Registry No. MC, 50-07-7.

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Renaturase and Ribonuclease H: A Novel Mechanism That Influences Transcript Displacement by RNA Polymerase II in Vitro†

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Received September 18, 1987; Revised Manuscript Received January 8, 1988

ABSTRACT: I have previously reported an activity in HeLa cells which facilitates transcript displacement by purified mammalian RNA polymerase II in vitro. I have shown that this activity copurifies with one of two separable ribonuclease (RNase) H activities in HeLa cells. The RNase H activity in question has characteristics similar to those reported for RNase H2b from calf thymus. RNase H proteins purified from several other sources including *Escherichia coli* also show renaturase activity. When the renaturase/RNase H protein is present during transcription by purified RNA polymerase II, transcripts are truncated close to the 5′ end, and the remainder of the transcript is displaced normally from its template by the polymerase. Since RNA polymerase II dependent transcripts in vivo normally require the presence of the 5′-triphosphate terminus for capping, the in vivo significance of RNase H as a renaturase factor is presently not understood. However, the in vitro action of renaturase/RNase H suggests that the mechanism of this reaction may involve R-loop displacement after formation of a short single-stranded region of DNA on the template strand following hydrolysis of a hybrid transcript oligonucleotide by RNase H.

To understand the molecular details of eukaryotic transcription, a system containing only purified components would be of considerable value. Recent studies have shown that there are a large number of genetic elements and protein factors needed in conjunction with purified RNA polymerase II for accurate and efficient transcription of different genes. Although the majority of the known factors are implicated in correct initiation of transcription, it is to be expected that additional factors will be important in the elongation and termination phases of transcription as well.

† Supported by NIH Grant RO1-GM34963 to Michael J. Chamberlin.

0006-2960/88/0427-3187$01.50/0

Previous studies of the transcription elongation reaction carried out by highly purified mammalian RNA polymerase II showed that such transcription is often defective, in that the nascent transcript remains hybridized to the DNA template strand, with continual displacement of the nontranscribed DNA strand on both linear and supercoiled templates (Sekeris et al., 1972; Lescure et al., 1978; Lavialle et al., 1982; Kadesch & Chamberlin, 1982; Dedrick & Chamberlin, 1985). This behavior is dependent on the particular polymerase protein, and bacterial polymerases invariably displace their transcripts under similar conditions and with similar templates (Dedrick & Chamberlin, 1985). These results suggested that the "renaturase" function of the bacterial RNA polymerase (Richardson, 1975) was missing from the mammalian enzyme and led us to search for activities in cell extracts that might carry out this essential function. Such an activity was detected and partially purified from HeLa cells (Kane & Chamberlin, 1985); it was designated renaturase. In the presence of partially purified renaturase, RNA polymerase II synthesizes undiminished amounts of RNA transcripts, all of which are efficiently displaced from the DNA template strand. A similar activity has been recently reported in *Drosophila* cells (Greenleaf et al., 1987; Price et al., 1987; Sluder et al., 1988).

This paper describes the further purification of HeLa cell renaturase and the characterization of its reaction mechanism. The activity appears to be inseparable from a ribonuclease H (RNase H)[1] activity, and the RNase H activity is implicated in the renaturase function itself.

Materials and Methods

Materials

Calf thymus RNA polymerase II was purified by a modification of the procedure of Hodo and Blatti (1977) as modified by Dedrick and Chamberlin (1985). Purified *Escherichia coli* RNase H was the generous gift of T. Ogawa and A. Kornberg, Stanford University. *Drosophila* RNase H was generously provided by R. DiFrancesco and I. R. Lehman, Stanford University, which they purified as described (DiFrancesco & Lehman, 1985). *E. coli* RNase H was also purchased from Bethesda Research Laboratories. *E. coli* RNA polymerase was purified as described by Gonzales et al. (1977). AMV reverse transcriptase was from Life Sciences Inc., S1 nuclease was from Boehringer Mannheim, RNase A and DNase I were from Worthington, and terminal deoxynucleotidyltransferase was from Ratliff Biochemicals (Los Alamos, NM). Restriction enzymes were used according to the manufacturer's instructions. DNase I was treated with bentonite to remove contaminating RNase (Maniatis et al., 1982). Bovine serum albumin was treated with acetic anhydride to inhibit contaminating DNase and RNase activities (Gonzales et al., 1977).

DEAE-cellulose (DE52, preswollen, microgranular) and phosphocellulose (P11) were from Whatman. DE52 was prepared according to the manufacturer's instructions. Phosphocellulose also was prepared according to the manufacturer's instructions except that the acid wash was eliminated prior to equilibration. Bio-Gel HTP and Bio-Gel A 0.5m were from Bio-Rad; Cibacron blue F3GA–agarose was from Pierce Chemical Co. (Rockford, IL). Double-stranded DNA–cellulose was prepared according to Alberts and Herrick (1971) with calf thymus DNA.

Ammonium sulfate and urea were ultrapure grade from Schwarz/Mann. Unlabeled nucleotides were from P-L Biochemicals and Boehringer Mannheim. [α-^{32}P]CTP was synthesized according to Symons (1977) or purchased from Amersham. [γ-^{32}P]ATP was synthesized according to Johnson and Walseth (1979). ^{32}P-Labeled inorganic phosphate was purchased from New England Nuclear (Boston, MA).

M13mp18 DNA was prepared from isolated phage as described (Messing, 1983). Plasmid DNA used for transcription templates was prepared by alkaline lysis (Birnboim & Doly, 1979) followed by two sequential CsCl/ethidium bromide equilibrium density gradients.

Methods

Many of the methods used have been previously described (Kane & Chamberlin, 1985). These include transcription reactions, preparation of DNA templates, and growth of tissue culture cells. Nucleic acid concentrations are given as nucleotides unless otherwise indicated.

Assay for Renaturase. Renaturase was assayed as an activity which converted transcripts produced by RNA polymerase II from an RNase A resistant to an RNase A sensitive form (Kane & Chamberlin, 1985). Briefly, transcription reactions carried out with or without renaturase were stopped with a solution containing 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 50 mM EDTA. Aliquots of this stopped reaction were incubated an additional 20 min in the presence or absence of 12 μg/mL RNase A. Nucleic acids in these reactions were precipitated with 4% trichloroacetic acid in the presence of carrier RNA, and the acid-insoluble material was recovered on Whatman GF/C filters (Chamberlin et al., 1979). Radioactive RNA was quantitated by liquid scintillation counting in BetaMax (West Chem Scientific). One unit of renaturase activity is defined as that amount which converts 40 pmol of transcript to an RNase A sensitive form in 10 min at 37 °C in the presence of 1 microunit (Dedrick & Chamberlin, 1985) of calf thymus RNA polymerase II. At least three different amounts of renaturase protein were assayed for each fraction throughout the purification. The linear range of the assay falls between 20% and 90% RNase A sensitive transcript; thus, unit calculations require extrapolation to determine the protein required to effect 100% RNase A sensitive transcript. When necessary, fractions were diluted into chilled buffer [10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM DTT (TED)] immediately prior to assay.

