On the mechanism of oligonucleotide-primed RNA synthesis. I. Model studies with deoxy-homopolymer templates and *Escherichia coli* RNA polymerase

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ABSTRACT

We have studied the effect of temperature, primer chain length and primer concentration on the oligonucleotide-primed transcription by *Escherichia coli* RNA polymerase. Our experiments with the homopolymer model systems poly(dT).oligo(A)_n, poly(dA).oligo(U)_n and poly(dA).oligo(dT)_n lead to three main conclusions. First, de novo chain initiation on single-stranded templates is preferentially suppressed at higher temperatures. Second, stable annealing of template and primer is neither a prerequisite nor does it stimulate the primer-dependent transcription. Third, formation of the ternary enzyme-template-primer complex is a rate-limiting step in the oligonucleotide-primed RNA synthesis. The maximal rate of primer-stimulated RNA synthesis, moreover, is strongly dependent on the nature of the primer and decreases in the order oligo(A)-primed poly(A) synthesis >> oligo(U)-primed poly(U) synthesis > oligo(dT)-primed poly(U) synthesis. We attribute this to differences in the rate at which the first nucleotide is added to the primer.

Raising temperature and primer concentration renders transcription in the model systems almost completely primer-dependent. This can be useful in a transcription approach to DNA sequence analysis.

INTRODUCTION

We are interested in the use of RNA transcripts for sequence analysis of DNA, using single-stranded DNA as template. In vitro transcription of single-stranded DNA by *Escherichia coli* RNA polymerase is known to be much less specific than the transcription of double-stranded DNA (1). This permits the transcription of regions otherwise not transcribed, but it also leads to a heterogeneous RNA product because of the multiple start and termination sites. In order to direct the RNA synthesis to specific parts of the template we have, therefore, started to look for conditions in which elongation of primers is strongly favoured over de novo chain initiation (2).

Stimulation of RNA synthesis by complementary ribo-oligonucleotide primers has been shown earlier for bacterial RNA poly-

merases in a number of systems using either ribohomopolymers or deoxyhomopolymers, mixed polymers or short synthetic polynucleotides as template (3-9). Evidence has been presented that the oligomers act as chain initiators and that they become covalently incorporated into the 5'-end of the newly-synthesized RNA (3-5, 9,10). Two conditions have been described for the E. coli polymerase which lead to suppression of chain initiation relative to chain elongation: low substrate concentrations and omission of sigma factor (4,5). Using poly(dT).oligo(A)₅ as model system, we have reported two additional conditions that help to render transcription primer-dependent (2), i.e. raising the incubation temperature and addition of rifampicin. The finding that poly(A) synthesis is maximal at high temperature in this particular system indicated that a pre-existing template-primer complex was not a prerequisite for primer stimulation, as had been suggested by others (11). In this paper we present a more detailed study of the effects of temperature, primer chain length and primer concentration on the oligonucleotide-primed transcription of deoxyhomopolymer templates. Some additional experiments on the action of rifampicin are also reported. In the accompanying paper we show that the results obtained in the model systems enabled us to synthesize on a natural DNA specifically-primed RNA copies, suitable for sequence analysis. Part of this work has been briefly summarized elsewhere (12).

MATERIALS AND METHODS

Template and primers

Poly(dT) (2.3-5.3 S), poly(dA) (3 S), poly(U) (molecular wt. $>10^5$ daltons) and the dephosphorylated ribo-oligonucleotides (A)₂ to (A)₆ were purchased from Miles; deoxyribo-oligonucleotides (dT)₅, (dT)₆, (dT)₈, (dT)₉, (dT)₁₆ and (dT)₁₇ from P.L.Biochemicals. The ribo-oligomers (U)₅ to (U)₁₅ were obtained by controlled alkaline hydrolysis of poly(U), following published procedures (13). This included at 37°C, hydrolysis for 5 min in 0.3 M KOH, 1 h incubation in 0.1 M HCl (hydrolysis of terminal cyclic phosphate groups) and 4 h incubation with E. coli alkaline phosphatase (10 µg/mg poly(U); Boehringer). After phenol extraction, fractionation of the (Up)_nU mixture was obtained on a DEAE-

