Hershey, J. W. B., Yanov, J., & Fakunding, J. L. (1979) *Methods Enzymol. 60*, 3–11.
Hopfield, J. J., & Yamane, T. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 585–596, University Park Press, Baltimore, MD.
Ikemura, T. (1981) *J. Mol. Biol. 146*, 1–21.
Jelenc, P. C., & Kurland, C. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3174–3178.
Khazaie, K., Buchanan, J. H., & Rosenberger, R. F. (1984) *Eur. J. Biochem. 144*, 491–495.
Kung, H. F., Spears, C., & Weissbach, H. (1975) *J. Biol. Chem. 250*, 1556–1562.
Kung, H. F., Bekesi, E., Guterman, S. K., Gray, J. E., Traub, L., & Calhoun, D. H. (1984) *MGG, Mol. Gen. Genet. 193*, 210–213.
Lagerkvist, U. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1759–1762.
Laughrea, M. (1981) *Biochimie 63*, 145–168.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Meza-Basso, L., Robakis, N., Cenatiempo, Y., Weissbach, H., & Brot, N. (1981) *Biochem. Biophys. Res. Commun. 101*, 459–463.
O'Farrell, P. H. (1978) *Cell (Cambridge, Mass.) 14*, 545–557.
Parker, J., & Friesen, J. D. (1980) *MGG, Mol. Gen. Genet. 177*, 439–445.
Parker, J., Pollard, J. W., Friesen, J. D., & Stanners, C. P. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1091–1095.
Pestka, S. (1968) *J. Biol. Chem. 243*, 2810–2820.
Plumbridge, J. A., Deville, F., Sacerdot, C., Petersen, H. U., Cenatiempo, Y., Cozzone, A. J., Grunberg-Manago, M., & Hershey, J. W. B. (1985) *EMBO J. 4*, 223–229.
Robakis, N., Cenatiempo, Y., Meza-Basso, L., Brot, N., & Weissbach, H. (1983) *Methods Enzymol. 101*, 690–706.
Sancar, A., Hack, A. M., & Rupp, W. D. (1979) *J. Bacteriol. 137*, 692–693.
Stüber, D., & Bujard, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 167–171.
Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77–90.
Thompson, R. C., & Stone, P. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 198–202.
Thompson, R. C., Dix, D. B., Gerson, R. B., & Karim, A. M. (1981) *J. Biol. Chem. 256*, 6676–6681.
Weissbach, H., Robakis, N., Cenatiempo, Y., & Brot, N. (1984) *Biotechniques 2*, 16–22.
Weissenbach, J., & Grosjean, H. (1981) *Eur. J. Biochem. 116*, 207–213.
Yarus, M. (1979) *Prog. Nucleic Acid Res. Mol. Biol. 23*, 195–225.
Yarus, M. (1982) *Science (Washington, D.C.) 218*, 646–652.
Yarus, M., & Thompson, R. C. (1983) *Cold Spring Harbor Monogr. Ser. No. 15*, 23–63.

Accessibility of the Leading End of Ribonucleic Acid in Transcription Complexes†

Susan L. Bernhard and Claude F. Meares*
Chemistry Department, University of California, Davis, California 95616
Received February 10, 1986; Revised Manuscript Received May 27, 1986

ABSTRACT: A photoaffinity-protection technique has been developed to study the accessibility of the leading (5′) end of nascent RNA as it passes through the transcription complex formed by *Escherichia coli* RNA polymerase and phage T7 DNA. The macromolecules contacted by the leading (5′) end of the growing RNA chain in the transcription complex have been determined previously by photoaffinity labeling experiments using aryl azides attached to the leading end of nascent RNA [Hanna, M. M., & Meares, C. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4238–4242]. By using thiols to reduce accessible photoprobes, we have modified the photoaffinity technique so that it tests the accessibility of the leading end of nascent RNA to small molecules in solution, as a function of RNA chain length. We examined in detail RNA molecules containing 11–50 nucleotides, whose 5′ ends label the β and β' enzyme subunits with good yield. The thiol's accessibility to the leading end of each transcript was determined by comparing the RNAs cross-linked to $\beta\beta'$ in thiol-treated samples to controls not treated with thiol. Incubation with 1 mM dithiothreitol for 5 min reduced approximately 36% of the 5′-azides on RNAs 11–13 bases long and approximately 43% on RNAs 28–37 bases long but practically none of the 5′-azides on RNAs 40–43 bases long. Also notable was the reduction of 34 ± 1% of the 5′-azides on RNA 12 bases long but only 14 ± 2% on the 14-base RNA; on the T7 A1 promoter, the leading end of the transcript diverges from the DNA template when the chain is between 12 and 14 bases long.

DNA-dependent RNA polymerases catalyze the synthesis of ribonucleic acid, using deoxyribonucleic acid as a template (Losick & Chamberlin, 1976; von Hippel et al., 1984; McClure, 1985). These enzymes are usually large, multisubunit assemblies; *Escherichia coli* RNA polymerase contains five major subunits, with a total M_r of 449K. The "core" enzyme consists of subunits β' (M_r 155 162; Ovchinnikov et al., 1982) and β (M_r 150 619; Ovchinnikov et al., 1981) and two α subunits (M_r 36 512; Ovchinnikov et al., 1977). The core enzyme is capable of elongating RNA but does not specifically initiate transcription at promoter sites on DNA. The RNA polymerase holoenzyme contains the core plus the

† Supported by Research Grant GM 25909 to C.F.M. from the National Institute of General Medical Sciences, National Institutes of Health.

0006-2960/86/0425-6397$01.50/0

dissociable subunit σ (M_r 70 263; Burton et al., 1981), which is required for specific initiation of transcription.

For some time we have been interested in the path that nascent RNA follows through the transcription complex (DeRiemer & Meares, 1981a,b; Hanna & Meares, 1983a,b). Using photoaffinity-labeling methods, with a light-activated aryl azide positioned at the leading (5′) end of nascent RNA, we have mapped that path at the level of resolution of the enzyme subunits and DNA, using the A1 promoter of bacteriophage T7 deletion mutants ΔD111 and ΔD123 (Hanna & Meares, 1983a,b). When the RNA is 3–12 bases long, the DNA template receives most of the photoaffinity labeling. In addition, for trinucleotide RNA, the β and σ subunits are just detectably labeled but not β' or α, and for 4–12 nucleotide RNA, the β and β' subunits are detectably labeled but not α or σ. For RNA more than 12 nucleotides long (and as long as 94 nucleotides on T7 ΔD123), the β and β' subunits are heavily labeled, and no significant labeling of the other macromolecules occurs (even for RNAs as long as 116 nucleotides).[1] These observations are consistent with many different experiments performed in other laboratories on binary enzyme–DNA or ternary enzyme–DNA–RNA complexes, which have been reviewed by von Hippel et al. (1984).

