Probing the Protein−DNA Contacts of a Yeast RNA Polymerase III Transcription Complex in a Crude Extract: Solid Phase Synthesis of DNA Photoaffinity Probes Containing a Novel Photoreactive Deoxycytidine Analog†

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ABSTRACT: A novel photoreactive deoxycytidine analog, 4-[*N*-(*p*-azidobenzoyl)-2-aminoethyl]-dCTP (AB-dCTP), has been synthesized and incorporated at specific sites within the *SUP4* $tRNA^{Tyr}$ gene. Immobilized single-stranded DNA was annealed to specific oligonucleotides and AB-dCMP incorporated into DNA by primer extension. DNA photoaffinity labeling with AB-dCMP was used to survey protein−DNA contacts in initiation and elongation complexes of RNA polymerase III (Pol III), and compared to DNA photoaffinity labeling using the previously described photoreactive deoxyuridine analog, 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]-dUMP (AB-dUMP) [Bartholomew et al. (1993) *Mol. Cell.Biol. 13*, 942−952]. In contrast to previous studies, we have used a crude protein fraction rather than highly purified preparations of Pol III and transcription factors TFIIIC and TFIIIB to examine if some component of the transcription complex is lost upon purification. Eleven nucleotide positions from bp −17 to bp +17 (+1 being the start site of transcription) on the nontranscribed strand were modified and shown to have little or no effect on transcription complex formation, initiation, or elongation as determined by multiple-round transcription assays. Efficient photoaffinity labeling by DNA containing AB-dCMP gave results comparable to that with AB-dUMP at proximal nucleotide positions and provided new evidence for the placement of the 160 and 31 kDa subunits of Pol III near the 5′ end of the transcriptional bubble in an elongation complex. A novel 40 kDa protein was cross-linked at bps −17, −9, and −8 in a TFIIIC-dependent manner that had not been previously detected.

Initiation of transcription in eukaryotes is elicited by a multitude of transcription factors in conjunction with RNA polymerase. Although nuclear eukaryotic RNA polymerases have 10−16 different subunits and a total molecular mass of 500−600 kDa, these enzymes lack the ability to recognize their respective DNA promoters to initiate transcription at the correct start site of the gene (Sentenac et al., 1992; Young, 1991). RNA polymerase III is responsible for transcribing tRNA, 5S rRNA, U6 snRNA, 7SL, 7SK RNAs, and a variety of other small RNA genes. In general, there are two or three transcription factors required for transcription by RNA polymerase III (Pol III),[1] which are commonly designated TFIIIA, TFIIIB, and TFIIIC (Geiduschek & Kassavetis, 1992; Kassavetis et al., 1994). TFIIIA is required only for transcription of the 5S rRNA gene. Both transcription factors TFIIIA and TFIIIC are specific DNA-binding proteins that are used to recruit TFIIIB, which in turn recruits Pol III to DNA (Kassavetis et al., 1990). TFIIIB in *Saccharomyces cerevisiae* has been shown to be the only transcription factor absolutely required for initiation of transcription. Although a minimal transcription complex has been demonstrated using recombinant TFIIIB, highly purified TFIIIC, and Pol III, this approach gives a reductionist view of the transcription complex (Kassavetis et al., 1995). There are several lines of evidence that suggest additional proteins may play an important role, possibly in regulating the activity of the basic machinery (Kassavetis et al., 1994). Of particular interest, a fourth transcription factor in yeast has been noted to be required for single and multiple rounds of transcription, although not required for "template commitment" (Dieci et al., 1993).

In order to study the mechanism of promoter recognition by Pol III, DNA photoaffinity labeling has been used to map the positions of transcription factors and Pol III subunits to specific sites in DNA in initiation (binary) and elongation (ternary) complexes (Bartholomew et al., 1990, 1991, 1993; Braun et al., 1993; Kassavetis et al., 1991). The photoreactive deoxyuridine analog, 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]-dUMP, was constructed into specific sites in DNA along with an α-^{32}P-labeled nucleotide. The photoreactive DNA was used to radioactively tag protein(s) associated near specific sites on DNA. DNA probe synthesis was done by primer extension using single-stranded M13

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[1] Abbreviations: Pol III, RNA polymerase III; BR500, the 500 mM KCl step fraction from an S-100 extract loaded onto a BioRex70 column; AB-dUMP, 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]deoxyuridine monophosphate; AB-dCMP, 4-[*N*-(*p*-azidobenzoyl)-2-aminoethyl]-deoxycytidine monophosphate; AB-dCTP, 4-[*N*-(*p*-azidobenzoyl)-2-aminoethyl]deoxycytidine monophosphate; daeCTP, N^4−(aminoethyl)deoxycytidine triphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BSA, bovine serum albumin; dNTP, deoxynucleotide triphosphate; NTP, ribonucleotide triphosphate; TLC, thin layer chromatography; PEI-F, poly(ethyleneimine) with fluorescent indicator; TEAB, triethylammonium bicarbonate; TE, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

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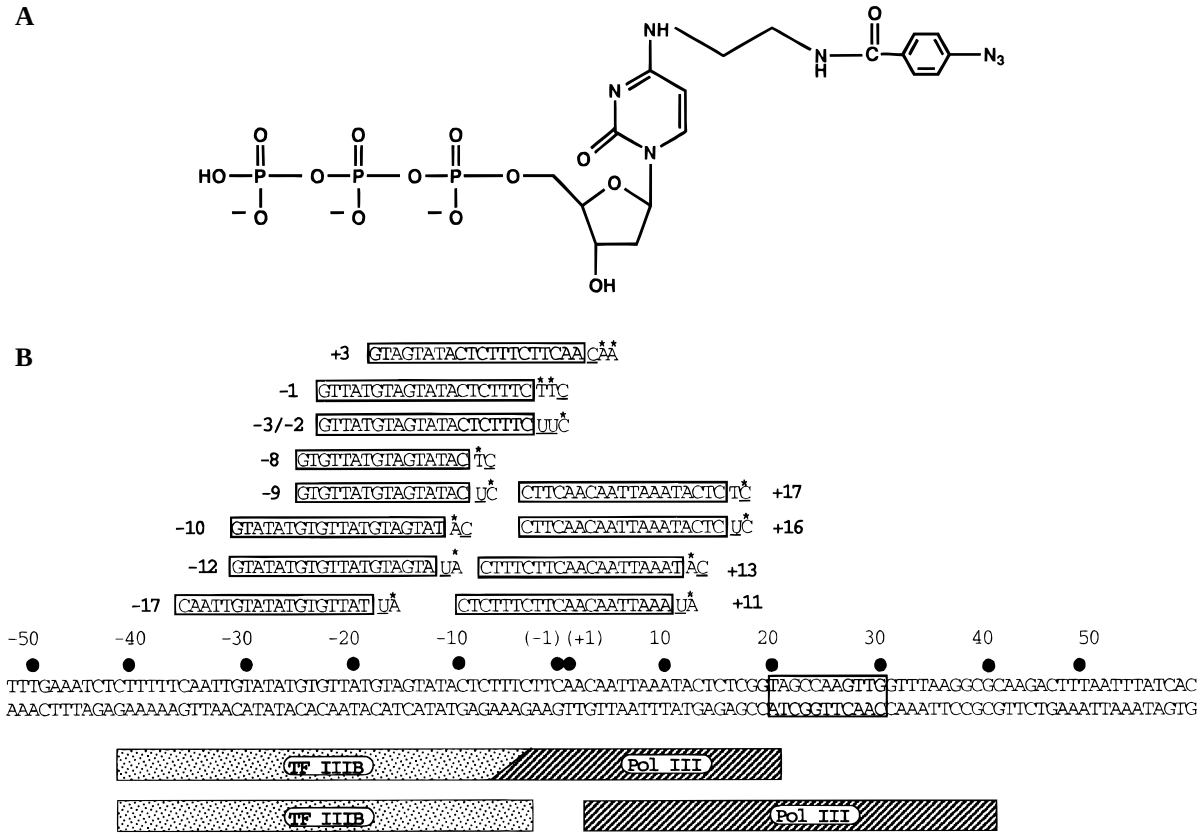

FIGURE 1: Site-directed modification of DNA. (A) Structure of 4-[*N*-(*p*-azidobenzoyl)-2-aminoethyl]-dCTP. (B) DNA photoaffinity probes. The sequences of the oligonucleotides used to direct the incorporation of a modified nucleotide are shown enclosed in boxes with the sequence of the *SUP4* $tRNA^{Tyr}$ gene displayed below extending from 52 base pairs upstream and 59 base pairs downstream of the transcriptional start site (designated +1). To the right of the oligonucleotides is shown the α-^{32}P-labeled nucleotide with an asterisk above and the modified nucleotide underlined (U is AB-dUMP and C is AB-dCMP) that are incorporated in the primer extension step. Each DNA photoaffinity probe is referred to by a number or pair of numbers indicating the location of AB-dUMP or AB-dCMP. The shaded boxes below the sequence indicate the DNase I footprint of TFIIIB and Pol III in binary (initiation) and ternary (elongation) complexes arrested at base pair +17. The diagonal boundary shown is indicative of the difficulty in assigning contacts to either Pol III or TFIIIB in the binary complex on the basis of DNase I footprinting alone. The boxed segment of the DNA sequence from +20 to +30 is the *box A* region of the *SUP4* $tRNA^{Tyr}$ gene.

