The Role of Subunits in Yeast DNA-Dependent Ribonucleic Acid Polymerase A

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The properties of RNA polymerase A*, which lacked the subunits of 48000, 37000 and 16000 mol.wt., were compared with those of RNA polymerase A by using native calf thymus DNA as the template. The results showed that: (1) the specific activity of RNA polymerase A* was about one-third that of RNA polymerase A; (2) more than 80% of RNA polymerase A, but only about 25% of RNA polymerase A*, made RNA; (3) initiation by RNA polymerase A*, but not by RNA polymerase A, began after a lag of 2 min; (4) the temperature-dependence for productive binding to DNA was greater for RNA polymerase A*; (5) the apparent K_m for UTP was greater for RNA polymerase A*. These results support the supposition that the subunits missing from RNA polymerase A* are involved in DNA binding [Huet, Dezélee, Iborra, Buhler, Sentenac & Fromageot (1976) *Biochimie* 58, 71–80] and show also that the loss of these subunits affects the elongation reaction.

Various forms of RNA polymerase A have been isolated from yeast that differ in their subunit composition. These differences are mainly confined to the subunits of low mol.wt. (16000–10000). However, in two instances well-defined differences occur in the composition of subunits in the mol.wt. range 37000–48000. Hager *et al.* (1977) obtained an enzyme lacking the subunits of 48000 and 44000 mol.wt. that were present in enzyme previously prepared by that group using a different procedure (Valenzuela *et al.*, 1976), and Huet *et al.* (1975) obtained by dissociation of RNA polymerase A an enzyme called RNA polymerase A* that lacked the subunits of 48000 and 37000 mol.wt.

Information about the function of the dissociable subunits may be obtained by comparing the properties of the various forms of RNA polymerase A. This approach has been used by Huet *et al.* (1975, 1976) and Sentenac *et al.* (1976), who have compared the ability of RNA polymerases A and A* to catalyse RNA synthesis and pyrophosphate exchange on various templates and the sensitivity of RNA synthesis by these enzymes to increasing ionic strength. The results suggested that the subunits were involved in the binding of the enzyme to DNA and/or in initiation. The present report describes the properties of two forms of RNA polymerase A that correspond closely to the enzymes isolated by Huet *et al.* (1975). For this reason, the nomenclature used by those authors was adopted. The results show that the RNA polymerase A* used in the present study is defective in both the DNA-binding and elongation reactions of RNA synthesis.

Experimental

Materials

[5-³H]UTP (10–30 Ci/mmol) and [³²P]P_i were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Nucleoside triphosphates, calf thymus DNA (type I, highly polymerized) and heparin (pig, grade 1) were from Sigma (London) Chemical Co., Poole, Dorset, U.K. Phosphocellulose (P-11) and DEAE-cellulose (DE-52) were from Whatman, Maidstone, Kent, U.K. γ -³²P-labelled nucleoside triphosphates were prepared as described by Glynn & Chappell (1964). *Saccharomyces cerevisiae* strain A364A (*a*, *ad*₁, *ad*₂, *ur*₁, *hi*₇, *ly*₂, *ty*₁, *ga*₁) was obtained from Dr. L. H. Hartwell, University of Washington, Seattle, WA, U.S.A. Yeast was grown with aeration and stirring at 29°C in a medium that contained 1% yeast extract (Oxoid, Basingstoke, Hants., U.K.), 2% Bacto-peptone (Difco, West Molesey, Surrey, U.K.), 2% glucose and 20 μ g of adenine and uracil/ml.

Purification of RNA polymerases A and A*

RNA polymerase A was purified from 200–300 g of yeast as described by Cooper & Quincey (1979). RNA polymerase A* was prepared from 25–30 g of yeast by a method similar to that of Dezélee *et al.* (1972). Yeast was disrupted and the extract was centrifuged in the same way as for RNA polymerase A. The resulting supernatant was adjusted to 160 ml with extraction buffer and fractionated with (NH₄)₂SO₄. Protein that precipitated between 33% and 68% saturation with (NH₄)₂SO₄ was redissolved in 40 ml

