Transcription of Double-stranded RNA in Virions of
Aspergillus foetidus Virus S

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SUMMARY

It is shown that the virion RNA polymerase of Aspergillus foetidus virus S, which has a genome consisting of three double-stranded (ds)RNA species, is a transcriptase. Synthesis of single-stranded RNA (ssRNA) in vitro continues for up to 48 h, during which time 6 to 8 full length transcripts are produced, on average, per molecule of dsRNA2, i.e. re-initiation of transcription occurs in this in vitro system. Full length ssRNA transcripts of dsRNA1 are also produced in smaller quantity, but no transcripts of dsRNA3 could be detected. In reactions containing 3H-UTP, label is also incorporated into dsRNA2 reaching maximum levels after 6 h, but no incorporation of labelled precursor into dsRNA1 or dsRNA3 could be detected. The ssRNA transcripts produced were released into the medium, but labelled dsRNA remained within the virions. The locations, in the AfV-S multicomponent system, of RNA polymerase activities promoting incorporation of 3H-UMP into the two ssRNA transcripts and into dsRNA2 have been determined.

INTRODUCTION

Two serologically distinct double-stranded RNA (dsRNA) viruses, AfV-S and AfV-F, have been isolated from the fungus Aspergillus foetidus (Ratti & Buck, 1972) and virions of both viruses have been found to exhibit RNA polymerase activity in vitro (Ratti & Buck, 1975). AfV-S contains two major dsRNA components, RNA1 (60 % G+C, mol. wt. 4.1 x 10^6) and RNA2 (47 % G+C, mol. wt. 2.6 x 10^6) and a smaller, minor dsRNA component, RNA3 (mol. wt. 0.27 x 10^6; Buck & Ratti, 1975, 1977). AfV-S is a multicomponent virus, consisting of four basic virions, two major, S1a and S2a, which contain RNA2 and RNA1 respectively, and two minor, S1b and S2b, which contain RNA2 plus RNA3 and RNA1 plus RNA3 respectively. In addition virus preparations contain small amounts of particles (S2x, S3 and S4) which contain RNA2, but are in order increasingly more dense than S1a particles. The capsids of all particles are constructed from the same polypeptides.

In the present paper the nature and rate of formation of the products of RNA synthesis in vitro catalysed by AfV-S virion associated RNA polymerase, and the distribution in the AfV-S multicomponent system of polymerase activity and products, are reported.

METHODS

Buffer solutions. Buffer A: 0.3 M-NaCl + 0.03 M-Na citrate, pH 7.0; buffer B: 0.2 M-NaCl + 10 mm-tris-HCl + 2.5 mm-Na phosphate + 5 mm-EDTA, pH 6.85.
Preparation and purification of virus. Virus preparations were obtained from *Aspergillus foetidus* Strain IMI 41871, grown in 60 l fermenters. Concentration of virus particles by polyethylene glycol precipitation and purification by velocity sedimentation in sucrose gradients and by DEAE-cellulose chromatography have all been described previously (Ratti & Buck, 1972, 1975; Buck & Ratti, 1975). Purified AfV-S was dialysed against 0·05 mM-tris-HCl buffer, pH 7·9, containing 0·15 M-NaCl and 0·1 mM-EDTA and stored in sterile vials at 4 °C or in the same buffer made 50 % by vol. in glycerol at -20 °C.

