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Synthesis of Sindbis Virus Nonstructural Polypeptides in Chicken Embryo Fibroblasts

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The identification of eight previously undescribed polypeptides in chicken embryo cells infected with Sindbis virus is reported. Seven of these polypeptides were distinguishable from the virus structural polypeptides and their precursors by their molecular weights and tryptic peptide maps. The eighth was closely related to pE2 (Schlesinger and Schlesinger, 1973), a precursor to one of the virus particle glycoproteins. Pulse-chase experiments and the use of an inhibitor of proteolytic cleavage allowed a division of the seven nonstructural (NS) polypeptides into three stable end products (NS p89, NS p82, and NS p60) and four precursors (p230, p215, p150, and p76). The labeling kinetics after synchronous initiation of translation indicated that synthesis of the NS polypeptides started at a single site and showed that the order of the genes coding for the NS polypeptides was (5′ → 3′) NS p60, NS p89, and NS p82. Short-pulse experiments under conditions of both synchronized and nonsynchronized translation suggested that cleavage of the primary translation product of the NS genes occurred only after its synthesis was completed and that the first cleavage removed the C-terminal polypeptide. From these and other experiments, we propose a detailed scheme for the synthesis and processing of Sindbis virus NS polypeptides.

The genome of alphaviruses such as Sindbis virus and Semliki Forest virus consists of single-stranded RNA that has a molecular weight of $4.2 \pm 0.2 \times 10^6$ and a sedimentation coefficient of about 42S (29, 23). In Sindbis virus particles, this RNA is encapsidated by core (C) protein in a nucleocapsid, which is surrounded by a lipoprotein envelope containing two glycoproteins (E1 and E2 [27]). In Semliki Forest virus particles, a third glycoprotein (E3) is present (15). Experiments in several laboratories have established that, in infected cells, a subgenomic RNA species (26S RNA; molecular weight, $1.8 \pm 0.2 \times 10^6$) acts as mRNA for the structural proteins (4, 6, 7, 30, 34). Studies in infected cells and with cell-free synthesizing systems have shown that the gene order (5′ → 3′) in 26S RNA is C, E3, E2, and E1 and that these proteins are synthesized from a single initiation site by a combination of nascent and post-translational cleavages (8, 9, 25, 28, 31).

Since the 42S RNA is infectious (13, 26), it must encode all the virus-specified polypeptides required for virus multiplication. These polypeptides comprise not only the structural proteins but also nonstructural (NS) polypeptides, some or all of which are components of the virus-specified RNA-dependent RNA polymerase (24, 32). Recent work has established that the nucleotide sequence of the 26S RNA is located inward from the 3′ end of the 42S RNA, and therefore the genes coding for the NS polypeptides must be situated in the 5′ terminal two-thirds of the genome (19, 34). In Semliki Forest virus-infected cells, several NS polypeptides have been identified (10, 17, 18, 20), and we have recently reported the presence of two virus-specified NS polypeptides in preparations of virus RNA polymerase purified from infected-cell lysates (11). Several lines of evidence, including cell-free translation studies with 42S RNA, as well as the kinetics of appearance of NS polypeptides in infected cells after synchronous initiation of translation (5, 10, 16, 21), suggest that synthesis of the NS polypeptides is initiated at a single site near the 5′ end of the genome and terminates internally before the start of the structural protein genes and that the primary translation product is then processed (5, 20). Many aspects of this overall scheme are, however, quite unclear. In particular, little is known about the processing of the primary translation product and, since almost all the available information has come from studies with Semliki Forest virus, we considered it pertinent to examine in some detail the expression of the NS genes of the 42S RNA of Sindbis virus.

In the present paper, we report the presence of three previously undetected NS polypeptides

in Sindbis virus-infected cells and show by tryptic peptide mapping and pulse-chase experiments that these polypeptides are synthesized via a series of precursors, the largest of which probably represents the entire translation product of the NS genes.

MATERIALS AND METHODS

Materials. Acrylamide, sodium dodecyl sulfate (SDS; especially pure grade), and molecular-weight markers (range, 53,000 to 265,000) were obtained from British Drug Houses Ltd; *N,N'*-methylenebisacrylamide from Eastman Organic Chemicals; trypsin (treated with L-1-tosylamido-2-phenylethylchloromethyl ketone) from Worthington Biochemical Corp.; *N-2*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Flow Laboratories Ltd; ovalbumin, bovine serum albumin, human transferrin, and phosphorylase *a* from Sigma Chemical Co; L-[^{35}S]methionine (350 to 500 Ci/mmol) from the Radiochemical Centre; and precoated silica gel plates for thin-layer chromatography from Schleicher and Schüll, Dassel, W. Germany. Actinomycin D was a generous gift from Merck, Sharpe and Dohme.

Virus. The wild-type AR339 strain of Sindbis virus was used throughout this work (1). Working stocks were prepared by infecting BHK, clone 13, cells in 2.5-liter roller bottles with (per cell) 0.1 PFU of virus that had been plaque-purified three times. After 1 h at 37°C the inoculum was replaced with 50 ml of medium 199 containing 2% calf serum, and incubation was continued for 24 h at 37°C. The extracellular fluid, which contained 0.5 to 1 × 10^9 PFU/ml, was stored at −70°C and used without further passage.

