Purification and Polypeptide Composition of Semliki Forest Virus RNA Polymerase

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SUMMARY

A purification method for Semliki Forest virus-specified RNA-dependent RNA polymerase from BHK cells is described. The procedure entails (i) the preparation of a crude cell lysate by Dounce homogenization of cells 3.5 h post-infection, (ii) differential centrifugation to give a 15,000 g ‘mitochondrial’ pellet, (iii) equilibrium centrifugation on discontinuous sucrose gradients (Friedman et al. 1972) to give a membranous band of density 1.16 g/ml, (iv) solubilization with Triton N-101 and velocity centrifugation to give a 25 S solubilized polymerase complex and (v) affinity chromatography through an oligo (dT)-cellulose matrix bearing immobilized 42 S virus particle RNA. The overall purification was approx. 360-fold with a 5% recovery of activity.

Of the various intermediate fractions in the purification procedure, only the relatively crude post-nuclear supernatant fraction was competent to synthesize the major single-stranded RNAs found in infected cells. Other fractions incorporated precursor only into replicative intermediate (RI) or replicative form (RF). Analysis of the product RF showed that it was of the same size and could bind to the same extent to oligo (dT)-cellulose as the RF isolated directly from lysates of infected cells. Displacement hybridization and ribonuclease digestion suggested that the purified polymerase could only complete previously initiated progeny positive strands using negative strands as template and, even in its most highly purified form, was still tightly bound to its template.

Analysis on polyacrylamide slab gels revealed the presence of three 35 S-labelled polypeptides in the purified polymerase preparation, but a polypeptide which had identical electrophoretic mobility to the lowest mol. wt. polypeptide of the purified polymerase was also present in material from mock-infected cells which had been taken through the purification procedure. From these results we conclude that only two virus-specified polypeptides are present in the polymerase. A scheme for the synthesis of these polypeptides is presented in the accompanying paper.

INTRODUCTION

Cells infected with alphaviruses such as Semliki Forest virus (SFV) and Sindbis virus contain a virus-specified RNA-dependent RNA polymerase (Lust, 1966; Martin & Sonnabend, 1967; Sreevalsan & Yin, 1969). In crude extracts this enzyme is capable of synthesizing virus-specific single-stranded RNA of both genome (42 S RNA) and 26 S RNA size (Michel & Gomatos, 1973). This latter RNA acts as mRNA for the structural
proteins of the virus particle (Cancedda, Swanson & Schlesinger, 1974; Simmons & Strauss, 1974; Clegg & Kennedy, 1975). The RNA polymerase is intimately associated with membranous structures termed cytopathic vacuoles which have been isolated by equilibrium centrifugation (Friedman et al. 1972).

As virion 42S RNA is infectious (Friedman, Levy & Carter, 1966; Sonnabend, Martin & Mécès, 1967) it is likely that it is translated directly to the polypeptide(s) of the RNA polymerase. Complementation studies with temperature-sensitive mutants of Sindbis virus indicate that at least two cistrons of the genome are involved in specifying RNA synthesizing functions (Burge & Pfefferkorn, 1966). This contention is supported by biochemical experiments on one of the Sindbis mutants which indicate that synthesis of the 42S RNA is independent of 26S RNA formation (Segal & Sreevalsan, 1974), suggesting that synthesis of these RNAs is catalysed by two distinct enzyme activities. With the exception of these observations, which at best provide circumstantial evidence for two polymerases, nothing is known about the properties of the virus-specified polymerase polypeptide(s) or the possible existence of host-specified components in the polymerase. In particular the size of the virus-specified polymerase polypeptide(s) is not known and therefore the fraction of the coding potential of the genome devoted to the polymerase genes cannot be estimated. For these reasons, therefore, it is pertinent to attempt to characterize the polymerase of SFV and to identify the virus-specified components.

In the present paper we present a purification method for SFV polymerase from the cytoplasm of infected hamster cells. This method entails equilibrium banding on sucrose gradients, non-ionic detergent solubilization, velocity centrifugation and a novel affinity chromatography technique involving binding of the RNA polymerase/template complex to immobilized 42S virion RNA. Analysis of the purified RNA polymerase on polyacrylamide gels revealed three labelled polypeptides, two of which were totally absent from purified extracts from mock-infected cells and are therefore strong candidates for virus-specified polymerase components.

METHODS

Materials. Brij 58, polyvinyl sulphate and Triton N-101 were obtained from Sigma Chemical Co., London. Dextran sulphate 500 was supplied by Pharmacia, London; oligo (dT)-cellulose (type T3) by Collaborative Research Inc., Waltham, Mass., U.S.A., and agarose by L’Industrie Biologique Française S.A., Gennevilliers, France. Pancreatic ribonuclease covalently bound to Sepharose was obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Actinomycin D was a generous gift from Merck, Sharpe and Dohme Research Laboratories, N.J., U.S.A. 8-3H-guanosine-5'-triphosphate ammonium salt (17 Ci/mmol), guanosine 5'-32P-triphosphate ammonium salt (1·04 to 3·66 Ci/mmol), L-35S-methionine (240 Ci/mmol) and 5-3H-uridine (25 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, Bucks. The radiolabelled guanosine triphosphates were lyophilized before use to remove ethanol. Before use acrylamide and N,N'-methylenebisacrylamide (both from Kodak Ltd, Liverpool) were re-crystallised (Bruton & Kennedy, 1975) and phenol, ethanol and ether redistilled. All other chemicals were the best grade available commercially.

