Niemann, H., Watanabe, K., Hakomori, S., Childs, R. A., & Feizi, T. (1978) *Biochem. Biophys. Res. Commun. 81*, 1286–1293.
Nudelman, E. D., Mandel, U., Levery, S. B., Kaizu, T., & Hakomori, S. (1989) *J. Biol. Chem.* (in press).
Okada, Y., Kannagi, R., Levery, S. B., & Hakomori, S. (1984) *J. Immunol. 133*, 835–842.
Stellner, K., Watanabe, K., & Hakomori, S. (1973a) *Biochemistry 12*, 656–661.
Stellner, K., Saito, H., & Hakomori, S. (1973b) *Arch. Biochem. Biophys. 155*, 464–472.
Stoffel, W., & Hanfland, P. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 21–31.
Svennerholm, L. (1965) *Acta Chem. Scand. 19*, 1506–1507.
Tai, T., Yamashita, K., & Kobata, A. (1975) *J. Biochem. (Tokyo) 78*, 679–686.
Taki, T., Matsuo, K., Yamamoto, K., Matsubara, T., Hayashi, A., Abe, T., & Matsumoto, M. (1988) *Lipids 23*, 192–198.
Uemura, K., Childs, R. A., Hanfland, P., & Feizi, T. (1983) *Biosci. Rep. 3*, 577–588.
Vliegenthart, J. F. G., Dorland, L., & van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209–373.
Watanabe, K., & Hakomori, S. (1976) *J. Exp. Med. 144*, 644–653.
Watanabe, K., Laine, R. A., & Hakomori, S. (1975) *Biochemistry 14*, 2725–2733.
Watanabe, K., Powell, M., & Hakomori, S. (1978) *J. Biol. Chem. 253*, 8962–8967.
Watanabe, K., Powell, M. E., & Hakomori, S. (1979a) *J. Biol. Chem. 254*, 8223–8229.
Watanabe, K., Hakomori, S., Childs, R. A., & Feizi, T. (1979b) *J. Biol. Chem. 254*, 3221–3228.
Young, W. W., Jr., Portoukalian, J., & Hakomori, S. (1981) *J. Biol. Chem. 256*, 10967–10972.
Zhu, B. C. R., & Laine, R. A. (1985) *J. Biol. Chem. 260*, 4041–4045.

Release of the σ Subunit from *Escherichia coli* RNA Polymerase Transcription Complexes Is Dependent on the Promoter Sequence†

Thomas M. Stackhouse,‡ Alice P. Telesnitsky,§ and Claude F. Meares*,‡

Department of Chemistry, University of California, Davis, California 95616, and Department of Biochemistry, University of California, Berkeley, California 94720

Received February 27, 1989; Revised Manuscript Received May 30, 1989

ABSTRACT: The σ subunit of bacterial RNA polymerase is required for the specific initiation of transcription at promoter sites. However, σ is released from the transcription complex shortly after transcription is initiated, and elongation proceeds in the absence of σ. In order to study the position of σ release, we have developed a method to quantify the photoaffinity labeling produced by an aryl azide positioned at the leading (5′-) end of nascent RNA, as a function of the transcript length [Stackhouse, T. M., & Meares, C. F. (1988) *Biochemistry 27*, 3038–3045]. Here we compare photoaffinity labeling of transcription complexes containing three natural bacteriophage promoters (λ P_R, λ P_L, and T7 A1) and two recombinant constructs, A1/P_R (T7 A1 promoter with the λ P_R transcribed region) and P_R/A1 (λ P_R promoter with the T7 A1 transcribed region). Significant photoaffinity labeling of the σ subunit was observed only on the templates containing the λ P_R promoter region, regardless of the sequence of the transcribed region. These results indicate the molecular interactions responsible for the position of σ release from the transcription complex mainly involve the nucleotide sequence of the promoter region—rather than the transcribed region—of the DNA template. Further studies on transcription complexes containing the A1/P_R and the P_R/A1 templates were performed, using polyclonal antibodies against the holoenzyme or against the σ subunit. These experiments corroborate the promoter dependence of σ release. They also show a correlation between the release of σ and stable binding of the transcript by the transcription complex.

Much of our understanding of the control of gene expression at the level of transcription has evolved from studies of *Escherichia coli* RNA polymerase (Burgess, 1976; Lewin, 1983). RNA polymerase from *E. coli* contains five major subunits, with a total molecular weight of 449 068. The primary structures of all the subunits have been determined: α (M_r 36 512; Ovchinnikov et al., 1977); β (M_r 150 619; Ovchinnikov et al., 1981); β' (M_r 155 162; Ovchinnikov et al., 1982); and σ (M_r 70 263; Burton et al., 1981). The *core enzyme* contains four subunits ($\alpha_2\beta\beta'$) and is capable of transcriptional elongation. However, specific initiation of a transcript at a promoter site requires the *holoenzyme*, which contains the core enzyme and the σ subunit. In addition to the predominant σ, other σ factors have been discovered in *E. coli* and other bacteria, all of which use the same core enzyme but require unique promoter DNA sequences to initiate transcription. The presence of another subunit (ω) has also been observed [Gentry & Burgess (1986) and references cited therein]; the function of ω is not yet established.

In vivo, only one-fourth to one-half of bacterial RNA polymerase is found as the holoenzyme. This observation, as well

†Supported by Research Grant GM25909 to C.F.M. from the National Institute of General Medical Sciences, NIH.
‡University of California, Davis.
§University of California, Berkeley.

0006-2960/89/0428-7781$01.50/0

as the experiments of Travers and Burgess (1969) analyzing transcription initiation from bacteriophage T7 and T4 DNA, led to the hypothesis that σ can be released shortly after the initiation of transcription and subsequently can bind to another core enzyme to initiate another transcript. Experiments using fluorescence depolarization spectroscopy support this hypothesis (Wu et al., 1975).

The binding of σ increases the specific binding of RNA polymerase to promoters and decreases nonpromoter binding (Hinkle & Chamberlin, 1972; Strauss et al., 1980; Kadesch et al., 1980; Hawley & McClure, 1982; Roe et al., 1984; Hawley & McClure, 1983; McClure, 1985). The association of σ with promoter DNA is evident from cross-linking experiments (Simpson, 1979; Chenchick et al., 1981; Park et al., 1982a,b); σ can be cross-linked to the region of the *lac* UV5 template between −37 and −35, and to −18, −17, −5, and −3. Genetic studies have shown that certain σ mutants are allele-specific suppressors of promoter mutations (Helmann & Chamberlin, 1988). This further supports a direct interaction of σ with DNA (Doi & Wang, 1986).

