Studies on Transcription of 3'-Extended DNA Templates by Mammalian RNA Polymerase II. Partial Purification and Characterization of a Factor from HeLa Cells That Facilitates Renaturation of the DNA Template†

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ABSTRACT: Transcription by purified mammalian RNA polymerase II in vitro leads to extensive formation of DNA-RNA hybrids between nascent RNA and the template DNA strand. This is especially clear during transcription of 3'-extended (dC-tailed) DNA templates where the nontranscribed DNA strand is progressively displaced as transcription proceeds [Kadesch, T. R., & Chamberlin, M. J. (1982) *J. Biol. Chem. 257*, 5286-5295]. Addition of small amounts of a HeLa cell extract to such a transcription system enhances renaturation of the template DNA and displacement of the nascent RNA, as measured by the sensitivity of the RNA to pancreatic ribonuclease. Using this latter assay, we have purified a protein factor (renaturase) 250-fold from HeLa cell extracts using chromatography on DEAE-cellulose, DNA-cellulose, and hydroxylapatite. Renaturase preparations facilitate complete renaturation of the template DNA duplex during transcription by RNA polymerase II and lead to concurrent displacement of the nascent RNA. Current preparations are free from all but traces of deoxyribonuclease or ribonuclease. The active component has a molecular weight of about 30 000 as estimated by preparative density gradient sedimentation. We have examined the structure of transcribing RNA polymerase II complexes in the presence and absence of renaturase, using the electron microscope and the Williams polylysine technique [Williams, R. C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2311-2315]. In the presence of renaturase, the DNA template is fully renatured, and a ternary complex in which the nascent RNA is displaced during transcription is seen. A number of nucleic acid binding proteins have been tested for renaturase activity and are inactive, including *Escherichia coli* single-stranded binding protein, ρ factor, *recA* protein, HU protein, T4 phage gene 32 protein, histone H2A and high mobility group proteins, 15S and 30S heterogeneous nuclear ribonucleoprotein particles, avian myeloblastosis virus reverse transcriptase, and *Xenopus* topoisomerase I.

Mammalian RNA polymerase II is a complex enzyme which contains 10-12 distinct polypeptide chains as it is usually purified (Roeder, 1976). These purified preparations are unable to initiate chains efficiently at eukaryotic promoter sites, unless additional factors are present that do not normally copurify with the polymerase itself (Matsui et al., 1980; Samuels et al., 1982; Davison et al., 1983; Dynan & Tjian, 1983; Parker & Topol, 1984). Transcription elongation by purified mammalian RNA polymerase II alone is also aberrant and leads to extensive formation of DNA-RNA hybrids between the nascent RNA and the DNA template strand with both linear (Sekeris et al., 1972; Lavialle et al., 1982; Kadesch & Chamberlin, 1982) and circular (Lescure et al., 1978) templates. This hybrid formation is most clearly seen during transcription of linear DNA templates which have been extended at the 3' end of the DNA chains by addition of short poly(dC) tails (Kadesch & Chamberlin, 1982). Such tailed templates allow rapid and efficient initiation of transcription by purified mammalian RNA polymerase II at nearly any DNA sequence and should be of considerable utility in the study of RNA chain elongation and termination by polymerase II.

Using such tailed DNA templates, we have shown that the failure of purified mammalian RNA polymerase II preparations to displace the nascent RNA during transcription is a feature of the polymerase protein itself and is not simply determined by the choice of reaction conditions. RNA polymerase II preparations from wheat germ and *Escherichia coli* core RNA polymerase both displace their nascent transcripts efficiently when tailed DNA templates are transcribed, while RNA polymerases II from calf thymus, HeLa cells, and *Drosophila* produce hybrid products with the same templates (Dedrick & Chamberlin, 1985).

RNA polymerases form a short region of transient RNA-DNA hybrid during transcription. It is known that the ability to displace the nascent RNA transcript from this hybrid is probably determined by a specific "renaturase" site on the polymerase molecule, at least for the *E. coli* enzyme (Richardson, 1975; Chamberlin, 1976). In addition, we found that transcription in HeLa extracts as well as early fractions in the calf thymus polymerase preparation [F_2; see Dedrick & Chamberlin (1985)] produced predominantly free RNA. This result suggested either that the purified polymerase II fractions which are unable to displace the nascent transcripts had been damaged in some way to destroy the renaturase function or that some essential factor had been separated from the polymerase during purification. The possibility that the renaturase function had been damaged during purification seemed the less likely of the two, since the purification procedure employed for the calf thymus, wheat germ, and *Drosophila* enzymes was quite different from that used for human polymerase (Dedrick & Chamberlin, 1985). Also, there was no change in the amount of hybrid product formed by the calf thymus RNA polymerase fractions through the last three steps in the purification. These results led us to test whether cell extracts might contain an activity that could restore the ability of purified calf thymus RNA polymerase II to displace its

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nascent RNA from the template. We report here the identification and purification of such a factor from HeLa cell extracts.

Materials and Methods

Materials. DEAE-cellulose (DE52) was obtained from Whatman (Clifton, NJ), and Bio-Gel HTP was obtained from Bio-Rad (Richmond, CA). Column materials were prepared according to the manufacturer's instructions. DNA–cellulose was prepared according to Alberts & Herrick (1971) with calf thymus DNA. Single-stranded DNA–agarose was prepared according to Schaller et al. (1972). [γ-^{32}P]ATP was synthesized by the method of Johnson & Walseth (1979), and [α-^{32}P]CTP was synthesized by the method of Symons (1977); ^{32}P-labeled inorganic phosphate was purchased from New England Nuclear (Boston, MA). The specific activity of [α-^{32}P]CTP used in transcription reactions was 400–1000 cpm/pmol. Unlabeled nucleoside triphosphates were obtained from P-L Biochemicals (Milwaukee, WI).

Enzymes and Other Proteins. Calf thymus RNA polymerase II was purified by a modification of the procedure of Hodo & Blatti (1977). *Pvu*II restriction endonuclease was obtained from BRL (Gaithersburg, MD), and nuclease digestion conditions were those recommended by the manufacturer. Purified terminal deoxynucleotidyl transferase (Chang & Bollum, 1971) was the kind gift of Dr. Robert Ratliff (Los Alamos, NM) and Dr. Robert Wells (University of Alabama). Pancreatic ribonuclease A (RNase A)[1] was obtained from Worthington (Freehold, NJ). T4 gene 32 protein was the kind gift of Dr. Bruce Alberts, University of California at San Francisco. *E. coli* single-stranded binding protein (ssb), purified as described by Weiner et al. (1975), was the gift of A. Kornberg, Stanford University. *E. coli* ρ protein, purified according to Morgan et al. (1983), was the gift of P. H. von Hippel, University of Oregon. *E. coli recA* protein, purified as described by Weinstock et al. (1981), was the gift of I. R. Lehman, Stanford University, *E. coli* HU protein, purified by a modification of the procedure of Rouviêre-Yaniv & Gros (1975), was supplied by M. Schmidt, University of California, Berkeley. AMV reverse transcriptase was from Life Sciences, Inc. *Xenopus* topisomerase I, purified according to Breaux, Stowers, and Benbow (personal communication), was the gift of R. M. Benbow, Johns Hopkins University. Histone H2A was from Worthington Biochemicals. HMG 14 and 17 proteins were purified from chicken erythrocytes as described by Weisbrod & Weintraub (1981). 15S and 30S heterogeneous nuclear ribonucleoprotein particles, prepared according to Martin et al. (1973), were the gift of T. Martin, The University of Chicago.