cellulose column in 7 M urea, as described (14). Except for the oligonucleotides (U)₁₂₋₁₅ separate peak fractions were pooled, desalted by reabsorption to DEAE-cellulose, eluted with saturated ammonium carbonate, evaporated to dryness and washed with twice-distilled water several times. All the oligo(U), oligo(A) and oligo(dT) primers used in the experiments were checked for purity by paper chromatography as described by Thach and Doty (15). To determine concentrations of polynucleotides and oligonucleotides, published molar extinction coefficients were used (13,16,17); indicated quantities are expressed in terms of the monomer units.

RNA polymerase, substrates and assay

RNA polymerase holoenzyme from E. coli B was purified as described before (2). It was analysed for purity and subunit stoichiometry by sodium dodecylsulphate polyacrylamide gel electrophoresis (18) and found to be over 98% pure. Compared with the correct stoichiometry of the core subunits ($\beta\beta'\alpha_2$), the sigma factor was present in low amounts: 0.23 mol sigma/mol core enzyme. The specific activity of the enzyme used was 230 units/mg protein.

Unlabelled ribonucleotides were obtained from Merck and ³H-labelled ribonucleotides were products from the Radiochemical Centre, Amersham, UK.

The standard (0.2 ml) reaction mixture for the polymerization assay contained 40 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 0.1 mM EDTA, 0.4 mM K₂HPO₄, 0.1 mM dithiothreitol, 1 µg poly(dT) or poly(dA) and 10 µg enzyme. Unlabelled and ³H-labelled ribonucleoside triphosphates were, like the primers, added as indicated in the legends. The reactions were started by adding the enzyme, after preliminary incubation of all other reagents for 5-10 min at the indicated temperature unless stated otherwise.

Incorporation of isotope was measured as acid-insoluble radioactivity after an incubation period of 1 min or 10 min as indicated. The reaction was stopped by adding a mixture of cold 5% trichloroacetic acid and 60 mM sodium pyrophosphate or by transferring 20-µl samples from the reaction mixture to the trichloroacetic acid solution. After addition of 100 µg bovine serum albumin as carrier, the precipitates were collected on Whatman glass-paper discs, washed extensively, dried and counted in a Nuclear

Chicago liquid scintillation counter.

Melting profiles and mixing curves

Thermal melting of the polymer-oligomer complexes under assay salt conditions was carried out in a Gilford recording spectrophotometer, model 2400, as described (19). For the lower temperatures the cuvette housing was cooled with a flow of cold nitrogen. The temperature was measured in a separate cuvette in the cuvette housing with a type-402 thermistor of Yellow Springs Instruments; 1-cm cuvettes (1.5 ml) with teflon stoppers were used.

Mixing curves were determined under assay salt conditions by the method of continuous variation (20), using the same technical setup. Before measuring the absorption at 260 nm, each sample was incubated for at least 2 h at the indicated temperature.

RESULTS

Kinetics of transcription

RNA synthesis with a variety of polymer templates is known to show a sigmoidal substrate concentration curve. This sigmoidicity is abolished by adding complementary primer oligonucleotides, which apparently overcome the rate-limiting initiation step (4,10). Under our conditions similar results were obtained for both poly(dT)-directed and poly(dA)-directed synthesis, although the effect was less pronounced for the latter (Figs 1a and 1b). The observed primer stimulation is maximal at low nucleotide concentration and increases exponentially below 100 μ M (Figs 1c and 1d).

To make the de novo initiation reaction rate-limiting without blocking net RNA synthesis too much, we have taken 50 μ M as substrate concentration for further experiments.

