
+SV
+BL
Δ2A
+SV
+BL
Δ2C
+SV
+BL
Δ2B
+SV
+BL
Δ2E
+SV
+BL
NSP
200 Kd
92.5 Kd
69 Kd
46 Kd
ΔP1234
ΔP123
ΔP12
nsP2
nsP3
ΔnsP2
nsP1

Fig. 6B

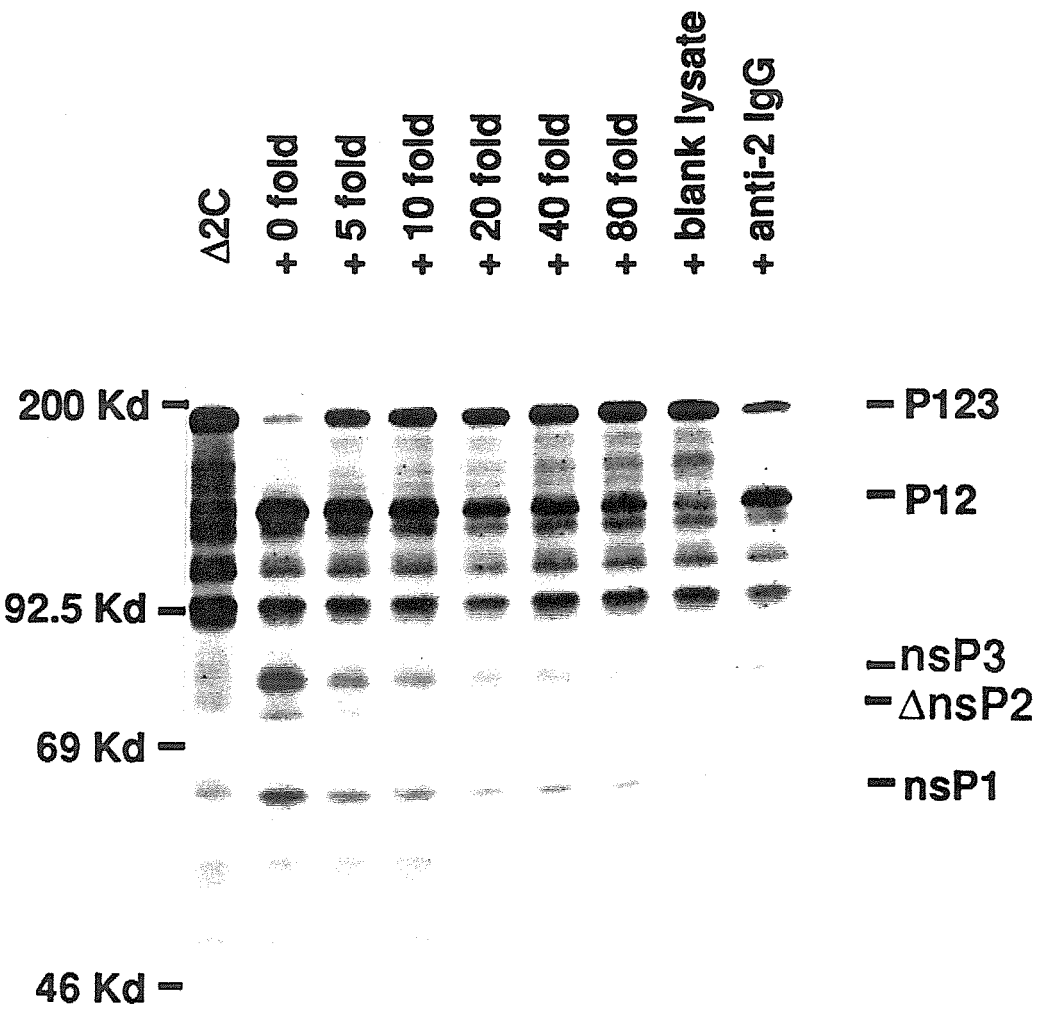

Stds.
Δ2F
+SV
+BL
Δ2G
+SV
+BL
Δ2H
+SV
+BL
200 Kd
92.5 Kd
69 Kd
46 Kd
ΔP123
ΔP12
nsP3
nsP1
Toto 1100.S
+SV
+BL
Controls
A.
B.
C.
D.
P123
P34
P12
nsP2
nsP3
nsP1

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 proteinase 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 which *cis*-processing still occurred. First, processing in *trans* was examined by incubating one (or more) 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 5-fold 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

**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 with either 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 an equal volume of blank lysate. In the last lane the unlabeled nonstructural proteins were preincubated on ice for 5 min with anti-nsP2 lgG 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.


Δ2C
+ 0 fold
+ 5 fold
+ 10 fold
+ 20 fold
+ 40 fold
+ 80 fold
+ blank lysate
+ anti-2 IgG
200 Kd
92.5 Kd
69 Kd
46 Kd
P123
P12
nsP3
ΔnsP2
nsP1

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 were present but most of the radioactivity was 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 nsPl 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 was in nsPl and nsP2 sequences (see the 26-min time point in Fig. 2). In a similar experiment to examine 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

**Fig. 8.** Effect of dilution upon processing of Sindbis 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 mixtures were 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. no effect of dilution upon the processing of P12 could be detected (data not shown).

Fig. 8A

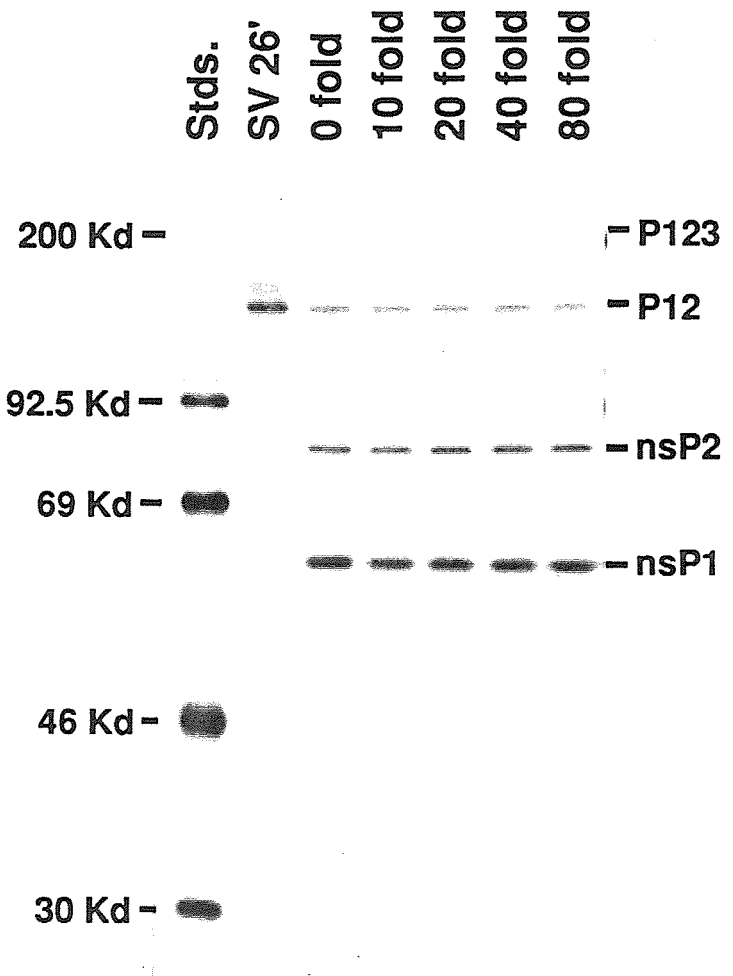
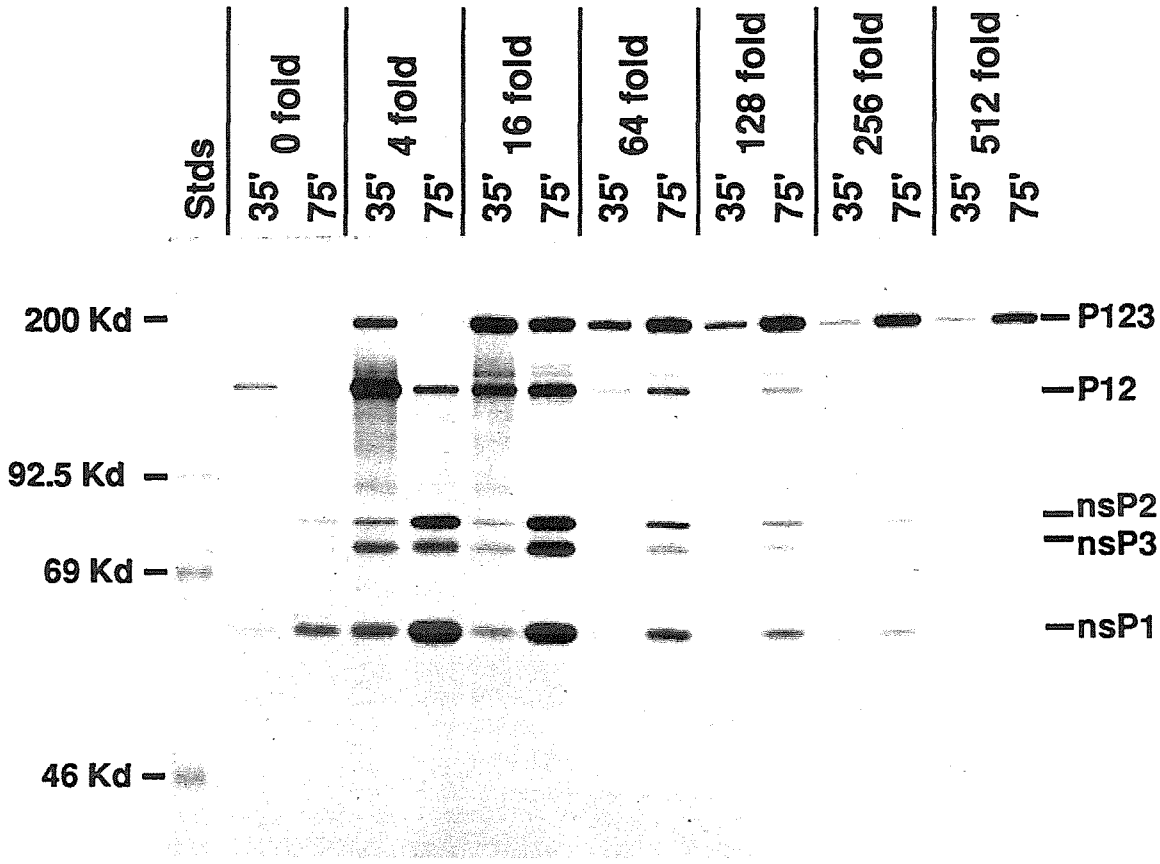

Stds.
SV 26'
0 fold
10 fold
20 fold
40 fold
80 fold
200 Kd
92.5 Kd
69 Kd
46 Kd
30 Kd
P123
P12
nsP2
nsP1

Fig. 8B


Stds
0 fold
4 fold
16 fold
64 fold
128 fold
256 fold
512 fold
35'
75'
200 Kd
92.5 Kd
69 Kd
46 Kd
P123
P12
nsP2
nsP3
nsP1

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 one 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, 43μg/ml, which was 3-fold 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. 8a). 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 the 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 nsPl 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. (20) 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 importantly 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 mutations 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 *Puu*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.**

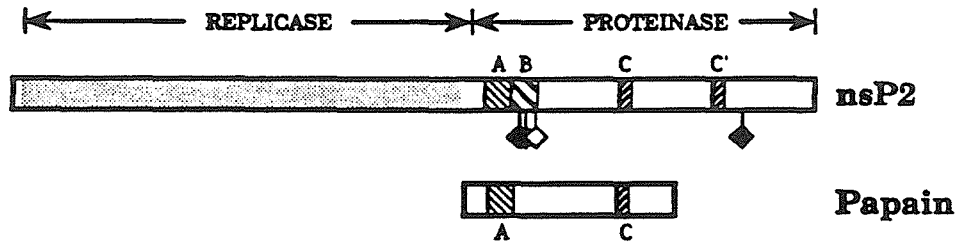
Recently, Ding and Schlesinger (13) proposed that nsP2 may be a metalloproteinase. We suggest, however, that it may be a thiol proteinase. First, $Zn^{2+}$ inhibits the processing of alphavirus precursors (6), which is a

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 $N^{\alpha}$-tosyl-L-lysine chloromethyl ketone (TLCK), 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 the 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

**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 that 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 that appears above the papainlike proteases is that of papain (12), while that for the alphavirus sequences is 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).




