

RNA Polymerase Components in Semliki Forest Virus-Infected Cells: Synthesis from Large Precursors

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SUMMARY

Two previously undescribed stable polypeptides (referred to as nsp 90 and nsp 63) appear in mammalian and avian cells infected with Semliki Forest virus. They are distinguishable from the virus structural proteins and their known precursors by their molecular weights and tryptic peptide maps, and are identical in size to two polypeptides found in purified preparations of virus-specific RNA polymerase. Data from pulse-chase experiments and from the use of inhibitors of proteolytic cleavage indicate that nsp 90 and nsp 63 are synthesized via a series of post-translational cleavages from three larger polypeptides, p200, p184 and p150. The labelling kinetics after synchronous initiation of protein synthesis are also consistent with the synthesis of nsp 90 and nsp 63 from a common initiation site, and show that nsp 63 is located close to this site. It is concluded that nsp 90 and nsp 63 are components of the virus-specific RNA polymerase, and are synthesized via a post-translational cleavage scheme entirely separate from that leading to the synthesis of the virus structural proteins.

INTRODUCTION

Recent work in several laboratories, using cell-free systems capable of translating exogenous mRNA, has shown that the structural proteins of alphaviruses are synthesized under the direction of sub-genomic RNA (26S RNA) which is found in substantial quantities in infected cells (Cancedda & Schlesinger, 1974; Cancedda, Swanson & Schlesinger, 1974; Clegg & Kennedy, 1974*a*, 1975*a*; Simmons & Strauss, 1974; Wengler, Beato & Hackemack, 1974). Initiation of synthesis on this messenger takes place at a single site located near the beginning of the virus core protein (Clegg & Kennedy, 1975*b*). As translation proceeds the core protein is cleaved from the nascent peptide chain (Clegg, 1975), and the remainder is cleaved before or after termination to yield one envelope protein (E1) and a precursor of the other two (E2 and E3), which in turn is cleaved to give the final products (Schlesinger & Schlesinger, 1972, 1973; Morser, Kennedy & Burke, 1973; Simons, Keränen & Kääriäinen, 1973; Morser & Burke, 1974). Although it is established that the three envelope proteins are glycosylated (Garoff, Simons & Renkonen, 1974), the extent of glycosylation of their precursors remains unclear. With the exception of E3, which is very small, these structural proteins and their precursors can be detected readily by polyacrylamide gel electrophoresis of extracts of infected cells labelled with radioactive amino acids.

As well as the structural proteins and their precursors, alphavirus-infected cells contain an RNA-dependent RNA polymerase activity which functions in the replication and

transcription of virus-specific RNA (Martin & Sonnabend, 1967). Since isolated virion RNA (42S; mol. wt. about 4.2×10^6) is infectious and isolated 26S RNA (mol. wt. about 1.8×10^6) is not (Scheele & Pfefferkorn, 1969), it is reasonable to conclude that any virus-specified component of the polymerase is synthesized under the direction of 42S RNA, using as template that part of its sequence which is not contained in 26S RNA. Since it has been shown that 26S RNA contains the nucleotide sequence inward from the 3'-terminus of 42S RNA (S. I. T. Kennedy, manuscript in preparation), the putative RNA polymerase genes must be located nearer the 5'-terminus in a region which the difference in mol. wt. between 42S RNA and 26S RNA indicates can potentially code for about 260000 daltons of polypeptide, if fully translated.

In cell-free systems 42S RNA has been found to direct the synthesis of material distinguishable from the structural proteins of the virus. These products have taken the form either of a set of several polypeptides higher in mol. wt. than the virus structural proteins (Simmons & Strauss, 1974; Cancedda *et al.* 1975), or of a set of tryptic peptides not found among those derived from the virus structural proteins (Kääriäinen *et al.* 1975). However, there have also been reports of the identification of virus-specific polypeptides in infected cells which are apparently unrelated to the structural proteins (Morser *et al.* 1973; Lachmi *et al.* 1975), but the significance of these results has been difficult to assess in the absence of information on the molecular composition of the RNA polymerase.

In this paper we show that in Semliki Forest virus (SFV)-infected cells two polypeptides exist, distinguishable from the virus structural proteins and their precursors by their electrophoretic behaviour and their tryptic peptide maps and which are synthesized from a common initiation site via a post-translational cleavage scheme completely separate from the one leading to the synthesis of the structural proteins of the virus. Furthermore, these two polypeptides are electrophoretically identical to two found associated with SFV RNA polymerase activity, and are thus probably virus-coded components of the nucleic acid synthesizing machinery of the virus.

METHODS

Materials. Acrylamide and sodium dodecyl sulphate (especially pure grade) were obtained from British Drug Houses Ltd, Poole, Dorset; *N,N'*-methylenebisacrylamide from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; trypsin, treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone, from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; canavanine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), ovalbumin, catalase, bovine serum albumin, human transferrin and β -galactosidase (from *E. coli*) from Sigma Chemical Co., London; L-³⁵S-methionine (300 to 400 Ci/mmol), L-³H-leucine (40 to 60 Ci/mmol) and L-³H-valine (1.5 Ci/mmol) from the Radiochemical Centre, Amersham; Soluene from Packard Instrument Co. Ltd, London; and pre-coated silica gel plates for thin layer chromatography from Schleicher and Schüll, Dassel, W. Germany. Actinomycin D was a gift of Merck, Sharpe and Dohme Research Laboratories, Rahway, N.J., U.S.A. Myosin was purified from rabbit skeletal muscle by the method described by Perry (1955).

Virus. A three-times plaque-purified wild-type strain of SFV was used as described previously (Kennedy & Burke, 1972; Clegg & Kennedy, 1974b).

Cells and media. Monolayer cultures of BHK cells, clone 13, were grown in glass scintillation vials or in 1 l glass flat-sided bottles in the Glasgow modification of Eagle's minimal essential medium, supplemented with 0.3 % tryptose phosphate broth and 10 % calf serum. During and after infection cells were maintained in medium 199 supplemented

with 2 % calf serum and 1 μ g actinomycin D/ml and buffered with 20 mM-HEPES sodium salt (maintenance medium). Radioactive precursors were supplied in Earle's salts solution supplemented with 2 % dialysed calf serum, 1 μ g actinomycin D/ml, and also buffered with 20 mM-HEPES sodium salt (HEDA).

Labelling of virus-specific proteins. Cells were cooled to 4 °C, washed once with cold phosphate-buffered saline, infected at an input multiplicity of approx. 100 with virus diluted in maintenance medium, and left for 1 h at 4 °C. The fluid was then replaced with pre-warmed maintenance medium and the cells incubated at 37 °C. After the appropriate time, specified relative to the end of the adsorption period, the medium was replaced with HEDA containing 10 to 50 μ Ci 35 S-methionine/ml. At the end of the labelling period, incorporation of radioactivity was stopped by removal of medium followed by immediate immersion in a methanol-solid CO₂ bath and addition of 5 % trichloroacetic acid containing 1 % casamino acids, or by replacement with maintenance medium supplemented with 1 mM-non-radioactive methionine and further incubation at 37 °C. This chase period was ended by freezing the cells as described above.

