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Survey of four different photoreactive moieties for DNA photoaffinity labeling of yeast RNA polymerase III transcription complexes

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ABSTRACT

In order to optimize the detection of protein–DNA contacts by DNA photoaffinity labeling, we attached four different photoreactive groups to DNA and examined their ability to crosslink yeast RNA polymerase III (Pol III) transcription complexes. Photoreactive nucleotides containing an aryl azide (AB-dUMP), benzophenone (BP-dUMP), perfluorinated aryl azide (FAB-dUMP) or diazirine (DB-dUMP) coupled to 5-aminoallyl deoxyuridine were incorporated into the *SUP4* tRNA^{Tyr} gene at bp –3/–2 or +11. Photo-crosslinking with diazirine revealed contacts of Pol III with DNA that are not detected by DNA photoaffinity labeling using an aryl azide, fluorinated aryl azide or benzophenone group attached to DNA. These novel contacts were of the 82 kDa subunit of Pol III with DNA at bp –3/–2 in the initiation complex and of the 82, 40(37) and 31 kDa subunits of Pol III with DNA at bp +11 in elongation complexes stalled at bp +17. These results provide evidence for the subcomplex of the 82, 34 and 31 kDa subunits of Pol III being positioned near the transcription bubble of actively transcribing Pol III, as all three proteins were crosslinked at bp +11 of the stalled transcription complex.

INTRODUCTION

The position of ~25 different proteins of the yeast RNA polymerase III (Pol III) transcription complex have been mapped by DNA photoaffinity labeling to specific sites on DNA (1–6). Crosslinking a protein at a specific nucleotide position indicates the proximity of the protein at that location on DNA; whereas, the lack of crosslinking shows the absence of a protein or the lack of reactivity of that particular protein surface with the photoreactive species. Photoreactive DNAs need to be designed with a highly reactive and non-specific photoreactive group in order to know if a protein is truly not present when not crosslinked at a particular site.

The original mapping of protein–DNA contacts was done with an aryl azide attached at specific sites in DNA. Aryl azides may not have been the best choice for a photoreactive group, because the singlet nitrene formed by photolysis rapidly rearranges by ring expansion to form a dehydroazepine. Dehydroazepines react slowly and preferentially with nucleophiles. A better nitrene-generating reagent can be made by flourination of aryl azide to help stabilize the nitrene and reduce the rate of rearrangement to dehydroazepine (at least 170 times slower with pentafluoroaryl azide) (7). Flourinated dehydroazepines are also many times more reactive than the corresponding non-fluorinated dehydroazepine. Benzophenone has been suggested to be potentially a more efficient crosslinker than aryl azides because of, (i) its inability to react with water, a key competitor of the photoaffinity labeling reaction and (ii) it preferentially reacts with fairly unreactive C-H bonds. Although benzophenone can be a more efficient crosslinker under appropriate conditions, it has a high selectivity due to the geometry required for H abstraction by the diradical (8). Diazirines upon photolysis generate carbenes that are more reactive than nitrenes and can react with all twenty different amino acid side chains (9). A certain percentage of the irradiated diazirine isomerizes to form a less reactive diazo compound that reacts with nucleophiles and can cause non-specific crosslinking to occur because of its long half life. Crosslinking by the diazo compound can be avoided by making the diazo group much less reactive or inert by introduction of a strong electron withdrawing group as in 3-trifluoromethyl-3-phenyl diazirine (10–12). 3-trifluoromethyl-3-phenyl diazirine has been shown to efficiently insert into C-H bonds and has proven to be an effective tool for studying membrane and ribosomal structures (10,13–17). Diazirines have the advantage of being photolyzed at ~350 nm and not being sensitive to reducing agents.

We examined the effect of these different photoreactive groups on the efficiency and selectivity of DNA photoaffinity labeling of yeast Pol III transcription complexes by tethering the four different photoreactive groups to the C-5 carbon of deoxyuridine. Diazirine containing DNA revealed new protein–DNA contacts at bp –3/–2 in initiation complexes, and at bp +11 in stalled elongation complexes of the *SUP4* tRNA^{Tyr} gene (+1 being the start site of transcription) that were not detected with photoreactive DNAs containing an aryl azide, perfluorinated aryl azide or benzophenone.