Assay for RNase H. HeLa RNase H activity was assayed by adding enzyme to a 50-μL reaction containing 30 mM Tris-HCl, pH 7.8, 1.5 mM 2-mercaptoethanol, 32 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 100 μg/mL acetylated bovine serum albumin, and 20–60 pmol of RNA as an RNA/DNA hybrid (23 μM M13mp18 DNA). Reactions were incubated for 15 min at 37 °C, and they were stopped by the addition of 200 μL of a solution containing 50 mM EDTA, 200 μg/mL Torula RNA (Sigma), and 0.1 M sodium pyrophosphate followed by 2.5 mL of 4% trichloroacetic acid. Acid-insoluble material was recovered by GF/C filtration as above, and the nondigested hybrid was quantitated by liquid scintillation counting. One unit of activity is that amount of RNase H needed to render 1 nmol of RNA in an RNA/DNA hybrid acid soluble in 15 min at 37 °C.

Substrate for RNase H assays was prepared by transcription of M13mp18 single-stranded DNA with *E. coli* RNA polymerase in reactions containing 44 mM Tris-HCl, pH 8, 14 mM

[1] Abbreviations: RNase, ribonuclease; DNase, deoxyribonuclease; AMV, avian myeloblastosis virus; DTT, dithiothreitol; SEVAG, chloroform/isoamyl alcohol, 24:1; NEM, *N*-ethylmaleimide; poly(rA), poly(riboadenylic acid); poly(dT), poly(thymidylic acid); SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PEG, poly(ethylene glycol); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); HTP, hydroxylapatite.

$MgCl_2$, 14 mM 2-mercaptoethanol, 20 mM NaCl, 2% glycerol, 35 μg/mL acetylated bovine serum albumin, 2.7 mM ATP, 1 mM GTP, 1.6 mM UTP, 0.6 mM [α-^{32}P]CTP (200–500 cpm/pmol), 0.23 mM M13mp18 DNA, and 10–30 μg/mL *E. coli* RNA polymerase. Reactions were incubated for 10 min at 37 °C; rifampicin was added to 50 μg/mL, and the incubation was continued for 5 min. This substrate was used without further treatment; no effect was seen on the assays for RNase H if the substrate was further treated with RNase A to remove nonhybridized RNA or if the substrate was purified by phenol/chloroform extraction and ethanol precipitation prior to RNase H assays.

Drosophila RNase H activity was assayed by R. DiFrancesco as described (DiFrancesco & Lehman, 1985). One unit of enzyme hydrolyzes 1 nmol of [^{3}H]poly(A) in a poly(A)·poly(dT) hybrid in 15 min at 37 °C. *E. coli* RNase H activity was assayed in 50-μL reactions containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 M KCl, 0.1 mM DTT, 0.1 mM EDTA, 5% glycerol, and 20–60 pmol of RNA as hybrid (described above). One unit of enzyme hydrolyzes 1 nmol of hybrid nucleotide in 15 min at 30 °C. In all cases, when RNase H activity was assayed, enough enzyme was added to digest no more than about 80% of hybrid in the reaction. When necessary, fractions were diluted into chilled TED buffer immediately prior to assay.

Assay for RNA Endonuclease Activity. RNA endonuclease activity was assayed by mixing purified renaturase in 1 μL with 2 μL containing 5–10 pmol of RNA in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (TE) prepared as described below. These mixtures were incubated 15 min at 37 °C; 8 μL of a solution containing 80% formamide, 50 mM Tris–borate, pH 8.3, 1 mM EDTA, and 0.04% bromphenol blue and xylene cyanol was added. Samples were heated at 90 °C for 5 min and resolved on a 5% polyacrylamide/8.3 M urea gel. Autoradiographic signals of RNAs incubated with or without purified renaturase were indistinguishable.

Radiolabeled RNAs for RNA endonuclease assays were prepared by transcribing supercoiled templates with *E. coli* RNA polymerase in the presence of radioactive nucleotide triphosphates as described (Reines et al., 1987). Transcripts were purified by phenol/SEVAG extraction and ethanol precipitation, and RNAs were suspended in TE.

S1 Nuclease Analysis and Quantitation of Protected Fragments. The DNA used as probe was from the 309 bp fragment resulting from *Hpa*II digestion of pBR322 (Maniatis et al., 1982) which spans the initiation site for transcription from the *Pvu*II-cut and poly(dC)-tailed template used in these experiments (Kane & Chamberlin, 1985). The fragment was isolated by electroelution following preparative polyacrylamide gel electrophoresis, treated with calf intestinal phosphatase, and labeled with polynucleotide kinase (Maniatis et al., 1982). Single-stranded probes were isolated by electroelution following strand separation on polyacrylamide gels (Maniatis et al., 1982), and the particular strand was identified by chemical sequencing of the isolated DNA (Maxam & Gilbert, 1980). Only one of the two separated strands was used as a probe in these experiments, and transcripts initiated at the tail–duplex junction of the template (Dedrick & Chamberlin, 1985) would protect a fragment of 256 nucleotides with this probe.

Transcription reactions were performed in the usual way except that nonradioactive nucleoside triphosphates were used as substrates, and the reactions were incubated for 25 min at 37 °C. Reactions were brought to 2 mM $CaCl_2$ and 25 μg/mL tRNA, and RNase-free DNase I was added to 12 μg/mL to degrade the DNA template. After incubation for 10 min at 37 °C, EDTA was added to 50 mM, and the RNA was extracted with phenol/SEVAG twice. Hybridization and S1 nuclease analysis were carried out as described by Gilman and Chamberlin (1983). Typically, 30000–100000 cpm of probe was used per reaction which assured probe excess over hybridizing transcripts.

Gel slices corresponding to protected fragments were excised and quantitated by liquid scintillation counting.

Heat Inactivation Analysis. Samples (10 μL) of fraction IV were placed at various temperatures for 1 min. Forty microliters of chilled TED was then added to each sample, and an aliquot was used to assay for both renaturase and RNase H activities. The untreated sample was diluted in the same fashion after being held on ice for 1 min. An aliquot of the untreated sample was enough to displace 93% of the transcript in a standard renaturase reaction and to degrade 13 of 28 pmol of CMP present as hybrid in a standard RNase H reaction.