Effect of primer chain length on the optimal temperature for RNA synthesis

Optimal stimulation of RNA synthesis has been reported to occur when the primer is present in about stoichiometric amounts relative to the template (7,8). In poly(U)-directed synthesis of poly(A) maximal stimulation was observed at temperatures near the melting temperature of the polymer-oligomer complexes (21). These observations have led to the conclusion that template-primer

Fig. 1. Effect of ribonucleoside triphosphate concentrations on poly(dT)-directed and poly(dA)-directed RNA synthesis. Incorporation was measured at 30°C in a 10-min incubation period as described in Methods. a) In the absence or presence of 1.8 nmoles oligo(A)₅; b) in the absence or presence of 2.5 nmoles oligo(U)₅. In the panels c) and d) the ratios of primer-stimulated synthesis over *de novo* synthesis are plotted against the ribonucleoside triphosphate concentrations.

annealing is an important step in the mechanism of oligonucleotide-primed RNA synthesis (11).

Fig. 2 shows the effect of primer chain length on the optimal temperature for the poly(dT)-directed system, in the presence of half to equimolar amounts of primer (in nucleotide residues). Because primed poly(A) synthesis is very rapid, incorporation is determined after 1 min to ensure that initial velocities are measured. Synthesis in the absence of primer is low, declines above 30°C and becomes zero at 50°C. Primer-dependent synthesis, on the other hand, is maximal at, respectively, 30°C and 40°C for (A)₂ and (A)₃ and continues to increase with temperature up to 50°C for the larger oligomers. Hence, at 50°C poly(A) synthesis is completely primer-dependent and the amount of incorporation increases with increasing primer chain length.

To see whether the differential effect of chain length was due to differences in stability of the polymer-oligomer complexes, melting profiles were determined under the conditions used for the assay of RNA synthesis. For the complexes with (A)₅ and (A)₆ this

Fig. 2. The effect of primer chain length on the optimal temperature for poly(dT)-directed poly(A) synthesis. Reaction mixtures were as described in Methods and contained 50 μ M ATP. Incorporation was measured in a 1-min incubation period at the temperature indicated in the absence of primer or in the presence of 3.1, 3.5, 1.8, 1.9 and 2 nmoles of the adenine oligonucleotides (A)₂ to (A)₆, respectively. The dashed lines were obtained by subtracting the values for the de novo reaction from the oligonucleotide primed reaction.

is shown in Fig. 3. The observed T_m 's of, respectively, 5°C and 8°C are at least 40°C below the optimal stimulation temperature for these primers. Therefore, stable annealing of template and primer cannot occur at this temperature. (Melting curves of complexes with the smaller adenine oligonucleotides could not be accurately measured because of the low T_m 's.) The observed T_m 's reflect the stabilities of triple-stranded structures of the type 2 poly(dT).oligo(A)_n, as indicated by the hyperchromicity at 284 nm (insert Fig. 3; and ref. 22) and confirmed by the mixing curve analysis in Fig. 4. No other thermal transitions could be detected for either complex up to 60°C.

For the poly(dA)-directed system similar results were obtained (Fig. 5). In the absence of primer, poly(U) synthesis declines at higher temperatures and becomes zero at 50°C. Upon addition of the primers (U)₅ to (U)₈ the optimal temperature gradually shifts towards 40°C and remains at about this value for the larger oligomers. Hence, also in this system synthesis becomes completely

Fig. 3. Melting profiles of the complexes of poly(dT) with oligo(A)₅ and oligo(A)₆. Mixtures containing 30 μ M poly(dT) and 20 μ M of the adenine oligonucleotide were melted under assay salt conditions as described in Methods. ● - ●, Oligo(A)₅; ○ - ○, oligo(A)₆.

Fig. 4. The composition of the poly(dT).oligo(A)₅ complex determined by mixing-curve analysis. The absorption of the mixtures at 260 nm was measured at 0°C under assay salt conditions as described in Methods. The starting solutions contained 46 μ M poly(dT) and 48 μ M oligo(A)₅.