MATERIALS AND METHODS

Synthesis and incorporation of photoreactive deoxyuridine analogs

The following procedures were all carried out under reduced lighting conditions. The photoreactive nucleotides 5-[*N*-(4-benzoyl-benzoyl)-3-aminoallyl]-deoxyuridine triphosphate (BP-dUTP), 5-[*N*-(4-azido-2,3,5,6-tetrafluorobenzoyl)-3-aminoallyl]-deoxyuridine triphosphate (FAB-dUTP) and 5-{*N*-[4-[3-(trifluoromethyl)-

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diazirin-3-yl] benzoyl]-3-aminoallyl}-deoxyuridine triphosphate (DB-dUTP) were synthesized similar to that described for 5-[*N*-(*p*-azidobenzoyl)-3-aminoallyl]-deoxyuridine triphosphate (AB-dUTP) (1,18). Reactions contained 100 µl of 100 mM 4-azido-2,3,5,6-tetrafluorobenzoate *N*-hydroxysuccinimide (FAB-NHS, Molecular Probes), 4-benzoylbenzoate *N*-hydroxy-succinimide (BP-NHS, Molecular Probes) or 4-[3-(trifluoro-methyl)diazirin-3-yl]benzoate *N*-hydroxysuccinimide [DB-NHS, prepared as described (12)], in dimethylsulfoxide (DMSO); and 100 µl of 20 mM 5-aminoallyl deoxyuridine triphosphate (5-aa-dUTP) in 100 mM sodium borate, pH 8.5. An additional 200 and 800 µl of DMSO was added to BP-NHS and DB-NHS containing reactions, respectively. Reactions were incubated at 25°C for 4 h for FAB-dUTP and BP-dUTP and 7.5 h for DB-dUTP.

The nucleotide analogs were purified by ion exchange chroma-tography with DEAE A-25 Sephadex as described (18). Column fractions were analyzed by PEI-F cellulose thin layer chroma-tography developed with 1 M LiCl. The R_f values for BP-dUTP, FAB-dUTP, DB-dUTP and 5-aa-dUTP were 0.01, 0.05, 0.01 and 0.23, respectively. The concentrations of BP-dUTP (9.87 mM^{-1}) and FAB-dUTP (18.00 mM^{-1}) were determined by UV spectrophotometry at 275 nm and of DB-dUTP (390 mM^{-1}) at 348 nm (12).

Optimal modified nucleotide concentrations were determined for each nucleotide analog in an enzymatic incorporation assay using an oligonucleotide primer annealed to a single-stranded DNA template. A sample containing 12.0 pmol of oligonucleotide pCTTCAACAATTAAATACTG and 10.0 µg of the single stranded p2A2D DNA (1) in 28 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) was incubated for 3 min at 90°C then 30 min at 37°C. Next, 28 µl of buffer C containing α-^{32}P-labeled dCTP (specific activity 3000 Ci/mmol, final concentration ~2.4 µM) and 150 µg/ml bovine serum albumin was added to the reaction. The sample was divided into 4.5 µl aliquots and 4.5 µl of either BP-dUTP, FAB-dUTP or DB-dUTP in buffer C at concentrations of 60, 20, 6, 2 and 0.6 µM were added along with 0.25 U exonuclease-free Klenow fragment of DNA polymerase I. The samples were briefly vortexed and incubated at 37°C for 5 min. From each sample, 2 µl were removed and added to 10 µl of 95% formamide with 0.1% xylene cyanol and bromophenol blue for analysis on a 10% polyacrylamide gel containing 8.3 M urea. All three novel photoreactive nucleotides were found to be efficiently incorporated into DNA, similar to AB-dUTP. A concentration of 3 µM for all four of the modified nucleotides is sufficient for efficient incorporation without read-through.

DNA probe synthesis, photoaffinity labeling of DNA–protein complexes and *in vitro* transcription assays

Synthesis of DNA photoaffinity probes at positions –3/–2 and +11, photoaffinity labeling of DNA–protein complexes and *in vitro* transcription assays were as described, except for irradiation conditions of DNAs containing BP-dUMP and DB-dUMP (5). After photolysis, the samples were digested with DNase I and S-1 nuclease, and analyzed on a 4–20% SDS–PAGE. Photoaffinity labeled proteins were detected by autoradiography of the dried gel. Pol III transcription complexes formed on DNA containing BP-dUMP or DB-dUMP were irradiated at a distance of 7 cm from a Fotodyne transilluminator Model 3-4400. A Pyrex glass casserole dish was placed between the samples and the transilluminator to filter out wavelengths <300 nm. Photoaffinity labeling with DNA containing DB-dUMP, reached its maximum level after only 2 min of irradiation, and DNA containing BP-dUMP after 15 min. All crosslinking experiments using DNAs containing DB-dUMP and BP-dUMP were irradiated in this manner for 5–7 min. Pol III purified to near homogeneity, (5) and prestained broad molecular weight range protein molecular mass standards (BioRad) served as molecular weight standards.

