The Replication of Semliki Forest Virus

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SUMMARY

Using polyacrylamide gel electrophoresis, column chromatography on CF11 cellulose and salt precipitation, together with ribonuclease treatment, we have identified, in chick cells infected with Semliki Forest virus, four species of virus-specified single-stranded RNA, three species of double-stranded RNA and a multi-stranded RNA species. The mol. wt. of the four single-stranded RNA species were $4.0 \times 10^6$, $3.1 \times 10^6$, $2.3 \times 10^6$ and $1.8 \times 10^6$.

The kinetics of virus-specified RNA synthesis were studied in virus-infected cells, both early in infection and when virus-specified RNA synthesis was at the maximum rate. The first species of virus RNA detected were the multi-stranded and double-stranded species of RNA. The first single-stranded species detected was the $42S$ virus particle RNA, at $1.5$ h after infection. The average time taken to synthesize a molecule of virus-specified RNA was between $1$ and $1.5$ min. Multi-stranded and double-stranded RNA both had the properties expected of an intermediate in RNA synthesis, and the number of single-stranded equivalents in the multi-stranded species was $4.3$. The data show that synthesis of virus RNA is mainly semi-conservative and that the virus messenger RNA is of the same polarity as the genome.

INTRODUCTION

Several new virus-specified RNA species are synthesized in cells infected with Group A togaviruses (alphaviruses). Three classes of RNA have been distinguished by centrifuging in sucrose gradients, with sedimentation coefficients of $42S$, $26S$ and $20S$ (Friedman, Levy & Carter, 1966; Sonnabend, Martin & Mecs, 1967; Cartwright & Burke, 1970). The $42S$ and $26S$ species are single-stranded, whereas the $20S$ species is largely, although not entirely, double-stranded. The $42S$ species appears to be identical in all respects with virus RNA; it has the same sedimentation coefficient, the same base composition (Sonnabend et al. 1967; Kääriäinen & Gomatos, 1969), and both are infectious (Friedman et al. 1966). Free $42S$ RNA with a nucleotide sequence complementary to virus RNA has not been found in the infected cell (Simmons & Strauss, 1972a). The infectivity of the virus RNA suggests that at least part of it is translated, to produce virus RNA polymerase, and that transfer of information to another nucleic acid is not required for polymerase synthesis (Baltimore, 1971).

The $26S$ species of RNA has little or no infectivity (Sreevalsan et al. 1968). Its mol. wt., as estimated by polyacrylamide gel electrophoresis, is $1.7 \times 10^6$ to $1.8 \times 10^6$ (Cartwright & Burke, 1970; Dobos & Faulkner, 1970; Levin & Friedman, 1971). Several theories have been proposed to account for $26S$ RNA. It has been suggested that it is a conformational
variant of 42 S RNA, and that virus RNA, released from the polymerase in the form of 26 S RNA, may be converted to the 42 S RNA form during incorporation into the virus particle (Sonnabend et al. 1967). However, this would require a radical conformational change, and the one report of this conversion being effected in vitro has not been confirmed (Sreevalsan et al. 1968). These authors suggested that 26 S RNA differed from 42 S RNA only by having a more open structure. However, if this were so, then the 26 S RNA species should move more slowly than 42 S RNA on polyacrylamide gel electrophoresis, whereas the reverse is the case (Cartwright & Burke, 1970). It has also been suggested that 26 S RNA is a precursor to 42 S RNA (Sreevalsan et al. 1968; Dobos & Faulkner, 1969; Cartwright & Burke, 1970). However, the basis of this suggestion, the conversion of 42 S RNA to 26 S RNA by treatment with dimethylsulphoxide, heat or urea, appears to be due to the presence of hidden breaks in the 42 S RNA, and if precautions are taken in the extraction of 42 S RNA, no conversion to 26 S RNA occurs under denaturing conditions (Arif & Faulkner, 1972).

Two other single-stranded virus RNA species have been detected in cells infected with Sindbis virus (Levin & Friedman, 1971). These have mobilities by polyacrylamide gel electrophoresis intermediate between those of 42 S and 26 S RNA, and have been designated 38 S and 33 S RNA, although precise sedimentation coefficients have not been determined by sedimentation. Their mol. wt., as estimated by their mobility on polyacrylamide gel electrophoresis, are $3.1 \times 10^6$ and $2.4 \times 10^6$, respectively.

Recently, analysis of the polyribosomes from infected cells has shown the presence of 26 S RNA and 33 S RNA (Kennedy, 1972; Mowshowitz, 1973) and it appears that the function of these RNA species is to act as messenger RNAs. Thus, the evidence suggests that 42 S, 33 S and 26 S RNA may be translated in infected cells, but the mechanism involved in the regulation of this translation is unknown, although there is evidence that the transcription of the 42 S and 26 S RNA species is controlled independently (Scheele & Pfefferkorn, 1970).

The 20 S double-stranded RNA species has been shown to be heterogeneous, containing a membrane-bound multi-stranded RNA (Friedman, 1968; Cartwright & Burke, 1970) and more than one species of double-stranded RNA (Levin & Friedman, 1971; Stollar, Shenk & Stollar, 1972). Thus, both the transcription and translation of togavirus RNA is complex.

The purpose of this investigation was to study further the mechanism of RNA replication of the togavirus, Semliki Forest virus (SFV). The results of an independent, but similar, investigation by Simmons & Strauss (1972a, b) on the replication of Sindbis virus have recently been published.

