Purification and Properties of the Ribonucleic Acid-Dependent Ribonucleic Acid Polymerase from *Halobacterium cutirubrum*

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1. The RNA-dependent RNA polymerase from *Halobacterium cutirubrum* was purified to electrophoretic homogeneity. 2. It requires a single-stranded molecule of RNA or polyribonucleotide as template. 3. Nearest-neighbour analyses of the products formed on random poly(A,U) or alternating poly(A-U) templates and base analysis of the product of synthesis directed by wheat-germ RNA prove that the template is copied accurately. 4. The enzyme initiates new chains with purine ribonucleoside triphosphates. 5. Sucrose-density-gradient analysis of the product indicates that it has a size distribution similar to that of the template. 6. Preliminary amino acid analysis of the RNA-dependent polymerase shows that it contains much less serine than either of the subunits of *H. cutirubrum* DNA-dependent RNA polymerase. 7. The RNA-dependent enzyme is unable to substitute for either subunit of the DNA-dependent polymerase, and both the latter are devoid of RNA-dependent activity.

We have described (Fitt & Louis, 1970; Louis & Fitt, 1971b) the isolation and partial purification of an RNA-dependent RNA polymerase from the extremely halophilic bacterium *Halobacterium cutirubrum*. The enzyme was readily separable from the DNA-dependent RNA polymerase [nucleoside triphosphate-RNA nucleotidyltransferase (DNA-dependent); EC 2.7.7.6] (Fitt & Louis, 1970; Louis & Fitt, 1971a,c,d, 1972a,b) of the bacterium and had mol. wt. 17000–18000 (Louis *et al.*, 1971). It required a high-molecular-weight RNA template and all four common nucleoside triphosphates to synthesize a ribonuclease-sensitive product, and the reaction was unaffected by deoxyribonuclease (Louis & Fitt, 1971b).

In the present paper, we describe the purification of the enzyme to electrophoretic homogeneity. We also present detailed evidence that it (i) requires a single-stranded polyribonucleotide template, (ii) copies the template accurately, (iii) initiates new polynucleotide chains and (iv) makes a product with a size distribution on sucrose density gradients similar to that of the template.

Experimental

Materials

Chemicals and substrates were purchased from the following suppliers: ^{14}C - and ^3H -labelled nucleoside triphosphates, Amersham/Searle Corp., Don Mills,

Ont., Canada; sodium dodecyl sulphate (specially pure), BDH (Canada) Ltd., Toronto, Ont., Canada; Bio-Gel HTP hydroxyapatite powder and P-60 polyacrylamide gel, Bio-Rad Laboratories, Richmond, Calif., U.S.A.; high-molecular-weight wheat-germ RNA, Calbiochem, Los Angeles, Calif., U.S.A.; Triton X-100, Canadian Laboratory Supplies Ltd., Montreal, P.Q., Canada; poly[d(A-T)], General Biochemical Inc., Chagrin Falls, Ohio, U.S.A.; ^{32}P -labelled nucleoside triphosphates, ICN Chemical and Radioisotope Division, Irvine, Calif., U.S.A.; poly(A), poly(U), poly(A,U) and poly(A)·poly(U), Miles Laboratories Inc., Kankakee, Ill., U.S.A.; Ficoll, Pharmacia (Canada) Ltd., Montreal, P.Q., Canada; unlabelled nucleoside triphosphates, P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; calf thymus DNA, yeast tRNA and high-molecular-weight yeast RNA, Sigma Chemical Co., St. Louis, Mo., U.S.A.

Buffers. The pH of buffers refers to the value at 25°C. High-salt buffer has the composition: 2.5M-KCl–1M-NaCl–10mM-tris–HCl, pH 8.6.

Cell culture. *H. cutirubrum* N.R.C. 34001 cells were grown as described by Peterkin & Fitt (1971), but were harvested after 66–72 h of growth, i.e. in late exponential or early stationary phase. (We previously referred to this strain as no. 9.)

Preparation of $\text{Zn}(\text{OH})_2$ gel. $\text{Zn}(\text{OH})_2$ gel was prepared by the method of Mahler (1955), on one-tenth the scale described, and was suspended in 0.01M-tris–HCl buffer, pH 7.4. The gel was left in the dark

at room temperature for 5 days before use. Excess of gel was discarded.