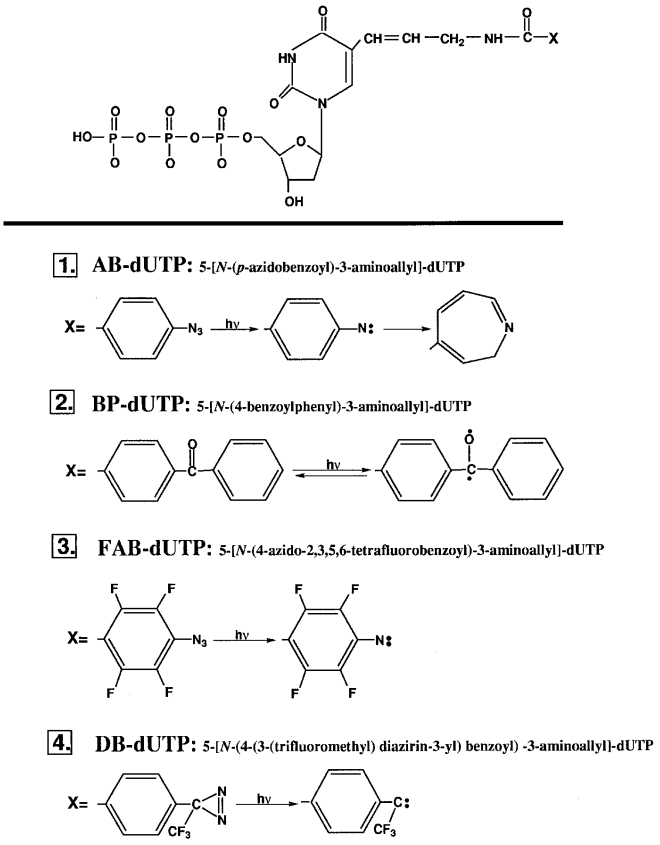


Figure 1. The structure of photoreactive deoxyuridine analogs containing an azidobenzoyl, benzophenyl, tetrafluorobenzoyl or diazirinyl moiety. The structure of four deoxyuridine analogs with different photoreactive groups attached to the C-5 position of uracil using an allyl amine linker is shown. These nucleotides were synthesized in the triphosphate form by coupling the *N*-hydroxysuccinimide esters of 4-azidobenzoic acid (Structure 1), 4-benzoyl benzoic acid (Structure 2), 4-azido-2,3,5,6-tetrafluorobenzoic acid (Structure 3), 4-(3-(trifluoromethyl)-diazirin-3-yl) benzoic acid (Structure 4) to 5-aminoallyl-2′-deoxyuridine tri-phosphate. The photoreactive intermediate generated upon photolysis for each compound is shown on the right.

RESULTS

We have examined the effect of the photoreactive group attached to DNA on the crosslinking of proteins assembled in a Pol III transcription complex. Four photoreactive deoxyuridine analogs were synthesized each having identical tethers attached to the C-5 position of uracil, but with different photoreactive groups (Fig. 1). One of these analogs, AB-dUTP (Fig. 1A), had been previously characterized extensively and has an aryl azide as its photoreactive group (18).