**METHODS**

*Materials.* Acrylamide and SDS (specially purified) were obtained from British Drug Houses Limited, Poole, Dorset. $N,N'$-methylenebisacrylamide and $N,N,N',N'$-tetramethyl-ethylenediamine (TEMED) were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. Acrylamide and $N,N'$-methylenebisacrylamide were recrystallized before use from chloroform and acetone, respectively (Loening, 1967). Tris (hydroxymethyl) aminomethane (tris), as Trizma base, bovine pancreatic ribonuclease, as ribonuclease 1-A (100 Kunitz units/mg), bovine albumin powder (BSA, Cohn Fraction V), and 2-mercaptopethanol were obtained from Sigma Chemical Company, St Louis, Mo., U.S.A. Diethylpyrocarbonate was purchased as Baycovin from Bayer AG, Leverkusen, Germany. Dimethylsulphoxide (DMSO) and phenol were obtained from Hopkin and Williams, Limited, Chadwell Heath, Essex. 2,5-diphenyloxazole (P.P.O.), 1,4-di(2-(5-phenyloxazolyl))-benzene (P.O.P.-
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O.P.) and naphthalene, both scintillation grade, were obtained from Nuclear Enterprises (GB) Limited, Edinburgh. Aerosil standard silica was obtained from Bush, Beach, Segner and Bayley Limited, London. Ether was obtained from May and Baker Limited, Dagenham, Essex. Phenol and ether were both redistilled before use. Agarose was obtained from l'Industrie Biologique Française S.A., Gennevières (Seine), France. Whatman cellulose CF11 was obtained from Griffin and George Limited, London, England. Polygram thin-layer plates were obtained from Mancherey Nagal and Company, Duren, West Germany. [5-3H]-uridine (24 Ci/mol) and [2-14C]-uridine (60 mCi/mol) were obtained from the Radiochemical Centre, Amersham, Bucks. Actinomycin D was a gift from Merck, Sharpe and Dohme Research Laboratories, Rahway, N.J., U.S.A. Calf serum was obtained from Biocult Laboratories Limited, Paisley, Scotland. Colomycin was obtained from Pharmax Limited, Crayford, Kent, and crystamycin was obtained from Glaxo Laboratories Limited Greenford, Middlesex. Deoxyribonuclease (ribonuclease-free, electrophoretically purified) was obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Earle's medium was obtained in power form from Oxoid Limited, London, Medium 199 was obtained from Wellcome Reagents Limited, Beckenham, Kent. Non-radioactive poly G was a gift from Dr M. Eaton of the Department of Molecular Sciences, Warwick University; it had a sedimentation coefficient of about 10S. [12C]-labelled poly C was a gift from Dr S. I. T. Kennedy of this department.

Cells. Primary chick embryo fibroblasts were prepared as described by Morser, Kennedy & Burke (1973).

Growth of virus. Semliki Forest virus (ts+) strain, supplied by Professor F. Fenner) was grown in suckling mouse brain and then passed once in high-density suspension cultures of chick embryo fibroblasts, as described by Kennedy & Burke (1972), before use. Virus infectivity and haemagglutinin titres were measured as described by Kennedy & Burke (1972).

Preparation of radioactive Semliki Forest virus. Virus was grown in suspension culture in the presence of actinomycin D (0.1 μg/ml) and [3H]-uridine (10 μCi/ml).

Infection of chick cells with virus. Chick cells were infected with 10 p.f.u./cell of virus, and time after infection was measured from the time of addition of virus.

Determination of radioactivity incorporated into chick cell monolayers. Incorporation of radioactivity into monolayers was determined as described by Skehel et al. (1967), except that cells were washed with unlabelled PBS instead of saline containing uridine.

Virus purification. Saturated ammonium sulphate (prepared in Earle's medium and adjusted to pH 7.5) was added dropwise to the clarified virus suspension at 4 °C to 60% (v/v) saturation. The opaque suspension was stirred for 15 min at 4 °C and the precipitate was collected by sedimentation at 15,000 g for 20 min. The supernatant fluid was discarded and the precipitate resuspended in one-twentieth the starting vol. of TNE buffer (0.05 M-tris, 0.1 M-NaCl, 0.001 M-EDTA (disodium salt) adjusted to pH 7.0 at 20 °C with HCl). The suspension was dialysed against 100 vol. of TNE buffer overnight at 4 °C. The dialysate was centrifuged at 15,000 g for 30 min, and the supernatant fluid collected. A discontinuous sucrose gradient was prepared by layering 2 ml of 20% (w/v) sucrose over 2 ml 45% (w/v) sucrose in a 50 ml centrifuge tube, solutions being made in TNE buffer containing 0.5% calf serum. The virus suspension (40 ml) was layered over the sucrose and centrifuged at 120,000 g for 3 h at 4 °C. The supernatant fluid was discarded, and the pellet suspended in 1.0 to 1.5 ml TNE buffer. This was then layered over a preformed 20 to 55% (w/v) linear sucrose gradient in TNE buffer containing 0.5% calf serum, which was then centrifuged at 75,000 g for 15 to 18 h at 4 °C. The opalescent band was diluted four- to fivefold in TNE...
buffer containing 0.5% calf serum and centrifuged at 150000 g for 3 h at 4 °C. The pellet was suspended in TNE buffer and used within a few hours. The overall yield (as measured by haemagglutinin titration) was 11%.

**Extraction of RNA from purified virus.** The suspension of purified virus was extracted with 1% 2-mercaptoethanol, 3.3% SDS and phenol. The phenol phase was re-extracted with TNE buffer containing 2% SDS, and the aqueous wash pooled with the first aqueous extract. Phenol was removed by ether extraction, and ether by bubbling with nitrogen.

**Preparation of radioactive virus-specified RNA.** The method was a modification of that described by Levin & Friedman (1970). Cells were infected with SFV at 10 p.f.u./cell and [3H]-uridine was added at the times indicated as an RNA precursor rather than [3H]-uridine and [3H]-adenosine. After extraction of nucleic acids, two vol. of ethanol were added, and the extract stored at −20 °C. When cells were extracted with SDS, large amounts of DNA were also extracted, giving viscous solutions. Viscosity was decreased by dissolving the ethanol-precipitated RNA in TNM buffer (0.01 M-tris, 0.1 M-NaCl, 0.002 M-MgCl₂, buffered to pH 7.5 with HCl), and treatment with deoxyribonuclease (10 μg/ml for 30 min). After treatment, two vol. of ethanol were added and the sample stored at −20 °C.

**Preparation of cellular RNA.** Cells were incubated with or without [14C]-uridine (2 μCi/culture) for 18 to 24 h before extraction of RNA with phenol in the absence of SDS.

**Sucrose density gradient sedimentation of RNA.** Linear gradients of sucrose were prepared in TNE buffer containing 1% SDS (TNES). Gradients were formed at room temperature and equilibrated at 20 °C. Up to 120 μg of RNA was layered on to the gradients which were unloaded by upward displacement with 50 to 65% sucrose.