DNA Templates. Plasmid DNA was isolated by using the method of Birnboim & Doly (1979) with minor modifications. Tailed template (pCpBR DNA) was prepared as described before (Dedrick & Chamberlin, 1985), using *Pvu*II-cleaved pBR322 DNA. All nucleic acid concentrations are expressed as nucleotide residues unless otherwise indicated.

Cells and Media. HeLa S3 cells were grown by suspension in F13 media (Gibco) supplemented with 10% calf serum (Microbiological Associates, Walkersville, MD). Cells were harvested at a density of (3–5) $\times$ 10^5 cells/mL.

Assays for RNA Polymerase and Renaturase. RNA polymerase II and the RNase A sensitivity of its products were assayed essentially as described previously (Dedrick & Chamberlin, 1985). Transcription reactions (50 μL) contained 70 mM Tris-HCl, pH 8.0, 50 mM $(NH_4)_2SO_4$, 20% glycerol, 6 mM $MgCl_2$, 5 mM spermidine, 1 mM dithiothreitol, 800 μM each of ATP, UTP, and GTP, 100 μM [α-^{32}P]CTP, and 0.5 μg of pCpBR DNA together with 1 microunit of purified calf thymus RNA polymerase II (as measured by using the pCpBR DNA template) (Dedrick & Chamberlin, 1985) and renaturase fractions where appropriate. Reactions were normally carried out for 10 min at 37 °C.

For renaturase assays, the fraction to be assayed is added to the reaction solution including RNA polymerase on ice, and the reaction is started by transfer to a 37 °C bath. After 10 min at 37 °C, the reaction is stopped by the addition of 0.5 mL of 10 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 0.1 M NaCl. Then, 200-μL samples are dispensed to two tubes. Tube a is brought to 13 μg/mL RNase A; tube b represents total transcript produced. Both tubes are incubated an additional 20 min at 37 °C. Acid-insoluble nucleic acids are recovered on GF/C filters following Cl_3CCOOH precipitation (Chamberlin et al., 1979). The percent total transcript sensitive to RNase A is calculated as 100 (1 – pmol of tube a/pmol of tube b). One unit of activity is defined as that amount of renaturase which converts 40 pmol of transcript to an RNase A sensitive form in 10 min at 37 °C in the presence of 1 microunit of calf thymus RNA polymerase II.

Gel Electrophoresis. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and visualized by staining with Coomassie Brilliant Blue R250 (Ames, 1974) or silver (Wray et al., 1981; Morrissey, 1981).

^{32}P-Labeled ribonucleic acid transcripts were resolved on 1% agarose gels following glyoxal treatment (McMaster & Carmichael, 1977) and were visualized by autoradiography at –80 °C with Kodak XAR film and Cronex Lightning Plus intensifying screens.

Glycerol Gradient Sedimentation. Glycerol gradients (15–30%) were prepared in 10 mM Tris-HCl, pH 7.9, 0.1 M $(NH_4)_2SO_4$, 1 mM EDTA, and 1 mM DTT. Samples in 10% glycerol were layered on top of the gradient and were sedimented at 45 000 rpm in an SW50.1 rotor for 16–18 h at 4 °C. Fractions were collected from the bottom of the tube, and mobilities of active renaturase fractions were compared to marker proteins sedimented in a separate gradient.

Protein Determinations. Protein concentration was determined according to Lowry et al. (1951) or Bradford (1976) with bovine serum albumin as a standard.

Electron Microscopy. Transcription reactions with and without renaturase were stopped by the addition of EDTA to 20 mM and were prepared for electron microscopy in one of two ways. (1) Double-stranded DNA was distinguished from single-stranded DNA by using formamide–cytochrome *c* spreads as described by Kadesch & Chamberlin (1982). (2) Protein–nucleic acid interactions were visualized by using the polylysine spreading technique described by Williams (1977). Single-stranded nucleic acid was distinguished from double-stranded nucleic acid on polylysine spreads in the following two ways: (a) by direct visualization; (b) by incubating re-

[1] Abbreviations: AMV, avian myeloblastosis virus; RNase A, ribonuclease A; SSC, standard saline citrate (0.15 M NaCl and 0.015 M sodium citrate); PMSF, phenylmethanesulfonyl fluoride; pCT7, T7 duplex DNA with poly(dCMP) extending from the 3′ ends (Kadesch & Chamberlin, 1982); DTT, dithiothreitol; pCpBEU50/*Hpa*I DNA, pBEU50 plasmid DNA cleaved with *Hpa*I restriction endonuclease with poly(dCMP) extending from the 3′ ends (Dedrick & Chamberlin, 1985); pCpBR/*Aha*III DNA, pBR322 plasmid DNA cleaved by *Aha*III restriction endonuclease with poly(dCMP) extending from 3′ ends (Dedrick & Chamberlin, 1985); HMG, high mobility group; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.

actions (prior to spreading them) with 4 μg/mL T4 gene 32 protein at 37 °C for 5 min to coat single-stranded nucleic acid.

Purification of Renaturase. All procedures were carried out at 4 °C unless otherwise described.

(*A*) *Preparation of Cell Extract.* Thirty-two liters of HeLa cells was harvested and washed 3 times with 1 × SSC buffer containing 10 mM Tris-HCl, pH 7.5, and 1 mg/mL $MgCl_2$. The washed cell pellet was suspended by vortexing in 160 mL of extraction buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, and 0.25 M sucrose brought to 1 mM PMSF immediately before mixing with cells). The suspension was homogenized in a Dounce homogenizer with five strokes of the B pestle. Nuclei were removed by low-speed centrifugation. The supernatant fluid was kept on ice while the nuclei were reextracted in the same way with 60 mL of extraction buffer. The supernatant fluids were combined and were centrifuged at 30000 rpm for 45 min in a type 30 rotor. The supernatant fluid was recovered as fraction I.

(*B*) *Ammonium Sulfate Precipitation.* Fraction I was brought to 35% saturation in ammonium sulfate by the addition of 19.4 g of solid ammonium sulfate per 100 mL of solution with constant stirring at 4 °C. The solution was kept neutral by the addition of small amounts of 1 N NaOH during salt addition and dissolution. After the solution was stirred for 30 min, the precipitate was removed by sedimentation at 9000 rpm for 20 min in a Sorvall GSA rotor. The supernatant fluid was adjusted to 65% saturation by adding 18.4 g of ammonium sulfate per 100 mL of 35% $(NH_4)_2SO_4$ saturated solution as described above. The precipitate was collected by centrifugation as above and was suspended in 30 mL of TED (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT) containing 0.2 M NaCl. The solution was then dialyzed against several changes of the same buffer overnight. The dialysate was centrifuged to remove a slight precipitate, and the supernatant fluid was designated fraction II.