N-Ethylmaleimide Treatment. N-Ethylmaleimide (NEM) (Sigma) was dissolved in distilled, deionized H_2O, filter-sterilized, and stored frozen. Aliquots of fraction IV and the 0.9 M NaCl eluate from the Cibacron blue column were dialyzed against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M NaCl, and 10% glycerol to remove reducing reagents. These aliquots were exposed to a final concentration of 0.1 or 0.5 mM NEM for 20 min at room temperature. Samples were then added to either the standard renaturase or the RNase H assay. Untreated samples received distilled, deionized H_2O instead of NEM.

Synthesis of Poly(dT)·Poly(rA). Poly(riboadenylic acid) was synthesized according to Chamberlin and Berg (1964) using *E. coli* RNA polymerase, M13mp18 DNA, and [α-^{32}P]ATP (300 cpm/pmol). The products were analyzed on polyacrylamide gels, and all were longer than 100 nucleotides. Twenty-two nanomoles of poly(riboadenylic acid) was mixed with 120 nmol of poly(thymidylic acid) [poly(dT), Sigma] in 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, and 0.03 M sodium citrate. The mixture was heated at 90 °C for 2 min and was placed at 65 °C for 25 min (DiFrancesco & Lehman, 1985). The hybridization mixture was then cooled by placing the reaction at room temperature. Treatment of the mixture with RNase H, RNase A, and DNase I confirmed that greater than 96% of the poly(riboadenylic acid) was present as an RNA/DNA hybrid.

Paper Chromatography of RNase H Digestion Products. Descending chromatography was performed as described (DiFrancesco & Lehman, 1985). Oligoribonucleotides of known chain length (provided by Barbara Dengler, University of California, Berkeley) were cochromatographed with each sample. Oligonucleotides of nine or longer remained at the origin. Oligonucleotides that cochromatographed with UV-absorbing material were excised and quantitated by liquid scintillation counting.

Polyacrylamide Gel Electrophoresis. Proteins were separated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and were visualized by silver staining (Wray et al., 1981; Morrissey, 1981).

Fragments recovered after S1 nuclease analysis were resolved on 8% polyacrylamide/8.3 M urea gels and were visualized by autoradiography at −80 °C with Kodak XAR film and Lightning Plus intensifying screens.

Protein Determination. Protein concentrations were determined with the Bio-Rad protein assay reagent using bovine serum albumin as a standard.

Purification of Renaturase. All procedures were carried out at 4 °C unless otherwise indicated. Both renaturase and

RNase H activities were determined at each step of the purification.

(*A*) *Cell Extract Preparation.* HeLa cells were harvested at a density of (5–6) × 10^5 cells/mL by centrifugation and were washed 3 times with a solution containing 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.15 M NaCl, and 0.015 M sodium citrate. Cells were swollen in 10 mM Tris-HCl, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT (about 10 mL/L of cells) and were lysed by Dounce homogenization. Nuclei were removed by centrifugation at 1000*g* for 10 min. The supernatant fluid was kept on ice while the nuclei were washed once in the homogenization buffer. The two supernatant solutions were combined (cytosolic fraction), brought to 1 mM phenylmethanesulfonyl fluoride (PMSF), and clarified by centrifugation at 12K*g* for 10 min. The low-speed supernatant fluid was stored frozen at −80 °C until material from 100–400 L of HeLa cells was collected. The solution was thawed quickly and centrifuged for 60 min at 55K*g* in a Beckman type 21 rotor. The supernatant fluid from this centrifugation is fraction I.

(*B*) *Ammonium Sulfate.* Fraction I was brought to 25% saturation in ammonium sulfate by the addition of 0.134 g/mL solid ammonium sulfate with stirring at 4 °C. Small amounts of 1 N NaOH were added as the salt dissolved to maintain neutral pH. After the solution was stirred for 60 min, the precipitate was removed by centrifugatioin at 13K*g* for 30 min. The supernatant solution was adjusted to 65% saturation in ammonium sulfate by the addition of 0.245 g/mL solid ammonium sulfate to the 25%-saturated solution as described above. The precipitate was collected by centrifugation as above and was dissolved in TED containing 0.2 M NaCl. If necessary, this material can be quickly frozen and stored at −80 °C prior to running the DE52 column without loss of activity. The solution was brought to 1 mM PMSF and then dialyzed against this same buffer overnight with several buffer changes. Dialyzed material was clarified by centrifugation, and the supernatant fluid was designated fraction II.

(*C*) *DE52.* A 6 × 25 cm DE52 column was poured and equilibrated in TED/0.2 M NaCl. Fraction II from 350 L of HeLa cells was loaded onto the column, and the flowthrough was collected. The column was washed with the same buffer until no protein was detected in the eluate. The flowthrough material containing renaturase/RNase H activity (fraction III) was brought to 1 mM PMSF, divided into six aliquots (each containing about 800 mg of protein), and quickly frozen for storage at −80 °C.

(*D*) *Cibacron Blue–Agarose.* Aliquots of fraction III were processed one at a time. Fraction III was quickly thawed, PMSF was added to 1 mM, and the solution was placed into rapid dialysis (Englander & Crowe, 1965) against TED containing 10 mM NaCl and 10% glycerol. After dialysis for 3 h with two buffer changes, the solution was clarified by centrifugation to remove a small precipitate and was loaded onto a 25-mL column of Cibacron blue–agarose equilibrated in the same buffer (the column previously had been washed with a solution containing 100 μg/mL acetylated bovine serum albumin followed by a wash with TED containing 1M KSCN). The column loaded with fraction III was washed until no protein was detected in the eluate. Then the column was eluted with TED containing 0.9 M NaCl and 10% glycerol followed by elution with TED containing 2 M NaCl and 10% glycerol. RNase H activity was recovered in both the 0.9 and 2 M NaCl wash fractions, but renaturase activity was recovered only in the 2 M NaCl wash fraction. Over 80% of the total RNase H activity was contained in the 0.9 M NaCl fraction. Before additional aliquots of fraction III were loaded, the column was washed with TED containing 1 M KSCN and 10% glycerol and reequilibrated. The 2 M NaCl fractions from six column procedures were pooled (633 mL) and were concentrated by placing the solution into dialysis bags surrounded with PEG 6000 (Union Carbide, Carbowax 8000) at 4 °C. The concentrated pool (160 mL) was dialyzed overnight against 5 L of TED containing 0.2 M NaCl and 10% glycerol with one buffer change; this was followed by rapid dialysis for 3 h against 2 L of TED containing 10 mM NaCl and 10% glycerol with one buffer change. The dialysate was clarified as above, and the supernatant fluid was designated fraction IV.

(*E*) *Double-Stranded DNA–Cellulose.* Fraction IV was loaded onto a 50-mL DNA–cellulose column (2 mg of DNA/mL of column bed) equilibrated in TED, 10 mM NaCl, and 10% glycerol. This column also had been washed with a solution containing 100 μg/mL acetylated bovine serum albumin followed by TED/1 M NaCl prior to final equilibration. The column loaded with fraction IV was washed with the equilibration buffer until no protein was detected in the flowthrough. Protein was eluted with sequential steps of TED/0.2 M NaCl/10% glycerol and TED/0.5 M NaCl/10% glycerol. Active fractions from the 0.5 M NaCl step were pooled and designated fraction V.