Fig. 5. The effect of primer chain length on the optimal temperature for poly(dA)-directed poly(U) synthesis. Reaction mixtures were as described in Methods and contained 50 μ M UTP. Incorporation was measured in a 10-min incubation period at the temperature indicated in the absence of primer or in the presence of 2.5 nmoles of the oligomers (U)₅ to (U)₁₁ and 2.8 nmoles of oligo(U)₁₂₋₁₅. The dashed lines were obtained by subtracting the values for the *de novo* reaction from the oligonucleotide-primed reaction.

primer-dependent at higher temperature. Table I shows the amount of incorporation at 20°C and 50°C in relation to the T_m 's of the polymer-oligomer complexes. Above 30°C none of the poly(dA).-oligo(U) complexes tested is stable. The rate of synthesis at 50°C, however, again increases with increasing primer chain length. At 20°C the larger oligonucleotides can interact with poly(dA) to form stable complexes while the smaller ones cannot. Nevertheless, almost exclusively *de novo* synthesis is measured at this temperature, because the observed primer stimulation is minimal or even negative. Apparently, UTP is a better primer than oligonucleotides under these conditions. Stable complexes between poly(dA) and poly(U) have been reported to exist only as a triple helix (22). By mixing curve analysis we have extended this observation to the polymer-oligomer complexes (results not shown).

TABLE I - EFFECT OF PRIMER CHAIN LENGTH IN POLY(dA)-DIRECTED POLY(U) SYNTHESIS AND THERMAL STABILITY OF THE CORRESPONDING POLYMER-OLIGOMER COMPLEXES

Incorporation results are from Fig. 5. Melting profiles were determined under assay salt conditions at 260 nm as described in Methods. Melting mixtures contained 28 μ M poly(dA) and about 30 μ M of the uracil oligonucleotides. The measured T_m 's, together with the T_m of the polymer-polymer complex, fell on a straight line when plotted against the reciprocal chain length.

Primer	T_m ($^{\circ}$ C) of triplex with poly(dA)	nmoles (3 H)UMP incorporated	
		at 20 $^{\circ}$ C	at 50 $^{\circ}$ C
None	-	0.17	0.001
(U) ₅	-40*	0.19	0.006
(U) ₆	-20*	0.28	0.028
(U) ₇	-7*	0.18	0.033
(U) ₈	4	0.12	0.082
(U) ₉	11	0.09	0.246
(U) ₁₁	23	0.10	0.330
(U) ₁₂₋₁₅	30	0.09	0.820

* Determined by extrapolation.

Therefore, the measured T_m 's reflect the thermal stabilities of triple-stranded complexes of the type poly(dA).2 oligo(U)_n.

Effect of primer concentration

We have studied the effect of primer concentration in both model systems, using different primer chain lengths. For each primer the effect was measured at two different temperatures. First at the optimal temperature as determined in the presence of stoichiometric amounts of primer (see Figs 2 and 5) and second, at 50 $^{\circ}$ C at which the de novo chain initiation is completely blocked. At the optimal temperatures, poly(A) synthesis with (A)₂ and (A)₃ reaches a plateau when the primer/template ratio (in nucleotide residues) is greater than 5 and 3, respectively (Fig. 6). Similarly, poly(U) synthesis at the optimal temperatures with the primers (U)₅ and (U)₁₁ becomes maximal above a ratio of 1.5 and 0.8, respectively (Fig. 7). At 50 $^{\circ}$ C, however, a more differential effect is observed. The very low poly(A) synthesis with equimolar amounts of (A)₂ increases over 40 times when the primer concentration is raised 100-fold, but even then no plateau is reached. With oligo-(A)₃ a plateau is reached, but only when the primer/template ratio is greater than 10. Using the longer oligomer (A)₆, a maximum is

Fig. 6. Effect of primer concentration on poly(dT)-directed poly(A) synthesis. Reaction mixtures were as described in Methods and contained 50 μ M ATP. Incorporation was measured in a 1-min incubation period. a) In the presence of increasing amounts of (A)₂ at 30°C and 50°C, as indicated; b) in the presence of increasing amounts of (A)₃ at 40°C and 50°C, as indicated.

reached above the ratio of 2 at either 50°C or 30°C and only the plateau values differ (50°C > 30°C; not shown). Similar results were obtained for poly(U) synthesis at 50°C. In this case synthesis with primer oligo(U)₁₁ even exceeds the plateau value found at 40°C when the primer/template ratio exceeds 4. In both model systems the concentration dependence of primer stimulation is most pronounced with the smaller primers.

