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

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Plasmids were constructed which 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 nsP1 and nsP2. Thus, although the rate of cleavage between nsP1 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 nsP1 and nsP2 (and nsP4) are relatively stable; nsP3, however, is unstable, with a half-life of about 1 h, 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.

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 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 nsP1, 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 which 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 to generate 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 repetoire 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 which 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

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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% CO_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, *trpL* 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 β-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 which 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 β-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 β-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 × *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 which 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 was 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 × *g* and 12°C for 3 min, the pellet was washed three times by suspension in TEN buffer containing 0.1% Nonidet P-40, followed by centrifugation, the final pellet was suspended in 700 μl of cracking buffer (0.01 M sodium phosphate [pH 7.2], 1% β-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 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 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 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 nsP1, nsP2, and nsP3, 80 μl 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 was 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 was 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, was 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 × *g* and 20°C for 10 min in an International centrifuge (International Equipment Co.). The pellet was suspended 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 × *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% β-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 was loaded per lane for discontinuous SDS-PAGE analysis.

RESULTS

Expression of *trpE* fusion proteins. Using the *trpE* expression system in *E. coli* and 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).

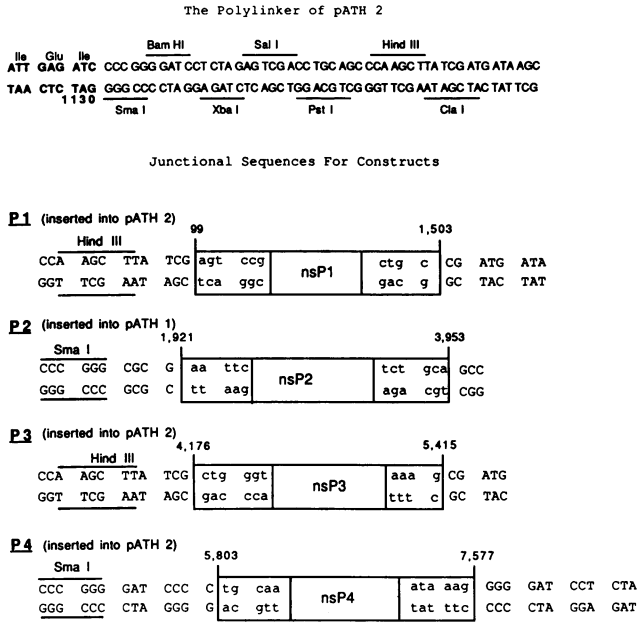


FIG. 1. The polylinker of pATH 2 and junctional sequences for constructs. The nucleotides in boldface correspond to sequences from the *trpE* gene of *E. coli* and are numbered in accordance with Yanofsky et al. (27). The lightface capital letters denote sequences within the polylinker of the pATH vector, 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 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 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 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.

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 β-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

TABLE 1. Properties of fusion proteins

Nonstructural protein	NT/AA[a]	Location of fusion protein insert[b]	Predicted % of protein represented	Predicted size of fusion protein (kDa)	Size of fusion protein from SDS-PAGE (kDa)
nsP1	1,620/540	99–1503	~87	90	83
nsP2	2,421/807	1921–3953	~84	113	105
nsP3	1,647/549	4176–5415	~75	84	98
nsP4	~1,848/~616	5803–7577	~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 the system of Strauss et al. (23).

known concentration to estimate the amount of fusion protein produced and to monitor the purification steps and protein integrity (Fig. 2). 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).