of buffer I (20mM-Tris/HCl, pH7.9 at 22°C, containing 10mM-2-mercaptoethanol, 1.0mM-EDTA and 10% glycerol). This solution was desalted by passage through a Sephadex G-25 column (bed volume 200ml) equilibrated with buffer I/0.05M-(NH₄)₂SO₄ and then applied to a column of DEAE-cellulose (5cm² × 10cm). RNA polymerase forms A and B were separated by elution with a 320ml linear gradient of 0.05–0.50M-(NH₄)₂SO₄ in buffer I. Fractions containing the form-A RNA polymerase were pooled, desalted and applied to a column of phosphocellulose (2cm² × 3.5cm). RNA polymerase A*, which was eluted at about 0.15M-(NH₄)₂SO₄, was recovered by using a 36ml linear gradient of 0.05–0.45M-(NH₄)₂SO₄ in buffer I/2% glycerol. RNA polymerase A* was then centrifuged in a glycerol density gradient as described for RNA polymerase A except that 0.1M-(NH₄)₂SO₄ was present instead of 0.05M-(NH₄)₂SO₄.

RNA polymerase enzymes were stored at –20°C after adjustment to 50% glycerol. RNA polymerases A and A* were pure as judged by electrophoresis in 5% polyacrylamide gels under non-denaturing conditions (Kedinger *et al.*, 1974). No ribonuclease was detected in preparations of RNA polymerase A or A*. Contamination of RNA polymerase A*, but not of RNA polymerase A, by polynucleotide kinase was observed occasionally. This contamination arose because polynucleotide kinase was eluted with RNA polymerase A* from phosphocellulose and was not always completely separated by the glycerol-gradient step. Preparations of RNA polymerase A* that were contaminated with polynucleotide kinase were not used for studies on initiation. No contamination of RNA polymerase A or A* by RNA polymerase C could be detected by chromatography of these enzymes on DEAE-Sephadex A-25.

Formation of enzyme–DNA complexes and initiated complexes

Enzyme–DNA complexes were made by incubating RNA polymerase A or A* with native calf thymus DNA (400µg/ml) for 10min at 30°C in 105mM-Tris/HCl, pH7.9 at 22°C, containing 60mM-(NH₄)₂SO₄ and 1.2mM-MnCl₂. Initiated complexes were formed by incubating RNA polymerase A or A* for 40min at 30°C in a reaction mixture containing 400µg of native calf thymus DNA/ml, 105mM-Tris/HCl, pH7.9 at 22°C, 60mM-(NH₄)₂SO₄, 2.4mM-MnCl₂, 0.3mM-ATP and -GTP and either 0.3mM-UTP and 4µM-CTP or 0.3mM-CTP and 4µM-UTP.

Measurement of RNA synthesis

RNA synthesis at 30°C in the presence of 1.5–1.7mM-2-mercaptoethanol was measured after adding unbound enzyme, enzyme–DNA complexes or initi-

ated complexes last to the reaction mixture. The template was always native calf thymus DNA. The specific activity (expressed as pmol of [³H]UMP incorporated into RNA/min per µg of protein) of RNA polymerases A and A* was determined by incubating each enzyme for 20min in a reaction mixture of 75µl that contained 20µg of DNA, 1.0mM-ATP, -GTP, -CTP, 0.2mM-[³H]UTP (0.10µCi/nmol) and various concentrations of (NH₄)₂SO₄ and MnCl₂ or MgCl₂. RNA synthesis by enzyme–DNA complexes was started by adding 50µl of the complexes to 35µl of a reaction mixture so that the final mixture of 85µl contained 60mM-Tris/HCl, pH7.9 at 22°C, 35mM-(NH₄)₂SO₄, 3.6mM-MnCl₂, 1.0mM-GTP, -ATP, -CTP, various concentrations of [³H]UTP and 20µg of DNA. RNA synthesis by initiated complexes in the absence of further initiation was achieved by two means. In the first method, 50µl portions of initiated complexes were added to 35µl of a reaction mixture composed to ensure that the final reaction mixture of 85µl was the same as that used for enzyme–DNA complexes except that 25–100µg of heparin/ml was also present. In the second method, initiated complexes were cooled to 0°C and 14µl of 1.6M-(NH₄)₂SO₄, pH7.9, was added per 50µl of initiated complexes. After 3min at 0°C, the mixture was warmed at 30°C for 1min before adding 64µl portions to 21µl of additional reaction mixture so that the final concentration of reactants was the same as before, except that 0.3M-(NH₄)₂SO₄ was present instead of heparin.