A question that follows from these studies is when does the nascent transcript become available to interact with other molecules in solution? Nascent RNA is thought to interact with a number of regulatory proteins while still in the transcription complex (Yanofsky, 1981; Chamberlin et al., 1986), and of course messenger RNA is destined eventually to bind to ribosomes. The results of Yanofsky and co-workers imply that nascent tryptophan synthetase mRNA in a transcription complex is available to bind ribosomes and guide protein synthesis under conditions where there is little separation between the ribosome and the trailing (3′) end of the polymerase-bound RNA [Landick et al. (1985) and references cited therein].

In earlier studies, Kumar and Krakow (1975) found that a 12-base fragment of nascent poly[r(A-U)] in an *Azotobacter vinelandii* RNA polymerase/poly[d(A-T)] transcription complex was protected from ribonuclease A digestion. Recently Levin (1985) has found that at least 18 nucleotides at the 3′ end of RNA in *E. coli* RNA polymerase/T7 ΔD111 transcription complexes are protected from ribonuclease A. These results imply that at least some regions of nascent RNAs just upstream from the RNA/DNA hybrid are accessible to other molecules in solution.

Since the 5′ ends of RNAs as long as 94 nucleotides can have significant contact with the β and β' subunits of RNA polymerase (Hanna & Meares, 1983b), we have devised a photoaffinity-protection experiment to test the accessibility of the leading end of nascent RNA to small molecules in solution. The rationale for this experiment is based on the fact that aryl azides can be reduced (and thereby deactivated) by thiol reagents under very gentle conditions (Staros et al., 1978). As schematically illustrated in Figure 1, it is possible to compare the accessibility of the leading end of RNA in the transcription complex at various chain lengths by (1) dividing the sample into two aliquots, (2) reducing the more accessible azides in one aliquot with a thiol before irradiation, (3) measuring the amount of RNA of each chain length photoattached to $\beta\beta'$, and (4) comparing the results for the thiol-treated aliquot with those for the untreated control.

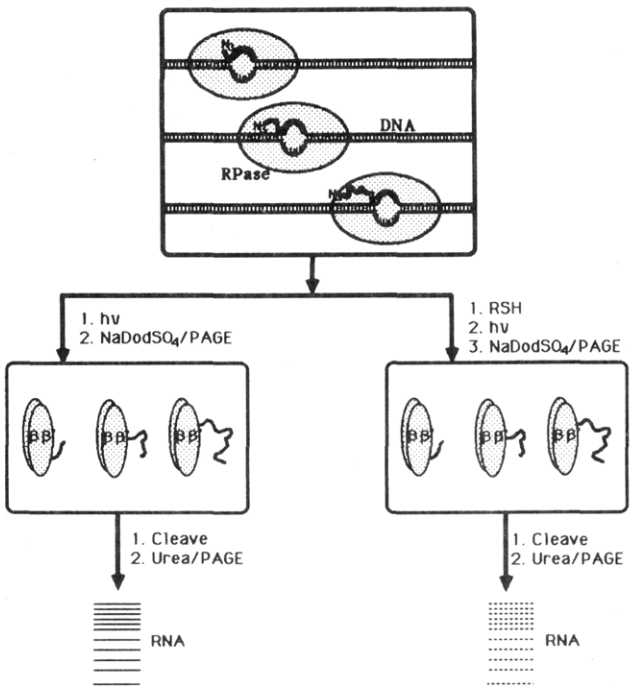

FIGURE 1: Diagram of experiments to test accessibility of the leading end of nascent RNA in transcription complexes. The experimental procedure is to first prepare transcription complexes containing a wide range of RNA lengths initiated with an aryl azide (N_3) photoaffinity probe, then divide the sample and treat half with a limited amount of a thiol compound (RSH) to reduce the azide groups on the more accessible RNA 5′ ends, and then irradiate both samples to cross-link intact azides to adjacent macromolecules, isolate the labeled β and β' enzyme subunits by NaDodSO$_4$/PAGE, cleave off the attached RNAs, and finally look for differences in the yield of RNA of each length photoattached to $\beta\beta'$ with and without the thiol treatment.

This procedure has some conceptual similarity to studies of protein binding to DNA by determining changes in the reactivity of sites on DNA, e.g., with respect to methylation by dimethyl sulfate (Mirzabekov et al., 1978; Siebenlist et al., 1980), ethylation by ethylnitrosourea (Siebenlist et al., 1980), or cleavage by methidiumpropyl-EDTA(Fe) (Van Dyke et al., 1982). In those studies, it is appropriate to compare DNA modification in the presence and absence of the protein. But here, the necessary comparison is $\beta\beta'$ photoaffinity labeling in the presence and absence of thiol. Since the photoaffinity-labeling yield is proportional to the amount of photoprobe, the percent decrease in this yield provides a direct measure of the percent of photoprobe lost by reaction with thiol. As described below, this quantity can be measured for a series of individual lengths of RNA.

We used the transcript from the A1 promoter of bacteriophage T7 ΔD111 DNA for these experiments. Preliminary evidence suggested that the ΔD111 transcript might form extensive secondary structure (J. R. Levin and M. J. Chamberlin, personal communication; Hanna, 1982), to which these experiments should be sensitive. The sequences of the ΔD111 and ΔD123 transcripts are identical for the first 23 nucleotides, and they give similar photoaffinity-labeling results (S. L. Bernhard, unpublished observations; Hanna, 1982; Hanna & Meares, 1983a,b).

Experimental Procedures

Materials

All reagents and solvents were of reagent grade and were

[1] Recent photoaffinity experiments using the λ P_R promoter show that the behavior of the σ subunit depends on the nature of the promoter (Bernhard & Meares, 1986). The λ P_R transcripts between 9 and 13 bases long clearly label σ. Otherwise the results are similar to those above; β and β' are heavily labeled by transcripts 11 or more bases long, and α is not labeled. The DNA was not examined for labeling.

used without further purification unless noted otherwise. Deionized, glass-distilled water was used throughout. Tris[2] was recrystallized from methanol, and acrylamide was recrystallized from chloroform before use. Ribonucleoside triphosphates, purchased from Sigma, were twice column purified before use (McClure et al., 1978). Kodak XAR film and Cronex Lightning-Plus intensifying screens were used for autoradiography, which was carried out at −80 °C.