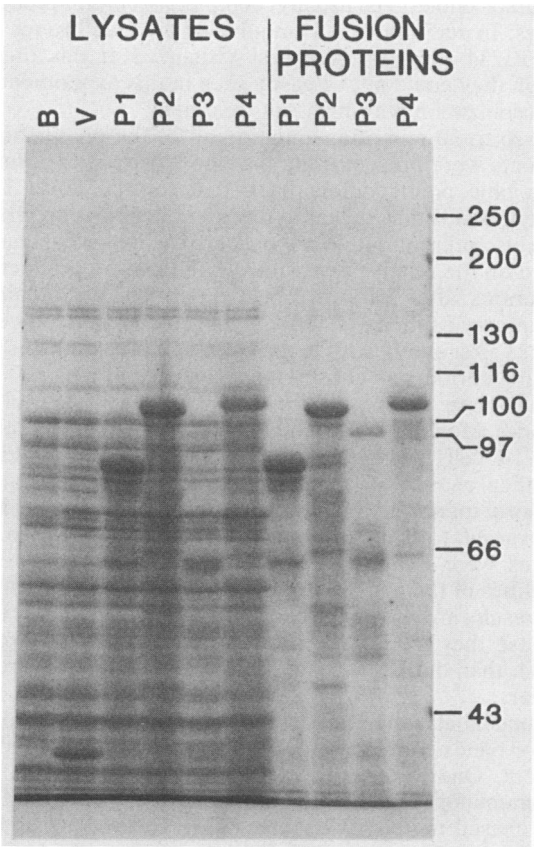


FIG. 2. Cultures of *E. coli* containing the hybrid gene fusions were induced with β-indoleacrylic acid. To obtain whole-cell lysates, the cells from 1 ml of culture were pelleted, suspended in 50 μl of cracking solution (0.01 M sodium phosphate [pH 7.2], 1% β-mercaptoethanol, 1% SDS, 6 M urea), and 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; P1, P2, P3, and P4, lysates or semipurified fusion proteins from cells containing the plasmids described in the legend to Fig. 1.

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, nsP1 (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 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-nsP1, 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).

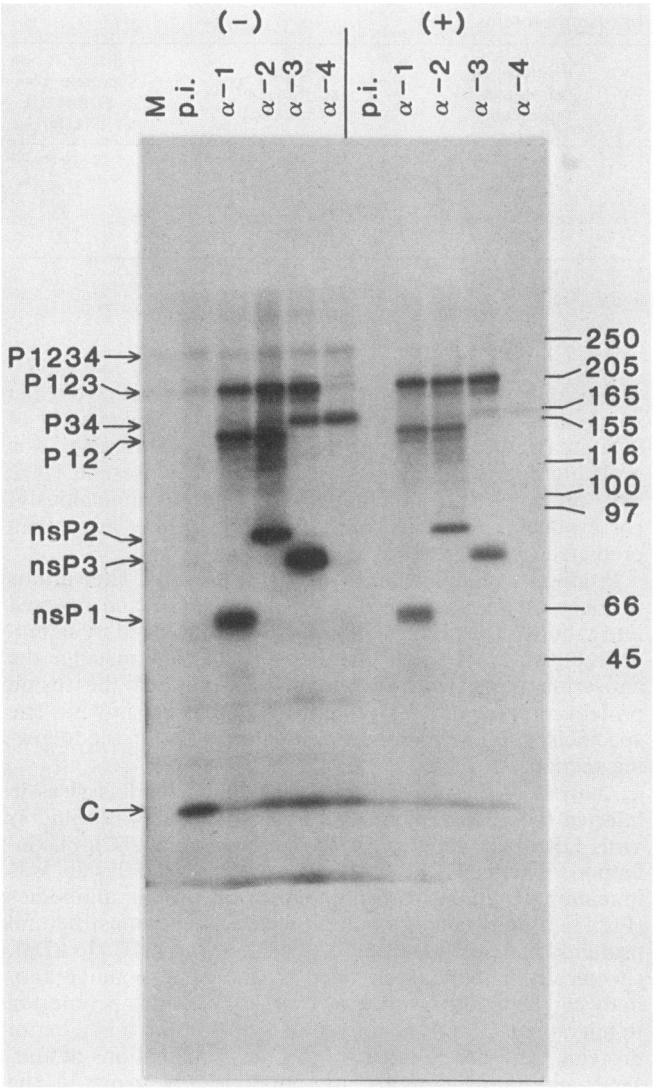


FIG. 3. Fluorogram of immunoprecipitated virus-specific nonstructural proteins of Sindbis virus. Duplicate monolayers of BHK cells were infected as described in the text. At 3.5 h postinfection the media over one set of monolayers were replaced with hypertonic media. At 4 h postinfection both sets of 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 nsP1, nsP2, nsP3, and nsP4, respectively; (+) and (−), cells with or without hypertonic treatment, respectively. Molecular mass standards are indicated as described in the legend to Fig. 2. C, Capsid protein.

However, host protein synthesis was reduced more than viral protein synthesis. After immunoprecipitation with α-nsP1, 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 anti-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 (Fig. 4). This suggests that the cleavage which 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, nsP1, 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 nsP12, 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.

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. Based on 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-nsP1, α-nsP2, and α-nsP3 and was chased more slowly than P123. The second appeared only in the nsP1 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.