(*C*) *DEAE-cellulose.* Fraction II (41 mL) was loaded onto a 100-mL DEAE-cellulose column which had been equilibrated in TED–0.2 M NaCl. The column was washed with the same buffer until no protein was detected in the effluent. Those pass-through fractions that contained renaturase activity were pooled and were dialyzed rapidly (Englander & Crowe, 1965) against TED, 10 mM NaCl, and 10% glycerol for 6–7 h with one buffer change. The dialysate (fraction III) was stored at 4 °C overnight.

(*D*) *DNA–Cellulose.* Fraction III (86 mL) was loaded onto a 20-mL DNA–cellulose column which had been equilibrated in TED, 10 mM NaCl, and 10% glycerol, and the column was washed with the same buffer until no protein was detected in the effluent. The column was washed sequentially with solutions of TED plus 10% glycerol, containing 0.2 M NaCl, 0.5 M NaCl, and lastly 1 M NaCl. Renaturase activity was recovered in the column pass-through and the 0.5 M NaCl wash. The activity in the pass-through was completely recovered in the pass-through of a second DNA–cellulose column. This activity was 5-fold lower in specific activity than fraction III, constitutes only 10–30% of the total units recovered from this column, and has not been studied further. Also, mixing the various column fractions does not reconstitute any renaturase activity above that expected for an additive effect. The active fractions recovered in the 0.5 M NaCl wash were pooled (fraction IV).

(*E*) *Bio-Gel HTP.* Fraction IV was dialyzed rapidly (Englander & Crowe, 1965) against a solution containing 25 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% glycerol for 2 h with one buffer change. The dialysate was loaded onto a 2-mL Bio-Gel HTP column equilibrated in the same buffer. The column was washed with the same buffer followed by sequential washes with the same buffer containing 0.1 M potassium phosphate and then 0.5 M potassium phosphate. Active fractions recovered in the 0.1 M potassium phosphate wash were pooled, dialyzed against storage buffer [TED, 0.1 M $(NH_4)_2SO_4$, and 50% glycerol], and stored at −20 °C (fraction V). Fraction V was stable under these conditions at least 4 months.

RESULTS

Identification and Purification of a Factor That Enhances the RNase A Sensitivity of Nascent RNA Polymerase II Transcripts. Transcription of a dC-tailed template such as pCT7 or pCpBR DNA with purified calf thymus RNA polymerase II leads to progressive displacement of the nontranscribed DNA strand and formation of a DNA–RNA hybrid between the nascent RNA and the transcribed DNA strand (Kadesch & Chamberlin, 1982). Studies of the biochemical properties of this reaction, and of the effect of the enzyme source on RNA strand displacement (Dedrick & Chamberlin, 1985), led us to the hypothesis that RNA strand displacement by mammalian RNA polymerase II might depend on a factor or factors, present in complete cell extracts, that are easily separated from the polymerase during purification.

To detect and to fractionate such factors, we have made use of a biochemical assay for RNA strand displacement based on the relative resistance of DNA–RNA hybrids to cleavage by pancreatic RNase A. During normal transcription of pCpBR DNA by purified calf thymus RNA polymerase II, over 60% of the RNA product is resistant to subsequent cleavage by RNase A (Dedrick & Chamberlin, 1985). A factor which leads to displacement of the nascent RNA and renaturation of the DNA template duplex would render all of the RNA product RNase A sensitive. This assay is rapid and semiquantitative and has been useful in studying the effect of enzyme and template structure on the DNA strand displacement reaction (Dedrick & Chamberlin, 1985). Hence, it is an acceptable assay for use in protein purification. However, the method is not very specific, and we describe controls which are needed to ensure that any activity detected that enhances RNase A sensitivity is not simply due to factors—such as certain ribonucleases or deoxyribonucleases—that might be unrelated to normal transcription.

We first obtained evidence for the enhancement of RNA strand displacement in DNA-free HeLa cell extracts (C. Kane, unpublished results). Although we have subsequently observed enhanced RNA strand displacement in calf thymus extracts (Dedrick & Chamberlin, 1985), these fractions contain substantial levels of inhibitors of transcription as well as RNA binding proteins which made assay for strand displacement difficult. Accordingly, we have continued to use HeLa cell extracts and found substantial levels of an activity (renaturase) that renders RNA polymerase II transcripts RNase A sensitive when renaturase is present during transcription. The extent of RNase A sensitivity is proportional to the amount of extract added (see below), from which it is possible to define a unit of activity. The activity is not simply due to the presence of a ribonuclease which can cleave the nascent transcript since the amounts of extract needed to obtain 100% RNase sensitivity of the transcript do not significantly reduce the yield of RNA formed.

This result led us to fractionate these extracts to purify the factor involved, which we have designated as renaturase. The renaturation activity remains in the supernatant fluid after

Table I: Purification of Renaturase[a]

fraction	units/mL	protein (mg/mL)	sp act. (units/mg)	x-fold purification	% recovery
(I) high-speed, supernatant fluid	500	7.3	68	1	
(II) ammonium sulfate	4000	13.7	292	4.3	100
(III) DEAE-cellulose	1667	4.3	388	5.7	90
(IV) DNA–cellulose	1250	0.32	3906	57	9
(V) hydroxylapatite	1667	0.1	16670	245	3

[a] The results of a purification from 32 L of HeLa cells are presented. Details are described under Materials and Methods.

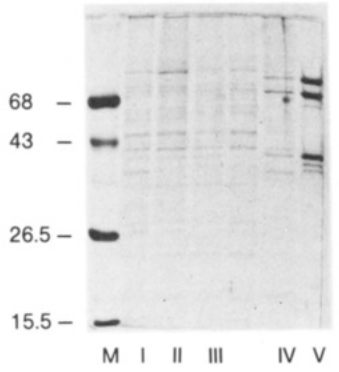

FIGURE 1: SDS–polyacrylamide gel of renaturase purification. One microgram of each indicated fraction from the renaturase purification was electrophoresed onto a 12.5% SDS–polyacrylamide gel and stained with Coomassie blue as described under Materials and Methods. Molecular weights of markers ($\times 10^{-3}$) are indicated in lane M.

nuclei have been removed by sedimentation. It has been purified by ammonium sulfate fractionation and passage through DEAE to remove nucleic acids, followed by chromatography on DNA–cellulose and then hydroxylapatite. The overall purification is about 250-fold with 3% overall recovery (Table I). However, the assay we have used is not necessarily quatitative especially in early steps in the fractionation, and hence, these are only approximate values.

SDS–polyacrylamide gel analysis of the hydroxylapatite fraction (fraction V) shows three major polypeptide components (Figure 1). At least two of these are likely to be contaminants since they are much larger than the maximum size estimated for renaturase (see below). However, we have not yet been able to obtain a further purification of renaturase activity due to substantial losses in activity when additional fractionation methods are tried. We suspect that this is due to the very low protein concentration of fraction V and to the possible tendency of the activity to stick to glass. It should be possible to solve these problems once we can scale the preparations up to 200 L of HeLa cells.