**Polyacrylamide gel electrophoresis of RNA.** 2-0% gels were prepared as described by Loening (1967). For preparation of gels containing agarose, the gel components, except for ammonium persulphate and TEMED, were mixed at double the final required concentration and brought to 45 °C. An equal vol. of melted 1% agarose at 45 °C was added, ammonium persulphate and TEMED added and the gels allowed to set in 20 cm perspex tubes. Before use, about 5 mm was sliced from the top end of the gel, to remove a region of agarose and unpolymerized acrylamide. Gels were pre-electrophoresed at 100 V for about 30 min before the sample (up to 50 μg of RNA) was applied in 100 μl of TNE buffer containing 0.1 or 0.2% (w/v) SDS, 5 to 10% (w/v) sucrose and about 0.01% (w/v) bromophenol blue. Electrophoresis was at room temperature at 6 V/cm with buffer recirculation. After electrophoresis, the gels were removed from the tubes and washed in distilled water for about 1 h. When unlabelled chick cell RNA was used as a marker, the RNA was visualized by laying the gels on a thin-layer plate impregnated with a fluorescent dye (Polygram CEL 300 UV₂₅₄), and observed under u.v. light. The RNA appeared as dark purple against a bright green background, and as little as 10 μg could be detected in this way. Gels were sliced and solubilized as previously described for determination of radioactivity (Kennedy & Burke, 1972).

**Chromatography of RNA on cellulose columns.** Single- and double-stranded RNA were fractionated by chromatography on CF11 cellulose as described by Bishop & Koch (1969). Columns (11 cm × 1.5 cm) were packed under gravity in TNE plus 35% (v/v) ethanol. Samples, containing not more than 0.5 mg/ml RNA, were loaded in 35% ethanol TNE buffer and eluted as described below at a rate of 1 ml/min at room temperature. Fractions (1 ml) were collected in tubes containing 10% (v/v) 2-mercaptoethanol (0.1 ml).

**Salt fractionation of RNA.** Single- and double-stranded RNA were fractionated by treating cell extracts with 1 M-NaCl in TE buffer (0.05 M-tris, 0.001 M-disodium salt of EDTA,
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Fig. 1. Polyacrylamide gel electrophoresis of the RNA species extracted from infected cells which had been labelled with [3H]-uridine from 2 to 6 h post-infection. The arrow shows the position of the 28S chick ribosomal RNA marker, and the labels the identity of the various species. Migration is towards the right.

adjusted to pH 7.0 at 20 °C with HCl) at -20 °C for 18 h by the method of Bishop & Koch (1969), or with 2 M-LiCl at 4 °C (Baltimore & Girard, 1966). In both cases, precipitates were collected by sedimentation at 1500 g for 20 min and washed with the corresponding salt. The precipitate was dissolved in TNE buffer, precipitated with 2 vol. of ethanol and stored at -20 °C.

Treatment with ribonuclease. Preliminary experiments showed that single-stranded RNA was completely digested to TCA-soluble material by treatment with 10 μg/ml of ribonuclease for 10 min at 37 °C in 3 × TNE, 3 × TN or SSC buffers (0.15 M-NaCl, 0.015 M-trisodium citrate, adjusted to pH 7 with HCl). Enzyme action was terminated by addition of BSA (100 μg/ml) and 5% TCA, sedimentation and washing with 5% TCA only, before determination of radioactivity. Washing with ethanol caused loss of precipitated radioactivity.

Denaturation of RNA with dimethylsulphoxide (DMSO). The method was based on that of Iglewski & Franklin (1967). Samples were dissolved in TE buffer and 6 vol. DMSO added. The solution was incubated at 37 °C for 2 min and quickly cooled on ice. One-tenth vol. of 10 × TNE buffer was added, and the RNA precipitated with two vol. of ethanol at -20 °C.

Annealing of RNA. Samples were dissolved in 2 × SSC or 3 × TNE buffer, and incubated at 65 °C in thin-walled, 2 ml glass tubes, fitted with screw-caps. After incubation, samples were cooled in ice. In order to estimate the time of annealing, samples were treated with ribonuclease (10 μg/ml ribonuclease A plus 34 units/ml ribonuclease T1 at 37 °C for 15 min in SSC or TNE buffer), before and after incubation. Ribonuclease-resistant material was estimated by precipitation with cold 5% TCA as described.
RESULTS

Separation and characterization of the virus-specified RNA species

General

Chick embryo cells were infected with SFV at a multiplicity of 10 p.f.u./cell in the presence of 1 μg/ml actinomycin D in order to suppress cellular RNA synthesis. The virus RNA species were labelled by addition of [3H]-uridine (100 μCi/culture of 10⁸ cells), extracted with phenol and SDS and fractionated by electrophoresis on polyacrylamide gels. A number of components were observed (Fig. 1), the pattern being similar to that reported by Levin & Friedman (1971). The different species were identified by electrophoresis of virus RNA under similar conditions, by fractionation of the RNA species by use of cellulose chromatography or salt precipitation, and by measuring the effect of ribonuclease on the different species, as described below.

Virus RNA was obtained by extraction from purified virus which had been grown in the presence of [3H]-uridine. Polyacrylamide gel electrophoresis showed that the component at fraction 60 in Fig. 1 was virus RNA.

Further information was obtained by fractionating the RNA species on cellulose columns. Franklin (1966) showed that when nucleic acids were chromatographed on a column of CF₁₁ cellulose, and eluted stepwise with decreasing concentrations of ethanol in a suitable buffer at pH 7, DNA and low mol. wt. RNA were eluted with 35% ethanol, the majority of the single-stranded RNA was eluted with 15% ethanol, and double-stranded RNA (RF) and multi-stranded RNA (RI) were eluted with buffer containing no added ethanol.