A

| | 470 | * | 480 | 490 | 500 |
|---|---|---|---|---|---|
| Papain | QGSCGS | CWA | FSA | | |
| Bromelain | .NP..A | ..G | .A. | | |
| Cathepsin B | ..... | ... | .G. | | |
| Cathepsin H | ..A... | ..T | ..T | | |
| Calpain | ..AL.D | ..L | LA. | | |
| SIN | SCKTNV | CWA | KALEPILATAGIVLTGC | | |
| SF | QN.A.. | ... | .S.V.V.D....R..AE | | |
| RR | QN.AK. | ... | .C.VQV.E....RM.AE | | |
| ONN | QN.A.. | ... | .C.V...D....K.SDR | | |
| VEE | QN.A.. | ... | ...V.V.K....DM.TE | | |

B

| | 510 | 520 |
|---|---|---|
| | ts 18 L | ts 17 T ts 7 N |
| SIN | QWSELFPQFADDKPHSAIYALDVICI | |
| SF | E..TIITA.KE.RAY.PVV..NE..T | |
| RR | E.DT-VLA.RE.RAY.PEV..NE..T | |
| ONN | ...QIVQA.KE.RAY.PEV..NE..T | |
| VEE | ..NT-VDY.ET..A...EIV.NQL.V | |

C

| | 155 | * | 165 |
|---|---|---|---|
| Papain | G---NKVD | HA | VAAVGY |
| Bromelain | K---D.LN | .. | .T.I.. |
| Cathepsin B | D---VMGG | .. | IRIL.W |
| Cathepsin H | HKTPD..N | .. | .L.... |
| Calpain | TFKKLVKG | .. | YSVTAF |

| | 615 | * | 625 |
|---|---|---|---|
| SIN | VPVNRNLP | HA | LVPEYK |
| SF | I.I..R.. | .. | ..A... |
| RR | L.S..R.. | .. | ..TS.Q |
| ONN | I.A..R.. | .S | ..A.HH |
| VEE | .....R.. | .. | ..L-HH |

C'

| | 705 | * | 715 |
|---|---|---|---|
| SIN | HHFQQCED | HA | ATLKTL |
| SF | ..Y...V. | .. | MK.QM. |
| RR | ..Y...V. | .S | MK.QM. |
| ONN | ..Y...V. | .. | MK.QM. |
| VEE | ..Y...E. | .. | IK.SM. |
| MID | ..Y...E. | .. | MK.QM. |

that show similarities in sequence to the 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 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 nsPl, 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 to ~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 (see 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, 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 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 to note 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 regulate replication differentially 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 18 concentrations of nsP4 are required or simply tolerated for efficient replication of some alphaviruses but not others is unknown.

## ACKNOWLEDGMENTS

We are grateful to E. Strauss for many helpful discussions and for considerable assistance in preparation of the manuscript, and to M. Schlesinger and F. Bazan for furnishing manuscripts before publication. This work was supported by Grants A110793 and AI20612 from NIH and Grant DMB8617372 from NSF.

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# Chapter 3

## Synthesis and Processing of the Nonstructural Polyproteins of Several Temperature-Sensitive Mutants of Sindbis Virus

This chapter is being prepared for submission.

# Synthesis and Processing of the Nonstructural Polyproteins of Several Temperature-Sensitive Mutants of Sindbis Virus

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## ABSTRACT

We have examined the synthesis and processing of nonstructural polyproteins by several temperature-sensitive mutants of Sindbis virus, representing the four known RNA-minus complementation groups. Four mutants that possess mutations in the C-terminal domain of nonstructural protein nsP2 all demonstrated aberrant processing patterns when cells infected with these mutants were shifted from a permissive to a nonpermissive temperature. Mutants *ts*l7, *ts*18, and *ts*24 showed a severe defect in processing of nonstructural polyproteins at the nonpermissive temperature, whereas *ts*7 showed only a minor defect in processing. Other mutations tested, including *ts*21, which is found in the N-terminal half of nsP2, *ts*11, which has a mutation in nsP1, and *ts*6, which has a mutation in nsP4, all demonstrated nonstructural polyprotein processing indistinguishable from that in wild-type-infected cells. These results support our conclusion based upon deletion mapping studies, that the C-terminal domain of nsP2 contains the nonstructural proteinase activity.


## INTRODUCTION

The type alphavirus, Sindbis virus, has a single strand RNA genome of 11.7 kB whose sequence is known (22). During infection this plus-strand RNA serves as a template for the transcription of full-length minus-strand RNA, which in turn serves as a template for the synthesis of both 26S subgenomic mRNA and additional genome-length RNA (reviewed in 23, 24). The subgenomic mRNA encodes the structural proteins of the virion, which include the capsid protein and two envelope glycoproteins, whereas the nonstructural proteins of the virus, which are presumed to be involved in viral RNA replication, are translated as two large polyprotein precursors

from the 5' terminal two-thirds of the genomic RNA. The smaller and more abundant polyprotein (~ 200 kDa) contains the sequences of nsP1, nsP2 and nsP3, while the larger precursor (~ 250 kDa), which is produced in smaller quantities by readthrough of an opal codon at the end of nsP3, contains, in addition, the sequence of the fourth nonstructural protein, nsP4 (8, 13, 21). Processing of these precursors occurs by a proteolytic activity within the C-terminal half of nsP2 (9).

Large numbers of temperature-sensitive (*ts*) RNA-minus mutants of Sindbis virus, which are defective in viral RNA synthesis at the nonpermissive temperature, have been isolated and assigned by complementation to four groups (A, B, F and G) (4, 5, 20). Recently, the amino acid changes responsible for the *ts* phenotype of members of each group have been identified within the viral nonstructural proteins, and from this mapping some of the functions of these proteins have been deduced (6, 7). The only mutant in group B, *ts*11, has a mutation in nsP1, implicating this protein in the initiation of minus-strand synthesis (7). This protein has also been implicated in methylation of the 5' terminal cap structure on virion RNAs (14). Three group F mutants have been mapped to nsP4 (6). The best characterized member, *ts*6, fails to synthesize any RNA upon shift from permissive to nonpermissive conditions (2, 11, 18) and this, together with the presence of the GDD motif found in a number of viral polymerases (10, 16), has led to the hypothesis that nsP4 is the major RNA polymerase of Sindbis virus.

Finally, members of group G (*ts*18 and *ts*7) and group A (*ts*17, *ts*21 and *ts*24) have all been found to map in nsP2 and thus the original complementation found between these groups was intracistronic (7). From the phenotypes of these various mutants, nsP2 has been implicated in the

initiation of 26S mRNA synthesis, the regulation of minus-strand synthesis, and the proteolytic processing of nonstructural polyproteins (7, 11, 17, 18, 19).

Previously, we have studied the kinetics of synthesis and processing of the nonstructural proteins of Sindbis virus using monospecific antibodies both *in vivo* (8) and *in vitro* (9). It seemed important at this point to use these monospecific antibodies to reexamine processing of the nonstructural proteins in cells infected with *ts* mutants whose lesions have been mapped to specific proteins. Furthermore, in the process of mapping, the *ts* lesions have been rescued into an otherwise uniform background, making it more possible to rule out the contributions of other changes in the genome to any aberrant processing observed. These results have proven useful not only for mapping *ts* lesions in the nonstructural proteinase but also for elucidating the processing pathways of the nonstructural polyproteins.

## MATERIALS AND METHODS

### Cell Cultures and Viruses:

The heat-resistant, small-plaque strain of Sindbis virus (SIN HR) and temperature-sensitive mutants *ts*6, *ts*7, *ts*11, *ts*17, *ts*18, *ts*21, and *ts*24 (4, 5) have been described (20). Virus stocks were prepared in primary chick embryo fibroblasts (15). The construction of hybrid viruses in which the *ts* lesion of different mutants was rescued into an otherwise uniform background (Toto1101) has been described (6, 7). The clones used in this work were Toto:*ts*17B1, Toto:*ts*18B1, and Toto:*ts*24B1, as well as *ts*17R, *ts*18R, and *ts*24R, the corresponding clones of revertants of each mutant.

**Labeling of Infected Cells**

Duplicate plates (60 mm or 100 mm) of confluent BHK 21 cells were infected at 30°C at a multiplicity of 50pfu/cell with SIN HR, a *ts* mutant, or a hybrid virus, as previously described (8). After 70 min the inoculum was removed and replaced with Eagle's Minimal Essential Medium containing 10% dialyzed fetal calf serum, 1 μg/ml actinomycin D, and 1/20 the normal concentration of methionine. At 5 hr postinfection this medium was removed and replaced with the same medium prewarmed to 30°C or 40°C and incubation was continued at 30°C and 40°C, respectively. One hour later the medium was removed and cells were labeled for 30 min in Eagle's Medium lacking methionine but containing 40 μCi/ml of [$^{35}$S]methionine (>800 Ci/mM, Amersham Corp.). Cell lysates were prepared as described (8) and stored at -70°C.

**SDS Polyacrylamide Gel Electrophoresis**

SDS polyacrylamide gel electrophoresis was performed on slab gels containing either 7.5% or 10% (wt/v) acrylamide (acrylamide to bisacrylamide ratio 30:0.4 wt/wt) and the gels were fixed and prepared for fluorography as described (8).

**Immunoprecipitation**

Whole cell lysates were prepared and immunoprecipitated as described (8).

## RESULTS

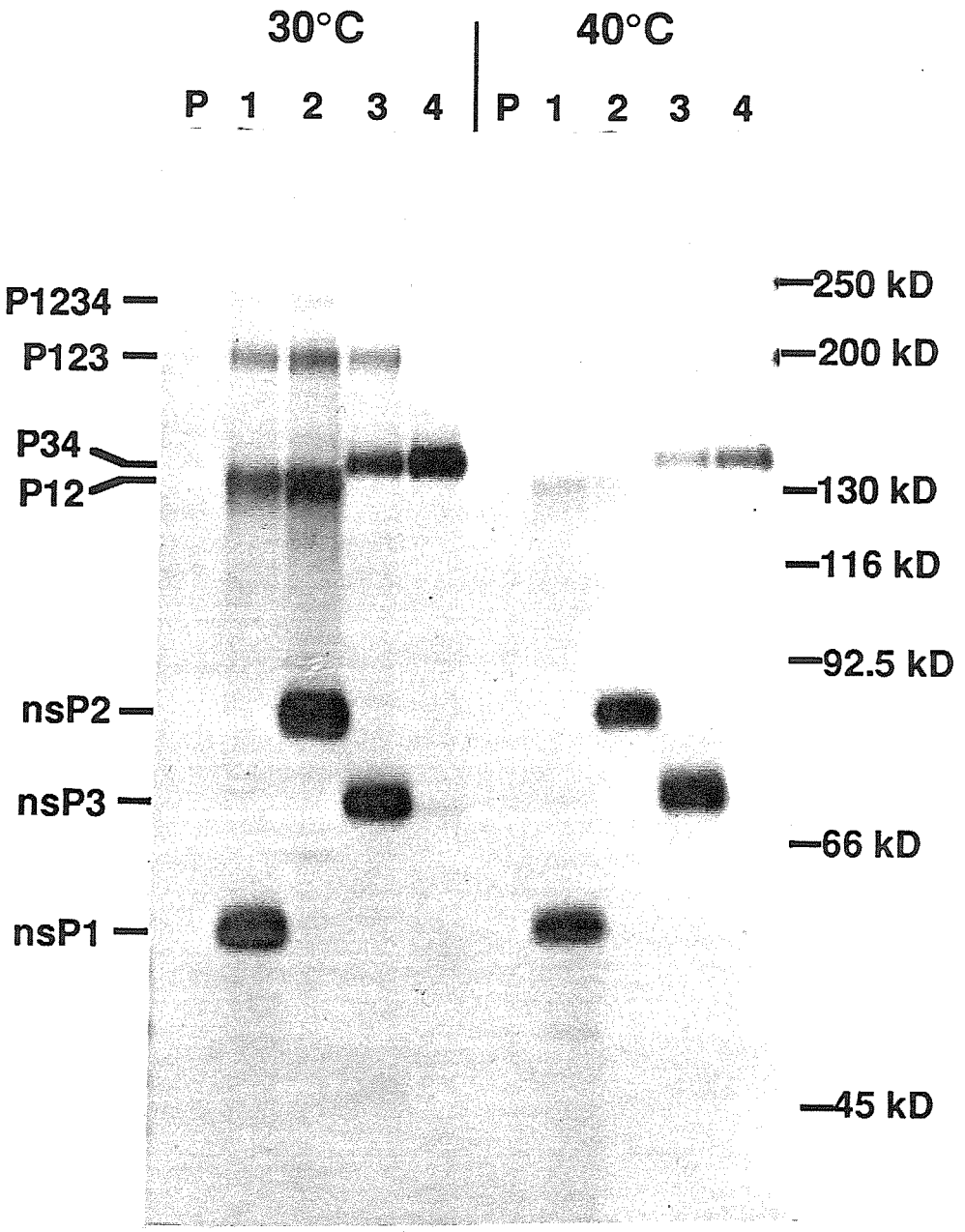
### Mutations in nsP1 and nsP4

Cells were infected with *ts*6, whose *ts* lesion is glycine-153 to glutamic acid in nsP4, with *ts*11, whose lesion is alanine-348 to threonine in nsP1, or with SIN HR, and labeled with [$^{35}$S]methionine at 30°C or 40°C, following a shift from 30°C. Labeled proteins were immunoprecipitated with antisera monospecific for nsP1, 2, 3, or 4 and the labeled proteins examined by polyacrylamide gel electrophoresis. In general, there was less labeled methionine incorporated into nonstructural proteins, and processing of the nonstructural polyproteins occurred more rapidly at 40°C than at 30°C. Otherwise, the protein patterns at either temperature were qualitatively similar (Fig. 1), consistent with previous findings (11, 13). These results are also consistent with our previous results, that the deletion of domains in nsP1 or nsP4 does not affect proteolytic processing *in vitro* (9).