Virus. Three-times plaque-purified wild-type ts+ SFV was used throughout and virus inocula prepared by a single passage in suspensions of chick embryo fibroblasts (Kennedy, 1974).

Cells. Monolayer cultures of BHK cells, clone 13, or on occasion, chick embryo fibro-
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blasts, were grown in 14 cm plastic Petri dishes or in 2.5 l smooth-walled roller bottles (Morser, Kennedy & Burke, 1973).

Preparation of labelled virus. Purified SFV labelled with either ³H-uridine or ³⁵S-methionine was prepared as described before (Kennedy, 1974).

Infection and labelling of cells for polymerase studies. Monolayer cultures were washed with maintenance medium (Morser et al. 1973) and infected with 20 to 100 p.f.u. of SFV per cell. Mock-infected cultures were treated in the same way except that maintenance medium replaced the virus inoculum. After adsorption at 37 °C, or occasionally 4 °C, for 1 h the fluids were replaced with maintenance medium (50 ml/roller bottle culture; 20 ml/ Petri dish culture) and incubated at 37 °C for 2 h when the fluids were again replaced with maintenance medium containing 1 µg/ml of actinomycin D. For time course experiments the actinomycin D was added with the virus inoculum. For labelling experiments actinomycin D was present for 5.5 h prior to the addition of isotope. Cultures labelled with ³⁵S-methionine were incubated for 18 h prior to infection with Glasgow modified MEM (GMEM) containing one-tenth of the normal methionine concentration and 10% dialysed calf serum, and during infection up to the labelling period with methionine-free GMEM containing 2% dialysed calf serum and 1 µg/ml of actinomycin D. Immediately prior to labelling, cultures were washed with Earle’s solution containing 1 µg/ml of actinomycin D and 2% dialysed calf serum (EDA), and then incubated with 10 ml of EDA containing either 500 µCi ³H-uridine or 750 µCi ³⁵S-methionine per roller bottle culture. The labelling period specified relative to the end of adsorption is given in individual experiments. At the end of the labelling period, cultures were rapidly cooled to 4 °C, washed four times with ice-cold PBS and the cells scraped off the glass and collected by centrifuging.

Preparation of crude cell lysates. Pelleted cells were rinsed and re-suspended in ice-cold sterile 10 mM-tris (pH 7.4) containing 10 mM-NaCl, 1.5 mM-MgCl₂ and 10 mM-2-mercaptoethanol (RSBM) to a density of approx. 1 × 10⁸ cells/ml. After 15 min at 4 °C the cells were disrupted with 28 strokes of a stainless steel Dounce homogenizer having a clearance of 0.002 in. The efficiency of cell breakage was estimated by phase microscopy to be >97%.

Assay of RNA-dependent RNA polymerase activity. Reactions were performed in 10 ml conical glass tubes containing 350 µl of reaction mixture comprising 100 µl of sample (at a protein concentration of up to 1 mg/ml) and 250 µl of the following components each dissolved in 100 mM-tris (pH 8.0) containing 10 mM-MgCl₂: 20 µg of pyruvate kinase, 5 µmol of phospho(enol) pyruvate, 50 nmol each of ATP, CTP and UTP, 2.5 µCi of ³H- or ³²P-labelled GTP in a total of 1 nmol, 1.5 µmol of dithiothreitol, 3.5 µg of dextran sulphate 500, 1 µg of actinomycin D and 4 µmol of KCl. Incubation was at 35 °C for 30 min at which time the reaction was stopped by adding 3.5 ml of ice-cold 6.5% (w/v) TCA containing 100 mM-Na₂P₂O₇ and 250 µg of bovine serum albumin as carrier. TCA-precipitable material was collected, washed and counted as described by Clegg & Kennedy (1974). All polymerase activities are expressed as ct/min of TCA-precipitable product.

Analysis of the polymerase products. For analysis of the RNA products of the polymerase reaction, subcellular fractions were prepared in RSBM containing 20 µg/ml of polyvinyl sulphate and the assay was scaled up to a total volume of 2.45 ml (700 µl of sample plus 1.75 ml of assay components). After incubation reaction mixtures were made 20 mM in EDTA, 1.95% (w/v) in SDS and 0.5% (v/v) in Brij 58 (Michel & Gomatos, 1973) and portions layered directly on to 12 ml 15 to 30% (w/v) linear sucrose gradients prepared in 50 mM-tris (pH 7.4) containing 100 mM-NaCl, 1 mM-EDTA and 0.1% SDS.
Centrifugation was for 16 h at 10 °C at 76000 g. Gradients were unloaded by upward displacement and fractions TCA precipitated and counted (Clegg & Kennedy, 1974). On occasion, after incubation, reaction mixtures were made 2 % (w/v) in SDS and 1 % naphthalene-1,5-disulphonate and RNA phenol extracted and alcohol precipitated (Clegg & Kennedy, 1974).

Isolation and characterization of replicative form (RF). The RF synthesized by the 25S solubilized fraction (see below) in vitro was isolated by LiCl fractionation and CF11 cellulose chromatography, and characterized with respect to size, poly A content and the radioactivity in component strands as described before (Bruton & Kennedy, 1975).