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 poly-

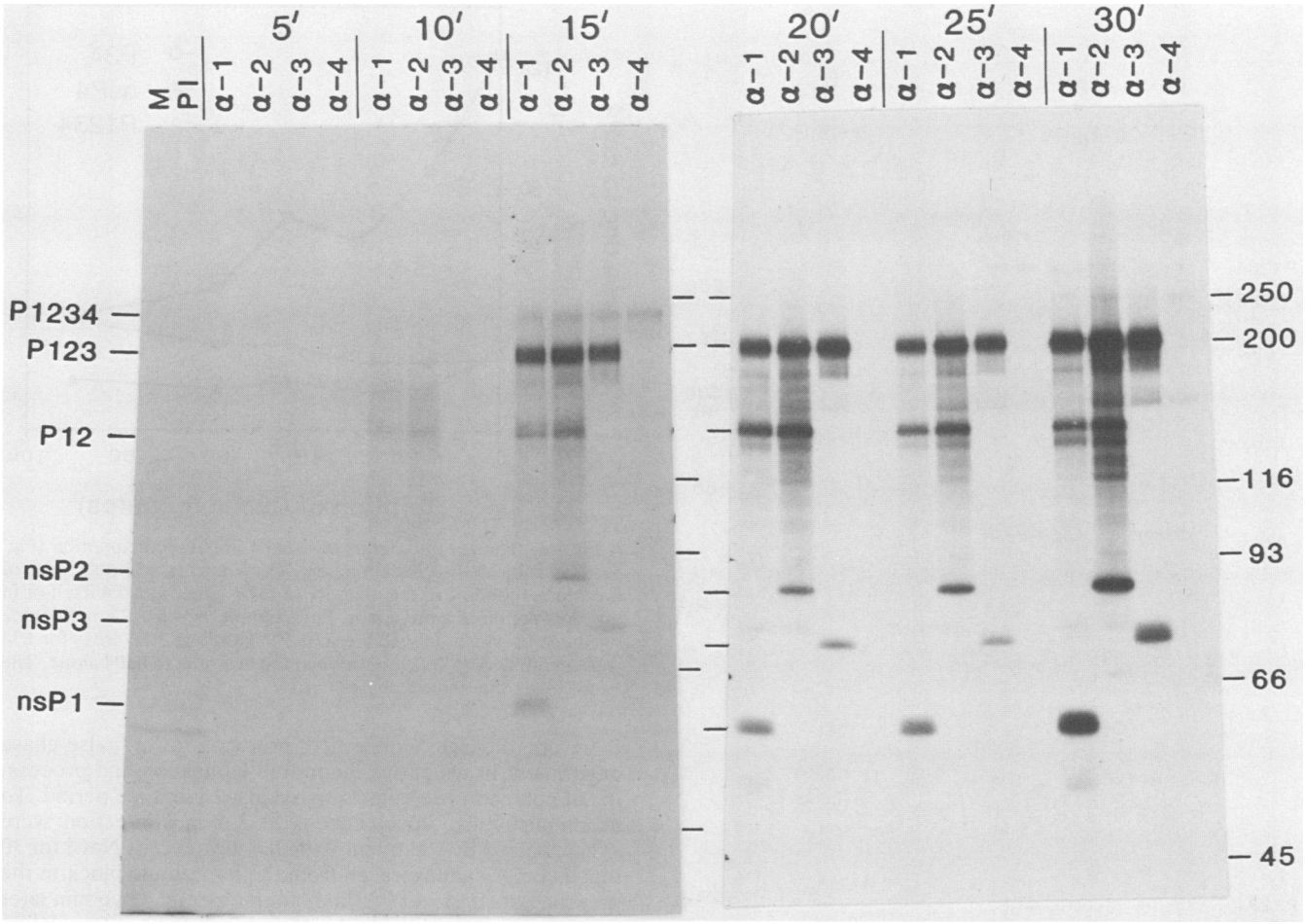


FIG. 4. Monolayers of BHK cells were infected with Sindbis virus HRSP as described in the text. At 3 h postinfection 5 M NaCl was added to the medium in each petri plate to give a final concentration of 220 mM NaCl in excess of that in the normal medium; 30 min later the hypertonic medium was removed 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 5-, 10-, and 15-min autoradiograms were exposed 2.5 times as long as the 20-, 25-, and 30-min ones.