**RNA polymerase assay.** Standard reaction mixtures contained: 0·05 M-tris-HCl buffer, pH 7·9; 0·15 M-NaCl; 0·1 mM-EDTA; 3 mM-MgCl₂; 0·15 mM-ATP; 0·15 mM-GTP; 0·15 mM-CTP; 0·15 mM-[^3]H-UTP (sp. act. 50 to 300 mCi/mmol); virus (80 to 360 μg/ml); actinomycin D, 125 μg/ml and, when included, bentonite, 800 μg/ml, prepared according to Fraenkel-Conrat et al. (1961). Incubation was at 30 °C, unless otherwise stated. Removal of bentonite from solutions was accomplished by centrifugation at 38000 g for ~ h; the supernatants were then filtered through 0·22 μm Millipore filters into sealed sterile vials. When necessary, the reaction, which is completely dependent on Mg²⁺ ions, could be stopped by adding EDTA to a final concentration of 10 mM and immediately chilling the sample on ice. Incorporation of[^3]H-UMP into acid insoluble material was determined by adding to 25 to 200 μl of reaction mixture, 3 ml of 10 % (w/v) trichloroacetic acid and allowing precipitation to occur at 0 °C for 30 min. The precipitate was collected on glass fibre filters (Whatman CF/F) and washed with 80 ml of 2 % trichloroacetic acid and then with ethanol. Filters were dried and radioactivity was determined by liquid scintillation counting in a toluene based scintillation fluid.

**Preparation of virus RNA.** Virus suspensions were made 1 % in sodium dodecyl sulphate and then extracted with phenol. The ethanol precipitated RNA was resuspended in the buffer required and then dialysed against the same buffer. For separating ssRNA and dsRNA two methods were used. (a) Chromatography on cellulose (Whatman CF11), performed as described by Franklin (1966). (b) Selective precipitation of ssRNA in 2 M-LiCl. RNA solutions were made 2 M in LiCl and stored at 4 °C for 24 h. The precipitate was collected by centrifugation, washed in 4 M-LiCl and dissolved in buffer and the solution was dialysed against the same buffer. DsRNA remaining in the 2 M-LiCl solution was precipitated with 3 vol. of ethanol at -20 °C overnight and resuspended in buffer and residual ssRNA was removed by two more cycles of precipitation in 2 M-LiCl in the presence of yeast RNA carrier.

**Analysis of RNA by electrophoresis.** For electrophoretic analysis of the RNA product of the polymerase reaction in 4 % polyacrylamide gels, samples were either prepared by phenol extractions or small amounts of reaction mixture were layered directly on to the gel; in the latter case release of RNA from within virus particles was achieved, when required, by heating at 60 °C in the presence of 1 % sodium dodecyl sulphate. Heat denaturation of dsRNA prior to electrophoresis was as described by Buck & Ratti (1977). Electrophoresis was carried out in 0·04 M-tris-0·02 M-acetate-2 mM-EDTA buffer, pH 7·8, and also in a system containing 8 M-urea (Buck & Ratti, 1977). Gels, cast in quartz tubes, were scanned directly at 260 nm in a Gilford Model 240 spectrophotometer equipped with a Model 2410-S gel scan attachment and subsequently were either stained with 0·01 % aqueous toluidine blue or sliced for radioactivity measurements. For the latter the dried gel slices were solubilized by soaking in 0·3 ml of hydrogen peroxide (30 % w/w), containing 1 % by vol. of aqueous ammonia (specific gravity 0·88) and incubating overnight at 37 °C in tightly capped vials. Five ml of 2-methoxyethanol and 10 ml of scintillation fluid were added and radioactivity was determined in a Beckman LS-230 scintillation spectrometer.
RNA hybridization assays. Two procedures were used in the course of this work. Good efficiency of annealing was obtained with either; however, method B is simpler and higher concentrations of nucleic acid can be obtained more easily.

Method A. This was a modification of the procedure described by Bruton & Kennedy (1975). Mixtures of dsRNA and ssRNA in the proportions required, dissolved in 20 mM-tris-HCl buffer, pH 7.3, containing 1 mM-EDTA, were diluted with 9 vol. of dimethyl sulphoxide and samples were incubated at 50 °C for 5 min. The solution concentrations were then adjusted to 45% dimethyl sulphoxide by vol., 0.3 mM-NaCl, 10 mM-tris-HCl and 0.5 mM-EDTA and incubation was continued at 60 °C for 4 h followed by 16 h at 37 °C. The RNA was precipitated with 3 vol. of ethanol at −20 °C and resuspended in buffer A.

