Convolution Analysis of Transcription by Yeast DNA-Dependent Ribonucleic Acid Polymerase A

A MATHEMATICAL METHOD FOR STUDYING RIBONUCLEIC ACID CHAIN ELONGATION

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The rate of initiation of RNA synthesis catalysed by yeast RNA polymerase A on native calf thymus DNA decayed exponentially with a half-life of about 4.3 min. The rate constant for initiation was unaffected by preincubating the enzyme with DNA, or by decreasing the concentration of GTP 4-fold. The rate of RNA synthesis was constant for 15–20 min and then decreased. Each enzyme molecule made no more than one RNA molecule. In this situation, initiation, elongation and total RNA synthesis are related by a convolution integral. Solution of the convolution integral revealed that the rate of elongation was apparently biphasic. Analysis of the size of the RNA product showed that this biphasic profile arose because most but not all of the enzyme stopped RNA synthesis soon after initiation.

The synthesis of RNA by DNA-dependent RNA polymerase (nucleoside triphosphate–RNA nucleotidyltransferase, EC 2.7.7.6) is the result of four temporally related experimentally separable steps: binding of the enzyme to DNA, ribonucleotide chain initiation, chain elongation and termination (Chamberlin, 1974; Losick & Chamberlin, 1976). However, the technique required to examine an individual step may be complex (e.g. Rhodes & Chamberlin, 1974).

In this paper we describe a novel method for investigating the elongation step in which the change in length with time of an average RNA molecule is calculated from the kinetics of initiation and total RNA synthesis. A convolution integral relating these parameters is obtained that is solved by using Laplace Transforms. Application of this analysis to the transcription of native calf thymus DNA by yeast RNA polymerase A provided information complementary to that obtained from measurements of chain length by other methods. The analysis is not valid if each enzyme molecule makes more than one RNA molecule.

Experimental

Materials

[5-³H]UTP and [³²P]P_i were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Nucleoside triphosphates and calf thymus DNA (type 1, highly polymerized) were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Phosphocellulose (P-11) and DEAE-cellulose (DE-52) were obtained from Whatman, Maidstone, Kent, U.K. γ -³²P-labelled nucleoside triphosphates were

prepared as described by Glynn & Chappell (1964). rRNA and tRNA were prepared respectively from a ribosome pellet and 110000g supernatant of an extract of *Escherichia coli* by treating with 2% (v/v) sodium dodecyl sulphate and phenol.

Saccharomyces cerevisiae strain A364A (a ad₁ ad₂ ur₁ hi₇ ly₂ ty₁ ga₁) 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, 2% (w/v) Bacto-peptone (Difco Laboratories, West Molesey, Surrey, U.K.), 2% (w/v) glucose and 20 μ g each of adenine and uracil/ml.

Assay for RNA synthesis and initiation

For RNA synthesis the reaction mixture (0.075 ml) contained 70 mM-Tris/HCl, pH 7.9, 1.6 mM-MnCl₂, 20 μ g of native calf thymus DNA, 0.2 mM each of CTP, GTP, ATP, 0.2 mM-[³H]UTP (0.1 μ Ci/nmol) and 40 mM-(NH₄)₂SO₄. Initiation was measured similarly, except that unlabelled UTP and a γ -³²P-labelled nucleoside triphosphate (0.1–0.2 μ Ci/nmol) were present. After incubation at 30°C, the reaction was terminated by adding, in order, 0.0075 ml of 0.5 M-EDTA, pH 8.0, 0.02 ml of 0.2 M-sodium pyrophosphate, pH 7.5, 0.8 ml of 50 mM-EDTA/25 mM-sodium pyrophosphate, pH 7.5, 100 μ g of *Torula* RNA (B grade; Calbiochem, Bishops Cleeve, Herts., U.K.) and 0.2 ml of 50% (w/v) trichloroacetic acid. After standing for 0°C for 30 min the RNA was spun down (2000 rev./min for 20 min) and the pellet was washed by resuspension in 4 ml of 5% trichloroacetic acid/30 mM-sodium pyrophosphate. The washed

pellet was dissolved in 1.1 ml of 50 mM-EDTA/25 mM-sodium pyrophosphate, pH 7.5, and extracted with 0.25 ml of phenol/*m*-cresol/water mixture (50:7:7, by vol.). After washing of the phenol layer with 1.1 ml of 50 mM-EDTA/25 mM-sodium pyrophosphate, the aqueous layers were combined and 0.5 ml of 50% trichloroacetic acid was added. After 30 min at 0°C, the precipitates were collected by filtration on glass-fibre discs and washed with 30 ml of 5% trichloroacetic acid/30 mM-sodium pyrophosphate. Radioactivity was measured in a Phillips liquid-scintillation analyser for 10 min at an efficiency of approx. 70% for ^{32}P and 32% for ^3H . The scintillant contained 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene.