Cells and media. All experiments were performed in chicken embryo cells set up in glass scintillation vials as described previously (25). To increase the incorporation of radioactive methionine, the cells were seeded and incubated overnight in Glasgow modified minimal essential medium (GMEM) containing 5% calf serum and one-tenth the normal amount of methionine. After infection, cells were incubated in GMEM lacking methionine but containing 2% dialyzed calf serum and 1 μg of actinomycin D per ml, buffered to pH 7.4 with 20 mM HEPES (GMEM-met).

Radioactive labeling of cells. Cells were washed once with GMEM-met and then infected with 100 PFU of virus per cell for 1 h at 37°C. The inoculum was then replaced with GMEM-met. At the times specified in individual experiments, the medium was replaced with Earle salt solution containing 2% dialyzed calf serum, 1 μg of actinomycin D per ml, and 50 μCi of [^{35}S]methionine per ml, buffered to pH 7.4 with 20 mM HEPES (labeling medium). In experiments involving a chase period, the labeling medium was replaced with identical medium but containing 2 mM unlabeled methionine in place of the isotope (chase medium). The pulse or chase was terminated by removing the medium and placing the vial in a freezing mixture of methanol/dry ice. The cells were then processed for gel electrophoresis as previously described (10).

SDS-polyacrylamide gel electrophoresis. Samples were run on slab gels (20 by 18.5 by 0.15 cm) with an apparatus similar to that described by Studier (33). Samples were analyzed on 7.5% acrylamide + 0.2% *N,N'*-methylenebisacrylamide with the buffer systems of Laemmli (22). After electrophoresis (for 16 h at 45 V), the gels were dried under vacuum at 90°C and autoradiographed on Kodirex X-ray film.

Molecular-weight determinations. The continuous-gel system of Fairbanks et al. (12) was used. For molecular weights greater than 100,000, samples were run on gels containing 3.5% acrylamide + 0.093% *N,N'*-methylenebisacrylamide. Molecular-weight markers in this instance consisted of a commercial product (from British Drug Houses) comprised of oligomers of a protein of molecular weight 53,000. Molecular weights of less than 100,000 were determined with a gel containing 7.5% acrylamide + 0.2% *N,N'*-methylenebisacrylamide. Markers were phosphorylase *a* (92,000), human transferrin (77,000), bovine serum albumin (68,000), and ovalbumin (45,000). Labeled virus-specified polypeptides and markers were subjected to coelectrophoresis on the same gel, which was stained with Coomassie blue to visualize the markers and then dried and autoradiographed to reveal the position of the labeled polypeptides.

Tryptic peptide mapping. p230 and p215 were prepared by labeling infected cells for 30 min at 4 h postinfection in the presence of 0.1 mM $ZnCl_2$. p150, p76, and pE2 were prepared by labeling cells for 10 min at 4 h postinfection. NS p89, NS p82, NS p60, NS p59, E1 + E2, and C were prepared by labeling cells for 1 h at 3.5 h postinfection and then incubating the cells in chase medium for a further 1 h. In all cases, the polypeptides were purified by gel electrophoresis and digested in situ with trypsin, and the peptides were eluted, oxidized with performic acid, and fingerprinted by two-dimensional thin-layer chromatography as described before (10).

RESULTS

Identification of novel virus-specified polypeptides. Replicate cultures of chicken cells, infected or mock-infected, were labeled with [^{35}S]methionine for 30 min at either 4 or 8 h postinfection, and the polypeptides were extracted and analyzed on SDS-polyacrylamide gels. The results of this experiment are shown in Fig. 1. Early in infection (lane b), a large number of host polypeptides were still being synthesized, but there were also certain new polypeptides with mobilities different both from host polypeptides (lane a) and the structural polypeptides and their precursors (p120, pE2, E1 + E2, and C). These new polypeptides are designated p150, NS p89, NS p82, and NS p60. Later in infection (lane c), host protein synthesis was largely shut off, with structural protein synthesis accounting for a large percentage of total protein synthesis. At this time, the new polypeptides were made in smaller amounts

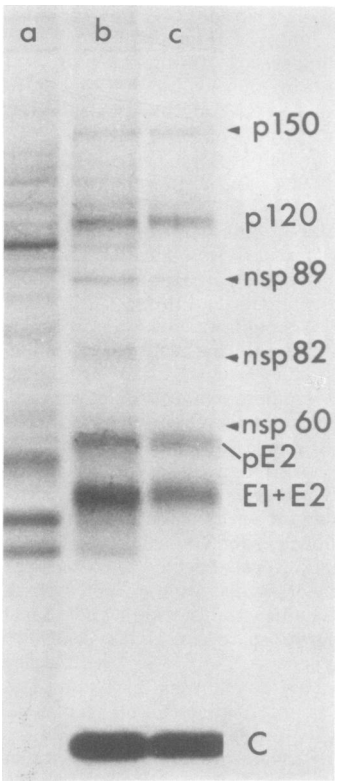

FIG. 1. *Synthesis of virus-specified polypeptides in chicken cells during a 30-min pulse. Infected and mock-infected replicate cultures of chicken cells were labeled with [^{35}S]methionine for 30 min and then processed for SDS-polyacrylamide gel electrophoresis. (a and b) Profiles from mock-infected and infected cells, respectively, labeled at 4 h postinfection. (c) Profile from infected cells labeled at 8 h postinfection. Migration in this and all subsequent electrophoretograms is from top to bottom. The identities of E1 + E2 and C were established from the positions of the structural proteins of purified virus particles run on a parallel gel (not shown).*