Glycerol gradient sedimentation of fraction V leads to limited recovery of activity and protein, probably due to loss of the protein on tube surfaces. However, the sedimentation coefficient was estimated by comparison with bovine serum albumin, ovalbumin, and egg white lysozyme and gave a value of 2.8 S. This corresponds to a molecular weight of about 32 000 for a native, globular protein (Martin & Ames, 1961).

Renaturase activity is lost when fractions are heated, as expected if a protein is involved. To test for optimal conditions for storage and handling of renaturase, samples of fraction IV were heated to 47 °C for 10 min in a variety of different solutions and then assayed for RNA displacement activity by the standard renaturase assay. In a solution containing 10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM NaCl, and 0.1 mM DTT, 50% of the activity is lost in 10 min. Components which stabilized the activity included salt (NH_4^+, K^+, or Na^+ and Cl^-, SO_4^{2-}, or PO_4^{2-}) at ionic strength over 0.1, 1–10 mM mercaptan (DTT or β-mercaptoethanol), and 50% glycerol. Components which did not stabilize included nonionic detergents (0.1–10% of NP40 or Triton X-100), BSA (up to 2 mg/mL), or ethylene glycol (to 50%). None of these components had any influence on the RNase A sensitivity of the RNA polymerase II transcript produced in the absence of renaturase. Optimal stability was obtained in a solution (storage solution) containing 0.1 M $(NH_4)_2SO_4$, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 50% glycerol. In this solution, about 80% of the renaturase remains active after 25 min at 47 °C.

Table II: Purified Proteins and Complexes That Do Not Displace the RNA Transcript[a]

E. coli ssb
E. coli ρ
E. coli recA
E. coli HU
T4 gene 32
AMV reverse transcriptase
Xenopus topoisomerase I
histone H2A
HMG 14 and 17
30S heterogeneous nuclear ribonucleoprotein particles
15S heterogeneous nuclear ribonucleoprotein particles

[a] Samples of the indicated proteins were added instead of renaturase fraction V to the renaturase assay described under Materials and Methods. All proteins were present in at least a 7-fold excess by protein weight (0.7–15 μg, depending on the protein) compared to fraction V (0.1 μg) in a parallel reaction.

Fraction V renaturase was assayed for several different enzymatic activities that might contaminate or be associated with the factor. Two units (~100 ng of protein) of the renaturase preparation had no detectable ATPase (less than 2 pmol cleaved in 10 min), topoisomerase (no alteration in topoisomer mobility of 200 ng of supercoiled SV40 DNA in 10 min), or DNA exonuclease activity (no solubilization of ^3H from 3 nmol of *E. coli* DNA, 10 000 cpm/nmol, in 10 min). Only traces of ribonuclease activity are seen; no release is detected of acid-solutble ^{32}P from 300 pmol of in vitro RNA (34 cpm/pmol), and no appreciable cleavage of long RNA chains occurs as judged by analytical gel electrophoresis (see below). Traces of DNA endonuclease activity are also detected.

Many proteins and protein complexes interact with nucleic acids in ways that potentially could affect RNA displacement and renature the template. We have tested several such proteins for their ability to replace renaturase in a transcription reaction with purified RNA polymerase II. The tested components are listed in Table II; none of them was capable of rendering the transcript RNase A sensitive.

Characterization of Purified HeLa Renaturase. (*i*) *Effect of Renaturase, DNA, and RNA Polymerase II Concentrations.* Using the RNase A sensitivity assay, we found that the amount of free as opposed to hybrid RNA transcribed by calf thymus RNA polymerase II is linearly proportional to the concentration of fraction V renaturase until 100% of the RNA becomes RNase A sensitive (Figure 2). The amount of renaturase required at saturation (60 ng of protein in Figure 2) is roughly proportional to the amount of RNA polymerase II in the reaction across at least a 10-fold range of polymerase

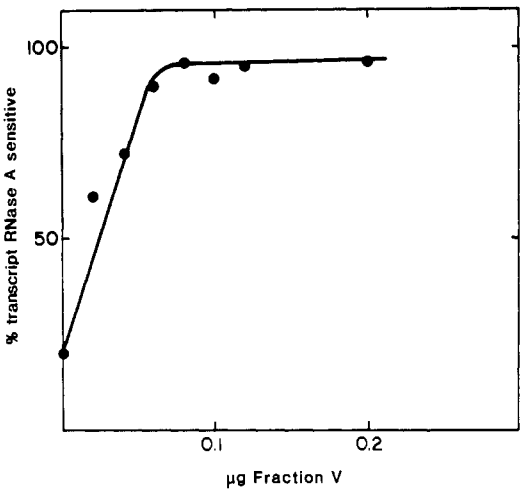

FIGURE 2: Proportionality of transcript displacement with amount of renaturase. Renaturase assays (50 μL) were run as described under Materials and Methods with 1 microunit of RNA polymerase II (50 ng) and the indicated amounts of renaturase fraction V.

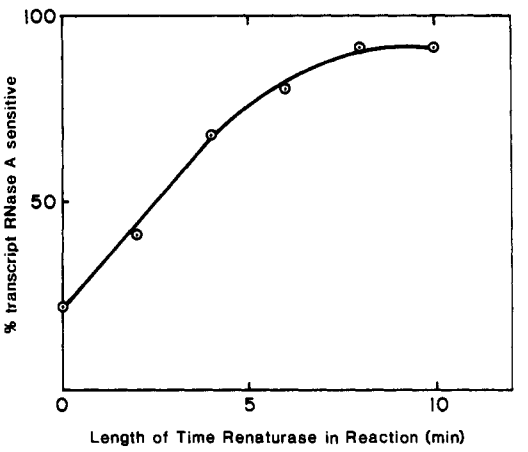

FIGURE 3: Extent of transcript displacement depends on time renaturase is present in reaction. Renaturase assays (50 μL) were as described under Materials and Methods except that renaturase fraction V (0.1 μg) was added to separate tubes either at the start (10 min) or at various times after starting the transcription reaction. These values were plotted along with results of a reaction where renaturase fraction V was not present (0 min).

concentration. This result could reflect a requirement for binding of renaturase to the polymerase or a proportionality between the amount of RNA product formed and the amount of renaturase required.

The amount of excess DNA template added to the reaction has little effect on the amount of renaturase needed to reach 100% RNase A sensitivity. Thus, transcription of 0.9 and 1.9 μg of pCpBR DNA (which gave the same picomoles of transcript when transcribed by a fixed amount of RNA polymerase II) required identical saturation concentrations of fraction V renaturase. Furthermore, addition of excess (20 μg) duplex calf thymus DNA lacking dC tails to a renaturase reaction had no inhibitory effect on renaturase action (data not shown). This result is interesting since renaturase activity binds to and elutes from duplex DNA–cellulose. This duplex DNA binding is apparently not sufficient to interfere with its action during transcription.