Method B. DsRNA was denatured by heating at 100 °C for 3 min in 10 mM-phosphate buffer, pH 7.0, containing 5 mM-EDTA; annealing was obtained by increasing the salt concentration to 0.2 M-NaCl and incubating at 70 °C for 4 h as described by Vandewalle & Siegel (1976). After annealing, the amount of 3H-dsRNA was determined as the amount of trichloroacetic acid precipitable radioactive material present after incubation for 1 h at 37 °C with ribonuclease A (dsRNA/enzyme ratio = 10 to 20) at high salt concentrations (buffer A or buffer B; Bellamy et al. 1967). Ribonuclease Tt was used together with ribonuclease A in some earlier experiments but since equivalent results were obtained, it was eliminated from the assay.

In all experiments the level of input 3H-RNA was determined in control samples containing the appropriate amount of labelled and unlabelled RNA and processed as described above, with the omission of the ribonuclease step. Hybridization was measured as the percentage of input counts that remained trichloroacetic acid precipitable after ribonuclease digestion.

Analytical ultracentrifugation. Equilibrium density gradient centrifugation of virus particles in cesium chloride solutions was carried out in a Beckman Model E ultracentrifuge equipped with a monochromator and a double-beam u.v. absorption optical system, with photoelectric scanner and multiplexer accessory. Samples were placed in cells with double-sector charcoal filled Epon centrepieces in the 4 place AN-F rotor, and centrifuged at 34,000 rev/min for 24 h at 25 °C. Densities of cesium chloride solutions were calculated from refractive indices (Brakke, 1967) and buoyant densities were calculated as described by Szybalski (1968).

Materials. Materials were obtained as follows: 3H-UTP (The Radiochemical Centre, Amersham, Bucks, U.K.); unlabelled nucleoside triphosphates (P-L Biochemicals, Milwaukee, Wisconsin, U.S.A. and Boehringer, Mannheim, W. Germany); actinomycin D (Merck, Sharp & Dohme, West Point, Pennsylvania, U.S.A.); ribonuclease A (Worthington Biochemical Corporation, New Jersey, U.S.A.); S-adenosyl methionine (AdoMet; Boehringer, Mannheim, W. Germany); acrylamide and N,N'-methylenebisacrylamide (Kodak Ltd, Kirkby, Liverpool, U.K.); tobacco mosaic virus (kindly donated by Dr R. Barton, Glasshouse Crops Research Institute, Littlehampton, U.K.).

RESULTS

Optimization of RNA polymerase reaction conditions

In a preliminary report on the virion associated RNA polymerase activity in AfV-S (Ratti & Buck, 1975), incorporation of 3H-UMP into RNA apparently ceased after 4 h. However further studies have shown that when bentonite was included in the reaction
mixture, as a ribonuclease inhibitor, or when the reagents were treated with bentonite prior to the reaction, incorporation of $^3$H-UMP into product RNA, after an initial faster stage of 4 h, continued at a steady rate for 44 h (Fig. 1). Usually after this period the reaction slows down considerably and cannot be significantly stimulated by addition of further nucleoside triphosphates; this is probably because of disruption of virus particles, which become less stable as a consequence of the reaction (data not presented).

If the RNA polymerase reaction is carried out in the presence of bentonite, the virus binds to it and may be easily removed from the reaction mixture by centrifugation. Comparison of the $^3$H-UMP incorporation in bentonite-washed reaction mixtures and in mixtures containing bentonite in suspension indicated that the adsorption of the virus did not impair polymerase activity nor was it responsible for the increased period of synthetic activity. The optimal concentration of Mg$^{2+}$ for the reaction was 3 mM (at nucleoside triphosphate concentrations of 0.15 mM) when determined in the presence or absence of bentonite, with short (1 h) and long (24 h) incubation periods and over a range of virus concentrations (80 to 360 µg/ml).