DNA constructs, and DNA polymerase, free nucleotides, and buffer components were removed at different steps by spin column gel filtration. DNA was digested with a restriction endonuclease and the DNA fragment purified by gel electrophoresis. A further extension of this method is reported here by (1) the introduction of a novel photoreactive deoxycytidine analog, 4-[*N*-(*p*-azidobenzoyl)-2-aminoethyl]-dCMP (AB-dCMP, see Figure 1A), to increase the number of DNA sites that can be probed by DNA photoaffinity labeling, (2) the refinement of photoreactive DNA synthesis using a DNA template immobilized to a magnetic support, and (3) using a crude protein extract to compare the composition and positioning of components in the transcription complexes formed with a potentially more "complete" system versus those with highly purified TFIIIC, TFIIIB, and Pol III.

DNA photoaffinity labeling using a crude protein extract and DNA probes containing AB-dUMP at bps +16, +11, −3/−2, −9, −12, and −17 gave results comparable to those with highly purified TFIIIC, TFIIIB, and Pol III. These results demonstrate the utility of DNA photoaffinity labeling in complex mixtures, where it can be extremely difficult to probe protein−DNA complexes. DNAs containing AB-dCMP efficiently labeled components of the transcription complex and gave results that were consistent with those using DNAs containing AB-dUMP at adjacent positions. A 40 kDa protein was photoaffinity labeled at bps −17, −9, and −8 that was not observed with purified components, suggesting that the 40 kDa protein could be a component of the transcription complex not found in purified TFIIIC, TFIIIB, and Pol III. Another new finding was the placement of the 160 and 31 kDa subunits of Pol III at the 5′ end of the transcriptional "bubble" in the stalled elongation complex at bp +3.

MATERIALS AND METHODS

Synthesis of 4-[N-(p-Azidobenzoyl)-2-aminoethyl]deoxycytidine Triphosphate, AB-dCTP. N^4-(Aminoethyl)deoxycytidine triphosphate (daeCTP) was synthesized by a bisulfite-catalyzed transamination reaction with diaminoethane following the procedure of Draper (1984). After the transamination reaction was complete, the reaction mixture containing 900 μL of the bisulfite−amine solution and 100 μL of 100 mM deoxycytidine 5′-triphosphate in 50 mM triethylammonium bicarbonate buffer (TEAB) at pH 8.0 was diluted with 25 mL of deionized water before loading onto a 1 × 8 cm DEAE A-25 Sephadex column (flow rate, 35 mL/h). TEAB was made as described (Bartholomew et al., 1995). The column was washed with 100 mM TEAB at pH 8.0 and eluted with a 40 mL 0.1 to 1.5 M TEAB pH 8.0 linear gradient. One milliliter fractions were collected (daeCTP eluted with 0.84−1 M TEAB), and UV-absorbing

fractions were evaporated to dryness by vacuum centrifugation and resuspended in 250 μL of deionized water. The fractions were again evaporated to dryness and resuspended in deionized water two more times to remove TEAB. Samples were resuspended in 100 mM sodium borate at pH 8.0, after which 1 μL of each was spotted on a PEI-F cellulose TLC plate (J. T. Baker, Baker-flex 20 × 20 cm) and developed with 1 M LiCl. The R_f value for daeCTP was 0.221 as compared to an R_f of 0.076 for dCTP. The appropriate fractions were pooled, and the concentration was determined by UV spectrophotometry ($\epsilon_{271} = 9.1$ mM^{-1}).

The following steps were conducted under reduced lighting. One hundred microliters of a 100 mM solution of 4-azidobenzoic acid *N*-hydroxysuccinimide (Sigma) in dimethylformamide (Aldrich) was added to an equal volume of 8.8 mM daeCTP in 100 mM sodium borate at pH 8.5. The reaction mixture was incubated at room temperature for 3 h, after which an equal volume of deionized water was added. The product, 4-[*N*-(*p*-azidobenzoyl)-2-aminoethyl]-deoxycytidine triphosphate, AB-dCTP, was purified by DEAE A-25 chromatography and then analyzed by TLC, and finally, TEAB was removed by vacuum centrifugation as described for daeCTP. The final resuspension was in TE [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] rather than in 100 mM sodium borate, and the R_f value for AB-dCTP was 0.05. The concentration of AB-dCTP was determined by UV spectrophotometry at 271 nm using the combined extinction coefficients of azidobenzoic acid (27.1 mM^{-1}) and daeCTP (9.1 mM^{-1}) for AB-dCTP (36.2 mM^{-1}). Enzymatic incorporation of AB-dCTP was checked by performing oligonucleotide primer extension assays.

Plasmids and Immobilized Single-Stranded DNA. Plasmid DNAs pTZ1 and pLNG56 containing *SUP4* tRNA^{Tyr} genes with promoter-up and promoter-down mutations inserted into pGEM1 have been described (Kassavetis et al., 1989). Biotinylated DNA containing the promoter-up version of the *SUP4* tRNA^{Tyr} gene was made by initially digesting pTZ1 with HindIII and filling in the 5′ overhangs with Bio-11-dUTP (Sigma Chemical Co.) and Bio-14-dATP (GIBCO/BRL). A typical 270 μL reaction mixture contained 200 pmole of linearized pTZ1, 20 μM Bio-14-dATP, dCTP, and dGTP, 25 μM Bio-11-dUTP, and 150 units of exonuclease-free Klenow fragment of DNA polymerase I (U.S. Biochemicals) in buffer A [10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT]. The unincorporated dNTPs were removed by spin column chromatography using Sephacryl S-200 resin (Pharmacia) equilibrated in buffer B [1 M LiCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% SDS]. The biotinylated DNA was precipitated by the addition of 2.5 volumes of ethanol. Biotinylated DNA was digested with *Eco*RI and *Rsa*I to generate a 315-base pair biotinylated DNA fragment containing the *SUP4* tRNA^{Tyr} gene. Biotinylated DNA was bound to Dynabeads M-280 Streptavidin (Dynal) following the procedures supplied by the manufacturer, after which the nonbiotinylated strand was removed by treatment with 0.1 M NaOH. The immobilized DNA was dephosphorylated by the addition of 20 units of shrimp alkaline phosphatase (Amersham/U.S. Biochemicals) to 40 pmol of DNA in a volume of 200 μL of buffer C [30 mM Tris-HCl (pH 8.0), 50 mM KCl, 7 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 0.05% Tween 20] and incubation at 37 °C for 60 min with constant vortexing. The shrimp alkaline phosphatase was removed by washing the beads with TE containing 0.1% SDS and any residual phosphatase inactivated by heating at 65 °C for 15 min.