Synchronization of initiation of protein synthesis. Infected cells were treated for 40 min with HEDA made hypertonic by addition of 225 mM-NaCl (Saborio, Pong & Koch, 1974; Clegg, 1975). Synchronous initiation took place on return to isotonic medium.

SDS-gel electrophoresis. Labelled cell monolayers were allowed to thaw, washed once in 5 % trichloroacetic acid containing 1 % casamino acids, washed twice in ethanol-ether (1:3, v/v) and air-dried. They were then dissolved in 2 % SDS, 1 % 2-mercaptoethanol, 50 mM-tris (pH 9.0), heated in a boiling water bath for 3 min, cooled to 37 °C, and iodoacetamide added to a concentration of 0.2 M. After 1 h the solution was dialysed against 0.1 % SDS, 40 mM-boric acid, 40 mM-tris (pH 8.6).

Proteins were analysed on slab gels containing 7.5 % acrylamide and 0.2 % methylene-bisacrylamide in an apparatus similar to that of Studier (1973), using either the discontinuous buffer system of Laemmli (1970) or the continuous system of Fairbanks, Steck & Wallach (1971), which contains 1 % SDS throughout. For mol. wt. determinations the latter system was used, with the mol. wt. markers myosin heavy chain (200 000), β -galactosidase (130 000), human transferrin (80 000), bovine serum albumin (68 000), catalase (58 000) and ovalbumin (43 000). The positions of these proteins were revealed by Coomassie blue staining, and those of radioactive proteins by autoradiography of the dried gels.

Tryptic peptide mapping. Strips of acrylamide gel containing 35 S-methionine-labelled polypeptides for mapping were cut from the dried gel. The polypeptides from a small fragment of gel were eluted into 0.1 % SDS and used to monitor the purity of the preparation by polyacrylamide gel electrophoresis. The remainder of the gel strip was washed overnight with continuous agitation in about 500 vol. of 20 % ethanol, 10 % acetic acid. After a further 60 min wash in this mixture, the gel was neutralized by two successive 30 min washes in 10 mM-NH₄HCO₃. The gel pieces were then mixed with about 10 vol. of 10 mM-NH₄OH containing 50 μ g trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) per ml and incubated for 6 h at 37 °C with occasional swirling. The fluid was removed, centrifuged at low speed to remove debris and lyophilized. After dissolution in water and further lyophilization, the tryptic peptides were performic acid-oxidized at -8 °C by the method of Bray & Brownlee (1973), again lyophilized, dissolved in a small vol. of 10 mM-NH₄OH and stored at -70 °C. The peptides were analysed on 20 \times 20 cm silica gel coated plates (0.25 mm layer thickness) by two-dimensional chromatography. The solvent in the first dimension was methyl acetate/isopropanol/25 % NH₄OH (3:2:2 by vol.), and in the second dimension was butan-1-ol/acetic acid/water (3:1:1

by vol.). The plates were dried at 80 °C for 30 min after the first dimension. To maintain comparability the dye xylene cyanol FF was run on every plate to a predetermined position. Radioactive peptides were located by autoradiography.

Assay of virus-specific RNA synthesis. Vial cultures of BHK cells were pre-incubated for 1 h at 37 °C in maintenance medium containing actinomycin D (1 µg/ml), then infected with 100 p.f.u. of SFV per cell for 1 h at 4 °C and incubated at 37 °C in maintenance medium. RNA was labelled by replacing the medium with HEDA containing ³H-uridine (2 µCi/ml). After 30 min the cells were frozen in a methanol-solid CO₂ bath, washed twice in 5 % trichloroacetic acid, twice in ethanol/ether (1:3, v/v), air-dried, and finally dissolved in Soluene. ³H-activity was measured by liquid scintillation spectrometry after addition of toluene scintillator.

RESULTS

Synthesis of virus proteins during the infectious cycle

In order to detect previously undescribed polypeptides synthesized in cells infected with SFV, the pattern of protein synthesis was surveyed at different times after infection using the high resolving power of the Laemmli (1970) gel system coupled to autoradiography. Monolayers of BHK cells in scintillation vials were first labelled with ³H-leucine (40 µCi/ml; 45 min), then infected with SFV, and labelled with ³⁵S-methionine (10 µCi/ml) for 30 min periods at different times after infection. The ³H-content of the protein extracts was measured by liquid scintillation spectrometry and used to load equivalent quantities of material from each time point onto the gel (Fig. 1). The ³H-labelled host polypeptides are not detectable by autoradiography under the conditions used. The pattern of polypeptides synthesized in mock-infected cells is shown in lane a. No qualitative change can be detected in this pattern within the time required for completion of the infectious cycle of SFV. Analysis of the infected cell extracts shows drastic changes in the polypeptides they synthesize. The proportion of labelled host-specified polypeptides decreases steadily from about 2 h post-infection (p.i.), until by 6 h, after virus release has reached its maximum rate (Bruton & Kennedy, 1975), the virus structural proteins and their precursors dominate the pattern. These are the core protein (C), the envelope protein E₁, and the precursor of E₂ and E₃, p68 (the nomenclature of the virus-specific polypeptides, other than the structural, is based on their mol. wt. determined as described below). Also visible are p120, a precursor of all three envelope proteins, and p136, which appears to contain the amino acid sequences of the virus envelope and core proteins (Keränen & Kääriäinen, 1975), although some doubt has been cast on its postulated role as a precursor (Clegg, 1975). These polypeptides are fairly prominent against the background of host material by 2 to 2.5 h p.i., and are first visible when labelled from 1.5 to 2 h. However, two further polypeptides, p200 and p184, become visible when the cells are labelled as early as 1 to 1.5 h after infection, and a third, p150, is also apparent from 1.5 to 2 h onwards. These products are present only at low levels, but it appears that their synthesis reaches a maximum about 2 h p.i. and declines thereafter, although they are still being synthesized as late as 6 to 6.5 h p.i.