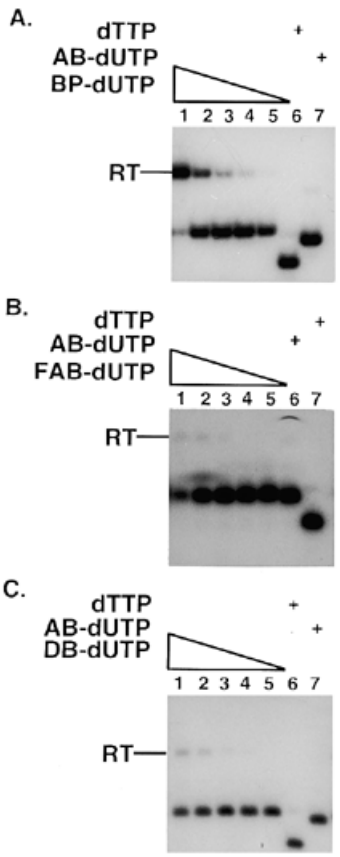


Figure 2. Efficient enzymatic incorporation of deoxyuridine analogs with different photoreactive groups. DNA primer extension reactions were performed as described in Materials and Methods using the oligonucleotide 5′-pCTTCAACAATTAAATACTG-3′ annealed to the M13 single-stranded DNA construct p2A2D. One modified deoxyuridine and one [^{32}P]deoxycytidine were incorporated per DNA template using the exonuclease-free form of the Klenow fragment of DNA polymerase I, and continued primer extension was prevented by the omission of dGTP. Samples were analyzed on a 10% polyacrylamide gel containing 8.3 M urea and visualized by autoradiography. Results shown contained (**A**) BP-dUTP (benzophenone), (**B**) FAB-dUTP (pentafluoro-aryl azide) and (**C**) DB-dUTP (diazirine), at concentrations of 30, 10, 3, 1 and 0.3 μM for lanes 1–5, respectively. In lanes 6 and 7 the reactions contained 3 μM dTTP or AB-dUTP. The position of the read-through (RT) product is indicated.

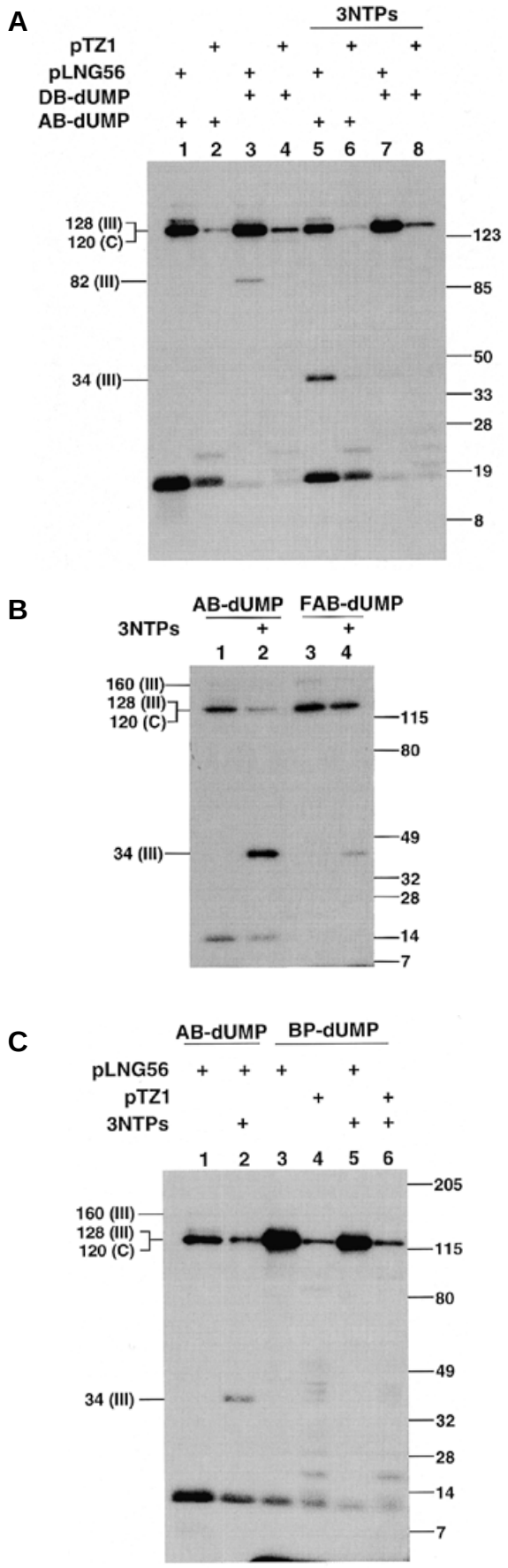
Optimal nucleotide concentration for incorporation of each modified nucleotide was examined by DNA primer extension assays using a range of nucleotide concentrations of 0.3–30 μM. All three novel photoreactive nucleotides were found to be efficiently incorporated into DNA, similar to that of dTTP and AB-dUTP. At higher concentrations, BP-dUTP was more prone to read-through than either FAB-dUTP or DB-dUTP (Fig. 2A, lanes 1–5). At 30 μM concentration, incorporation of FAB-dUMP was partially inhibited in contrast to the other nucleotide analogs (Fig. 2B, lane 1). An effective concentration for incorporation of all 4 nt was found to be ~3 μM for eliminating possible read-through or inhibition, and was used for DNA photoaffinity probe synthesis.