The sum of the background radioactivity and the residual radioactivity from reactions stopped at zero time amounted to 10 d.p.m. for ^{32}P and 200 d.p.m. for ^3H . The ^3H count rate was corrected for internal quenching by RNA and loss of RNA (5%) during this procedure. One unit of enzyme activity was defined as the amount of enzyme required to incorporate 1 pmol of [^3H]UMP into trichloroacetic acid-insoluble RNA/min.

Purification of yeast RNA polymerase A

Enzyme was purified from 100 g of yeast. The initial stages of the purification were carried out as described by Buhler *et al.* (1974), although the yeast was disrupted in 20 g portions by shaking at 4°C with 20 g of glass beads (0.4 mm diameter) in a Braun disintegrator for 4 min. The suspension was then sonicated for 6×10 s in an MSE sonicator (probe diameter 1.0 cm) operating at 20 000 Hz; 70–80% of the yeast was disrupted.

Centrifugation and phosphocellulose batch purification were completed as described by Buhler *et al.* (1974). The phosphocellulose filtrate in Buffer I [20 mM-Tris/HCl (pH 7.9)/10 mM-2-mercaptoethanol/0.5 mM-EDTA/10% (v/v) glycerol] containing 0.4 M-(NH_4) $_2\text{SO}_4$ was thoroughly mixed in a beaker with 45 g wet wt. of DEAE-cellulose that had been equilibrated with Buffer I containing 0.1 M-(NH_4) $_2\text{SO}_4$. The (NH_4) $_2\text{SO}_4$ concentration was lowered to 0.1 M by adding 3 vol. of Buffer I. The suspension was stirred for 30 min and the DEAE-cellulose then collected in a Buchner funnel. The cake was resuspended in 100 ml of Buffer I containing 0.1 M-(NH_4) $_2\text{SO}_4$ and poured into a glass tube. The resulting column of DEAE-cellulose was washed with 150 ml of Buffer I containing 0.1 M-(NH_4) $_2\text{SO}_4$ before elution with a 150 ml linear gradient of 0.1–0.3 M-(NH_4) $_2\text{SO}_4$ in Buffer I. RNA polymerase was eluted as a single peak at 0.14 M-(NH_4) $_2\text{SO}_4$. Fractions containing most of the activity were pooled and the solution was adjusted to 0.09 M-(NH_4) $_2\text{SO}_4$ by adding Buffer I. The enzyme was applied to a phosphocellulose

column (1.5 cm $^2 \times$ 4.0 cm) that was then washed with 20 ml of Buffer II [20 mM-Tris/HCl (pH 7.9)/10 mM-2-mercaptoethanol/0.5 mM-EDTA/2% glycerol] containing 0.05 M-(NH_4) $_2\text{SO}_4$. Elution was carried out with a 40 ml linear gradient of 0.05–0.45 M-(NH_4) $_2\text{SO}_4$ in Buffer II. A single symmetrical peak of RNA polymerase was eluted at about 0.24 M-(NH_4) $_2\text{SO}_4$ [RNA polymerase A*, examined separately, was eluted at about 0.125 M-(NH_4) $_2\text{SO}_4$]. Portions (1 ml) containing RNA polymerase A were loaded on 11.6 ml linear gradients of 10–30% glycerol in Buffer I containing 0.05 M-(NH_4) $_2\text{SO}_4$ and spun at 196 000 g_{av} for 17 h at 1°C in a 6×14 ml swing-out rotor ($r_{\text{av}} = 11.1$ cm) in an MSE Superspeed 65 centrifuge. Peak fractions were pooled and adjusted to approx. 50% glycerol before storage at –20°C.