Buffers. These were as follows: buffer A, 80 mM Tris-HCl, pH 7.9, 5 mM 2-mercaptoethanol, 0.1 mM Na_2EDTA, and 50% glycerol; buffer B, 10 mM Tris-HCl, pH 8.0, and 1 mM Na_2EDTA; buffer C, 50 mM Tris-HCl, pH 7.9, 5 mM 2-mercaptoethanol, 10 mM NaCl, 10 mM $MgCl_2$, and 5% (v/v) glycerol; buffer D, 0.5 mL of buffer F, 2.9 mL of 9–10 M urea, 0.5 g of sucrose, 12 mg of $NaDodSO_4$, 0.1 mL of 0.1% (w/v) bromphenol blue, and 10 mM DTT (added just before use); buffer E, in 1 L, 4.4 mL of ethanolamine, 4.5 g of glycine, and 1.0 g of $NaDodSO_4$, pH 9.7; buffer F, 18.6 mL of triethanolamine, 8 mL of concentrated HCl, and 96 g of urea in 200-mL total volume, pH 7.5; buffer G, 50 mM NaCl, 5 mM Tris-HCl (pH 8.0), 12 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 μg/mL bovine serum albumin (added just before use).

Cleavage Solution. First, 0.1% (w/v) $NaDodSO_4$ is saturated with phenylmercuric acetate by shaking with an excess of the pure solid for a minimum of 4 h at room temperature. Then the mixture is briefly centrifuged to settle the precipitate, and the precipitate is discarded. Just before use, 1 mg/mL soluble RNA is added as a carrier.

Methods

RNA polymerase was purified from *E. coli* MRE 600 cells according to the method of Burgess and Jendrisak (1975) as modified by Lowe et al. (1979). The enzyme was dialyzed against buffer A and stored at −20 °C. Protein concentrations were determined by the method of Bradford (1976) or Sedmak and Grossberg (1977).

DNA Template. Bacteriophage T7 ΔD111 stock and the *E. coli* C_1A host strain were kindly provided by Judith Levin and Michael Chamberlin. The phage was grown and harvested essentially according to the method of Yamamoto et al. (1970). The phage particles were concentrated with poly(ethylene glycol) and banded in a CsCl step gradient in a Beckman L2-65B ultracentrifuge (Maniatis et al., 1982). After isolation, phage particles were disrupted with $KDodSO_4$ (Schleif & Wensink, 1981); the partially purified DNA was extracted with phenol, dialyzed against buffer B, and stored at 4 °C. DNA concentration was determined by measuring absorbance at 260 nm (one A_{260} unit = 50 μg/mL).

Dinucleotide Photoaffinity Probe. 5′-[[(4-Azidophenacyl)thio]phosphoryl]adenylyl(3′–5′)uridine (N_3RSpApU, Figure 2) was synthesized as reported by Hanna and Meares (1983a).

Preparation for Transcription Experiments. A total of 12 different transcription reactions were run in a single experiment to give a distribution of RNA chain lengths (3–80-mers). For each of four sets of reactions, one base-specific RNA chain terminator was used at three concentrations to generate short, medium, and long RNA transcripts.

[2] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; NTP, a mixture of nucleoside triphosphates; GSH, glutathione; RPase, RNA polymerase; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; $KDodSO_4$, potassium dodecyl sulfate; ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; GTP, guanosine 5′-triphosphate; UTP, uridine 5′-triphosphate.

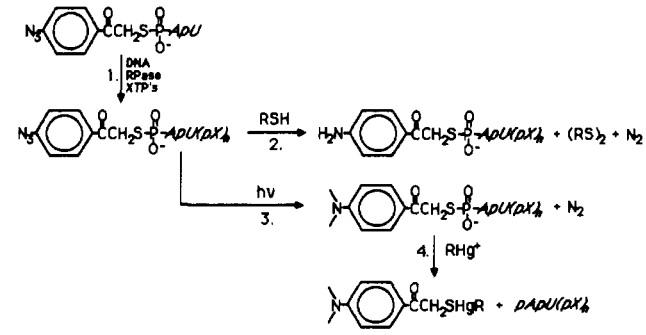

FIGURE 2: Chemical reactions employed in these experiments. Reaction 1: the dinucleotide probe (upper left) is incorporated into the 5′ end of RNA in a transcription complex. Reaction 2: accessible azides may be reduced by thiols. Reaction 3: irradiation converts azides into nitrenes, which nonselectively insert into chemical bonds on neighboring macromolecules. Reaction 4: the P–S bond is hydrolyzed in the presence of a mercurial, releasing the RNA from the cross-link.

First, two master mixes were prepared: an initiating NTP mix and an elongating NTP mix.

The initiating NTP mix was prepared from unlabeled ATP, CTP, GTP, and [α-^{32}P]GTP (note that UTP was omitted at this step). Once prepared and thoroughly mixed, this was aliquoted into 12 vials and stored on ice. Concentrations of the initiating NTPs were chosen to be 1 μM for ATP and 3 μM for CTP and GTP, after addition to an aliquot of the ternary complex mixture (described below). The amount of radioactivity used per reaction varied from 15 to 50 μCi, depending on how many thiol concentrations were being tested and whether the final RNA gels were to be analyzed qualitatively (visual inspection of autoradiograms) or quantitatively (cutting and counting gel slices).

The elongating NTP mix was composed of unlabeled ATP, CTP, GTP, and UTP. Twelve aliquots (three for each of the four base-specific terminators) were put into separate vials. The amount of substrate was chosen so that each reaction would contain 15 μM NTPs in a final reaction volume of 80–90 μL.