Application of this technique to the virus RNA species which had been labelled between
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Table 1. Ribonuclease resistance of SFV RNA fractionated by treatment with 1 M-NaCl and chromatography on CF11 cellulose

<table>
<thead>
<tr>
<th>Fraction</th>
<th>RNase resistance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 RNA soluble in 1 M-NaCl</td>
<td>≥90</td>
</tr>
<tr>
<td>2 RNA insoluble in 1 M-NaCl, and chromatographed on CF11 cellulose: (a) eluted with 15% ethanol</td>
<td>1.4</td>
</tr>
<tr>
<td>(b) eluted with buffer</td>
<td>36</td>
</tr>
</tbody>
</table>

* Samples were treated with ribonuclease (10 μg/ml) for 10 min at 37 °C in 3×TNE buffer. The radioactivity of TCA-insoluble material was then determined.

Fig. 3. Fractionation by polyacrylamide gel electrophoresis of the material eluted with buffer containing 15% ethanol from CF11 cellulose (see Fig. 2). The positions of Hela nucleolar 45 S, 41 S, 32 S and 28 S RNA are indicated by arrows. Migration is towards the right.

2 and 6 h after infection in the presence of 1 μg/ml of actinomycin D gave the result shown in Fig. 2. A negligible amount of radioactivity was eluted with buffer containing 35% ethanol, and the majority with buffer containing 15% added ethanol. The material eluted from CF11 cellulose with 15% ethanol was completely sensitive to ribonuclease (Table 1), and the RNA was therefore single-stranded. When this material was analysed on a polyacrylamide gel, only those species whose electrophoretic mobility was greater than or equal to virus RNA were seen (Fig. 3, which is taken from Kennedy, 1972). Two major peaks (at fractions 17 and 45) and two minor peaks (at fractions 21 and 35) of intermediate mobility were detected. An additional peak of lower mol. wt. material (about 20 S) was also occasionally observed. The mobilities and proportions of these species were similar to those of the 42 S, 38 S, 33 S and 26 S RNA species described by Levin & Friedman (1971).
in cells infected with Sindbis virus. Sucrose gradient sedimentation of the material eluted with 15% ethanol showed two peaks of radioactivity with sedimentation coefficients of 42S and 26S. The faster-sedimenting peak coincided with that of added virus RNA. The two major peaks thus correspond to virus RNA and the interjacent RNA described by Martin (1967).

Further fractionation was obtained by a combination of salt fractionation and cellulose column chromatography. Montagnier & Sanders (1963) showed that double-stranded RNA was soluble in 1 M-NaCl, while single-stranded RNA was insoluble, and Bishop & Koch (1969) found that poliovirus RI, which has the properties of both single- and double-stranded RNA, was co-precipitated with single-stranded RNA by 1 M-NaCl. However, single-stranded RNA can be separated from other forms of RNA by chromatography on CF11 cellulose. Thus, a combination of salt fractionation and chromatography on CF11 cellulose provides a way, in principle, of separating double-stranded, multi-stranded and single-stranded RNA.

**Double-stranded RNA**

The RNA soluble in 1 M-NaCl was characterized by polyacrylamide gel electrophoresis as follows. RNA was extracted from infected cells which had been labelled with [3H]-uridine
between 2 and 6 h after infection in the presence of actinomycin (1 μg/ml). The RNA was precipitated with ethanol and redissolved in TNE buffer before addition of an equal vol. of 2 M-NaCl. The precipitate was removed by sedimentation and RNA recovered from the supernatant fluid by addition of ethanol. When the salt supernatant RNA, labelled with [14C] was treated with ribonuclease (10 μg/ml, 37 °C, 15 min, in 3 × TNE buffer), and co-electrophoresed with untreated salt supernatant RNA labelled with [3H], both materials yielded a major peak which had very similar mobility (Fig. 4). The small
Table 2. Comparison of the proportions of the different forms of RNA, estimated by polyacrylamide gel electrophoresis (PAGE), CF11 cellulose chromatography and treatment with 1 M-NaCl

<table>
<thead>
<tr>
<th>% radioactivity in:</th>
<th>Page*</th>
<th>CF11†</th>
<th>1 M-NaCl‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-stranded RNA</td>
<td>8.1</td>
<td>22.0</td>
<td>9.8</td>
</tr>
<tr>
<td>Multi-stranded RNA</td>
<td>13.5</td>
<td>22.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Single-stranded RNA</td>
<td>78.4</td>
<td>78.0</td>
<td>90.2</td>
</tr>
</tbody>
</table>

* Polyacrylamide gel electrophoresis. RNA from cells labelled 4 h to 5 h 10 min after infection.
† CF11 cellulose chromatography. RNA from cells labelled 4 h to 5 h after infection.
‡ Treatment with 1 M-NaCl. RNA from cells labelled 2 to 6 h after infection.

Table 3. Molecular weights of SFV single-stranded RNA species

<table>
<thead>
<tr>
<th>Species</th>
<th>Mol. wt. (× 10⁻⁶)*</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>42S</td>
<td>4.0 ± 0.08</td>
<td>15</td>
</tr>
<tr>
<td>38S</td>
<td>3.1 ± 0.07</td>
<td>11</td>
</tr>
<tr>
<td>33S</td>
<td>2.3 ± 0.04</td>
<td>12</td>
</tr>
<tr>
<td>26S</td>
<td>1.8 ± 0.02</td>
<td>15</td>
</tr>
</tbody>
</table>

* ± standard error of the mean.

difference in mobility of the major peak may have been due to the presence of two species present in different proportions, not resolved here but resolved in Fig. 5b.

The ribonuclease-treated material showed another peak of higher mobility at 43 mm on the gel, while the salt supernatant RNA contained only a trace amount of this species. The small peak at 56 mm was not seen on other occasions. In addition, the salt supernatant RNA contained a small amount of material which did not enter the gel, and was probably RI. It thus appeared that the RNA soluble in 1 M-NaCl was double-stranded, and this was confirmed by treatment with ribonuclease (Table 1).