We were particularly interested in the results with *ts*11 because Waite (1973) had previously reported that a polypeptide of approximately 133 kDa accumulated in cells infected with *ts*11 at the nonpermissive temperature and comigrated with the uncleaved structural polyprotein from *ts*13-infected cells (25). No evidence for such a band was seen in the patterns of the immunoprecipitated nonstructural proteins. To determine whether this polyprotein might represent an uncleaved structural precursor, we immunoprecipitated proteins from *ts*11-infected cells, labeled at the nonpermissive temperature, with antisera to the structural proteins C, E1 and PE2, and precipitated a polypeptide of approximately 133 kDa. Thus, it appears that *ts*11 also has a lesion in the capsid proteinase which leads to the accumulation of the structural polyprotein, but which is not dominant ,

**Fig. 1.** Fluorograms of immunoprecipitated nonstructural proteins from cells infected with SIN HR, *ts*6, or *ts*11. Duplicate monolayers of BHK cells were infected with 50 pfu/cell for 70 minutes at 30°C. At 5 hours post-infection, medium prewarmed to either 30°C or 40°C was added and the cultures were returned to 30°C, or shifted to 40°C, respectively. At 6 hours postinfection monolayers were labeled at either 30°C or 40°C for 30 minutes in medium containing [$^{35}$S]methionine, after which the cells were lysed and prepared for immunoprecipitation. Immunoprecipitations were carried out using antiserum specific for each nonstructural protein of Sindbis virus or preimmune serum (**P**), and the protein patterns were analyzed on 7.5% discontinuous SDS-polyacrylamide gels. A) SIN HR. B) *ts*6. C) *ts*11.

Fig. 1A


30°C
40°C
P 1 2 3 4
P 1 2 3 4
P1234
P123
P34
P12
nsP2
nsP3
nsP1
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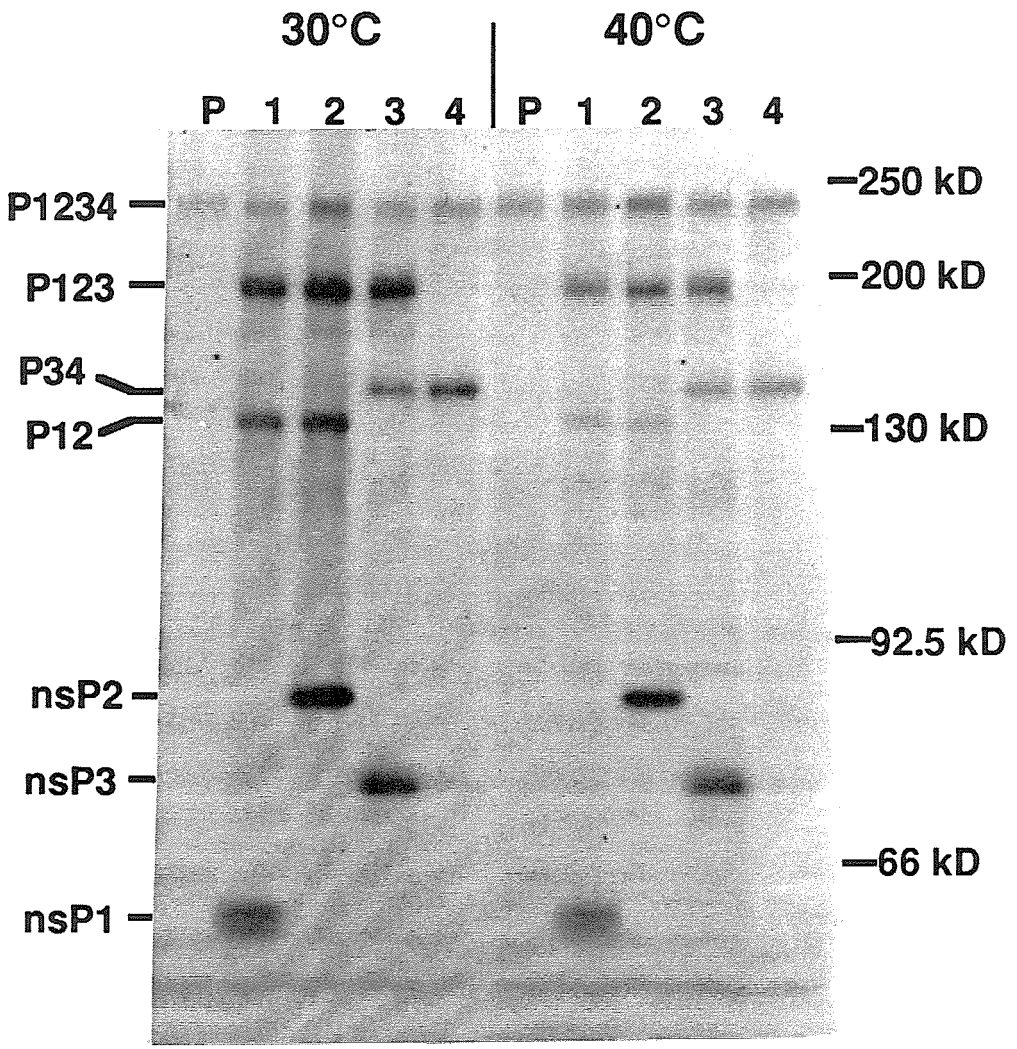
200 kD
130 kD
116 kD
92.5 kD
66 kD
45 kD

Fig. 1B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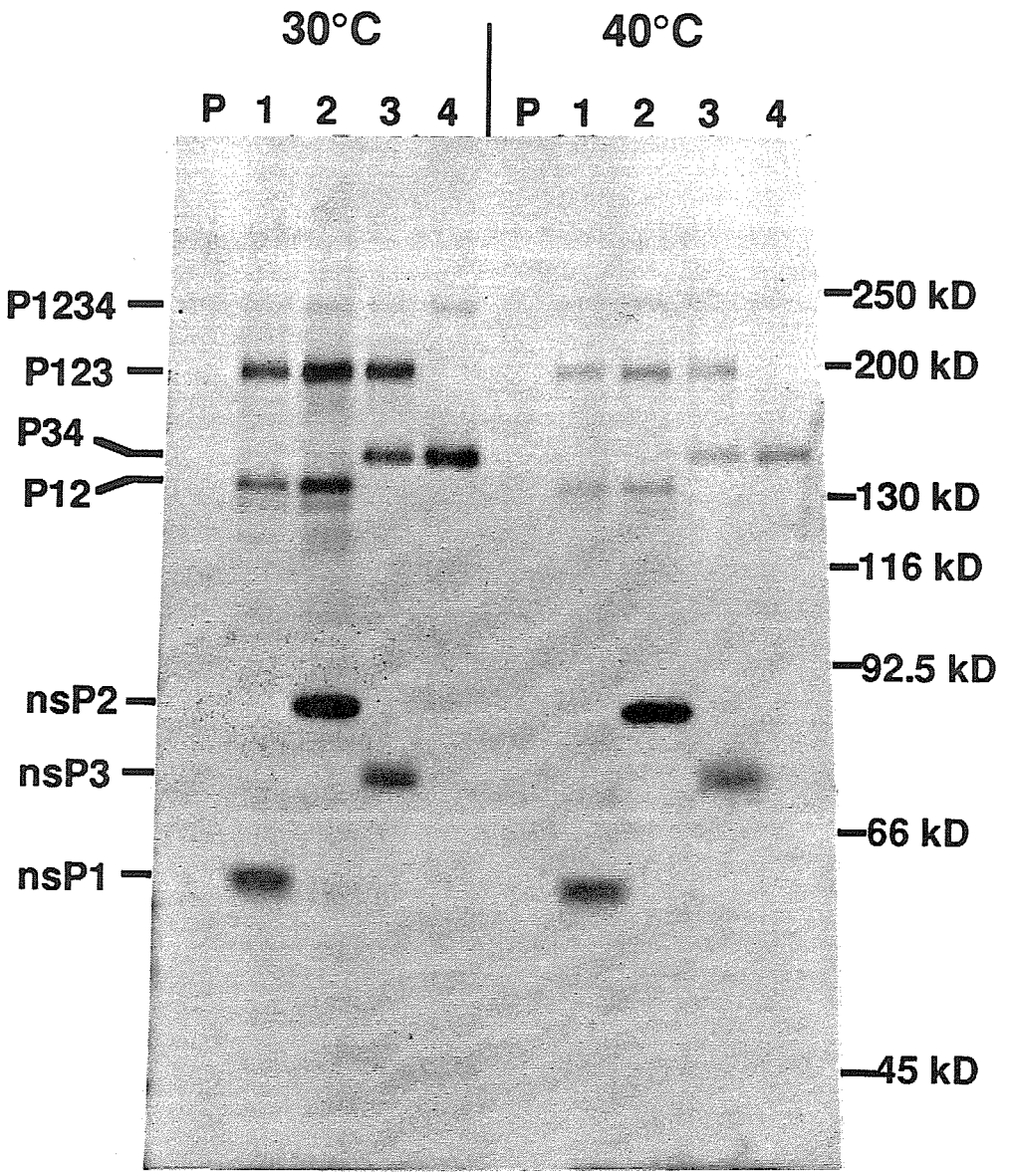
40°C
P 1 2 3 4 P 1 2 3 4
P1234
P123
P34
P12
nsP2
nsP3
nsP1
250 kD
200 kD
130 kD
92.5 kD
66 kD

Fig. 1C


30°C
40°C
P 1 2 3 4 P 1 2 3 4
P1234
P123
P34
P12
nsP2
nsP3
nsP1
250 kD
200 kD
130 kD
116 kD
92.5 kD
66 kD
45 kD

since large amounts of capsid protein and other structural proteins are produced in *ts*11-infected cells at 40°C (data not shown).

**Mutations in nsP2**

Five mutations in nsP2 were examined: *ts*17 (alanine-517 to threonine), *ts*21 (cysteine-304 to tyrosine), and *ts*24 (glycine-736 to serine) of complementation group A; and *ts*7 (aspartic acid-522 to asparagine) and *ts*18 (phenylalanine-509 to leucine) of group G (*ts*7 also has an unrelated mutation in nsP3, phenylalanine-312 to serine) (7). The polyprotein-processing pattern for *ts*21 was identical to that for wild-type (data not shown), consistent with our deletion-mapping results (9), that the N-terminal domain of nsP2 is not required for proteolysis. The remaining four mutations, all found within the C-terminal domain of nsP2, affected the polyprotein patterns observed (Fig. 2). In the case of *ts*7 the effects upon the pattern observed are minor; the large polyprotein precursors P123 and P1234 are processed, but there is an accumulation of P23, which contains the sequences of nsP2 and nsP3. This polyprotein is not found in wild-type-infected cells in which the dominant pathway is cleavage between nsP2 and nsP3 to produce P12 and nsP3, followed by cleavage of P12 into nsP1 and nsP2 (Fig. 1A) (8).

Mutations in *ts*17, *ts*18, and *ts*24 had a more profound effect upon the polyprotein pattern found at the nonpermissive temperature (Fig. 2). All three mutants accumulated high molecular weight precursors, P123 and P1234, and demonstrated reduced amounts of processed precursors P12 and P34, as well as mature nonstructural proteins, in comparison with SIN HR-infected cells at 40°C. Furthermore, these mutants accumulated large amounts of polyprotein P23 at 40°C (this polyprotein of ~175 kDa is not well

**Fig. 2.** Fluorograms of nonstructural proteins immunoprecipitated from cells infected with mutants in nsP2. As in Figure 1, BHK cells were infected with a *ts* mutant at 30°C, and at 5 hours postinfection either kept at 30°C or shifted to the nonpermissive temperature. At six hours post-infection, the cells were labeled in media containing [$^{35}$S]methionine for 30 minutes at either 30°C or 40°C, and harvested. Infected cell lysates were immunoprecipitated with preimmune serum or antiserum to one of the nonstructural proteins and the labeled products separated on 7.5% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis. A) *ts*7. B) *ts*17. C) *ts*18. D) *ts*24.