Synthesis Of DNA Photoaffinity Probes. A site-specific 5′-phosphorylated oligonucleotide (see Figure 1B) was annealed to the single-stranded *Eco*RI and *Hind*III fragment (containing the promoter-up mutant of the *SUP*4 tRNA^{Tyr} gene) immobilized on paramagnetic particles. The general annealing reaction mixture contained 1 pmol of the *Eco*RI and *Hind*III fragment bound to paramagnetic beads, 4 pmol of oligonucleotide primer, and bovine serum albumin (BSA, Boehringer Mannheim) at 100 μg/mL in a 20 μL volume of buffer C. The annealing mixture was heated at 90 °C for 3 min and incubated, with constant vortexing, for 30 min at 37 °C. The photoreactive and radioactive nucleotides were incorporated at different stages of the DNA probe synthesis. In the case of DNA photoaffinity probes at bps −10, −8, −1, +13, and +17; α-^{32}P-labeled dATP or dTTP (specific activity of 2000−3000 Ci/mmol, ∼3 pmol) and 0.25 unit of exonuclease-free Klenow fragment of DNA polymerase I (Amersham/U.S. Biochemicals, diluted from a 10 U/μL stock concentration to 0.25 U/μL with buffer containing 2-mercaptoethanol in order to reduce the final concentration of dithiothreitol in the probe synthesis reaction mixture to approximately 7.5 μM) were added to the reaction mixture and incubated for 5 min at 37 °C with constant vortexing. The immobilized DNA was washed with buffer C to remove free nucleotides and resuspended in 20 μL of buffer C, after which the photoreactive nucleotide analog, either AB-dCTP (10 μM) or AB-dUTP (5 μM), plus an additional 0.25 unit of exonuclease-free Klenow fragment were added to the immobilized DNA and incubated for 5 min at 37 °C. A 0.5 μL aliquot was removed from the reaction sample and added to 10 μL of 95% formamide with 0.1% xylene cyanol and bromophenol blue for later analysis on a 10% polyacrylamide gel containing 8.3 M urea to check for proper incorporation of photoreactive and radiolabeled nucleotide. DNA photoaffinity probes at bps −17, −12, −9, −3/−2, +3, +11, and +16 had the photoreactive nucleotide incorporated first at the concentrations indicated above, followed by the removal of unincorporated nucleotide and the incorporation of the α-^{32}P-labeled deoxynucleotide.

The primer was extended by the addition of 2.5 μL of 5 mM unlabeled dNTPs (Pharmacia, FPLC grade) in buffer C and incubation at 37 °C for 5 min. After the 5 min incubation, a 0.5 μL aliquot of the sample was removed for subsequent analysis of the reaction product. The chase reaction was stopped by the addition of 1 μL of 5% sodium dodecyl sulfate (SDS).

The magnetic beads were washed sequentially with buffer D ([1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% Triton X-100], TE + 0.1% SDS, and buffer C in order to remove unincorporated nucleotides and DNA polymerase. BSA (100 μg/mL) and 9 pmol of a second oligonucleotide (5′-CTCTAGAGGATCCTTTAG-3′) complementary to a region upstream of the first oligonucleotide were added to the immobilized DNA resuspended in 25 μL of buffer C. The oligonucleotide was allowed to anneal to the template for 30 min at 37 °C with a constant gentle vortex. The primer was extended by the addition of 2.5 μL of 5 mM dNTPs and 5 units of T4 DNA polymerase (New England Biolab). After 10 min at 37° C, the DNA probes were ligated by the addition of ATP (0.35 mM) and 1 unit of T4 DNA ligase with incubation at 37 °C for 60 min. A 0.5 μL aliquot

was taken from the sample for later analysis. T4 DNA ligase was removed by washing with TE + 0.05% SDS and then with buffer C and any residual ligase inactivated by heating at 65 °C for 15 min.

The DNA was released from the magnetic beads by digestion with *Bam*HI adding 1.5 μL of 2 M KCl and 20 units of *Bam*HI (U.S. Biochemicals) to the sample and incubating, with a constant vortex, at 37 °C for 60 min. After the restriction digestion, a 0.5 μL aliquot was removed for analysis. The supernatant was removed and the beads were washed with 20 μL of buffer C. One microliter of each sample was spotted onto DE-81 Whatman filter paper and analyzed by scintillation counting to quantify the amount of radiolabeled DNA, and another 1 μL of sample was analyzed on a 4% polyacrylamide gel.

The samples were extracted first with phenol/chloroform (1:1) and then with chloroform, DNA precipitated by the addition of $^1/_{10}$ volume of 10 M LiCl and 2.5 volumes of ethanol, and then stored at −20 °C for at least 6 h. The DNA probes were centrifuged at 16000*g* for 30 min at 4 °C and pellets washed with ethanol. The supernatant was poured off and the pellet dried for 10 min by vacuum centrifugation and resuspended in TE + 0.05% Tween 20 to a final concentration of 2 fmol/μL. The DNA photoaffinity probes were wrapped in foil and stored at 4 °C.

Proteins. An S-100 extract was prepared from *S. cerevisiae* strain BJ926 according to the methods of Schultz et al., (1991). The S-100 extract was precipitated in 35%- and 70%-saturated $(NH_4)_2SO_4$, and the dialyzed 70% $(NH_4)_2SO_4$ pellet was fractionated on a BioRex 70 column as described by Kassavetis et al., (1989). The 500 mM KCl step fraction contains RNA polymerase III and transcription factors TFIIIC and TFIIIB and was referred to as BR500. Transcription assays were performed to determine the optimal amount of BR500 extract for transcription of the *SUP4* $tRNA^{Tyr}$ gene (Kassavetis et al., 1989).

RNA polymerase III was purified from *S. cerevisiae* strain NZ16 kindly provided by Nick Zecherle and Benjamin Hall (University of Washington). Six consecutive histidine residues followed by four tandem repeats of the octapeptide FLAG sequence (IBI/Kodak) were added onto the N-terminus of the second-largest subunit of RNA polymerase III. The preparation of BR500 extract from this strain was the same as for strain BJ926. The BR500 extract of the NZ16 strain was further purified by DEAE A-25 ion exchange chromatography (Kassavetis et al., 1989) and to apparent homogeneity by Ni-NTA (Qiagen) affinity column chromatography.

In Vitro Transcription Assays of DNA Photoaffinity Probes. A typical 34 μL transcription reaction mixture contained 500 ng of pGEM1 plasmid DNA (Promega) cut with *Eco*RI, 12 fmol of the particular DNA photoaffinity probe or pTZ1 DNA, and BR500 extract in buffer E [40 mM Tris-HCl (pH 8.0), 7.5 mM $MgCl_2$, 100 mM NaCl, 0.5 mM EDTA, and 5 mM 2-mercaptoethanol]. Samples were vortexed and incubated at 25 °C for 30 min. Transcription was initiated by adding 1.5 μL of 2 mM ATP, CTP, and UTP and 1 μL [α-^{32}P]GTP (2000−3000 Ci/mmol) and the mixture incubated at 25 °C for 20 min. Next, 2 μL of 1 mM GTP in buffer E was added, and samples were incubated for an additional 15 min. DNase I was added to a final concentration of 50 μg/mL and the mixture incubated at 25 °C for 20 min. Nuclease digestion was terminated by the addition of 160 μL of stop solution [0.5% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 8.0), and 5 mM EDTA]. Reaction mixtures were extracted with phenol/chloroform (1:1), and the phenol layer was back-extracted with 100 μL of stop solution. The two aqueous layers were combined and extracted with chloroform. The nucleic acids were precipitated by the addition of 2.5 volumes of ethanol, $^1/_{10}$ volume of 10 M LiCl, and 2.5 μg of pGEM1 DNA and stored at −20 °C for at least 1 h. Samples were centrifuged at 4 °C for 30 min. The supernatant was poured off and the pellet washed with ethanol. Nucleic acid pellets were dried by vacuum centrifugation for 15 min and resuspended in 5 μL of TE + 0.05% Tween 20. Once the pellet was resuspended, 10 μL of dye mix (95% formamide, 0.1% xylene cyanol, and 0.1% bromophenol blue) was added to each sample. Next, samples were heated at 90 °C for 3 min and analyzed by gel electrophoresis on a 10% denaturing polyacrylamide gel containing 8.3 M urea. The yields of the transcripts were determined by scintillation counting of excised gel pieces.

Photoaffinity Labeling of DNA−Protein Complexes. Reaction mixtures contained 2 fmol of DNA photoaffinity probe, 500 ng of pLNG-56 or pTZ1 linearized with *Eco*RI, and 1−4 μL of a BR500 extract in 20 μL of buffer E; and were incubated at 25 °C for 30 min. Either 1 μL of sterile deionized water or 1 μL of NTP mix containing 2 mM ATP, CTP, and UTP (Boehringer Mannheim, HPLC grade) in buffer E was added to the reaction mixtures, and the mixture was incubated at 25 °C for 15 min for formation of arrested ternary complexes. TFIIIC and RNA polymerase III were released from DNA in nontranscribing complexes by the addition of heparin to 100 μg/mL. Samples were photocross-linked and digested with nuclease as described previously (Bartholomew et al., 1991) with the following modification. After the S-1 nuclease digest, the pH was readjusted to approximately 7 with the addition of 0.5 M Tris-base and the samples were denatured in 5% (vol/vol) 2-mercaptoethanol, 2% SDS, and 70 mM Tris-HCl (pH 6.8) at 90 °C for 3 min. Samples were analyzed on a 4 to 20% gradient SDS−polyacrylamide gel (SDS−PAGE). Prestained broad range protein molecular mass standards (BioRad) or purified RNA polymerase III served as molecular mass markers for estimating relative molecular masses. Quantitation of radiolabeled protein bands was done using the Molecular Dynamics Phosphorimager Model 445.