Replicative intermediate

The RNA in the 1 M-NaCl precipitate was fractionated by cellulose chromatography before polyacrylamide gel fractionation. The salt precipitate was redissolved in TNE buffer, and ethanol added to 35 %. Chromatography on CF11 cellulose gave a result similar to that shown in Fig. 2, except that there was less material eluted with 0 % ethanol. The majority of the radioactivity was eluted with 15 % ethanol (fraction A) and the remainder was eluted with buffer containing no added ethanol (fraction B). When fraction B was electrophoresed, radioactivity was only found in the first few fractions of the gel (Fig. 5a). However, when this RNA was treated with ribonuclease (10 μg/ml, 37 °C, 15 min in 3 × TNE buffer), and co-electrophoresed on a gel with RNA soluble in 1 M-NaCl, three peaks were observed (Fig. 5b). The first two of these peaks corresponded to the major peak in Fig. 4 (at 35 to 36 mm), and the third to the minor peak at 43 mm. Thus, a species of RNA was extracted from infected cells which was precipitated by 1 M-NaCl, which is a property of single-stranded RNA, but chromatographed on CF11 cellulose in the way expected of double-stranded RNA. The material was also partially resistant to ribonuclease (Table 1), and thus had the properties of a replicative intermediate (RI). It was concluded that the RNA which failed to enter the gel was not an artefact caused by aggregation of RNA, but represented a complex, multi-stranded structure of RNA.
Because the multi-stranded RNA only entered the first few gel fractions, it was important to test whether or not the different species of virus-specified RNA were recovered in the same proportions by polyacrylamide gel electrophoresis as by other techniques. In order to do this, the relative amounts of radioactivity in multi-stranded, double-stranded and single-stranded RNAs were compared with the relative recoveries of these species by CFI cellulose chromatography and by salt precipitation (Table 2). It was found that the proportions of the different types of RNA were approximately the same, when estimated by polyacrylamide gel electrophoresis, or estimated from the recoveries using the preparative techniques. Therefore, polyacrylamide gel electrophoresis can be used to estimate the relative amounts of the different types of RNA found in SFV-infected cells.

Thus, four species of single-stranded virus RNA, three species of double-stranded virus RNA and a multi-stranded virus RNA, which yielded the same three double-stranded species, were found.

**Molecular weights of the virus-specified single-stranded RNA species**

The mol. wt. were determined by comparison of the electrophoretic mobility of the virus RNA species with those of the chick ribosomal RNA species, it being assumed that there is a linear relationship between mobility and log (mol. wt.) (Bishop, Claybrook & Spiegelman, 1967). Values of $1.58 \times 10^6$ and $0.7 \times 10^6$ were taken for the mol. wt. of the 28S and 18S chick ribosomal RNA species (Loening, 1968). [$^3$H]-labelled virus-specified RNA extracted from infected cells was mixed with [$^{14}$C]-labelled RNA from uninfected
Fig. 7. Polyacrylamide gel electrophoresis in 2.0 % gels containing 0.5 % agarose of SFV-specified RNA, labelled at 5 h after infection for (a) 1 min, (b) 2 min and (c) 5 min with [3H]-uridine. Migration is towards the right.
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Fig. 8. Incorporation of [3H]-uridine into (a) total virus-specified RNA and (b) the different species of virus RNA during short pulses, estimated from the electrophoretic profiles shown in Fig. 7.

chick cells, and the mixtures were co-electrophoresed on 2-0 % acrylamide gels. The mean values for the mol. wt. of the four single-stranded species are shown in Table 3.

The kinetics of virus RNA synthesis

In order to determine which species of virus RNA were synthesized first after infection, chick cell monolayers were treated with 1 μg/ml of actinomycin D for 1 h before infection with virus. Cultures were then pulsed with [3H]-uridine for 30 min periods starting from the addition of virus until 11 h after infection. RNA was extracted from the cells after each pulse, and analysed by polyacrylamide gel electrophoresis (Fig. 6).

After an initial lag of about 1 h, double-stranded (RF) and multi-stranded (RI) were the first species to be detected (Fig. 6c) and their rate of synthesis was found to increase at a linear rate (data not shown). No synthesis of single-stranded species was detected for another 30 min, after which time 42S RNA was observed (Fig. 6d). The 26S species could be detected within the next 30 min (Fig. 6e) and the 33S and 38S species at slightly later times (data not shown).

Infected cells were also labelled with [3H]-uridine for short periods at 5 h after infection, when synthesis of virus-specified RNA was at its maximum rate (unpublished data). Analysis of the product by polyacrylamide gel electrophoresis showed that when the cells were labelled for 1 or 2 min, most radioactivity was found in RI or RF (Fig. 7a, b). When the period of labelling was 5 min or longer, however, most radioactivity was found in a single-stranded species of RNA (Fig. 7c). Measurement of the radioactivity associated with each species of virus-specified RNA enabled an estimate to be made of the rate of labelling of the total virus-specified RNA and of each RNA species (Fig. 8a, b). The radioactivity in 26S was approx. 1-8 times that in 42S RNA in all the labelling periods (Table 4). A similar result was obtained with longer labelling times of 15, 30 and 70 min (results not shown). Assuming that these species are labelled to the same specific activity in the cell, this means that 26S RNA molecules are being synthesized at 3-7 times the rate
Table 4. Ratios of radioactivity in 26S RNA and 42S RNA, and in RI and single-stranded (SS) RNA after short exposures of SFV-infected cells to [3H]-uridine*

<table>
<thead>
<tr>
<th>Time of addition of [3H]-uridine (min)</th>
<th>Radioactivity in</th>
<th>Ratio of</th>
<th>Ratio of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26S RNA</td>
<td>42S RNA</td>
<td>26S RNA : 42S RNA</td>
</tr>
<tr>
<td>1</td>
<td>176</td>
<td>97</td>
<td>1:8:1</td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>37</td>
<td>2:1:1</td>
</tr>
<tr>
<td>5</td>
<td>885</td>
<td>705</td>
<td>1:3:1</td>
</tr>
<tr>
<td>10</td>
<td>1890</td>
<td>954</td>
<td>2:0:1</td>
</tr>
</tbody>
</table>

* SFV-infected cells were pulse-labelled 5 h after infection.

of the 42S RNA molecules, since 42S RNA is approx. 2.3 times the size of the 26S RNA (Table 3.) Extraction in the presence of diethylpyrocarbonate, as described by Öberg & Phillipson (1971), had no effect on the relative proportions of double- and multi-stranded RNA.