Base-specific terminators were added to aliquots of the elongating NTP mix in the following manner: Short RNA chain lengths (up to 20-mers) were prepared by using terminator/NTP ratios of 125/1 for 3′-deoxy-ATP/ATP, 100/1 for 3′-*O*-methyl-CTP/CTP, and 50/1 for 3′-*O*-methyl-GTP/GTP. Medium RNA chain lengths (21 to ~60-mers) were prepared by using ratios of 45/1 for 3′-deoxy-ATP/ATP and 3′-*O*-methyl-CTP/CTP, 20/1 for 3′-*O*-methyl-GTP/GTP, and 40/1 for 3′-*O*-methyl-UTP/UTP. Longer RNA chains were made by using 10/1 ratios for 3′-deoxy-ATP/ATP, 3′-*O*-methyl-CTP/CTP, and 3′-*O*-methyl-UTP/UTP and 5/1 for 3′-*O*-methyl-GTP/GTP.

Detailed Description of Transcription Experiments. For proper timing, the three A-terminated reactions were carried to completion (including irradiation), then the C-terminated reactions, and so on.

A ternary complex mix was prepared, containing 15 nM T7 ΔD111 DNA (15 nM A1 promoter), 100 nM *E. coli* RNA polymerase, and 100 μM N_3RSpApU, in buffer C. Once prepared and thoroughly mixed, this was aliquoted into four vials, one for each set of base-specific terminator reactions, and stored on ice in reduced light until needed. Ternary complex mixtures were incubated for 10 min at 37 °C in reduced light. At the end of this time, each ternary complex was aliquoted into three vials containing initiating NTPs (ATP, CTP, GTP); then elongating NTPs (premixed with terminator) were added to individual vials at 0.5, 1.0, and 1.5 min to

generate short, medium, and long RNA chains. Elongation was allowed to proceed for approximately 10 min at 37 °C. At the end of the elongation period, a 5-μL aliquot was removed from each reaction (this aliquot was run on an RNA sequencing gel to confirm the lengths of RNA made). The three reaction mixtures for a given terminator were pooled and then redivided as either control or thiol test samples. In our hands, the combination of three separate elongation reactions in this manner produced the best overall distribution of RNA lengths (reaction 1 in Figure 2).

For the thiol tests (reaction 2 in Figure 2), sulfhydryl reagents were added as tenfold concentrated stock solutions. An equal volume of water was used instead of thiol in untreated control reactions. Both thiol-treated and control samples were incubated at 25 °C for 5 min under reduced light. At the end of this time an aliquot was removed from each for an unirradiated control sample, and the remainder of the complex was transferred to a borosilicate tube and irradiated for 2 min in a Rayonet photochemical reactor ($\lambda > 300$ nm). Following irradiation (reaction 3 in Figure 2), each sample was denatured by addition of an equal volume of buffer D and allowed to stand in the dark at room temperature for 1 h. Samples then were frozen at −20 °C or loaded directly onto a protein gel.

Protein Gels. The protein gel used in these experiments was the $NaDodSO_4$–urea system of Wu and Bruening (1971). The gel was a 40 cm × 15 cm × 0.75 mm polyacrylamide step gradient. The four layers of the gel were a 1-cm 20% bottom layer, a 25-cm 7% lower layer, a 10-cm 3.5% upper layer, and a stacking layer. This preparative gel separated photoaffinity-labeled β and β' subunits of RNA polymerase from the DNA template and the other polymerase subunits but not from each other. The analyses that follow are based on the combined $\beta\beta'$ subunits. Gels were run for 8–10 h at 10–15 mA or until the bromphenol blue tracking dye reached the 20% polyacrylamide layer. After electrophoresis the gels were autoradiographed for a minimum of 12 h.

Electroelution and Cleavage. Photoaffinity-labeled $\beta\beta'$ subunits, located by autoradiography and by comparison with stained marker lanes, were excised from the protein gels and transferred to 1.5-mL polypropylene Eppendorf centrifuge vials. Gel slices in vials were counted (Cerenkov radiation) in a Beckman LS 6800 scintillation counter prior to electroelution.

Electroelution was carried out by placing individual gel slices plus 200 μL of buffer E into pieces of dialysis tubing. The dialysis bags were placed in a tray containing buffer E, and electroelution was performed at 50 mA, 4 °C, for 4–6 h. This procedure eluted roughly 40–50% of the ^{32}P from protein gel slices.

The eluted protein–RNA conjugates were transferred to Eppendorf vials. Each dialysis bag was rinsed with 200 μL of 1 mg/mL aqueous carrier RNA. The rinse was pooled with the original eluant and counted for ^{32}P and then applied to a 1-mL Sephadex G-50–80 gel filtraton column equilibrated with 0.1% $NaDodSO_4$. The column was centrifuged according to the method of Penefsky (1979) to elute the labeled macromolecules in 0.1% $NaDodSO_4$. The eluted sample (usually recovered with 80% efficiency) was Cerenkov counted and then treated with an equal volume of freshly prepared cleavage solution. Cleavage (reaction 4 in Figure 2) was allowed to proceed to completion (18–24 h) at room temperature in the dark. Samples were lyophilized (8 h, 20–50 mtorr) and then resuspended in 15 μL of 8 M urea (2 h at room temperature). Marker dyes were added [0.05% (w/v) bromphenol blue and xylene cyanol] and 10-μL aliquots were loaded onto RNA sequencing gels.

RNA Sequencing Gels. These were 40 cm × 15 cm × 0.75 mm 25% acrylamide gels containing 89 mM Tris–borate (pH 8.3), 1 mM EDTA, 7 M urea, and 1/29 methylenebis(acrylamide)/acrylamide (Carpousis & Gralla, 1980). Gels were allowed to polymerize overnight and were preelectrophoresed for 6 h at 1000 V before use. For analysis of short to medium RNA chain lengths, RNA gels were run at 1000 V for 12 h or until the bromphenol blue tracking dye was 27.5 cm from the bottom of the wells. For longer chain lengths, gels were run at 1300 V for 12 h or until the xylene cyanol tracking dye was 27.5 cm for the bottom of the wells. After electrophoresis, the gels were autoradiographed. For some experiments, calibration strips containing graded concentrations of ^{32}P were included so that film response could be related to radioactivity.

Quantitation of Photoaffinity Labeling. For both the 1 mM DTT experiments and the untreated controls, the extent of photoaffinity labeling was quantitated by cutting and counting gel slices. Lanes on the gels, visualized by autoradiography, were cut into 3 mm × 1 cm wide slices; the slices were transferred into Eppendorf vials and counted (Cerenkov radiation). For each length of RNA shown in Figure 5, the ratio of counts per minute in the treated and untreated gel bands was calculated; this yields $[azide]/[azide]_0$ for each RNA length. Corrections were made for differences in electroelution efficiency and recovery from the gel filtration columns of the photoaffinity-labeled $\beta\beta'$ subunits from which the RNA samples were derived. Standard deviations due to counting statistics were calculated by standard methods (Bevington, 1969).