Fig. 2A

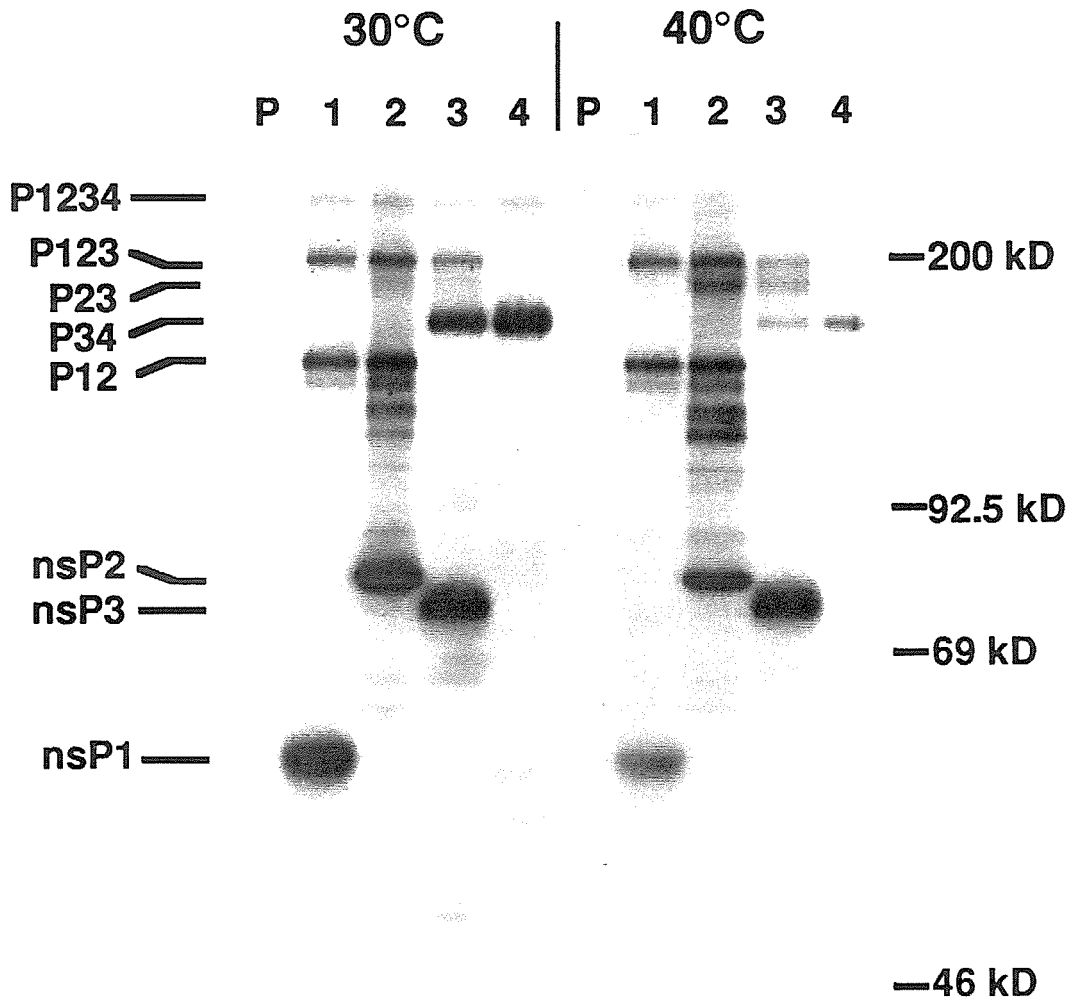

30°C
40°C
P 1 2 3 4 P 1 2 3 4
P1234
P123
P23
P34
P12
nsP2
nsP3
nsP1
200 kD
92.5 kD
69 kD
46 kD

Fig. 2B

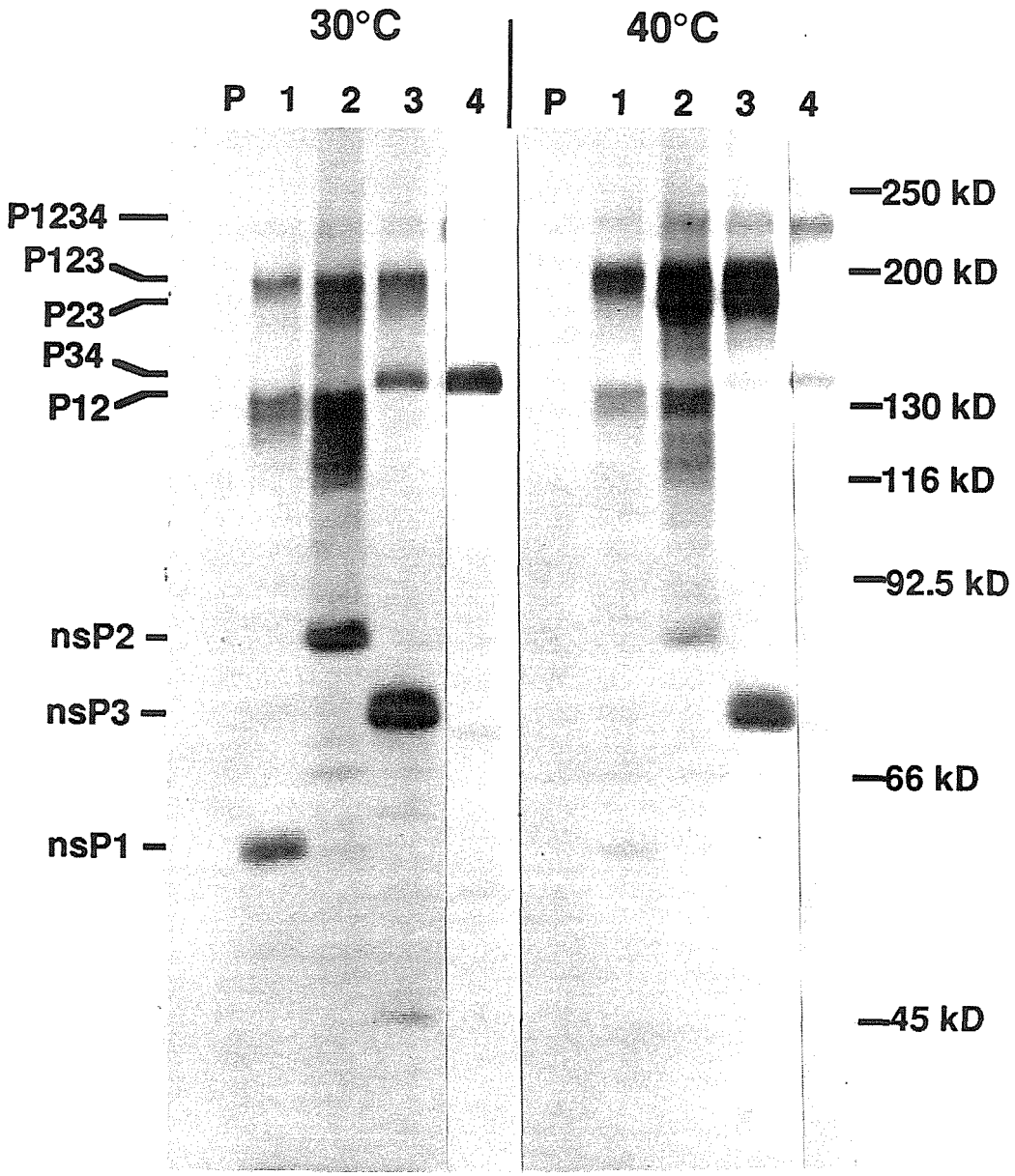

30°C
40°C
P 1 2 3 4
P 1 2 3 4
P1234
P123
P23
P34
P12
nsP2
nsP3
nsP1
250 kD
200 kD
130 kD
116 kD
92.5 kD
66 kD
45 kD

Fig. 2C

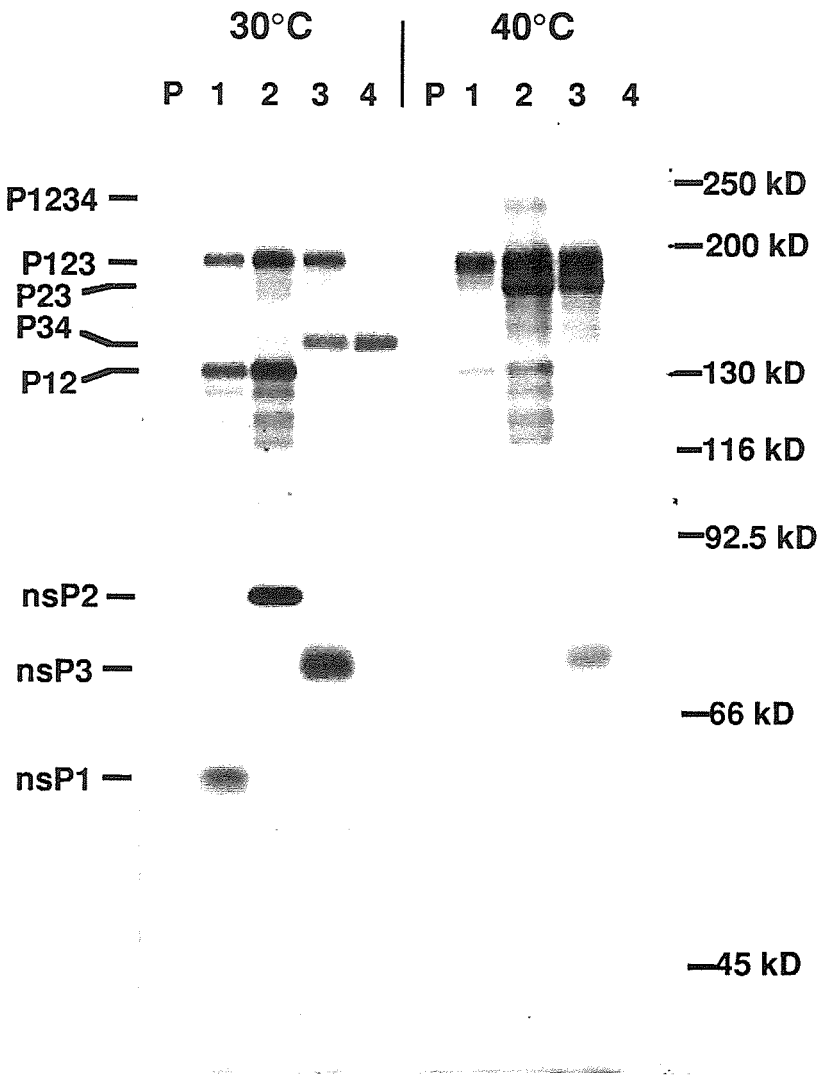
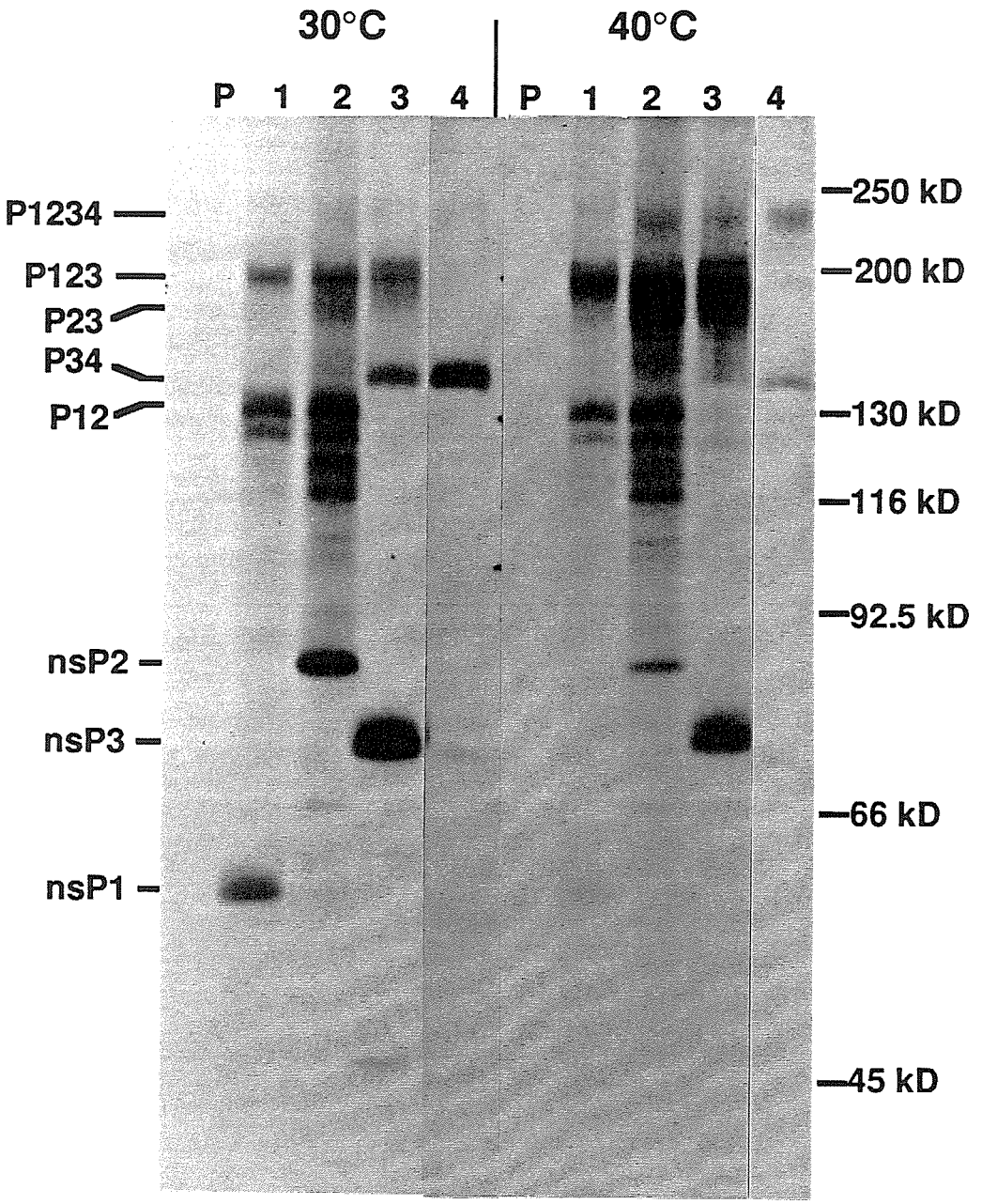