An approximate calculation of the average time to synthesize a molecule of RNA can be made by determining the pulse time required to give equal amounts of radioactivity in RI and single-stranded RNA (Baltimore, 1969). The ratio of the radioactivities of RI and single-stranded RNA determined from polyacrylamide gels is shown in Table 4. This ratio needs to be corrected for the slow equilibration of [3H]-uridine with the intracellular pool, and this correction may be made from the data of Fig. 8 a. In this way a value of between 1 and 1.5 min for the average synthesis time for all species of virus RNA was found. However, the radioactivity in RI only approaches that in single-stranded RNA after a single synthesis time as $N_p$ tends to infinity, where $N_p$ is the number of polymerase molecules per RI. For one synthesis time and any given value of $N_p$, the ratio is greater than one if the newly synthesized strands of RNA are displaced by the next polymerase molecule, the difference from unity being greater the smaller the value of $N_p$. The ratio is correspondingly less than one if the newly synthesized strands are detached from the RI on completion. For $N_p = 8$, the value found here (see below), the ratio is changed by ±20 %, and since we are not able to choose between the two models for displacement of newly synthesized strands, an additional error is introduced to the calculation of the time of synthesis.

The molar ratios of the three species of RF (RF I:RF II:RF III, Fig. 5 b) under different conditions were determined by treatment of purified RI or RF with ribonuclease and separation of the three species on polyacrylamide gels. The mol. wt. found by Simmons & Strauss (1972 b) were used to calculate the molar ratios. Values of 1:0:0:62:0:58 were found when label was present from 2 to 6 h after infection (average of four determinations). These ratios are similar to but somewhat higher than the ratios of the three forms found in purified RF, and analysed without ribonuclease treatment, when ratios of 1:0:42:0:39 were found after a 1 min pulse, and as low as 1:0:14:0:14 after pulsing from 2 to 6 h after infection.

Annealing properties of the free single-stranded virus RNA species

Experiments were performed to detect the presence of labelled RNA, whose nucleotide sequence was complementary to that of 42S virus RNA. As a positive control, [14C]-labelled polycytidylic acid (poly C) was annealed to unlabelled polyguanylic acid (poly G).
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Table 5. Annealing of single-stranded RNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration* (µg/ml)</th>
<th>Time at 65 °C (h)</th>
<th>Ribonuclease resistance (% TCA-insoluble radioactivity) (a) before annealing</th>
<th>(b) after annealing</th>
<th>Annealing† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]-poly C+poly G</td>
<td>300</td>
<td>1</td>
<td>0.1</td>
<td>96.3</td>
<td>96.2</td>
</tr>
<tr>
<td>[3H]-42 S RNA</td>
<td>400</td>
<td>8</td>
<td>3.8</td>
<td>3.1</td>
<td>0</td>
</tr>
<tr>
<td>Unlabelled 42 S RNA + [3H]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>single-stranded RNA labelled:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 2 to 6 h</td>
<td>29</td>
<td>3</td>
<td>0.9</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>(b) 2½ to 3 h</td>
<td>200</td>
<td>5</td>
<td>0.4</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>(c) for 1 min at 5 h</td>
<td>150</td>
<td>5</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Concentration of unlabelled RNA, except in the case of [3H]-42 S RNA.
† % annealing was taken as % ribonuclease-resistant radioactivity after incubation, minus that before incubation.

The optimal conditions for hybridization were calculated from the initial and final concentrations of single-stranded RNA, and an estimated value of the rate constant K, the inverse of time required for half reaction.

Table 6. Ribonuclease resistance of multi-stranded RNA

<table>
<thead>
<tr>
<th>Labelling period after virus infection</th>
<th>Ribonuclease resistance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 to 6 h</td>
<td>30</td>
</tr>
<tr>
<td>2½ to 3 h</td>
<td>31</td>
</tr>
<tr>
<td>1 min at 5 h</td>
<td>70</td>
</tr>
</tbody>
</table>

* 10 µg/ml of ribonuclease A plus 34 units/ml of ribonuclease T 1 at 37 °C for 15 min in SSC.

Before annealing, [14C]-poly C was about 0.1 % resistant to ribonuclease treatment, while after annealing it was 96 % resistant (Table 5). By contrast, less than 1 % self-annealing could be detected with 42 S virus RNA (Table 5). In order to prepare virus-specified RNA for annealing, infected cells were labelled with [3H]-uridine between 2 and 6 h after infection, between 2½ and 3 h after infection and for 1 min at 5 h after infection. RNA was extracted from the cells, and single-stranded RNA was purified by salt precipitation and chromatography on a CFI cellulose column. The labelled, single-stranded RNA obtained by this procedure was incubated under annealing conditions with known amounts of unlabelled 42 S virus RNA. No significant annealing could be detected between any of these labelled preparations and 42 S virus RNA (Table 5). Thus, very little (less than 1 %) RNA complementary to SFV particle RNA occurs either in the virus particle, or free in the infected cell from the earliest time in the infectious cycle that sufficient single-stranded RNA could be prepared for the annealing assay.

Ribonuclease resistance of multi-stranded RNA

In order to determine the ribonuclease resistance of multi-stranded RNA, purified preparations were required. RNA was prepared from cells which had been labelled between 2 and 6 h after infection (continuously labelled RNA) or for 1 min at 5 h after infection (pulse-labelled RNA). After extraction of the RNA from the cells, it was purified by salt precipitation and three cycles of chromatography on CFI cellulose. The purified preparations were treated with ribonuclease, and the proportions of radioactivity which remained insoluble in TCA are shown in Table 6.
The single-stranded RNA content of a labelled multi-stranded RNA may be estimated from its ribonuclease resistance, \( R \), using the equation (Baltimore, 1969):

\[
R = \frac{\text{proportion of radioactivity in hydrogen-bonded form}}{\text{total radioactivity}}.
\]

If the multi-stranded RNA is uniformly labelled, there will be the equivalent of two single-stranded RNA molecules in the ribonuclease-resistant 'core'. If double-stranded RNA is 100 % resistant to ribonuclease, and if single-stranded RNA is 0 % resistant to ribonuclease, then the number of single-strand equivalents, \( N \), is given by the equation:

\[
N = \frac{100 \times 2}{2 + N}.
\]

However, double-stranded RNA was found to be about 93 % resistant to the conditions of ribonuclease digestion used, and about 1 % single-stranded RNA remained TCA-insoluble under the same conditions. Using these values, the equation becomes

\[
N = \frac{186 - 2R}{R - 1},
\]

Substituting the value of \( R \) (30 %) for continuously labelled multi-stranded RNA, \( N \) was found to be 4.3.
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The number of polymerase molecules per RI (*Np*) can also be calculated from its ribonuclease resistance as follows. If the newly synthesized RNA chains are not detached from the RI until the next polymerase molecule discharges them, two extreme arrangements of the polymerase molecules can be envisaged (Fig. 9), where *Np* = 4.