As an additional approach to measure $\beta\beta'$ labeling, autoradiograms were scanned with a Zeineh SL-504-XL soft laser scanning densitometer equipped with a Hewlett-Packard 3390A integrator. Areas under peaks were compared with the results of scintillation counting, and generally good qualitative agreement was found. However, because the film showed a somewhat nonlinear response, only the scintillation counting results are reported here.

Runoff Transcription Experiments. For evidence to support the hypothesis that the transcript formed stable hairpin structure(s), we determined that the RNA could photoaffinity label itself. A 519 base pair restriction fragment of T7 ΔD111 DNA that included the A1 promoter sequence, and allowed a 61-base runoff transcript, was prepared by digesting intact T7 ΔD111 DNA with *Rsa*I in buffer G at 37 °C. Restriction fragments produced by the enzyme digest were separated on a 40-cm 5% nondenaturing polyacrylamide gel. A *Hin*cII digest of phage φX174 DNA served as a length marker. The gel was stained briefly with ethidium bromide to permit detection of the bands by ultraviolet light. The 519 base pair band was excised from the gel, electroeluted into 45 mM Tris–borate (pH 8.3)/0.5 mM EDTA, phenol extracted, and ethanol precipitated. The purified fragment was stored in buffer B at 4 °C. Concentration of the stock solution was estimated by ethidium bromide fluorescence (Maniatis et al., 1982).

Runoff transcripts initiated on this restriction fragment were prepared by a single round of transcription in the following way: 10 nM A1 promoter, 100 nM *E. coli* RNA polymerase, and 150 μM N_3RSpApU initiator were preincubated in buffer C for 5 min at 37 °C under reduced light. Initiating NTPs (ATP, CTP, GTP, and UTP, including 50 μCi of [α-^{32}P]ATP) were added to a final concentration of 5 μM each. After 1.25 min, heparin was added (0.1 mg/mL final concentration)

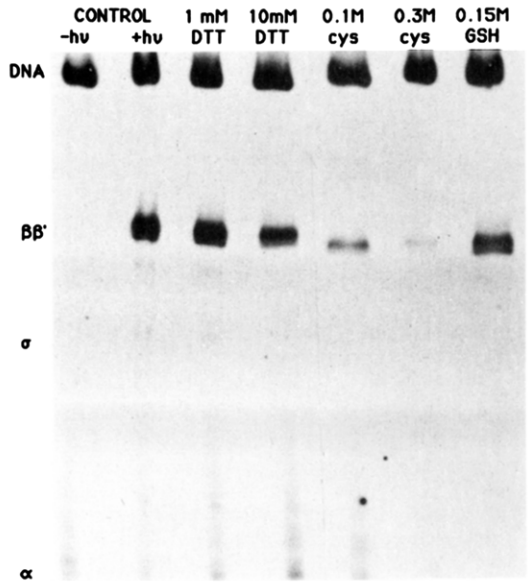

FIGURE 3: $NaDodSO_4$–urea polyacrylamide gel showing the effects of thiol treatment on photoaffinity-labeling yield. The unirradiated control lane shows only DNA (labeled by a side reaction), while the irradiated control lane shows maximum $\beta\beta'$ photoaffinity labeling. The other lanes (all irradiated) show the effects of 5-min treatments with the indicated thiols.

to the reaction mixture to prevent reinitiation. Elongation of the transcript was allowed to proceed for 10 min at 37 °C. A mixture of all four unlabeled ribonucleoside triphosphates (20 μM each, final concentration) was added, and an additional 5 min was allowed to complete the runoff transcripts. The final reaction volume was 60 μL, which was then split into six equal aliquots.

After transcription, samples that were to be photolyzed were transfered to borosilicate tubes; irradiation was for 2 min. All samples were denatured with 0.1% (w/v) $NaDodSO_4$, either with or without phenylmercuric acetate. All denatured samples were quenced with 100 μM DTT and allowed to stand in the dark at room temperature for 1 h. RNA gel electrophoresis and autoradiography were carried out as described above.

Results and Discussion

Effect of Thiol Structure and Concentration on Photoaffinity-Labeling Yield. The reduction of $\beta\beta'$ labeling in transcription complexes treated with DTT (a small neutral dithiol, M_r 154), cysteine (a monothiol, M_r 121), or glutathione (a negatively charged tripeptide monothiol, M_r 307) is illustrated in Figure 3. These reagents were chosen to explore the effect of changes in size, charge, and number of SH groups on azide reduction. In agreement with the work of Staros et al. (1978), the number of thiols proved to be the most significant. Figure 3 indicates that a 5-min incubation with 10 mM DTT has roughly the same effect as a 5-min incubation with 0.15 M glutathione; treatment with 0.1 M cysteine is somewhat more effective at reducing the photoaffinity-labeling yield. Because it was effective at the lowest concentrations, DTT was used for the more detailed experiments described below.

Staros et al. (1978) studied the reduction of several small aryl azides by thiol compounds; our findings are consistent with theirs. In particular, their second-order rate constant for reduction of 3-nitro-4-[(aminoethyl)amino]phenyl azide with DTT (k_2 = 0.41 M^{-1} s^{-1} at pH 8.0, 30 °C) provided a useful preliminary estimate of the extent of reduction to be expected in our experiments with transcription complexes.

Table I: Kinetics of Azide Reduction by DTT[a]

	[azide]/[azide]$_0$ ± standard deviation		
	1 mM DTT	10 mM DTT	n[b]
A lanes	0.583 ± 0.015	0.280 ± 0.004	4
C lanes	0.609 ± 0.005	0.198 ± 0.007	4
G lanes	0.531 ± 0.067	0.450 ± 0.094	4
U lanes	0.829 ± 0.030	0.430 ± 0.014	4
av	0.638 ± 0.022	0.340 ± 0.028	16
predicted from eq 2		0.011 ● 0.004	

[a] $\beta\beta'$ labeling exhibited by photoprobes at the leading ends of nascent RNAs in transcription complexes. Reductions were allowed to proceed for 5 min at room temperature, pH 7.9, before irradiation. [b] n is the number of experiments included in each determination.