Methods

Purification of *H. cutirubrum* RNA-dependent RNA polymerase. The enzyme was prepared by a modification of the method of Louis & Fitt (1971b). All operations were performed at 0–5°C and $(\text{NH}_4)_2\text{SO}_4$ saturation was calculated by the procedure of Green & Hughes (1955).

The crude extract (Table 1) was prepared from 32 g wet wt. of bacteria and the pH4 precipitation was performed as described by Louis & Fitt (1972a). The pellet after acid precipitation was suspended in the minimum volume of high-salt buffer and was dialysed against the latter (200 vol.) until no more solid dissolved (24–36 h). Undissolved material was discarded and the solution was dialysed for a total of 4 h against two changes (100 vol. each) of 10 mM-tris-HCl buffer, pH 8.6. Solid $(\text{NH}_4)_2\text{SO}_4$ was then added to 80% saturation; the precipitate was removed by centrifugation and was discarded, and the supernatant was dialysed overnight against high-salt buffer. The resulting solution (20–30 ml) was passed at 40 ml/h by upward flow through a column (5 cm × 85 cm) of Bio-Gel P-60 prepared, stabilized and operated as described by Louis & Fitt (1972a). The combined active fractions (100–130 ml) were concentrated to approx. 1 ml by dialysis against 50% (w/v) Ficoll in high-salt buffer. $\text{Zn}(\text{OH})_2$ suspended in 0.01 M-tris-HCl buffer, pH 7.4 (17 mg/ml; 0.5 vol.), was added, and the mixture was stirred thoroughly and left for 20 min. The solid was then removed by centrifugation for 5 min in a clinical centrifuge and the supernatant was dialysed against two changes (100 vol. each) of high-salt buffer for a total of 4 h.

The purified enzyme was electrophoretically homogeneous in polyacrylamide gels (see below).

Preparation of *H. cutirubrum* nucleic acids. DNA and RNA were prepared from *H. cutirubrum* as described by Louis & Fitt (1972a).

Enzyme assays. *H. cutirubrum* RNA-dependent RNA polymerase activity was measured by the standard assay (Louis & Fitt, 1971b, 1972a). The assay medium (0.1 ml) contained: tris(hydroxymethyl)-methylaminopropanesulphonic acid-NaOH buffer, pH 9.5, 10 μmol ; high-molecular-weight RNA, 75 μg ; [^{14}C]ATP, 5 nmol (about 10000 d.p.m./nmol); CTP, GTP and UTP, 20 nmol each; MgCl_2 , 10 μmol ; MnCl_2 , 1 μmol ; KCl, 150 μmol ; NaCl, 60 μmol ; enzyme, up to 3 units.

H. cutirubrum DNA-dependent RNA polymerase was assayed as described by Louis & Fitt (1972a). The assay medium (0.1 ml) contained: glycine-NaOH buffer, pH 9.5, 10 μmol ; native *H. cutirubrum* DNA, 30 μg ; [^{14}C]ATP, 5 nmol (about 10000 d.p.m./

nmol); CTP, GTP and UTP, 15 nmol each; MgCl_2 , 10 μmol ; MnCl_2 , 1 μmol ; KCl, 150 μmol ; NaCl, 60 μmol ; enzyme. (Crude extracts may require up to 100-fold dilution for this assay to be linear, because crude preparations of the DNA-dependent enzyme have a bell-shaped enzyme-saturation curve.)

For both assays, incubation was at 37°C for 1 h. Samples (up to 85 μl) were then streaked on strips (1.5 cm × 3 cm) of Whatman no. 3 filter paper. The latter were collected in 7% (w/v) HClO_4 (10 ml/strip) and swirled for 7 min. The acid was aspirated and the washing procedure repeated successively with 7% (w/v) HClO_4 , 1% (w/v) HClO_4 and ethanol. The strips were air-dried and their radioactivity was determined in a Beckman LS-230 liquid-scintillation counter, with 0.8% (w/v) 2,5-diphenyloxazole in toluene (10 ml/strip) as scintillation fluid. Suitable control experiments were performed in all cases and the results were corrected for quenching as described by Louis & Fitt (1971a). A unit was defined as the amount of enzyme catalysing the incorporation of 1 nmol of [^{14}C]ATP into acid-insoluble material/h.