RESULTS

Incorporation of AB-dCMP at Specific Sites within the SUP4 $tRNA^{Tyr}$ Gene. The photoreactive deoxycytidine analog, 4-[*N*-(*p*-azidobenzoyl)-2-aminoethyl]deoxycytidine triphosphate (AB-dCTP, Figure1 A), was synthesized and characterized by PEI-F cellulose TLC and shown to be efficiently incorporated into DNA at concentrations ranging from 3 to 200 μM (results not shown). The nucleotide AB-dCMP was incorporated at single-base pair positions in the nontranscribed strand of the *SUP4* $tRNA^{Tyr}$ gene from −10 to +17. The site-directed incorporation of AB-dCMP was accomplished by using one of several oligonucleotide primers (Figure 1B) annealed to an immobilized single-stranded DNA template. The DNA template was immobilized by biotinylation of the 3′ end of the DNA and binding to streptavidin coupled to paramagnetic particles. Immobilization of the DNA template has greatly reduced the time required for DNA

probe synthesis by eliminating the need for gel filtration and purification by gel electrophoresis. This approach also affords greater control in the incorporation of the modified and radioactive nucleotides than previously, because of the ease and efficiency of removing free nucleotides for a stepwise incorporation of modified and then radioactive nucleotide (or vice versa).

Analysis of the incorporation of the photoreactive nucleotide AB-dCMP or AB-dUMP and [α-^{32}P] dNMP was done for nucleotide positions −10, −9, −8, −3/−2, and −1 (Figure 2A). The correct incorporation of photoreactive and radioactive nucleotides and the absence of read-through were demonstrated by their relative mobility and the presence of single radioactively labeled bands (Incorporate, lanes A−E). After complete extension in the presence of a large excess of all four deoxynucleotide triphosphates (Extend, lanes F−J), the Klenow fragment of DNA polymerase I and unincorporated nucleotides were removed by washing the immobilized DNA template. A second oligonucleotide was annealed to a region upstream of the initial oligonucleotide and extended in the presence of all four dNTPs using T4 DNA polymerase in order to synthesize the upstream region. The two DNA fragments bound to the complementary template strand were ligated together using T4 DNA ligase (Ligate, lanes K−O) with an efficiency of >80%. Finally, the DNA probe was released from the paramagnetic particles by digestion with *Bam*HI (Digest, lanes P−T) and analyzed on a 4% native polyacrylamide gel (Figure 2B, lanes A−E) to confirm the presence of a uniform product with the expected mobility. All of the other DNA photoaffinity probes used were analyzed in a similar fashion.

In Vitro Transcription Assays of SUP4 tRNA^{Tyr} Gene Photoaffinity Probes. The effect of the site-directed modification of DNA on the formation, initiation, and elongation of Pol III transcription complexes was examined by measuring the transcriptional efficiency of the *SUP*4 tRNA^{Tyr} gene containing AB-dCMP or AB-dUMP or no modified nucleotide. The amount of RNA transcripts obtained using DNA probes with photoreactive nucleotides incorporated at bps −17 to +17 ranged from 50 to 94% of that obtained with unmodified pTZ1 DNA. The amount of transcription detected with a particular DNA probe fluctuated due to the inherent variability in the recovery of RNA by phenol/chloroform extraction and ethanol precipitation. Within the reproducibility of the transcription assay, DNAs containing the single- or double-point alterations in the *SUP4* tRNA^{Tyr} gene had only slightly lowered transcriptional activity as compared to unmodified DNA. We concluded that these DNA modifications did not significantly interfere with binding of transcription factors and Pol III or transcriptional initiation or elongation. Previously, the incorporation of AB-dUMP at bps −17, −12, −9, −3/−2, +6/+7, +11, and +16 had been shown not to interfere with transcription complex formation by gel-shift and DNA competition assays using highly purified Pol III, TFIIIC, and TFIIIB (Bartholomew et al., 1993). The only modified DNA found to be significantly less transcriptionally active (<10% of that of pTZ1) had AB-dUMP at bps +6 and +7. Because of the apparent interference of the DNA modification at bp +6/+7, this position was not examined further by DNA photoaffinity labeling.

DNA Photoaffinity Labeling of the Pol III Transcription Complex in a Crude Extract. DNA photoaffinity labeling should not be effected by the presence of a large excess of nontranscriptional proteins given that the DNA is efficiently assembled into active transcription complexes. DNA photoaffinity labeling using BR500 extract was done at DNA positions in the *SUP4* tRNA^{Tyr} gene that had been previously examined using purified components for comparison. The specificity of photoaffinity labeling of the *SUP4* tRNA^{Tyr}

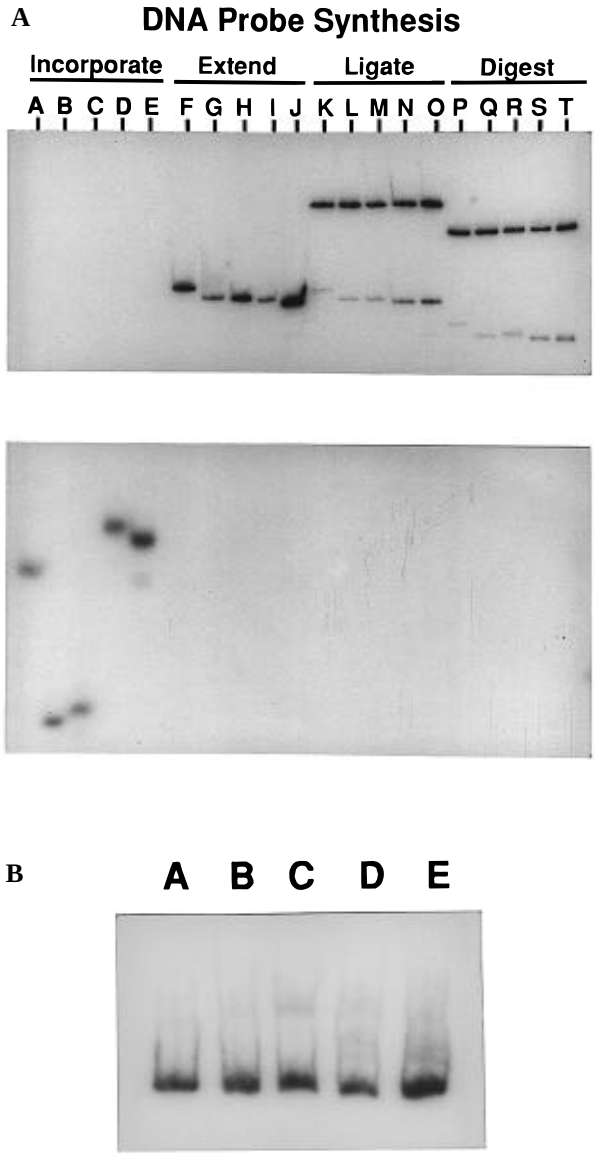

FIGURE 2: Analysis of DNA probe synthesis at bps −10, −9, −8, −3/−2, and −1. (A) Analysis of the various steps of DNA probe synthesis by 10% denaturing polyacrylamide gel electrophoresis (PAGE) with only the top and bottom portions of the autoradiogram shown. Samples were taken after (1) the incorporation of AB-dCMP or AB-dUMP and [α-^{32}P]dNMP (lanes A−E, Incorporate), (2) the full length extension of oligonucleotide primers in the presence of all four dNTPs (lanes F−J, Extend), (3) addition of upstream oligonucleotide, primer extension, and ligation of the two contiguous DNA fragments (lanes K−O, Ligate), and (4) digestion with *Bam*HI (lanes P−T, Digest). Lanes A, F, K, and P are from the DNA probe at nucleotide −10. Lanes B, G, L, and Q are from the DNA probe at nucleotide −9. Lanes C, H, M, and R are from the DNA probe at nucleotide −8. Lanes D, I, N, and S are from the DNA probe at nucleotides −3 and −2. Lanes E, J, O, and T are from the DNA probe at nucleotide −1. (B) Analysis of DNA probes by nondenaturing 4% PAGE. Samples were prepared before phenol extraction and ethanol precipitation of DNA probes and loaded onto a 20 × 20 cm × 0.8 mm 4% polyacrylamide gel as described. The DNA probes contained in lanes A−E were at bps −10, −9, −8, −3/−2, and −1, respectively.