The presence of AdoMet causes a 60-fold increase in the rate of RNA synthesis by the
dsRNA dependent transcriptase associated with virions of cytoplasmic polyhedrosis virus (Furuichi, 1974). This effect is independent of methylation, since a similar stimulation is observed with S-adenosylhomocysteine, and appears to be due to an induced conformational change in the enzyme (Furuichi, 1978). Therefore the effect of AdoMet on AfV-S RNA polymerase activity was examined. Standard reaction mixtures containing $10^{-3}\,\text{mM}$, $10^{-2}\,\text{mM}$, $10^{-1}\,\text{mM}$ and $1\,\text{mM}$ AdoMet, when incubated at $30\,\text{°C}$ for $2\,\text{h}$, yielded approximately the same amount of acid insoluble labelled product as similar mixtures containing no AdoMet. In a second experiment the amounts of label incorporated at various times, between $1\,\text{h}$ and $24\,\text{h}$, were determined in parallel for a standard reaction mixture and a mixture containing $1\,\text{mM}$-AdoMet. It was found that addition of AdoMet had little or no effect on the rate of the reaction.

*Analysis and mol. wt. of reaction products*

The RNA products of an $18\,\text{h}$ standard reaction (see Methods) were isolated by phenol/sodium dodecyl sulphate extraction and examined by electrophoresis in polyacrylamide gels with and without $8\,\text{M}$-urea. Gels were scanned at $260\,\text{nm}$ and then sliced and the amounts
Fig. 3. Mol. wt. determinations on the in vitro products. SsRNA products for an 18 h RNA polymerase reaction were extracted as described in the Methods, mixed with ssRNA mol. wt. markers (1, tobacco mosaic virus RNA, mol. wt. = 2.1 x 10^6; 2, phage MS2 RNA, mol. wt. = 1.2 x 10^6; 3 and 4, Escherichia coli 23S and 16S ribosomal RNAs, mol. wt. = 1.05 x 10^6 and 5.3 x 10^5, respectively) and subjected to electrophoresis for 6 h in 8 M-urea-4 % polyacrylamide gels, as described by Buck & Ratti (1977). I, ssRNA1; II, ssRNA2.

of label in the slices were determined. Typical results for a gel without urea are shown in Fig. 2. The gel scan showed, in addition to bands corresponding to template dsRNAs 1 and 2, a major band of newly synthesized RNA of greater mobility; however, label was found, not only in this major band, but also in the band of dsRNA2. If the RNA products were incubated with ribonuclease A in buffer A prior to electrophoresis the major band of newly synthesized RNA was no longer observed, but the amount of label in the band of dsRNA2 was unchanged. It is concluded that the major product of the polymerase reaction is ssRNA, but that ^3H-UMP is also incorporated into dsRNA2.

8 M-Urea reduces the amount of secondary structure in ssRNA and electrophoresis of ssRNA in urea gels gives much sharper bands of RNA with consequent increased sensitivity and is more suitable for mol. wt. determination than electrophoresis in gels without urea (Floyd et al. 1974). Under these conditions the RNA polymerase product was shown to contain, in addition to the major band of ssRNA, a second minor band of ssRNA (about 3 % of the major band). In the gel without urea, shown in Fig. 2, this minor RNA component was not separated from dsRNA2; however, in urea gels good separation was obtained (see Fig. 7). The mol. wt. of the major and minor ssRNA components (designated ssRNA2 and ssRNA1 respectively), determined from plots of log mol. wt. versus mobility with internal ssRNA standards (Fig. 3) were 1.35 x 10^6 and 2.1 x 10^6, i.e. about half the mol. wt. of dsRNA2 and dsRNA1. Further co-electrophoresis experiments showed that ssRNA2 and ssRNA1 had mobilities identical to those of heat denatured dsRNAs 2 and 1 respectively. No labelled products corresponding to dsRNA3 or heat denatured dsRNA3 were detected.