Previously (Louis & Fitt, 1971b) we used tris-HCl buffer, pH 9.5, in the standard assay of the RNA-dependent enzyme. Recently, for a period of several months, we obtained high blanks with tris buffers, owing apparently to the presence in the public water supply of a steam-distillable oxidizing agent that caused rapid oxidation of Mn^{2+} in the presence of tris buffer. This difficulty was eliminated by using either tris (hydroxymethyl) methylaminopropanesulphonic acid-NaOH or cyclohexylaminopropanesulphonic acid-NaOH buffers, pH 9.5. The problem has now disappeared and we have confirmed that use of these buffers instead of tris buffer makes no difference to either the activity or the requirements of the enzyme.

Protein assays. Enzyme protein concentration was assayed spectrophotometrically (Warburg & Christian, 1942).

Micro-disc polyacrylamide-gel electrophoresis. Micro-disc electrophoresis (Neuhoff, 1968) in 8, 10 and 12% (w/v) polyacrylamide gels at pH 7.0, 8.0 or 9.4 was performed as described by Louis & Fitt (1972a).

Results and Discussion

Purification of H. cutirubrum RNA-dependent RNA polymerase

Table 1 shows the results of a typical purification of *H. cutirubrum* RNA-dependent RNA polymerase from 32 g wet wt. of bacteria. The small residual DNA-dependent RNA polymerase activity present in the precipitate from the acid precipitation step was

Table 1. Purification of *H. cutirubrum* RNA-dependent RNA polymerase

The enzyme was purified from 32 g wet wt. of bacteria as described in the Experimental section. Activity was measured by the standard assay. Numbers in parentheses represent units of DNA-dependent RNA polymerase activity determined by the assay described in the Experimental section.

Fraction	Activity (units)	Sp. activity (units/mg)
Crude extract	234 (1584)	0.28
pH4 precipitate	228 (6)	0.34
(NH ₄) ₂ SO ₄ supernatant	600 (0)	24.1
Bio-Gel P-60 concentrated pool	570 (0)	1294
Zn(OH) ₂ supernatant	444 (0)	1345

eliminated at the next stage and a contaminant of high electrophoretic mobility still present after gel filtration was removed by adsorption to Zn(OH)₂ gel. As previously observed (Louis & Fitt, 1971*b*) a large increase in the total number of units occurs after (NH₄)₂SO₄ fractionation, presumably owing to a removal of degradative enzymes such as nucleases and phosphatases. The final product was electrophoretically homogeneous at pH 7.0, 8.0 and 9.4, in each case in 8%, 10% and 12% polyacrylamide gels and was free of DNA-dependent RNA polymerase activity (Table 1). This is therefore the second enzyme from an extreme halophile to be purified to homogeneity, according to these criteria, as we have previously described (Louis & Fitt, 1972*a*) the isolation of the electrophoretically pure subunits of *H. cutirubrum* DNA-dependent RNA polymerase. It seems probable that the methods we have used (Peterkin & Fitt, 1971; Louis & Fitt, 1971*a,b*, 1972*a*) for the purification in good yield of enzymes from extremely halophilic bacteria will be generally applicable, with minor modifications, and thus permit a more penetrating study of the properties of such enzymes than has been possible before. However, it is important to ensure that the enzymes are fully detached from membranes, or membrane particles, before extensive purification is attempted, as we have found that both the polynucleotide phosphorylase of *H. cutirubrum* (Peterkin & Fitt, 1971) and the DNA-dependent RNA polymerase of *Halobacterium halobium* (P. S. Fitt & P. I. Peterkin, unpublished work), although of low molecular weight, may be closely associated with membrane material in crude extracts prepared by ultrasonic disruption of the bacterial cells. These crude membrane complexes are difficult, if not impossible, to purify, and give incorrect, high molecular weights for the enzymes by gel filtration.

Table 2. Amino acid analysis of *H. cutirubrum* RNA-dependent RNA polymerase

A sample of the enzyme, the product of one preparation, was hydrolysed in 6M-HCl at 100°C for 22h and the hydrolysate was chromatographed on a Beckman model 120C amino acid analyser. Peaks were manually integrated by the half-height approximation and the results were compared with those obtained with standard mixtures of amino acids. No correction was made for amide N, cysteine or tryptophan. The results for *H. cutirubrum* DNA-dependent RNA polymerase α and β subunits are from Louis & Fitt (1972*a*).