All buffers were adjusted to pH 7.9 at 22°C and all stages of the purification were carried out at 0–4°C.

Size of the RNA product

Preparation of [^3H]RNA. [^3H]RNA was made in a 3-fold reaction mixture by using 6 pmol of enzyme. The reaction was stopped after 20 min by adding 0.01 ml of 100 mM-EDTA (all components of the reaction mixture, except enzyme, were extracted with phenol and then ether to destroy any ribonuclease). Secondary structure was disrupted by adding 0.8 ml of formamide and incubating at 37°C for 5 min (Marmur & Ts'o, 1961). The mixture was frozen at –80°C, freeze-dried until about 0.1 ml remained and then made up to 0.5 ml with water.

Sucrose-density centrifugation. A 0.3 ml portion of the above material was layered on an 11.5 ml linear gradient of 10–30% sucrose in 20 mM-Tris/HCl buffer (pH 7.9)/10 mM-2-mercaptoethanol/1 mM-EDTA/0.05 M-(NH_4) $_2\text{SO}_4$. After spinning for 12 h at 144 000 g_{av} in a 6×14 ml swing-out rotor ($r_{\text{av}} = 11.1$ cm) in an MSE Superspeed 65 centrifuge at 10°C, the contents of each tube were fractionated into 0.4 ml portions by pumping from the bottom of the gradient. Then 0.25 ml of each fraction was added to 0.03 ml of *Torula* RNA (5 mg/ml) and precipitated by adding 1 ml of 10% trichloroacetic acid/60 mM-sodium pyrophosphate. The precipitate was collected and measured for radioactivity as described for the assay for RNA synthesis.

Sedimentation of unlabelled rRNA and tRNA from *E. coli* was monitored by pumping the contents through a flow cell attached to an LKB Uvicord I set at 254 nm and linked to a chart recorder.

Ribonuclease was removed from sucrose solutions by boiling with 0.05% diethyl pyrocarbonate for 10 min.

Estimation of rate constants

Rate constants for initiation with GTP (k_G) were estimated by fitting the data to a function of the form

$$I_G[t_i] = e_G\{1 - \exp(-k_G t_i)\}$$

(see the Results section for definitions) by using an iterative method that minimizes the sum of the squares of residuals. A residual is defined as the difference between the data point $I_G[t_i]$ at a given time, t_i , and the value of $I_G[t_i]$ calculated from the above equation.

Other methods

Non-denaturing 5% polyacrylamide-gel electrophoresis was performed as described by Keding *et al.* (1974); 15% polyacrylamide slab gels were run in the presence of sodium dodecyl sulphate, as described by Laemmli (1970) and Valenzuela *et al.* (1976). The assay for polynucleotide kinase was similar to the assay for initiation of RNA synthesis, except that a single nucleoside triphosphate was present ($[\gamma\text{-}^{32}\text{P}]\text{GTP}$ or $[\gamma\text{-}^{32}\text{P}]\text{ATP}$).

Ribonuclease activity was determined by using the reaction mixture described for $[\text{H}^3]\text{UMP}$ incorporation, except that the DNA was replaced by $2.82\text{ }\mu\text{g}$ of $[\text{H}^3]\text{RNA}$ ($0.019\text{ }\mu\text{Ci}/\mu\text{g}$). The reaction was started by adding enzyme (0.5–1.5 pmol of RNA polymerase A) and continued for 125 min at 30°C . The reaction was terminated by adding, in order, 0.005 ml of 1 M MgCl_2 , 0.1 ml of *Torula* RNA (5 mg/ml), 1.62 ml of water and 0.2 ml of 50% trichloroacetic acid. After 30 min at 0°C the RNA was spun down and 1.0 ml of the supernatant was mixed with 10 ml of toluene/Triton scintillant and counted for radioactivity in a Philips liquid-scintillation analyser for 10 min. Solubilization of 0.1% of the RNA could be detected.

Protein was estimated by the micro tannin turbidity procedure of Mejbaum-Katzenellenbogen & Dobryszewska (1959), with bovine serum albumin as a standard. The concentration of bovine serum albumin was estimated by assuming that the A_{279} of a 1 mg/ml solution was 0.667.