For small molecules diffusing freely in solution, the rate of reduction of azides by DTT is first order with respect to each (Staros et al., 1978) so that the kinetic rate law is given by

$$-\mathrm{d[azide]}/\mathrm{d}t = k_2[\mathrm{DTT}][\mathrm{azide}] \quad (1)$$

If the DTT is present in great excess, its concentration remains nearly constant during the reaction. For small molecules in solution, the fraction of azide remaining at time t will be

$$[\mathrm{azide}]/[\mathrm{azide}]_0 = \exp(-k_2[\mathrm{DTT}]t) \quad (2)$$

Equation 2 predicts that raising the DTT concentration tenfold should reduce the fraction of azide remaining by a *power* of 10, for reactions between small molecules. On the other hand, we might expect that, for azides incorporated into RNA in transcription complexes, the environments of some would be different from others. If azides in transcription complexes differ in their accessibility to DTT, they would be expected to have different rate constants, so that k_2 would be replaced by a sum of rate constants. Such a heterogeneous population of azides would fail to obey eq 2.

As shown in Table I, reduction of azides at the 5′ end of nascent RNA does not follow the pseudo-first-order kinetics observed for small molecules. On comparison of the results of treatment with 1 and 10 mM DTT, it appears that in all cases there is a subpopulation of nascent RNAs in which the azide group is somewhat protected from the reagent. Use of the exact expression for second-order kinetics (Laidler & Meiser, 1982) leads to the same conclusion. Further, there are reproducible differences between the RNAs prepared with different base analogues as chain terminators; these are due to the relative amounts of various chain lengths produced in these reactions, which may be seen in Figure 4.

Relation between RNA Chain Length and Reduction of Photoaffinity Labeling. The data in Table I imply that the cross-linking of RNAs to enzyme subunits is sensitive to prior treatment with thiol, in a way that can vary with the chain length of RNA in the transcription complex. A unique feature of our experiments is that cross-linked RNAs can be quantitatively cleaved from the enzyme subunits and analyzed for length and abundance. Figure 4 shows an RNA gel autoradiogram comparing the populations of RNAs cleaved from $\beta\beta'$, with and without DTT treatment. RNAs from 11 to >50 bases long are represented. For each length of RNA, changes in intensity due to thiol treatment are directly related to the accessibility of the 5′ end. Notably, bands 40G and 41U are not diminished by the thiol treatment, while bands for RNAs 28–37 bases long have clearly reduced intensities.

Differences in accessibility are best revealed by quantitative analysis. Figure 5 shows the values of [azide]/[azide]$_0$ for all RNA lengths that could be reliably quantitated for 1 mM DTT-treated samples. There are variations in azide acces-

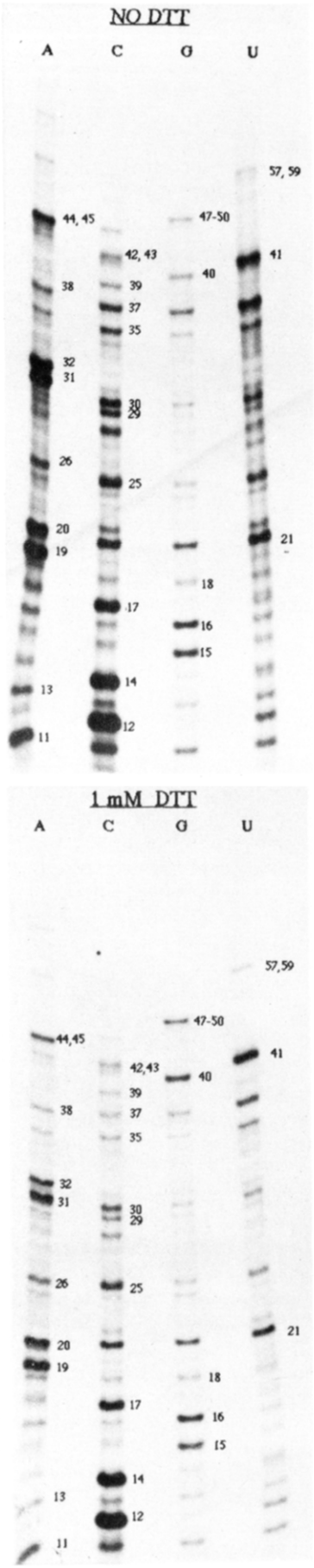

FIGURE 4: RNA gel autoradiograms showing the distribution of RNA chain lengths released from cross-links with $\beta\beta'$ by treatment with phenylmercuric acetate. Four base-specific chain terminators were used (A, C, G, U). Treatment with 1 mM DTT decreases the intensity of all the bands but not equally (e.g., 40G and 41U remain dark, while bands containing 28–37 bases are dimmed). See Figure 5.

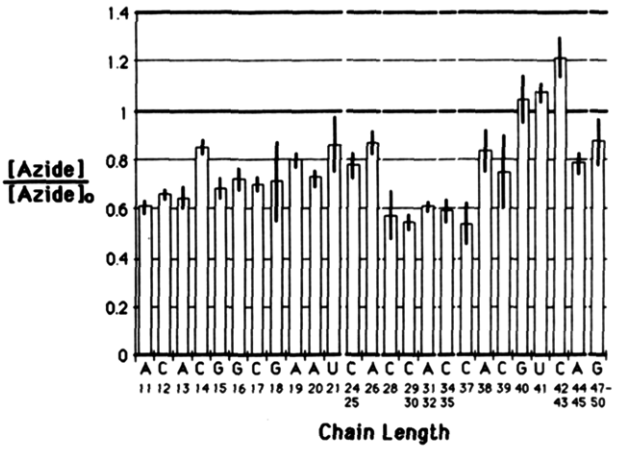

FIGURE 5: Quantitative comparison of the relative accessibility of the leading end of RNA in transcription complexes, as a function of chain length. As described under Methods, [azide]/[azide]$_0$ was determined by cutting and counting the RNA gels in Figure 4. Error bars indicate one standard deviation due to counting statistics.

sibility over the entire range of RNA lengths examined. In particular, for azides at the leading ends of RNAs 28–37 bases long, [azide]/[azide]$_0$ = 0.57 ± 0.03, indicating that the thiol reduces ≈40% of the azide. RNAs 40–43 bases long stand out clearly with [azide]/[azide]$_0$ = 1.12 ± 0.09, indicating that the 5′-azide is protected from reduction. Several independent experiments corroborate these results. Treatment with 10 mM DTT exerts a stronger effect, but the reduction in photoaffinity labeling leads to less reliable radioactive counting statistics (data not shown).