At the beginning of transcription, the RNA polymerase–DNA transcription complex enters an abortive initiation phase where short oligonucleotides are transcribed and released from the complex. It is believed the abortive initiation phase ceases when the σ subunit is released from the initiation complex, forming a stable *elongation complex* containing the $\alpha_2\beta\beta'$ core enzyme, DNA template, and nascent RNA (Hansen & McClure, 1980). The elongation complex may subsequently bind other factors involved in control of transcription and translation (Chamberlin, 1974; McClure, 1985; Greenblatt et al., 1987; Barik et al., 1987). Therefore, it is important to understand the interactions responsible for σ release during transcription and their role in controlling gene expression.

To investigate the sequence dependence of RNA interactions with the σ subunit, and thereby study the transcript position at which σ ceases to be associated with the ternary transcription complex, Stackhouse and Meares (1988) developed a *quantitative* analysis of photoaffinity labeling. This allows a direct comparison of the photolabeling of RNA polymerase subunits when different DNA templates are used. We have applied this procedure, along with a complementary set of immunoprecipitation experiments, to investigate the nucleic acid sequence dependence of σ release from *E. coli* transcription complexes containing several natural and recombinant DNA sequences.

Experimental Procedures

Materials

Reagents. All reagents and solvents were the purest available and were used without further purification unless otherwise noted. Nanopure water (Barnstead) was used throughout. *E. coli* MRE 600 cells were purchased from Grain Processing Corp. Bactotryptone and yeast extract were from Difco. HPLC-purified ribonucleoside triphosphates were purchased from ICN. Ultrapure urea and acrylamide were purchased from Schwartz-Mann. Soluble RNA (yeast tRNA), phenylmercuric acetate, dithiothreitol, tetramethylethylenediamine, bis(acrylamide), cordycepin triphosphate (3′-deoxy-ATP, a chain-terminating ATP analogue), agar, Tris,[1] ethidium bromide, and agarose were purchased from Sigma. *p*-Azidophenacyl bromide was from Pierce, adenosine 5′-*O*-thiophosphate was from Boehringer-Mannheim, and 3′-*O*-methyl-UTP, 3′-*O*-methyl-CTP, and 3′-*O*-methyl-GTP (RNA chain terminators) were from P-L Biochemicals. [α-^{32}P]GTP and [α-^{32}P]CTP were purchased from Amersham. The pUC19 plasmid was from BRL. RNasin was from Promega-Biotec. Acetylated bovine serum albumin (BSA), T4 polynucleotide kinase, T4 ligase, calf intestinal phosphatase (CIP), the Klenow fragment of DNA polymerase I, and the restriction endonucleases with their respective buffers were purchased from Bethesda Research Laboratories. Protein A–Sepharose 4B was obtained from Sigma. Kodak XAR films were used without intensifying screens for autoradiography. The photoprobe N_3RSpApU was synthesized as reported by Hanna and Meares (1983a).

Buffers. These were as follows: *buffer A* contained 80 mM Tris-HCl (pH 7.9), 5 mM 2-mercaptoethanol, 0.1 mM Na_2EDTA, and 50% (v/v) glycerol; *buffer B* contained 10 mM Tris-HCl, pH 8.0, and 1 mM Na_2EDTA; *buffer C* contained 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* contained (in a final volume of 4 mL) 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 dithiothreitol (added just prior to use); *buffer E* contained, 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* contained 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* contained 89 mM Tris–borate (pH 8.3), 1 mM Na_2EDTA, 7 M urea; *buffer H* contained 15 mM Tris-HCl, pH 8.5, 10 mM glycine, 0.06% $NaDodSO_4$; *buffer I* contained 50 mM Tris-HCl, pH 7.4, 150 mM NaCl; *buffer J* contained 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween, 2 mM DTT, 30 units of RNasin, and 25 mg/mL phenylmethanesulfonyl fluoride; *buffer K* contained, in buffer G, 0.1% (w/v) bromphenol blue and xylene cyanol; *buffer L* contained 89 mM Tris–borate (pH 8.3) and 1 mM Na_2EDTA.

Methods

RNA Polymerase. The 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). It was dialyzed against buffer A and stored at −20 °C. Protein concentration was determined by the method of Burgess (1976).

σ. The σ overproducer plasmid pMRG8 was a gift from the laboratory of Richard R. Burgess. The growth and purification of σ was carried out according to the method of Gribskov and Burgess (1983). The concentration of σ was determined by using the method of Lowe et al. (1979).

DNA Templates. The DNA containing the λ P_R and λ P_L templates was obtained from plasmid pGW7, which was a gift from the laboratory of William Konigsberg. The T7 A1 template was obtained from plasmid pAR1707 (Studier & Rosenberg, 1981). Each plasmid was transformed into an *E. coli* JM83 host. The growth of the bacteria containing pGW7 was at 28 °C, whereas the growth of pAR1707 was at 37 °C. Both plasmids were isolated by alkali lysis and a CsCl density gradient (Maniatis et al., 1982). Isolated pGW7 was digested with *Eco*RI and *Hin*dIII, and the fragments containing the λ P_R (−565 to +495, where the start site of transcription is +1) and the λ P_L (−5069 to +1313) wild-type templates were isolated by electrophoresis in 0.8% agarose followed by electroelution and extraction with phenol/chloroform. Isolated pAR1707 was restricted with *Sal*I, and the linearized plasmid containing the T7 A1 promoter and early transcribed region

[1] Abbreviations: DTT, dithiothreitol; Na_2EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; N_3RSpApU, 5′-[(4-azidophenacyl)thiophosphoryl]adenylyl(3′–5′)uridine.

(−4337 to +501) was also purified by electrophoresis in 0.8% agarose followed by electroelution and extraction with phenol/chloroform. The DNA concentration was determined by measuring absorbance at 260 nm (one A_{260} unit = 50 μg/mL). The DNA was stored in buffer B, at −20 °C.