Theory

The amount of RNA made is a function of the number of RNA molecules and their length. These parameters, expressed as a function of time, are related by a convolution integral, provided that each enzyme molecule makes no more than one RNA molecule.

The number of RNA chains begun in a small time dt_i at time t_i after the start of the reaction is $I'[t_i]dt_i$ where $I'[t_i]$ is the first derivative of the initiation function $I[t_i]$. Representing the length of each RNA chain at time $t-t_i$ after initiation as $E[t-t_i]$, the RNA produced by these molecules is given by $E[t-t_i]I'[t_i]dt_i$. Assuming that no reinitiation occurs this expression can be integrated to give the convolution integral:

$$T[t] = \int_0^t E[t-t_i]I'[t_i]dt_i \quad (1)$$

where $T[t]$ is the amount of RNA made at time t after the start of the reaction. Thus $E[t-t_i]$ may be calculated if $I[t_i]$ and $T[t]$ are determined.

Results

Properties of RNA polymerase A

The enzyme migrated as a single band on a non-denaturing 5% polyacrylamide gel. Analysis on a 15% polyacrylamide slab gel in the presence of sodium dodecyl sulphate showed that the subunit composition of the enzyme was similar to that observed by Buhler *et al.* (1976) in the molecular-weight range 12200–190000. The enzyme also contained traces of the 16000-dalton subunit detected by Hager *et al.* (1977). Thus a total of 13 subunits were observed. All calculations were made by assuming a molecular weight for the enzyme of 650000 (Valenzuela *et al.*, 1976).

The specific activity of the enzyme was 12 units/ μg . However, when measured in the presence of Mg^{2+} as described by Buhler *et al.* (1974) the specific activity increased to 30 units/ μg , the same as observed by those authors. Chromatography on DEAE-Sephadex A-25 showed that the enzyme was free from RNA polymerase C. RNA polymerase A had the same sensitivity to α -amanitin as observed by Huet *et al.* (1975). No ribonuclease or polynucleotide kinase activity was detected.

Initiation

Initiation by yeast RNA polymerase A on native calf thymus DNA occurs at single-stranded gaps in the template (Dezelee *et al.*, 1974). The amount of initiation with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (Fig. 1) increased with time according to the equation:

$$I_G[t_i] = e_G\{1 - \exp(-k_G t_i)\} \quad (2)$$

where $I_G[t_i]$ is initiation as a function of time t_i , e_G is the asymptotic value of initiation and k_G is the first-order rate constant for initiation (the subscript G indicates that initiation is with GTP; elsewhere the subscript A is used to denote initiation with ATP). k_G increased from 2.45 ms^{-1} to 3.15 ms^{-1} (each the average of two determinations) when the enzyme was preincubated with DNA, and was 2.6 ms^{-1} when the concentration of GTP was lowered 4-fold. These differences were within the error of determination of the rate constant by this method. e_G was unaffected by preincubating the enzyme with DNA, but decreased from 0.65 to 0.43 pmol when the concentration of GTP was lowered 4-fold. The latter result is ex-

Fig. 1. Initiation characteristics of RNA polymerase A on native calf thymus DNA

[γ - 32 P]GTP incorporation was measured by using a 7–8-fold reaction mixture from which 0.075 ml samples containing 1.0 μ g (1.5 pmol) of enzyme were removed at various times. □, No preincubation: 0.2 mM-[γ - 32 P]GTP. A 'cocktail' containing all reaction components except enzyme was warmed at 30°C for 5 min before the reaction was started by adding the enzyme. ○, Preincubation: 0.2 mM-[γ - 32 P]GTP. Enzyme was added to a prewarmed reaction mixture lacking nucleoside triphosphates. After 15 min at 30°C the reaction was started by adding prewarmed nucleoside triphosphates (0.02 ml/0.055 ml of enzyme–DNA complex). ●, Preincubation: 0.05 mM-[γ - 32 P]GTP. Conditions were as for 'preincubation: 0.2 mM-[γ - 32 P]GTP' except that the final concentration of [γ - 32 P]GTP was 0.05 mM.

plained if lowering the concentration of GTP promoted initiation by ATP. Initiation with [γ - 32 P]ATP was studied less intensively, but fewer RNA molecules were started with ATP (Table 1). The rate constant for initiation with ATP was estimated to be about 1.3 ms $^{-1}$, approximately half the value of k_G . An insignificant number of RNA molecules started with UTP and CTP.