peptides with apparent molecular masses of 79 to 113 kDa appeared. These polypeptides were precipitated only with anti-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, 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 which was 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 neglible 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 protein 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 depend upon the stage of completion of the polypeptide. We

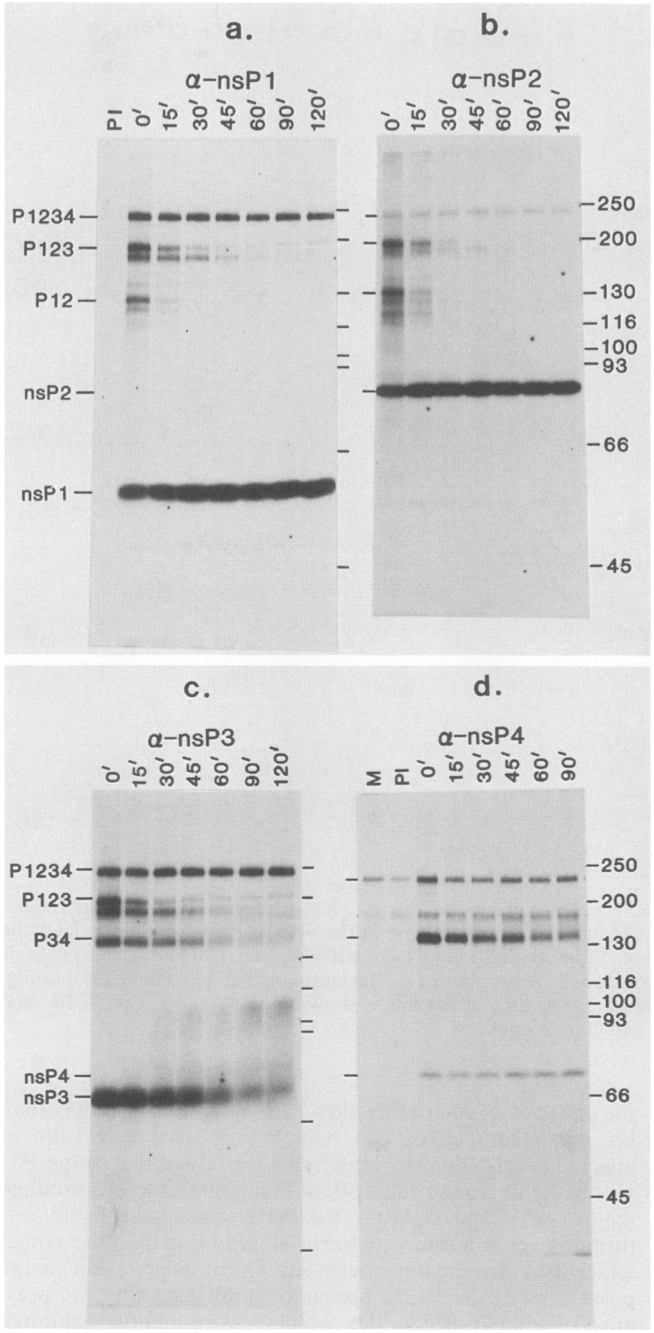


FIG. 5. Monolayers of chick cells were infected with Sindbis virus HRSP and labeled beginning at 3 h postinfection for 30 min in medium containing 40 μCi of [^{35}S]methionine per ml. The cells were then chased for 0, 15, 30, 45, 60, 90, and 120 min (') in medium containing a 20-fold excess of nonradioactive methionine. The cells were lysed, the lysates were immunoprecipitated, and the immunoprecipitates were analyzed by PAGE. The samples in panels a, b, c, and d were immunoprecipitated with α-nsP1, α-nsP2, α-nsP3, and α-nsP4, respectively. The time of chase 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.