Location of the ssRNA and dsRNA reaction products

When, after reaction, the virus particles were removed by centrifugation together with the bentonite, examination of the RNA in the supernatant by gel electrophoresis showed that the ssRNA products had been released from the virions into the medium. RNA remaining within virus particles was released by incubation of the bentonite pellet with sodium dodecyl
Aspergillus foetidus virus S

Fig. 4. Hybridization of AfV-S RNA polymerase product to virus template RNA. 3H-labelled, ssRNA product (18,000 cts/min) was hybridized (Method A) with increasing amounts of unlabelled virus dsRNA (dsRNA2 content of this sample was 50% as estimated by gel electrophoresis). Hybridization is expressed as the percentage of input ssRNA label which becomes ribonuclease resistant in high salt.

sulphate (free RNA did not bind to bentonite in the conditions used) and examined by gel electrophoresis. The results showed that most of the dsRNA labelled during the polymerase reaction remained within virions.

Hybridization of ssRNA product with genome RNA

In order to prove that the ssRNA product arises by transcription of the virus template dsRNA, the 3H-ssRNA product of an 18 h standard polymerase reaction was isolated, either by precipitation with 2 M LiCl or by chromatography on Whatman CF11 cellulose and annealed (Method A) with increasing amounts of denatured unlabelled AfV-S dsRNA. The extent of hybridization which occurred was measured by the amount of label which remained insoluble after treatment with ribonuclease A and ribonuclease T1 in buffer A. The proportion of product capable of hybridizing to the virus template RNA increased with the amount of virus dsRNA added up to 93% (Fig. 4).

The annealing procedure was also used to determine whether one or both strands of the template dsRNA are transcribed in vitro. The 3H-labelled ssRNA product was incubated in annealing conditions (Method B) at a concentration of 17 μg/ml. Control experiments with denatured dsRNA showed that at this concentration duplex formation would be expected to increase the ribonuclease resistance of the single-stranded product to 90% if both strands were equally represented. However, only 3.5% of the incubated RNA remained acid precipitable after ribonuclease digestion in buffer B. A value of 2.7% was obtained when a non-incubated control sample was treated with ribonuclease in identical conditions.

Kinetics of labelling of ssRNA and dsRNA

During the early part of the polymerase reaction 3H-UMP incorporation into dsRNA2 increased in parallel with that into ssRNA2. However, whereas synthesis of ssRNA2 continued for periods of up to 48 h, incorporation of 3H-UMP into dsRNA2 reached maximum levels after 6 h reaction (Fig. 5). Rates of incorporation of 3H-UMP into ssRNA2
Fig. 5. Incorporation of $^3$H-UTP into dsRNA$_2$ and synthesis of ssRNA$_2$ by AfV-S particles. At the indicated times during an RNA polymerase reaction 100 µl samples were taken and the reaction was stopped with EDTA. The ssRNA released into the medium and the dsRNA within the virus particles were analysed by gel electrophoresis as described in the Methods. The amounts of ssRNA$_2$ were estimated from the areas in the scans at 260 nm and expressed as molar ratios (ssRNA$_2$:dsRNA$_2$) using as reference the area obtained from dsRNA$_2$ at zero time. (The values were corrected by assuming that the extinction coefficient of dsRNA is 4/5 that of ssRNA.) The amount of UTP incorporated into dsRNA$_2$ was estimated from the ct/min present in the dsRNA$_2$ peak and converted into ng of RNA (1 pmol of UTP corresponds to 1.378 ng of RNA in which all four nucleotides are present in equal proportions). RNA synthesis in dsRNA$_2$ is expressed, by analogy with the ssRNA curve, as the molar amount of one strand synthesized per mole of dsRNA$_2$ input, ○—○, ssRNA; ●—●, dsRNA.

and dsRNA$_2$ were unaffected by inclusion of AdoMet (1 mM) in reaction mixtures. The amount of $^3$H-UMP incorporated into dsRNA$_2$ after 6 h reaction varied from 350 to 750 pmol per pmol of dsRNA$_2$ with different virus preparations (equivalent to the synthesis of 40 to 80 % of one strand per dsRNA$_2$ molecule, making the assumption that the newly-synthesized strand contains 25 % U). The molar amount of ssRNA$_2$ formed after 48 h
Aspergillus foetidus virus S