A random population of RIs will generate the average of these extremes of numbers of single-strands, i.e.

\[
N = \frac{1}{2} \left[ \sum_{n=1}^{Np-1} n + \sum_{n=1}^{Np} n \right]
\]

\[
= \frac{1}{2} \left[ 2 \sum_{n=1}^{Np-1} n + Np \right]
\]

\[
= \frac{Np-1}{Np} \sum_{n=1}^{Np} n + 0.5;
\]

and since

\[
\sum_{n=1}^{Np-1} n = \frac{Np}{2} (Np - 1),
\]

\[
N = \frac{Np}{2}.
\]

Thus

\[Np = 8.6.\]

However, if the newly synthesized RNA chains are detached from the RI on completion, rather than by displacement by the next polymerase molecule, equation (3) has to be modified, and this assumption gives a value of *Np* between 10 and 11, rather than between 8 and 9. Thus the number of polymerase molecules per RI depends on the way in which the nascent strands are displaced from RI. However, the number of single-stranded equivalents *N*, derived from equation (2), is independent of the model used for displacement.

**Attempted separation of strands of virus-specified double-stranded RNA**

An attempt was made to separate the strands of double-stranded RNA by treatment with DMSO. Double-stranded RNA was extracted from infected cells, purified by salt precipitation of single- and multi-stranded species of RNA, and further purified by chromatography on CF11 cellulose three times. Before treatment with DMSO, the double-stranded RNA was 93% resistant to ribonuclease, but only about 1% resistant after treatment. Polyacrylamide gel electrophoresis of the product showed a series of peaks, the largest of which was 42 S RNA. The peaks of lower mol. wt. could not be identified with certainty.

**DISCUSSION**

The separation techniques used in this study, polyacrylamide gel electrophoresis, chromatography on CF11 cellulose and salt precipitation, made possible the separation of a number of virus-specified RNA species extracted from cells infected with SFV. Four species of single-stranded RNA were found. These corresponded with the 42 S, 38 S, 33 S and 26 S species identified by Levin & Friedman (1971) in cells infected with the related Sindbis virus. The mol. wt. of these species, determined by use of polyacrylamide gel electrophoresis, were 4.0 x 10^6, 3.1 x 10^6, 2.3 x 10^6 and 1.8 x 10^6. These values are close to those estimated by Levin & Friedman (1971) and Kennedy (1972), who both also used
polyacrylamide gel electrophoresis. The mol. wt. of the 42S and 26S species of Sindbis virus have also been estimated by sedimentation on sucrose gradients containing DMSO or formaldehyde, and by competition hybridization. The values obtained by these techniques were $43.3 \pm 0.3 \times 10^6$ for 42S RNA, and $1.6 \times 10^6$ for 26S RNA (Simmons & Strauss, 1972a). Thus, the mol. wt. values estimated for the 42S and 26S RNA species were close to those determined by other physical techniques.

Three species of double-stranded RNA (replicative form, RF I, RF II and RF III) were obtained by ribonuclease treatment of the RI. No accurate values could be obtained for their mol. wt., due to breakdown of the RNA on chain separation, although the data suggest that the largest RF was a duplex of 42S RNA with a consequent mol. wt. for the RF of $8 \times 10^6$. Using sucrose gradient sedimentation, Simmons & Strauss (1972b) have recently estimated that the mol. wt. of the double-stranded species of RNA from BHK cells, infected with Sindbis virus, are RF I $8.8 \times 10^6$, RF II $5.6 \times 10^6$ and RF III $2.9 \times 10^6$. They concluded that the largest and smallest of these are duplexes of Sindbis virus RNA and Sindbis 26S RNA, respectively.

The relative proportions of these three species of RF have also been determined. Stollar et al. (1972), using chick cells infected with Sindbis virus, found that the proportions varied as a function of time after infection. The labelling ratios of RF I:RF II:RF III were approx. 1:0:4:0:4 in the first 6 h after infection, and approx. 1:0:6:0:6 from 6 to 11 h after infection. They also found different values of the ratios in infected BHK and mosquito cells. Simmons & Strauss (1972b), using Sindbis virus-infected chick and BHK cells, found that the molar ratio of RF I:RF II fell early in infection to about 0:4 but then rose to about 1:6 at 12 h after infection. They found the molar ratio of RF II:RF III to be 1:0 throughout infection. These observations were made by treatment of the total RNA extract with ribonuclease before separation of the RFs, and the double-stranded species were therefore derived from both RI and RF. We treated both isolated RI and RF with ribonuclease, and found that the molar ratios for both were 1:0:62:0:58 at 6 h after infection, in contrast to the Simmons & Strauss (1972b) values of 0:4:1:1 (Fig. 3 of their paper). They found molar ratios of 1:0:2:2 after a 1 min pulse while we found 1:0:42:0:39. Thus we find a 1:1 ratio of RF II:RF III at short and long labelling times, whereas Simmons & Strauss (1972b) only find a 1:1 ratio after long labelling times. Our results also differ from those of Simmons & Strauss (1972b) in that we find a smaller figure for the ratio of RF I:RF II (1:0:4 instead of 0:4:1). When free RF was analysed very little RF III was found. The relatively small amount of the two smallest RFs has consequences for the mechanism of RNA replication which are discussed below.