Effect of deoxyribo-oligonucleotide primers

The RNA polymerase of *E. coli* has been shown to catalyze the covalent attachment of ribonucleotides to 3'-OH ends of DNA chains (23,24). Also stimulation of ribohomopolymer-directed RNA synthesis by deoxyribosyl oligonucleotides has been reported and this process was recently shown to result in the covalent incorporation

Fig. 7. Effect of primer concentration on poly(dA)-directed poly(U) synthesis. Reaction mixtures were as described in Methods and contained $50\ \mu\text{M}$ UTP. Incorporation was measured in a 10-min incubation period. a) In the presence of increasing amounts of $(\text{U})_5$ at 32°C and 50°C , as indicated; b) in the presence of increasing amounts of $(\text{U})_{11}$ at 40°C and 50°C , as indicated.

of the deoxy primer into the newly-synthesized RNA as well (11,25).

To compare the stimulatory effect of ribosyl and deoxyribosyl primers we have studied poly(dA)-directed poly(U) synthesis in the presence of several (dT) oligonucleotides. The effects of temperature and primer concentration are similar in this system to those observed in the oligo(U)-primed system. At stoichiometric primer concentration the optimal temperature for poly(U) synthesis depends on the primer chain length (Fig. 8) and at this optimal temperature synthesis rapidly reaches a plateau when the primer/-template ratio is increased (Fig. 9).

The main difference between the oligo(dT) $_n$ -primed and oligo-(U) $_n$ -primed poly(U) synthesis is that the deoxyoligomers are much less efficient primers. Table II shows that at the optimal temperature primer-dependent poly(U) synthesis is 3-5 times lower with the deoxy primers than with the corresponding uracil oligomers. Moreover, whereas some priming is found with oligo(U) $_5$ at 50°C

Fig. 8. The effect of deoxy primer chain length on the optimal temperature in poly(dA)-directed poly(U) synthesis. Reaction mixtures were as described in Methods and contained 50 μM UTP. Incorporation was measured in a 10-min incubation period at the indicated temperature in the presence of 2.5 nmoles of the deoxyoligonucleotides (dT)₅, (dT)₆ and (dT)₈ and 2.6 nmoles of oligo(dT)₁₇. The dashed lines were obtained by subtracting the values for the *de novo* reaction (not shown) from the oligonucleotide-primed reaction.

Fig. 9. Effect of primer concentration on poly(dA)-directed poly(U) synthesis. Reaction mixtures were as described in Methods and contained 50 μM UTP. Incorporation was measured in a 10-min incubation period with increasing amounts of; a) oligo(dT)₅ at 30°C and 50°C, as indicated; b) oligo(dT)₆ at 32°C and 50°C, as indicated; c) oligo(dT)₈ at 38°C and 50°C, as indicated, and d) oligo(dT)₁₇ at 50°C.

TABLE II - COMPARISON OF RIBOSYL AND DEOXYRIBOSYL PRIMERS IN THE STIMULATION OF POLY(dA)-DIRECTED POLY(U) SYNTHESIS

Incorporation data, optimal temperatures and T_m 's for the uracil oligonucleotides are from Fig. 5 and Table I. For the oligo(dT)-stimulated poly(U) synthesis incorporation data and optimal temperatures are from Fig. 8. Melting profiles of the poly(dA)-oligo(dT) complexes were determined under assay salt conditions at 260 nm as described in Methods. Melting mixtures contained 28 μ M poly(dA) and about 30 μ M of the oligomers (dT)₆, (dT)₈, (dT)₉ and (dT)₁₆, respectively. The measured T_m 's, together with the T_m of the polymer-polymer complex, fell on a straight line when plotted against the reciprocal chain length.