The original purification scheme included this column immediately after the DE52 column (Kane & Chamberlin, 1985). In that purification scheme, 10–30% of the total renaturase units were reproducibly recovered in the flowthrough fraction of the DNA–cellulose column. In the current purification, no activity was detected in the flowthrough of this column.

(*F*) *Bio-Gel HTP.* Fraction V was rapidly dialyzed against 25 mM potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% glycerol, and the dialysate was clarified as above. The dialysate was loaded onto a 6-mL BioGel HTP (hydroxylapatite) column equilibrated in the same buffer. Prior to final equilibration, the column material had been washed with a solution containing 100 μg/mL acetylated bovine serum albumin followed by 1 M potassium phosphate, pH 7.5, 1 mM EDTA, and 1 mM DTT. The column loaded with dialyzed fraction V was washed with the equilibration buffer until no protein was detected in the eluate. The activity was eluted from the column with 0.2 M potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10% glycerol, and the active fractions were individually dialyzed into storage buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 M $(NH_4)_2SO_4$, and 50% glycerol and were stored at −20 °C. These samples were designated fraction VI. As discussed in the text, during the large-scale purification, there was a significant activity loss in the first 24 h at −20 °C; the fractions were then stable for more than 6 months. This activity loss was not seen in smaller scale purifications. The final extent of purification of the renaturase activity is calculated from the assay data before storage at −20 °C since the observed activity loss was not accompanied by a loss in protein from the fractions. However, the overall renaturase recovery drops from 30% to 8% (Table I).

(*G*) *A 0.5M Sizing.* Samples of each stored fraction were also chromatographed on a Bio-Gel A 0.5M column equilibrated in 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 0.1 M $(NH_4)_2SO_4$, 0.05% Brij 58, and 10% glycerol. The elution properties of the column had previously been determined by chromatography of a mixture of blue dextran, bovine serum albumin, lactoglobulin, cytochrome *c*, and [^{14}C]leucine. Active fractions from this column have been

Table I: Purification of Renaturase from HeLa Cells[a]

	sp act. (units/mg)		renaturase purity	renaturase recovery (%)	RN:H ratio
	RN	H	(x-fold)		
cytosol (I)	158	13	1		12
25–65% AS[d] (II)	132	64	1	100	2
DE52 (III)	118	102	1	72	1.2
Cibacron (IV)	3660	66	23	59	55
dsDNA (V)	78100	1690	490	50	46
HTP 14	412000	10700	2610		39
HTP 15	589000	12600	3730	30	47
HTP 16 (VI)[b]	120000	3750	760		32
HTP 14	60600	1740	380	8	35
HTP 15	150000	4000	950		38
HTP 16 (VI)[c]	74400	1630	470		46

[a] Renaturase was purified from 350 L of HeLa cells as described under Materials and Methods. All fractions were also assayed for RNase H activity, and the specific activities calculated from each assay are indicated (RN = renaturase, H = RNase H). The ratios of the specific activities of renaturase to RNase H are also presented. HTP 14, 15, and 16 refer to active fractions stored individually. The results of assays of these fractions off Bio-Gel HTP are shown both before and after storage for 24 h at −20 °C; the loss of activity in this preparation was not reproducible. The significance of this result is described in the text. [b] Before 24-h storage at −20 °C. [c] After 24-h storage at −20 °C. [d] AS, ammonium sulfate.

designated fraction VII; however, this procedure resulted in a 2–6-fold loss in activity with only a modest increase in specific activity (no increase to a 2-fold increase). It was thus used to determine native molecular weight, but it was not included in the purification scheme itself. Although this procedure removed several polypeptides which were present in fraction VI, multiple proteins were still visible on silver-stained SDS–polyacrylamide gels (data not shown).

Results

RNase H Activity Copurifies with Renaturase Activity. We previously reported upon an activity—designated renaturase—that allows efficient displacement of nascent transcripts by RNA polymerase II in vitro (Kane & Chamberlin, 1985). Because of the potential importance of this activity in allowing normal in vitro transcription by RNA polymerase II, a substantial effort was made to purify the factor to homogeneity and to determine its mechanism of action. The renaturase activity was fractionated through six steps beginning with large quantities (100–400 L) of HeLa cells. The most purified fraction had a specific activity 3700-fold higher than the initial, clarified cytosolic fraction. This fraction contained no detectable DNase nor single-stranded RNase activities (data not shown).

During this purification, it became evident that an RNase H activity was present in even the most highly purified fractions of HeLa renaturase. To explore the relationship of the two activities, the purification was repeated, and both renaturase and RNase H activities were assayed in all fractions. As expected from previous studies [see Crouch and Dirksen (1982) for a review], two separable RNase H activities were observed that will be referred to as RNase H1 and RNase H2, using the nomenclature that distinguishes the enzymes by molecular weight (Busen & Hausen, 1975; Wyers et al., 1976; Cathala et al., 1979; Busen, 1980a). RNase H1 is the larger of the two enzymes.

One of these HeLa RNase H activities, H2, copurified with renaturase through many different procedures (Table I). The

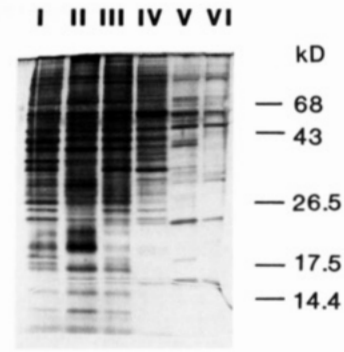

FIGURE 1: SDS–polyacrylamide gel of renaturase purification. Two micrograms of protein from the indicated fractions was resolved on a 12.5% polyacrylamide gel as described under Materials and Methods. Molecular masses (in kilodaltons) of marker proteins run on the same gel are indicated.

two HeLa RNase H activities separate during Cibacron blue chromatography, and only RNase H2 partitions with the renaturase activity. The ratio of renaturase to RNase H2 remains constant through all subsequent purification steps (Table I).

The duplicate sets of purification data for the hydroxylapatite column report specific activities before and after storage for 24 h at −20 °C. In this particular case, there was a very large (75%) loss in activity on storage (similar losses were not seen with previous purifications in which this fraction had a lower protein concentration). Despite this extensive inactivation, both the renaturase and RNase H activities declined to the same extent; that is, the ratios of the activities remained the same before and after storage. The copurification and constant activity ratios led to the hypothesis that the renaturase activity and RNase H activity might be associated with the same protein.