The stability of these three large polypeptides was examined during chase periods of different length after labelling them from 2 to 2.5 h p.i. (Fig. 2). Within a chase period of 1 h the p68 found immediately after the 30 min pulse (lanes b and e) is converted almost completely to E₂, which is well resolved from E₁ in the discontinuous system (lane c), and presumably to E₃, which is not visible on these gels. At the same time the large polypeptides

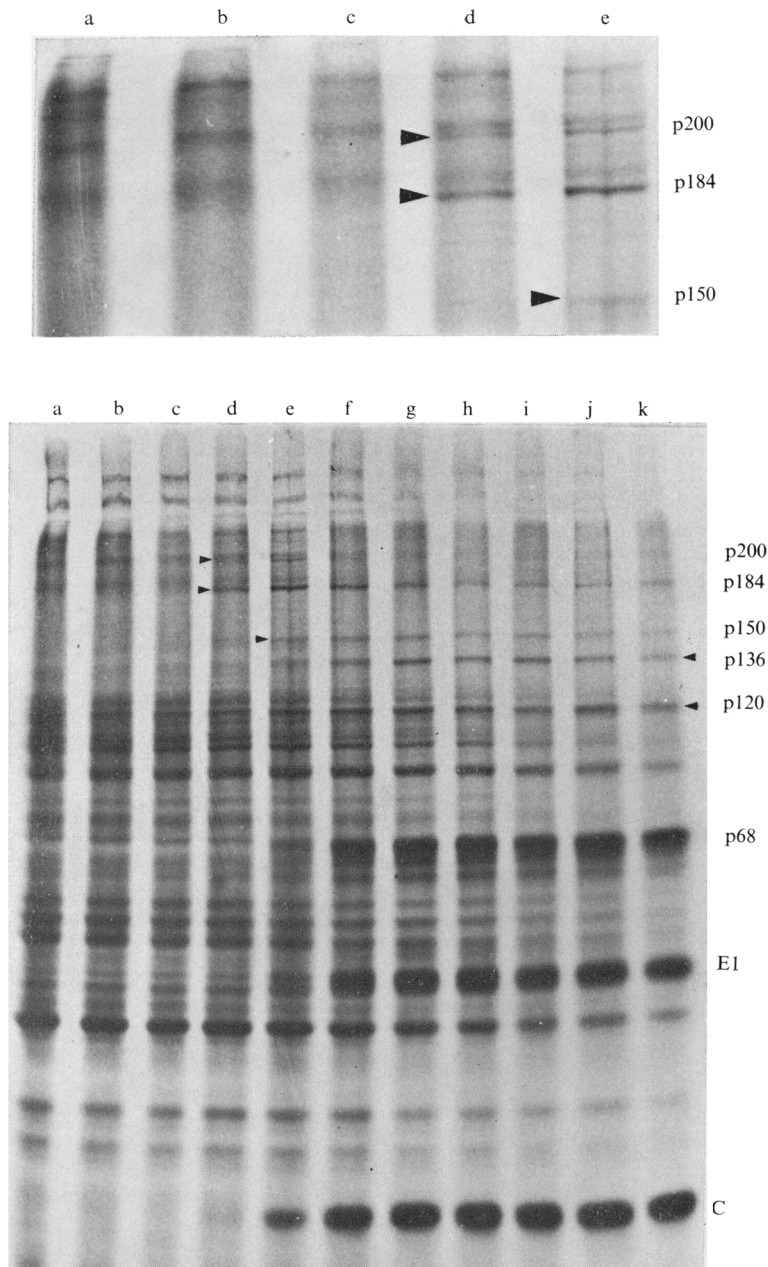


Fig. 1. Development of virus protein synthesis during infection. Vial cultures of BHK cells pre-labelled with ^3H -leucine were infected with SFV and then labelled for 30 min periods with ^{35}S -methionine starting at the indicated times. Samples of solubilized proteins containing equal ^3H -activities were analysed on a discontinuous gel. ^{35}S -labelled polypeptides were detected by autoradiography. a, mock-infected cells. SFV-infected cells labelled from: b, 0 h; c, 0.5 h; d, 1 h; e, 1.5 h; f, 2 h; g, 2.5 h; h, 3 h; i, 3.5 h; j, 4 h and k, 6 h after infection. The upper panel shows an enlarged view of the top of the resolving gel containing lanes a to e.

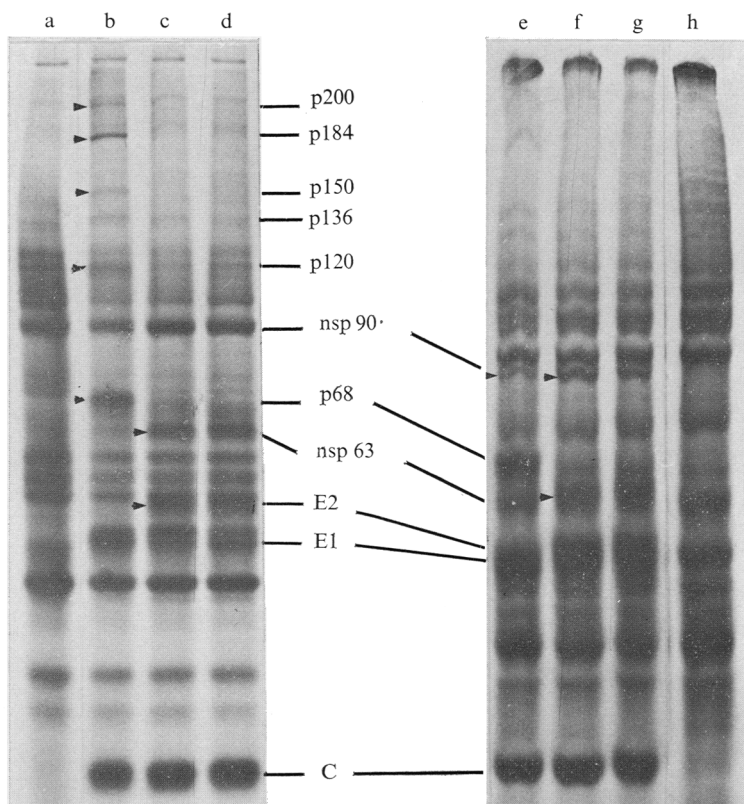


Fig. 2. Stability of virus-specific proteins. Vial cultures were labelled with ^{35}S -methionine from 2 to 2.5 h after infection with SFV, and proteins analysed immediately, or after chase periods of 1 h or 3.5 h. Equal activities of ^{35}S -labelled protein were analysed on a to d, a discontinuous gel, and e to h, a continuous gel. a and h, mock-infected cells. SFV-infected cells with b and e, no chase, c and f, 1 h chase, d and g, 3.5 h chase.

p200, p184 and p150 disappear completely, while another polypeptide nsp 63, migrating somewhat faster than p68, increases markedly in intensity (lane c). Analysis of the same extracts in the continuous gel system shows that there is a similar increase in intensity of another polypeptide, nsp 90 (lane f), which in discontinuous gels is masked by host cell material. The prefix 'nsp' stands for non-structural polypeptide, meaning one not related to the virus structural proteins or their precursors (see the tryptic peptide map data below). A chase of 3.5 h results in a pattern of polypeptides identical with that seen after a 1 h chase (lane d and g), thus indicating that nsp 90 and nsp 63, once formed, are stable for the remainder of the infectious cycle. All five of the novel proteins p200, p184, p150, nsp 90 and nsp 63 can also be seen in extracts of SFV-infected primary chick cells labelled in the same way as the BHK cells described above. Polypeptides comparable but not identical in size have also been observed in BHK and chick cells infected with wild-type Sindbis virus (results not shown).