Initiation with GTP ceased after 40 min (Figs. 1 and 2), and after 60 min the number of RNA mole-

cules started with ATP and GTP corresponded to 84% of the enzyme added (Table 1). These observations suggested that each enzyme molecule made one RNA molecule. Any model in which recycling of enzyme occurred was unlikely, because this would mean that both the molar equivalence of enzyme and product and the simple first-order decay of initiation were fortuitous. Nonetheless, because this was an important point, the possibility that recycling might occur on extensive single-stranded regions was eliminated by measuring initiation on DNA from which single-stranded regions had been removed by passage through nitrocellulose (Dezelee & Sentenac, 1973). The kinetics of initiation were similar to those shown in Fig. 1 and the amount of initiation was identical with that observed with untreated DNA.

Under different experimental conditions repeated initiation by enzyme occurred. Thus adding 1 mM-spermine 45 min after transcription of native calf thymus DNA had started caused total initiation to exceed the amount of enzyme (result not shown). Recycling of enzyme also occurred when denatured calf thymus DNA was transcribed (Table 1).

RNA synthesis

The rate of transcription of native calf thymus DNA was constant for 15–20 min (Fig. 2). Thus for this period

$$T[t] = at \quad (3)$$

where a is the rate of RNA synthesis calculated after correcting [3 H]UMP incorporation for the base composition of the DNA template (see Table 1).

Elongation on native DNA in the absence of recycling

Solution of the convolution integral. $I[t_i]$ comprised two components, $I_G[t_i]$ and $I_A[t_i]$, which represented initiation by GTP and ATP respectively. $I_G[t_i]$ was

Table 1. Incorporation of [3 H]UMP, [γ - 32 P]ATP and [γ - 32 P]GTP into RNA

The incubation mixture (0.075 ml) contained either 20 μ g of native DNA or 10 μ g of denatured DNA. The reaction was started by adding 0.52 μ g (0.8 pmol) of yeast RNA polymerase A and stopped after 20 min or 60 min. 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), ignoring initiation with UTP and CTP. Initiation with UTP and CTP amounted to less than 5% of the total when native calf thymus DNA was used as the template.

DNA template	Time (min)	Incorporation of labelled nucleotide (pmol)			Av. RNA chain length (nucleotides)
		[3 H]UMP	[γ - 32 P]ATP	[γ - 32 P]GTP	
Native DNA	20	60	0.14	0.37	420
	60	124	0.22	0.46	640
Denatured DNA	20	105	0.85	5.05	63
	60	205	1.28	8.70	72

Fig. 2. Initiation and RNA synthesis on native calf thymus DNA

The kinetics of $[^3\text{H}]\text{UMP}$ incorporation ($\bullet$) during the first 16 min of RNA synthesis are shown in (a). (b) shows the data for $[^3\text{H}]\text{UMP}$ ($\bullet$) and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ ($\circ$) incorporation, from which values of k_G (2.6 ms^{-1}), e_G (0.49 pmol) and a (0.217 pmol of nucleotide/s) were calculated for use in the convolution analysis (see the text for definitions). These data were obtained on the same occasion by using $0.52\text{ }\mu\text{g}$ of enzyme/ 0.075 ml . In both (a) and (b) a 6–7-fold reaction mixture from which 0.075 ml samples were later withdrawn was warmed at 30°C for 5 min before the reaction was started by adding the enzyme.

defined in eqn. (2); $I_A[t_i]$ had a similar form. If it is assumed that $k_A = k_G$, the mathematics is simplified. In this case,

$$I[t_i] = (e_G + e_A) \{1 - \exp(-k_G t)\} \quad (4)$$

and

$$I'[t_i] = k_G(e_G + e_A) \exp(-k_G t) \quad (5)$$

Substituting for $I'[t_i]$ and $T[t]$ (see eqn. 3) in eqn. (1) and using Laplace Transforms (Stephenson, 1961; Bellman & Cooke, 1963) to solve this equation for $E[t-t_i]$ gives:

$$E[t-t_i] = \frac{a}{k_G(e_G + e_A)} \{1 + k_G(t-t_i)\} \quad (6)$$

When the different rate of initiation with ATP is taken into account, initiation and total RNA synthesis are related by the convolution integral:

$$T[t] = \int_0^t E[t-t_i] (I'_G[t_i] + I'_A[t_i]) dt_i \quad (7)$$

Using Laplace Transforms to solve this equation for $E[t-t_i]$ gives:

$$E[t-t_i] = C_1(t-t_i) + C_2 - C_3 + (C_4 - C_2 + C_3) \exp\{-C_5(t-t_i)\} \quad (8)$$

where

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$$\begin{aligned} C_1 &= \frac{a}{e_A + e_G} \\ C_2 &= \frac{a(k_A + k_G)}{k_A k_G (e_A + e_G)} \\ C_3 &= \frac{a(e_G k_G + e_A k_A)}{k_G k_A (e_G + e_A)^2} \\ C_4 &= \frac{a}{e_A k_A + e_G k_G} \\ C_5 &= \frac{k_A k_G (e_A + e_G)}{e_A k_A + e_G k_G} \end{aligned}$$

When eqn. (6) was applied to the data in Fig. 2(b), with e_A obtained by multiplying e_G by the ratio of $[\gamma\text{-}^{32}\text{P}]\text{ATP}/[\gamma\text{-}^{32}\text{P}]\text{GTP}$ incorporation at 60 min (Table 1), the RNA appeared to be 130 nucleotides long at time $(t-t_i) = 0$ (Fig. 3). If eqn. (8) was used with $k_A = 1.3\text{ ms}^{-1}$, the RNA was apparently 150 nucleotides long at time $(t-t_i) = 0$ (Fig. 3). A more realistic interpretation of the elongation profile that allows for the fact that $I[t_i]$, $T[t]$ and hence also $E[t-t_i]$ are only good approximations is shown by the broken line in Fig. 3.

This elongation function is consistent with a bi-phasic elongation reaction in which each molecule becomes longer rapidly at first and then more slowly (model 1). An alternative explanation is that there are two populations of enzyme–DNA complexes: one that makes RNA for a short time before stopping and

Fig. 3. Theoretical elongation functions

The lower line was calculated from eqn. (6). Values for k_G , a and e_G were taken from data in Fig. 2(b). e_A was calculated by multiplying e_G by the ratio of $[\gamma\text{-}^{32}\text{P}]\text{ATP}/[\gamma\text{-}^{32}\text{P}]\text{GTP}$ incorporation after 60 min (Table 1). The upper line was calculated from eqn. (8) by using the same values but with $k_A = 1.3 \text{ ms}^{-1}$, half the value of k_G . If k_A was increased to 2.6 ms^{-1} the line obtained from eqn. (8) was identical with that obtained from eqn. (6). The broken line shows a more realistic interpretation of the elongation function calculated from eqn. (6) (see the text).

another that remains active (model 2). In this case, an apparently biphasic elongation profile arises because the elongation function is an average obtained by dividing the RNA made after time $t-t_i$ by the number of enzyme molecules initiating in time dt_i . When some of these molecules stop growing, their contribution to the RNA made also ceases and the average rate of elongation decreases. These models were distinguished by measuring the size of the RNA product.

Size of the $[\text{H}]$ RNA produced after 20 min of RNA synthesis. In this period 70% of the RNA molecules are begun in the first 7 min. If model 1 is correct, these molecules will be 415–560 nucleotides long after 20 min (see line defined by eqn. 8, Fig. 3), and the rest will be shorter. If model 2 is correct and most of the enzyme is active for a short time only, most of the RNA molecules will be 130–150 nucleotides long, but some will be longer.