(*ii*) *Effect of Time of Addition on Renaturase Action.* Renaturase functions in transcription reactions even when added after the reaction has started. Initiation of transcription by polymerase II is complete after about 1 min with dC-tailed DNA templates (Kadesch & Chamberlin, 1981) while the amount of free RNA formed in a 10-min reaction increases almost linearly with the length of time renaturase is present during transcription (Figure 3). When added late in the reaction, renaturase displaces more picomoles of transcripts than are synthesized during its time present in the reaction, and thus it may also be effective in displacing previously synthesized RNA.

(*iii*) *Renaturase Action Does Not Depend on the DNA Template.* Different DNA templates can give very different amounts of free RNA transcript as compared to a RNA–DNA hybrid with calf thymus RNA polymerase II (Dedrick & Chamberlin, 1985). This depends primarily on the nature of the sequences at the end of the duplex. Renaturase fraction V gives 100% RNase A sensitive RNA with all of the DNA templates we have tested, including pCpBR/*Pvu*II DNA, pCpBEU50/*Hpa*I DNA, and pCpBR/*Aha*III DNA as well as plasmids containing cloned adenovirus or human histone genes (M. Hanna, personal communication). When transcription of one template gives 4 times the free transcript RNA as transcription from a second template, then the second template requires 4 times the amount of renaturase to give 100% RNase A sensitive transcript. In addition, transcription of total calf thymus DNA by RNA polymerase II, which normally gives about 80% hybrid, is rendered over 70% sensitive to RNase A by renaturase fraction V (data not shown). Hence, there does not seem to be any evidence of template specificity nor a requirement for dC tails for renaturase action.

Renaturase Catalyzes Progressive Displacement of Intact RNA Chains from the Ternary Polymerase Complex. The initial purification and characterization of renaturase employed primarily the RNase A sensitivity assay. We were confident from appropriate control experiments that renaturase was not an RNase itself, simply able to cleave RNA from DNA–RNA hybrids, since it does not reproducibly inhibit incorporation of nucleotides into RNA. Similarly, renaturase is not a DNase able to release free RNA hybrids by cleavage of the DNA strand, since the template remains intact after transcription (see below). However, we have employed two additional methods that do not involve RNase A sensitivity to show that transcription by calf thymus RNA polymerase II in the presence of renaturase closely resembles normal transcription elongation, in which RNA chains grow processively and are displaced continuously from the DNA as elongation proceeds.

First, we have followed the growth of RNA chains by RNA polymerase II in the presence and absence of the factor using agarose gel electrophoresis (Figure 4). With pCpBR/*Pvu*II DNA as template, we see that RNA chains grow progressively for at least 10 min, reaching about 4300 nucleotides after 10 min, as expected from a mean elongation rate of 7 nucleotides/s (Kadesch & Chamberlin, 1982). This rate is not significantly altered by the presence of excess renaturase nor is the size of the RNA greatly changed, although there is a slight increase in the amount of the smaller species, as expected if traces of ribonuclease still are present in the preparation. Thus, RNA polymerase II continues to elongate large RNA chains in the presence of enough renaturase to render all of these chains sensitive to RNase A. RNA polymerase II processively elongates RNA chains even in the presence of renaturase. Therefore, these chains are being displaced constantly from the template strand during transcription. This is shown by the fact that elongation and rewinding both proceed normally in the presence of excess heparin (60 μg/mL) which quantitatively inhibits free RNA polymerase.

A second method of following transcription elongation involves use of the electron microscope to visualize transcription

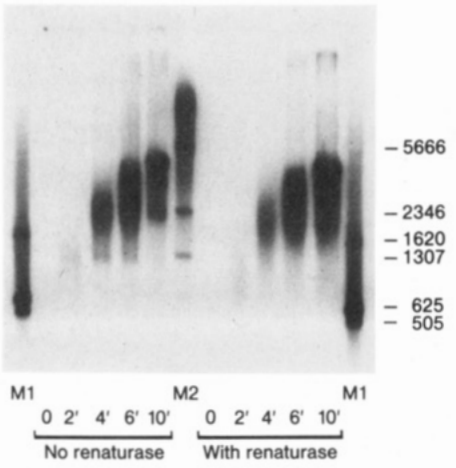

FIGURE 4: Transcription kinetics of calf thymus RNA polymerase II with and without renaturase. Transcription reactions (150 μL) containing 0.2 μg of RNA polymerase II were incubated at 37 °C with or without renaturase fraction V (0.3 μg). At the indicated times, 25-μL aliquots were withdrawn; the nucleic acids were purified, treated with glyoxal, and then resolved on 1% agarose gels as described under Materials and Methods. The nucleotide lengths of RNA markers (lanes M1 and M2) are as indicated.

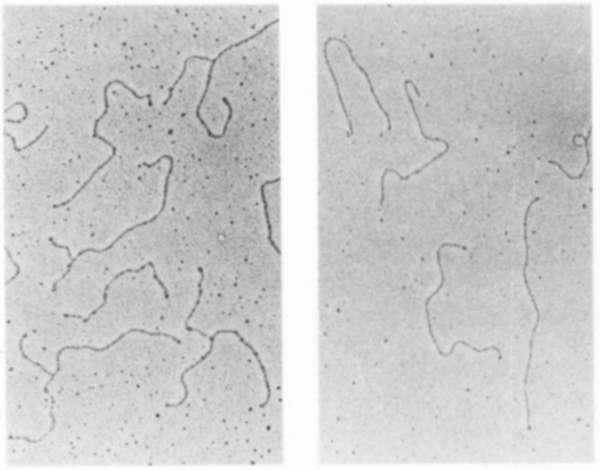

FIGURE 5: Electron micrographs of transcription reactions spread by using formamide–cytochrome *c*. Transcription reactions (50 μL) containing 0.3 μg of RNA polymerase II were incubated at 37 °C with or without renaturase fraction IV (0.2 μg). After 10 min, reactions were brought to 20 mM EDTA, diluted to 0.5 μg/mL DNA, and spread by the formamide–cytochrome *c* technique described under Materials and Methods. (a) No renaturase; (b) with renaturase.

complexes as elongation proceeds. This kind of procedure has been used to show that transcription elongation proceeds with progressive displacement of the nascent RNA strand in vitro and in vivo for both prokaryotic and eukaryotic RNA polymerases (Miller et al., 1970; Beyer et al., 1980; Delius et al., 1973; Kadesch & Chamberlin, 1982). When transcription complexes of calf thymus RNA polymerase II transcribing a dC-tailed template are visualized by using the formamide–cytochrome *c* method, characteristic forked structures are seen due to the displacement of the nontranscribed DNA strand from the end of the template during transcription (Kadesch & Chamberlin, 1982; see Figure 5a). In contrast, when transcription complexes acting in the presence of renaturase are visualized, no forked structures are evident. Instead, only full-length DNA molecules are seen (Figure 5b). Failure to see nascent RNA chains is not unexpected since these collapse in cytochrome *c* spreads. However, the observation that all template DNA molecules remain intact indicates clearly that