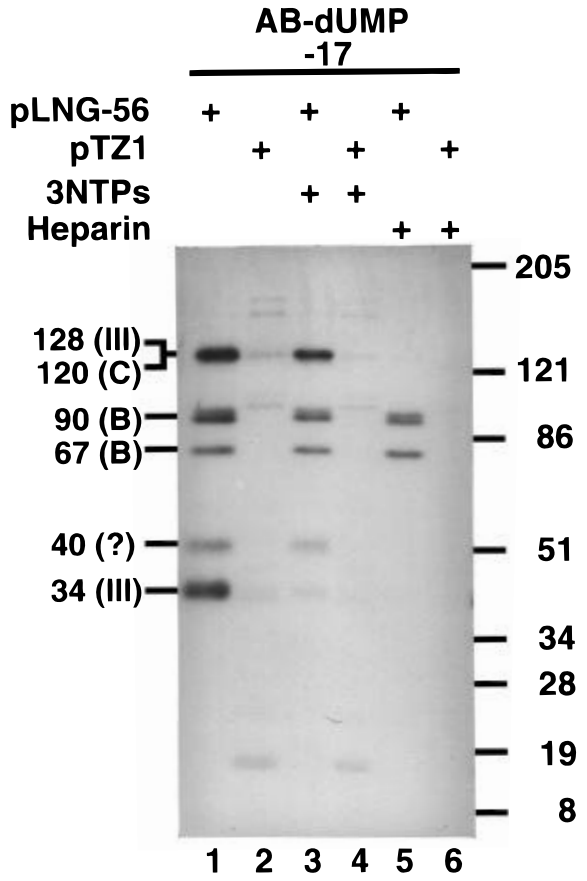

FIGURE 3: DNA photoaffinity labeling of a 40 kDa protein at bp −17 is TFIIIC-dependent and heparin-sensitive. Samples were analyzed on a 4−20% SDS−PAGE, and the autoradiogram of the dried gel is shown. The relative mobilities of the prestained protein molecular mass markers (in kilodaltons) are indicated to the right, and relative mobilities of the photoaffinity-labeled 128 and 34 kDa subunits of Pol III (III), the 67 and 90 kDa subunit of TFIIIB, and the 120 subunit of TFIIIC (C) are indicated to the left. The addition of excess pLNG56 (promoter-down) or pTZ1 (promoter-up) DNA, of 100 μM ATP, CTP, and UTP, or of heparin to a final concentration of 100 μg/mL to the reaction mixture is indicated above each lane.

gene was determined by the following three controls: (1) DNA competition using specific vs nonspecific DNAs containing either the up or down mutant promoter of the *SUP*4 $tRNA^{Tyr}$ gene (pTZ1 and pLNG56 DNAs, respectively), (2) conversion of the initiation complex into a stalled elongation complex paused at bp +17 by the addition of ATP, CTP, and UTP, and (3) heparin resistance of TFIIIB in initiation or elongation complexes and of Pol III only in elongation complexes. Photoaffinity labeling experiments using the crude extract were shown to efficiently label proteins that were specific and in terms of their apparent molecular mass correlated very well with previous results for highly purified Pol III, TFIIIC, and TFIIIB. The proteins photoaffinity labeled in the BR500 extract were identified as subunits of TFIIIB, TFIIIC, or Pol III by their apparent molecular mass and the specificity of photoaffinity labeling.

Four of the five polypeptides labeled at bp −17 in a promoter-dependent manner correspond by relative molecular mass to the 90 and 67 kDa subunits of TFIIIB, the 128 kDa subunit of Pol III or the 120 kDa subunit of TFIIIC, and the 34 kDa subunit of Pol III and were previously detected using purified components [Figure 3, lanes 1 and 2, compared to Figure 2 in Bartholomew et al., (1993)]. The 90 and 67 kDa proteins were photoaffinity labeled in the presence of heparin in a TFIIIC-dependent manner consistent with their being subunits of TFIIIB (lanes 5 and 6). The addition of nucleotides significantly reduced photoaffinity labeling of the 34 kDa protein, as had been seen with purified components, due to translocation of Pol III on DNA (lanes 3 and 4). The 90 kDa subunit of TFIIIB was photoaffinity labeled to a greater degree and the 34 kDa subunit of Pol III to a lesser degree in the BR500 extract with respect to the other labeled proteins at bp −17 than seen with purified components.

The translocation of the 34 kDa subunit of Pol III in the stalled elongation complex had been shown with purified components by the shift of photoaffinity labeling of the 34 kDa subunit from bp −17 to −3/−2. This result was faithfully replicated not only at bp −17 but also at bp −3/−2 with BR500 extract as shown by strong labeling of the 34 kDa protein at bp −3/−2 with the addition of ATP, CTP, and UTP (Figure 4A, lanes 7 and 8). Four out of five proteins specifically photoaffinity labeled (34, 67, 128/120, and 160 kDa) at bp −9 correspond by relative molecular mass to subunits of Pol III and TFIIIB that had been previously photoaffinity labeled with purified components [Figure 4A, lane 3, compared to Figure 6B in Bartholomew et al. (1993)]. The 160 and 34 kDa subunits of Pol III were not significantly labeled at bp −9 upon formation of elongation complexes, consistent with prior results and the translocation of Pol III due to elongation of the transcription complex (lane 4). Upon addition of ATP, CTP, and UTP, a 128 kDa protein was efficiently photoaffinity labeled at bp +16 in a heparin-resistant manner (Figure 4B, lane 8, and result not shown); consistent with it corresponding to the second-largest subunit of Pol III.

Five polypeptides were also photoaffinity labeled at bp +16 in the initiation complex corresponding by relative molecular mass to the 74 kDa subunit of TFIIIC and the 27, 53, 82, and 160 kDa subunits of Pol III (Figure 4B, lane 7). This labeling pattern closely resembles that noted for purified components [see Figure 10, lane 3, of Bartholomew et al., (1993)], except that the relative labeling intensity of the 160 kDa subunit of Pol III was more and that of the 27 kDa subunit of Pol III less than labeling of the 74 kDa subunit of TFIIIC. Two proteins with relative molecular masses of 128 and 160 kDa were efficiently photoaffinity labeled at bp +11; whereas proteins corresponding to the 27, 53, and 82 kDa subunits of Pol III and the 74 kDa subunit of TFIIIC were much less efficiently labeled (Figure 4C, lane 7), similar to that seen previously with purified components [see figure 9 of Bartholomew et al. (1993)].

These results demonstrate that the subunits of TFIIIC, TFIIIB, and Pol III were specifically and efficiently photoaffinity labeled in a BR500 extract and could be correlated to prior results using purified components. In the case of the 120 kDa subunit of TFIIIC and the 128 kDa subunit of Pol III, it is difficult to identify which one is labeled because of their similar mobilities on SDS−PAGE. The 128 kDa subunit of Pol III can be distinguished from the 120 kDa subunit of TFIIIC in ternary complexes, because Pol III is bound to DNA whereas TFIIIC is released in the presence of heparin. A comparison of the results obtained using BR500 extract and purified Pol III, TFIIIC, and TFIIIB is summarized in Table 1.