Purification of the polymerase. Unless otherwise stated, all steps in the purification were performed at 3 to 5 °C and all solutions were sterilized by autoclaving at 121 °C for 10 min. Step 1: preparation of 15000 g (P15) fraction. The crude cell lysate was centrifuged at 700 g for 10 min to remove nuclei. The post-nuclear supernatant fraction was then centrifuged at 15000 g for 20 min and the pellet gently resuspended in RSBM to a protein concentration of 1 mg/ml. Step 2: equilibrium centrifugation. Into a 25 ml polycarbonate centrifuge tube was placed 3 ml of 65 % sucrose, 4 ml of 45 % sucrose, 4 ml of 40 % sucrose, 5 ml of P15 made 30 % in sucrose, 4 ml of 25 % sucrose and 3 ml of RSBM. All sucrose solutions were w/w in RSBM. Centrifugation in an MSE 3 × 25 ml rotor was at 96000 g for 17 to 20 h. The gradient was unloaded by aspiration (Clegg & Kennedy, 1974) to give approx. 35 fractions and portions of each fraction were assayed for polymerase activity and radioactivity. The density of selected fractions was determined from refractive index measurements. Fractions rich in polymerase activity were pooled and the enzyme recovered by diluting with RSBM to < 5 % (w/w) sucrose and centrifuging at 56000 g for 1 h. Step 3: solubilization and velocity sedimentation. Pelleted material from step 2 was resuspended in 0-5 ml RSBM, dispersed by gentle homogenization in a loose-fitting glass Dounce homogenizer and 20 % (v/v) Triton N-101 in RSBM added slowly to a final concentration of 1 % (v/v). The sample was then immediately layered over a 5 to 40 % (w/v) linear sucrose gradient prepared in RSBM containing 1 % Triton N-101. Centrifugation was at 100000 g for 3-5 h in an MSE 6 × 14 ml titanium rotor. The gradient was unloaded by aspiration to give about 25 fractions and a portion of each was assayed for polymerase activity. Enzyme-rich fractions were pooled and made 0-2 M in NaCl. Step 4: affinity chromatography. A jacketed 60 × 5 mm column of oligo (dT)-cellulose was prepared and washed at 30 °C with 50 ml of 50 mM-tris (pH 7-4) containing 0-2 M-NaCl, 1 mM-EDTA and 0-1 % Triton N-101 (binding buffer). About 200 μg of 42S RNA from purified SFV (Martin & Burke, 1974) in a volume of 1-2 ml of binding buffer was then applied to the column at 4 °C which was washed with binding buffer at 4 °C until all the unbound RNA (about 25 % of the total applied; Clegg & Kennedy, 1974) was eluted. The solubilized enzyme preparation was then applied to the column at 4 °C and the column maintained at this temperature for 1 h with periodic resuspension of the matrix. The column was then washed at 4 °C with 10 ml of binding buffer and bound material eluted with 5 ml of 10 mM-tris (pH 7-4) containing 0-1 % Triton N-101 at 30 °C. Fractions of 500 μl were taken and 10 to 40 μl assayed for polymerase activity and radioactivity. Enzyme-rich fractions were stored at −70 °C or at −20 °C in the presence of 50 % (v/v) glycerol.

Polyacrylamide gel electrophoresis. Samples taken during the purification procedure for analysis by polyacrylamide gel electrophoresis were precipitated by adding NH4HCO3 to 0-1 M and 9 vol. of acetone. After 3 h at −20 °C the precipitates were collected by centrifuging at 10000 g for 20 min at 4 °C, washed in ethanol:ether (1:3, v/v) and ether, dissolved in 2 ml of 0-5 % SDS and lyophilized twice. Samples were reduced, alkylated and analysed
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on 7.5 % (w/v) acrylamide plus 0.2 % bisacrylamide slab gels (Clegg & Kennedy, 1975) using the Laemmli (1970) gel system. After electrophoresis gels were dried under vacuum at 90 °C and autoradiographed in Kodirex X-ray film.

Protein determinations. The concentration of protein in subcellular fractions was determined by the method of Lowry et al. (1951) using bovine serum albumin (fraction V) as standard. Corrections were made for interference by sucrose and Triton N-101.

RESULTS

Levels of polymerase activity during virus multiplication

Chick embryo fibroblasts and BHK cells were infected with SFV and cultures harvested at various times post-infection (p.i.). PI5 fractions were prepared and assayed for polymerase activity, which was just detectable above the respective mock-infected values 30 min after the end of the adsorption period (Fig. 1). In BHK cells the activity rose rapidly reaching a maximum at about 3.5 h p.i. Thereafter, the activity remained almost constant until 5.5 h p.i. at which time it began to fall, probably due to cell lysis. In chick cells, maximum activity was reached slightly earlier than in BHK cells (at 2.5 h p.i.), but the value reached was only about 17 % of that reached in BHK cells. This difference may reflect the synthesis of more polymerase in BHK cells than in chick fibroblasts, or an intrinsically higher metabolic activity in the faster growing cultured hamster cells. In any event, the lower polymerase activity in chick fibroblasts does not appear to impair the production of progeny extracellular virus; the yields from the two cell systems being essentially the same.