Molecular weight determinations

Using the mol. wt. markers mentioned in Methods, it was established that the continuous gel system of Fairbanks *et al.* (1971) with a concentration of 7.5% acrylamide gives a

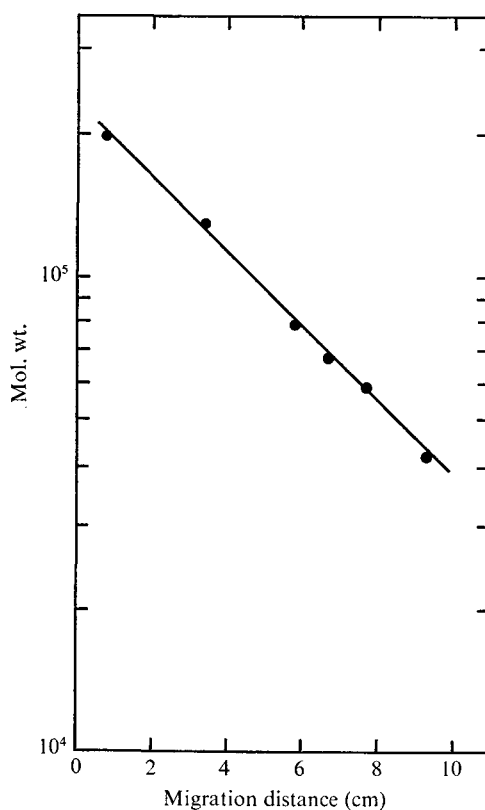


Fig. 3. Calibration of continuous gels with markers of known mol. wt. Mixtures of the proteins described in the text were analysed in the gel system of Fairbanks *et al.* (1971), and their positions revealed by staining with Coomassie blue. The positions of virus-specific proteins in adjacent lanes were determined by autoradiography of the dried gels.

Table 1. *Molecular weights of virus-specific proteins*

	Designation of protein	Mol. wt. $\times 10^{-3}$
Polypeptides related to the structural proteins	p136	136
	p120	120
	p68	68
	E1	49
RNA polymerase components and precursors	p200	200
	p184	184
	p150	150
	nsp90	90
	nsp63	63

linear relationship between the logarithm of the mol. wt. and mobility in the range between 200000 and 40000 (Fig. 3). Although the discontinuous gels have better resolution in the high mol. wt. region, the linear relationship does not hold over such a wide range. The continuous gel system was therefore used to determine the apparent mol. wt. of the virus polypeptides (other than the structural proteins) which are summarized in Table 1, and

which form the basis of the nomenclature used here for the virus-specific proteins not found in the virus particle.

Tryptic peptide maps

The fact that the five novel proteins p200, p184, p150, nsp 90 and nsp 63 can be detected in SFV-infected cells of both avian and mammalian origin, and are absent from both types of uninfected cell, indicates that they are probably coded for by the virus genome. Tryptic peptide mapping by two-dimensional chromatography was used to investigate the relationship between some of these novel polypeptides and the structural proteins of the virus. Unfortunately, it has not yet been possible to prepare sufficient quantities of the three large unstable polypeptides for tryptic peptide mapping, but adequate quantities of nsp 90, nsp 63, C, E1, E2 and p68 were purified by SDS-gel electrophoresis. To reduce contamination by labelled host material the cells were exposed to ^{35}S -methionine from 4 to 5 h p.i., by which time the virus-induced shut-off of host protein synthesis was pronounced. After the labelling period the cells were incubated with medium containing excess non-radioactive methionine to allow accumulation of stable products. Polypeptides of mock-infected cells were also separated on gels and the regions corresponding in mobility to nsp 90 and nsp 63 cut out and processed. The results are illustrated in Fig. 4. Each polypeptide gives a characteristic pattern of radioactive tryptic peptides, and the technique shows, as expected, that the virus structural proteins C, E1 and E2 have unrelated amino acid sequences. However, there are many correspondences between the peptides of E2 and p68, which demonstrate the close relationship that has been found in previous studies (Schlesinger & Schlesinger, 1972; Simons *et al.* 1973). Comparison of the map of nsp 90 with that of the similarly migrating polypeptide from uninfected BHK cells shows that the maps are completely different; it is clear that the nsp 90 map is free of many of the prominent peptides of its host cell counterpart, so it follows that the nsp 90 map shown is representative of virus-specific material. Similar arguments apply to the nsp 63 map and that of the corresponding region in gels of extracts of uninfected cells.

Comparison of the nsp 90 map with those of the virus structural proteins shows that there is a complete lack of correspondence between them, indicating that nsp 90 contains amino acid sequences not found in the structural proteins, and that no sequences of the latter are found in nsp 90. Similar comparisons show that the map of nsp 63 contains peptides of two classes. One class consists of peptides which are characteristic of p68 and in some cases of E2, and the other class (indicated by arrows) consists of peptides not found in any structural protein, nor in nsp 90. While this result can be interpreted to mean that nsp 63 contains amino acid sequences characteristic of p68 as well as non-structural sequences, this is improbable for two reasons. Since nsp 63 is smaller than p68 it is unlikely that it will have a greater sequence complexity. Also, it is difficult to envisage synthesis of a polypeptide containing the p68 amino acid sequence linked to one not found in structural proteins, since the gene for p68 is located between those for the core protein and E1 (Clegg, 1975). A more probable explanation is that the nsp 63 preparation is contaminated with material containing p68 sequences. Since contamination by p68 itself at the level indicated by the peptide map would have been apparent in the electrophoretic re-analysis of the purified protein, it is possible that the contaminant is a non- or sub-glycosylated version of p68, which might be expected to migrate about the position of nsp 63. In any event the tryptic peptide maps show that nsp 90 and nsp 63 do not contain common amino acid sequences and are the result of the expression of genetic information other than that coding for the structural proteins of the virus.