The recombinant constructs were made by hybridizing two synthetic oligonucleotides (produced from a DNA synthesizer) with 12 base pairs of 3′-terminal complementarity, according to Oliphant and Struhl (1987). One ss oligonucleotide contained the promoter sequence of the template (λ P_R or T7 A1), and the other contained the transcribed sequence of the template (T7 A1 or λ P_R), respectively (Figure 2). The single-stranded regions of the hybridized oligonucleotides were filled in by using the Klenow fragment of DNA polymerase, and the products were treated with polynucleotide kinase to add the 5′-phosphate. The double-stranded DNA fragments containing the desired sequences (from −60 through +30 for A1/P_R and −57 to +29 for P_R/A1) were purified by electrophoresis through 10% polyacrylamide, followed by electroelution and extraction with phenol/chloroform. The DNA concentrations were determined by measuring the absorbance at 260 nm. The DNA was stored in buffer B, at −20 °C. Each of the two fragments was then blunt-end-ligated into a pUC19 vector, which had been restricted with *Sma*I, and transformed into an *E. coli* JM83 host. The insertion was verified by restriction mapping. The plasmids were isolated by using alkali lysis and CsCl centrifugation and restricted with *Pvu*II. The *Pvu*II fragments (−173 to +252 for A1/P_R, −170 to +251 for P_R/A1) were purified by electrophoresis through 0.8% agarose, followed by electroelution and extraction with phenol/chloroform. The sequence of the insert was verified by the method of Maxam and Gilbert (1977). The DNA was stored in buffer B, at −20 °C. The purified 425 bp fragments, one containing the P_R/A1 template and the other containing the A1/P_R template, were used in the transcription experiments described below.

Preparation of Transcription Reactions. All transcription reactions were carried out in reduced light. Four separate transcription reactions were carried out, one each to terminate the transcript at adenylate, uridylate, guanidylate, or cytidylate residues. The main goal was to produce an evenly distributed set of RNA lengths in the transcript mix so that all were represented for photoaffinity labeling or immunoprecipitation.

First, the *preinitiation mix* was prepared by combining (in a total volume of 33.0 μL) 0.61 μM RNA polymerase, 0.15 μM DNA template, and 470 μM N_3RSpApU, in buffer C. This was incubated at 37 °C for 10 min to allow the formation of a complex. Depending on the template, a *pulsed mix* was made by adding 10 μL of [α-^{32}P]GTP or CTP (>3000 Ci/mmol) in buffer C to the preinitiation mix to bring the GTP or CTP concentration to 3 μM and incubating at 37 °C for 30 s. A 7-μL aliquot from the *pulsed mix* was added to each of four elongation mixes (see below), forming four complete 15-μL reaction mixtures.

For transcription from templates containing the λ P_R transcribed region, each final elongation mix contained 18 μM ATP, 18 μM UTP, 18 μM CTP, and 6 μM [α-^{32}P]GTP (>500 Ci/mmol) in buffer C. The concentrations of the RNA chain terminators were as follows: elongation mix A, 1.0 mM cordycepin triphosphate; elongation mix U, 0.83 mM 3′-*O*-methyl-UTP; elongation mix C, 0.83 mM 3′-*O*-methyl-CTP; elongation mix G, 0.33 mM 3′-*O*-methyl-GTP.

For transcription from the templates containing the T7 A1 or λ P_L transcribed regions, each elongation mix contained 18 μM ATP, 18 μM UTP, 18 μM GTP, and 6 μM [α-^{32}P]CTP (>500 Ci/mmol) in buffer C. The concentrations of the RNA chain terminators were as follows: elongation mix A, 1.2 mM cordycepin triphosphate; elongation mix U, 0.80 mM 3′-*O*-methyl-UTP; elongation mix G, 0.70 mM 3′-*O*-methyl-GTP; elongation mix C, 0.33 mM 3′-*O*-methyl-CTP. These complete reaction mixtures were incubated for 10 min at 37 °C. At the end of this elongation period, samples were removed for protein and RNA analysis as described by Bernhard and Meares (1986), except that a Bio-Rad transblot apparatus was used for electroelution into buffer H. Quantification of transcripts photolabeled to the protein subunits of the transcription complex, by cutting and counting gels, was carried out as described by Stackhouse and Meares (1988).

Immunoprecipitation. Rabbit antisera against either holo RNA polymerase or the σ subunit were produced by Antibodies Inc. (Davis, CA). The antisera were characterized by using an enzyme-linked immunoassay (ELISA) and Western blotting. The anti-σ antiserum did not react with subunits α, β, β′, or ω under the conditions used here (data not shown).

The procedures for immunoprecipitation were essentially those of Barik et al. (1987). Transcription complexes were formed as described above except that the photoaffinity probe was replaced by the dinucleotide pApU and the concentration of the DNA was 2.4 μM. Ten microliters of each transcription reaction was added to 100 μL of chilled buffer J containing a 1:250 dilution of antiserum. Each antiserum mix (anti-σ or anti-holo) was treated identically, and each of the four terminated transcription mixes was analyzed separately. Therefore, each template had four anti-σ reactions (terminated at A, U, C, or G), and four anti-holo reactions for a complete analysis. The 110-μL mixtures were incubated on ice for 15 min and then transferred to 30 μL of a 50% (w/v) suspension of protein A–Sepharose 4B in buffer I. This was incubated on ice for 45 min with mild vortex mixing every 5 min to resuspend the resin. The suspension was then centrifuged for 2 min in a Beckman microcentrifuge, and the protein A–Sepharose pellet was washed three times with 200 μL each of ice-cold buffer J. The suspension was then washed a final time with 20 μL of buffer J and recentrifuged. As a check for complete washing, the last supernatant was added to an equal volume of buffer K containing 0.05% SDS and applied to an RNA sequencing gel. The pellet was treated with buffer K containing 0.05% SDS, vortex mixed, and heated to 37 °C for 30 min followed by 90 °C for 1 min. The samples were applied to the RNA sequencing gel and electrophoresed as described above.

The RNA sequencing gels from the immunoprecipitation experiments were analyzed on an AMBIS radioisotope scanner according to the manufacturer's instructions. The amount of radioactivity from each transcript length in the anti-holo precipitate was compared to those of the same transcript length in the total transcription reaction prior to immunoprecipitation, thus giving the percent yield of RNA in the precipitated complexes. This provides information on the stability of the complexes as a function of transcript length. The radioactivity from each transcript length in the anti-σ precipitate, compared to that in the unfractionated transcription reaction, gave the percent yield of RNA in the complexes containing the σ subunit. This provides a measure of the relative retention of σ as a function of transcript length but does not attempt to correct for the large number of abortively initiated transcripts at short RNA lengths present in the mixture.