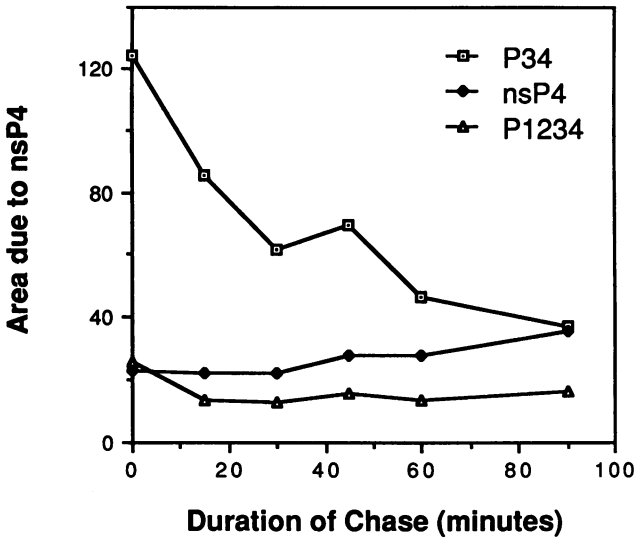
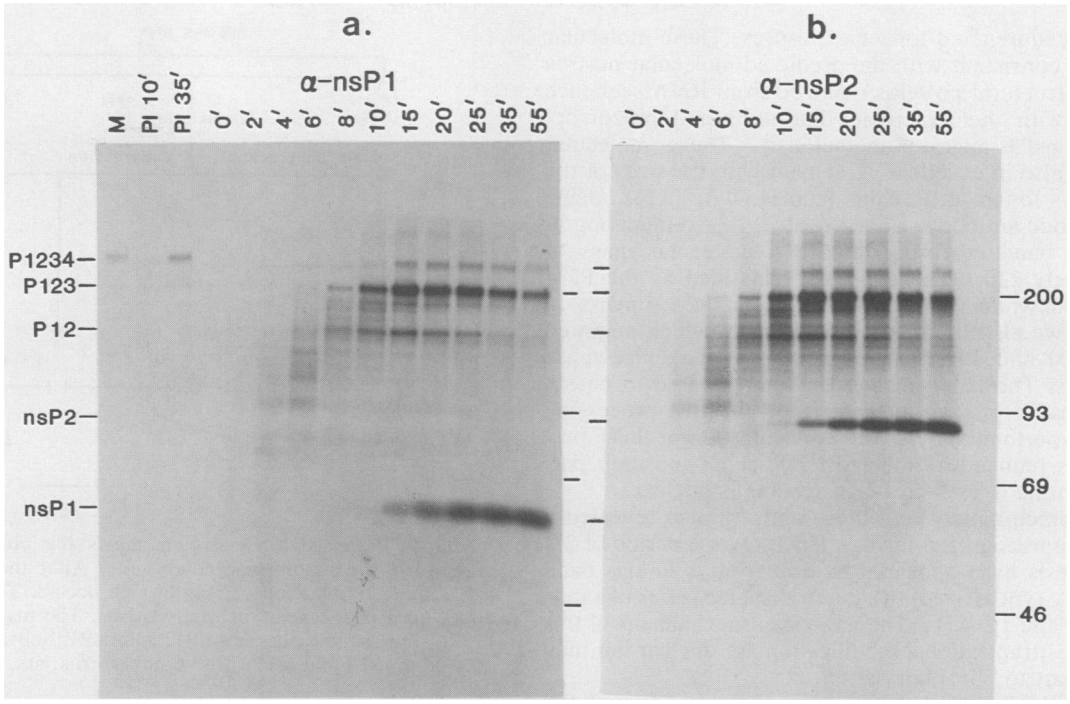


FIG. 6. The autoradiogram of α-nsP4 immunoprecipitation (Fig. 5d) was subjected to densitometry. The *y* axis is arbitrary units of area corresponding to the quantity of nsP4 either in nsP4 itself or in the nsP4 region of a precursor. For example, P34 has 27 methionine residues, of which only 16 are 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.

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) was 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 predominantly nsP3 was labeled; a small amount of label was also found in nsP2, and no label was observed in nsP1. Because the order of proteins in the polyprotein precursor is NH_2-nsP1-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