Effect of DNA modification at bp –3/–2 and +11 on active Pol III transcription complexes was examined by measuring *in vitro* transcription activity of modified versus unmodified DNA templates of the *SUP*4 tRNA^{Tyr} gene (data not shown). Previously, DNA containing AB-dUMP at these and several other positions in the *SUP*4 tRNA gene, had been shown to have little or no effect on transcriptional activity (5). Similarly, the difference in the photoreactive group appeared to have no significant effect on complex formation, initiation or elongation of transcription.

Photoaffinity labeling at bp –3/–2 of the *SUP4* tRNA gene

Pol III initiation complexes were photoaffinity labeled with DNA containing DB-dUMP, FAB-dUMP or BP-dUMP at bp –3/–2, and compared to that with DNA containing AB-dUMP (Fig. 3). All four DNA probes labeled an ~125 kDa protein that corresponds to the 120 kDa subunit of TFIIIC and the 128 kDa subunit of Pol III which are not resolved by gel electrophoresis (Fig. 3A–C, lanes 1 and 3). Labeling of the ~125 kDa protein by the four different photoreactive DNAs was shown to be TFIIIC-dependent by competition with specific competitor DNA (pTZ1) containing an up mutant *SUP*4 tRNA promoter (Fig. 3A lanes 2, 4, 6 and 8; Fig. 3C lanes 4 and 6 and results not shown). The non-specific competitor DNA (pLNG56) used is identical to pTZ1 except for a 2 bp change that results in a defective *box B* promoter element causing a 3000 lower affinity for TFIIIC binding (1). Pol III and TFIIIB cannot assemble onto DNA without proper binding of TFIIIC to template DNA, thus, the non-specific competitor DNA is unable to efficiently compete for binding of IIIC, IIIB or Pol III. The ~125 kDa protein was most efficiently labeled by DNA containing the benzophenone photoreactive group, i.e. BP-dUMP (Fig. 3C). Photoaffinity labeling of a protein with an apparent molecular weight corresponding to the third largest subunit of Pol III (82 kDa) was only detected using DB-dUMP (Fig. 3A, lane 3). Photoaffinity labeling of the ~80 kDa polypeptide was shown to be dependent on a functional *box B* element by competition with pTZ1 DNA (Fig. 3A, lane 4).

Next, initiation complexes were converted into stalled elongation complexes by the addition of ATP, CTP and UTP (100 μM) to produce a 17 nt long RNA transcript and further elongation prevented by omission of GTP. Previously, elongation complexes stalled at bp +17 on the *SUP4* tRNA^{Tyr} gene have been well characterized by DNase I and permanganate footprinting, and analysis of the RNA transcripts (19,20). DNase I footprinting showed a shift in protection at the start site of transcription to sequences further downstream upon conversion of the initiation complex to a stalled elongation complex with the addition of ATP, CTP and UTP. Analysis of the radiolabeled RNA transcripts from these complexes showed a single RNA transcript corresponding to the 17 nt RNA. Movement of the transcription bubble consistent with stalling of the elongation complexes has also been shown by permanganate footprinting. These results demonstrate that stalled elongation complexes can be almost quantitatively formed without significant read-through. A protein corresponding to the 34 kDa subunit of Pol III was crosslinked in the elongation complex at bp –3/–2 with AB-dUMP (Fig. 3A, lane 5). Translocation of the 34 kDa subunit of Pol III due to elongation of the transcription complex was previously shown by crosslinking the 34 kDa subunit at bp –17 in initiation and not stalled elongation complexes, and at bp –3/–2 in stalled elongation and not initiation complexes (3). The 34 kDa subunit of Pol III was much less efficiently crosslinked using the diazirine or fluorinated



aryl azide groups than with the simple aryl azide, and not labeled by benzophenone-modified DNA (Fig. 3A, compare lanes 5 and 7; Fig. 3B, lanes 2 and 4; Fig. 3C, lanes 2 and 5). Crosslinking of the ~80 kDa polypeptide or 82 kDa subunit of Pol III was eliminated by elongation of the transcription complex consistent with the ~80 kDa protein being transported to a site on DNA downstream of bp –3/–2 as part of the elongating Pol III complex (Fig. 3, lane 7).