than at earlier times. Over the duration of the experiment, there were no apparent changes in the pattern of polypeptides from mock-infected cells.

Product-precursor relationships among the novel polypeptides. Recently, evidence has been presented which suggests that a second cleavage scheme, distinct from the one that produces the structural proteins, operates for the NS proteins of Semliki Forest virus (10, 20). To see whether a similar scheme operates in Sindbis virus-infected cells, two experiments were performed. First, two infected and two mock-infected cultures were labeled for 10 min at 4 h postinfection, and then one of each type of culture was chased for a further 1 h. Extracts were prepared from these cultures and analyzed on an SDS-gel. Figure 2 shows the result of this pulse-chase experiment. The infected sample prepared immediately after the pulse (lane a) contained p150, NS p89, and two additional polypeptides (p215 and p76) not present in the respective mock-infected extract (lane c). After a chase in infected cells (lane b), radioactivity was found in NS p89, NS p82, and NS p60, whereas p215 had disappeared, and the amounts of p150 and p76 had decreased. In addition, an additional polypeptide, NS p59, with a mobility close to that of pE2, was detected after a chase. Together, these results suggest that NS p89, NS p82, NS p60, and NS p59 are stable end products and that p215, p150, and p76 may be precursors to them. In the second experiment, zinc ions, which have been reported to cause accumulation of uncleaved high-molecular-weight precursors (3), were added to infected and mock-infected cultures,

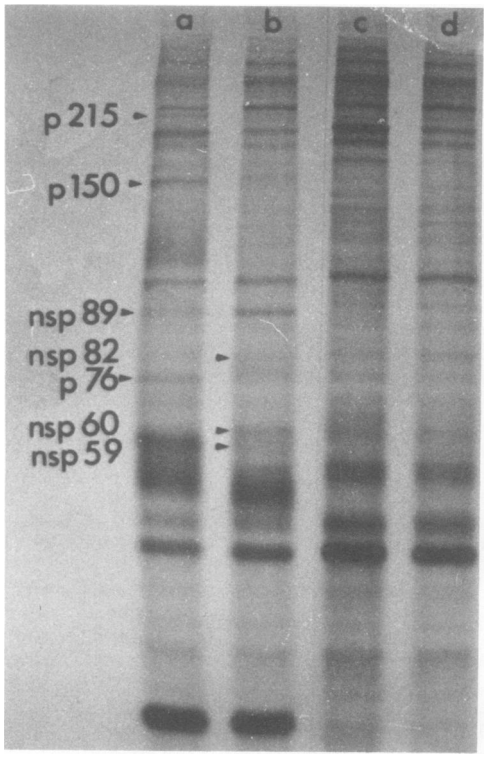

FIG. 2. *Synthesis of virus-specified polypeptides in chicken cells during short-pulse and pulse-chase conditions. (a and b) Infected and (c and d) mock-infected chicken cells were labeled for 10 min with [^{35}S]methionine at 4 h postinfection. One sample of each was processed immediately (a and c), while the others had labeled medium replaced with chase medium for a further 1 h (b and d).*

which were then pulsed with [^{35}S]methionine, and their proteins were analyzed (Fig. 3). Over the range of zinc chloride concentrations used in this experiment, there was no detectable change in the polypeptide profiles from mock-infected cells. With low concentrations of zinc (less than 0.01 mM), cleavage in infected cells appeared to occur normally (lanes a and b), but as the concentration was increased, the amounts of p150, NS p89, NS p82, and NS p60 decreased, while at the same time the amount of p215 increased and a further high-molecular-weight polypeptide, p 230, became clearly visible (lanes c and d). This result is entirely consistent with the tentative conclusions drawn from the pulse-chase experiments and suggests that p230 may be the precursor of all the stable end products.