Results

Photoaffinity Labeling of σ. Figure 1 shows the photoaffinity labeling yields on the σ subunit as a function of transcript

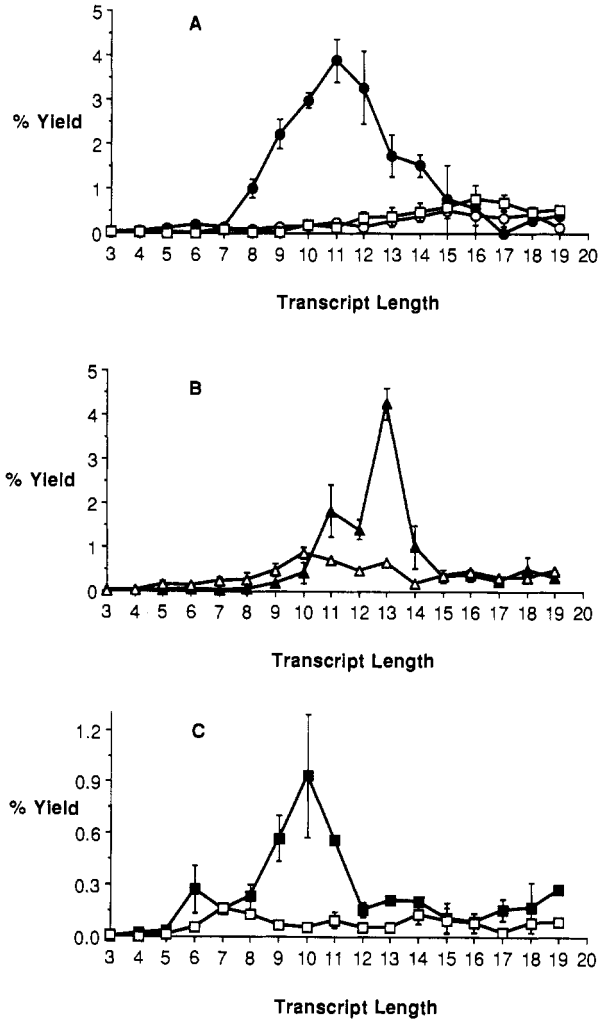

FIGURE 1: Percent photoaffinity labeling yield on σ as a function of transcript length, analyzed from transcription complexes containing the following: (A) λ P_R (filled circles), T7 A1 (open circles), or λ P_L (open squares) DNA; (B) P_R/A1 (filled triangles) or A1/P_R (open triangles) DNA. (C) Percent yield of RNA immunoprecipitated with polyclonal antibodies against the σ subunit, analyzed as a function of transcript length from transcription complexes containing the recombinant templates P_R/A1 (filled squares) or A1/P_R (open squares). In each case, the results of two independent experiments are shown (bars) along with their average.

length, for several DNA templates. This provides a quantitative analysis of what was seen on an autoradiogram of the RNA sequencing gel (data not shown). Figure 1A shows the results for transcription initiated from the three natural templates containing T7 A1, λ P_R, or λ P_L promoters. When transcribing from the λ P_R template, the σ subunit was labeled most heavily by transcript lengths of 8–14, with a maximum yield of 4% at 11 nucleotides. When transcription takes place from the λ P_L or the T7 A1 template, the yields from σ for all RNA lengths observed remained below 0.7%. A statistical t test indicated significant differences between the templates, with a confidence limit of 95% (Weinberg et al., 1981). These results show clearly that the photoaffinity labeling of the σ subunit is dependent on the nucleotide sequence of the DNA.

To determine the *region* of the template responsible for the difference in photolabeling, two recombinant templates were constructed. One of these contained the λ P_R promoter with the T7 A1 transcribed region (P_R/A1) and the other contained the T7 A1 promoter with the λ P_R transcribed region (A1/P_R), as shown in Figure 2. Comparing the photolabeling of σ on these hybrid templates allowed a determination of the relative influence of promoter sequences upstream from the transcription start site versus sequences in the initial transcribed region. Figure 1B shows the photoaffinity labeling yields from the σ subunit for the two recombinant templates A1/P_R and P_R/A1. The σ subunit was labeled most heavily by transcript lengths of 11, 12, and 13 when transcribing from the P_R/A1 template, with a maximum yield of 4% at a transcript length of 13 nucleotides. The σ subunit was labeled to a lesser extent on the A1/P_R template by transcripts with lengths of 9, 10, and 11, reaching a maximum yield of 1% at a transcript length of 10 nucleotides. These results indicate that the most important interactions leading to the photoaffinity labeling of σ involve the promoter region of the template, rather than the transcribed region.

Two lines of evidence from the photoaffinity experiments indicate that the nucleotide sequence within the transcribed portion of the template may play a minor role in controlling σ release. First, the transcript length at which the maximum yield of photoaffinity labeling was observed was not the same for the two templates containing the λ P_R promoter. Second, based on a paired t test with 95% confidence, the percent yields on σ for the two templates containing the T7 A1 promoter were statistically different; the wild-type T7 A1 showed less photo-cross-linking of the message to σ than did the recombinant A1/P_R.

Photoaffinity Labeling of the β/β' Subunits. Panels A and B of Figure 3 show the photoaffinity yields from β/β' (analyzed together) as a function of transcript length, when transcription was initiated with the wild-type templates (T7 A1, λ P_R, and λ P_L) or the recombinant templates (A1/P_R and P_R/A1). The photoaffinity labeling yields were initially similar for all five templates, remaining below 1% for transcripts 3–10 nucleotides long. All of the templates showed a dramatic increase in the percent yield on β/β', reaching a maximum at a transcript length of $\approx$14 nucleotides. However, the final yields on β/β' from the RNA containing 14–19 nucleotides were higher when transcribing from the T7 A1, λ P_R, and λ P_L templates ($\approx$15%) than when transcribing the recombinants A1/P_R and P_R/A1 ($\approx$9%). The possible reasons for this difference in photolabeling of β/β' are discussed below. The α subunit was not detectably labeled under these conditions.

Immunoprecipitation. Two possible causes could lead to the observed difference in photolabeling of the σ subunit when different promoter sequences were used to initiate transcription. First, the positioning of σ could be different on the complexes such that only the λ P_R template had σ in close proximity to the 5′-end of nascent RNA, leading to cross-linking of the message to σ. Alternatively, release of the σ subunit from the transcription complexes containing the T7 A1 or λ P_L promoters before the transcript is 8 nucleotides long could explain the lack of photoaffinity labeling, while retention of (at least some) σ on transcription complexes containing the λ P_R promoter until the transcript was at least 13 nucleotides long would lead to the observed results.