When the incorporation of [³H]UMP was measured, the reaction was stopped by adding 1ml of cold 10% trichloroacetic acid/60mM-Na₄P₂O₇. The precipitate was filtered on to glass-fibre discs, washed with 50ml of cold 5% trichloroacetic acid/30mM-Na₄P₂O₇, dried and measured for radioactivity in a Philips scintillation counter after being immersed in 10ml of toluene scintillant [4.0g of 2,5-diphenyloxazole and 0.05g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/litre of toluene]. When [³²P]GTP or [³²P]ATP incorporation was measured, the reaction was stopped with EDTA and Na₄P₂O₇, carrier *Torula* RNA was added and the nucleic acids were precipitated with trichloroacetic acid. Subsequent processing included a phenol extraction step (Cooper & Quincey, 1979). This method was also used when the ratio of [³H]UMP to [³²P]ATP and [³²P]GTP incorporation was obtained to calculate the average chain length.

Other methods

Protein was estimated by the micro-tannin turbidity procedure of Mejbaum-Katzenellenbogen & Dobryszcka (1959), by using bovine serum albumin as a standard and assuming that $A_{1\text{cm}}^{1\%} = 6.67$ at 279nm. Electrophoresis in the presence of sodium

dodecyl sulphate in a 15% polyacrylamide slab gel (12cm×15cm×0.15cm) was as described by Valenzuela *et al.* (1976).

All other materials and methods, including those for measuring ribonuclease and polynucleotide kinase activity, were as described by Cooper & Quincey (1979).

Results and Discussion

Characterization of RNA polymerases A and A*

Analysis of the subunit composition of the RNA polymerase A used in this work showed that this was the same as described by Buhler *et al.* (1976), except that a trace of a subunit of 16000 mol.wt. was also present (Fig. 1). RNA polymerase A* used in the present work lacked the subunits of 48000, 37000 and 16000 molecular weight. The subunit composition of these two enzymes was identical with that of the RNA polymerases A and A* isolated by Huet *et al.* (1975) except that both of the latter enzymes lacked two of four subunits of 16000–12200 molecular weight.

These differences in the content of the subunits of 16000–12200 mol.wt. appeared not to influence the properties of the enzymes where these were compared. RNA polymerases A and A* were eluted from phosphocellulose at 0.24M- and 0.15M-(NH₄)₂SO₄ respectively, but migrated together when 4μg of each enzyme was subjected to electrophoresis in a non-denaturing 5% polyacrylamide gel. RNA synthesis by RNA polymerase A* was 4–5 times more sensitive to inhibition by 50–100μg of α-amanitin/ml when measured as described by Huet *et al.* (1975) than that by form A. In all these respects, RNA polymerases A and A* behaved exactly like the enzymes described by those authors. RNA polymerase A and the RNA polymerase A that was studied by Buhler *et al.* (1974) had similar specific activities (27 and 30 units/μg of protein respectively) when measured in the same way in the presence of 5mM-Mg²⁺ and 10mM-(NH₄)₂SO₄. Provided that less than 25mM-(NH₄)₂SO₄ was present, RNA polymerase A was about 3 times more active than RNA polymerase A* with 3.6mM-Mn²⁺ or 15mM-Mg²⁺ as the bivalent cation. At greater concentrations of (NH₄)₂SO₄ the difference in the activity of the two enzymes was more pronounced, especially when Mg²⁺ was present. These results are consistent with those obtained by Huet *et al.* (1976), who employed somewhat different reaction conditions.