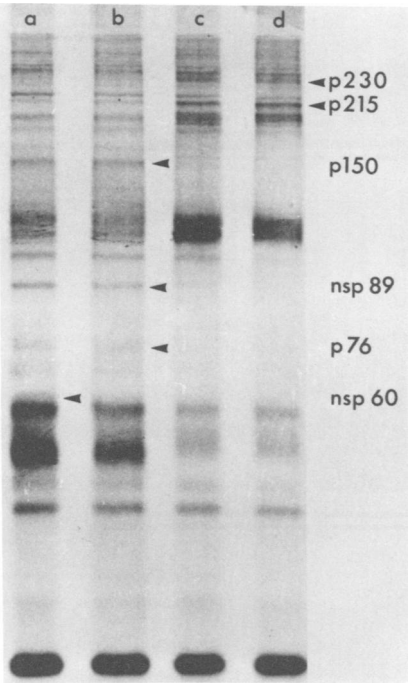

FIG. 3. *Effects of zinc ions on the synthesis of virus-specified polypeptides. Replicate cultures of chicken cells were infected and, at 3.5 h postinfection, the medium was replaced with medium containing various concentrations of zinc chloride. At 4 h postinfection, the medium was replaced with labeling medium containing the appropriate concentration of zinc. After a further 30 min, the cells were processed for electrophoresis. (a) 0.001 mM Zn^{2+}; (b) 0.01 mM Zn^{2+}; (c) 0.05 mM Zn^{2+}; and (d) 0.1 mM Zn^{2+}.*

Molecular weights of the novel polypeptides. The molecular weights of all the novel polypeptides, both stable end products and their putative precursors, were determined by using the continuous-gel system of Fairbanks et al. (12). The determinations were carried out in two parts as described in Materials and Methods. The two calibration curves are shown in Fig. 4. On the basis of these determinations, the novel polypeptides were designated p230 (molecular weight 230,000), p215 (molecular weight 215,000), p150 (molecular weight 150,000), NS p89 (molecular weight 89,000), NS p82 (molecular weight 82,000), p76 (molecular weight 76,000), NS p60 (molecular weight 60,000), and NS p59 (molecular weight 59,000).

Tryptic peptide mapping of the novel polypeptides. To further investigate possible precursor-product relationships, virus-specified polypeptides were isolated from infected cells and compared by the technique of tryptic peptide mapping. The conditions for the labeling of the polypeptides were essentially those described above for analytical gels, except that 10 vial cultures were used for each experiment. The peptide maps are shown in Fig. 5.

Several points emerge from this experiment.

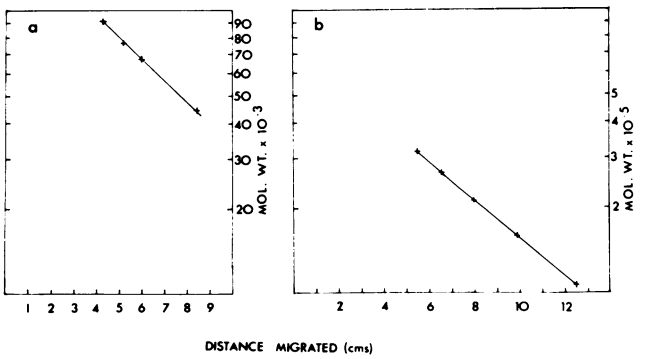

FIG. 4. *Calibration curves for the determination of the molecular weights of the novel polypeptides. The calibration curve for proteins with molecular weights of less than 100,000 (a) or more than 100,000 (b) were determined with commercial markers on 7.5 and 3.5% acrylamide gels, respectively.*