To distinguish between these possibilities, we examined the RNA from those transcription complexes stable enough to be immunoprecipitated by polyclonal antibodies to either holo RNA polymerase or the σ subunit, using the templates P_R/A1 and A1/P_R for comparison. Transcripts precipitated by anti-σ are shown Figure 1C. The results in Figure 1C show that less than 0.3% of any given length of the RNA synthesized from A1/P_R was precipitated by anti-σ. However, when transcription was initiated from the P_R/A1 template, a statistically different percentage of the transcripts (based on a paired t test analysis with 95% confidence) was precipitated by anti-σ at

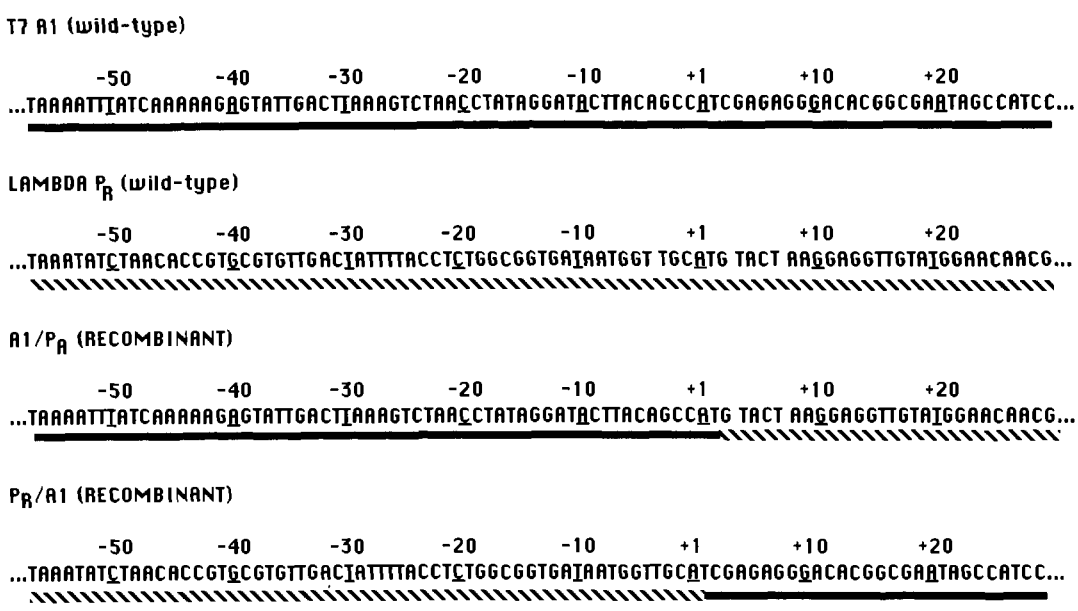

FIGURE 2: Nucleotide sequence of the promoter and early transcribed sequences of the T7 A1 (wild type, solid bar), λ P_R (wild type, hatched bar), and the two recombinant templates, $A1/P_R$ and $P_R/A1$. The bars below the sequences indicate the template of origin; for example, $A1/P_R$ contains the T7 A1 promoter and the λ P_R transcribed region. Details are given under Experimental Procedures.

RNA lengths of 9, 10, and 11, reaching a maximum of 1.1% at a transcript length of 10 nucleotides.

Formation of a Stable Elongating Transcription Complex. The abortive initiation reaction of RNA polymerase causes the production and release of large amounts of short oligonucleotides (Johnston & McClure, 1976; McClure & Cech, 1978; Carpousis & Gralla, 1980). In our experiments, the precent yield is a ratio of transcripts within the complexes, whether photo-cross-linked to the protein or immunoprecipitated by the antibodies, to the *total* RNA present in the transcription mixture. Abortive initiation may therefore have a large effect on percent yields where short oligonucleotides are concerned.

It has been generally assumed that the elongating transcription complex does not become stable to abortive initiation until it has released σ (Hansen & McClure, 1980). Since the above experiments make it clear that the release of σ is dependent on the nucleotide sequence of the promoter, it was logical to ask if the abortive initiation reaction is also template dependent. In order to investigate this question, the yields of RNA from transcription complexes stable enough to be precipitated by anti-holo were examined as a function of transcript length for the two templates $A1/P_R$ and $P_R/A1$ (Figure 3C). When transcription was initiated from the $A1/P_R$ template, the yield of immunoprecipitated transcripts remained below 0.5% for RNA <8 nucleotides long and then rose to a maximum of approximately 6% for RNA ≥8 nucleotides long. However, when transcription was initiated from the $P_R/A1$ template, the change in yield of immunoprecipitated RNA took place at a length of 10 nucleotides, rather than 8. The percent yields observed for the $A1/P_R$ and $P_R/A1$ complexes at transcript lengths of 8 and 9 nucleotides were statistically different, as determined by the paired *t* test with 95% confidence limits.

The results in Figure 3C show that the ternary complexes containing the $A1/P_R$ template become more stable when the RNA reaches a length of 8 nucleotides, whereas when transcribing from the $P_R/A1$ template, a similar change in stability of the complex does not occur until the transcript is 10 nucleotides long. This implies that, with *E. coli* RNA polymerase, the abortive initiation cycle is dependent on the nucleotide sequence of the DNA template, as first suggested by Hansen and McClure (1980), but does not differentiate between the influence of promoter sequences and sequences in the initial transcribed region.

Discussion

We have photoaffinity labeled transcription complexes on various natural and synthetic templates in order to assess the influence of different template regions on determining the position of σ factor release. A cleavable dinucleotide photoaffinity probe (N_3RSpApU) was used in these experiments to initiate transcripts beginning with AU. Details of the probe's synthesis and its use in transcription experiments have been reported elsewhere (Hanna & Meares, 1983a). By using N_3RSpApU to initiate transcription, and chain-terminating 3′-*O*-methyl derivatives of the ribonucleotides in the proper ratio to the normal substrates (one of which was labeled with α-^{32}P), it was possible to form a mixture of complexes that contain RNAs of discrete lengths, with aryl azides positioned at their 5′-ends. This mixture of arrested transcription complexes was then irradiated with UV light (wavelength λ ≥ 300 nm) to convert the aryl azide to a highly reactive nitrene. This nitrene (or its derivatives) can insert itself into chemical bonds within collision distance (Bayley & Staros, 1984; Bayley & Knowles, 1977; Knowles, 1971). Electrophoresis through a sodium dodecyl sulfate/urea polyacrylamide gel separated the photolabeled protein subunits α, σ, and β/β′ (photoaffinity labeled β and β′ comigrate in this gel system) from each other, and from the DNA template and any free transcripts. Electroelution from the gel matrix and subsequent cleavage at the sulfur–phosphorus bond of the probe released the radioactive transcript as the 5′-monophosphate. The RNA that had been released from a particular subunit was then compared to the total RNA present in the original transcription mix. Through quantitative procedures described by Stackhouse and Meares (1988), the percent yields of transcripts photo-cross-linked to the protein were determined as a function of transcript length.

