The C-terminal domain (CTD) of RNA polymerase II (RNAP II) is essential for the assembly of RNAP II into preinitiation complexes on some promoters such as the dihydrofolate reductase (DHFR) promoter. In addition, during the transition from a preinitiation complex to a stable elongation complex, the CTD becomes heavily phosphorylated. In this report, interactions involving the CTD have been examined by protein-protein cross-linking.

As a prelude to the study of CTD interactions, the effect of recombinant CTD on in vitro transcription was examined. The presence of recombinant CTD inhibits in vitro transcription from both the DHFR and adenosine 2 major late promoters, suggesting that the CTD is involved in essential interactions with a general transcription factor(s). Factors in the transcription extract that interact with the CTD were identified by protein-protein cross-linking. Recombinant CTD was phosphorylated at its casein kinase II site, at the C terminus of protein cross-linking. Recombinant CTD was phosphorylated at its casein kinase II site, at the C terminus of protein cross-linking.

The CTD of RNAP II (RNAP II) contains an unusual structure known as the CTD (C-terminal domain). This highly repetitive domain located at the end of the largest RNAP II subunit consists of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser repeated 52 times in mammalian cells. Genetic studies have established that the CTD is essential for cell viability (Nonet et al., 1987; Allison et al., 1988; Bartolomei et al., 1988; Zehring et al., 1998). The conspicuous presence of the CTD in eukaryotes in addition to its unique nature has generated considerable attention in recent years.

Basal levels of transcription catalyzed by RNAP II require the concerted action of the general transcription factors (TF) IIA, IIB, IIE, IIF, and IIH and TATA-binding protein (TBP) (for a review, see Zawel and Reinberg (1993)). Activated transcription occurs in the presence of the multisubunit TFIIID complex (TBP and TBP-associated factors) (Dylacht et al., 1993; Tanese et al., 1991) and sequence-specific TFs whose binding sites may be located proximal or distal to the promoter (Mitchell and Tjian, 1989).

In vivo, the CTD is either unphosphorylated (RNAP IIA) or heavily phosphorylated (RNAP IIo). A CTD-less enzyme (RNAP IIB) is found only in purified preparations of RNAP II as a result of facile proteolytic cleavage of the CTD. Transcription studies have shown that although RNAP IIA assemblies into the preinitiation complex, transcription elongation is catalyzed by RNAP IIo (for a review, see Dahmus (1994)). However, the role or even the significance of CTD phosphorylation during the transition from initiation to elongation has yet to be established. The CTD’s function also appears to be promoter-specific. During transcription from the dihydrofolate reductase (DHFR) promoter, the CTD is necessary for the assembly of the preinitiation complex (Kang and Dahmus, 1993). Transcription from the viral adenosine 2 major late promoter (Ad2-MLP), however, proceeds efficiently in the absence of the CTD (Kim and Dahmus, 1989; Kang and Dahmus, 1993). Other studies have demonstrated that the CTD is involved in the transcriptional activation of some but not all genes (Scafe et al., 1990; Liao et al., 1991; Gerber et al., 1995).

One approach to the analysis of CTD function is to define the interactions in which the CTD participates during the course of transcription. Both genetic and biochemical studies have implicated a functional interaction between the CTD and the multisubunit TFIIID (Koleske et al., 1992; Conaway et al., 1992). Indeed, a direct interaction between the CTD of RNAP IIA and TBP has been demonstrated (Usheva et al., 1992).
Identification of CTD Interactions by Photocross-linking

TFIIE, via its 56-kDa subunit, has also been shown to selectively interact with RNP II A and thus to be sensitive to the phosphorylation state of the CTD. However, a direct and stable interaction between the CTD and TFIIE could not be rigorously established (Maxon et al., 1994). Genetic evidence suggests a functional interaction between the CTD and SIN1, a negative regulator of transcription (Peterson et al., 1991). Genetic evidence also suggests a functional interaction between the CTD and SRB5 (Thompson et al., 1993). Finally, recent studies have established that yeast RNP II holoenzyme is comprised of core RNP II and a large multiprotein complex consisting of these SRBs as well as some of the general TFs (Thompson et al., 1993; Kim et al., 1994; Koleske and Young, 1994). The CTD may be directly involved in the interaction of RNP II with this macromolecular complex termed the mediator (Kim et al., 1994).

A novel approach to defining CTD interactions during transcription is to photolabel CTD-interacting proteins with a cleavable, radioactive azide-based photoprobe attached to the CTD itself. These studies were patterned after previous studies that utilized azide photochemistry to cross-link Escherichia coli RNA polymerase to nascent RNA transcripts (Hanna and Mares, 1983a, 1983b). In this report, recombinant CTD is first labeled by phosphorylation with casein kinase II in the presence of [35S]ATP-S. Only the most C-terminal serine is flanked by acidic residues and is phosphorylated by CKII. The probe is then coupled to the 35S-thiophosphate. This procedure results in the positioning of the photoprobe at a unique site at the very C terminus of the CTD. The probe consists of an azide, which can be photoincubated of crosslinking CTD to nearby proteins in the presence of ultraviolet light. Following cleavage by phenylmercuric acetate, the 35S is transferred from the CTD to the protein to which it was cross-linked. Therefore, the term “photolabel” is used here to indicate the transfer of radioactive label from the CTD to CTD-interacting proteins and is distinct from the term “photocross-link.” The studies presented here demonstrate that the CTD interacts with a limited number of proteins in a HeLa cell transcription extract and that some of these proteins comigrate on SDS-polyacrylamide gel electrophoresis with a subset of the general TFs.