Renaturase and RNase H Activities Reside in the Same Polypeptide. The proteins from different fractions during the purification were analyzed by SDS–polyacrylamide gel electrophoresis (Figure 1). Although the renaturase and RNase H activities copurify, there are still multiple polypeptides in the final preparation. Information provided by such stained gels could not resolve the question of whether renaturase and RNase H were actually the same polypeptide. Two attempts were made to renature peptides excised from unstained SDS gels by procedures shown to be successful with many other proteins (Hager & Burgess, 1980; Konigsberg & Henderson, 1983). However, neither renaturase nor RNase H activity has been recovered from any gel fractions taken through such renaturation protocols.

To probe this question by a different approach, several highly purified RNase H preparations from other sources were tested for possible renaturase activity. These other enzymes were purified by different procedures from *E. coli* and *Drosophila*. Cloned RNase H purified to homogeneity from *E. coli* (Ogawa and Kornberg, unpublished procedure), commercially obtained *E. coli* RNase H (BRL) (data not shown), and *Drosophila* RNase H (DiFrancesco & Lehman, 1985) all contained renaturase activity (Table II). Note that the specific activity as renaturase of the homogeneous *E. coli* RNase H is even higher than that of the HeLa renaturase, a result consistent with the two activities residing in the same polypeptide. This result also suggests that the HeLa enzyme requires another 10-fold purification to achieve homogeneity. Each of these proteins was assayed for RNase H under conditions optimized for that activity; renaturase activity was determined under conditions optimal for transcription by RNA polymerase II. Thus, renaturase:RNase H activity ratios are

Table II: Proteins Purified as RNase H Contain Renaturase Activity[a]

enzyme source	sp act. (units/mg) RNase H	renaturase
E. coli	1×10^6	1.3×10^6
Drosophila	1.8×10^5	9×10^3
HeLa VI	4.6×10^3	1.2×10^5

[a] *E. coli* RNase H was the gift of Ogawa and Kornberg and was purified by them from a strain overexpressing the cloned gene (unpublished procedure). *Drosophila* RNase H was the gift of DiFrancesco and Lehman (1985). RNase H activities were assayed under conditions optimal for each separate enzyme as described under Materials and Methods; renaturase activity was assayed under conditions optimal for transcription by RNA polymerase II.

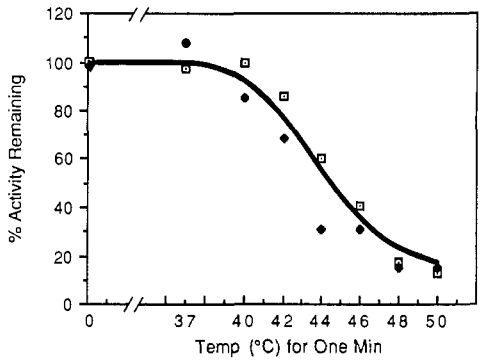

FIGURE 2: Heat inactivation of RNase H and renaturase. Samples of fraction IV renaturase were heated for 1 min at the indicated temperatures and then placed on ice. Assays for both renaturase and RNase H were performed immediately, and activity is expressed as the percent of that in the untreated sample. The untreated fraction contained 625 units/mL renaturase, 26 units/mL RNase H, and 0.36 mg/mL protein. (⊡) Renaturase activity; (♦) RNase H activity.

probably not meaningful in comparison to those found for the HeLa activity. Recently, renaturase activity was purified from *Drosophila* by a different procedure, and this preparation also contained RNase H activity (Greenleaf et al., 1987; Price et al., 1987; Sluder et al., 1988). These results provide support for the idea that RNase H proteins have an intrinsic renaturase activity.

Other biochemical evidence also suggests that the two activities reside in the same protein. As mentioned above, both activities declined to the same extent upon storage at −20 °C. Furthermore, RNase H activity declines at the same rate as renaturase activity upon heat treatment (Figure 2). In addition, both renaturase activity and the associated RNase H activity are completely inhibited by treatment with low concentrations (0.1 mM) of *N*-ethylmaleimide (see also Table III). Finally, both activities cochromatograph on sizing columns, in a position expected for a globular protein with a molecular weight of 30 000 (fraction VII; see Materials and Methods).

RNase H2, Not RNase H1, Is Associated with Renaturase Activity. We have characterized two HeLa RNase H activities that partition by Cibacron blue chromatography. Only one of the two, fraction IV, is associated with the renaturase activity. The other RNase H activity elutes from the Cibacron column with 0.9 M NaCl, well ahead of renaturase. Two RNase H activities have previously been purified from eukaryotic and viral sources (Crouch & Dirksen, 1982) including calf thymus (Busen & Hausen, 1975; Stavrianopoulos & Chargaff, 1978), yeast (Wyers et al., 1976; Iborra et al., 1979), *Tetrahymena* (Tashiro et al., 1976), chick embryos (Kitihara et al., 1982), rodent cells (Sekeris & Roewekamp, 1972; O'Cuinn et al., 1973; Cooper et al., 1974; Sawai et al., 1978; Cathala et al., 1979; Muller et al., 1980), and retroviruses (Gerard & Grandgenett, 1975). RNase H activity also has been reported previously in HeLa cells. Although only a single activity was described (Ferrari et al., 1980), there is immunological evidence for two distinct forms (Busen, 1980a), which is consistent with our purification results.

Table III: Distinguishing the RNase H Activities That Partition on Cibacron Blue Chromatography[a]

fraction	chemical	concn	pmol released
0.9M	NEM	0.5 mM	0
		0.1 mM	12
		0	10
fraction IV	NEM	0.5 mM	0
		0.1 mM	0
		0	6
0.9M	ssDNA	77 μM M13	2
		10 μM M13	3
		1 nM M13	4
		0	6
fraction IV	ssDNA	77 μM M13	8
		10 μM M13	8
		1 nM M13	9
		0	9
0.9M	dsDNA	0.5 μM CT DNA	3
		50 pM CT DNA	2
		0	6
fraction IV	dsDNA	0.5 μM CT DNA	10
		50 pM CT DNA	9
		0	9

[a] RNase H activity was assayed as described under Materials and Methods for NEM-treated fractions; activity was assayed according to Cathala et al. (1979) for assessing nucleic acid inhibition. Fractions were pretreated with NEM as described under Materials and Methods; the indicated nucleic acids were added directly to the reaction. NEM = *N*-ethylmaleimide; ssDNA = single-stranded DNA from M13mp18; dsDNA = double-stranded DNA from calf thymus (CT). 0.9M refers to the RNase H activity which elutes from Cibacron blue–Sepharose with 0.9 M NaCl; fraction IV refers to the renaturase-containing fraction which elutes from the same column with 2 M NaCl.