Role of the Promoter Sequence in the Release of σ. The data presented here show that the major interactions leading to the release of σ from ternary transcription complexes lie within the promoter region of the DNA template. Figure 4 shows a summary of the results of photoaffinity and immunoprecipitation experiments, in cartoon form. When tran-

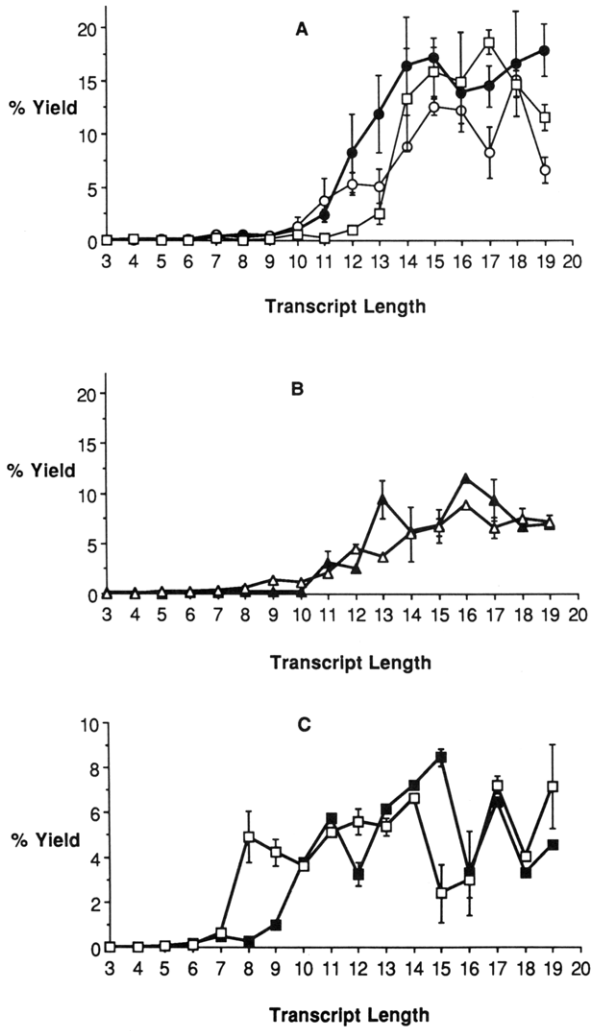

FIGURE 3: Percent photoaffinity labeling yield on β/β' as a function of transcript length, analyzed from transcription complexes containing the following: (A) λ P_R (filled circles), T7 A1 (open circles), or λ P_L (open squares) DNA; (B) P_R/A1 (filled triangles) or A1/P_R (open triangles) DNA. (C) Percent yield of RNA immunoprecipitated with polyclonal antibodies against the holoenzyme, analyzed as a function of transcript length from transcription complexes containing the recombinant templates P_R/A1 (filled squares) and A1/P_R (open squares). In each case, the results of two independent experiments are shown (bars) along with their average.

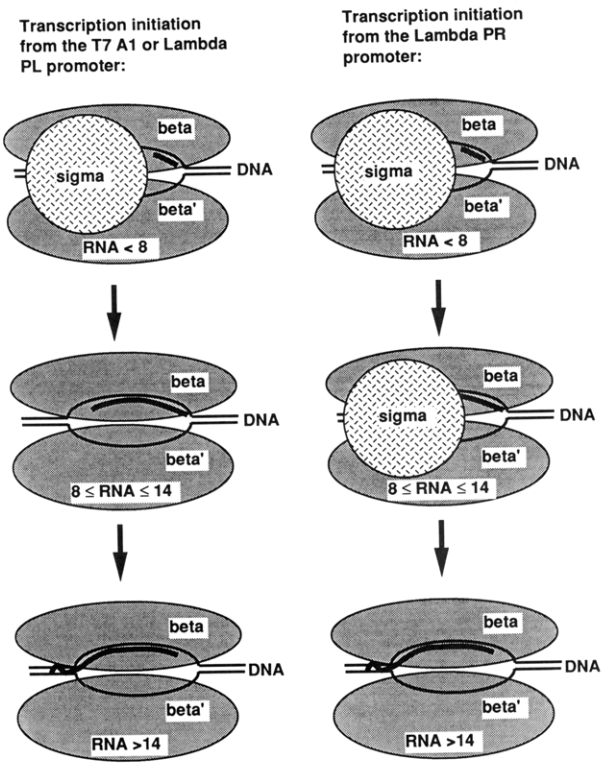

FIGURE 4: Model of σ release based on the data from the photoaffinity and immunoprecipitation experiments. The model shows the β,β', and σ subunits of *E. coli* RNA polymerase and the DNA template. Initially, all complexes contain the σ subunit. As transcription begins and the RNA is elongated, the complexes containing the λ P_L or the T7 A1 promoter release their σ earlier than the complexes containing the λ P_R promoter.

scription took place from templates containing the λ P_R promoter, the release of the σ subunit was not complete until the transcript reached a length of 14 nucleotides. When transcription was initiated from templates containing either the λ P_L or the T7 A1 promoter, the σ subunit of RNA polymerase was released quantitatively from the complex shortly after the formation of the first few phosphodiester bonds—apparently before the transcript reached a length of 8 nucleotides (Figure 3C). Note that σ has been shown to be required for transcription initiation from T7 DNA (Travers & Burgess, 1969; Wu et al., 1975; Shimamoto et al., 1986).

Before these experiments were undertaken, a qualitative initial survey revealed that three other templates containing the T7 A1 promoter, and two containing the *tac* promoter, showed a similar lack of photoaffinity labeling on σ regardless of the sequence of the initial transcribed region (data not shown).