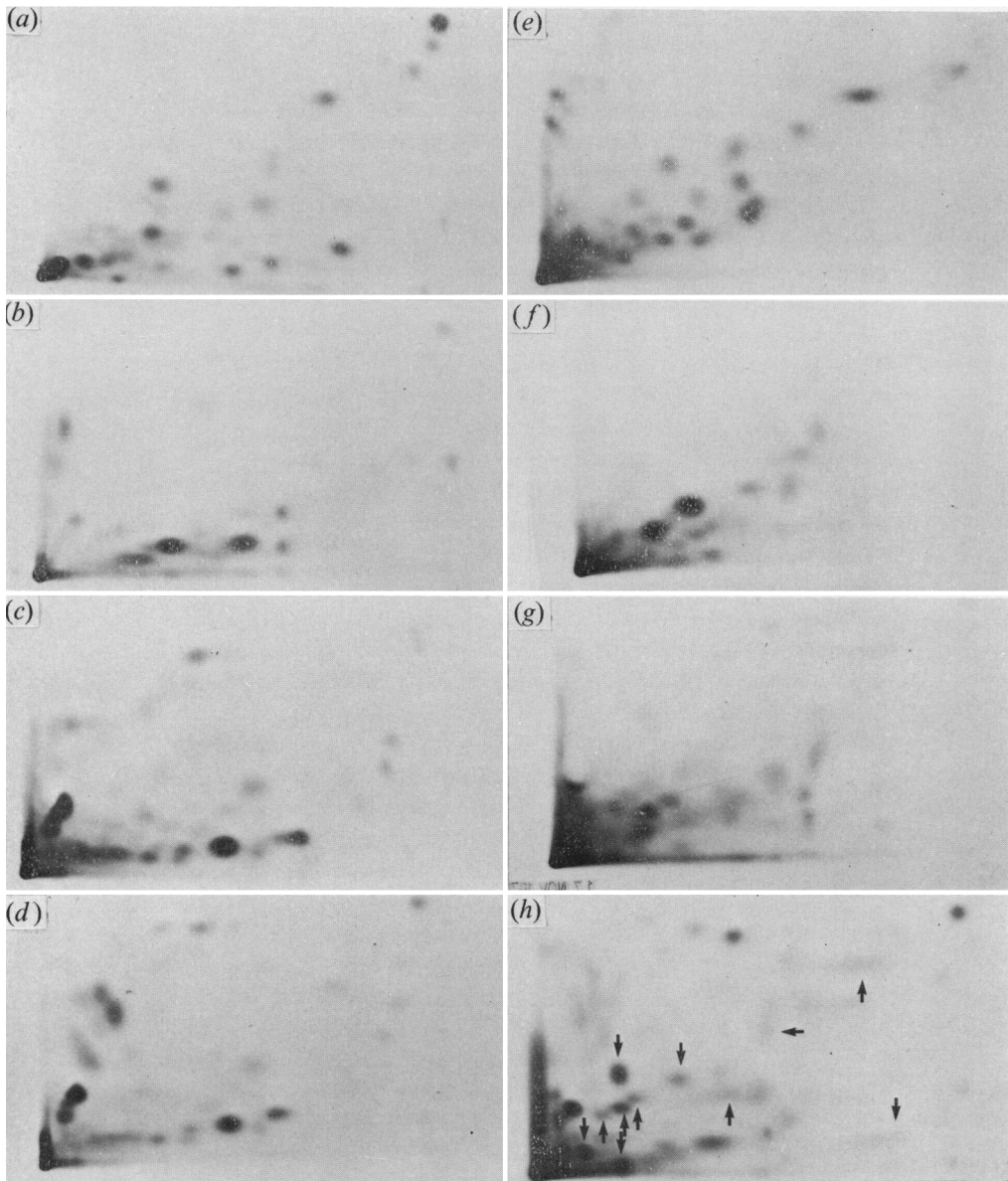


Fig. 4. Tryptic peptide maps of virus-specific proteins. Proteins from infected cells were isolated on preparative polyacrylamide gels, digested with trypsin, and the performic acid-oxidized peptides analysed by two-dimensional thin layer chromatography. A xylene cyanol FF dye marker was run to the same pre-determined position in each case, and ^{35}S -methionine-labelled peptides detected by autoradiography. The first dimension is from left to right, the second from bottom to top. (a) C, (b) E1, (c) E2, (d) p68, (e) protein from mock-infected cells with same mobility as nsp 90, (f) nsp 90, (g) protein from mock-infected cells with same mobility as nsp 63, (h) nsp 63. Positions of some peptides found only in nsp 63 are indicated by arrows.

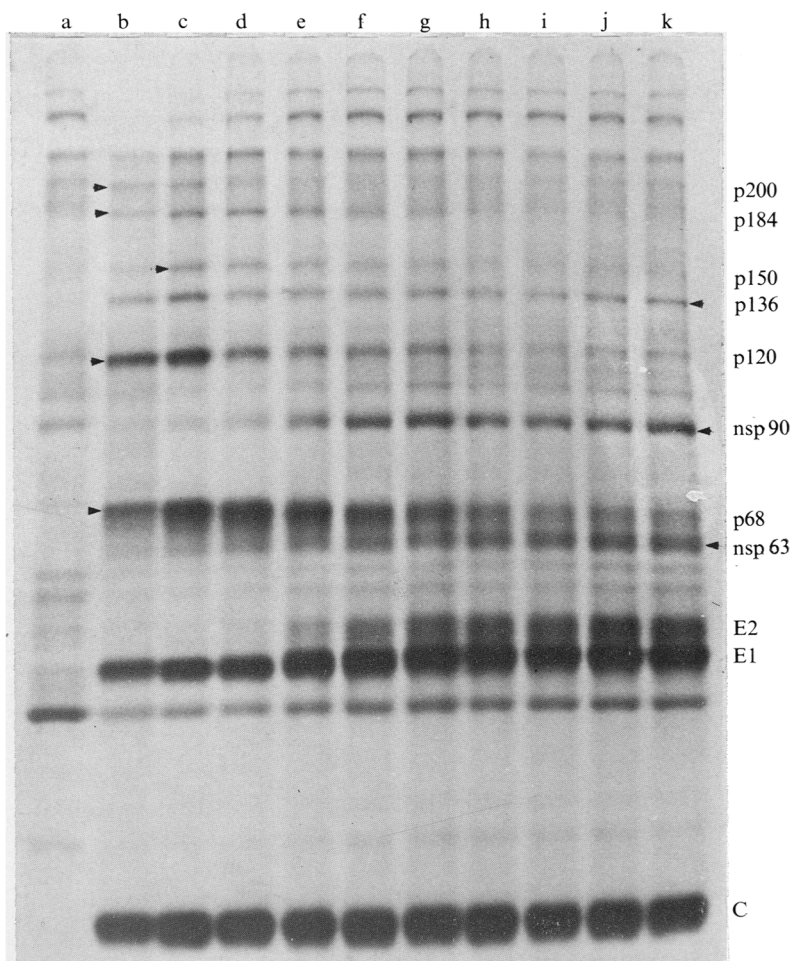


Fig. 5. Pulse-chase analysis of virus-specific proteins. Vial cultures of cells pre-labelled with ^3H -valine were labelled with ^{35}S -methionine for 5 min, starting 2.5 h p.i. with SFV. The cultures were then incubated in maintenance medium containing excess non-radioactive methionine for periods up to 45 min. Protein extracts containing equal ^3H -activities were analysed on a discontinuous gel. a, Mock-infected cells. SFV-infected cells chased for: b, 0 min; c, 5 min; d, 10 min; e, 15 min; f, 20 min; g, 25 min; h, 30 min; i, 35 min; j, 40 min and k, 45 min.