Digestion of the calf thymus DNA template with nuclease S1 in the presence of 0.2M-NaCl to remove single-stranded gaps (Germond *et al.*, 1974) decreased 5-fold RNA synthesis catalysed by RNA polymerase A when 3.6mM-Mn²⁺ was present. Similar results were obtained by Dezélee *et al.* (1974) with Mg²⁺ instead of Mn²⁺ as the bivalent cation. Thus on this

Fig. 1. Subunit composition of RNA polymerases A and A*. A sample (7–10μg) of each enzyme was subjected to electrophoresis in a 15% polyacrylamide slab gel containing 0.1% sodium dodecyl sulphate. Segments of the gel were scanned in a Gilford recording spectrophotometer with the full-scale deflection (f.s.d.) set at an absorbance of 1.25 or 0.20. The numbers in the Figure represent the values obtained by Huet *et al.* (1975) and Buhler *et al.* (1976) for the molecular weights ($\times 10^{-3}$) of the various subunits. The assignments were based on the use of the trace as a 'finger-print' and on the mobility of ox heart cytochrome c (Cyt. c; mol.wt. 12384).

template initiation occurs in a predominantly unphysiological manner in the presence of Mg²⁺ or Mn²⁺. In contrast, with yeast DNA some selective transcription of genes for rRNA may be demonstrated in the presence of Mg²⁺, but not Mn²⁺ (Holland *et al.*, 1977).

Formation of initiated complexes

The partial reactions of RNA synthesis needed to be examined to gain an insight into the nature of the differences between RNA polymerases A and A*. This was achieved by comparing RNA synthesis started by adding unbound enzyme, enzyme-DNA complexes or initiated complexes. The accumulation of active initiated complexes in good yield was difficult, because the time taken for most of the enzyme to begin RNA synthesis was greater than the period of RNA synthesis by most enzyme molecules (Cooper & Quincey, 1979). This problem was overcome by allowing the reaction to proceed for 40–60 min with a limiting concentration (4 μ M) of one of the nucleoside triphosphates used for elongation but not initiation. Under these conditions, the rate of initiation was nearly normal, but RNA synthesis was greatly decreased (Fig. 2). When the concentration of the limiting nucleoside triphosphate was increased to 0.2 mM, the rate of RNA synthesis increased 10-fold

and the amount of initiation by about 30%. Since no recycling of the enzyme occurs in these reaction conditions (Cooper & Quincey, 1979) about one-third of the enzyme must have been free in solution or bound to DNA but not engaged in transcription before the CTP was added, unless part of the increment in [γ - 32 P]GTP incorporation was due to chain extension by initiated complexes whose RNA was too small to be acid-precipitable.

Additional initiation was eliminated by adding 25–100 μ g of heparin/ml, or by pre-treating initiated complexes with 0.4 M-(NH₄)₂SO₄. These manipulations prevented RNA synthesis by unbound enzyme and enzyme-DNA complexes, but did not prevent elongation by initiated complexes, although (NH₄)₂SO₄ markedly decreased the activity of initiated complexes containing RNA polymerase A* (Table 1; Fig. 3). This effect of (NH₄)₂SO₄ on the elongation reaction catalysed by RNA polymerase A* might contribute to the enhanced sensitivity to (NH₄)₂SO₄ of RNA synthesis by this enzyme that Huet *et al.* (1976) interpreted as indicating a defect in DNA binding.

Initiated complexes were not formed when Mn²⁺ was replaced by Mg²⁺.

Initiation by RNA polymerases A and A*

Initiation of RNA synthesis with GTP by RNA polymerase A* started after a lag of about 2 min and ceased after about 30 min. Initiation by RNA polymerase A occurred without any lag (Fig. 4). The cause of this difference was investigated by looking at the temperature-dependence of RNA synthesis by RNA polymerases A and A* and by enzyme-DNA and initiated complexes formed with these two enzymes. By this criterion, the difference between RNA polymerase A and A* was abolished after the enzyme had bound to DNA (Table 2). The greater temperature-dependence for the binding to DNA of RNA polymerase A* suggested that the enthalpy of activation for this step was larger for this enzyme than for RNA polymerase A, which could explain the lag in the kinetics of initiation observed for RNA polymerase A* but not for form A.