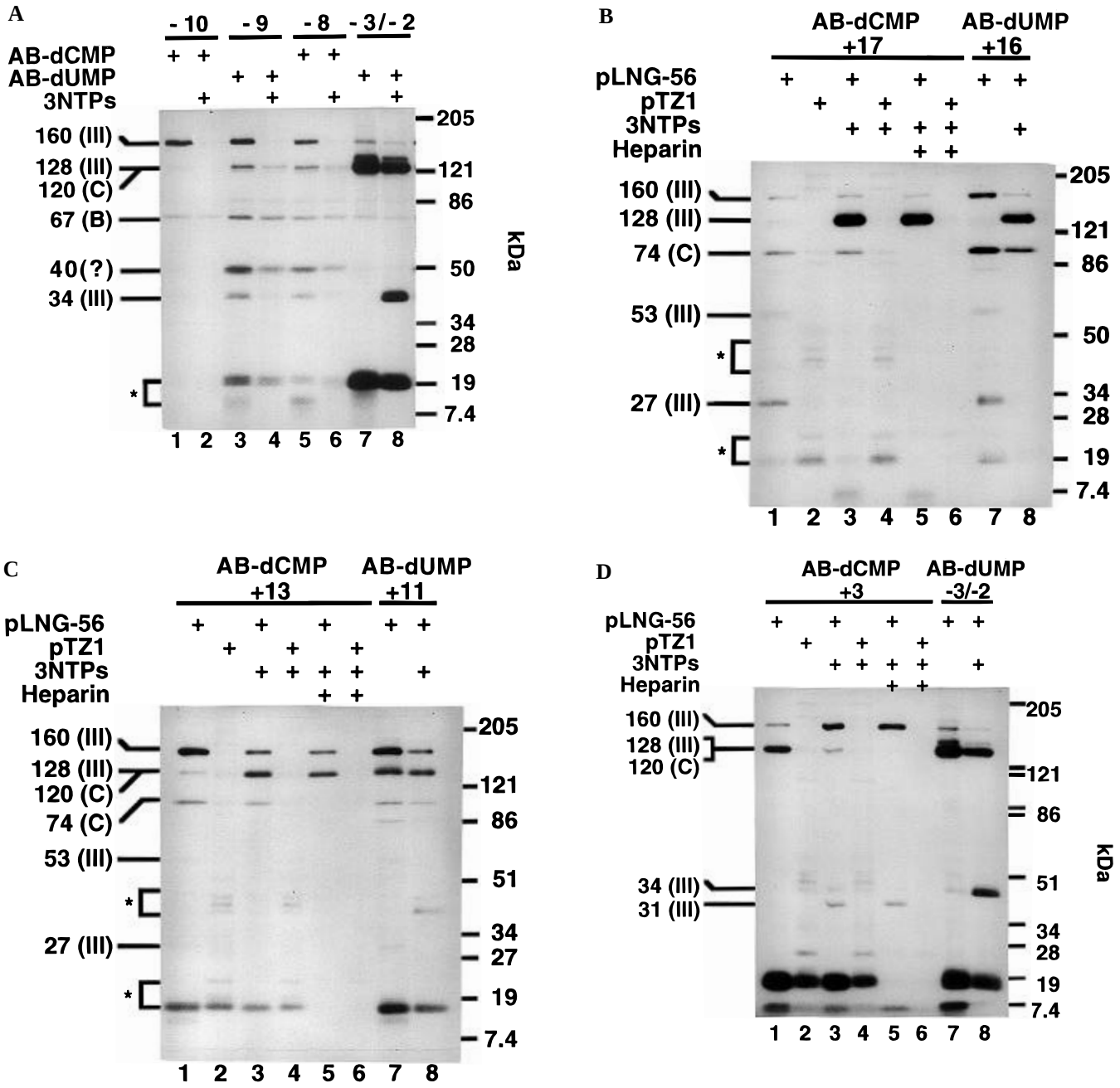

FIGURE 4: Comparison of protein−DNA cross-linking in Pol III transcription complexes with DNA photoaffinity probes containing AB-dCMP or AB-dUMP. Photoaffinity labeling results are shown in (A) with DNA containing AB-dCMP at bp −10 (lanes 1 and 2) and −8 (lanes 5 and 6) and containing AB-dUMP at bps −9 (lanes 3 and 4) and −3/−2 (lanes 7 and 8), (B) with DNA containing AB-dCMP at bp +17 (lanes 1−6) and AB-dUMP at bp +16 (lanes 7 and 8), (C) with DNA containing AB-dCMP at bp +13 (lanes 1−6) and AB-dUMP at bp +11 (lanes 7 and 8), and (D) with DNA containing AB-dCMP at bp +3 (lanes 1−6) and AB-dUMP at bp −3/−2 (lanes 7 and 8). Samples were analyzed on a 4−20% SDS−PAGE, and the autoradiograms of the dried gels are shown. The relative mobilities of the prestained protein molecular mass markers (in kilodaltons) are indicated to the right, and relative mobilities of the photoaffinity-labeled 160, 128, 53, 34, 31, and 27 kDa subunits of Pol III (III), the 67 kDa subunit of TFIIIB, and the 120 and 74 kDa subunits of TFIIIC (C) are indicated to the left. The addition of excess pLNG56 (promoter-down) or pTZ1 (promoter-up) DNA, of 100 μM ATP, CTP, and UTP, or of heparin to a final concentration of 100 μg/ml to the reaction mixture is indicated above each lane. The third-largest subunit of TFIIIC has a relative molecular mass of 95 kDa, whereas the calculated molecular mass is 74 kDa as indicated. Proteins that are nonspecifically photoaffinity-labeled are indicated by the (asterisks) to the left of the autoradiogram.

A Novel 40 kDa Protein was Photoaffinity Labeled at bps −8, −9, and −17 in a TFIIIC-Dependent Manner with BR500 Extract. One significant difference found between the BR500 extract and purified components was photoaffinity labeling of a 40 kDa protein at bps −17 and −9 (Figure 3, lane 1, and Figure 4A, lane 3). The 40 kDa protein labeled at bps −17 and −9 was not as efficiently displaced from DNA as was the 34 kDa protein by the addition of ATP, CTP, and UTP (Figure 3, lanes 1 and 3, and Figure 4A, lanes 3 and 4). Labeling of the 40 kDa protein was TFIIIC-dependent and eliminated by the addition of heparin (Figure 3, lanes 2 and 5, and results not shown). The labeling requirements of the 40 kDa protein suggest that it is a subunit neither of Pol III (not efficiently translocated with the 160 and 34 kDa subunits of Pol III) nor TFIIIB (lack of heparin resistance).

Detection of the 40 kDa polypeptide at bps −17 and −9 prompted an investigation of other positions for additional

Table 1: Summary of DNA Photoaffinity Labeling Using BR500 Extract and Highly Purified TFIIIC, TFIIIB, and Pol III[a]

nucleotide position base pair(s)	initiation complex: photoaffinity-labeled protein (kDa)										
	Pol III							IIIC	IIIB		other
	160	128 or 120*	82	53	34	31	27	74	90	67	40
−17 (BR500)		++			++				+	+	+
−17 (purified)		++			+++					+	
−12 (BR500)	++	++			+					++	
−12 (purified)	++	+			+					+	
−10 (BR500)	+++									+	
−9 (BR500)	+++	+			+				+	+	++
−9 (purified)	++	+++ (120)			+					+++	
−8 (BR500)	++	+			+				+	+	+
−3/−2 (BR500)	+	+++							+	+	
−3/−2 (purified)	+	+++			+					+	
−1 (BR500)	+	++									
+3 (BR500)	+	++									
+6/+7 (purified)	+++	++			+		+				
+11 (BR500)	+++	++	+	+			+	+			
+11 (purified)	++	++	+	+			+	+			
+13 (BR500)	+++	+						+			
+16 (BR500)	++		+	+			+	++			
+16 (purified)	+	+	+	+			++	++			
+17 (BR500)	+	+		+			+	+			
	elongation complex										
−3/−2 (BR500)		+			++						
−3/−2 (purified)		+			+++						
−1 (BR500)		+			++						
+3 (BR500)	++					+					
+6/+7 (purified)	+++	+	+		+++	+					
+11 (BR500)	++	++			+						
+11 (purified)	+	+			++						
+13 (BR500)	++	++									
+16 (BR500)	+	+++									
+17 (BR500)	+	+++									

[a] The relative amount of photoaffinity labeling of Pol III, TFIIIB, and TFIIIC subunits are indicated by +++ for strong, ++ for moderate, and + for weak labeling. The * indicates that it was not possible to distinguish the 128 kDa subunit of Pol III from the 120 kDa subunit of TFIIIC, and therefore, they were grouped together. The (120) indicates that only the 120 kDa subunit of TFIIIC was labeled at this position as determined using purified components. The elongation complex refers to the heparin-stripped elongation complex stalled at bp +17. The label (BR500) indicates the these results are from this paper using the BR500 extract and the label (purified) refers to the results of Bartholomew et al. (1993) using highly purified TFIIIC, TFIIIB, and Pol III.