Amino acid	Amino acid composition (mol/100mol)		
	<i>H. cutirubrum</i> RNA-dependent RNA polymerase	<i>cu tirubrum</i> DNA-dependent RNA polymerase	
		Protein α	Protein β
Asp	8.2	7.0	6.3
Thr	6.7	5.3	5.0
Ser	6.9	15.9	16.1
Glu	11.0	14.2	13.4
Pro	3.7	4.2	4.1
Gly	17.6	15.6	17.8
Ala	9.9	8.0	8.2
Val	4.5	3.6	3.6
Met	0.8	0.6	0.5
Ile	2.5	1.8	2.2
Leu	4.6	3.1	4.0
Tyr	2.0	1.8	1.9
Phe	2.1	1.4	1.8
Lys	12.0	11.1	9.0
His	3.8	3.1	3.4
Arg	3.6	3.2	2.7

However, they appear to be readily dissociated by nonionic detergents.

The evidence that the RNA-dependent RNA polymerase has a molecular weight in the range 17000 to 18000 has been presented (Louis *et al.*, 1971) and the fact that the enzyme migrates as one band during micro-disc electrophoresis in nine different combinations of pH and polyacrylamide-gel concentration indicates that it is almost certainly a single protein chain. The general requirements and stability of the homogeneous enzyme are unchanged from those described after partial purification (Louis & Fitt, 1971*b*) and are unaffected by use of either tris (hydroxymethyl) methylaminopropanesulphonic acid-NaOH or cyclohexylaminopropanesulphonic acid-NaOH buffers in place of tris-HCl buffer.

Amino acid analysis of the enzyme was performed on the product from one preparation (Table 2). Insufficient material was available for determination of cysteine or tryptophan, and the results are uncorrected for amide N or minor peaks of unhydrolysed oligopeptides. The analysis is compared in Table 2 with those obtained (Louis & Fitt, 1972a) for the α and β subunits of *H. cutirubrum* DNA-dependent RNA polymerase. The RNA-dependent enzyme contains remarkably less serine than either of the other two proteins, but other differences between them are not great.

Template specificity of H. cutirubrum RNA-dependent RNA polymerase

Louis & Fitt (1971b) found that *H. cutirubrum* RNA-dependent RNA polymerase required a high-molecular-weight RNA as template for maximum activity; tRNA was a relatively poor template, and became even less effective after removal of its terminal C-C-A group, and the enzyme was inactive with either native or denatured DNA. The results in Table 3 show that the nature of the high-molecular-weight polyribonucleotide template is not important provided that it is not double-stranded. Thus, poly(U) was a template for the incorporation of ATP, whereas both ATP and UTP were incorporated in the presence of the random co-polymer poly(A,U), but poly(A)·poly(U) was not a template.

The rate of reaction in the presence of wheat-germ RNA was unaffected by a concentration of rifampicin that inhibits *H. cutirubrum* DNA-dependent RNA polymerase completely (Louis & Fitt, 1972a).

No acid-insoluble product was formed when the enzyme was incubated with RNA as template in a

standard assay mixture containing the four deoxy-ribonucleoside triphosphates instead of the ribonucleoside triphosphates.

Template transcription and chain initiation by H. cutirubrum RNA-dependent RNA polymerase

Louis & Fitt (1971b) found that the product formed during *H. cutirubrum* RNA-dependent RNA polymerase-catalysed incorporation of ribonucleotides in the presence of a high-molecular-weight RNA template was ribonuclease-sensitive. The experiments described in Tables 4, 5 and 6 show that this product is an accurate copy of the polyribonucleotide template, and the results in Table 7 prove that the enzyme catalyses the initiation of new polymer chains.

Table 4 shows the results of a nearest-neighbour analysis of the product formed with the random 1:1 co-polymer poly(A,U) as template and with unlabelled CTP, GTP and UTP and [α - 32 P]ATP as substrates. After alkaline hydrolysis of the labelled product the 32 P was equally distributed between 2'(3')-AMP and 2'(3')-UMP, as would be expected from the random composition of the template.