Another difference was also observed. After 60 min of transcription, the number of RNA molecules started with ATP and GTP made by RNA polymerase A* corresponded to one-quarter of the number of RNA polymerase A* molecules, whereas the number made by RNA polymerase A corresponded to 85% of the number of molecules of that enzyme (Table 3). So most of RNA polymerase A made RNA, since no re-initiation occurs in these reaction conditions (Cooper & Quincey, 1979). In contrast, most of RNA polymerase A* either made no RNA or started RNA molecules with CTP and UTP. This latter possibility was inherently unlikely and certainly did not occur

Fig. 2. Effect of CTP on initiation and RNA synthesis by RNA polymerase A

Two reaction mixtures of 0.60 ml each contained 4.0 μ g of RNA polymerase A, 160 μ g of DNA, 70 mM-Tris/HCl, pH 7.9, 40 mM-(NH₄)₂SO₄, 1.6 mM-MnCl₂, 0.2 mM-ATP, 4 μ M-CTP and either 0.2 mM-[³H]UTP (0.1 μ Ci/nmol) and 0.2 mM-GTP, or 0.2 mM-[γ -³²P]-GTP (0.5 μ Ci/nmol) and 0.2 mM-UTP. At 60 min after addition of unbound enzyme to start the reaction, the concentration of CTP was increased to 0.2 mM by adding 5 μ l of 3.2 mM-CTP per 75 μ l of reaction mixture. The incorporation of [³H]UMP (△) and [γ -³²P]GTP (○) into RNA at 30°C was measured by removing 75 μ l portions before 60 min and 80 μ l portions after that time.

Table 1. *Effects of heparin and (NH₄)₂SO₄ on RNA synthesis*

For heparin, the procedure was as described for initiated complexes in the Experimental section. RNA synthesis was for 12min at 30°C by using 1.0μg of RNA polymerase A with 0.2mM-[³H]UTP (0.10μCi/nmol), or 0.25μg of RNA polymerase A* with 20μM-[³H]UTP (1.0μCi/nmol). In the absence of heparin, the amount of [³H]UMP incorporated by RNA polymerase A and A* added in the unbound form was respectively 46pmol and 7.6pmol. For (NH₄)₂SO₄, the procedure was as described in the Experimental section for initiated complexes, except that either 14μl of water or 1.6M-(NH₄)₂SO₄ was added per 50μl portion of unbound enzyme, enzyme-DNA complex or initiated complex so that the concentration of (NH₄)₂SO₄ during the preincubation was 47mM or 0.40M. RNA synthesis was for 12min at 30°C, with 0.2mM-[³H]UTP (0.10μCi/nmol) and either 0.5μg of RNA polymerase A or 0.6μg of RNA polymerase A*. The amount of [³H]UMP incorporated into RNA in 35mM-(NH₄)₂SO₄ by RNA polymerase A and A* added in the unbound form was 54pmol and 39pmol respectively. In both experiments, each value is the mean of two determinations.

State of RNA polymerase added to assay	³ H]UMP incorporated (%)				
	Heparin (μg/ml)			(NH ₄) ₂ SO ₄ (mM)	
	0	25	100	35	300
RNA polymerase A					
Unbound enzyme	100	1	—	100	0
Enzyme-DNA complexes	59	3	2	72	2
Initiated complexes	118	83	43	—	48
RNA polymerase A*					
Unbound enzyme	100	4	—	100	0
Enzyme-DNA complexes	97	6	2	67	2
Initiated complexes	76	53	51	—	19

Fig. 3. *Effect of (NH₄)₂SO₄ on RNA synthesis by initiated complexes*
RNA synthesis for 10min at 30°C in the presence of 25μg of heparin/ml and 0.2mM-[³H]UTP (0.1μCi/nmol) was by initiated complexes formed from 1.0μg of RNA polymerase A (○) or 0.7μg of RNA polymerase A* (Δ). The incorporation of [³H]UMP at 35mM-(NH₄)₂SO₄ by initiated complexes made from RNA polymerases A and A* was 47pmol and 24pmol respectively.

Fig. 4. *Kinetics of initiation of RNA synthesis with [³²P]GTP*
Unbound enzyme was added to a reaction mixture containing 70mM-Tris/HCl, pH7.9, 40mM-(NH₄)₂SO₄, 1.6mM-MnCl₂, 267μg of DNA/ml, 0.2mM-ATP, -CTP, -UTP and -[³²P]GTP (0.2-0.5μCi/nmol). [³²P]GTP incorporation into RNA at 30°C was measured by removing 75μl of reaction mixture containing 0.5μg of RNA polymerase A (○) or RNA polymerase A* (Δ). The results for RNA polymerase A* are shown as the means ± s.e.m. of the values obtained in three experiments.