Fig. 6. Distribution of RNA polymerase activity in the AfV-S multicomponent system. (a) An AfV-S preparation (60 ml, A_{260} 1) was loaded on to a 400 ml gradient of 20 to 50 % (w/w) sucrose in 0.03 M-Na phosphate buffer, pH 7.6, containing 0.15 M-NaCl, in an MSE B XIV zonal rotor and centrifuged at 47,000 rev/min for 1.5 h. Five ml fractions were collected and their A_{260} was measured. Fractions corresponding to the S1 peak (I), S2 peak (II) and S3 plus S4 peaks (III) were pooled as indicated. (b), (c) and (d) Combined fractions I, II and III were each concentrated by binding to and elution from DEAE-cellulose, loaded on to 50 ml gradients of 20 to 50 % (w/w) sucrose in the phosphate buffer and centrifuged at 24,000 rev/min in a Beckman SW25.1 rotor for 3 h. Fractions (0.5 ml) were collected and dialysed against 0.05 M-tris-HCl buffer, pH 7.9, containing 0.15 M-NaCl and 0.1 mM-EDTA. ●—●, A_{260}; ○—○, 3H-UMP incorporation into acid insoluble product, assayed in standard 18 h RNA polymerase reactions with 50 μl of each fraction. Sp. act. of 3H-UTP was 150 mCi/mmol.

incubation was 6 to 8 times that of its dsRNA2 template as estimated by comparison of the peak areas in gel scans at 260 nm as described in the legend to Fig. 5 or by calculating the amount of newly synthesized RNA from the number of pmol of UMP incorporated, assuming that ssRNA2 contains 25 % U.

**Location of RNA polymerase activities in the AfV-S multicomponent system**

In order to determine the location of RNA polymerase activities synthesizing ssRNA1, ssRNA2 and dsRNA2 in the AfV-S multicomponent system, AfV-S preparations were
Table 1. $^3$H-UMP incorporation, and particle and dsRNA composition of AfV-S fractions

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>$^3$H-UMP incorporation per unit $A_{260}$†</th>
<th>Particle composition (%)‡</th>
<th>dsRNA composition (mole %)§</th>
</tr>
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<tr>
<td></td>
<td>(ct/min x 10^{-4})</td>
<td>S1a</td>
<td>S1b</td>
</tr>
<tr>
<td>I. 4</td>
<td>56·3</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>II. 6</td>
<td>5·6</td>
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* Fig. 6(b) and 6(c).
† Values in Fig. 6(b) and 6(c) divided by the $A_{260}$ of the fraction.
‡ Determined by analytical caesium chloride equilibrium density gradient centrifugation (Ratti & Buck, 1972; Buck & Ratti, 1975). S2a particles, which contain RNA1, and S2x particles, which contain RNA2, have similar buoyant densities and are measured together.
§ Determined from u.v. scans of gels after electrophoresis.

centrifuged in linear gradients of sucrose. Fractions corresponding to the peaks of S1a, S2a, and S3+S4 particles respectively were pooled as indicated in Fig. 6(a) and subjected to a further cycle of sucrose density gradient centrifugation and fractionation. The $A_{260}$ and $^3$H-UMP incorporation in 18 h RNA polymerase reactions for each fraction are shown in Fig. 6(b), (c) and (d). Fractions I.4 (Fig. 6b) and II.6 (Fig. 6c), which corresponded to the major peaks of RNA polymerase activity, were selected for further study. The $^3$H-UMP incorporation per unit $A_{260}$, together with the particle and dsRNA composition, for these two fractions are given in Table 1.