Distribution of polymerase activity in subcellular fractions

Infected cultures of BHK cells were harvested at 4 h p.i., the cells disrupted by Dounce homogenization to give a crude cell lysate which was then fractionated by low speed centrifugation (700 g; 10 min) into a nuclear fraction and a post-nuclear supernatant fraction. The post-nuclear fraction was then centrifuged at 15000 g for 20 min to give a ‘mitochondrial’ pellet (PI5) and a post-mitochondrial supernatant fraction. The protein concentration of each fraction was determined and portions, containing approximately the same amount of protein, were assayed for polymerase activity. Table 1 shows that the highest specific activity was associated with PI5. This observation is at variance with the result of Michel & Gomatos (1973) who reported that 75 to 90 % of the polymerase activity was found in the large particle fraction (nuclear pellet) from infected BHK cells. Their result may have been due to inadequate separation of membranes from nuclei so causing the polymerase, which has been shown to be closely associated with membranes (Friedman et al. 1972; see also later), to remain associated with the nuclei. Certainly the nuclei described in Table 1 were shown by phase microscopy to be heavily contaminated with cytoplasmic membranes which were only partially removed by a second Dounce homogenization and centrifugation. Attempts to further fractionate and purify the polymerase by differential centrifugation did not result in a significant increase in specific activity; rather there was loss of activity.

Competence of the polymerase present in the post-nuclear supernatant and in PI5 to synthesize single-stranded RNA

The polymerase should, in vitro, be capable of synthesizing the RNA species found in the infected cell. These are 42S and 26S single-stranded RNAs (the major species), the
Fig. 1. Levels of polymerase activity during virus multiplication. Pairs of Petri dish cultures of BHK cells and chick fibroblasts were either infected with SFV at a m.o.i. of 50 or mock-infected using maintenance medium. After adsorption for 1 h at 37 °C the fluids were replaced with maintenance medium and pairs of cultures harvested at the times indicated. From each pair of cultures a P15 fraction was prepared and assayed for enzyme activity and protein content as described in Methods. From these the specific activities in: ○—○, infected chick cells; ●—●, infected BHK cells; △—△, mock-infected chick cells and ▲—▲, mock-infected BHK cells were determined.

Table 1. Distribution of polymerase activity in subcellular fractions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Subcellular fraction</th>
<th>Sp. act.* (ct/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dounce homogenization</td>
<td>Crude cell lysate</td>
<td>14853</td>
</tr>
<tr>
<td>Centrifugation at 700 g for 10 min</td>
<td>Nuclear pellet</td>
<td>13043</td>
</tr>
<tr>
<td>Centrifugation at 15000 g for 20 min</td>
<td>Post-nuclear supernatant</td>
<td>67800</td>
</tr>
<tr>
<td>Dounce homogenization and centrifugation of the nuclear pellet</td>
<td>Mitochondrial pellet (Pt5)</td>
<td>97396</td>
</tr>
<tr>
<td></td>
<td>Post-mitochondrial supernatant</td>
<td>4928</td>
</tr>
<tr>
<td></td>
<td>Second nuclear pellet</td>
<td>10389</td>
</tr>
<tr>
<td></td>
<td>Second post-nuclear supernatant</td>
<td>3881</td>
</tr>
</tbody>
</table>

* Values are the mean of triplicate assays.

replicative intermediate (RI, a multi-stranded species) and replicative form(s) (RF, wholly double-stranded species). We therefore examined the competence of selected fractions from Table 1 to synthesize products recognizable by their sedimentation on sucrose gradients. As Fig. 2 shows, the post-nuclear supernatant fraction was competent to synthesize 42S, 26S and also RI/RF which sediments at about 20 to 23S (Bruton
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Fig. 2. Sedimentation analysis of the polymerase products. A post-nuclear supernatant fraction and a P15 fraction were prepared from 10 roller bottle cultures of BHK cells infected with 50 p.f.u./cell of SFV, and harvested 4 h p.i. These two fractions were incubated with the polymerase assay components, and the products extracted (Michel & Gomatos, 1973), and analysed by sedimentation through 15 to 30 % (w/v) linear sucrose gradients as described in Methods. Sedimentation is from left to right. The arrows a, b and c indicate the positions of RF, 26S and 42S RNA respectively, isolated from infected cells and run on a parallel gradient; ○—○, product synthesized by post-nuclear supernatant; ●—●, product synthesized by P15.

& Kennedy, 1975). About 30 % of the product was resistant to digestion with pancreatic (10 μg/ml) and T₁ (50 Units/ml) ribonucleases in high salt (0.4 M). This value which reflects the percentage of RF and RI cores (Bruton & Kennedy, 1975) indicates that single-stranded RNA accounts for over 75 % of the product. In contrast however, the mitochondrial fraction (P15), although rich in polymerase activity, was not able to synthesize either 42S or 26S RNA (Fig. 2), RF/RI being the only recognizable product. Several explanations can be proposed to explain this loss of single-stranded synthesizing activity. Firstly, centrifugation to prepare P15 may remove a component from the polymerase complex which is essential for single-stranded RNA synthesis. Secondly, the preparation of P15 may in itself damage the polymerase complex and destroy its ability to synthesize single-stranded RNA, possibly by exposing the complex to endogenous RNase which digests the single-stranded products. Thirdly, if the single-stranded products of the polymerase have to be 'actively removed' from the RNA synthesizing complex, the 26S RNA for translation, the 42S RNA for encapsidation, then preparation of P15 may dislocate this process and release of single-stranded products may be prevented. Although we have not been able to determine which of these explanations, if any, is correct, we would tentatively exclude the first, since the addition of the post-mitochondrial supernatant to P15 did not restore the competence of the polymerase to synthesize single-stranded RNA. Moreover, at no stage in the
subsequent purification of the polymerase was the ability to synthesize single-stranded RNA regained although more highly purified preparations, e.g. the detergent-solubilized 25S complex, were demonstrably free of RNase (see later). This suggests that RNase digestion may not be the primary cause of loss of single-stranded RNA synthesizing activity.