Centrifugation of the RNA product through a 10–30% sucrose gradient (Fig. 4) showed that more than 50% of the $[\text{H}]$ RNA sedimented at about 4.7S. This corresponds to a chain length of about 120 nucleotides (Spirin, 1961). The remainder of the product sedimented between 4.7S and 16S. Sedimentation of tRNA, which migrated as a narrow band (Fig. 4), showed that the broad profile for $[\text{H}]$ RNA was due to the presence of RNA of different sizes and not to zone broadening. Thus $[\text{H}]$ RNA of at least 1500 nucleotides was also present. This size distribution is consistent with

Fig. 4. Sedimentation of RNA through 10–30% sucrose gradients

Sedimentation was of $[\text{H}]$ RNA synthesized in 20 min *in vitro* ($\circ$) and of $47 \mu\text{g}$ of tRNA (A_{254} ; ----). The arrows show the positions of 16S and 23S rRNA spun at the same time. The bracket shows the approximate position of RNA of 415–560 nucleotides calculated by using the equation obtained by Spirin (1961) relating molecular weight to s value. Centrifugation at 10°C was for 12 h at $144000g_{\text{av}}$. Gradients containing $[\text{H}]$ RNA were fractionated and the RNA was precipitated with acid. Gradients containing tRNA and rRNA were pumped through a flow cell attached to a recording spectrophotometer.

model 2, but not with model 1, which predicts that the modal size of the RNA product should be 415–560 nucleotides and that larger RNA should be absent. The result is also consistent with the observation by Buhler *et al.* (1974) of two populations of RNA molecules that differed in size, if it assumed that the difference in size of the two populations in the present study was less marked as a result of the different reaction conditions.

Essentially no degradation of 16S and 23S rRNA could be detected after sucrose-density-gradient centrifugation of $90 \mu\text{g}$ of rRNA which had been incubated for 20 min at 30°C in an RNA-synthesis reaction mixture that was complete except for nucleoside triphosphates. So the profile observed for $[\text{H}]$ RNA did not arise as a result of the action of a trace of ribonuclease on larger RNA molecules.

Discussion

The study of elongation is facilitated by techniques that allow initiation to be by-passed and/or inhibited. These include the use of high ionic strength to inhibit initiation (Anthony *et al.*, 1966), binding of initiated complexes to membrane filters (Anthony *et al.*, 1969), the use of oligonucleotides to by-pass initiation (Niyogi & Stevens, 1965), the use of inhibitors of initiation (Gissinger *et al.*, 1974) and the isolation of initiated complexes (Rhodes & Chamberlin, 1974). These methods suffer from several disadvantages. Firstly, the number of active enzyme molecules may not be known, because of incomplete initiation or

inactivation of an unknown fraction of the enzyme during the isolation of initiated complexes. Secondly, formation of initiated complexes may not allow the investigation of the early events between initiation and the formation of the initiated complex. Thirdly, inhibitors of initiation may also affect elongation. When a single round of initiation occurs these problems can be overcome, in principle, by using the mathematical analysis presented here. The incorporation of [γ - ^{32}P]nucleoside triphosphate into trichloroacetic acid-insoluble RNA gives a direct indication of the number of active enzyme molecules, and the early stages of elongation after the appearance of RNA in an acid-insoluble form can be studied. In the present instance a biphasic profile revealed by convolution analysis was caused by most enzyme molecules stopping soon after initiation. The molecular basis of this effect is unknown.

Quite apart from the result of the mathematical analysis was the contrast between the kinetics of initiation catalysed by RNA polymerase A at single-stranded gaps and by *E. coli* RNA polymerase holoenzyme at promoter sites. The rate of initiation by RNA polymerase A decayed exponentially with a half-life of about 4.3 min. A similar time course for initiation was observed by Schafer *et al.* (1973) for *E. coli* RNA polymerase transcribing bacteriophage-T4 and -T5 DNA. Slow initiation by the holoenzyme was probably the result of promoter searching (Chamberlin & Hinkle, 1972) or promoter 'melting' events (Mangel & Chamberlin, 1974a,b) because initiation was rapid if the holoenzyme and bacteriophage-T7 DNA were incubated together at 37°C before the reaction was started by adding nucleoside triphosphates (Rhodes & Chamberlin, 1975). During this rapid initiation by preformed enzyme-DNA complexes the rate-limiting step is thought to be the binding of the 5'-terminal nucleoside triphosphate (Rhodes & Chamberlin, 1975). In contrast, the rate constant for initiation by yeast RNA polymerase A was unaffected by preincubating the enzyme and DNA at 30°C and was independent of the concentration of the 5'-terminal nucleoside triphosphate. This may reflect a real difference in the nature of physiological initiation by these enzymes, or may result from the non-physiological initiation by yeast RNA polymerase A (Dezelee *et al.*, 1974; Hager *et al.*, 1977).

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