when poly[d(A-T)] was the template, because ATP-dependent pyrophosphate exchange in the presence of 0.5 mM-UTP proceeded at the same rate regardless of whether ATP or the $\alpha\beta$ -methylene analogue of ATP was present (Sentenac *et al.*, 1976). Thus most of RNA polymerase A* was presumed to make no RNA. The failure to make RNA was more likely to be due to some event such as unproductive binding to DNA than to inactivation of the enzyme before the experiment, because the specific activity of RNA polymerase A* was similar to that of the RNA polymerase A* isolated by Huet *et al.* (1975), which was as active as RNA polymerase A when RNA synthesis or pyro-

phosphate exchange was measured with poly[d(A-T)] as the template. This finding adequately explains the difference in the ability of RNA polymerases A and A* to catalyse pyrophosphate exchange on native calf thymus DNA in spite of the equal affinity of these enzymes for the initiating nucleoside triphosphate (Sentenac *et al.*, 1976). However, the defect that causes the change in the apparent K_m for UTP (see below) could also influence the rate of pyrophosphate exchange on this template.

Elongation by RNA polymerases A and A*

The values of the apparent K_m for UTP for initiated complexes formed from RNA polymerases A and A* were different. This effect of the subunits was shown by adding $(\text{NH}_4)_2\text{SO}_4$ and $[^3\text{H}]\text{UTP}$ to initiated complexes made in the presence of 4 μM -UTP and allowing elongation to proceed for 6 min in the presence of 3.6 mM- Mn^{2+} , 1.0 mM-ATP, -GTP, -CTP and various concentrations of $[^3\text{H}]\text{UTP}$. Under these conditions, the rate of incorporation of $[^3\text{H}]\text{UMP}$ was not constant and after 6 min was 72% of the amount predicted by extrapolation of the initial rate. But this deviation from linearity was the same at 20 μM - and 80 μM -UTP for both RNA polymerases A and A*, which meant that the amount of $[^3\text{H}]\text{UMP}$ incorporated after 6 min could be used as a measure of the initial rate. The results showed that the values for the apparent K_m for UTP were 20 μM and 45 μM for RNA polymerases A and A* respectively (Fig. 5).

When elongation is the only reaction that is measured and one substrate is varied with the other three in excess, as in the present case:

$$K_m(\text{app.}) = \frac{f_u \cdot K_{mu}}{k_{3u} \cdot \sum_{\alpha} \frac{f_{\alpha}}{k_{3\alpha}}}$$

where f_u and f_{α} are the mole fractions of UMP and nucleotide α in the RNA product, K_{mu} is the K_m for UTP and k_{3u} and $k_{3\alpha}$ are the composite rate constants for phosphodiester-bond formation and translocation

Table 2. Temperature-dependence of transcription by RNA polymerases A and A*

The final reaction mixture containing 0.5 μg of RNA polymerase A or 0.6 μg of RNA polymerase A* was as described for enzyme-DNA complexes (see the Experimental section), with 0.2 mM- $[^3\text{H}]\text{UTP}$ (0.15 $\mu\text{Ci}/\text{nmol}$). The reaction was started by adding unbound enzyme, enzyme-DNA complexes or initiated complexes and was continued for 6 min at 10°C or 30°C. Heparin (25 $\mu\text{g}/\text{ml}$) was present when RNA synthesis by initiated complexes was measured. The amount of $[^3\text{H}]\text{UMP}$ incorporated into RNA was measured in duplicate. At 30°C these values were 30–80 pmol. The results of two or three experiments are shown.

State of RNA polymerase added to assay	$\frac{[^3\text{H}]\text{UMP incorporation at 30}^\circ\text{C}}{[^3\text{H}]\text{UMP incorporation at 10}^\circ\text{C}}$	
	RNA polymerase A	RNA polymerase A*
Unbound enzyme	6.5	11.2
	5.5	10.0
Enzyme-DNA complexes	6.2	7.9
	7.3	7.4
	6.3	
Initiated complexes	4.7	3.7
	4.5	3.6
		5.5