Synthesis of nsp 90 and nsp 63 via p200, p184 and p150

The data described in Fig. 2 suggest that the stable products nsp 90 and nsp 63 are formed as a result of the processing of p200, p184 and p150, i.e. that these three large polypeptides are precursors of the smaller ones. This possibility is supported by the results of a more detailed pulse-chase experiment (Fig. 5). BHK cells, pre-labelled with ^3H -valine before infection as described above, were labelled with ^{35}S -methionine for 5 min starting 2.5 h p.i., and then further incubated in the presence of excess non-radioactive methionine for the times indicated. The discontinuous gel was loaded with equal quantities of ^3H -label. It is clear that p120 and p68, precursors of the virus envelope proteins, behave as expected during the chase period, declining in intensity as the chase proceeds, while

one of their products, E2, becomes more and more prominent. The comparable increase in intensity of E1 is to some extent masked by the large quantity of this polypeptide which is found immediately after labelling, presumably as a result of cleavage before, or immediately after, completion of translation of p120. The small glycoprotein, E3, also derived from p68, migrates with the buffer front in these gels. However, p136, which contains the amino acid sequence of all the structural proteins of the virus (Keränen & Kääriäinen, 1975), appears to remain largely stable during the chase period. This polypeptide may thus be a dead-end product, not contributing significantly to the pool of virus structural proteins. Turning now to the novel polypeptides, p200 and p184, visible immediately after the 5 min pulse, are more clearly apparent after a 5 min chase, probably due to the completion of nascent chains, and p150 increases markedly after a 5 min chase. When the chase period is increased to 10 min the intensity of p200 declines rapidly, while the intensities of p184 and p150 fall at a lower rate, becoming barely visible after a chase period of 30 min. Immediately after the 5 min pulse nsp 90 and nsp 63 are only slightly labelled, but as the chase proceeds the intensities of these two proteins rise together with kinetics approximating very closely the decline of p184 and p150. Thus, the labelling behaviour of these polypeptides is entirely consistent with the flow of label from p200 through p184 and p150 to give nsp 90 and nsp 63.

This hypothesis was tested further by examining the effects of addition of inhibitors of proteolytic cleavage to the infected cells. Those chosen were zinc ions, which have been shown to inhibit the processing of the proteins of some picornaviruses (Butterworth & Korant, 1974), and the arginine analogue canavanine. The presence of this analogue impairs the synthesis of the virus envelope proteins, possibly by an effect on cleavage from their precursors (Ranki, 1972), and also completely inhibits the appearance of virus-specific RNA polymerase activity when added early after infection (Ranki & Kääriäinen, 1970). After 1.75 h of infection by SFV, cultures of BHK cells were given HEDA containing 0.1 mM- or 0.8 mM-ZnCl₂, or 2 mM-canavanine, and after a further 15 min incubation ³⁵S-methionine was added to a concentration of 10 µCi/ml. Incorporation was stopped after 30 min either by freezing the cells, or by replacing the medium with chase medium containing excess non-radioactive methionine and the same inhibitors. These cultures were chased for 30 min. Proteins were prepared and analysed on both discontinuous and continuous gels as shown in Fig. 6. It is clear that in the absence of inhibitors the polypeptides p200, p184 and p150 visible immediately after the labelling period (lane b) completely disappear after a 30 min chase, while there is a rise in the labelling intensities of nsp 90 and nsp 63 (lanes c and i). However, when both labelling and chase are carried out in the presence of zinc ions or canavanine, the synthesis of p150 is markedly reduced and the appearance of nsp 90 and nsp 63 is completely blocked (lanes d to f, j to l). The presence of p200 and p184 after labelling and chase under such conditions indicates that the processing of these polypeptides has been severely impaired at the same time. The most economical explanation of these results is that the stable polypeptides nsp 90 and nsp 63 are end products of a series of proteolytic cleavages acting on their precursors p200, p184 and p150, and that this processing system is susceptible to inhibition by zinc ions and canavanine. Certain polypeptides labelled in the presence of canavanine, notably C and E1, migrate more slowly than their normal counterparts in the discontinuous gel system. Since this is not the case in the continuous system it may reflect an electric charge effect which manifests itself in 0.1 % SDS, but which is masked in 1 % SDS.

One implication of such an origin of nsp 90 and nsp 63 which is amenable to experimental test is that both should be synthesized from a single site of initiation of protein synthesis,

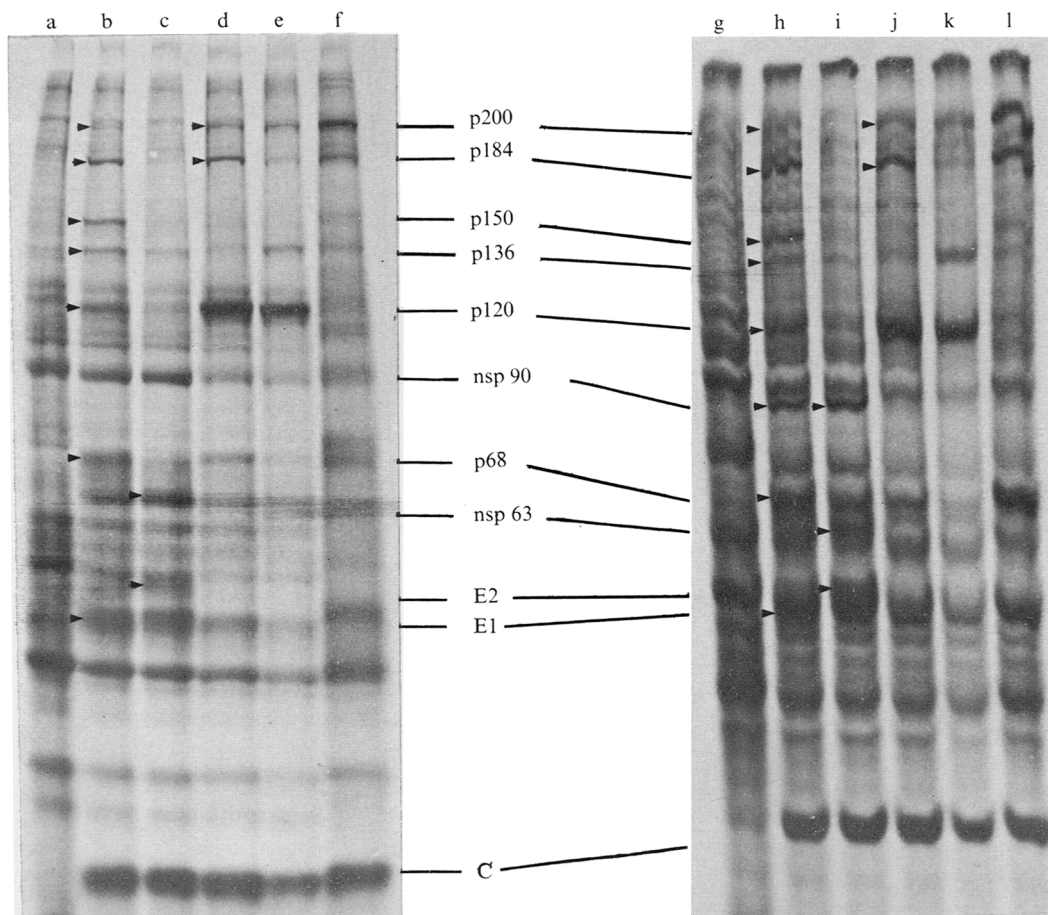


Fig. 6. Effect of inhibitors of proteolytic cleavage on virus-specific protein synthesis. Vial cultures of cells were exposed to the inhibitor indicated 1.75 h p.i. with SFV, and ^{35}S -methionine was added 15 min later. After a further 30 min the medium was replaced with maintenance medium containing excess non-radioactive methionine and the same inhibitor, and incubation continued for a further 30 min. Protein extracts containing equal quantities of radioactivity were analysed on a to f, a discontinuous or g to l, a continuous gel. a and g, Mock-infected cells. SFV-infected cells with: b and h, no inhibitor, no chase; c and i, no inhibitor, with chase; d and j, label and chase with 0.1 mM- Zn^{2+} ; e and k, label and chase with 0.8 mM- Zn^{2+} ; f and l, label and chase with 2 mM-canavanine.