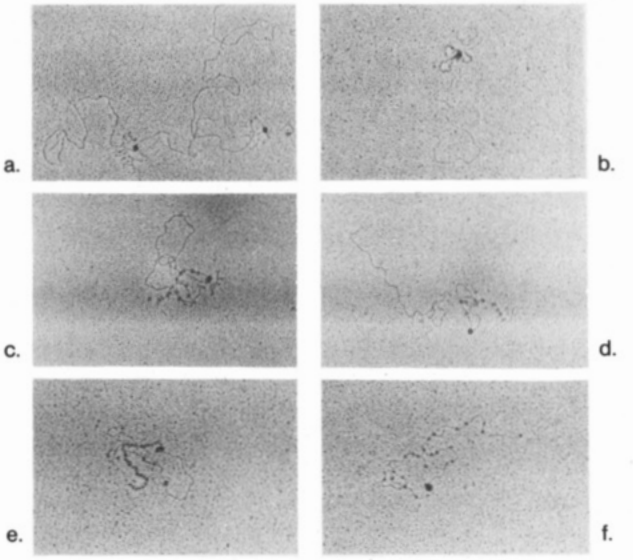

FIGURE 6: Electron micrographs of transcription reactions spread by using polylysine. Transcription reactions (50 μL) containing 100 ng of RNA polymerase II were incubated at 37 °C with or without renaturase fraction V (0.2 μg). After 10 min, reaction samples were brought to 20 mM EDTA and were further incubated for 15 min at 37 °C with or without 40 μg/mL RNase A. T4 gene 32 protein was added to 4 μg/mL where indicated, and all reactions were incubated for 5 min at 37 °C before being spread for electron microscopy by using the polylysine technique (Williams, 1977). (a) RNA polymerase II only; (b) RNA polymerase II and T4 gene 32 protein; (c–f) reactions also containing renaturase fraction V; (c) renaturase added; (d and f) reactions treated with RNase A; (e and f) reactions incubated with T4 gene 32 protein.

renaturation of the DNA template is taking place during transcription in the presence of renaturase. The procedure also serves as a sensitive assay for DNA endonucleases in the renaturase preparation.

To determine directly whether or not the displaced transcript extends from the polymerase in an "authentic" ternary complex and to distinguish templates involved in transcription from those which are not, we spread the same reactions using the polylysine technique. This procedure allows direct visualization of the RNA polymerase molecule on its template and of single-stranded RNA as well. We could identify template molecules involved in transcription by counting only those that have an internal RNA polymerase molecule attached from which single-stranded nucleic acid extended. (Since RNA polymerase II can also bind nonproductively to the ends of template molecules, templates with polymerase bound at the ends were not included in the evaluation as we were unable to distinguish nonproductive from productive end binding.) The single-stranded nucleic acid was visualized in two ways. When spread with the polylysine technique, single-stranded DNA and RNA collapse into a bushlike structure (Figure 6a). Alternatively, if the stopped transcription reactions were incubated with T4 gene 32 protein prior to spreading of samples, the single-stranded nucleic acid was visualized as a protein-coated extension (Figure 6b). To determine whether the extended, single-stranded nucleic acid was RNA or DNA, we treated examples of the stopped transcription reactions with RNase A prior to spreading for electron microscopy.

The results of this electron microscopy are presented in Figure 6 and Table III. Micrographs of the reactions with RNA polymerase II alone display the morphology of single-stranded nucleic acid with (Figure 6b) and without (Figure 6a) gene 32 proteins. The remaining micrographs are examples of transcription reactions containing renaturase (Figure 6c–f) followed by incubation with (Figure 6d,f) or without

Table III: Electron Microscopy of Transcription Complexes[a]

	when does single-stranded nucleic acid extend from transcription complexes			
	visualized with gene 32[b]		visualized without gene 32[b]	
				
RNA polymerase II	30	1	17	1
RNA polymerase II, RNase A	36	17	49	37
RNA polymerase II, renaturase	18	34	11	20
RNA polymerase II, renaturase, RNase A	1	31	1	30

[a] Reactions containing 1.7 units (0.2 μg) of renaturase fraction V and 2 microunits (100 ng) of RNA polymerase II were prepared for electron microscopy as described in the text and in the legend to Figure 4. [b] Numbers refer to the template molecules that contain an internal RNA polymerase II molecule.

(Figure 6c,e) RNase A. Single-stranded nucleic acid was visualized with (Figure 6e,f) or without (Figure 6c,d) T4 gene 32 protein. From the results in Table III, we see that in the absence of renaturase, 57–68% of the single-stranded nucleic acid molecules extending from RNA polymerase II are resistant to RNase A. This nucleic acid is therefore single-stranded DNA and is indicative of the presence of an RNA–DNA hybrid. On the other hand, in the presence of renaturase, at least 97% of the RNA polymerase II molecules have no single-stranded molecules extending from them after RNase A treatment. In this experiment, 65% of the polymerase molecules have no single-stranded nucleic acid extending from them in the presence of renaturase even without RNase A treatment. In another experiment, 30% of the internal polymerase molecules had no single-stranded nucleic acid extending from them. This result may be due to release of transcripts during spreading, since renaturase does not reduce the transcript yield as evaluated by acid-precipitable material nor does it lead to extensive cleavage of chains (Figure 4). Clearly, those transcription complexes formed in the presence of renaturase contain RNA, not DNA, extending from the polymerase molecule, and thus, these are transcription complexes resembling those seen in vivo and in vitro with polymerases that carry out progressive elongation of the nascent RNA strand.

Discussion

Purified bacterial RNA polymerase holoenzymes acting alone are able to carry out selective initiation and termination of RNA chains in vitro for certain transcription units. However, it has been known for some time that accessory transcription factors that are not tightly bound to the polymerase during purification are essential for many transcription units in vivo (Chamberlin, 1976). Recent studies suggest that similar accessory factors such as the *E. coli nusA* and *nusB* proteins may be needed for elongation and termination with all bacterial transcription units in vivo (Greenblatt & Li, 1981; Ward & Gottesman, 1981; Nakamura & Uchida, 1983).

Eukaryotic RNA polymerases appear to be even more dependent on accessory transcription factors than the bacterial enzymes. Purified eukaryotic RNA polymerase II preparations require several such factors for effective initation at all known promoter sites (Matsui et al., 1980; Samuels et al., 1982; Dynan and Tjian, 1983; Davison et al., 1983; Parker & Topol, 1984), although the exact identity and number of factors involved are still not well-known. Moreover, efficient RNA chain elongation by RNA polymerase II probably also requires accessory factors since elongation by purified RNA polymerase II is much slower than the in vivo process, and much of the nascent RNA remains bound to the transcribed DNA strand as a DNA–RNA hybrid.