The results in Figure 5 provide useful insight about the surroundings of nascent RNA in transcription complexes. The leading end of the shortest RNA examined, 11 bases long, is clearly accessible to DTT. The experiments of Levin (1985) showed that RNA at least 18 nucleotides long is protected from ribonuclease A digestion in transcription complexes on the same DNA. The observed difference is consistent with the expectation that the small, neutral DTT molecule can more readily penetrate into restricted spaces on the transcription complex.

Previous photoaffinity experiments using the same DNA template have shown that the leading end of RNA 12 bases long is in contact with DNA, β, and β', while RNAs 14 or more bases long contact only β and β' (Hanna & Meares, 1983b). Our data indicate that the 14-mer is significantly protected from reduction, relative to the 12-mer. It may be that the 14-mer interacts with the proposed "rewindase" site (Gamper & Hearst, 1982), which acts to displace the RNA from the template and re-form the DNA double helix.

As the RNA elongates beyond the 14-mer, there are further changes in its accessibility, but no clear pattern emerges until the transcript reaches a length of 28 nucleotides. Figure 5 shows that the 5′ ends of chains 28–37 bases long are the most susceptible to reduction of all the lengths examined; this may indicate that they are not tightly bound to the surface of the enzyme. Protection increases markedly for chains 40–43 bases long, which are essentially unaffected by the 1 mM DTT treatment.

It is interesting to compare these results with the experiments of Levin (1985), who used calf intestine alkaline phosphatase treatment to study the accessibility of [^{32}P]-phosphate at the 5′ end of nascent RNA in similar transcription complexes. Levin found that the 5′-phosphate was not accessible to phosphatase until the RNA chain was at least 60 nucleotides long. Thus the 5′ ends of all the RNA chains examined here would be protected from phosphatase action.

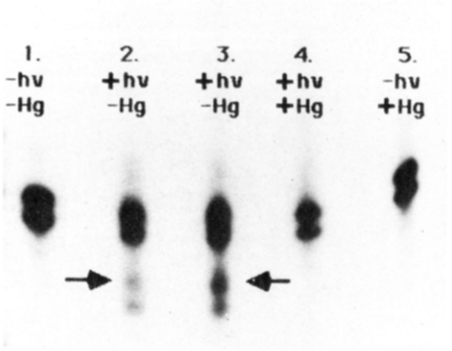

FIGURE 6: RNA gel autoradiogram showing self-labeling of runoff transcript. The normal runoff transcript (see Methods) migrates as a doublet in all the lanes. Lanes 1 and 5 contain unirradiated transcripts, untreated (lane 1) or treated with phenylmercuric acetate (lane 5), to remove the photoaffinity group. Lanes 2 and 3 contain irradiated but uncleaved transcripts and show the formation of new species. The faster migrating doublet produced by photoaffinity labeling (arrows) is converted to normal mobility by treatment with phenylmercuric acetate to cleave the P–S bond (lane 4).

Because they deal with photoaffinity-labeled β and β' subunits, our experiments provide information about the environment of RNA as it passes along the interface between those subunits. These results indicate that the 5′ ends of the shortest chains examined are acessible to DTT; this includes the 11- and 12-mers, for which the 5′ end of the RNA chain has not yet diverged from the DNA template (Hanna & Meares, 1983b).

There are a number of physical models that might account for the variation in accessibility to DTT that is observed for longer RNA chains. It is important to remember that the observed effects may be due to secondary structure in the RNA, to simple steric hindrance by other components of the transcription complex, or to some combination of these. Rigorously distinguishing among these causes will require (at least) knowledge of the particular sites on β and β' labeled by individual RNA chains.

A possible explanation for the observed protection of the 40–43 nucleotide transcript is suggested by the sequence of the first 30 nucleotides, which contains regions of dyad symmetry. This sequence allows a thermodynamically stable stem–loop structure (a hairpin) to form. The first 50 bases of the transcript have the sequence 5′-AUCGAGA*GGGA*CAC*GGC*GAAUA*GCCAUCCC*AA-UCCACACGUCCAACGGGG, where the italicized bases can form the stem of a hairpin. According to the rules of Tinoco et al. (1973), $\Delta G° \approx -15$ kcal/mol for formation of this stem–loop structure. Since the RNA–DNA hybrid in the transcription complex is thought to occupy about 12 base pairs (Hanna & Meares, 1983b; von Hippel et al., 1984), the 40–43 base RNA is approximately long enough to form both the complete hybrid duplex and a 30-base hairpin. For example, for a transcript 43 nucleotides in length, if bases 8–30 form the stem and loop of the hairpin, then on the 3′ side of the hairpin up to 13 bases (31–43) could remain base paired to the coding strand of the DNA; on the other side, the probe at the 5′ end of the transcript could presumably be placed in different surroundings by formation of such a structure.

Indirect evidence for the formation of such a hairpin by the T7 ΔD111 transcript has been observed by Levin (1985), who noted strong transcriptional pause sites at positions 37, 39, and 40. Transcriptional pausing has been correlated with the synthesis of a stable hairpin structure on many templates (Adhya & Gottesman, 1978; Rosenberg & Court, 1979; Yanofsky, 1981; Farnham & Platt, 1981; Platt, 1981; Reisbig & Hearst, 1981; Landick & Yanofsky, 1984; Levin, 1985), though other factors are also cited. Additional evidence for secondary structure in the T7 ΔD111 transcript has been provided by earlier experiments from this laboratory (Hanna, 1982; Hanna & Meares, 1983b), in which there was some indication that probes on the 5′ ends of ΔD111 transcripts approximately 45–50 nucleotides long could contact the DNA template.

To search for further evidence concerning the existence of secondary structure in this RNA sequence, we examined a 61-nucleotide runoff transcript, initiated with the photoaffinity probe N_3RSpApU. Any folded RNA structures can form intramolecular cross-links upon irradiation, and RNAs containing cross-links will have altered mobility on RNA sequencing gels; furthermore, cleavage of the P–S bond in the cross-link with phenylmercuric acetate should approximately restore the original mobility. As shown in Figure 6, irradiation of this RNA does indeed lead to the appearance of new bands, which are eliminated by treatment with phenylmercuric acetate. Such self-labeling is not observed with all RNA photoprobes, but neither is it uncommon in our experience. We interpret these results to indicate the formation of RNA secondary structure but not to prove that a particular structure is formed. Futher experiments, including the identification of the site(s) of RNA self-labeling, might serve to narrow the range of possibilities.