Photoaffinity labeling of stalled elongation complexes at bp +11 of the *SUP4* tRNA gene

Before photo-crosslinking and 15 min after the addition of ATP, CTP and UTP, heparin was added to a final concentration of 100 µg/ml to disrupt binding of TFIIIC and non-transcribing Pol III, so as to crosslink only Pol III that is engaged in transcription elongation. The polyanion heparin has been shown to efficiently strip off TFIIIC and Pol III, but not TFIIIB from the initiation complex (21). After formation of the elongation complex, Pol III becomes more stably bound to DNA and is not as effectively stripped from DNA by heparin as in the initiation complex. The ~80 kDa protein was crosslinked at bp +11 in the stalled elongation complexes with DB-dUMP, and not with AB-dUMP, FAB-dUMP or BP-dUMP (Fig. 4, compare lane 3 to lanes 1, 2 and 4). Photoaffinity labeling of the 80 kDa polypeptide was dependent on a functional *box B* promoter element (lane 7). Heparin resistant labeling of the ~80 kDa protein at bp +11 required the formation of an active stalled elongation complex, i.e. the addition of ATP, CTP, UTP (result not shown). Contact of the third largest subunit of Pol III with template DNA in the elongation complex was only detected by crosslinking with carbenes.

Other polypeptides corresponding to the 160, 128, 40(37), 34 and 31 kDa subunits of Pol III were also photoaffinity labeled at bp +11 with DB-dUMP in a promoter-dependent manner (Fig. 4, lane 3). Overall, carbene mediated crosslinking was shown to be most effective for photoaffinity labeling of the 160, 82, 40(37) and 31 kDa subunits of Pol III, whereas the simple aryl azide was the best for photoaffinity labeling of the 34 kDa subunit of Pol III at bp +11. Flourinated aryl azide or benzophenone mediated crosslinking was much less intense than that with carbene or the simple aryl azide (lanes 1 and 2 versus 3 and 4).

Figure 3. Photoaffinity labeling of Pol III transcription complexes at bp –3/–2 of the *SUP4* tRNA gene with an aryl azide, diazirine, fluorinated aryl azide or benzophenone moiety. (**A**) Selective labeling of an 82 kDa polypeptide in the Pol III initiation complex by diazirine at bp –3 and –2. Reactions contained photoreactive DNA with DB-dUMP, (lanes 3, 4, 7 and 8) or AB-dUMP (lanes 1, 2, 5 and 6) incorporated at bp –3/–2 and linearized plasmid DNA, as either a non-specific (pLNG56, odd numbered lanes) or specific DNA competitor (pTZ1, even numbered lanes). Elongation complexes stalled at bp +17 were made by the addition of ATP, CTP and UTP to a final concentration of 100 µM (lanes 5–8). (**B**) Photoaffinity labeling with DNA containing FAB-dUMP (lanes 1–4) was compared to DNA containing AB-dUMP. Initiation (lanes 1 and 3) and stalled elongation complexes (lanes 2 and 4) were formed as described with modified DNA. (**C**) Photoaffinity labeling of the Pol III transcription complexes with DNA containing BP-dUMP (lanes 3–8) was compared to that with DNA containing AB-dUMP (lanes 1 and 2). The relative mobilities of photoaffinity labeled polypeptides allowed them to be identified as subunits of TFIIIC (C) or Pol III (III) as indicated on the left, and were visualized by autoradiography. The relative mobilities of prestained protein molecular weight markers are indicated on the right.