The role of double-stranded RNA in virus replication has been studied in cells infected with poliovirus, and it has been found that the synthesis of RF follows that of single-stranded RNA and RI in a way that suggests that it is a consequence rather than an intermediate of RNA replication (Baltimore & Girard, 1966; Noble & Levintow, 1970). However, we have found that RF was labelled more rapidly than RI early in infection (Fig. 6) and almost as rapidly with short pulses of $[^3H]$-uridine when virus RNA synthesis was at its maximum rate (Fig. 8b). We have considered three alternative suggestions to explain the rapid labelling of RF. First that RF is produced by the effect of cellular ribonuclease on RI, producing material with very short or no single-stranded tails. This explanation cannot be excluded, but is unlikely since we have found that the ratio of RF/RI is constant in a large number of experiments using different times after infection and different pulse lengths (Fig. 8b). The second possibility is that it is a product of RI turnover. This can readily be excluded, for the maximum amount of radioactivity expected in RF would be
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\[ I/(1 + N) \times S \], where \( S \) is the radioactivity in single-stranded RNA and \( N \) is the number of single-strand equivalents per RI, that is about 20% of \( S \), since \( N \) is 4.3. Thus even if the turnover time of RI were as low as the average time for synthesizing a molecule of RNA (about 70 s), (and poliovirus RI has a turnover time of about 10 min; Baltimore, 1969) then the most radioactivity one would expect to find in RF is about 20% of that in single-stranded RNA, whereas after a 1 min pulse, the radioactivity in RF was 130% of that in single-stranded RNA. The third possibility, and this we consider to be the most likely, is that RF plays an intermediate role in RNA synthesis, possibly as a precursor of RI. Similar results have recently been published by Michel & Gomatos (1973).

SFV RNA replication is mainly semi-conservative, since the RI obtained from infected cells which had been pulse-labelled with \([^{3}H]\)-uridine for 1 min was 70% ribonuclease-resistant. In semi-conservative or non-conservative replication, the newly synthesized portions of the nascent strands would be expected to remain in close association with the complementary strand. If replication were conservative, very little radioactivity would be expected to be ribonuclease-resistant after labelling for a period less than that required to synthesize a molecule of RNA, unless the newly synthesized portions of the nascent strands are hydrogen-bonded to the template. However, in order to generate such high ribonuclease resistance nearly all the complementary strand (a calculation gives over 80%) would have to be bound, thus giving effectively a semi-conservative mechanism. The non-conservative mechanism, in which each strand is displaced alternately thus producing a product containing equal proportions of complementary strands, is also excluded since no such complementary RNA was found in the free single-stranded RNA.

Finally, a mechanism for virus replication should be considered. The mechanism must explain the existence of four species of single-stranded RNA, three of which (42S, 33S and 26S) are probably messenger RNAs (Kennedy, 1972; Mowshowitz, 1973). It must also explain the existence of three species of double-stranded RNA, and the relative kinetics of labelling of the RI and RF.

The three RNA species which have messenger function are readily explained by a model similar to that proposed by Simmons & Strauss (1972b), in which the 26S and 33S RNA species are arranged end-to-end to form the 42S RNA species. The mol. wt. for these various species fit this well, but the model does not explain the origin of the 38S RNA species, a RNA of unknown function but which does not appear to be a messenger RNA, since it has not been found associated with polysomes. One possible explanation is that 38S RNA is formed by initiation or termination at some other point in the genome.

The largest RF, RF I, is very probably a duplex of 42S RNA. Simmons & Strauss (1972b) suggested that the two smaller RFs, RF II and RF III, were formed by nuclease cleavage of the RI responsible for 26S and 33S RNA production by removal of single-stranded tails and cleavage of the negative 42S RNA strand at the internal termination and initiation point. This explanation was supported by the finding that the ratio of RF II/RF III was unity, and that the ratio of RF I/RF III changed during the course of infection, reflecting the different rates of production of 42S and 26S RNA. However, the relative amounts of RF I, II and III found in this work are not consistent with this suggestion. We also find that the ratio of RF II/RF III is 1, but that the ratio of RF I/RF III is 2:1 6 h after infection, despite the fact that 26S RNA is being produced at this time at a much faster rate than 42S RNA. This means that the relative proportion of RF I/RF III cannot reflect the relative proportion of the RIs making 42S RNA and 26S RNA.

The other evidence for the formation of RF III from the RI responsible for production of 26S RNA comes from hybridization data (Simmons & Strauss, 1972b). However, their
Fig. 10. A proposed model for the replication of SFV RNA. A and B represent initiation sites for RNA synthesis on the template strand, and C and D termination sites. Diagonal lines represent nascent molecules of RNA.

Data can also be interpreted as showing that RF II contains some of the nucleotide sequences in 26S RNA. Simmons & Strauss (1972b) also state that 26S RNA contains all the information in the plus strand of RF III. They showed that non-radioactive 26S RNA would compete out labelled 26S RNA and 49S RNA, but their conclusion does not follow unless it had been shown that 100% of the minus strand of RF III will hybridize with 26S RNA. We conclude that although the mol. wt. and the molar ratios of RF II and RF III suggest that they are derived from a single nucleolytic cleavage of RF I, there is no convincing evidence that RF II and RF III are derived from the RIs involved in the production of 26S and 33S RNA. This conclusion is supported by recent experiments by S. I. T. Kennedy (personal communication) who found that when mouse-brain-grown SFV was used as an inoculum for BHK cells, little or no RF II or RF III was produced, but that when virus was passed several times in BHK cells, the proportion of the smaller RFs rose. Since more non-infectious virus is produced under the latter conditions, this suggests that the formation of the smaller RFs may be due to an abortive infection.

If the two smaller RFs are not derived from RIs involved in normal RNA transcription, then other alternatives for the arrangement of the RNA transcripts are possible, e.g. an overlapping pattern as shown in Fig. 10. In this model, synthesis of 42S is initiated at site A, under the direction of a protein which normally recognizes site D as a termination signal, but which occasionally recognizes site C as a termination signal, generating 33S RNA. The synthesis of 26S RNA is initiated at site B, and the protein that controls this
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normally recognizes site C as a termination signal, but occasionally reads through to site D, generating 38S RNA. The relative amounts of the single-stranded species are controlled by the relative efficiencies of initiation at sites A and B and termination at sites C and D. In Fig. 10, 33S is shown as region AC and 38S as region DB, but this choice is arbitrary.

There is one clear difference between the two models that could be tested experimentally. In the Simmons & Strauss model, synthesis of 42S RNA and 26S RNA is initiated at the same point, at one end of the template, whereas in this model, synthesis of 26S RNA is initiated internally.

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