Several groups have reported identification of factors that enhance the rate of in vitro RNA chain elongation by RNA polymerase II (Seifart et al., 1973; Horikoshi et al., 1984; Spindler, 1979; Sawadogo et al., 1981; Ueno et al., 1979; Natori, 1982; Sekimizu et al., 1982). At least one of these is implicated in transcription in vivo, since antibody to the factor inhibits transcription in isolated nuclei (Ueno et al., 1979) and cell extracts (Sekimizu et al., 1982). However, the mechanism of action of this factor is not yet understood.

The factor that we have isolated from HeLa cell cultures—renaturase—allows transcription in vitro by mammalian RNA polymerase II preparations to mimic the normal process in that all of the RNA synthesized is displaced from the DNA template and the template DNA duplex is re-formed. However, we do not yet have any direct evidence that implicates renaturase in transcription in vivo. Purification of renaturase to homogeneity will be an essential first step in elucidating its function and should allow preparation of specific antibodies and ultimately cloning of the gene or genes involved. Such experiments will allow direct testing of its possible role in cellular transcription.

Although we are not certain of the cellular function of renaturase, the biochemical reaction it mediates is interesting and unusual. The strand displacement reaction involved is similar to that in which the *E. coli recA* protein effects displacement of a DNA strand from a duplex by a homologous DNA single strand (Shibata et al., 1979; McEntee et al., 1979), although *recA* protein will not displace RNA from a DNA–RNA duplex (I. R. Lehman, personal communication). While renaturase binds well to duplex DNA–cellulose columns, it does not bind to single-stranded DNA columns run under the same conditions. It is plausible to speculate that this preferential binding to the duplex might, somehow, facilitate renaturation of the template DNA. In support of this possibility, renaturase preparations contain a component that may bind cooperatively to DNA duplexes as is evident in electron microscopic experiments such as those in Figure 6. It remains to be seen, however, whether renaturase binding to DNA is important in its effects on transcription.

In vitro, RNA–DNA hybrids are generally less stable than equivalent DNA–DNA duplexes (Chamberlin & Berg, 1964), and this instability is amplified when re-formation of the DNA duplex releases RNA, which can form its own tight RNA–RNA secondary structure. Hence, re-formation of the DNA template duplex should be strongly favored by thermodynamic considerations in transcription reactions with RNA polymerase II. However, the rates of such coil–helix displacement reactions are slow compared to the rate of transcription (Chamberlin & Patterson, 1965). R loops, which are directly analogous to the products formed by RNA polymerase II, can be formed by strand displacement by free RNA on a DNA duplex in formamide solutions; however, these R-loop structures are only slowly lost when the formamide is diluted away (Davis et al., 1971), and in the case of RNA polymerase ternary complexes, the protein probably interferes with direct RNA strand displacement at the growing point as well. Thus, the DNA–RNA hybrids formed by RNA polymerase II are stable in transcription reactions for some time (Kadesch & Chamberlin, 1982).

One might suspect, since release of the nascent RNA is thermodynamically favored, that proteins that preferentially

bind single-stranded RNA or duplex DNA (von Hippel & McGhee, 1972; Geider & Hoffman-Berling, 1981) might catalyze the re-formation of the DNA duplex and hence mimic renaturase action. However, we have tested a large number of candidates without finding such activity. This result encourages us to think that the renaturase reaction involves a complex and possibly specific process and may play an essential role in nuclear transcription.

The formation of RNA–DNA hybrids during in vivo transcription has been previously reported (Mandel & Borkowska, 1964; Girard et al., 1974). In fact, *E. coli* RNA polymerase seems to switch from making free to making hybrid transcript during the initiation of colEl replication (Itoh & Tomizawa, 1980; Hillenbrand & Straudenbauer, 1982; Kornberg, 1982). Possible purposes for nonreplicative hybrid formation have been suggested for eukaryotic cells (Frenster, 1965; Britten & Davidson, 1969; Sekeris et al., 1972; Wyers et al., 1976). However, if the nascent hybrid were to have more than a transient existence, this would lead to a nonproductive mode of RNA synthesis, as the RNA polymerase II transcript presumably would be unavailable for processing and export to the translation machinery. Such nonproductive formation of hybrids might still be functional. For example, the ability to modulate message availability for processing and translation would be an effective way of regulating gene expression. In fact, 80% of the potential mRNA transcripts produced in the nucleus are degraded and not exported (Davidson & Britten, 1979), which makes it very likely that mechanisms for modulation of transcript availability exist and are important in gene regulation. Such a mechanism has been reported for α_1-acid glycoprotein (Vannice et al., 1984).

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Registry No. RNA polymerase, 9014-24-8.

REFERENCES

Alberts, B., & Herrick, G. (1971) *Methods Enzymol. 21*, 198–217.

Ames, G. F. L. (1974) *J. Biol. Chem. 249*, 634–644.

Beyer, A. L., Miller, O. L., Jr., & McKnight, S. L. (1980) *Cell (Cambridge, Mass.) 20*, 75–84.

Birnboim, H. C., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513–1523.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Britten, R. J., & Davidson, E. H. (1969) *Science (Washington, D.C.) 165*, 349–357.

Chamberlin, M. J. (1976) in *RNA Polymerase* (Losick & Chamberlin, Eds.) pp 17–67, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Chamberlin, M., & Berg, P. (1964) *J. Mol. Biol. 8*, 297–313.

Chamberlin, M. J., & Patterson, D. L. (1965) *J. Mol. Biol. 12*, 410–428.

Chang, L. M. S., & Bollum, F. J. (1971) *J. Biol. Chem. 246*, 909–916.

Davidson, E. H., & Britten, R. J. (1979) *Science (Washington, D.C.) 204*, 1052–1059.

Davis, R. W., Simon, M., & Davidson, N. (1971) *Methods Enzymol. 21*, 413–428.

Davison, B. L., Egly, J.-M., Mulvihill, E. R., & Chambon, P. (1983) *Nature (London) 301*, 680–686.

Dedrick, R. L., & Chamberlin, M. J. (1985) *Biochemistry* (preceding paper in this issue).

Delius, H., Westphal, H., & Axelrod, N. (1973) *J. Mol. Biol. 74*, 677–687.

Dynan, W. S., & Tjian, R. (1983) *Cell (Cambridge, Mass.) 32*, 669–680.

Englander, S. W., & Crowe, D. (1965) *Anal. Biochem. 12*, 579–584.

Frenster, J. H. (1965) *Nature (London) 206*, 1269–1270.

Geider, K., & Hoffmann-Berling, H. (1981) *Annu. Rev. Biochem. 50*, 233–260.

Girard, M., Marty, L., & Manteuil, S. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1267–1271.

Greenblatt, J., & Li, J. (1981) *Cell (Cambridge, Mass.) 24*, 421–428.

Hillenbrand, G., & Staudenbauer, W. L. (1982) *Nucleic Acids Res. 10*, 833–853.

Hodo, H. G., & Blatti, S. P. (1977) *Biochemistry 16*, 2334–2343.

Horikoshi, M., Sekimizu, K., & Natori, S. (1984) *J. Biol. Chem. 259*, 608–611.

Itoh, T., & Tomizawa, J.-I. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2450–2454.