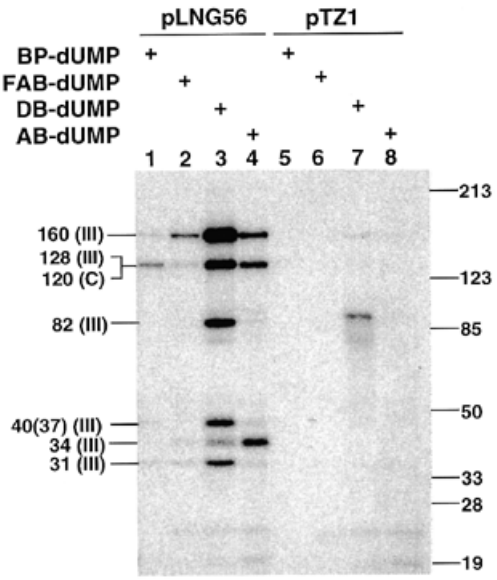


Figure 4. DNA containing DB-dUMP at bp +11 was more effective for photoaffinity labeling of stalled elongation complexes than DNAs containing an AB-dUMP, BP-dUMP or FAB-dUMP. The reactions contained 100 µM ATP, CTP and UTP to form a stalled elongation complex and heparin was subsequently added to remove TFIIIC and transcriptionally inactive or non-elongating Pol III. Photoreactive DNAs used in these reactions contained BP-dUMP (lanes 1 and 5), FAB-dUMP (lanes 2 and 6), DB-dUMP (lanes 3 and 7) or AB-dUMP (lanes 4 and 8). The protein molecular weight markers are the same as described in Figure 3. TFIIIC-dependent photoaffinity labeling was eliminated by competition with pTZ1 DNA as seen in lanes 5–8.

The photoaffinity labeled ~80 kDa protein can be shown to be the 82 kDa subunit of Pol III based on three criteria. First the relative molecular weight (Mr), as shown by SDS–PAGE, corresponds to the third largest subunit of Pol III with a slight retardation of mobility due to the small DNA fragment crosslinked to the protein. There are no known subunits of TFIIIC or TFIIIB that are of an equivalent Mr. Second, the photoaffinity labeling of the ~80 kDa protein requires the binding of the transcription factor TFIIIC and is therefore a specific component of the Pol III transcription complex. Third, labeling of the ~80 kDa protein is dependent on transcription; either stalling of the elongation complex assembly for cross-linking of the protein at bp +11 (6 nt away from the 3′-end of the RNA transcript) or the lack of transcription for crosslinking at bp –3/–2 in the preinitiation complex, and therefore, labeling of the ~80 kDa protein corresponds to the translocation of Pol III along the DNA template. The heparin resistant labeling of the ~80 kDa protein at bp +11, further eliminates the possibility of it being a previously unidentified subunit of TFIIIC. Labeling of the 160, 128, 40(37), 34 and 31 kDa proteins crosslinked at bp +11 with DB-dUMP were also shown to be Pol III subunits in a manner similar to that of the 82 kDa subunit of Pol III, and labeling of the 160, 128 and 34 kDa subunits of Pol III had been shown previously with AB-dUMP at bp +11 using highly purified TFIIIC, TFIIIB and Pol III (3).

DISCUSSION

We have compared the photoreactivity of (i) an aryl azide (AB-dUMP), (ii) benzophenone (BP-dUMP), (iii) perfluorinated aryl azide (FAB-dUMP) and (iv) diazirine (DB-dUMP) for protein–DNA crosslinking of Pol III transcription complexes. The photoreactive groups were placed in the major groove of the *SUP4* tRNA gene at bp –3/–2 or +11 by the enzymatic incorporation of a modified deoxyuridine with the photoreactive moiety attached at the C-5 position with a 3-amino allyl linker. The photoreactive group projects to the edge of the major groove as the C-5 carbon of deoxythymidine is located at the bottom of the major groove in B-DNA.

Crosslinking specificity of BP-dUMP appeared to make it a poor choice for protein–DNA crosslinking. Although DNA containing the benzophenone derivative, BP-dUMP, was the most efficient at crosslinking the 120/128 kDa proteins at bp –3/–2, it failed to photoaffinity label the other proteins at bp –3/–2 and +11 that were crosslinked with DNAs containing AB-dUMP, FAB-dUMP or DB-dUMP. The enhanced crosslinking efficiency of the 120/128 kDa protein was probably caused by the close proximity of a hydrophobic region of this protein that can provide C-H bonds with the appropriate geometry for H abstraction by the photoactivated benzophenone. Likewise the inability to crosslink other proteins with BP-dUMP is probably due to the chemical nature of those surfaces proximal to that site on DNA.