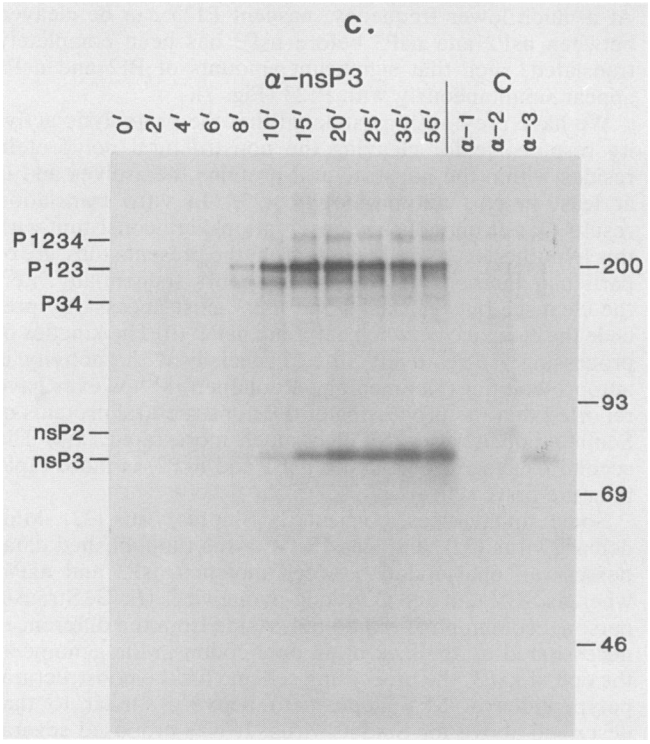


FIG. 7. Monolayers of chick cells were infected at high multiplicity with Sindbis virus HRSP. At 3 h postinfection NaCl was added to inhibit initiation of new polypeptide chains; 30 min later the hypertonic media were removed and Eagle medium containing 50 μCi of [^{35}S]methionine per ml was added. 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-nsP1 (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-nsP1, α-nsP2, or α-nsP3 as indicated. M and PI are defined in the legend to Fig. 3.

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 after the addition of pactamycin, mature nsP1 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-nsP1 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 nsP1 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 nsP1 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 nsP1 (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 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 which 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.

Based on 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 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.

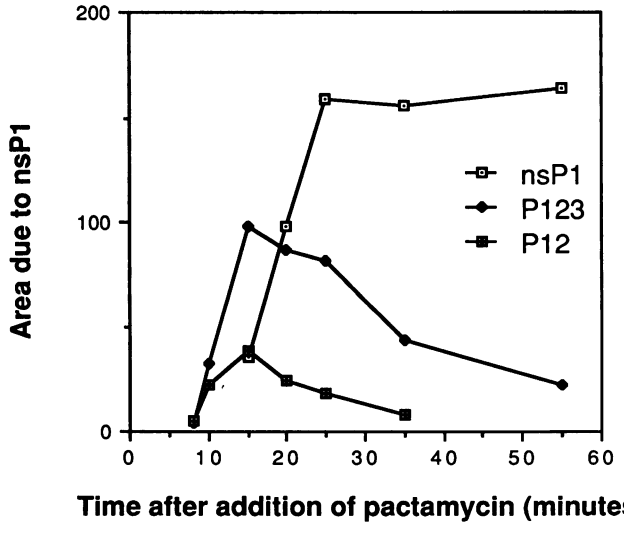


FIG. 8. The autoradiogram of immunoprecipitation by α-nsP1 (Fig. 7a) was subjected to densitometry to quantitate the amount of nsP1 and its precursors present at various times after pactamycin addition. Areas have been normalized as described in the legend to Fig. 6.

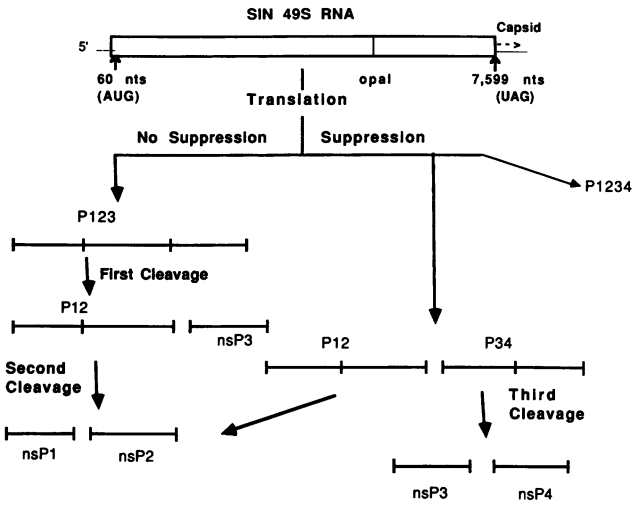


FIG. 9. Proposed model which shows the processing of the polyprotein precursors of Sindbis virus. After the translation of nsP3, either termination or readthrough occurs. Termination produces most of the nonstructural proteins. The major precursor is P123, which is first cleaved to P12 and nsP3. Subsequently, P12 is cleaved to nsP1 and nsP2. SIN, Sindbis virus; nts, nucleotides.

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 nsP1 and nsP2. (ii) The kinetics of processing are relatively slow, especially if the activity is autoproteolytic. (Keränen 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 nsP1 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 nsP1 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 which 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.

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