Table 3. Initiation and RNA synthesis by RNA polymerases A and A*

RNA synthesis was measured in a reaction mixture of 75 μl containing 70 mM-Tris/HCl, pH 7.9, 40 mM- $(\text{NH}_4)_2\text{SO}_4$, 20 μg of DNA, 1.6 mM- MnCl_2 and 0.2 mM-ATP, -GTP, -CTP and -UTP. In addition, either 1.5 μCi of $[^3\text{H}]\text{UTP}$ or 2.5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was present. The reaction was started by adding 0.52 μg of RNA polymerase A or 0.95 μg of RNA polymerase A* in the unbound state. Incubation was for 60 min at 30°C. Each value is the average of two determinations. Average chain length was calculated by assuming that 28.4% of the RNA bases were uracil (Chargaff, 1955) and that initiation with UTP and CTP was insignificant. The molecular weights of RNA polymerases A and A* were taken to be respectively 650 000 and 520 000 (Valenzuela *et al.*, 1976).

Enzyme	Incorporation of labelled nucleotide (pmol)			Average RNA chain length (nucleotides)
	$[^3\text{H}]\text{UMP}$	$[\gamma\text{-}^{32}\text{P}]\text{ATP}$	$[\gamma\text{-}^{32}\text{P}]\text{GTP}$	
RNA polymerase A (0.8 pmol)	124	0.22	0.46	640
RNA polymerase A* (1.8 pmol)	136	0.15	0.31	1040

for the incorporation of UMP and nucleotide α respectively into RNA (Hyman & Davidson, 1970). The mole fraction of each nucleotide in the RNA product may be regarded as being the same as in DNA, but with [U] equalling [T]. So the differences in the apparent K_m for UTP will be entirely due to differences in K_{mu} only if the term $k_{3u} \cdot \sum_{\alpha} (f_{\alpha}/k_{3\alpha})$ is the same for each enzyme. Since the value of this term is unknown, the conclusion is that K_{mu} and/or $k_{3u} \cdot \sum_{\alpha} (f_{\alpha}/k_{3\alpha})$ are different for RNA polymerases A and A*.

The determination of the apparent K_m for UTP by initiated complexes was performed at 0.3M-(NH₄)₂SO₄, which inhibits elongation by RNA polymerase A*, but not by form A. This effect of (NH₄)₂SO₄ could be caused by dissociation of some initiated complexes and/or by inhibition of one or more of the reactions involved in elongation. In this case, a difference in the apparent K_m for UTP might not be observed when the ionic strength was less. However, a difference in the response of RNA

polymerases A and A* to UTP was also apparent during RNA synthesis at low ionic strength (Fig. 6). This difference was again attributed to an effect on elongation, as UTP is used only in this reaction [cf. Anthony *et al.* (1969), who concluded that this assumption was valid for RNA synthesis catalysed by RNA polymerase from *Escherichia coli*].

A potential hazard in these experiments was that the values for the apparent K_m for UTP might be affected by the ribonuclease-H activity of RNA polymerases A and A*. This activity, which is suppressed by nucleoside triphosphates complementary to the template (Huet *et al.*, 1977), might cause the lines in Figs. 5 and 6 to curve upwards at low concentrations of UTP. However, there is no evidence of this, nor was such an effect expected, as the ribonuclease-H activity is non-processive, concealed within RNA polymerase molecules engaged in transcription and drastically inhibited by as little as 50mM-(NH₄)₂SO₄ and a 4-fold excess of DNA template over RNA·DNA hybrid (Huet *et al.*, 1977).

Fig. 5. Effect of UTP on RNA synthesis by initiated complexes formed from RNA polymerases A and A*

Initiated complexes formed in the presence of 4μM-UTP were treated with 0.4M-(NH₄)₂SO₄ to prevent further initiation when RNA synthesis was stimulated by adding more UTP. Each 85μl reaction mixture contained 2.5μCi of [³H]UTP (10–30Ci/nmol) and either 1.0μg of RNA polymerase A (○) or 0.6μg of RNA polymerase A* (Δ). RNA synthesis was for 6min at 30°C. Each value of [UTP]/[³H]UMP incorporated into RNA for RNA polymerase A was multiplied by 15 to improve the clarity of the presentation.

Fig. 6. Effect of UTP on RNA synthesis by RNA polymerases A and A*

RNA synthesis was measured in a reaction mixture of 75μl containing 20μg of DNA, 70mM-Tris/HCl, pH7.9, 40mM-(NH₄)₂SO₄, 15mM-MgCl₂, 1.0mM-ATP, -GTP, -CTP, various concentrations of UTP, and 1.5μCi of [³H]UTP (10–30Ci/nmol). The reaction was started by adding 0.7μg of RNA polymerase A (○), or 0.5μg of RNA polymerase A* (Δ) in the unbound form. Incubation was for 10min at 30°C.