Reaction requirements and chemical inhibition studies clearly distinguished among the different RNase H activities. Mouse RNase H1 is inhibited by a variety of nucleic acids whereas RNase H2 is unaffected (Cathala et al., 1979). Calf thymus, rat, and mouse RNase H2 enzymes are more sensitive to the disulfide reagents *N*-ethylmaleimide and *p*-chloromercuricbenzoic acid than are the RNase H1 enzymes (Busen & Hausen, 1975; Cathala et al., 1979; Sawai et al., 1980a; Muller et al., 1980). Both RNase H1 and RNase H2 require a divalent cation for activity. However, RNase H2 is most active with Mg^{2+} as the divalent cation whereas RNase H1 is active with either Mn^{2+} or Mg^{2+} (Busen & Hausen, 1975; Cathala et al., 1979; Sawai et al., 1980a; Muller et al., 1980).

These criteria also apply to distinguish the two HeLa RNase H activities, the 0.9 M NaCl fraction vs fraction IV (Table III). The 0.9 M fraction is inhibited by nucleic acids, and fraction IV is the more sensitive to NEM. Fraction IV requires Mg^{2+} for its RNase H activity and is inactive in the presence of Mn^{2+}, whereas the 0.9 M fraction is active with either divalent cation. On the basis of these cation requirements and chemical inhibition studies, the renaturase-associated RNase H has the biochemical characteristics of RNase H2.

This assignment is further supported by sizing column chromatography of the two activities. The native molecular weight of mammalian RNase H1 (80 000 to over 100 000 in different organisms) differs substantially from that of mammalian RNase H2 (30 000–40 000) (Cathala et al., 1979; Sawai et al., 1980a; Busen, 1980a,b). Both HeLa renaturase and its associated RNase H activity cochromatograph through an A 0.5M column, with an apparent molecular weight of about 30 000, as reported for RNase H2. In addition, the HeLa renaturase activity was previously shown to sediment

in glycerol gradients at a position consistent with a globular protein of about 30000 daltons (Kane & Chamberlin, 1985).

As demonstrated here, RNase H enzymes from a variety of sources show renaturase activity. This poses the question of why RNase H1 fails to display renaturase activity. One possible explanation is that RNase H1 is simply not active in the transcription reactions. In fact, the RNase H activity in the 0.9 M Cibacron blue fraction is inhibited 90–95% under transcription conditions (data not shown) possibly due to the DNA concentration (Cathala et al., 1979; Table III) or the spermidine (Sawai et al., 1980). All the same, when large amounts of this fraction are assayed under transcription conditions, RNase H activity easily can be detected. However, no corresponding renaturase activity is detected.

A similar situation is found with the AMV reverse transcriptase, which has an associated RNase H activity but fails to function as a renaturase. At the current time, we cannot establish decisively whether the failure of these RNase H enzymes to show renaturase activity is due to their poor activity under transcription conditions or to the fact that only certain RNase H peptides can carry out renaturase function. However, the model proposed below for the action of RNase H as a renaturase in vitro may require a very efficient RNase H activity; thus, conditions that disfavor RNase H activity may give little or no renaturase activity.

How Is RNase H Activity Related to the Mechanism of Renaturase Transcript Displacement? Our initial characterization of transcription in the presence of renaturase utilized electron microscopy, agarose gel electrophoresis of large transcripts, and acid precipitation of transcripts (Kane & Chamberlin, 1985). These previous studies indicated that the transcription reaction was normal in the presence of renaturase; that is, the transcript was continuously displaced from its template and could be recovered at the end of the reaction in intact form as judged by its mobility in gel electrophoretic analysis. However, traces of single-strand-specific RNase remained in these fractions and made analysis of the exact start sites and size of transcripts unreliable. Once the renaturase fractions had been freed of detectable single-stranded ribonuclease activity, the 5′ end of the transcript could be examined more carefully using S1 nuclease analysis (Gilman & Chamberlin, 1983). This analysis demonstrated that transcripts formed in the presence of renaturase had been cleaved in the region of their 5′ ends near the start site (Figure 3).

The sizes of the protected fragments resulting from the S1 nuclease analysis exactly map the position of the transcript 5′ ends in such an experiment. Fragments labeled 6 (Figure 3) identify the transcription start sites for RNA polymerase II on this *Pvu*II-cut, poly(dC)-tailed pBR322 template; these correspond to start sites near the tail–duplex junction previously characterized for initiation by RNA polymerase II [Dedrick & Chamberlin, 1985; fragments 4 and 5 are likely due to interference with S1 digestion near the 5′ end of the hybrid as also seen by Dedrick and Chamberlin (1985)]. In the presence of renaturase, the amount of these protected fragments at the start site is dramatically reduced, and the relative amount of smaller protected fragments increases (e.g., fragments 9, 12, 15, and 16; Table IV).

Since many of these specific smaller protected fragments are present in low proportions in the absence of renaturase, they could reflect S1-sensitive sites in the RNA/DNA hybrid. We would expect these sites to be more S1 sensitive when positioned near the 5′ end of a truncated transcript due to increased "breathing" near the end of the hybrid molecule. Thus, although the transcripts themselves could be truncated

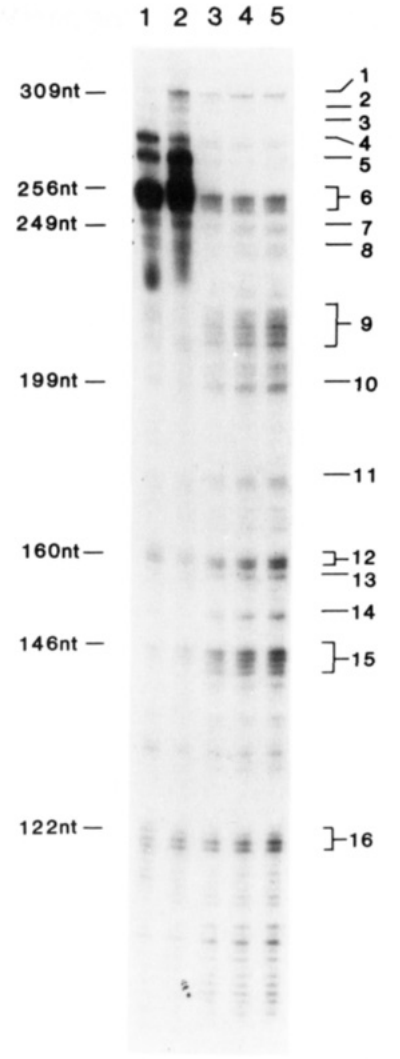

FIGURE 3: S1 nuclease analysis of transcripts synthesized in the presence and absence of renaturase. S1 analysis was performed as described under Materials and Methods. Protected fragments are resolved on an 8% polyacrylamide/8.3 M urea gel. Transcripts mapped were from reactions containing (lane 1) RNA polymerase II, (lane 2) *E. coli* core RNA polymerase, (lane 3) RNA polymerase II plus 1.2 units of renaturase, (lane 4) RNA polymerase II plus 2.1 units of renaturase, and (lane 5) RNA polymerase II plus 2.9 units of renaturase. Numbers on the right correspond to protected fragments quantitated in Table IV. Numbers on the left refer to the sizes (number of nucleotides) of the protected fragments indexed with a sequencing ladder run next to these samples on a separate gel.

by renaturase at random locations (see below), specific protected fragments would be recovered on these gels. Such specific protected fragments were excised and used for quantitation (Table IV).