In the experiment described in Table 5, *H. cutirubrum* DNA-dependent RNA polymerase was used to synthesize the unlabelled alternating copolymer poly(A-U) with poly[d(A-T)] as template. It has been shown by Louis & Fitt (1972a) that the product of this reaction is an accurate copy of the poly[d(A-T)] and that the DNA-dependent enzyme copies one strand of a double-stranded template only (so a double-stranded product is unlikely). Further, rifampicin is known to inhibit initiation of new chains by the DNA-dependent enzyme (Louis & Fitt, 1972b),

Table 3. *Template specificity of H. cutirubrum RNA-dependent RNA polymerase*

Enzyme activity was measured by the standard assay (see the Experimental section) with the indicated nucleic acid (75 μ g) as template. All four nucleoside triphosphates were present in every case.

Nucleic acid	Labelled nucleoside triphosphate	[14 C]- or [3 H]-Nucleoside triphosphate incorporated (nmol)
None	[14 C]ATP	0.00
Yeast high-molecular-weight RNA	[14 C]ATP	0.97
Wheat-germ high-molecular-weight RNA	[14 C]ATP	1.04
Wheat-germ high-molecular-weight RNA +rifampicin (1.16nmol)	[14 C]ATP	1.09
<i>H. cutirubrum</i> RNA	[14 C]ATP	1.04
Poly(U)	[14 C]ATP	1.06
Poly(A,U)	[14 C]ATP	1.06
Poly(A)·poly(U)	[14 C]ATP	0.00
Poly(A)·poly(U)	[3 H]UTP	0.00
Poly[d(A-T)]	[14 C]ATP	0.00

Table 4. *Nearest-neighbour analysis of the product of poly(A,U)-directed RNA synthesis by H. cutirubrum RNA polymerase*

A large-scale (0.5 ml) assay mixture containing the random 1:1 co-polymer poly(A,U) as template and all four nucleoside triphosphates, with [α - 32 P]ATP instead of [14 C]ATP, was incubated at 37°C for 2 h. A sample (25 μ l) was withdrawn and counted for radioactivity on paper (see the Experimental section) to determine the total incorporation. Poly(A,U) carrier (250 μ g in 0.5 ml of water) was added to the remaining bulk solution. The [32 P]RNA was isolated by phenol extraction (Bolton, 1966) and hydrolysed with 0.2 M-KOH, and the nucleotides were separated by paper chromatography as described by Litvak *et al.* (1970). The positions of the individual 2'(3')-nucleoside monophosphates were determined under u.v. light by comparison with standards, the chromatogram was cut up and the radioactivity present in each nucleotide determined by liquid-scintillation counting as in the standard assays.

	[32 P]Nucleoside monophosphate incorporated (nmol)	% of total incorporation
Total incorporation of 5'-[32 P]AMP units	2.01	
Recovery of 2'(3')-[32 P]AMP	0.836	41.6
Recovery of 2'(3')-[32 P]UMP	0.886	44.1
Total recovery of 2'(3')-[32 P]AMP and 2'(3')-[32 P]UMP	1.722	85.7
Ratio of U:A in product	1.06	

Table 5. *Nearest-neighbour analysis of the product formed by H. cutirubrum RNA-dependent RNA polymerase on a poly(A-U) template synthesized in situ*

The initial incubation mixture (0.5 ml) for the synthesis of poly(A-U) contained: tris-HCl buffer, pH 9.5, 50 μ mol; poly[d(A-T)], 150 μ g; ATP, CTP, GTP and UTP, 75 nmol each; MgCl₂, 50 μ mol; MnCl₂, 5 μ mol; KCl, 750 μ mol; NaCl, 300 μ mol; *H. cutirubrum* DNA-dependent RNA polymerase, 2 units. Incubation was at 37°C. After 2 h, rifampicin (1.16 nmol) was added and incubation was continued for a further 30 min. *H. cutirubrum* RNA-dependent RNA polymerase (2 units) and sufficient high-specific-radioactivity [α - 32 P]ATP to give a final specific radioactivity of 150 000 d.p.m./nmol (based on the original unlabelled ATP used) were added and incubation was continued for 1 h. A sample (25 μ l) was withdrawn for determination of the total incorporation, and isolation, hydrolysis and further analysis of the bulk product were performed as described in Table 4, using 1 mg of poly(A,U) as carrier. A control without RNA-dependent RNA polymerase was also performed and the experimental values were corrected accordingly (incorporation in the control was <0.1% of the experimental values).