30°C
40°C
P 1 2 3 4 P 1 2 3 4
P1234
P123
P23
P34
P12
nsP2
nsP3
nsP1
250 kD
200 kD
130 kD
116 kD
92.5 kD
66 kD
45 kD

Fig. 2D


30°C
40°C
P 1 2 3 4 P 1 2 3 4
P1234
P123
P23
P34
P12
nsP2
nsP3
nsP1
250 kD
200 kD
130 kD
116 kD
92.5 kD
66 kD
45 kD

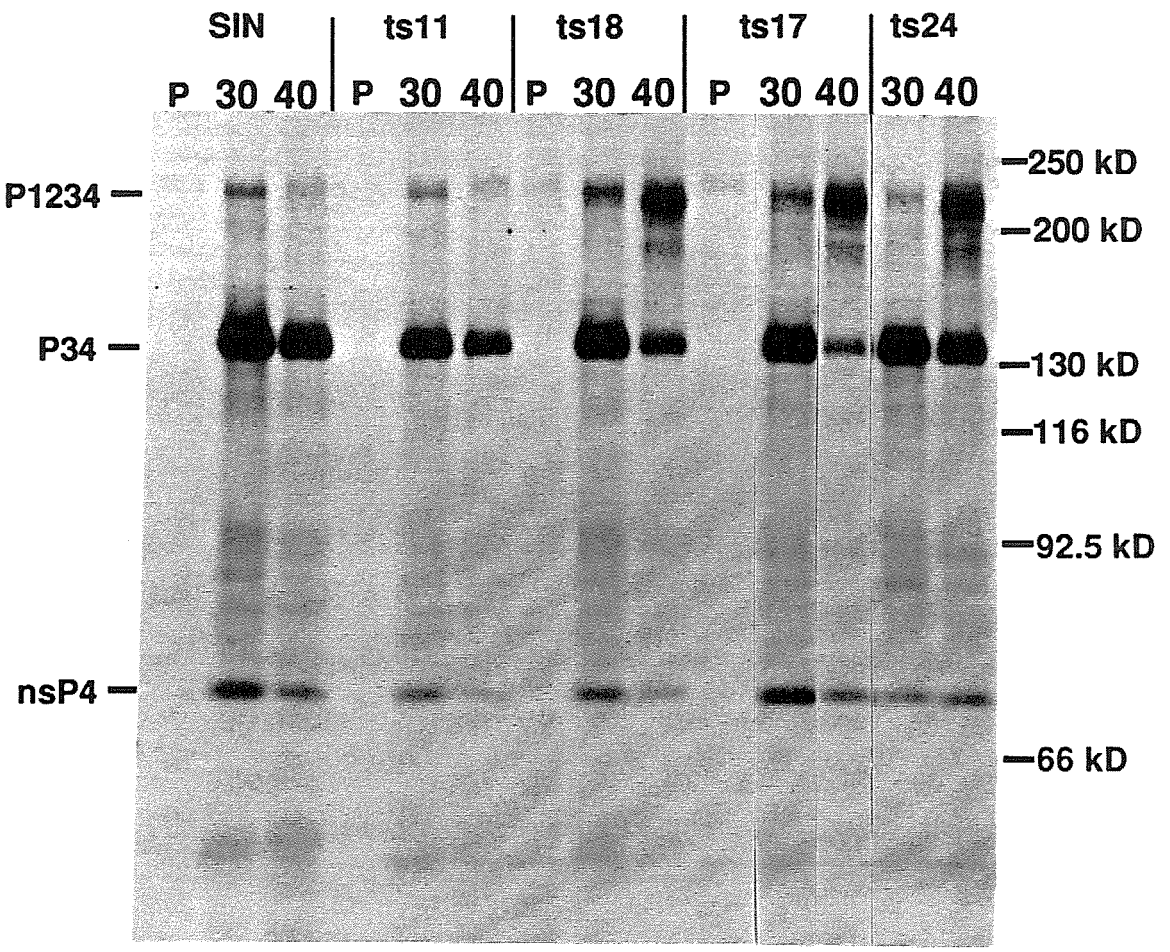
resolved in the patterns of *ts*17 and *ts*24). However, the fact that very little nsP1 is detected, much less than would be expected from the amount of P23 present, suggests that the turnover of nsP1 is abnormally high under these conditions. A second possibility is that cleavage to produce the P23 precursor is abnormal and that the nsP1 released is either unstable or less reactive with the antiserum to nsP1.

The amount of nsP3 found in these protein patterns is also puzzling. Although the major polypeptides found are P123 and P23, significant amounts of nsP3 accumulate, more so in *ts*24 and *ts*17 than in *ts*18. From the amounts of nsP3 present we would expect corresponding amounts of either P12 or the cleaved products nsP1 and nsP2, which are not seen. It is possible that nsP1, as discussed above, and nsP2 are both unstable and are degraded. Since the ts lesion is in nsP2, abnormal folding of this protein could lead to instability, but the situation for nsP1 is less clear. It seems possible that the amounts of some of the Sindbis nonstructural proteins are regulated (by the proteinase?) and that the abnormal nsP2 leads to more rapid turnover of both nsP1 and nsP2.

From the large amounts of P23 present and the significant amounts of nsP3 present it seems clear that the mutant proteinase is still able to cleave both the 1-2 and the 2-3 bonds. However, the processing pathways are altered quantitatively, if not qualitatively.

To examine cleavage of the 3-4 bond more carefully, a larger amount of labeled material was analyzed and longer exposure times were used (Fig. 3). For SIN HR and the *ts* mutants that are not defective in processing, more mature nsP4 and its precursors, P34 and P1234, are present at 30°C than at 40°C. In mutants that are temperature-sensitive for processing,

**Fig. 3.** nsP4 and its precursors in infected cells. For certain *ts* mutants an additional 7.5% discontinuous SDS-polyacrylamide gel was run in which one and one-half times the amount of sample (immunoprecipitated by anti-nsP4 in the above experiment) was loaded per lane. An equivalent amount of lysate from infected cells labeled at 40°C and immunoprecipitated with preimmune serum was run alongside to serve as a control. Because of the small quantity of nsP4 present, a longer exposure time was used.


SIN
ts11
ts18
ts17
ts24
P 30 40 P 30 40 P 30 40 P 30 40 30 40
P1234
P34
nsP4
250 kD
200 kD
130 kD
116 kD
92.5 kD
66 kD

*ts*17, *ts*18, and *ts*24, P1234 accumulates and reduced amounts of P34 are produced at 40°C. Despite this, the relative amount of nsP4 present at 30°C or at 40°C for these mutants is very similar to that found in SIN HR. This suggests either that cleavage between nsP3 and nsP4 occurs to the same extent in the mutants as it does in the wild-type, even though cleavage at the other sites is inhibited to a greater or lesser degree, or that the concentration of nsP4 is regulated at a level other than the rate and extent of proteolytic cleavage from its precursors.

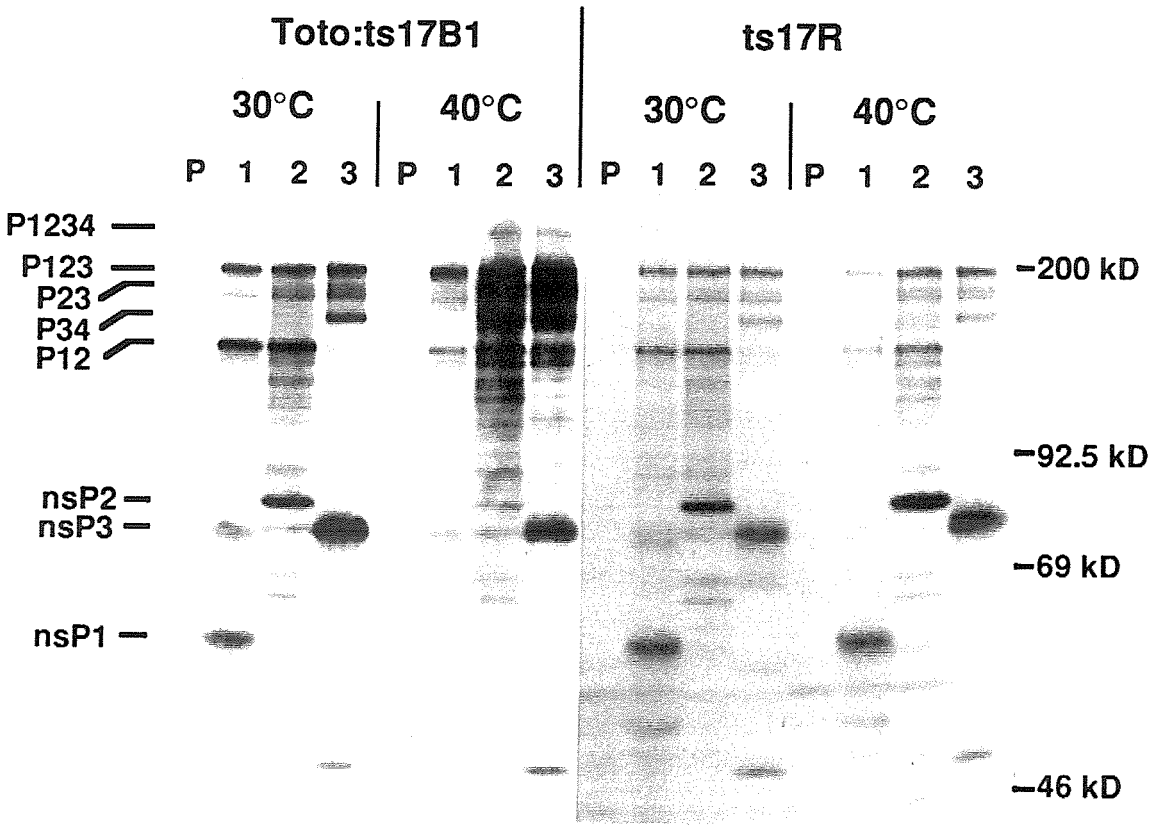
Previously, mutations in *ts*17, *ts*18, and *ts*24 were rescued into a uniform background, Toto1101, as were same-site revertants, *ts*17R, *ts*18R, and *ts*24R, and these hybrid viruses were characterized (7). In order to rule out the possible contribution of unmapped lesions in the viral genome to the aberrant processing patterns we observed, we also examined the polyproteins synthesized and processed by these rescued mutants and revertants (Fig. 4). The results were essentially identical with those obtained with the parental *ts* mutants. In Toto:*ts*17B1, Toto:*ts*18B1, and Toto:*ts*24B1, large amounts of P123 and P23, and larger than wild-type amounts of P1234 are present at 40°C, whereas nsP1 and nsP2 are under-represented. Revertant viruses *ts*17R, *ts*18R, or *ts*24R gave patterns similar to SIN HR. Thus it is clear that the aberrant polyprotein processing observed results from the *ts* lesions mapped in nsP2.

## DISCUSSION

We have examined the processing of nonstructural polyproteins in cells infected with a number of *ts* mutants of Sindbis virus whose lesions have been mapped to specific amino acids in the nonstructural proteins. The use of monospecific antisera allowed the identification of polyproteins present

**Fig. 4.** Nonstructural proteins immunoprecipitated from cells infected with the rescued mutants. BHK cells were infected, labeled and harvested under conditions identical to those used for the parental viruses. Preimmune serum or antiserum to each of the nonstructural proteins of Sindbis was used for the immunoprecipitations and the labeled proteins were resolved on 10% SDS-polyacrylamide gels. A) Toto:*ts*17B1 or *ts*17R. B) Toto:*ts*18B1 or *ts*18R. C) Toto:*ts*24B1 or *ts*24R.