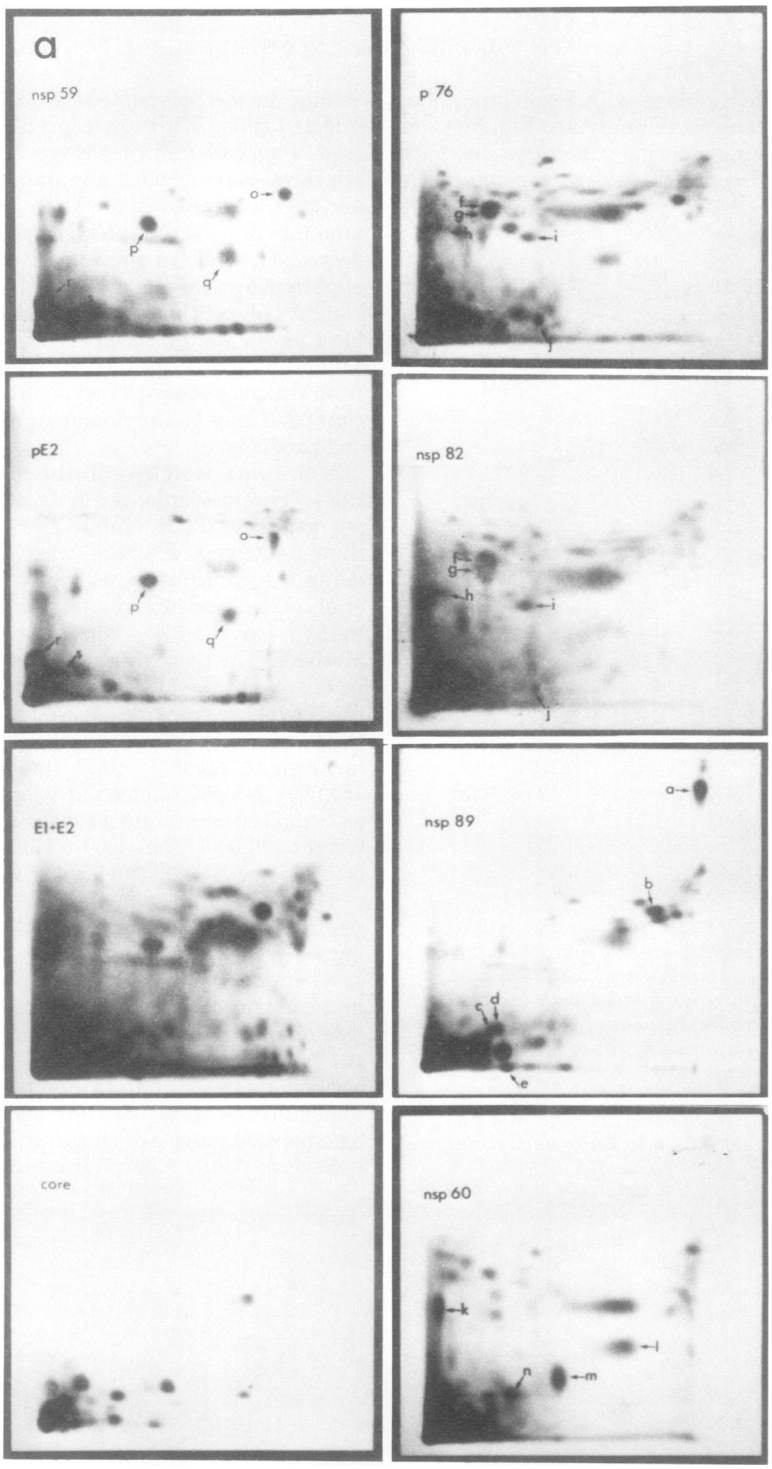

FIG. 5. *Tryptic peptide maps of virus-specified polypeptides. Each of the virus-specified polypeptides was labeled as described in Materials and Methods, purified by polyacrylamide gel electrophoresis, and digested with trypsin, and the performic acid-oxidized peptides were analyzed by two-dimensional chromatography. The first dimension is from left to right; the second is from bottom to top. In general, 1 cpm/dalton was applied. (a) Fingerprints of the structural proteins and their precursors, together with the putative NS polypeptides and p76. (b) Fingerprints of the NS polypeptides and their putative precursors. To aid comparisons, some of the most prominent spots in each map have been lettered (a–o). The significance of spot x is described in the text.*

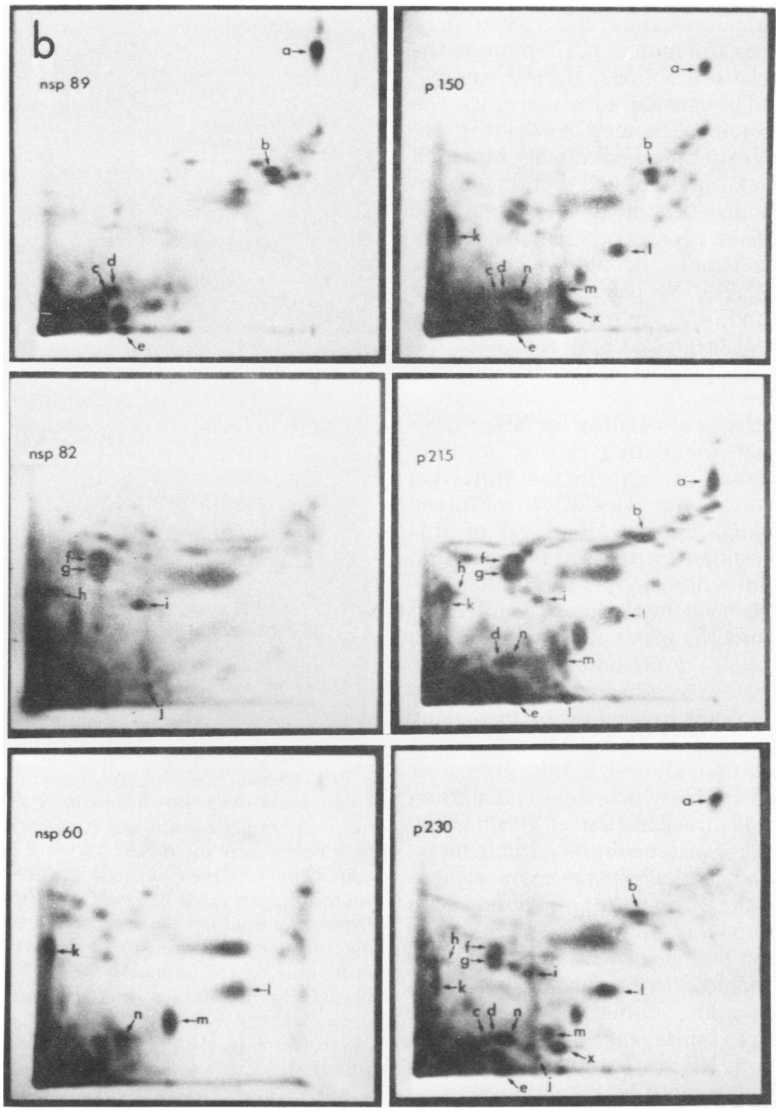

FIG. 5b.