Characterization of the polymerase complex by equilibrium centrifugation

In order to further characterize and purify the polymerase complex we used the technique of equilibrium banding first described by Friedman et al. (1971) for fractionating cytoplasmic extracts of chick cells infected with SFV. Applying this method to P15 fractions we observed (Fig. 3a) that polymerase activity was located at a buoyant density of 1.16 g/ml (36% [w/v] sucrose). No activity was present in this region of the gradient when a mock-infected extract was analysed. Visual inspection of the gradients of infected and mock-infected extracts revealed extensive differences in the position and intensity of the bands (inset of Fig. 3a) confirming previous reports of substantial alterations in membrane structure.

Fig. 3. Characterization of the polymerase complex by equilibrium centrifugation. A P15 fraction was prepared from 40 roller bottle cultures of BHK cells infected with 50 p.f.u. of SFV per cell and harvested 4 h p.i. This fraction, together with exactly analogous material from mock-infected cells, was then fractionated on equilibrium sucrose gradients as described in Methods. (a) The gradient containing material from infected cells was unloaded and fractions assayed for polymerase activity. The inset shows the positions of the major membranous bands from infected (I) and mock-infected (U) extracts. (b) A P15 fraction was prepared and fractionated on equilibrium sucrose gradients exactly as described in (a) except that three of the cultures were labelled for 5 min with 100 μCi [3H]-uridine immediately prior to harvesting. The gradient was unloaded and the radioactivity in each fraction determined. (c) The experiment depicted in (a) was repeated using, as inoculum, purified virus labelled with [3H]-uridine (total of 5 x 10^3 cts/min) or [35S]-methionine (total of 7 x 10^6 cts/min) at an m.o.i. of 10. The panel is a composite showing the distribution of: O—O, polymerase activity, •—•, [3H]-uridine radioactivity and △—△, [35S]-methionine radioactivity in fractions from the equilibrium gradients. In all gradients the top is at the left and the arrow indicates a density of 1.16 g/ml determined from refractive index measurements.
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in SFV-infected cells (Grimley et al. 1972). In order to determine the position on the gradient of RNA newly synthesized in vivo, we pulse-labelled the cells with ³H-uridine for 5 min immediately prior to harvesting, and analysed the distribution of radioactivity on an equilibrium gradient. (Under these labelling conditions most of the incorporated radioactivity can be extracted from the cells in the form of RI/RF; Martin & Burke, 1974). As Fig. 3(b) shows, the in vivo incorporated radioactivity also bands at a buoyant density of 1.16 g/ml, indicating that the polymerase present at this density is likely to be in the form of a membrane-bound polymerase complex, possibly the cytopathic vacuoles (CPV-1) described by Friedman et al. (1972) which are believed to be the site of virus-specified RNA synthesis. We now determined if any component of input virus, notably core protein and/or RNA, was present in the 1.16 g/ml band. When purified virus labelled with ³H-uridine was used there was a low, but reproducible, level of radioactivity in the 1.16 g/ml band (Fig. 3c). Most of this radioactivity was present in 42S RNA. In contrast, no radioactivity was present in the band derived from cells infected with purified ³⁵S-labelled virus. This experiment suggests that, of the components of the input virus, only the genome is present in the polymerase complex where its function is presumably to act as template for the synthesis of negative strands. However, we have to consider this idea as tentative because it is possible that the radioactivity in the 1.16 g/ml band is present not in input 42S RNA but in de novo synthesized RNA labelled with ³H-uridine from degraded 42S RNA. Because of the low level of radioactivity in the 1.16 g/ml band it was not possible to directly investigate this possibility.

Detergent solubilization of the polymerase

The 1.16 g/ml band was removed from the equilibrium gradient and the polymerase complex recovered by centrifugation at 56000 g for 1 h. The sedimentation coefficient of the polymerase complex was now determined on 5 to 40 % (w/v) linear sucrose gradients using virus nucleocapsids (150S) as a marker. In this experiment the polymerase complex had been labelled in vivo for 5 min with ³H-uridine immediately before harvesting and polymerase assays of gradient fractions were performed using α-³²P-GTP as precursor. As Fig. 4(a) shows there was coincidence between the positions of the in vivo label and polymerase activity. Indeed a visible membrane band was often seen in the gradient and this band contained over 90% of polymerase activity recovered from the gradient. From the position of the nucleocapsid marker, we estimated the S value of the polymerase complex to be 260S (average of 4 determinations). Several combinations of detergents were now evaluated in an attempt to solubilize the polymerase complex from membranous material. These included non-ionic detergents such as NP-40, Triton X-100, Triton N-101 and Brij 35 (all at 0.5 to 1 %) and ionic detergents such as deoxycholate. Of these, deoxycholate, either by itself or in combination with Triton N-101 (0.5 % and 1 % respectively), although solubilizing the polymerase complex as judged by velocity centrifugation of in vivo labelled material (see later), also rendered polymerase activity extremely labile, so much so that no polymerase activity could be detected after velocity centrifugation. Of the non-ionic detergents, Triton N-101 proved to be the most satisfactory both in terms of reproducibility of effect and stability of the polymerase activity. Fig. 4(b) shows a velocity gradient analysis of polymerase activity and of in vivo labelled equilibrium banded polymerase complex after treatment with 1 % (v/v) Triton N-101 as described in Methods. Centrifugation was under identical conditions to those used to examine the non-detergent-treated complex except that the gradient contained 1 % Triton N-101. For reasons that are unclear the sedimentation of the marker nucleocapsids increased slightly in the presence of Triton.
Fig. 4. Sedimentation of the equilibrium banded polymerase complex before and after detergent treatment. The 1.16 g/ml band from an equilibrium gradient of the P15 fraction prepared from 10 roller bottles of SFV-infected BHK cells labelled as described in the legend to Fig. 3(b), was recovered by centrifuging and resuspended in 1 ml of RSBM. Half of this material was then layered directly on to a 15 to 40 % (w/v) linear sucrose gradient (a). The other half was made 1 % (v/v) in Triton N-101 as described in Methods and then layered on to an identical gradient containing 1 % Triton N-101 (b). Centrifugation which was for 3.5 h at 100000 g is from left to right. Fractions collected by upward displacement were assayed for: ○—○, 3H-uridine radioactivity; ●—●, polymerase activity. The arrows indicate the position of marker 15S virus nucleocapsids labelled with 35S-methionine and prepared as described by Kääriäinen, Simons & von Bonsdorff (1969).