Fig. 4A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ts17R
30°C
40°C
30°C
40°C
P 1 2 3 P 1 2 3 P 1 2 3 P 1 2 3
P1234
P123
P23
P34
P12
nsP2
nsP3
nsP1
200 kD
92.5 kD
69 kD
46 kD

Fig. 4B

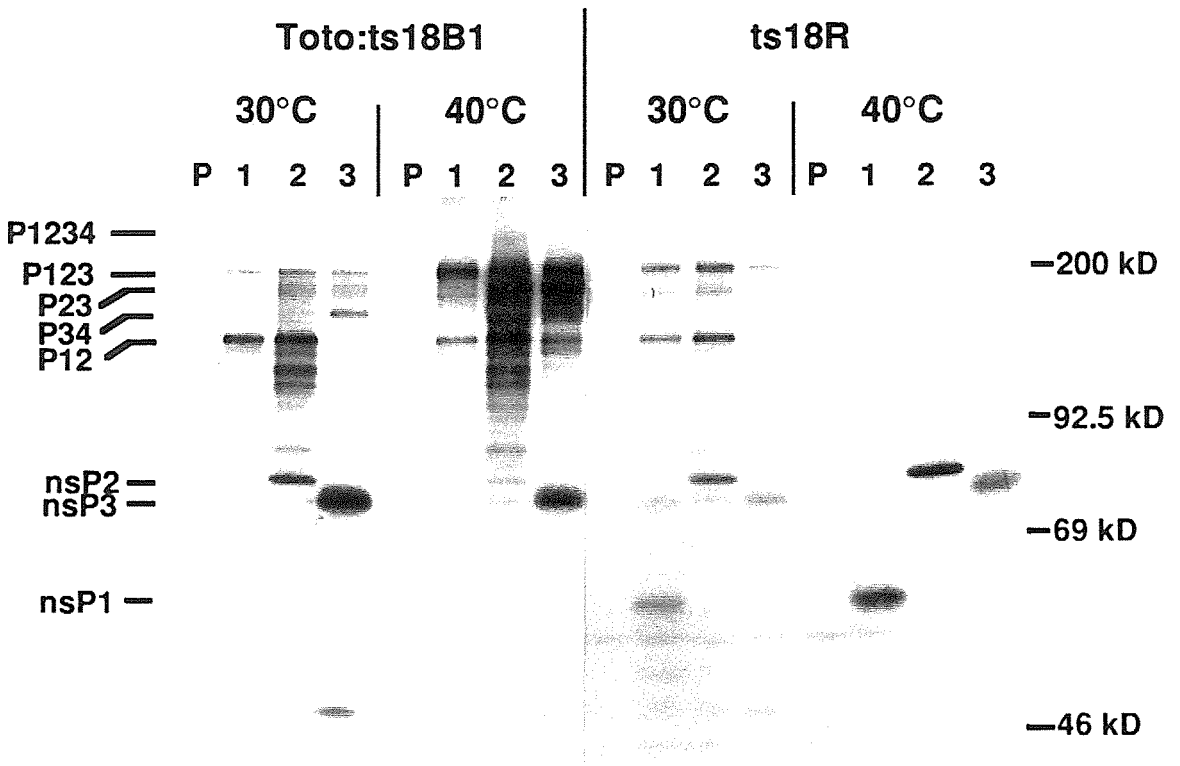
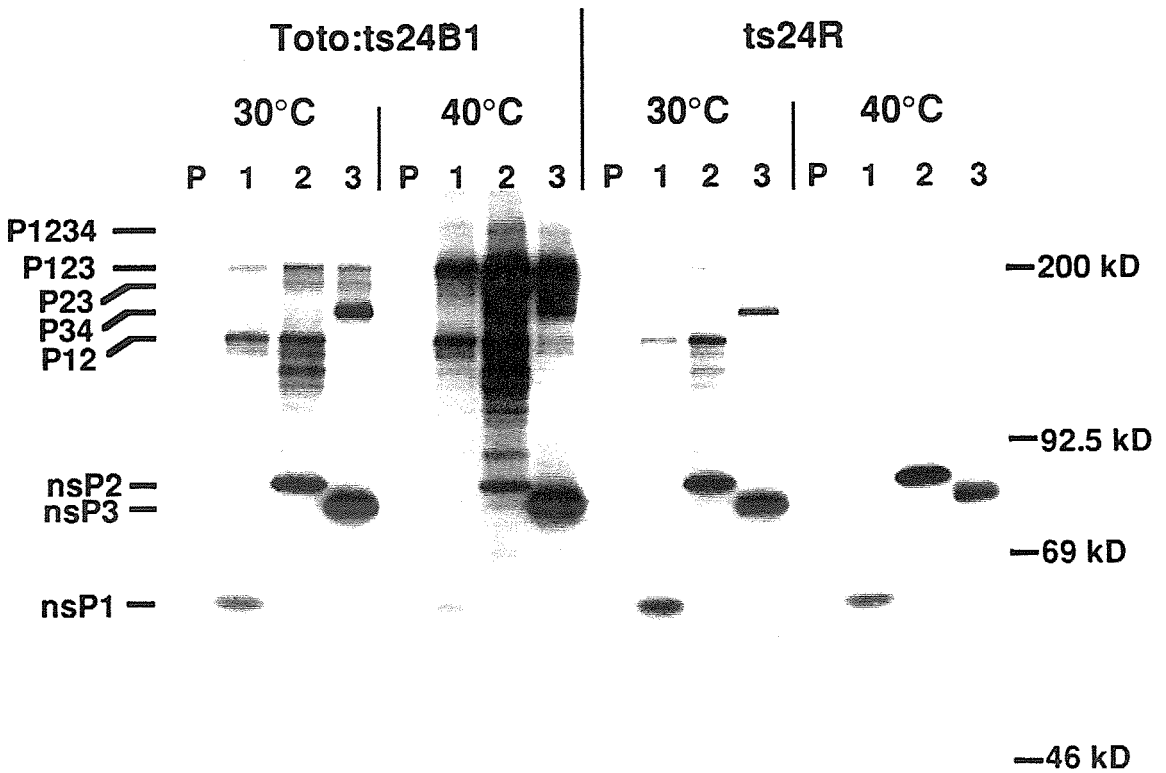

Toto:ts18B1
ts18R
30°C
40°C
30°C
40°C
P 1 2 3 P 1 2 3 P 1 2 3 P 1 2 3
P1234
P123
P23
P34
P12
nsP2
nsP3
nsP1
200 kD
92.5 kD
69 kD
46 kD

Fig. 4C


Toto:ts24B1
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30°C
40°C
30°C
40°C
P 1 2 3 P 1 2 3 P 1 2 3 P 1 2 3
P1234
P123
P23
P34
P12
nsP2
nsP3
nsP1
200 kD
92.5 kD
69 kD
46 kD

on the basis of the sequences they contain rather than simply on the basis of molecular weight and therefore allowed the precise identification of each polyprotein. Furthermore, by using mutants whose lesions had been mapped, and in the case of mutants which affect polyprotein processing severely, the use of hybrid viruses in which these mutations had been transferred into an otherwise uniform background, we have been able to define precisely the *ts* lesions that result in aberrant processing. Lesions in nsP1, nsP4 or in the N terminus of nsP2 did not affect processing, whereas four mutations in the C terminus of nsP2 all affected processing to a greater or lesser extent, consistent with our deletion mapping studies that the nonstructural proteinase is in the C terminus of nsP2 (9).

Bracha et al. (3) had reported that the cleavage of the precursor P123 (nsP230) was temperature-sensitive in *ts*21-infected cells. In this paper we found that polyprotein processing in *ts*21-infected cells was identical to that in SIN HR-infected cells, in agreement with the results of Keränen and Kääriäinen (11) and of Sawicki and Sawicki (17). We have also shown that the polyprotein that accumulates in *ts*11-infected cells at the nonpermissive temperature first reported by Waite (25) results from the failure of structural polyprotein processing rather than of nonstructural polyprotein processing.

The finding that significant amounts of P1234 accumulated in cells infected with mutants *ts*17, *ts*18 and *ts*24 at the nonpermissive temperature is in agreement with previous results in which we found that when sequences essential for proteolytic activity were deleted from the polyprotein or when processing was inhibited with antiserum to nsP2, P1234 was produced in much larger amounts upon translation *in vitro* (9). *In vivo* or *in vitro*, cleavage of P1234 appears to occur very rapidly, probably while the

polypeptide is nascent. Cleavage occurs either at the 2-3 site, leading to the production of P12 and P34, or at the 3-4 site, leading to the production of P123 and nsP4 (which appears to be degraded or lost) (8, 9).

We have found that the level of nsP4 produced by mutants *ts*17, *ts*18 and *ts*24 at the nonpermissive temperature was similar to that in wild-type virus, despite the relative abundance of P1234 and the lack of P34. This could imply either that cleavage of the 3-4 bond is not affected by the *ts* lesion in the proteinase, or that it is inhibited but is masked by some other process that regulates the concentration of nsP4 after its synthesis. For instance, if the concentration of nsP4 was maintained at some steady-state level, such that any excess nsP4 resulting from processing is degraded, a sharp reduction in processing might go unnoticed. Another possibility is that the viral proteinase is responsible for both the processing and the degradation of nsP4 and that while the lesion in nsP2 interferes with processing, it also allows more nsP4 to escape degradation. In this regard it is interesting that in Semliki Forest virus-infected cells, nsP4 appears to be less stable than the other three nonstructural proteins, and this difference is even more pronounced in cells infected with *ts*1, a mutant of Semliki Forest virus, which overproduces the nonstructural proteins at the nonpermissive temperature (12).

We have also observed that nsP1 is apparently unstable in cells infected with *ts*17, *ts*18, and *ts*24. There is reason to believe that nsP1 and nsP2 might interact with one another to form a functional complex. Several plant viruses, including tobacco mosaic virus, possess proteins that are homologous to nsP1 and nsP2, but in these plant viruses one polypeptide contains both the nsP1 and nsP2 domains (1). It is possible that free nsP1, not complexed with nsP2, is unstable. Similarly, free nsP2 might also be

unstable. In this regard it is noteworthy that the predominant processing pathway in wild-type Sindbis virus, to produce nsP1 and nsP2, is autoproteolysis of the P12 precursor (9), since this could imply that nsP1 and nsP2 are associated with one another from the moment of cleavage.

In all four mutants examined that are temperature-sensitive in processing, the processing pathways were changed quantitatively, if not qualitatively. P23 is a dominant polypeptide at the nonpermissive temperature in the mutants but is not seen in wild-type infection. Since the phenotype of a temperature-sensitive mutant is believed to result from changes in the folding of a polypeptide which occur upon a shift in temperature, it is possible that different conformations of the proteinase may result in different site and substrate specificities. This is also suggested by experiments that examined processing *in vitro*, where it appeared that cleavage between nsP2 and nsP3 was more susceptible to the deletion of sequences outside the C-terminal domain of nsP2 than was the cleavage between nsP1 and nsP2 (9).

We have also found that although P12 will autoproteolyze to form nsP1 and nsP2 fairly rapidly, precursor P123 will not, and, in fact, is the dominant product when virion RNA is translated at very low concentrations *in vitro* (9). Once again, the conformation of the proteinase must be different in P12 than in P123. Thus, it would appear that the proteolytic activities of the nonstructural proteins, and even their concentrations, are regulated by conformational changes and interactions with one another. This may also suggest that the various polyprotein precursors perform a function in viral replication which is different from that of their final products.

In a preliminary attempt to investigate the possible activities of these polyproteins, we have examined the nonstructural proteins made *in vivo* at 2 hours postinfection and have found that increased amounts of P123 and P1234 are present (unpublished data). Given the relative abundance of these precursors early in infection, it is possible that they play some role in minus strand synthesis. Further efforts are now under way to determine the functions of these proteins and their proteolytic specificities, using mutant viruses in which we have abolished proteolytic cleavage at certain sites by site-specific mutagenesis.

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# Chapter 4

## The Phosphorylation of Sindbis Virus Nonstructural Protein nsP3

This chapter is being prepared for submission as part of a collaborative effort with the laboratory of Charles M. Rice[1].

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## ABSTRACT

When Sindbis virus-infected chick cells were pulse-labeled with [$^{35}$S]methionine, then chased in the presence of unlabeled methionine for various periods of time, and the labeled products examined by immunoprecipitation with antiserum to nsP3 of Sindbis virus, three different forms of nsP3 were detected. Initially, nsP3 appeared as a 76 kDa band, but, over two to three hours this species was chased to two major high molecular-mass forms of approximately 79 kDa and 106-113 kDa. In similar experiments using $^{32}$P-inorganic phosphate, only the two larger forms of nsP3 became labeled with $^{32}$P, suggesting that nsP3 becomes phosphorylated posttranslationally. Phosphorylation of nsP3 was also examined in Sindbis virus-infected mosquito cells, and in chick cells using Middelburg virus, a closely related alphavirus. The results suggest that phosphorylation of nsP3 is a general feature in alphavirus infection and occurs in a wide variety of host organisms.

## INTRODUCTION

When Sindbis virus-infected chicken cells are labeled with [$^{35}$S]methionine, then chased in the presence of unlabeled methionine for various periods of time, and the labeled products are examined by immunoprecipitation with antibodies monospecific for each of the nonstructural proteins, nsP3 exhibits anomalous behavior (6). Initially, nsP3 appears as a single labeled species with an apparent molecular mass of 76 kDa. Then, over a two-hour period this species gradually chases to predominantly two higher molecular-mass forms of ~79 kDa and ~106-113 kDa. If Sindbis-infected chick cells are continuously labeled with $^{32}$P-inorganic phosphate for 3 hours, only the 79 kDa and 106 kDa forms of nsP3 become labeled, suggesting that nsP3 is being phosphorylated posttranslationally (6).