The products of RNA polymerase reaction with fractions I.4 and II.6 were analysed by electrophoresis in polyacrylamide gels containing 8 M-urea. The only ssRNA product formed with fraction I.4 had a mobility identical to that of the major ssRNA product obtained with unfractionated virus (ssRNA2). dsRNA was also labelled during the reaction (Fig. 7a). The relative mobilities of dsRNA2 and ssRNA2 in these gels were reversed compared with gels without urea (Fig. 1). Inclusion of 8 M-urea into gels has little effect on the secondary structure or mobility of dsRNA, but causes a substantial decrease in the mobility of ssRNA as a result of the reduction in the amount of its secondary structure (Floyd et al. 1974). Analysis of the reaction products of all the sucrose density gradient fractions in Fig. 6(b) showed that the activities promoting incorporation of $^3$H-UMP into ssRNA2 and into dsRNA2 had similar profiles and both reached maximum values in fractions I.4 and I.5.

The product of RNA polymerase reaction with fraction II.6 contained two ssRNA components, with mol. wt. of $2.1 \times 10^6$ and $1.35 \times 10^6$ and electrophoretic mobilities in urea gels identical to those of ssRNA1 and ssRNA2 formed with unfractionated virus (Fig. 7b). In this case, however, ssRNA1 was a major product. The molar ratio of ssRNA1:ssRNA2, calculated from the areas of the peaks in scans of gels stained with toluidine blue, was about 1:5:1. More than 95% of this ssRNA product was rendered resistant to ribonuclease A by annealing with an excess of denatured dsRNA1 plus dsRNA2 (Method B). Control experiments established the absence of any self-annealing of the ssRNA product. DsRNA2 was also labelled in the RNA polymerase reaction, but no $^3$H-UMP incorporation into dsRNA1 could be detected following electrophoresis in gels with or without urea.
DISCUSSION

It has been shown that the product of RNA polymerase reaction with unfractionated AfV-S contains a major and a minor species of ssRNA, both of which are released from the virions. This product RNA hybridized to an extent of 90% with genomic dsRNA (Fig. 4), showing that the major species (ssRNA2) arises by transcription of virion dsRNA. The proportion of the minor species (ssRNA1) in this experiment was too small to allow definitive conclusions with regard to this RNA, but further hybridization experiments showed that the RNA product from fraction II.6, which contains ssRNA1 as a major component (Fig. 7b) hybridized to an extent of 95% with virion dsRNA, proving that
ssRNA1 is also a dsRNA transcript. The mol. wt. of ssRNA2 is exactly half that of dsRNA2, suggesting that it was formed by complete transcription of dsRNA2, rather than by partial transcription of dsRNA1. This was confirmed by the formation of ssRNA2 from fraction I.4 (Fig. 7a), which contained only dsRNA2 together with the much smaller dsRNA3. The mol. wt. of ssRNA1 is exactly half that of dsRNA1 and it was formed by those virus fractions which contained dsRNA1 (e.g. II.6), but not by those which contained only dsRNA2 plus dsRNA3 (e.g. I.4). It is therefore concluded that ssRNA1 molecules are complete transcripts of dsRNA1.

From the results in Fig. 6 and the proportions of the four particle density classes in unfractionated AfV-S, it was calculated that about 90% of the AfV-S RNA polymerase activity resided in the S1 particle class with about 9% in the S2 class and less than 1% in the S3 plus S4 classes. Similar values were obtained when the RNA polymerase incubations were carried out for 1 h instead of 18 h, indicating that the values represent differences in overall activity rather than differences in particle and enzyme stability during the course of the reaction. The peak of RNA polymerase activity in the S1 particle class (Fig. 6b) corresponded with the peak of S1a particles, indicating that most of the transcription of dsRNA2 took place in those particles. The possibility that some transcriptions of dsRNA2 took place in S1b particles cannot be excluded. It was also deduced that transcription of dsRNA1 took place in S2a and S2b particles. However, no transcription of dsRNA3 from either S1b or S2b particles could be detected.