Replacing an aryl azide with a perfluorinated aryl azide did not significantly increase the number of different proteins photoaffinity labeled, nor the efficiency of labeling. At bp –3/–2 the same proteins were labeled with equivalent efficiency using FAB-dUMP or AB-dUMP, except for the less efficient labeling of the 34 kDa subunit of Pol III by FAB-dUMP. The more efficient labeling of the 34 kDa protein with AB-dUMP than with FAB-dUMP, BP-dUMP or DB-dUMP, suggests that the chemical nature of the surface of the 34 kDa subunit contacting DNA at bp –3/–2 promotes its more preferential crosslinking by an aryl azide. The more efficient labeling of the 34 kDa subunit is probably not caused by increased reactivity as DB-dUMP and FAB-dUMP generate a carbene or a more stabilized nitrene, respectively, and would be expected to be more reactive than AB-dUMP. However, the less reactive dehydroazepine formed by ring expansion of the aryl azide would be more efficient at crosslinking nucleophilic regions because of (i) the longer half life of the reactive species providing more time to find its preferred target and (ii) diminished competition of the reactive species for non-nucleophilic regions.

DNAs containing DB-dUMP were generally found to photoaffinity label the transcription complex as efficiently as AB-dUMP, and labeled additional proteins associated at bp –3/–2 and +11 in the transcription complex were not detected using AB-dUMP. A protein that corresponds by electrophoretic mobility and enzymatic properties to the third largest subunit (82 kDa) of Pol III was crosslinked at bp –3/–2 in the initiation complex with only the diazirine containing DNA. After formation of the stalled elongation complex, the 82 kDa subunit of Pol III was not labeled at bp –3/–2, but was crosslinked at bp +11 by diazirine-modified DNA. These results indicate that the 82 kDa subunit of Pol III is displaced from DNA immediately upstream of the start site of transcription upon elongation of the transcription complex, and that it is in close proximity to DNA within the transcription bubble (+3 to +19) of the stalled elongation complex. Earlier

DNA photoaffinity labeling studies with AB-dUMP did not reveal the placement of the 82 kDa subunit of Pol III upstream of the start site of transcription in the initiation complex, nor was there any indication of its movement associated with the elongating Pol III (3). Two other proteins with Mr corresponding to the 40(37) and 31 kDa subunits of Pol III, were only effectively detected at bp +11 in stalled elongation complexes with DNA containing DB-dUMP. Crosslinking of the 82 kDa subunit of Pol III at bp –3/–2 in initiation complexes was previously observed with DNAs containing 4-S-dTMP (22).

The 82 kDa subunit of Pol III serves an important role in the initiation of Pol III transcription, as indicated by the selective inhibition of promoter-dependent transcription of Pol III by 82 kDa subunit-specific antibodies (23). The 82 kDa subunit has no known sequence homology with subunits from other RNA polymerases and along with the 53, 34 and 31 kDa subunits are enzyme-specific subunits unique to Pol III (24). A subcomplex of the 82, 34 and 31 kDa subunits has been demonstrated to exist by (i) the loss of these three subunits from Pol III upon native electrophoresis, (ii) the release of these subunits upon inactivation of a zinc binding domain in the 160 kDa subunit and (iii) two-hybrid analysis indicating that these three subunits interact with each other (25–27). DNA photoaffinity labeling had shown that the 34 kDa subunit of Pol III was closely associated with DNA bound to the Brf subunit of TFIIIB at bp –17 (3). The two-hybrid system also demonstrated an interaction of the 34 kDa subunit of Pol III with the Brf subunit (26). Crosslinking of the 82 kDa subunit with DB-dUMP downstream of the site for the 34 kDa subunit provides information of the orientation of this subcomplex of Pol III while in the initiation complex. Photoaffinity labeling of the 82, 34 and 31 kDa subunits of Pol III 6 bp upstream of the 3′-end of the RNA transcript in stalled elongation complexes, demonstrates that this subcomplex is closely associated with DNA inside the transcription bubble.

We have shown that the choice of the photoreactive group used for photoaffinity labeling is an important factor in designing DNA photoaffinity labeling experiments. The coupling of 3-trifluoromethyl-3-phenyl diazirine to DNA has rendered DNA more photoreactive than by coupling of aryl azide, perfluorinated aryl azide or benzophenone to specific sites in DNA, and has revealed protein–DNA contacts missed with DNA photoaffinity labeling with aryl azides. Diazirines will also make it possible to do kinetic analysis of DNA–protein complexes by flash photolysis, because of the very short half life of the reactive carbene (nanoseconds) as compared to that of aryl azides (milliseconds).

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