DNA contacts of this protein. No labeling of the 40 kDa protein was detected at bp −12 or at positions upstream of bp −17 (results not shown). Additional probing was done at positions surrounding bp −9 using AB-dCMP incorporated at either bp −10 or −8. Photoaffinity labeling of the 160 kDa subunit of Pol III was shown to be most intense at bps −10 and −9 and progressively decreases at bps −8 and −3/−2 (Figure 4A, lanes 1, 3, 5, and 7). Three polypeptides with apparent molecular masses of 128/120, 40, and 34 kDa were photoaffinity labeled at bps −9 and −8, but not at bp −10. Labeling of 160, 128/120, 40, and 34 kDa proteins was shown to be TFIIIC-dependent by specific DNA competition (results not shown). Photoaffinity labeling of the 160 kDa subunit of Pol III at bps −10, −9, and −8 and the 34 kDa subunit of Pol III at bps −9 and −8 was greatly diminished, whereas labeling of the 128/120 and 40 kDa proteins at bps −9 and −8 was only slightly reduced with the addition of ATP, CTP, and UTP (Figure 4A, lanes 2, 4, and 6). Labeling of the 128 and 40 kDa polypeptides in the elongation complex was eliminated by the addition of heparin (results not shown). Photoaffinity labeling at bps −9, −8, and −3/2 results in nonspecific labeling of a 19 kDa polypeptide that is not dependent on a functional *box B* promoter element. The absence of photoaffinity labeling at bps −12 and −10 and labeling at bps −17, −9, and −8 on the nontranscribed strand suggests that the 40 kDa polypeptide is located on one side of the DNA helix. As shown in the following sections, the 40 kDa protein was not cross-linked in any position farther downstream than bp −8 using either AB-dUMP or AB-dCMP.

DNAs Containing AB-dCMP Efficiently Photoaffinity Labeled Pol III Transcription Complexes Comparable to That with DNAs Containing AB-dUMP at Adjacent Positions. The difference of photoaffinity labeling at bp −10 vs −9 could have been due to a difference between AB-dCMP and AB-dUMP, although this would not be consistent with the similarity of photoaffinity labeling at bps −9 and −8. Other positions were examined to more effectively compare DNA photoaffinity labeling with AB-dCMP vs AB-dUMP. The previously characterized DNA probes containing AB-dUMP at bps +16, +11, and −3/−2 (Bartholomew et al., 1993) were compared to novel DNA probes containing AB-dCMP at the proximal nucleotide positions of +17, +13, +3, and −1, all in the nontranscribed strand of the *SUP4* $tRNA^{Tyr}$ gene. Photoaffinity labeling with DNA probes containing AB-dCMP at bp +17 and AB-dUMP at bp +16 were compared (Figure 4B). Two polypeptides corresponding to the 53 and 27 kDa subunits of Pol III were equally photoaffinity labeled at both positions in the initiation complex (Figure 4B, compare lanes 1 and 7). Two larger polypeptides corresponding to the 74 kDa subunit of TFIIIC and the 160 kDa subunit of Pol III were cross-linked at both

positions in the initiation complex but were more efficiently labeled at bp +16 than at +17. Photoaffinity labeling of these four polypeptides was demonstrated to be TFIIIC-dependent (lanes 1 and 2 and data not shown). The movement of Pol III on DNA upon elongation of the transcription complex to bp +17 with the addition of ATP, CTP, and UTP was shown by changes in photoaffinity labeling (compare lanes 3 and 8 with lanes 1 and 7). Both DNA probes very efficiently labeled a polypeptide with a mobility corresponding to that of the 128 kDa subunit of Pol III in the halted elongation complex. The 128 kDa polypeptide was labeled 2.5 times more intensely at bp +17 than at bp +16, as determined by phosphorimaging. Addition of heparin prevents the photoaffinity labeling of the 74 kDa polypeptide, while retaining equivalent efficiency of labeling of the 160 and 128 kDa polypeptide (lane 5 and result not shown), consistent with the labeled 160 and 128 kDa proteins being the two largest subunits of Pol III in an elongation complex and the 74 kDa protein being a subunit of TFIIIC.

Photoaffinity labeling with DNA probes containing AB-dCMP at bp +13 was compared to that with AB-dUMP at bp +11 (Figure 4C). Four polypeptides were observed to be photoaffinity labeled with both probes in initiation complexes; three of these polypeptides correlate by mobility to the 160 and 128 kDa subunits of Pol III and the 74 kDa subunit of TFIIIC (Figure 4C, lanes 1 and 7). The fourth polypeptide (apparent molecular mass of 15 kDa) was not labeled in a TFIIIC-dependent manner as demonstrated by the addition of pTZ1 DNA, in contrast to the other three labeled polypeptides (compare lanes 1 and 2 and data not shown). The high labeling efficiency of the 160 kDa polypeptide in the initiation complex was the same with either probe as determined by phosphorimaging. The labeling intensity of the 128 kDa polypeptide was 10 times higher with probe +11 than with probe +13. Addition of ATP, CTP, and UTP caused a 10-fold increase in labeling of the 128 kDa polypeptide with probe +13, whereas no significant change in labeling intensity was observed for probe +11 (compare lanes 1 and 7 with lanes 3 and 8). Labeling of the 160 kDa polypeptide was decreased 4−5-fold for both DNA probes by the addition of the three nucleotide triphosphates. The persistent labeling of the 160 and 128 kDa polypeptides in the presence of heparin with the addition of ATP, CTP, and UTP is consistent with both polypeptides being subunits of Pol III in a stalled elongation complex (lane 5 and result not shown). Labeling of the 74 kDa subunit of TFIIIC was however not seen after the addition of heparin, consistent with stripping of TFIIIC from DNA by heparin. Nonspecific labeling of the 15 kDa polypeptide was also eliminated by the addition of heparin.

Previously, the 34 kDa subunit of Pol III had been shown to be weakly photoaffinity labeled at bp +11 in the paused elongation complex (Bartholomew et al., 1993) and can be seen in lane 8 of Figure 4. The 34 kDa subunit of Pol III was not however detected at bp +13 (Figure 4C, lanes 3 and 5), +16, and +17 (Figure 4B, lanes 3, 5, and 8) after the addition of 3 NTPS, suggesting that +11 may closely define the 3′ boundary of the 34 kDa subunit of Pol III in the paused elongation complex.

The efficient labeling of the 160 kDa polypeptide from bp +17 to +11 and the significantly less efficient labeling of the 128 kDa polypeptide from +17 to +13 in the initiation complex suggest that the largest subunit of Pol III makes close contact with the nontranscribed strand from bp +17 to +11, whereas the second-largest subunit of Pol III begins to come in closer contact only at bp +11. Dramatic changes in the placement of the two largest subunits of Pol III were observed by photoaffinity labeling upon initiation of transcription and formation of the paused elongation complex. The second-largest subunit of Pol III almost exclusively appears to be in close proximity to bps +16 and +17, and the two largest subunits of Pol III are close to the nontranscribed strand at nucleotides +11 and +13 in the halted elongation complex.

Two Polypeptides with an Apparent Molecular Mass Corresponding to the 160 and 31 kDa Subunits of Pol III Were Cross-Linked to the 5′ End of the Transcriptional Bubble in the Stalled Elongation Complex. Photoaffinity labeling by AB-dCMP incorporated at bp +3 (Figure 4D) or −1 (result not shown) was compared to that of a DNA probe containing AB-dUMP at bps −3 and −2 (−3/−2). In contrast to photoaffinity labeling at +11, +13, and +16, the 128 kDa protein was much more intensely labeled at +3, −1, and −3/−2 in the initiation complex than was the 160 kDa polypeptide (Figure 4D, lanes 1 and 7, and result not shown). Upon initiation of transcription, the photoaffinity labeling patterns at bps +3 and −1 or −3/−2 were found to be very dissimilar. Photoaffinity labeling of the 160 kDa polypeptide at bp +3 increased 3.9-fold, whereas labeling of the 128 kDa polypeptide decreased 10-fold upon the addition of ATP, CTP, and UTP (compare lanes 1 and 3). Labeling of the 160 and 128 kDa proteins at bp −1 decreased slightly with a large increase in labeling of a protein with a relative mobility expected for that of the 34 kDa subunit of Pol III, similar to that seen at bp −3/−2 (lane 8 and result not shown). A 31 kDa protein was photoaffinity labeled at bp +3 with the addition of ATP, CTP, and UTP, and to a much lesser extent, a 34 kDa protein was also labeled (Figure 4D, lane 3).