Recently, phosphorylated nsP3 was detected in BHK-21 cells infected with Semliki Forest virus (SFV), an alphavirus closely related to Sindbis virus, using antisera monospecific for the nonstructural proteins of SFV (14). However, unlike Sindbis virus, phosphorylation did not cause a change in the migration of nsP3 on SDS-polyacrylamide gels, nor did multiple phosphorylated forms of nsP3 appear. Phosphoamino acid analysis indicated that both serine and threonine residues, but not tyrosine residues, were modified. Since it had been previously shown that viral RNA polymerase activity in SFV-infected cells is found exclusively in the mitochondrial pellet fraction (P15) (16), mitochondrial pellet (P15) and supernatant fractions (S15) were prepared from SFV-infected cells labeled with [$^{35}$S]methionine and $^{32}$P-inorganic phosphate. When the $^{35}$S/$^{32}$P ratio of nsP3 was determined following immunoprecipitation and SDS-polyacrylamide gel electrophoresis, the nsP3 associated with the P15

fraction was found to possess an average of twice as many phosphates as the nsP3 from the S15 fraction, suggesting that phosphorylation of nsP3 may be important for RNA replication.

In eucaryotic organisms, protein phosphorylation performs a diverse role in cellular regulation, affecting protein synthesis (13) and cell division (11), and modulating the activity of many enzymes involved in central metabolism (2, 8). Phosphorylation of certain viral proteins has also been observed for a wide variety of animal and insect viruses (9), as well as for one plant virus (5), but, in only a few cases has the functional significance of this phosphorylation been demonstrated. In these cases, it appears to affect the interaction of the phosphoprotein with nucleic acid. Since examples of the fortuitous phosphorylation of viral proteins are known (7), the significance of viral protein phosphorylation during infection must be established.

We now present evidence that phosphorylation of nsP3 occurs in chick cells infected with Middelburg virus as well as with Sindbis virus. The generality of this phosphorylation in alphavirus infection is further supported by the presence of large amounts of phosphorylated nsP3 in Sindbis virus-infected mosquito cells, the primary vector of Alphaviruses.

## MATERIALS AND METHODS

### Cells and Virus Strains

Virus stocks were prepared in primary monolayers of chicken embryo fibroblasts (CEF) (15). The heat-resistant, small-plaque strain of Sindbis virus (SIN HR) used in these experiments has been described previously (17, 18) and, to a lesser extent, Middelburg virus (MID) (kindly provided by Dr. Phillip Marcus, University of Connecticut) has also been described (12,

17). The Aedes albopictus cells (Clone C6/36) were obtained from the American Type Culture Collection and were propagated as described previously (4).

**Labeling of CEF Cells Infected with Sindbis and Middelburg Viruses**

Duplicate sets of confluent monolayers of secondary CEF cells grown in 35 mm plates were washed once with room-temperature phosphate-buffered saline (PBS)(3) lacking divalent cations and infected at high multiplicity (50 pfu/cell) with SIN HR in PBS containing $Ca^{2+}$and $Mg^{2+}$,1 μg/ml Actinomycin D, antibiotics and 1% dialyzed fetal calf serum. Virus absorption was allowed to occur at 37°C for 70 min, and the inoculum was removed. The plates were washed once with room-temperature PBS to remove unabsorbed virus, and the incubation was continued at 37°C in Eagle's medium (EMEM) containing 3% dialyzed fetal calf serum, 1 μg/ml Actinomycin D, antibiotics, and 1/10th and 1/20th the normal concentration of phosphate and methionine, respectively. At three hours postinfection the medium was removed from both sets of plates and the cells were either continuously labeled for one, two, or three hours in phosphate-free EMEM containing 50 μCi/ml $^{32}$P-inorganic phosphate or pulse-labeled for 30 minutes in methionine-free EMEM containing 50 μCi/ml [$^{35}$S]methionine followed by a one-, two-, or three-hour chase in EMEM containing a 20-fold excess of cold methionine. The monolayers were then washed twice with ice-cold PBS and lysed with 250 μl of lysis solution (0.5% SDS, 5mM EDTA and 2mM PMSF).

The labeling of monolayers infected with Middelburg virus was performed similarly with the following exceptions: 1) the cells were maintained at 30°C throughout the experiment; 2) labeling occurred at six

hours, instead of three hours, postinfection; and 3) instead of a performing a pulse-chase analysis, labeling with [$^{35}$S]methionine was carried out continuously for one, two or three hours. All lysates were kept frozen at -70°C until ready for use.

**Labeling of Sindbis Virus-infected Mosquito and CEF cells**

Confluent monolayers of secondary CEF cells or Aedes albopictus cells grown in 35 mm plates were infected with 50 pfu/ml of SIN HR at 30°C for 70 minutes as described above, and the incubation was continued at 30°C in EMEM containing 10% dialyzed fetal calf serum. (The normal concentration of methionine was used in this medium to avoid the effects of methionine deprivation in mosquito cells.) At three hours' postinfection the medium was removed and the cells were continuously labeled for one, two or three hours in either methionine-free EMEM containing 50 μCi/ml [$^{35}$S]methionine or phosphate-free EMEM containing 50 μCi/ml $^{32}$P-inorganic phosphate. The monolayers were washed and lysed as previously described and the lysates were kept frozen at -70°C until ready for use.

**Immunoprecipitation of Whole Infected Cell Lysates**

Infected cell lysates were prepared and immunoprecipitated with monospecific antisera to nsP1-4 of Sindbis virus (6). For comparing the phosphorylation of nsP3 in CEF cells versus mosquito cells, 50 μl of lysate were diluted with 250 μl of binding buffer (50 mM Tris-Cl (pH 7.4), 0.4 M NaCl, 1 mM EDTA, 0.5% Triton X-100, 500 μg/ml bovine serum albumin, 0.2 mM phenylmethylsulfonyl fluoride), and 10 μl of nsP3 antiserum or preimmune serum were added to each immunoprecipitation.

Immunoprecipitations of MID-infected cell lysates were performed with 120 µl of lysate diluted with 420 µl of binding buffer and 10 µl of antiserum to nsP4 or preimmune serum.

### Discontinuous SDS Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out on 10% (wt/vol) acrylamide gels {30:0.4 and 37.5:1 (wt./wt.) acrylamide:bisacrylamide ratio} as previously described (6). Gels were treated with 1M sodium salicylate for fluorography (1) and exposed on prefogged X-Omat R (Eastman Kodak) or Cronex 4 (Dupont) film at -70°C.

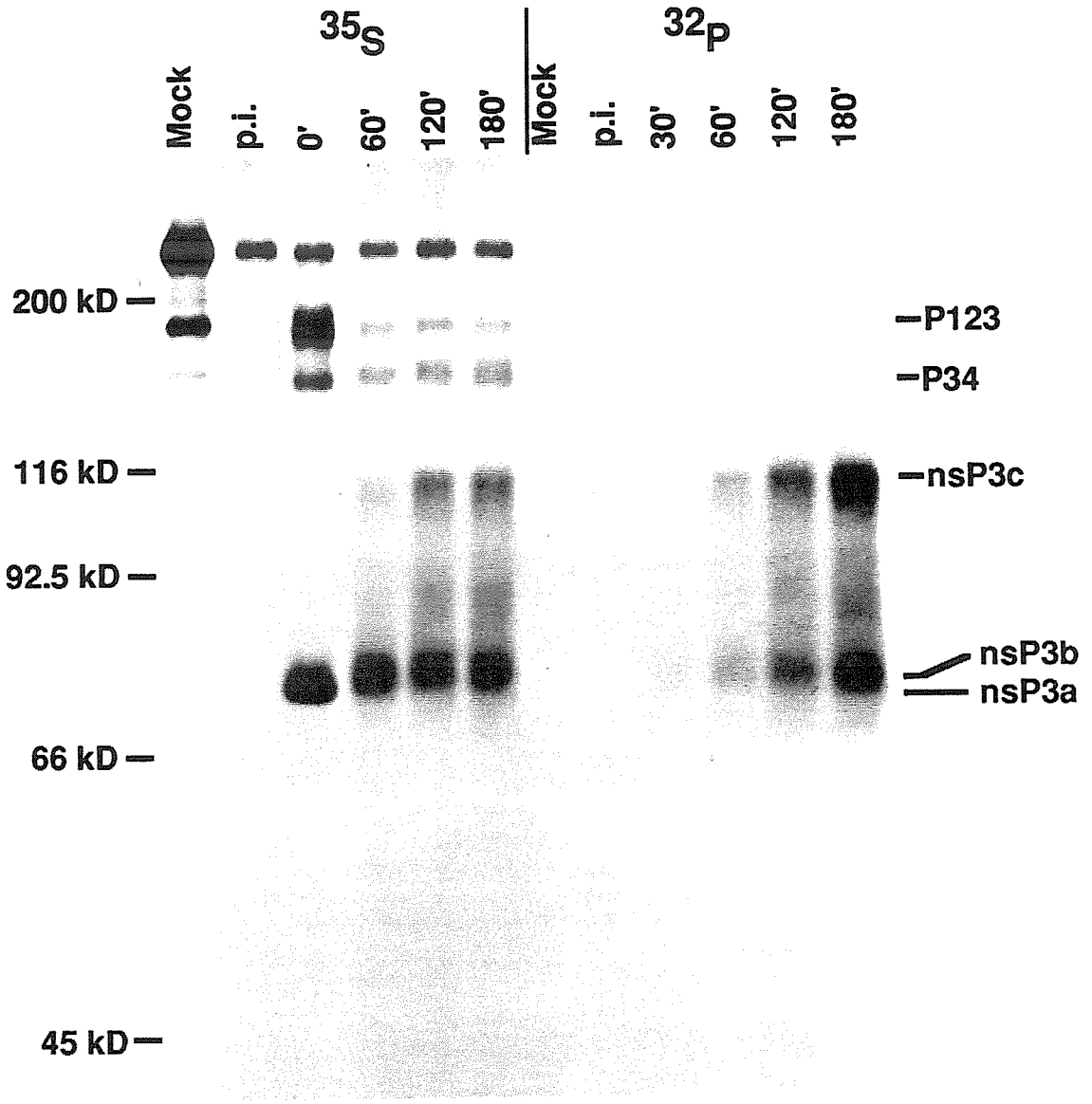
## RESULTS

### Phosphorylation of nsP3 in Sindbis and Middelburg Virus-Infected Cells

Confluent monolayers of CEF cells infected with Sindbis virus were either labeled for one, two or three hours in media containing $^{32}$P-inorganic phosphate, or pulse-labeled for 30 minutes in media containing [$^{35}$S]methionine and chased for one, two or three hours in medium containing an excess of unlabeled methionine. The cells were then lysed and the labeled products immunoprecipitated with antiserum monospecific for nsP3 of Sindbis virus or with preimmune serum. As a control, lysates of mock-infected CEF cells that had been either labeled with [$^{35}$S]methionine for 30 minutes, then chased for 60 minutes, or labeled continuously with $^{32}$P-inorganic phosphate for 60 minutes, were also immunoprecipitated. The immunoprecipitated products were then resolved by SDS-PAGE (see Fig. 1).

Following the pulse, nsP3 appeared as a single species (nsP3a) with an apparent molecular mass of 76 kDa. However, within two to three hours

**Fig. 1.** Phosphorylation of nsP3. At three hours postinfection Sindbis virus-infected CEF cells were either pulse-labeled for 30 minutes with [$^{35}$S]methionine, then chased for various periods of times in the presence of an excess of unlabeled methionine, or else labeled continuously with $^{32}$P-inorganic phosphate for 30 to 180 minutes. Lysates from these cells were then immunoprecipitated with antiserum to nsP3 of Sindbis virus or preimmune serum and the proteins were separated on a 10% SDS-polyacrylamide gel. As a control, mock-infected lysates were also prepared and immunoprecipitated with nsP3 antiserum.