Johnson, R. A., & Walseth, T. F. (1979) *Adv. Cyclic Nucleotide Res. 10*, 135–167.

Kadesch, T. R., & Chamberlin, M. J. (1982) *J. Biol. Chem. 257*, 5286–5295.

Kornberg, A. (1982) *1982 Supplement to DNA Replication*, pp S176–S177, W. H. Freeman, New York.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lavialle, C., Sekura, R., Madden, M.-J., & Salzman, N. P. (1982) *J. Biol. Chem. 257*, 12458–12466.

Lescure, B., Chestier, A., & Yaniv, M. (1978) *J. Mol. Biol. 124*, 73–85.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mandel, P., & Borkowska, I. (1964) *Biochim. Biophys. Acta 91*, 683–686.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.

Martin, T., Billings, P., Levey, A., Ozarslan, S., Quinlan, T., Swift, H., & Urbas, L. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 921–932.

Matsui, T., Segall, J., Weil, P. A., & Roeder, R. G. (1980) *J. Biol. Chem. 255*, 11992–11996.

McEntee, K., Weinstock, G. M., & Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2615–2619.

McMaster, G. K., & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835–4838.

Miller, O. L., Jr., Hamkalo, B. A., & Thomas, C. A., Jr. (1970) *Science (Washington, D.C.) 169*, 392–395.

Morgan, W. D., Bear, D. G., & von Hippel, P. H. (1983) *J. Biol. Chem. 258*, 9553–9564.

Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.

Nakamura, Y., & Uchida, H. (1983) *Mol. Gen. Genet. 190*, 196–203.

Natori, S. (1982) *Mol. Cell. Biochem. 46*, 173–187.

Parker, C. S., & Topol, J. (1984) *Cell (Cambridge, Mass.) 37*, 273–283.

Richardson, J. P. (1975) *J. Mol. Biol. 98*, 565–579.

Roeder, R. G. (1976) in *RNA Polymerase* (Losick & Chamberlin, Eds.) pp 285–330, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Rouviere-Yaniv, J., & Gros, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3428–3432.

Samuels, M., Fire, A., & Sharp, P. A. (1982) *J. Biol. Chem. 257*, 14419-14427.
Sawadogo, M., Lescure, B., Sentenac, A., & Fromageot, P. (1981) *Biochemistry 20*, 3542-3547.
Schaller, H., Nusslein, C., Bonhoeffer, F. J., Kurz, C., & Nietzschmann, I. (1972) *Eur. J. Biochem. 26*, 474-481.
Seifart, K. H., Kuhasz, P. P., & Benecke, B. J. (1973) *Eur. J. Biochem. 33*, 181-191.
Sekeris, C. E., Schmid, W., & Roewekamp, W. (1972) *FEBS Lett. 24*, 27-31.
Sekimizu, K., Yokoi, H., & Natori, S. (1982) *J. Biol. Chem. 257*, 2719-2721.
Shibata, T., Das Gupta, C., Cunningham, R. P., & Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1638-1642.
Spindler, S. R. (1979) *Biochemistry 18*, 4042-4048.
Symons, R. H. (1977) *Nucleic Acids Res. 4*, 4347-4355.
Ueno, K., Sekimizu, K., Mizuno, D., & Natori, S. (1979) *Nature (London) 277*, 145-146.
Vannice, J. L., Taylor, J. M., & Ringold, G. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4241-4245.
von Hippel, P. H., & McGhee, J. D. (1972) *Annu. Rev. Biochem. 41*, 231-300.
Ward, D. F., & Gottesman, M. E. (1981) *Nature (London) 292*, 212-215.
Weiner, J. H., Bertsch, L. L., & Kornberg, A. (1975) *J. Biol. Chem. 250*, 1972-1980.
Weinstock, G. M., McEntee, K., & Lehman, I. R. (1981) *J. Biol. Chem. 256*, 8829-8834.
Weisbrod, S., & Weintraub, H. (1981) *Cell (Cambridge, Mass.) 23*, 391-400.
Williams, R. C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2311-2315.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197-203.
Wyers, F., Sentenac, A., & Fromageot, P. (1976) *Eur. J. Biochem. 69*, 377-383.

Base-Pairing Properties of N^4-Methoxydeoxycytidine 5'-Triphosphate during DNA Synthesis on Natural Templates, Catalyzed by DNA Polymerase I of *Escherichia coli*†

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ABSTRACT: N^4-Methoxydeoxycytidine 5'-triphosphate (mo^{4}dCTP) was synthesized by reaction of dCTP with methoxyamine and then purified by high-performance liquid chromatography (HPLC) and used to analyze the specificity of mo^{4}dCMP incorporation during polymerization on natural templates, catalyzed by DNA polymerase I of *Escherichia coli*. Elongation of synthetic 5'-^{32}P-labeled primers, annealed to single-stranded DNA of bacteriophage M13, was carried out in the presence of only three of the four normal dNTPs; then, reaction products were displayed by high-resolution gel electrophoresis and visualized by autoradiography. By measuring primer elongation in each of the four "minus" reactions with and without added mo^{4}dCTP, we examined the specificity of mo^{4}dCMP incorporation at different positions along the M13 template. The results of this experimental approach indicated that (i) mo^{4}dCTP is utilized most readily (although at low efficiency) in place of dTTP during DNA synthesis, (ii) the analogue can also replace dCTP during primer elongation, although at barely detectable efficiency, and (iii) the ease at which both mo^{4}C·A and mo^{4}C·G pairs are formed during DNA synthesis on natural templates is markedly influenced by the nucleotide sequence of the template.

Many mutagenic agents are believed to act by producing chemical modifications of purine and pyrimidine residues on the DNA template or dNTP[1] precursors. Altered or ambiguous base-pairing specificities of these residues during DNA replication can lead to heritable changes in nucleotide sequence (Drake, 1970; Drake & Baltz, 1976, Singer & Kusmierek, 1982; Singer & Grunberger, 1983). We recently developed an electrophoretic assay of misincorporation that can be used to directly examine the base-pairing specificity of chemically modified dNTPs during DNA synthesis catalyzed by purified DNA polymerases on natural DNA templates (Hillebrand et al., 1984; Revich et al., 1984). The primer-template employed in this approach consists of a discrete restriction fragment or synthetic oligonucleotide (labeled with ^{32}P at the 5'-terminus), annealed to a circular template strand (extracted from a single-stranded DNA bacteriophage). The rate of elongation of 5'-^{32}P-labeled primer in the presence of only three of the four normal dNTPs (monitored by electrophoresis/autoradiography) reflects the rate of misincorporation in place of the missing dNTP. To examine the specificity of incorporation of a chemically modified nucleotide during DNA synthesis, each of the four "minus" reactions is carried out in the absence

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[1] Abbreviations: dNTP, 2'-deoxynucleoside 5'-triphosphate; HPLC, high-performance liquid chromatography; mo^{4}C, N^4-methoxycytosine; ho^{4}C, N^4-hydroxycytosine; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; UV, ultraviolet; ddNTP, 2',3'-dideoxynucleoside 5'-triphosphate.

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