Currently the major interactions believed to be responsible for the specific binding of proteins to DNA involve the hydrogen bond donors and acceptors within the grooves of the nucleic acid and the complementary hydrogen bond donors and acceptors in the active (binding) site of the protein (Seeman et al., 1976; von Hippel et al., 1982). Many interactions take place during transcription initiation. Although the details of such interactions remain to be elucidated, one mechanism does seem plausible for the differences reported here concerning the release of σ. The binding of RNA polymerase to the λ P_R promoter could lead to a transcription complex with a relatively large number of favorable interactions between the DNA and σ, or between the core polymerase and σ. The resulting increased attraction of σ to the DNA or the core enzyme could permit σ to remain with the complex during promoter clearance.

Photoaffinity Labeling vs Antibody Precipitation. Using the P_R/A1 template, the maximum photoaffinity yields on σ occurred when the transcript was 10–13 nucleotides long, yet the anti-σ immunoprecipitation indicated that the majority of the σ had been released from these complexes (Figure 1B vs Figure 1C). Since it is likely that the (5′-) end of the RNA was still base paired to the DNA at these lengths (Hanna & Meares, 1983b), this maximum yield at 13 nucleotides could have been caused by the approach of the 5′-probe to σ as the DNA/RNA duplex was elongated. However, photoaffinity labeling of σ is complicated by two other events: abortive initiation, which serves to reduce the labeling yield at short transcript lengths; and dissociation of σ, which reduces the yield at long transcript lengths. The occurrence of these events one after the other is the likely cause of the observed maximum in the yield curve.

It should also be noted that the photoaffinity cross-linking was performed within 11 min after formation of terminated complexes, whereas the immunoprecipitation required up to 2 h on ice. If σ was only marginally or transiently stable in transcription complexes containing RNA lengths of 10–13

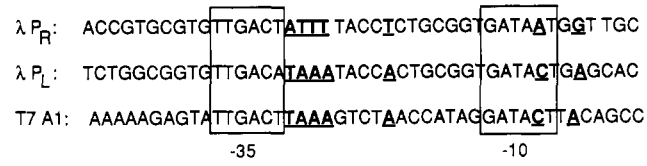

FIGURE 5: A sequence comparison of promoters from λ P_R, λ P_L, and T7 A1. The sequences are aligned for maximum overlap of their −10 and −35 regions (boxed). Positions where the nucleotides are identical between T7 A1 and λ P_L, but different from λ P_R, are underlined.

nucleotides, then the longer workup time of the antibody experiment might have allowed the antibody−σ complex to be released from the transcription complexes containing the RNA. Such a possibility seems consistent with the work of Shimamoto et al. (1986), who proposed a slow release of σ after a fast triggering step.

Interaction of β/β' with the Nucleotides in the Transcription Complex. The photoaffinity labeling yields on β/β' by transcripts longer than 13 nucleotides were somewhat higher when the complexes were transcribing from the wild-type templates containing λ P_R, T7 A1, and λ P_L than the recombinant templates P_R/A1 and A1/P_R. The interactions responsible for this difference remain to be determined, but at least two possibilities should be considered. First, interaction of β/β' with the nucleotides of both the promoter and the transcribed region of the template may have affected the path of the 5′-end of the message. Additionally, the regions of P_R/A1 and A1/P_R located upstream of −60 and downstream of +30 were identical with each other, but different from the wild-type templates. Interaction of these regions of the DNA with RNA polymerase could have altered the accessibility of the (5′-) probe to the β/β' subunits. DNA bending and looping during interactions with RNA polymerase and other macromolecules have been proposed (Ptashne, 1986; Mossing & Record, 1986).

Comparison of Promoter Sequences. Figure 5 shows the promoter sequences of λ P_R, T7 A1, and λ P_L, aligned by their −10 and −35 regions. Comparing those sequence positions that are the same in T7 A1 and λ P_L but different in λ P_R reveals positions −5 (A → G), −8 (C → A), −20 (A → T), and transversion of the sequence at −25 through −28 (TAAA → ATTT). All but one of these differences lie outside the hexanucleotide consensus sequences at −35 and −10; the difference at position −8 (C → A) is the only one found within a known conserved region. These nucleotide differences may or may not be responsible for the observed template dependent release of σ; they are likely candidates for future mutational analysis.

These experiments have allowed us to identify a region of the DNA that plays a major role in controlling the release of the σ subunit during the early stages of transcription. Using similar techniques in the future, we hope to be able to identify the nucleotide(s) responsible and to examine the roles of other transcriptional factors important to elongation and termination.

ACKNOWLEDGMENTS

We thank Susan Bernhard, Jeffrey Marx, Blaine Bartholomew, Utpala Ramesh, Michael Chamberlin, and Barbara Krummel for helpful comments and discussions.

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

REFERENCES

Barik, S., Ghosh, B., Whalen, W., Lazinski, D., & Das, A. (1987) *Cell 50*, 885–899.

Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–114.

Bayley, H., & Staros, J. V. (1984) Photoaffinity Labeling and Related Techniques. In *Azides and Nitrenes* (Scriven, E. F., Ed.) pp 433–490, Academic Press, Orlando, FL.

Bernhard, S. L., & Meares, C. F. (1986a) *Biochemistry 25*, 5914–5919.

Bernhard, S. L., & Meares, C. F. (1986b) *Biochemistry 25*, 6397–6404.

Burgess, R. R. (1976) Purification and Physical Properties of *E. coli* RNA Polymerase. In *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 69–100, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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

Burgess, R. R., Travers, A. A., Dunn, J. J., & Bautz, E. K. F. (1969) *Nature (London) 221*, 43–46.

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. (1974) *Annu. Rev. Biochem. 43*, 721–775.

Chenchick, A., Beabealashvilli, R., & Mirzabekov, A. (1981) *FEBS Lett. 128*, 46–50.

Doi, R. H., & Wang, L. H. (1986) *Microbiol. Rev 50*, 227–243.

Gentry, D. R., & Burgess, R. R. (1986) *Gene 48*, 33–40.

Greenblatt, J., Horwitz, R. J., & Li, J. (1987) in *RNA Polymerase and the Regulation of Transcription* (Reznikoff, W. S., Burgess, R. R., Dahlberg, J. E., Gross, C. A., Record, M. T., Jr., & Wickens, M. P., Eds.) pp 357–366, Elsevier, New York.

Gribskov, M. R., (1985) *Structure of Bacterial RNA Polymerase Sigma Factors*, Ph.D. Thesis, The University of Wisconsin, Madison.

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.