Fifty to sixty percent of the transcript 5′ ends produced in the presence of renaturase are recovered within 150 nucleotides of the transcription start site when enough renaturase is used to displace 100 percent of the transcripts (Table IV). Polyacrylamide gel electrophoresis of transcripts of 600–2000 nucleotides also suggested that renaturase reduced the transcript size by about 50–200 nucleotides (data not shown). Primer extension experiments (Maniatis et al., 1982) confirmed that this truncation was at the 5′ end of the transcript and that the cleavage leading to the truncation apparently was not sequence dependent and occurred randomly across the hybridized transcript (data not shown).

The most plausible explanation for these results is that renaturase function involves an early cleavage of an RNA/DNA hybrid formed by the RNA polymerase II near the start

Table IV: Quantitation of 5′ Ends of Transcripts Produced by RNA Polymerase II in the Presence and Absence of Renaturase[a]

protected fragment	% of each fragment			
	no renaturase	4 μL of renaturase	8 μL of renaturase	12 μL of renaturase
1	2	7	9	10
2	4	4	3	3
3	3	3	2	2
4	11	7	3	3
5	13	5	2	2
6	54	37	15	14
7	2	4	4	4
8	2	2	3	3
9	3	8	13	15
10	0.6	1	3	3
11	1	2	4	3
12	1	3	7	7
13			2	4
14	0.3	2	5	3
15	2	8	15	14
16	2	8	12	12
% sensitive	23	62	100	100

[a] Protected fragments are numbered according to those indicated in Figure 3. The percent of each protected fragment was determined by excising appropriate regions from a gel like that shown in Figure 3 and counting the gel slices in a liquid scintillation counter as described under Materials and Methods. Percentage of each fragment = (cpm in fragment n/total cpm in fragments 1–16) × 100; % sensitive refers to those transcripts in parallel reactions which are completely digested by RNase A and are therefore displaced transcripts. The amount of renaturase used in each reaction is indicated at the top of each column; this fraction contained 417 units/mL renaturase.

site (see discussion below). An alternative explanation for the S1 nuclease results could be initiation by RNA polymerase II at alternative, random start sites on the template in the presence of renaturase. However, this latter explanation seems less likely; renaturase can be added after initiation has taken place, and although the transcript yield is reduced relative to adding renaturase along with the polymerase (due to degradation of the hybrid transcript already formed), all of the transcript elongated after renaturase addition is displaced; i.e., it is sensitive to RNase A digestion (Kane & Chamberlin, 1985).

Quantitation of the S1 data (Table IV) demonstrated that only 10–15% of full-length transcripts were recovered from reactions containing enough renaturase to assure complete displacement of transcripts during transcription (compare fragments labeled 6 in Figure 3, lanes 1 and 3–5). This result would be predicted from our transcription assays; 10–20% of the transcript is displaced by purified RNA polymerase II in the absence of renaturase, and this amount of transcript would be expected to be completely resistant to digestion by renaturase/RNase H throughout the reaction.

During transcript displacement, the RNase H associated with renaturase could act as either an endonuclease or an exonuclease. To test the mechanism of the RNase H, poly(dT)·poly(rA) was digested with renaturase/RNase H, and the products were resolved by paper chromatography and visualized by autoradiography. Digestion with varying concentrations of enzyme produced oligomers of rA (Table V). Greater than 80% of the digestion products were larger than seven nucleotides, consistent with an endonucleolytic mechanism of attack or with an exonuclease whose action produces large oligomers rather than mononucleotides.

Discussion

We previously described an unusual feature of in vitro transcription by highly purified mammalian RNA polymerase

Table V: Digestion Products of Renaturase/RNase H2[a]

product	% $(rA)_n$	
	reaction I	reaction II
longer than $(rA)_7$	96	88
$(rA)_5$	2.5	7.7
$(rA)_3$	1.1	3.1
$(rA)_2$	0.3	0.7
rA	0.1	0.2

[a] Fraction VII renaturase/RNase H (see Materials and Methods) was incubated with poly(rA)·poly(dT), and the digestion products were resolved by paper chromatography as described under Materials and Methods. Fraction VII contained 2600 units/mg of RNase H. $(rA)_n$ = AMP-containing oligoribonucleotides of length n. Reaction I contained 33 ng of fraction VII and a total of 533 pmol of poly(rA); reaction II contained 83 ng of fraction VII and a total of 490 pmol of poly(rA). For an untreated sample, 497 pmol of poly(rA) was recovered at the origin.

II; that is, the nascent transcript is not efficiently displaced from the template strand. Instead, with the majority of transcribing complexes, a long RNA/DNA hybrid is formed during transcription with progressive displacement of the nontranscribed DNA strand (Kadesch & Chamberlin, 1982; Dedrick & Chamberlin, 1985; Kane & Chamberlin, 1985). This hybrid formation occurs on both linear and supercoiled templates and is not simply a function of the tailed template. In addition, wheat germ RNA polymerase II and also *E. coli* RNA polymerase are capable of displacing nascent transcripts efficiently from this template although the enzymes from calf thymus, HeLa cells, and *Drosophila* do not (Dedrick & Chamberlin, 1985).

Since it is evident that transcription in vivo involves transcript displacement, it seemed likely that some factor or factors in addition to purified RNA polymerase II might provide this displacement function. Thus, the detection of an activity in HeLa extracts that facilitated transcript displacement during in vitro transcription (Kane & Chamberlin, 1985) led to a substantial purification effort in order to determine whether this activity would have a similar role in the cell. During further purification of this factor, it was noticed that all of the purified renaturase fractions contained RNase H activity. These two activities continued to copurify through several procedures. Although the HeLa factor in this report is not homogeneous, the ability of highly purified *E. coli* RNase H to promote template renaturation provides strong evidence that the two activities can reside in the same polypeptide. In addition, RNase H activities from several sources purified with different procedures all contain renaturase activity. The finding that RNase H copurifies with renaturase was of great interest. A regulatory model was postulated wherein displacement would result in synthesis of productive RNA and the lack of displacement would result in degradation of the transcript as the hybrid.