Primer	Optimal incorporation temperature (°C)	Incorporation* at optimal temperature (nmoles (³ H)UMP)	T_m (°C) [∇] of complex with poly(dA)
(U) ₅	32	0.20	-40 [§]
(U) ₆	32	0.43	-20 [§]
(U) ₈	40	0.69	4
(dT) ₅	30	0.045	-10 [§]
(dT) ₆	30	0.075	6
(dT) ₈	38	0.220	25

* Poly(U) synthesis corrected for *de novo* synthesis.

[∇] The complexes between poly(dA) and oligo(U) are triple-stranded (see text), those between poly(dA) and oligo(dT) are double-stranded under our assay conditions (17).

[§] Determined by extrapolation.

(Fig. 7), none is found with oligo(dT)₅ (Fig. 9).

Finally, also in this system optimal stimulation is observed at temperatures far above the T_m of the corresponding polymer-oligomer complex (Table II).

Effect of temperature on poly(dT)-directed poly(A) synthesis

Fig. 10 shows that the overall rate of poly(dT)-directed poly(A) synthesis increases at higher temperature. When reactions started at 30°C are shifted to 50°C, a marked increase in the amount of AMP incorporated is observed. The same is seen if the initiation inhibitor rifampicin (26) is added just before the temperature shift. This indicates that declining poly(A) synthesis at higher temperatures, as measured with primers (A)₂ and (A)₃ in Fig. 2, cannot be due to increased termination of the polymerization reaction.

Specificity and mis-incorporation

We have shown previously that the primer dependence of transcription at high incubation temperature is not merely due to sta-

Fig. 10. Effect of temperature on poly(dT)-directed poly(A) synthesis. Reaction mixtures were as described in Methods and contained 50 μ M ATP. Reactions were started at 30°C by adding enzyme. After 10 min (arrow) rifampicin was added (2×10^{-5} M) as indicated and reactions were either further incubated at 30°C or shifted to 50°C. In the control reaction 1.8 nmoles (A)₅ was present and rifampicin was added before the enzyme. Incorporation was measured by taking samples from the reaction mixtures at the times indicated. ● - ●, Plus 50°C shift, minus rifampicin; ■ - ■, plus 50°C shift, plus rifampicin; ▲ - ▲, minus temperature shift, minus rifampicin; control; X - X, plus 50°C shift, plus rifampicin.

bilization of the polymerase against heat inactivation (2). Moreover, synthesis at 50°C is completely DNA-dependent and only complementary primers can stimulate the incorporation of complementary ribonucleotides (Van Kreijl, C.F., unpublished). The fidelity of transcription at 50°C is checked in Fig. 11 by assaying the incorporation of complementary and non-complementary ribonucleoside triphosphates in the poly(dT).oligo(A)₅ system. Poly(A) synthesis, as already shown in Fig. 2, is rapid under these conditions and reaches a plateau because the ATP is limiting. No misincorporation of either GTP or CTP could be detected during a period of 60 min. From the specific activities used we calculate that less than 1 GMP and 0.2 CMP residues are incorporated per 1000 AMP molecules. The incorporation of UTP observed was shown to be directed by the rapidly synthesized poly(dT).poly(A) hybrid which can act as template for *de novo* poly(U) synthesis, even at

Fig. 11. Incorporation of complementary and non-complementary ribonucleoside triphosphates during poly(dT)-directed poly(A) synthesis at 50°C. Reaction mixtures were as described in Methods and contained 50 μM of either ^3H -labelled or unlabelled ATP and 1.8 nmoles of the oligomer (A)₅. Incorporation was measured by taking samples at the times indicated. ▲ - ▲, $^3\text{H-ATP} + 100 \mu\text{M GTP}$; □ - □, ATP + 100 $\mu\text{M } ^3\text{H-GTP}$; X - X, ATP + 50 $\mu\text{M } ^3\text{H-CTP}$; ● - ●, ATP + 100 $\mu\text{M } ^3\text{H-UTP}$.