Under these detergent conditions the S value of the polymerase was found to be approx. 25S (average of 6 determinations). In addition the 25S material was faintly yellow, and the bulk of the protein, probably aggregates, was found in the bottom one-sixth of the gradient. Thus, detergent treatment converts the 260S membranous polymerase complex to a 25S structure which appears relatively free of membrane material, but which contains not only active enzyme but also, presumably, template.

Characterization of template and products of the solubilized polymerase

At this stage in the purification, the template and products of the 25S polymerase were investigated. As mentioned earlier the only recognizable product synthesized by the 25S
polymerase was RF/RI (over 80 % RF), and we therefore examined the size, structure and \textit{in vitro} labelling pattern of the component strands of the product RF isolated by LiCl fractionation and CF11 cellulose chromatography (Bruton & Kennedy, 1975). Fig. 5(a) shows that the size of the \textit{in vitro} synthesized RF was indistinguishable from that of the RF isolated from lysates of infected cells. Displacement of the positive strand of purified \textit{in vitro} synthesized RF with 42S RNA (Bruton & Kennedy, 1975) showed that
incorporation was almost entirely (over 95%) into the component positive strands of the RF and that these positive strands ranged in S value from 5S to about 40S (data not presented). In addition the in vitro RF product bound to oligo (dT)-cellulose to essentially the same extent (64%) as RF isolated from infected cells. We interpret these data as indicating that the solubilized polymerase continues the synthesis of previously initiated positive strands. Some of these positive strands appear to contain poly A and may therefore be complete.

To examine the nature of the template of the 25S polymerase complex it was first essential to show that this material was free of endogenous RNase. To do this we incubated %P-RNA with the 25S polymerase for 20 min at 35 °C, re-extracted the RNA with phenol and analysed it by gradient centrifugation (Fig. 5b). Little or no degradation was observed. This shows that the 25S polymerase complex is free of RNase. We now investigated the effect of added RNase on the polymerase activity. To do this the NaCl concentration of the 25S polymerase complex was increased to 0.3 M and half of the sample was incubated at 35 °C for 20 min with 10 μg/ml of pancreatic ribonuclease coupled to Sepharose 4B. The remaining half of the sample acted as a control and was incubated with Sepharose 4B alone. At the end of the incubation the Sepharose matrix was removed by centrifugation at 5000 g for 20 min and polyvinyl sulphate added to the supernatant to 20 μg/ml. (Removal/inactivation of the RNase was checked by incubating a portion of the treated sample with rRNA as described before. Fig. 5(b) shows that there was essentially no digestion – less than 10%). When these samples were assayed for polymerase activity it was found that incorporation by the RNase-treated sample was only 23% (average of 4 essays) of that of the control sample. This result indicates that the polymerase template (presumably in the main 42S negative strands; Bruton & Kennedy, 1975) is accessible even to immobilized RNase and is largely single-stranded.