35S
32P
Mock
p.i.
0'
60'
120'
180'
Mock
p.i.
30'
60'
120'
180'
200 kD
116 kD
92.5 kD
66 kD
45 kD
P123
P34
nsP3c
nsP3b
nsP3a

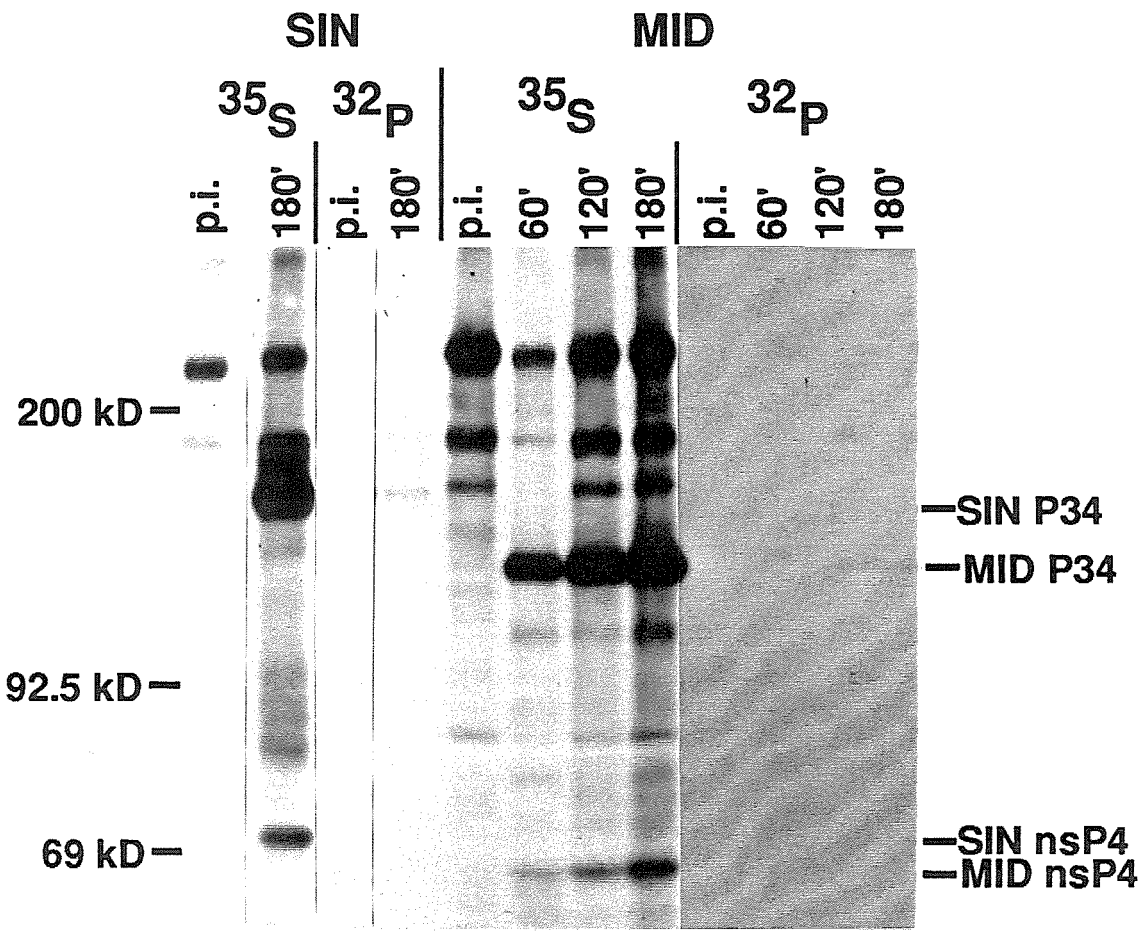
this species was converted to two major higher molecular-mass forms (nsP3b and nsP3c) of approximately 79 kDa and 106-113 kDa, which label with $^{32}$P-inorganic phosphate, as well as to a smear of intermediate phosphorylated species of heterogeneous molecular masses. Judging from the fluorographic pattern, the ratio of $^{35}$S/$^{32}$P label in the various products suggests that nsP3c is more highly phosphorylated than nsP3b.

Two faint bands of approximately 150 kDa and 185 kDa are also present in the lanes containing $^{32}$P-labeled products (most evident in the lane marked **180'**). Immunoprecipitations using antiserum to nsP4 have confirmed that the lower band is phosphorylated P34 (data not shown). From its position in the gel, it is probable that the other band is a phosphorylated form of precursor P123.

Phosphorylation of nsP3 was also examined in CEF cells infected with Middelburg virus (MID), a closely related alphavirus. Although antiserum specific for SIN nsP3 did not cross-react with nsP3 of MID, and although antisera to the nonstructural proteins of MID were not available, we were able to examine the phosphorylation of nsP3 indirectly by examining the P34 precursor. This was possible using antiserum to SIN nsP4, which has been observed to cross-react with nsP4 of several related alphaviruses including O'nyong-nyong, Ross River and Semliki Forest (unpublished observations).

CEF cells infected with MID were incubated at 30°C. Beginning at 6 hours postinfection, the cells were labeled for one, two or three hours in medium containing either [$^{35}$S]methionine or $^{32}$P-inorganic phosphate. Lysates prepared from these cells were immunoprecipitated with antiserum to SIN nsP4 or with preimmune serum and the immunoprecipitated products were examined by SDS-PAGE (see Fig. 2).

**Fig. 2.** Phosphorylation of Precursor P34 of Middelburg Virus. CEF cells were infected with Sindbis virus or Middelburg virus and incubated at 30°C. At six hours postinfection the cells were labeled with either [$^{35}$S]methionine or $^{32}$P-inorganic phosphate for various periods of time and then lysed. The lysates were immunoprecipitated with antiserum to nsP4 of Sindbis or with preimmune serum and the labeled products were examined on a 10% gel by SDS polyacrylamide gel electrophoresis.


SIN
MID
35S
32P
35S
32P
p.i.
180'
p.i.
180'
p.i.
60'
120'
180'
p.i.
60'
120'
180'
200 kD
92.5 kD
69 kD
SIN P34
MID P34
SIN nsP4
MID nsP4

After three hours of continuous labeling, a large amount of $^{35}$S-labeled P34, as well as some nsP4, were present in both the SIN- and MID-infected CEF cells. In addition, a very faint band representing phosphorylated P34 was also visible in the 180 minute lanes of $^{32}$P-labeled products. Thus, P34, and presumably nsP3, of MID is phosphorylated during MID infection.

**Phosphorylation in CEF Versus Mosquito Cells**

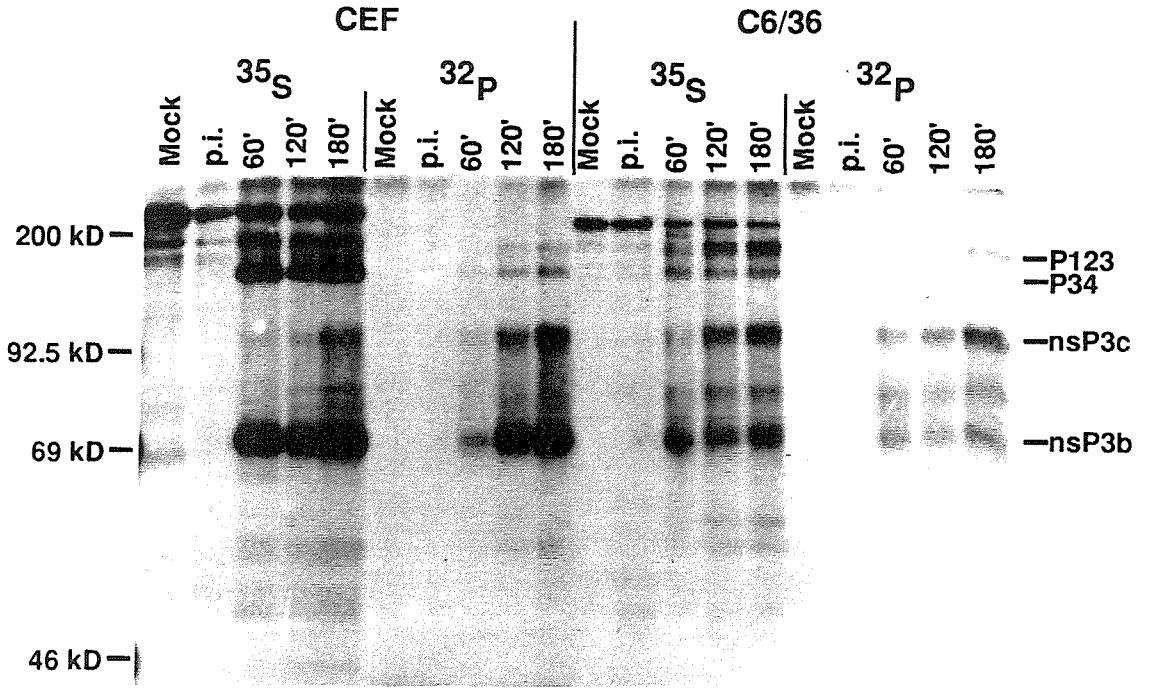
Monolayers of CEF cells or mosquito cells were infected at 30°C with Sindbis virus HRSP. At three hours postinfection the cells were labeled for one, two or three hours with either $^{32}$P-inorganic phosphate or [$^{35}$S]methionine. The labeled proteins were immunoprecipitated with antiserum to nsP3 or preimmune serum and subjected to discontinuous SDS-PAGE. Once again, immunoprecipitations were performed on mock-infected cell lysates to serve as a control.

Similar to the patterns from SIN-infected CEF cells, two phosphorylated forms of nsP3, nsP3b and nsP3c, appear in mosquito cells infected with SIN (see Fig. 3). However, in mosquito cells larger amounts of nsP3c appear more quickly (see **60'** time point in the lanes marked **$^{32}$P**). In addition, a distinct band of approximately 84 kDa is more prominent in the patterns of $^{32}$P-labeled products from mosquito cells. The presence of phosphorylated P34 was confirmed by immunoprecipitation using antiserum to nsP4 (data not shown).

## DISCUSSION

Phosphorylated forms of nsP3 have been found in both chick and mosquito cells infected with Sindbis virus as well as in chick cells infected with Middelburg virus, an alphavirus closely related to Sindbis virus.

**Fig. 3.** Phosphorylation of nsP3 in Mosquito Cells. Monolayers of CEF or mosquito cells were infected with Sindbis virus and incubated at 30°C. At three hours' postinfection the cells were labeled with either [$^{35}$S]methionine or $^{32}$P-inorganic phosphate for various periods of time, then lysed. These lysates were then immunoprecipitated with antiserum to nsP3 of Sindbis and the proteins were resolved on a 10% gel by SDS polyacrylamide gel electrophoresis. Immunoprecipitation of mock-infected lysates served as a control.


CEF
C6/36
35S
32P
35S
32P
Mock
p.i.
60'
120'
180'
Mock
p.i.
60'
120'
180'
Mock
p.i.
60'
120'
180'
Mock
p.i.
60'
120'
180'
200 kD
92.5 kD
69 kD
46 kD
P123
P34
nsP3c
nsP3b

Since phosphorylation of nsP3 has also been detected in baby hamster kidney cells infected with Semliki Forest virus (14), it appears that phosphorylation is a general feature of alphavirus infection and may be important for some aspect of the regulation and/or synthesis of viral RNA. This raises several questions concerning the nature, timing, and function of phosphorylation during infection. For example, is phosphorylation of nsP3 essential for the replication of viral RNA? Is phosphorylation coupled to RNA synthesis? What residues are phosphorylated? And what is the nature of the kinase responsible?

Independent experiments performed in collaboration with Li et al. (10) have provided further details. If nsP3b and nsP3c are incubated with calf intestinal alkaline phosphatase prior to SDS-polyacrylamide gel electrophoresis they are converted to a form that migrates identically to nsP3a, suggesting that they are phosphorylated forms of nsP3. Furthermore, when nsP3b and nsP3c were subjected to phosphoamino acid analysis, phosphoserine and phosphothreonine, but not phosphotyrosine, were found(10), consistent with the results obtained with nsP3 of Semliki Forest virus (14).

In Semliki Forest virus-infected cells labeled under similar conditions, only one form of phosphorylated nsP3 has been observed, and its apparent migration on SDS-polyacrylamide gels appears to be identical to that of the unmodified form of nsP3 (14). While no data are available on the number of phosphate groups present in either nsP3b or nsP3c, the large shift of approximately 30 kDa in apparent migration of nsP3c must be due to a change in the conformation or charge of nsP3 induced by the phosphates rather than to an actual increase in molecular mass. Since nsP3 of Sindbis and Semliki Forest virus contains 90 versus 74 serine plus threonine

residues, respectively, and since over half of these are located in the nonconserved region of nsP3 of Sindbis, versus only a third for nsP3 of Semliki Forest virus, it is probable that the anomalous behavior of nsP3c reflects a difference in the location of phosphorylation sites, as well as in the degree of phosphorylation.

From our results, small amounts of P34 appear to be phosphorylated in chick cells infected with Sindbis; this was confirmed using a mutant of Sindbis virus, which possesses a serine codon in place of the in-frame stop codon between nsP3 and nsP4, and which overproduces P34 (10). In light of these findings, phosphorylation of the P34 precursor in cells infected with Middelburg virus suggests that nsP3 is phosphorylated, too.

While phosphorylation of nsP3 appears to be a general phenomenon in cells infected with alphaviruses, its role in replication remains unclear. In Semliki Forest virus-infected cells, Peränen et al. (14) have found that the nsP3 associated with the mitochondrial pellet fraction (P15), which contains all of the viral RNA polymerase activity, is more heavily phosphorylated than that found in the supernatant fraction (S15). Since about 80% of all nsP3 in infected cells is found in the P15 fraction, it suggests that there may be a preference for phosphorylated nsP3 in viral replication complexes. On the other hand, experiments performed with Sindbis virus have demonstrated that the phosphorylation of nsP3 is neither coupled to viral RNA synthesis nor is it necessary for replication (10). Thus, while it is still possible that phosphorylation of nsP3 occurs fortuitously in infected cells, the results do not exclude some role for phosphorylated nsP3 in the temporal or quantitative regulation of viral RNA synthesis. Some obvious regulatory steps include the cessation of minus strand synthesis at three and a half hours' postinfection and the

regulation of 49S genomic RNA versus 26S subgenomic RNA synthesis. Further studies using mutants in which nsP3 is hypophosphorylated will be necessary to examine the effects of phosphorylation upon these aspects of RNA synthesis.

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