First, comparison of the maps of NS p89, NS p82 and NS p60 show them to be unrelated to the virus structural proteins or their precursors (pE2, E1, E2, and C; Fig. 5a); therefore, these novel polypeptides can indeed be regarded as NS. Second, the map of NS p59 (Fig. 5a) shows a large number of spots (o, p, q, r, and s) that are also seen in pE2, so it is likely that NS p59 is some portion of the pool of pE2 that remains stable during chase (Fig. 2). A similar phenomenon has been observed in Semliki Forest virus-infected cells, in which there exists a protein that is apparently related to pE2 but which does not undergo cleavage to E2 + E3 (17). Third, comparison of the maps of NS p82 and p76 (Fig. 5a) shows a high degree of similarity (spots f, g, h, i, and j), suggesting that these proteins have a similar amino acid sequence. The apparent increase in molecular weight could be explained by a post-translational modification of p76, and experiments are in progress to determine exactly what this might be. There are, therefore, three stable NS proteins present in Sindbis virus-infected chicken cells. Fourth, the map of p150 (Fig. 5b) shows those spots characteristic of NS p89 and NS p60 (a, b, c, d,

e, k, l, m, and n) but not those of NS p82 (f, g, h, i, and j), whereas the map of p215 contains the spots characteristic of NS p89, NS p82, and NS p60 and so could be considered as a precursor to all three NS proteins. The map of p230 (Fig. 5b) shows a very high degree of similarity to that of p215, with the exception of one prominent spot (x). This spot is also present in the map of p150 and is absent from all the other maps. Taken together, these results strongly suggest that NS p89, NS p82, and NS p60 arise by cleavage of p230, p215, and p150. Indeed, with a molecular weight of 230,000, p230 may represent the entire translation product of the NS genes of the 42S RNA.

Ordering of the genes coding for NS p89, NS p82 and NS p60. Incubating cells in medium containing high salt prevents further initiation of protein synthesis but does allow continued polypeptide elongation (9). Removal of this block by replacement with isotonic medium, after all protein synthesis has terminated, results in synchronous initiation. To order NS p89, NS p82, and NS p60, salt synchrony was employed as follows. Four hours postinfection, initiation of protein synthesis was blocked by adding medium made hypertonic by increasing the concentration of NaCl by 225 mM. Ribosome runoff was then allowed to take place over a period of 40 min, by which time translation, as measured by incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material, had stopped. The block was then released by replacing the hypertonic medium with isotonic medium containing [^{35}S]methionine. After increasing periods of time, the label was removed and replaced with chase medium for a further 1 h to allow stable end products to accumulate. The results, shown in Fig. 6, indicate that the first NS protein to be synthesized was NS p60, which could be seen after a 4-min pulse (lane b); next to appear was NS p89, which was visible by 6 min (lane c); and last to appear was NS p82, which was not visible until 10 min (lane d) after release of the block. The gene order (5′ → 3′) is therefore NS p60, NS p89, and NS p82. These results are supported by the peptide maps, which demonstrate that NS p89 and NS p60 are adjacent to one another (in p150) and that NS p82 must therefore be terminal in p215 (and p230).

Orders of cleavage of the NS polypeptides from their precursors. As we have already observed, in a short pulse (Fig. 2, lane a) p76 and p150 are clearly visible, whereas only very small amounts of NS p89 can be seen (NS p60 is obscured by pE2). This suggests that translation of the entire NS genes occurs before cleavage of p230 commences and that the first cleavage is C-terminal, producing p76 and p150. To explore this possibility further, a modification of the salt synchronization technique was employed. After release from the salt block, cells were labeled for 2, 4, 6, 8, 10, and 12 min and then immediately processed for gel electrophoresis. The result of this experiment is shown in Fig. 7. Up to 8 min after synchronous initiation, no discernible NS polypeptides or their precursors were seen. However, after 10 min of incubation, p150 became visible and after 12 min, p215 and p150 were seen. By contrast, the C protein that is known to be nascently cleaved from the translation product of the 26S RNA (9) was visible by 4 min of incubation. This result clearly confirms the suggestion that cleavage only commences after translation of the NS genes has been completed.