Affinity chromatography of the polymerase/template complex

We now attempted to develop a specific affinity chromatographic technique for the further purification of the polymerase/template complex. We reasoned that by exploiting the observations that the template for the 25S solubilized polymerase was principally of negative polarity and at least partly single-stranded and accessible to RNase (see last section), it might be possible to specifically hydrogen-bond the polymerase/template complex to immobilized 42S virus particle RNA. Fig. 6 shows that this is indeed possible and, moreover, that the polymerase can be recovered from the affinity matrix in an active form. Recovery was 40 to 55% of the total polymerase activity applied to the matrix. By contrast only about 5% of the total %S-methionine-labelled protein material bound. Although we do not know if the above postulate is correct in explaining the mechanism of action of the affinity column, three control experiments suggest that it is at least feasible. Firstly, no attachment was observed to oligo (dT)-cellulose itself. Secondly, no attachment (less than 4% of the total activity applied) was observed if the 25S solubilized polymerase complex was pre-incubated with the assay components before being applied to the column. We interpret this as indicating that during pre-incubation the polymerase product – positively-stranded RNA – collapses onto the negative strand template strand, rendering it unable to hydrogen-bond with the immobilized 42S RNA. This idea is supported by the finding that the product of the pre-incubation was over 90% resistant to ribonuclease, indicating that it is extensively hydrogen-bonded. Thirdly, no attachment was observed using immobilized 42S RNA from Sindbis virus, another alphavirus.
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Fig. 6. Affinity chromatography of the 25 S solubilized polymerase complex. A 60 x 5 mm column of washed oligo (dT)-cellulose was prepared and charged with about 180 µg of 42 S RNA from purified SFV as described in Methods. To this column at 4 °C was then applied the 25 S solubilized polymerase complex from 15 roller bottle cultures of SFV-infected BHK cells labelled from 0 to 4.5 h p.i. with 300 µCi 35 S-methionine per culture. After periodic resuspension for 1 h, unbound material was removed with 10 ml of high salt binding buffer. The temperature of the column was raised to 30 °C and bound material eluted with low salt elution buffer. Fractions of 500 µl were taken and portions assayed for: O---O, polymerase activity and ••••, 35 S-methionine radioactivity. The arrow indicates the change to elution buffer.

Evaluation of the purification of the polymerase

Because of the anticipated specificity of the affinity column technique, we investigated the number of labelled polypeptides present in the purified polymerase complex by polyacrylamide gel electrophoresis. We also examined the spectrum of labelled polypeptides in samples taken during the purification procedure and in samples taken during a purification of material from mock-infected cells. The result of this gel analysis is shown in Fig. 7. Only three labelled polypeptides could be detected in the affinity column purified polymerase preparation (lane j). The mol. wt. of these are 90000, 63000 and 40000 respectively (see accompanying paper). Only the 40000 mol. wt. polypeptide was present in material purified from mock-infected cells. Thus, the 90000 and 63000 mol. wt. polypeptides (hereafter referred to as nsp 90 and nsp 63 respectively) are very likely to be components of the virus-specified polymerase. The status of the 40000 mol. wt. polypeptide is less clear. It may be a host-derived component of the polymerase, or it may be a host polypeptide which co-purifies with the polymerase but which plays no active role in either polymerase structure or activity. Both nsp 90 and, to a lesser extent, nsp 63 could be discerned in samples taken from the P15 fraction (lane d), the 1.16 g/ml equilibrium-banded material (lane f) and the 25 S solubilized polymerase (lane h). In most cases there were no prominent host bands of the same electrophoretic mobilities in the analogous fractions from the 'mock purification'. Because of the complexity of the polypeptide profile of the post-nuclear supernatant fraction from infected cells (lane a) it was not possible to detect either nsp 90 or nsp 63. However, as is discussed in the accompanying paper, it is possible to readily
Fig. 7. Polyacrylamide gel electrophoresis of samples taken during the polymerase purification procedure. A set of 10 roller bottle cultures of BHK cells were infected with SFV at an m.o.i. of 100. A second set of cultures were mock-infected. Both sets of cultures were labelled from 0 to 4.5 h p.i. with 300 μCi 35S-methionine per culture. Extracts prepared from these two sets of cultures were then taken through the purification procedure and samples of the post-nuclear supernatant fractions (lanes a and b, 281,000 and 274,000 ct/min respectively), the P15 fractions (lanes c and d, 210,000 and 243,000 ct/min respectively), the equilibrium banded fractions (lanes e and f, 160,000 and 153,000 ct/min respectively), the 25S solubilized material (lanes g and h, 140,000 and 167,000 ct/min respectively), and the affinity column eluate (lanes i and j, 32,000 and 68,000 ct/min respectively) were analysed by electrophoresis on a 16.5 cm 7.5% (w/v) polyacrylamide slab gel as described in Methods. Electrophoresis is from top to bottom. Autoradiography was for 5 days. Lanes a, c, e, g and i are the electrophoretograms of the samples taken during the purification of material from mock-infected cells.
Table 2. Quantitative analysis of the polymerase purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total polymerase activity* (ct/min/mg protein)</th>
<th>Total protein (mg)</th>
<th>Sp. act. (ct/min/mg protein)</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>1.42</td>
<td>73.24</td>
<td>19372</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>P15</td>
<td>2.0</td>
<td>10.81</td>
<td>184781</td>
<td>141</td>
<td>9.5</td>
</tr>
<tr>
<td>Equilibrium banding</td>
<td>0.89</td>
<td>2.17</td>
<td>410184</td>
<td>63</td>
<td>21.2</td>
</tr>
<tr>
<td>25S solubilized material</td>
<td>0.16</td>
<td>&lt;0.01</td>
<td>564293</td>
<td>11</td>
<td>29.1</td>
</tr>
<tr>
<td>Affinity column eluate</td>
<td>0.07</td>
<td>&lt;0.01</td>
<td>&gt;70000000</td>
<td>5</td>
<td>&gt;361</td>
</tr>
</tbody>
</table>

* Expressed as ct/min of 3H-GTP incorporated into TCA-precipitable material/30 min incubation $\times 10^{-4}$.

detect both nsp 90 and nsp 63 using a different labelling regime. Under these conditions there are no detectable host polypeptides of identical electrophoretic mobility to nsp 90 and nsp 63.