	[32 P]Nucleoside monophosphate incorporated (nmol)	% of total incorporation
Total incorporation of 5'-[32 P]AMP	1.930	
Recovery of 2'(3')-[32 P]AMP	0.001	0.05
Recovery of 2'(3')-[32 P]UMP	1.790	92.9
Total recovery of 2'(3')-[32 P]AMP and 2'(3')-[32 P]UMP	1.791	93.0

although the elongation reaction continues for a further 20 min in the standard conditions in the presence of the antibiotic. Therefore, rifampicin was added to the reaction mixture after 2 h, and incubation was continued for a further 30 min. The RNA-dependent enzyme, for which poly[d(A-T)] is not a template (Table 3) and which is unaffected by rifampicin (Table 3), was then added, together with [α - 32 P]-ATP. After a further 2 h of incubation, the product of the reaction was isolated by phenol extraction and was subjected to nearest-neighbour analysis. Virtually all the incorporated 32 P was recovered in 2'(3')-

UMP, as would be expected if the poly(A-U) prepared *in situ* had been accurately copied by the RNA-dependent RNA polymerase.

In a further experiment (Table 6), the base composition of the polyribonucleotide product was compared with that of the wheat-germ RNA used as template in its synthesis. The results show that the product had a base composition complementary to that of the template. (The latter was present in great excess, so significant recopying of the product would not be expected.)

Finally, the enzyme was shown (Table 7) to initiate

Table 6. *Base composition of the product of wheat-germ RNA-directed RNA synthesis by H. cutirubrum RNA-dependent RNA polymerase*

The ratios of CTP, GTP and UTP in the product formed by the RNA-dependent RNA polymerase on wheat-germ RNA as template were obtained from experiments in which [^3H]CTP, [^3H]GTP or [^3H]UTP was included in the standard assay together with [^{14}C]ATP. The results were corrected for quenching and for simultaneous counting of the two radioisotopes by the external-standardization method and were used to calculate the base composition of the product. The base composition of the wheat-germ RNA was determined by the method of Yoshida & Shibata (1969).

[^3H]Nucleoside triphosphate	Product			Base composition (%)		
	[^3H]Nucleoside triphosphate incorporated (nmol)	[^{14}C]ATP incorporated (nmol)	Nucleoside triphosphate/ATP ratio			
				Base	Product	Template
UTP	1.55	2.27	0.683	A	28.4	20.5
GTP	2.53	2.32	1.09	U	19.4	30.0
CTP	1.95	2.60	0.750	G	30.9	22.3
				C	21.3	27.2

Table 7. *Incorporation of γ - ^{32}P -labelled nucleoside triphosphates into RNA*

The rate of initiation of new RNA chains by *H. cutirubrum* RNA-dependent RNA polymerase was measured by the method of Travers & Burgess (1969) by including the appropriate γ - ^{32}P -labelled nucleoside triphosphates in an assay mixture containing [^{14}C]ATP (see the Experimental section) and 20nmol each of the four nucleoside triphosphates. The template was wheat-germ RNA and the results were corrected for quenching and for ^{32}P counted in the ^{14}C channel of the liquid-scintillation counter.

[γ - ^{32}P]Nucleoside triphosphate	Chain elongation	Chain initiation	$^{14}\text{C}/^{32}\text{P}$ ratio
	([^{14}C]ATP incorporation, nmol)	([^{32}P]nucleoside triphosphate incorporation, pmol)	
ATP	1.04	8.0	130
CTP	0.94	<0.01	—
GTP	1.04	7.3	142
UTP	0.97	<0.01	—

new chains by measurement of the incorporation of γ - ^{32}P -labelled nucleoside triphosphates into the product (Travers & Burgess, 1969; Maitra *et al.*, 1965). As with the DNA-dependent RNA polymerases of *Escherichia coli* (Maitra & Hurwitz, 1965), *Azotobacter vinelandii* (Krakow & Horsley, 1967) and *H. cutirubrum* (Louis & Fitt, 1972a), the RNA-dependent RNA polymerase initiates new chains with purine nucleoside triphosphates only. However, the initiation process is not rifampicin-sensitive in this case (Table 3).