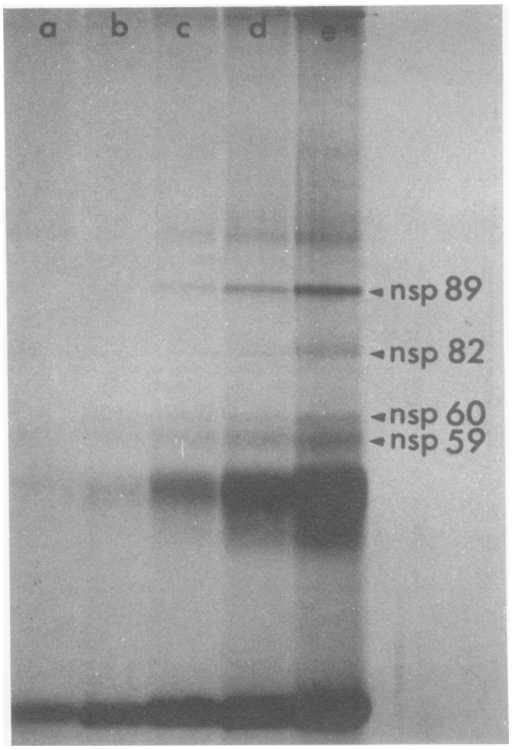

FIG. 6. *Synthesis of virus-specified polypeptides under pulse-chase conditions after synchronous initiation of protein synthesis. Replicate cultures of infected cells were incubated until 4 h postinfection, at which time the medium was replaced with medium containing an extra 225 mM NaCl for 40 min. The hypertonic medium was then replaced with labeling medium for (a) 2 min, (b) 4 min, (c) 6 min, (d) 10 min, and (e) 15 min. At the end of the labeling period, the medium was replaced with chase medium for a further 1 h, and the cells were extracted for gel electrophoresis. Approximately equal quantities (30 μg) of each extract were analyzed.*

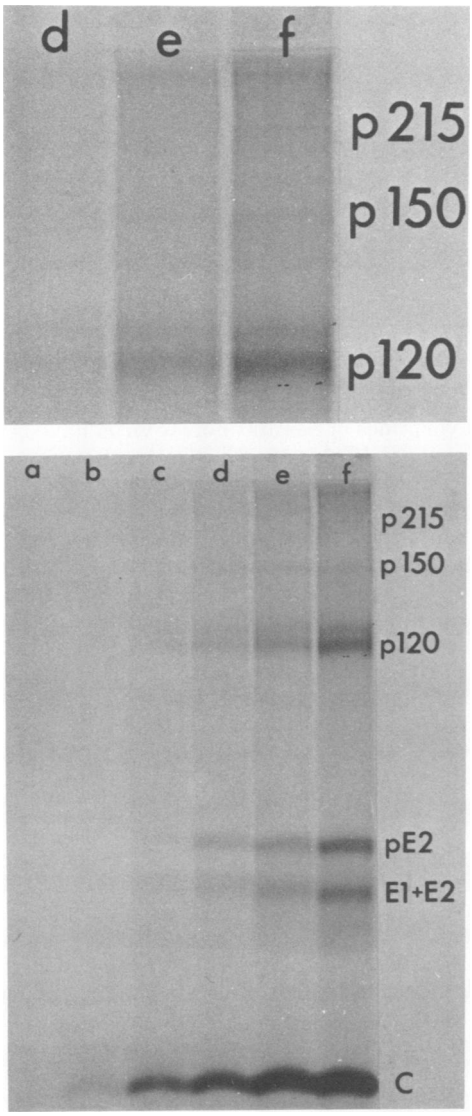

FIG. 7. *Synthesis of virus-specified polypeptides under short-pulse conditions after synchronous initiation of protein synthesis. Replicate cultures of infected cells were incubated until 4 h postinfection, at which time the medium was replaced with medium containing an extra 225 mM NaCl for 40 min. The hypertonic medium was then replaced with labeling medium for (a) 2 min, (b) 4 min, (c) 6 min, (d) 8 min, (e) 10 min, and (f) 12 min. After the pulse, incorporation was immediately stopped, as described in Materials and Methods, and extracts were prepared and analyzed by gel electrophoresis. Approximately equal quantities (50 μg) of each extract were analyzed. The upper panel is a magnified view of the top right-hand corner of the lower panel.*

DISCUSSION

The present work identifies eight novel polypeptides synthesized in chicken cells after infection with Sindbis virus. Most of these polypeptides are synthesized in proportionally greater amounts early in the virus multiplication cycle. Peptide mapping demonstrates that, of these eight polypeptides, only one, NS p59, is related to the structural proteins of the virus particle. NS p59 is closely related to pE2, possibly a subglycosylated variant of it. The remaining seven polypeptides are therefore NS and, as such, represent translation products of the 5′-terminal two-thirds of 42*S* RNA, i.e., that part of the 42*S* not represented in 26*S* RNA. Additional evidence that these seven polypeptides are indeed virus specified is provided by the observations that (i) they are readily detectable in infected but not uninfected hamster cells and (ii) in chicken fibroblasts infected with several RNA-negative mutants, a high-molecular-weight polypeptide, identical by tryptic peptide mapping with p215, accumulates at the nonpermissive, but not at the permissive, temperature (data not presented). Pulse and pulse-chase experiments (Fig. 1 and 2), together with the use of an inhibitor of post-translational cleavage (Fig. 3), permit a division of these seven polypeptides into three stable end products (NS p89, NS p82, and NS p60) and four unstable putative precursors (p230, p215, p150, and p76). Tryptic peptide mapping (Fig. 5) clearly confirms this precursor-product hypothesis and suggests the cleavage scheme shown in Fig. 8 for the expression of the NS genes of Sindbis virus. In this scheme, translation of the NS genes, which is initiated at or near the 5′ end of the genome (5, 16), produces p230. Although we cannot be certain that p230 represents the entire translation product of the NS genes, its molecular weight (230,000) is close to the theoretical coding capacity of the NS region of the 42*S* RNA (approximately 250,000, assuming that the total coding capacities are 420,000 for the 42*S* RNA and 175,000 for the 26*S* RNA). We propose that there are three cleavage sites (A, B, and C) in p230. Cleavage at C generates p150 and p76. Alternatively, p230 may be cleaved at A, generating p215 and fragment X. A careful comparison of the tryptic peptide maps of p230 and p215 in Fig. 5b shows a single peptide (x), which is present in p230 but absent from p215, and this peptide may therefore be characteristic of fragment X. This also indicates that fragment X is terminal in p230. Since the peptide characteristic of fragment X is present in p150, then this fragment must be at the extreme N-terminal end of p230. The fate of fragment X is, however, unclear. Analysis on gradient gels designed to resolve polypeptides in the molecular-weight range expected of X (7,500 to 20,000) have so far failed to provide evidence that fragment X is a stable end product and it may,