Table 2 shows a quantitative analysis of the purification of the polymerase. Because of the undetectable amount of protein in the affinity column eluate it was not possible to accurately measure the overall purification. However, taking 10 $\mu$g as the lower limit of protein detection in our assay then the overall purification was about 360-fold. Of the individual steps used, the affinity chromatography step was by far the most successful. This single step effectively selected three labelled polypeptides from over 40 present in the 25S solubilized complex (Fig. 7; lanes h and j).

**DISCUSSION**

The results described in this paper detail a method for the purification of the SFV-specified RNA polymerase from infected BHK cells. Maximum polymerase activity in BHK cells is reached about 3.5 to 4 h p.i. In chick cells and also in rabbit kidney and HeLa cells (unpublished results) maximum activity is only between 10 and 20% of that found in BHK cells. Early steps in our purification procedure, namely those up to and including the equilibrium banding step, are modifications of the techniques described by Friedman et al. (1972) for the characterization of the SFV polymerase found in chick fibroblasts. As these authors found, the BHK cell-derived polymerase bands at a density of about 1.16 g/ml and is associated with a discrete band of membranous material (Fig. 3), probably smooth membrane modified during virus multiplication. The sedimentation coefficient of this membranous polymerase complex can be reduced from 260S to about 25S by treatment with Triton N-101. The polymerase present in this solubilized complex can then be further extensively purified by affinity chromatography through oligo (dT)-cellulose bearing immobilized 42S virus particle RNA. Gel analysis of the purified polymerase showed that it contained three labelled polypeptides, one of which was almost certainly of host cell origin.

Several points about the purification procedure are worth noting. Firstly, it is relatively rapid: it can be completed within two days of infecting the cells. Secondly, it is somewhat 'scale dependent'. This means that whereas it should be possible from a consideration of enzyme activity to scale down our routine procedure which starts with 10 to 12 roller bottles of BHK cells, i.e. a total of about $3 \times 10^9$ cells to say 1 to 2 roller bottles, in fact, very poor recoveries are obtained if less than 5 bottles are used. Two steps in the procedure appear to be affected by the scale. These are the equilibrium banding step where wide variations can occur in the recovery of active polymerase, and the detergent step where
we have recorded extremely low recoveries either by separately processing several portions of the recovered 1.16 g/ml band from 12 bottles or processing the 1.16 g/ml band derived from two bottles of cells. Attempts to improve recoveries by, e.g. adding cold carrier protein (bovine serum albumin) or glycol, have been unsuccessful. Thirdly, the detergent step has to be carefully controlled. The Triton N-101 must be added very slowly and the treated preparation immediately centrifuged on the velocity gradient. Failure to observe either of these two aspects of the procedure results in very low recoveries of active polymerase. Fourthly, the enzyme can be stored at --70 °C at various steps in the purification. Harvested cells and the P15 fraction can be stored for up to 3 months without detectable loss of activity. The recovered 1.16 g/ml band from the equilibrium gradient can be stored for 6 weeks with less than a 10 % drop in activity. By contrast the 25S solubilized polymerase complex is unstable. It has a half-life of less than 48 h at 4 °C, is completely destroyed by freezing and thawing and should therefore be chromatographed through the affinity column as soon as it is prepared. The final product is also relatively unstable though less so than the 25S solubilized complex. It can be stored at --20 °C in the presence of 50 % (v/v) glycerol for 2 weeks with about a 40 % loss in activity. Attempts to store it by other procedures were unsuccessful.

Although the purified polymerase is enzymically active it is only able, at best, to complete previously initiated positive strands. These strands do not appear to be released and, probably as a consequence, the enzyme seems unable to re-initiate. Certainly the presence of 42S positive strands either in the eluate from the affinity column or added to the 25S solubilized complex before affinity chromatography, fails to stimulate polymerase activity (data not shown). Thus, although we observe two labelled polypeptides completely unique to the purified polymerase we have to consider the possibility that during purification another virus-specified polypeptide(s) is lost and that this loss accounts for the incompetence of the purified polymerase to synthesize single-stranded RNA and/or to re-initiate. If this is not the case, and the present study describes all the virus-specified polymerase components, then it will be of considerable interest to determine how the synthesis of 42S and 26S RNA is controlled by nsp 90 and nsp 63. In this context it is also pertinent to consider the role of host cell-derived components in the polymerase protein. With the exception of the 40000 mol. wt. host component (Fig. 7) no other polypeptide bands were visualized by Coomassie blue staining of the slab gel after autoradiography. This, however, probably just reflects the very small amount of protein present in the purified polymerase (< 10 μg). If a host component of the polymerase were to turn over slowly in the cell then our labelling procedure, i.e. from addition of virus to 3-5 h p.i., might not label it. A more rigorous approach would be to pre-label the cells for, say, three generations before infection.

Subtracting the mol. wt. of the 26S RNA (1.8 × 10^6), the messenger for the structural proteins (Clegg & Kennedy, 1975), from that of the virus 42S RNA genome (4.2 × 10^6) leaves a mol. wt. of about 2.4 × 10^6. This therefore, is the mol. wt. of the ‘non-structural’ region of the genome which encodes the polymerase polypeptides. If all of this coding potential is expressed then the total mol. wt. of the non-structural polypeptides would be approx. 240000. The combined mol. wt. of nsp 90 and nsp 63 (Fig. 7) is 153000, which suggests that the polymerase polypeptides described in the present work account for about two-thirds of the non-structural coding potential of the virus RNA. The mechanism whereby this coding potential is expressed in the infected cell is the basis of the accompanying paper.
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REFERENCES


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