and thus should be labelled with different kinetics after synchronous initiation in infected cells. Such a system, involving treatment of the infected cells with hypertonic medium followed by release from the consequent initiation block, has been used to establish the mode and order of synthesis of the structural proteins of SFV (Clegg, 1975; Clegg & Kennedy, 1975*b*). After incubation in hypertonic HEDA for 40 min, the cells were returned to isotonic HEDA at 2.5 h p.i. and labelled with ^{35}S -methionine (50 $\mu\text{Ci}/\text{ml}$) for periods of 2, 4, or 6 min. They were then transferred to maintenance medium containing excess non-radioactive methionine and incubated until 4 h p.i. Cell extracts were analysed in the discontinuous and continuous gel systems (Fig. 7), where it is clearly shown that

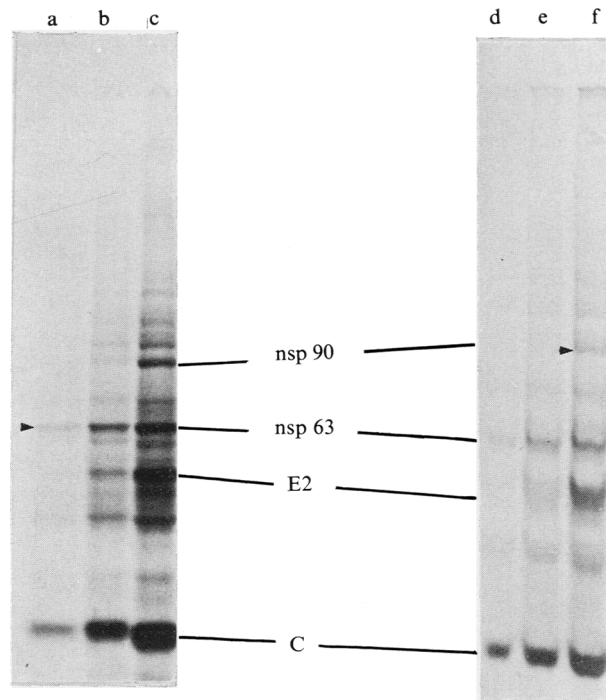


Fig. 7. Synthesis of virus-specific polypeptides after synchronous initiation of protein synthesis. Vial cultures of SFV-infected cells were treated for 40 min with HEDA made hypertonic by addition of 225 mM-NaCl, starting 1 h 50 min p.i. At 2.5 h p.i. this medium was replaced with isotonic HEDA containing ^{35}S -methionine and the cultures labelled for short periods as indicated, and chased in maintenance medium containing excess non-radioactive methionine until 4 h p.i. Approximately equal quantities of cell extracts were analysed on a to c, a discontinuous, or d to f, a continuous gel. The cells were labelled for: a and d, 2 min; b and e, 4 min; c and f, 6 min.

2 min after initiation nsp 63 is labelled in the absence of detectable nsp 90 (lanes a and d). Nsp 90 is detectable by 4 min after initiation (lanes b and e) and becomes quite prominent by 6 min (lanes c and f). These observations are entirely consistent with a model involving cleavage of both nsp 63 and nsp 90 from a common precursor, and further indicate that nsp 63 is located close to the initiation site for protein synthesis.

Identification of nsp 90 and nsp 63 as components of the virus RNA polymerase

Synthesis of virus RNA in alphavirus-infected cells is only sensitive to inhibition of protein synthesis early in the infectious cycle (Scheele & Pfefferkorn, 1969; Friedman & Grimley, 1969; Wengler & Wengler, 1975), suggesting that the components of the RNA polymerase synthesized early after infection continue to function during the whole cycle. It was shown by Ranki & Kääriäinen (1970) that the sensitivity to canavanine of RNA synthesis in SFV-infected cells varied in a similar way; although it prevented RNA synthesis altogether when given up to 1 h p.i.; when applied 3 h p.i. it had very little effect. We have tested the effect of addition of cycloheximide, canavanine and zinc ions at different times after infection on the subsequent level of synthesis of RNA. It is apparent from the data in Table 2 that both cycloheximide and canavanine only have an inhibitory

Table 2. *Effect of inhibitors of protein synthesis or cleavage on virus-specific RNA synthesis*

Inhibitor	Acid-precipitable ^3H -activity (ct/min) Time of addition of inhibitor*	
	1 h	3 h
None	97 700	128 574
Cycloheximide (0.03 mM)	35 484	147 550
Zinc ions (0.1 mM)	12 500	142 830
Canavanine (2 mM)	18 762	142 545

* From the end of the adsorption period.

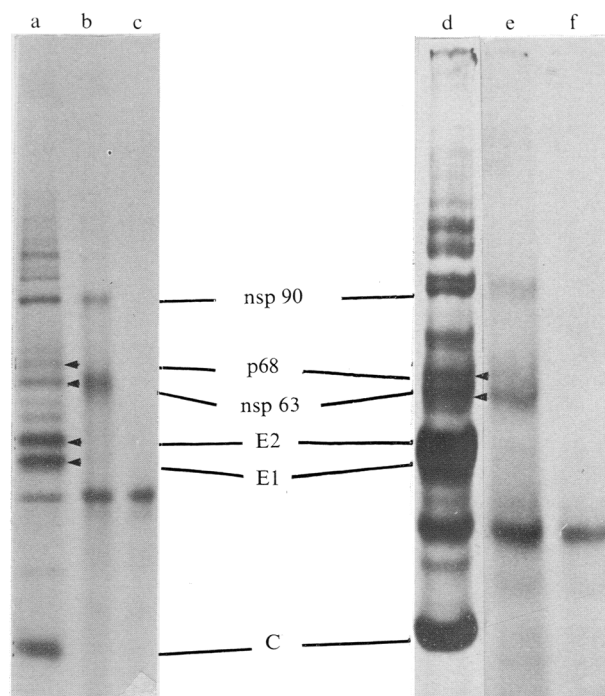


Fig. 8. Co-electrophoresis of nsp 90 and nsp 63 with purified virus-specific RNA polymerase. Vial cultures were labelled with ^{35}S -methionine from 2 to 2.5 h after infection with SFV, and chased for a further 1 h in maintenance medium containing excess non-radioactive methionine. Cell extracts were run in: a to c, a discontinuous, or d to f, a continuous gel, together with samples of purified SFV-specific RNA polymerase. a and d, Infected cell extract; b and e, SFV-specific RNA polymerase; c and f, preparation from mock-infected cells after purification by the same procedure as for the RNA polymerase.

effect when added at 1 h p.i.; by 3 h p.i. RNA synthesis has become refractory to these inhibitors, as shown by the authors cited above.