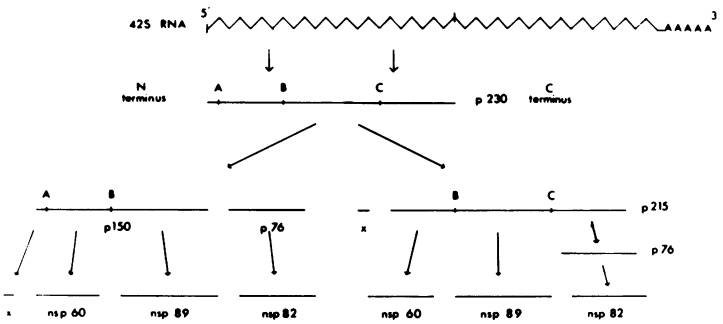

FIG. 8. *Proposed scheme for the synthesis and processing of Sindbis virus NS polypeptides.*

therefore, be degraded. We shall consider a possible role for fragment X later in the discussion. Cleavage of p150 at A and B generates fragment X, NS p60, and NS p89, and cleavage of p215 at B and C generates NS p60, NS p89, and p76. From short-pulse experiments either in unsynchronized (Fig. 2, lane a) or salt-synchronized cells (Fig. 7), it seems clear that the several cleavages outlined above only occur after translation of the NS genes has been completed. This situation contrasts with the one that operates during the synthesis of the structural proteins, in which there is evidence from both in vitro and in vivo studies that the C protein (the N-terminal protein) is nascently cleaved from the translation product of the 26S RNA (4, 6, 7, 9). The final step in the processing scheme converts p76 to NS p82. The nature of this conversion is unclear. The tryptic peptide maps of p76 and NS p82 are very similar (Fig. 5), and our evidence that p76 is indeed the precursor to NS p82 rests on pulse-chase data (Fig. 2). A possible modification of p76 that could result in an apparent increase in molecular weight is glycosylation, and experiments are in progress to investigate this and other possibilities. Salt synchronization experiments (Fig. 6) show that the gene order (5′ → 3′) of the NS genes in 42S RNA is NS p60, NS p89, and NS p82.

Since 42S RNA is infectious (13, 26) some, if not all, of the NS polypeptides described in the present work are extremely likely to be components of the virus-specified RNA-dependent RNA polymerase. The observation that this enzyme is associated with smooth membranes (14) raises the question of how the NS polypeptides could become associated with membrane. One explanation could be that fragment X plays a role analogous to the "signal sequence" present at the N-terminus of several proteins that are either components of, or are destined to cross, cellular membranes (2). Thus, the role of fragment X might be to anchor the NS polypeptide precursors in smooth membrane.

Recently, the synthesis and processing of NS polypeptides of Semliki Forest virus have been described (10, 17, 18, 20, 21). A comparison of the data in these reports with our present observations suggests that at least two of the Sindbis virus NS polypeptides, namely, NS p89 and NS p60, have counterparts in Semliki Forest virus-infected cells. Moreover, in one case, the NS polypeptides (designated NS p90 and NS p63) have been shown to be components of purified RNA polymerase (10). This observation, therefore, supports the idea mentioned earlier that NS p89 and NS p60 may be components of Sindbis virus polymerase. However, in addition to this correspondence, there is the observation of a third NS polypeptide (NS p82) in Sindbis virus-infected chicken cells, which Clegg et al. (10) did not detect in Semliki Forest virus-infected BHK cells. Four NS polypeptides have been identified or inferred in chicken cells infected with a temperature-sensitive mutant of Semliki Forest virus (21). Attempts to detect additional NS polypeptides in Sindbis-infected chicken cells by, for example, extending the labeling time of infected cells to 25 min after release from hypertonic block (more than twice the time required to label NS p82, the C-terminal polypeptide in p230) have not been successful. Because of the possibility that NS polypeptides might be masked by host or other virus-specified polypeptides, notably the structural polypeptides or their precursors, the final solution of not only the question of the number of NS polypeptides, but also the question of their function(s), may have to await the reconstruction of a membrane-associated polymerase capable of synthesizing 42S and 26S RNA of positive polarity and 42S RNA of negative polarity.

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