Some transcription of dsRNA2 also took place in S2x particles (fraction II.6, Table 1) which contain dsRNA2, but have a buoyant density and other physical properties similar to those of S2a particles, which contain dsRNA1 (Buck & Ratti, 1975). Since the molar ratio of dsRNA1:dsRNA2 in this fraction is about 8:1, whereas the ratio of ssRNA1:ssRNA2 formed was about 1.5:1, it appears that transcription of dsRNA2 in S2x particles occurs more efficiently, on average, than that of dsRNA1 in S2a plus S2b particles. S1a and S2x particles may represent different stages of the transcription process. It may be expected that the density of S1a particles would increase during transcription, due to the presence of nascent RNA strands and would reach a maximum prior to the release of the completed transcript. Such a situation has been observed in the case of reovirus, in which it has been found that transcribing cores, containing nascent ssRNA molecules, are more dense than non-transcribing cores (Skehel & Joklik, 1969).

The production of 6 to 8 molecules of ssRNA2 per molecules of dsRNA2 template after 48 h reaction shows that re-initiation of transcription occurs repeatedly in this in vitro system. However, the overall rate of RNA synthesis is slow. The initial time taken to achieve the synthesis of one strand of ssRNA2 per molecule of dsRNA2 was 1 h (Fig. 5), corresponding to an average rate of addition of 1 nucleotide/s/molecule of dsRNA2 template. Overall rates of nucleotide addition for transcription of AfV-S dsRNA1 and of P1 dsRNA in virus-like particles isolated from the yeast Saccharomyces cerevisiae (Herring & Bevan, 1977) were even lower (180 nucleotides/h and 18 nucleotides/h respectively). The overall slow rates of reaction could be the result of (a) a slow rate of reaction in all particles, (b) a wide range of activities in different particles or (c) only a small proportion of particles having activity. Possibility (a) can be eliminated for AfV-S because complete transcripts of dsRNA2 (4000 nucleotides per strand) and dsRNA1 (6000 nucleotides per strand) could be detected after 0.5 and 1 h reaction respectively, but the available evidence does not allow (b) and (c) to be distinguished. In the case of reovirus transcription in vitro it was found that the average (linear) rate of chain extension, based on the time taken to achieve a onefold net synthesis of ssRNA, was about 2 nucleotides/s; however, based on the time the first
Aspergillus foetidus virus S

transcripts could be detected, a rate of 60 nucleotides/s was calculated (Banerjee & Shatkin, 1970). In a laser light scattering study of the reovirus RNA transcription reaction, Bellamy & Harvey (1976) showed that all, or most, of the reovirus cores were active in transcription and, in order to explain the above results, suggested that a wide range of polymerase activities might be present in the population of reovirus cores.

The incorporation of \(^3\)H-UMP into dsRNA2 during the first 6 h of the AfV-S RNA polymerase reaction (Fig. 5) could be due to: (a) ‘filling-up’ of ssRNA tails on predominantly dsRNA molecules by a ss\textto dsRNA polymerase; such an activity has been found to be associated with bacteriophage \(\phi 6\) virions (Van Etten et al. 1973) and with virus-like particles isolated from rapidly growing yeast cells (Bevan & Herring, 1976); (b) complete replication of dsRNA within a proportion of the virus particles, as has been described in the case of virions of Penicillium stoloniferum virus S (Buck, 1975) or (c) transcription by semi-conservative strand displacement of dsRNA, so that the newly synthesized strand becomes part of the duplex, as is thought to occur \textit{in vivo} in the replication of bacteriophage \(\phi 6\) dsRNA (Coplin et al. 1975). S1a particles, which account for most of the activity promoting \(^3\)H-UMP incorporation into dsRNA2, contain one molecule of dsRNA2, the gel electrophoretic mobility of which remains unaltered after incubation with ribonuclease A (buffer A), indicating the absence of any, except possibly very short, ssRNA tails (Buck & Ratti, 1975). Hence the substantial amount of \(^3\)H-UMP incorporation into dsRNA2 (equivalent to the synthesis of 40 to 80 \% of one strand on average) excludes possibility (a). The co-sedimentation of the activities promoting incorporation of \(^3\)H-UMP into ssRNA2 and dsRNA2 (mainly with S1a particles) is consistent with possibility (c), but does not exclude (b).

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REFERENCES


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