Hansen, U. M., & McClure, W. R. (1980) *J. Biol. Chem. 255*, 9564–9570.

Hawley, D. K., & McClure, W. R. (1982) *J. Mol. Biol. 157*, 493–525.

Hawley, D. K., & McClure, W. R. (1983) *Nucleic Acids Res. 11*, 2237–2255.

Hellman, J. D., & Chamberlin, M. J. (1988) *Annu. Rev. Biochem. 57*, 862–890.

Hinkle, D. C., & Chamberlin, M. J. (1972) *J. Mol. Biol. 70*, 157–185.

Johnston, H. M., & McClure, W. R. (1978) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 413–420, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kadesch, T. R., Williams, R. C., & Chamberlin, M. J. (1980) *J. Mol. Biol. 136*, 65–93.

Knowles, J. R. (1971) *Acc. Chem. Res. 5*, 155–160.

Levin, J. R., Krummel, B., & Chamberlin, M. J. (1987) *J. Mol. Biol. 196*, 85–100.

Lewin, B. (1983) *Genes*, Chapter 10 John Wiley & Sons, New York.

Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344–1352.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.

Maxam, A. W., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–567.

McClure, W. R. (1985) *Annu. Rev. Biochem. 54*, 171-204.
McClure, W. R., & Cech, T. R. (1978) *J. Biol. Chem. 253*, 8949-8956.
Mossing, M. C., & Record, M. T., Jr. (1986) *Science 233*, 889-892.
Oliphant, A. R., & Struhl, K. (1987) *Methods Enzymol. 155*, 568-582.
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.
Park, C. S., Hillel, Z., & Wu, C.-W. (1982a) *J. Biol. Chem. 257*, 6944-6949.
Park, C. S., Wu, F. Y.-H., & Wu, C.-W. (1982b) *J. Biol. Chem. 257*, 6950-6956.
Ptashne, M. (1986) *Nature 322*, 697-701.
Roe, J. H., Burgess, R. R., & Record, M. T., Jr. (1984) *J. Mol. Biol. 176*, 495-521.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804-808.
Shimamoto, N., Kamigochi, T., & Utiyama, H. (1986) *J. Biol. Chem. 261*, 11859-11865.
Simpson, R. B. (1979) *Cell (Cambridge, Mass.) 18*, 277-285.
Stackhouse, T. M., & Meares, C. F. (1988) *Biochemistry 27*, 3038-3045.
Straney, D. C., & Crothers, D. M. (1987) *J. Mol. Biol. 193*, 267-278.
Strauss, H. S., Burgess, R. R., & Record, M. T., Jr. (1980) *Biochemistry 19*, 3504-3515.
Studier, F. W., & Rosenberg, A. H. (1981) *J. Mol. Biol. 153*, 503-525.
Travers, A. A., & Burgess, R. R. (1969) *Nature (London) 222*, 537-540.
von Hippel, P. H. (1979) in *Biological Regulation and Development* (Goldberger, R. F., Ed.) Vol. 1, pp 279-347, Plenum, New York.
von Hippel, P. H., Bear, D. G., Winter, R. B., & Berg, O. G. (1982) in *Promoters: Structure and Function* (Rodriguez, R. L., & Chamberlin, M. J., Eds.) pp 3-33, Praeger, New York.
Weinberg, G. H., Schumaker, J. A., & Oltman, D. (1981) in *Statistics: An Intuitive Approach*, pp 252-277, Brooks/Cole Publishing, Monterey, CA.
Wu, C.-W., Yarbrough, L. R., Hillel, Z., & Wu, F. Y.-H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3019-3023.
Wu, G. J., & Bruening, G. E. (1979) *Virology 46*, 596-612.

Characterization and Partial Purification of an Estrogen Type II Binding Site in Chick Oviduct Cytosol†

Charles L. Densmore,*,‡ Barry M. Markaverich,§ Bert W. O'Malley,‡ and James H. Clark‡
Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, and Center for Biotechnology, Baylor College of Medicine, The Woodlands, Texas 77381

Received April 14, 1989; Revised Manuscript Received June 9, 1989

ABSTRACT: An estrogen binding site of moderate affinity (K_d ~10 nM) and high capacity (~25-70 pmol/g of tissue) was measured in DES-stimulated chick oviduct cytosol. Saturation analysis by [^{3}H]estradiol exchange demonstrated that this binding site displayed sigmoidal binding characteristics suggesting a cooperative binding mechanism. Competition analysis with a number of compounds demonstrated that the bioflavonoid luteolin was a better competitor for binding to type II sites in chick than either estradiol or DES. Steroid specificity was demonstrated by the inability of 17α-estradiol, progesterone, testosterone, corticosterone, and the triphenylethylene antiestrogen nafoxidine (U-1100A) to compete for [^{3}H]-17β-estradiol binding to chick oviduct cytosol preparations. In addition, the binding site appeared to be sensitive to sulfhydryl reducing reagents as evidenced by a 75% reduction in binding activity in the presence of dithiothreitol. Both prelabeling and postlabeling procedures used in conjunction with Sephacryl S-300 chromatography resulted in a single major peak of type II binding activity representing a molecular weight in the 40 000 range. Type II binding activity was recoverable after precipitation with ammonium sulfate, and this material was subjected to a variety of column chromatography procedures in order to achieve further purification of the type II site. Significant purification of the site was achieved with a bioflavonoid–Sepharose (quercetin–Sepharose) affinity matrix. The purified type II sites eluted from quercetin–Sepharose displayed the same sigmoidal binding curves characteristic of native cytosol.

Previous reports have described multiple classes of estrogen binding sites in rat uterus (Clark et al., 1978; Eriksson et al., 1978), mouse mammary tumors (Watson & Clark, 1980), human breast cancer (Syne et al., 1982a,b; Lopes et al., 1987), mullerian duct (MacLaughlin et al., 1983), ventral prostate (Pliner & Swaneck, 1985), and hepatocarcinoma cells (Tam et al., 1986). These components have been designated type I (estrogen receptor; K_d ~1 nM) and type II (K_d ~10-20 nM) (Markaverich & Clark, 1979). Although the estrogen (type I) receptor has long been the subject of extensive investigation by many laboratories and its detailed structure has recently

† Supported by NIH Grant HD-08436. C.L.D. received NIH Postdoctoral Fellowship 1-F32-HD07196-01.
‡ Department of Cell Biology.
§ Center for Biotechnology.

0006-2960/89/0428-7788$01.50/0