In future applications, this experimental approach should permit the detection of molecular properties not otherwise revealed by photoaffinity labeling. For example, the binding of auxiliary proteins could change the accessibility of the 5′ end of RNA without materially changing the photoaffinity-labeling pattern. It will be interesting to investigate RNA accessibility in transcription complexes on other promoters, such as the A1 promoter of phage T7 ΔD123, whose transcript should differ in secondary structure (Levin, 1985), or λ P_R, whose transcript interacts with regulatory macromolecules (Lewin, 1978).

ACKNOWLEDGMENTS

We thank Michelle Hanna, Judith Levin, and Michael Chamberlin for gifts of material and helpful discussions on many aspects of this work, Michael McCall for preparation of the photoaffinity probe, Michael Dahmus and Deborah Cadena for help with the scanning densitometer, and Gloria Marquez for excellent secretarial assistance.

Registry No. DTT, 3483-12-3; GSH, 70-18-8; N_3RSpApU, 85977-19-1; L-Cys, 52-90-4.

REFERENCES

Adhya, S., & Gottesman, M. (1978) *Annu. Rev Biochem. 47*, 967–996.

Bernhard, S. L., & Meares, C. F. (1986) *Biochemistry 25*, 5910–5914.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, Chapter 4, McGraw-Hill, San Francisco.

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

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4636.

Burton, Z., Burgess, R. R., Lin, J., Moore, D., Holder, S., & Gross, C. A. (1981) *Nucleic Acids Res. 9*, 2889–2903.

Carpousis, A. J., & Gralla, J. D. (1980) *Biochemistry 19*, 3245–3253.

Chamberlin, M. J., Arndt, K., Briat, J.-F., Helmann, J., Reynolds, R. R., Schmidt, M. C., & Singer, V. L. (1986) *Welch Foundation Conferences on Chemical Research*, Vol.

XXIX, Welch Foundation, Houston (in press).
DeRiemer, L. H., & Meares, C. F. (1981a) *Biochemistry 20*, 1606–1612.
DeReimer, L. H., & Meares, C. F. (1981b) *Biochemistry 20*, 1612–1617.
Farnham, P. J., & Platt, T. (1981) *Nucleic Acids Res. 9*, 563–577.
Gamper, H. B., & Hearst, J. E. (1982) *Cell (Cambridge, Mass.) 29*, 81–90.
Hanna, M. M. (1982) Ph.D. Dissertation, University of California, Davis.
Hanna, M. M., & Meares, C. F. (1983a) *Biochemistry 22*, 3546–3551.
Hanna, M. M., & Meares, C. F. (1983b) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4238–4242.
Kumar, S. A., & Krakow, J. S. (1975) *J. Biol. Chem. 250*, 2878–2884.
Laidler, K. J., & Meiser, J. H. (1982) *Physical Chemistry*, p 356, Benjamin/Cummings, Menlo Park, CA.
Landick, R., & Yanofsky, C. (1984) *J. Biol. Chem. 259*, 11550–11555.
Landick, R., Carey, J., & Yanofsky, C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4663–4667
Levin, J. R. (1985) Ph.D. Dissertation, University of California, Berkeley.
Lewin, B. (1978) *Gene Expression*, Vol. 3, Chapter 4, Wiley, New York.
Losick, R., & Chamberlin, M., Eds. (1976) *RNA Polymerase*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Lowe, P. A., Hager, E. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McClure, W. R. (1985) *Annu. Rev. Biochem. 54*, 171–204.
McClure, W. R., Cech, C. L., & Johnston, D. E. (1978) *J. Biol. Chem. 253*, 8941–8948.
Mirzabekov, A. D., Shick, V. V., Belyavsky, A. V., Karpov, V. L., & Bavykin, S. G. (1978) *Cold Spring Haror Symp. Quant. Biol. 42*, 149–155.
Ovchinnikov, Yu. A., Lipkin, V. M., Modyanov, N. N., Chertov, O. Yu., & Smirnov, Yu. V. (1977) *FEBS Lett. 76*, 108–111.
Ovchinnikov, Yu. A., Monastyrskaya, G. S., Gubanov, V. V., Guryev, S. O., Chertov, O. Yu., Modyanov, N. N., Grinkevich, V. A., Makarova, I. A., Marchenko, T. V., Polovnikova, I. N., Lipkin, V. M., & Sverdlov, E. D. (1981) *Eur. J. Biochem. 116*, 621–629.
Ovchinnikov, Yu. A., Monastyrskaya, G. S., Gubanov, V. V., Guryev, S. O., Salomatina, I. S., Shuvaeva, T. M., Lipkin, V. M., & Sverdlov, E. D. (1982) *Nucleic Acids Res. 10*, 4035–4044.
Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.
Platt, T. (1981) *Cell (Cambridge, Mass.) 24*, 10–23.
Reisbig, R. R., & Hearst, J. E. (1981) *Biochemistry 20*, 1907–1918.
Rosenberg, M., & Court, D. (1979) *Annu. Rev. Genet. 13*, 319–353.
Schleif, R. F., & Wensink, P. C. (1981) *Practical Methods In Molecular Biology*, pp 100–101, Springer Verlag, New York.
Sedmak, J. J., & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.
Siebenlist, U., Simpson, R. B., & Gilbert, W. (1980) *Cell (Cambridge, Mass.)* 20, 269–281.
Staros, J. V., Bayley, H., Standring, D. N., & Knowles, J. R. (1978) *Biochem. Biophys. Res. Commun. 80*, 568–572.
Tinoco, I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M., & Gralla, J. (1973) *Nature (London), New Biol. 246*, 40–41.
Van Dyke, M. W., Hertzberg, R. P., & Dervan, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5470–5474.
von Hippel, P. H., Bear, D. G., Morgan, W. D., & McSwiggen, J. A. (1984) *Annu. Rev. Biochem. 53*, 389–446.
Wu, G. J., & Bruening, G. E. (1971) *Virology 46*, 596–612.
Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L., & Treiber, G. (1970) *Virology 40*, 734–744.
Yanofsky, C. (1981) *Nature (London) 289*, 751–758.