The persistent labeling of the 160 and 31 kDa proteins at bp +3 in arrested elongation complexes treated with heparin was consistent for subunits of Pol III engaged in transcription (Figure 4D, lane 5). The residual labeling of the 128 kDa polypeptide at bp +3 in the presence of ATP, CTP, and UTP appears to be due to transcriptionally inactive Pol III or labeling of the 120 kDa subunit of TFIIIC, since it was eliminated by the addition of heparin. Photoaffinity labeling of the 160 and 31 kDa polypeptides at bp +3 were shown to be TFIIIC-dependent (lanes 4 and 6). Weak nonspecific labeling of a 34 kDa protein at bp +3 in elongation complexes was removed by the addition of heparin. Nonspecific labeling of a 15 kDa protein was detected at bp +3. A polypeptide of similar size had previously been detected with purified TFIIIC and TFIIIB at bp −3/−2 (Bartholomew et al., 1991).

The data suggest that, beginning at bp +11, the second-largest subunit begins to come in closer contact with the nontranscribed strand of DNA in the initiation complex and is centered around the start site of transcription (i.e. −3/−2, −1, and +3) as indicated by it being intensely photoaffinity labeled at these positions. Upon initiation and limited elongation of Pol III, the second-largest subunit moves with the transcription bubble and remains centered over the site of RNA synthesis (i.e. +16 and +17, and to a lesser extent +11 and +13) and becomes less accessible to cross-linking

to DNA at +3. Placement of the 160 and 31 kDa subunits of Pol III on the nontranscribed strand in the stalled elongation complex was however shown to be in the vicinity of bp +3, at the extreme 5′ end of the transcription bubble.

DISCUSSION

The technique of site-directed DNA photoaffinity labeling to survey the contacts of transcription factors and RNA polymerase III had been previously restricted to deoxythymidine nucleotide positions (Bartholomew et al., 1990, 1991, 1993), but in these experiments, we have synthesized and utilized the photoreactive deoxycytidine analog, 4-[*N*-(p-azidobenzoyl)-2-aminoethyl]deoxycytidine triphosphate or AB-dCTP, to expand the number of DNA sites that can be examined. The modified deoxycytidine has a short alkyl chain attached through the N-4 amino group of cytidine to a photoreactive aryl azide (see Figure 1A) with an approximate distance of 9 Å. AB-dCTP readily substitutes for dCTP in oligonucleotide primer extension reactions, making it possible to enzymatically incorporate the photoreactive nucleotide at specific sites in DNA. Incorporation of the modified deoxycytidine at bps −10, −8, −1, +3, +13, and +17 on the nontranscribed strand of the *SUP4* $tRNA^{Tyr}$ gene appeared not to significantly interfere with transcription complex formation, initiation, or elongation of transcription as demonstrated by transcription assays using the modified DNA templates.

Most DNA photoaffinity labeling studies have utilized highly purified protein preparations, whereas photoaffinity labeling ideally can selectively label its specific protein target present in a crude extract. We have investigated the use of photoreactive DNA probes containing either AB-dCMP or AB-dUMP for probing the structure of *S. cerevisae* Pol III transcription complexes in a crude protein extract and have compared these results to previous ones using highly purified TFIIIC, TFIIIB, and Pol III (Bartholomew et al., 1991, 1993). Many of the positions in DNA investigated gave results similar to those obtained previously. Consistent with previous results, the 128 kDa subunit of Pol III was cross-linked near the site of RNA catalysis at bp +16 and +17 in the paused elongation complex. The 160 kDa subunit of Pol III was found to be located in positions flanking the second-largest subunit of Pol III (128 kDa). The translocation of the 34 kDa subunit of Pol III from bp −17 to −3/−2 upon initiation of transcription was also observed. The great similarity of the photoaffinity labeling results obtained with the crude protein extract given in this report with that reported before with highly purified TFIIIC, TFIIIB, and Pol III demonstrated the utility of DNA photoaffinity labeling for examining protein−DNA contacts in a potentially more complete but less-purified system.

Photoaffinity labeling in the region spanning nucleotides −17 to −8 on the nontranscribed strand revealed the contacts of a 40 kDa protein, which had not been detected using the highly purified system. Evidence is given for the specific association of this 40 kDa protein to the *SUP*4 $tRNA^{Tyr}$ gene by specific DNA competition. The position selectivity of cross-linking the 40 kDa subunit suggests that it is localized within ∼10 base pairs on DNA in close proximity to DNA near the 3′ end of the TFIIIB binding site and may be located on one side of the DNA helix as shown by it being cross-linked at bps −17, −9, and −8 (approximately one helical turn apart) and no cross-linking at bps −12 and −10 on the nontranscribed strand. The 40 kDa protein may potentially be the TFIIIE factor reported by Dieci et al. (1993) that is involved in effective utilization of stable transcription complexes, but not in template commitment. Gel filtration and glycerol gradient analysis indicated that TFIIIE has a relative molecular mass of 30 or 60 kDa, respectively, which would be consistent with the size of the 40 kDa protein identified in this report. The placement of the 40 kDa protein at this position in the transcription complex puts it at a pivotal position for access to Pol III, TFIIIB, and TFIIIC near the start site of transcription, and it could therefore conceivably regulate the activity of the transcription complex.

Another possibility is that the 40 kDa protein could be a missing subunit of Pol III that may not be essential in a highly purified system, but that normally facilitates the binding of Pol III to the transcription complex. The composition of highly purified Pol III has had some variability with regards to the presence of a 37 kDa subunit which is often present in submolar ratios or even absent (Sentenac et al., 1992). The 37 kDa subunit is one of only two subunits (the other is an 11 kDa protein) of Pol III that has not been cloned; the other 14 subunits have all been cloned and found to be essential for cell viability (Sadhale & Woychik, 1994; Sentenac et al., 1992). The preparation of Pol III used in previous studies appeared not to contain significant amounts of the 37 kDa subunit, thereby raising the possibility of the photoaffinity-labeled 40 kDa protein as a subunit of Pol III. If the 40 kDa polypeptide is a subunit of Pol III, it is somewhat surprising that upon initiation and elongation of Pol III the 40 kDa protein appears to remain associated with the region upstream of the start site of transcription. The identification of the labeled 40 kDa protein awaits further experiments of possibly tracking the protein through subsequent protein purification steps with DNA photoaffinity labeling or through immunoprecipitation of the labeled protein as the antibodies to TFIIIE or of 37 kDa subunit of Pol III become available.

The additional sites that can be probed in the transcription complex using AB-dCMP helps generate a more complete view of the topography of the Pol III transcription complex and are summarized and compared to other results in Table 1. Previously, the nontranscribed strand around the start site of transcription (−1 to +5) could not be probed using the photoreactive nucleotide AB-dUMP, because of its A- and C-containing sequence. Probing at bp +3 in the elongation complex shows results very different than those at bps −1 and −3/−2 with no significant labeling of either the 128 or 34 kDa subunits of Pol III in the ternary complex. However, the 160 kDa and to a lesser extent the 31 kDa subunits of Pol III are photoaffinity labeled at bp +3 in the ternary complex. In our experiments, the 31 kDa subunit of Pol III was not observed to be cross-linked at bps −1 or −3/−2 or at any of the other base pairs examined. Previously, the 31 kDa subunit of Pol III had been observed to be cross-linked at bps +6/+7 in the ternary complex, but to a much lesser degree than the 34 kDa subunit.

The transcription bubble in the *SUP4* $tRNA^{Tyr}$ gene has been positioned at bps −11 to +11 and +3 to +19 in initiation and elongation complexes by permanganate footprinting, respectively (Kassavetis et al., 1990, 1992). Contacts of the 31 and 160 kDa subunits of Pol III with the 5′ edge of the transcription bubble suggests that they might

function to help displace RNA transcript from DNA template and/or to facilitate the reannealing of the transcribed and nontranscribed strands of DNA as the transcription bubble is translated downstream. Other data that support this idea is the suppression of a conditionally defective 31 kDa subunit mutant by high-level expression of the *DED1* gene encoding a putative helicase (Thuillier et al. 1995). Evidence for interactions of the 31 kDa subunit with the 160 kDa subunit of Pol III is also shown by suppression of this same mutant containing the defective 31 kDa subunit by overexpression of the 160 kDa subunit of Pol III. This observation correlates well with our results of the 160 and 31 kDa subunits of Pol III together making contacts at bp +3 in the elongation complex.

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