However, the purified renaturase activity apparently acts in vitro through the hydrolytic action of the associated RNase H. The renaturase/RNase H activity truncates the 5′ end of the primary transcript in vitro; this removes the nucleoside 5′-triphosphate from which comes the nucleoside diphosphate required for the capping reaction (Venkatesan & Moss, 1980; Venkatasan et al., 1980). The cap on the 5′ end of the message is required both for efficient splicing (Konarska et al., 1984; Edery & Sonenberg, 1985; Ohno et al., 1987) and for subsequent translation (Shatkin, 1985; Sonenberg, 1987) in eukaryotic cells. Thus, a process that involves truncation of the 5′ end of the primary transcript is probably not used by the cell for generating precursors for productive translatable message. Whether or not such shortened transcripts might

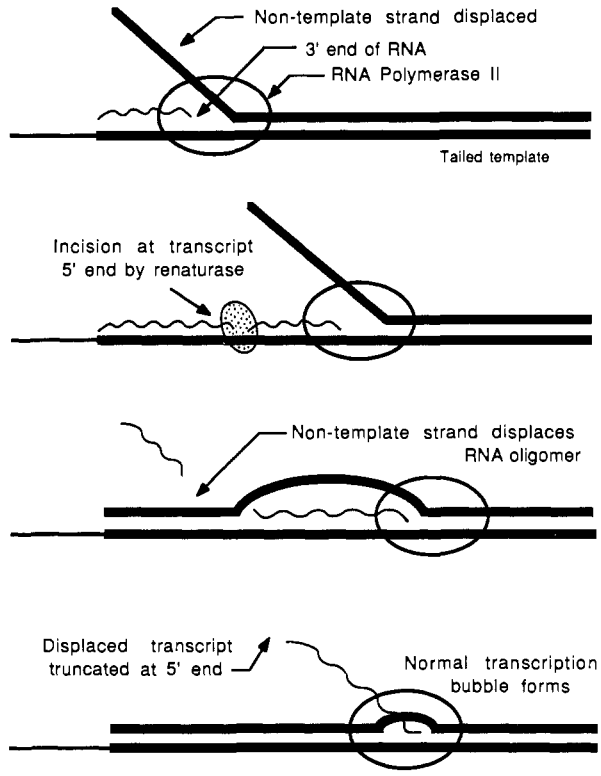

FIGURE 4: Role for RNase H in transcript displacement by RNA polymerase II in vitro.

serve other cellular functions is not known.

All these results provide us with a model for transcript displacement by renaturase in vitro (Figure 4). RNA polymerase II initiates transcription on the template at the tail–duplex junction. As the polymerase progresses into the transcription unit making an RNA–DNA hybrid (Kadesch & Chamberlin, 1982; Dedrick & Chamberlin, 1985; Kane & Chamberlin, 1985), renaturase/RNase H cleaves the transcript endonucleolytically, perhaps adjacent to the RNA polymerase. This cleavage apparently results in initiating the displacement of the transcript from the template strand; this may occur after the RNA oligomer dissociates and the nontranscribed DNA strand reanneals 5′ to the transcribing polymerase. Subsequently, the polymerase can continue to displace the RNA throughout the remainder of the transcription unit.

What function might such an RNase H activity serve in the cell? Despite extensive investigation, the cellular functions of the various RNase H activities have not been clearly established (Crouch & Dirksen, 1982). In DNA replication, such an activity may act to remove the RNA primer portion of Okazaki fragments. In accordance with this, RNAse H levels have been reported to rise and fall in concert with replication in whole cells (Busen et al., 1977; Sawai & Tsukada, 1977; Sawai et al., 1978, 1980b). Purified yeast RNase H stimulates yeast DNA polymerase A on nicked and gapped templates (Karwan et al., 1983). In a *Drosophila* system, purified RNase H interacts with a purified polymerase–primase complex to stimulate DNA synthesis, probably by increasing the recycling rate of the polymerase–primase complex (DiFrancesco & Lehman, 1985). The *Drosophila* RNase H exhibits some species specificity in this reaction as *E. coli* RNase H does not stimulate DNA synthesis (DiFrancesco & Lehman, 1985). Although the *Drosophila* RNase H polypeptide can hydrolyze RNA primers (DiFrancesco & Lehman, 1985), the stimulation of DNA synthesis by yeast or *Drosophila* RNase H apparently occurs through a mechanism that does not involve the hydrolysis reaction.

In addition, RNase H can influence transcription. Whereas RNase H1 levels increase along with replication in concanavalin A treated lymphocytes, RNase H2b activity increases along with transcription (Busen et al., 1977). Also, RNase H stimulates transcription by RNA polymerase II on native DNA and on isolated chromatin templates (Sekeris et al., 1972; Tashiro et al., 1976). In experiments with whole cells, the increase and decrease of RNase H and RNA polymerase I activities have been correlated (Tsukada et al., 1978; Sawai et al., 1980). Further, RNase H copurifies with RNA polymerase I from yeast and stimulates transcription by the purified polymerase on nonspecific DNA templates (Huet et al., 1975; 1977; Iborra et al., 1979). These diverse experiments suggest that RNase H proteins may play physiological roles in nucleic acid synthesis that may not be directly dependent on the hydrolytic activity of the enzyme.

Presently, we are left with the question of why RNA polymerase II purified from several sources inefficiently displaces its nascent RNA during transcription in vitro. Bacterial RNA polymerase apparently displaces its transcript effectively in vitro against a thermodynamic energy barrier that favors hybrid formation (Richardson, 1975); thus, some feature of the transcription machinery in the eukaryotic cell must perform the same function.

Transcription in the cell differs in many respects from transcription with purified RNA polymerase II. The eukaryotic template is actually nucleoprotein and not purified DNA. In addition, the primary transcript is capped and assembled into ribonucleoprotein complexes as transcription occurs. Any of the proteins associated with these complexes or processes might act to initiate transcript displacement. It also cannot be ruled out that one or more of the factors involved in promoter recognition or activation might also act to initiate transcript displacement.

If a separable accessory factor is involved in the displacement reaction, the substantial renaturase activity of RNase H2 in cell extracts may mask a second renaturase activity that might perform this function in the cell. Information about such an alternative factor might be derived by specifically inhibiting RNase H2 during early fractionation steps (such as with antibody). While the in vivo role of renaturase/RNase H2 remains to be defined, further examination of this unusual feature of RNase H2 action during in vitro transcription may provide us with some insight into the transcript displacement mechanism actually used by RNA polymerase II in the cell.

ACKNOWLEDGMENTS

I am grateful to T. Ogawa, A. Kornberg, R. DiFrancesco, and I. R. Lehman for the gifts of purified RNase H, to Barbara Dengler for the oligoribonucleotides of adenylic acid, and to Roberta Johnson and Letha Bradley for culturing HeLa cells. I thank Drs. Alexis Franzusoff and Lilian Hsu for critical reading of the manuscript.

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