50°C (Van Kreijl, C.F., unpublished). Therefore, mis-incorporation of UTP - although unlikely - could not be excluded in this experiment.

Protection against rifampicin by oligonucleotide primers

An alternative tool to render transcription primer-dependent is the use of rifampicin, a drug known to inhibit specifically chain initiation but not chain elongation (9,26). Using poly(dT).-oligo(A)₅ as model system, we have shown before that preincubation of enzyme with template and primer can effectively protect subsequent poly(A) synthesis against this drug (2). Fig. 12 shows comparable results for the poly(dA)-directed system, using either (U)₅ or (U)₁₂₋₁₅ as primer. Significant protection is found by preincubation with both primers at low temperature. This is emphasized by the fact that early in the reaction rifampicin-insensitive poly(U) synthesis clearly exceeds the *de novo* reaction (-prim, -rif). Stable annealing of primer with poly(dA) only occurs with (U)₁₂₋₁₅ under the preincubation conditions used (Table I). This does not seem to increase, however, the amount of rifampicin-protected poly(U) synthesis.

Fig. 12. Protection of poly(dA)-directed poly(U) synthesis against rifampicin by oligonucleotide primers. a) With (U)₅ at 35°C; b) with (U)₁₂₋₁₅ at 40°C. Reaction mixtures were as described in Methods and contained 50 μM UTP. Stoichiometric amounts of primer were added as indicated. Reaction mixtures without rifampicin and UTP were preincubated at 0°C for 30 min. After addition of rifampicin (2×10^{-5} M), as indicated, all reactions were started by adding UTP and transferring the tubes to either 35°C or 40°C. Incorporation was measured by taking samples at the times indicated. Δ - Δ , No primer, no rifampicin; $\blacksquare$ - $\blacksquare$, with primer and rifampicin; $\bullet$ - $\bullet$, with primer, no rifampicin; X - X, no primer, with rifampicin.

DISCUSSION

The transcription reaction catalysed by *E. coli* RNA polymerase is a complicated process. It can be divided into at least four discrete steps, each in itself complex (27). Each step contributes to the overall rate of RNA synthesis, but responds to variation of conditions in a different way. Attempts to develop assays for the detailed and separate studies of these steps have only recently begun (28-31). The complex reaction kinetics observed for RNA synthesis can in part be explained by the fact that initiation of synthesis is a second-order reaction with respect to substrate concentration, whereas chain elongation is a first-order reaction (10). Hence, at low substrate concentration chain initiation becomes rate-limiting and this limitation can be overcome by adding an appropriate primer oligonucleotide.

The inhibition of the *de novo* RNA synthesis at higher temperatures is clearly an effect on chain initiation rather than on chain elongation. This follows from Fig. 10 and from the marked stimulation of RNA synthesis by oligonucleotide primers at higher

temperatures. Analogous results were observed with single-stranded natural DNA as template (accompanying paper). Moreover, with other homopolymer systems the optimal temperature for RNA synthesis has also been found to shift to higher temperatures when either the rNTP concentration is raised (2,5,32) or when primers are added at low substrate concentration (4,5,21).

For the purpose of this discussion we simplify the process of primed chain initiation to two reactions:

P_n is the oligonucleotide primer containing n nucleotides, T is the template, E the enzyme and PP_i pyrophosphate. The effects of primer chain length, primer concentration and temperature can be simply rationalised from these reactions. At a given temperature an increase in primer concentration or primer chain length will drive reaction -1- to the right and will lead to an increase in RNA synthesis until a maximal rate is reached which is determined by the maximal rate of reaction -2-. An increase in temperature in a given template-primer system will lead to an increase in the rate of reaction -2- and further chain elongation, but it will also destabilize the ternary complex of enzyme, template and primer (reaction -1-) and, hence, lead to a decrease in the rate of primed chain initiation. These two opposing effects result in the bell-shaped curves shown in Figs 2, 5 and 8. The shape of the curve and the position of the maxima are dependent on the template-primer couple and the primer concentration.