Assignment of function

On the basis of the data that are presented, no function can unambiguously be assigned to any one of the subunits of 48000, 37000 and 16000mol.wt., nor is it clear whether these subunits act together or independently of each other. However, the subunit of 16000mol.wt. was probably not responsible for the relative inability of RNA polymerase A* to bind productively to DNA, because the RNA polymerase A* studied by Huet *et al.* (1975, 1976) and Sentenac *et al.* (1976) was similarly impaired, but had the same subunit composition as RNA polymerase A in the mol.wt. range 20000–12200. On the other hand, this defect might arise from the loss of the subunit of 48000mol.wt., as this protein, isolated independently as ribonuclease H1, binds to DNA and stimulates the transcription of bacteriophage-T7 DNA by both RNA polymerases A and B (Dezélée *et al.*, 1977). This possibility could be explored by testing the effect of this protein on transcription by RNA polymerase A*, although any conclusion about the function of this or any other subunit on events before initiation is from data obtained by using a non-physiological template and remains tentative until the effect on specific transcription of rRNA genes is defined.

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References

- Anthony, D. D., Wu, C. W. & Goldthwait, D. A. (1969) *Biochemistry* **8**, 246–256
- Buhler, J.-M., Sentenac, A. & Fromageot, P. (1974) *J. Biol. Chem.* **249**, 5963–5970
- Buhler, J.-M., Iborra, F., Sentenac, A. & Fromageot, P. (1976) *J. Biol. Chem.* **251**, 1712–1717
- Chargaff, E. (1955) in *The Nucleic Acids* (Chargaff, E. & Davidson, J. N., eds.), vol. 1, pp. 308–371, Academic Press, New York
- Cooper, C. S. & Quincey, R. V. (1979) *Biochem. J.* **177**, 825–831
- Dezélée, S., Sentenac, A. & Fromageot, P. (1972) *FEBS Lett.* **21**, 1–6
- Dezélée, S., Sentenac, A. & Fromageot, P. (1974) *J. Biol. Chem.* **249**, 5971–5977
- Dezélée, S., Wyers, F., Darlix, J.-L., Sentenac, A. & Fromageot, P. (1977) *J. Biol. Chem.* **252**, 8935–8944
- Germond, J. E., Vogt, J. M. & Hirt, B. (1974) *Eur. J. Biochem.* **43**, 591–600
- Glynn, I. M. & Chappell, J. B. (1964) *Biochem. J.* **90**, 147–149
- Hager, G. L., Holland, M. J. & Rutter, W. J. (1977) *Biochemistry* **16**, 1–8
- Holland, M. J., Hager, G. L. & Rutter, W. J. (1977) *Biochemistry* **16**, 16–24
- Huet, J., Buhler, J.-M., Sentenac, A. & Fromageot, P. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3034–3038
- Huet, J., Dezélée, S., Iborra, F., Buhler, J.-M., Sentenac, A. & Fromageot, P. (1976) *Biochimie* **58**, 71–80
- Huet, J., Buhler, J.-M., Sentenac, A. & Fromageot, P. (1977) *J. Biol. Chem.* **252**, 8848–8855
- Hyman, R. W. & Davidson, N. (1970) *J. Mol. Biol.* **50**, 421–438
- Kedinger, C., Gissinger, F. & Chambon, P. (1974) *Eur. J. Biochem.* **44**, 421–436
- Mejbaum-Katzenellenbogen, M. & Dobryszcka, W. (1959) *Clin. Chim. Acta* **4**, 515–522
- Sentenac, A., Dezelee, S., Iborra, F., Buhler, J.-M., Huet, J., Wyers, F., Ruet, A. & Fromageot, P. (1976) in *RNA Polymerase: Cold Spring Harbour Monograph Series* (Losick, R. & Chamberlin, M., eds.), pp. 763–778, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Valenzuela, P., Weinberg, F., Bell, F. & Rutter, W. J. (1976) *J. Biol. Chem.* **251**, 1464–1470