The results discussed above show that the enzyme is a true RNA-dependent RNA polymerase or replicase, that it both initiates new polymer chains and copies the RNA template accurately, and that it is not merely a terminal-addition enzyme. Like the much larger replicases induced by bacteriophages Q β and MS2 (Spiegelman *et al.*, 1968; August, 1969) it requires a single-stranded template, but unlike the virus-induced

enzymes it has a broad template specificity provided that this structural requirement is satisfied (Table 3).

Two reports of other possible RNA replicases in uninfected cells have so far appeared in the literature. Neuhoﬀ *et al.* (1970) have presented preliminary evidence for the presence of such an enzyme in competent macrophages, and Astier-Manifacier & Cornuet (1971) have isolated from Chinese-cabbage leaves an RNA polymerase that is most active in the presence of poly(U,C), but will also accept various RNA species and denatured DNA as template.

Nature of the product formed with H. cutirubrum RNA as template

The ratio of the rate of chain elongation to the rate of chain initiation shown in Table 7 corresponds to a product with an average chain length of 250–300

Fig. 1. Sucrose-density-gradient centrifugation of product RNA

H. cutirubrum RNA was obtained (Louis & Fitt, 1972a) as an undifferentiated fraction by selective propan-2-ol precipitation of DNA from a crude nucleic acid extract. DNA and protein were absent. Samples of this RNA (150 μ l, 0.5 mg/ml) were layered on to aq. 5–20% (w/v) sucrose gradients (4.2 ml) in centrifuge tubes (11.1 mm $\times$ 60.3 mm). Standard assay mixtures with [α - 32 P]ATP instead of [14 C]ATP were incubated with the *H. cutirubrum* RNA as template, and the product was isolated by phenol extraction (Bolton, 1966), precipitated with ethanol, redissolved in 150 μ l of water and layered on similar gradients. After centrifugation for 135 min at 55000 rev./min (Beckman SW56 rotor, L2-65B centrifuge), the tubes were pierced and 8-drop fractions were collected, giving 28–31 fractions/gradient. The E_{260} of 1:7 aqueous dilutions was read against a water blank. Radioactivity were determined as in the standard assay by streaking 100 μ l of each fraction on paper strips, drying the strips and counting their radioactivity. ●, *H. cutirubrum* RNA template, E_{260} ; ○, radioactive product, c.p.m. Duplicate gradients are shown in each case. The arrows indicate the location of 23S and 16S *E. coli* ribosomal RNA added as marker to one of the gradients containing the radioactive product.

nucleotide units (since only one γ - 32 P-labelled purine nucleoside triphosphate at a time was included in the assay mixture and therefore only about half the true rate of initiation was determined). This corresponds to an average mol. wt. of approx. 100000. Fig. 1 shows a more detailed study by sucrose-density-gradient centrifugation of the size distribution of the products from two independent experiments in which the template was *H. cutirubrum* RNA isolated as described by Louis & Fitt (1972a). For comparison the results of duplicate density-gradient analyses of the sample of *H. cutirubrum* RNA are plotted on the same axes. The enzyme appears to copy all the molecular species present in the somewhat heterogeneous RNA sample. (No detailed study of the physical properties of RNA of extreme halophiles has been published as yet, so it is not possible to state if the sample used was representative of undegraded *H. cutirubrum* RNA, although it was an effective template.)

Relationship of the RNA-dependent RNA polymerase to *H. cutirubrum* DNA-dependent RNA polymerase

The electrophoretically homogeneous *H. cutirubrum* RNA-dependent RNA polymerase was substituted for either the α or the β subunit of the DNA-dependent enzyme in the standard assay for the latter, but no net incorporation of nucleoside triphosphates was observed in either case. Neither the α nor the β protein had any detectable RNA-directed activity in the standard assay for the RNA-dependent enzyme, nor did they affect the activity of the latter. As previously reported (Louis & Fitt, 1971b), the activity of the RNA-dependent polymerase is decreased in the presence of the complete DNA-dependent enzyme ($\alpha\beta$), possibly because of the greater affinity of the latter for nucleoside triphosphates or through competition for the bivalent metal ions, but the RNA-dependent enzyme had no effect on the activity of the DNA-dependent RNA polymerase. These results make it extremely unlikely that

there is any direct functional relationship between the two enzymes. Further studies will be necessary to determine if the RNA-dependent RNA polymerase serves *in vivo* as a general amplifier of RNA transcribed by the DNA-dependent enzyme or whether it has some more specific role in the nucleic acid metabolism of the cell.

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