The maximal rate of RNA synthesis at excess primer concentration is governed by reaction -2- and strongly dependent on the base composition of the primer used. In oligo(A)-primed poly(A) synthesis on poly(dT) template maximal rates are reached that are up to 50% of the known rate of chain elongation by E. coli RNA polymerase (30). The maximal rates in oligo(U)-primed poly(U) synthesis are about 100-fold lower. This large difference is not a difference in the affinity for the template, because under our conditions (enzyme/template ≤ 1) nearly all enzyme molecules will rapidly bind to either poly(dT) or poly(dA) (33). There are also

no indications that chain elongation in poly(U) synthesis is much slower than in poly(A) synthesis. We, therefore, conclude that the rate at which the first nucleotide is added to the primer (reaction -2-) is much lower in oligo(U)-primed synthesis than in oligo(A)-primed synthesis. Apparently the strong preference of RNA polymerase for purines in initiation also extends to oligonucleotide primers. Oligo(dT) is even less effective as primer than oligo(U) and again the most plausible interpretation is that reaction -2- is rate-limiting.

In the model systems used by us, stimulation by equimolar amounts of primer is optimal at temperatures far above the melting points of the polymer-oligomer complexes. Stable annealing of template and primer, therefore, is neither a prerequisite nor does it increase primer-dependent transcription (Tables I and II). In this respect oligonucleotide-primed RNA synthesis with E. coli RNA polymerase is markedly different from oligonucleotide-primed DNA synthesis (34). Apparently, RNA polymerase is able to stabilize the template-primer complex. Our results suggest that stabilization is related to the efficiency of reaction -2-, because the difference between the optimal priming temperature at a primer/template ratio = 1 and the T_m of the complex decreases in the order poly(dT).oligo(A)_n, poly(dA).oligo(U)_n, poly(dA).oligo(dT)_n. The earlier observations of optimal stimulation near the melting temperature (11,21) may be fortuitous and due to the use of low primer concentrations.

In a given template-primer system, as indicated by our results, transcription can be made predominantly primer-dependent by raising temperature and primer concentration when low substrate concentrations are used. Thus, RNA synthesis under these conditions can be directed to specific parts of the template by choosing a proper primer. To our knowledge, no extensive studies on the effect of high temperature on the E. coli RNA polymerase have been presented so far. Indirect evidence indicates that the core enzyme is relatively stable against heat inactivation when bound to denatured DNA (35). We have shown in this paper that primed transcription is not only specific but proceeds at 50°C with high fidelity. The very low percentages of mis-incorporation of GTP and CTP are in good agreement with recent fidelity studies (36). Moreover, in

the accompanying paper we report the synthesis of specific primer-initiated RNA copies on single-stranded natural DNA, which were subsequently used for sequence analysis.

Finally, we have shown that in the poly(dA)-directed system also rifampicin can be used to restrict transcription to the elongation of primers. However, stable annealing of primer and template under the preincubation conditions used did not result in more rifampicin-protected poly(U) synthesis than without stable annealing. Compared with the primed reaction in the absence of inhibitor, rifampicin-protected synthesis was very low with both primers even after short times of synthesis. This suggests that the polymerase molecules do not preferentially form rifampicin-insensitive ternary E-T-P_n complexes upon preincubation. The same holds for the rifampicin-protected poly(A) synthesis observed before (2). Net synthesis of poly(A), however, is higher in this case because of the slippage phenomenon. Rifampicin, therefore, turns out to be much less efficient in obtaining primer-dependent transcription than raising temperature and primer concentration.

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