Furthermore, the effect of zinc ions on RNA synthesis has an identical temporal dependence. Since the results indicate that none of these agents directly affect the activity of the polymerase, they must exert their inhibitory action at early times through inhibition of the synthesis of the polymerase components themselves. Since we have shown above that both canavanine and zinc ions prevent the appearance of nsp 90 and nsp 63, it is an

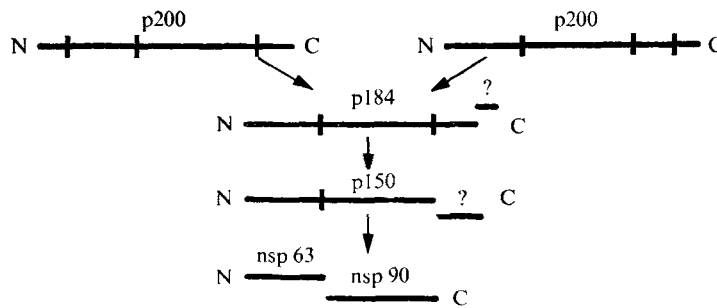


Fig. 9. Proposed alternative cleavage schemes for the synthesis of virus-specified components of the SFV-specific RNA polymerase.

attractive conclusion that either or both of these polypeptides are components of the virus-specific RNA polymerase.

Recently a more direct approach to identifying the functions of nsp 63 and nsp 90 became available with the purification of enzymically active virus-specific RNA polymerase from SFV-infected cells (see previous paper, Clewley & Kennedy, 1976). The polymerase was purified from infected cells labelled with ^{35}S -methionine and analysed in the discontinuous and continuous gel systems, together with a crude cell extract containing labelled nsp 90 and nsp 63. It is shown in Fig. 8 that as well as a host cell protein, which is also found in material purified by the same method from uninfected cells, the polymerase activity is associated with two polypeptides co-migrating in both gel systems with nsp 90 and nsp 63. This evidence suggests very strongly that these are viral RNA polymerase components, although we have not so far been able to prepare ^{35}S -labelled active RNA polymerase of sufficiently high specific activity to allow proof of this point by tryptic peptide mapping.

DISCUSSION

The evidence presented above suggests that nsp 90 and nsp 63, two previously undescribed proteins from SFV-infected cells, are components of the virus RNA polymerase. Their synthesis appears to be directed by virus genes distinct from those specifying the structural proteins, and it is thus presumed that they are the products of the expression of the genetic information carried by that part of the 42S RNA genome which is not represented in 26S RNA. It is also apparent that they are synthesized via a common set of high mol. wt. precursors by a process of proteolytic cleavage, a possible model for which is shown in Fig. 9. It is proposed that nsp 90 and nsp 63 are both the products of cleavage of p150, a possibility which is suggested by the sum of the molecular weights of the polymerase components. The location of nsp 63 at the N-terminal end of p150 is indicated by the labelling kinetics after synchronous initiation. We also propose that p150 is derived from p184, which is in turn derived from p200, by two other post-translational cleavages as shown. As shown in Fig. 9, the location of the 16000 mol. wt. fragment removed in converting p200 to p184 is not clear. Since this fragment has not yet been observed the labelling kinetics after synchronous initiation do not permit discrimination between the two alternatives. Support for such a model comes from the pulse-chase data, the effects of addition of proteolytic cleavage inhibitors, and also from a consideration of the amount of genetic information available. The coding capacity of the virus genome, after the structural proteins have been accounted for, is far too small to direct the synthesis of such

a set of independent polypeptides, while one of mol. wt. 200000 can be comfortably accommodated.

The fate and nature of the fragments detached in converting p200 to p150 through p184 is currently unclear. Although analysis of cell extracts on gels suitable for resolving proteins of the expected size (34000 and 16000 mol. wt.) has not given any positive results it seems unlikely that products of translation of this size, together representing some 25 % of the amino acid sequences of the known non-structural proteins, would be degraded immediately after synthesis without fulfilling any useful function. In view of the relative locations of the genes for the polymerase components and the virus structural proteins on the 42S RNA there is a remote possibility that the fragment of about 34000 mol. wt. which is removed in the conversion of p184 to p150 is identical with the virus core protein, and that p200 and p184 are the result of translational read-through of the protein synthesis initiation site normally used for structural protein synthesis. This possibility can be tested by examination of tryptic peptide maps of the large non-structural polypeptides.

Synthesis of the putative precursors of the RNA polymerase components becomes detectable about 1 h p.i., reaches a peak between 2 and 3 h p.i., and declines slowly thereafter, although they are still being made near the end of the infectious cycle. The kinetics of appearance of these polypeptides, and presumably of nsp 90 and nsp 63 themselves, are thus similar to the kinetics with which synthesis of virus-specific RNA begins (Martin & Burke, 1974; Bruton & Kennedy, 1975) and with which RNA polymerase activity appears in extracts of infected BHK cells (see accompanying paper, Clewley & Kennedy, 1976).

Since both major species of single-stranded RNA continue to be made when accumulation of polymerase components is blocked late in infection by cycloheximide or puromycin (Friedman & Grimley, 1969; Scheele & Pfefferkorn, 1969; Wengler & Wengler, 1975), or by canavanine (Ranki & Kääriäinen, 1970), it is unlikely that any essential polymerase component turns over rapidly, at least late in infection. Also the rate of RNA synthesis in infected cells reaches a plateau level rather than constantly increasing (Bruton & Kennedy, 1975). Together these observations suggest that the polymerase components made late in the infectious cycle may play no active role in the replication or transcription of the virus RNA.

The cleavage model outlined above provides an adequate description of the synthesis of the only two components so far associated with the SFV RNA polymerase. However, there are some aspects of the replication and transcription of RNA in alphavirus-infected cells which are not readily explained using so simple a model. Firstly, it is quite clear that the rate of synthesis of negative-strand RNA is under strict temporal control (Bruton & Kennedy, 1975). Secondly, the depression of 26S RNA synthesis following treatment of cells with puromycin or cycloheximide 1.5 to 2 h after infection with alphaviruses (Scheele & Pfefferkorn, 1969; Grimley *et al.* 1972) has been attributed to the low level, at early times after infection, of a protein required for the synthesis of 26S RNA, but not for that of 42S RNA. It is difficult to see how the proposed cleavage model could explain such temporal regulation of RNA synthesis, since both the known polymerase-associated components are necessarily synthesized simultaneously and in equimolar quantities. It is thus likely that work on the regulation of RNA synthesis in alphavirus-infected cells will lead to some elaboration of the model of RNA polymerase synthesis proposed here.

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