**EXPERIMENTAL PROCEDURES**

**Materials—**Glutathione-agarose, human threonin, benzamidine-Sepharose, azidophenacyl bromide, phenylmercuric acetate, phospho-rylase b, and phosphorylase kinase were purchased from Sigma. [35S]ATP-S, [32P]ATP, and En3Hance were purchased from DuPont NEN. [32P]CTP (800 Ci/mmol) was purchased from Amersham Corp. Protease K was purchased from Boderinnger Mannheim. Ultrapure ribonucleotides and protein molecular weight markers were purchased from Pharmacia Biotech Inc. Diagnostic film was purchased from Fujix BAS1000. Materials—Glutathione-agarose, human thronbin, benzamidine-Sepharose, azidophenacyl bromide, phenylmercuric acetate, phospho-rylase b, and phosphorylase kinase were purchased from Sigma. [35S]ATP-S, [32P]ATP, and En3Hance were purchased from DuPont NEN. [32P]CTP (800 Ci/mmol) was purchased from Amersham Corp. Protease K was purchased from Boderinnger Mannheim. Ultrapure ribonucleotides and protein molecular weight markers were purchased from Pharmacia Biotech Inc. Diagnostic film was purchased from Fujix BAS1000.

**Preparation of 35S-pCTD**—Purified rCTD (195 pmol) was incubated with 2 μM [35S]ATP-S (>1300 Ci/mmol) in the presence of 45 units of T4 polynucleotide kinase, which was added previously to the reaction. The reaction was incubated for 45 min at 24°C to allow preinitiation complex assembly. Reactions were stopped by addition of 15 μg of RNase A and 15 μg of pronase. Quantitation of specific run-off transcripts was carried out by phosphor-imaging analysis (Fuji BAS1000).

**Preparation of [35S]pCTD**—Purified rCTD (195 pmol) was incubated with 2 μM [35S]ATP-S (>1300 Ci/mmol) in the presence of 45 units of T4 polynucleotide kinase, which was added previously to the reaction. The reaction was incubated for 45 min at 24°C to allow preinitiation complex assembly. Reactions were stopped by addition of 15 μg of RNase A and 15 μg of pronase. Quantitation of specific run-off transcripts was carried out by phosphor-imaging analysis (Fuji BAS1000).

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![Diagram showing inhibition of transcription by recombinant CTD.](Image)

**A**

**Results**

Recombinant CTD Inhibits In Vitro Transcription—To determine whether the CTD of RNAP IIA is involved in important and perhaps critical interactions during transcription, increasing amounts of rCTD were added to transcription reactions containing either the Ad2-ML or DHFR promoter. Run-off transcription produces 560-nucleotide and 295-nucleotide transcripts from the purified templates containing the Ad2-ML and DHFR promoters, respectively. The DE0.25 transcription extract used for these transcription reactions requires the addition of exogenous RNAP II for transcriptional activity (Kang and Dahmus, 1993). Recombinant CTD was expressed as a fusion protein with GST in *E. coli* cells. With the addition of increasing amounts of GST-CTD, transcription diminished from each promoter (Fig. 1A, lanes 1–4 and 6–9). At the highest concentration of GST-CTD added, inhibition was 67% from the Ad2-MLP (lane 4) and 77% from the DHFR promoter (lane 9). The fact that an equimolar amount of GST at the highest concentration did not inhibit transcription from either promoter suggests that the inhibition is specifically caused by the CTD (Fig. 1A, lanes 5 and 10).

To confirm that the CTD moiety is responsible for the inhibition of transcription, purified rCTD was added to transcription reactions containing either the Ad2-ML or DHFR promoter. Because the GST-CTD fusion protein contains a genetically engineered recognition site for thrombin between the two proteins, the CTD moiety was released by digestion of the fusion protein with thrombin and purified as described under “Experimental Procedures.” The presence of increasing amounts of purified rCTD, up to 6.2 pmol, results in a 74% inhibition of transcription from the Ad2-MLP (Fig. 1B, lanes 1–3). Curiously, the addition of 18.6 pmol of rCTD or a 3-fold higher amount caused only a 30% inhibition (Fig. 1B, lane 4). Although the reason for the biphasic response is not known, this effect was reproducible. The presence of increasing amounts of rCTD, up to 26.0 pmol, results in a 58% inhibition of DHFR transcription (Fig. 1B, lanes 5–8). Thus, the presence...
of excess rCTD leads to an inhibition of transcription, suggesting that an essential TF(s) is being sequestered by the rCTD.

N$_3$R$_{35}$SpCTD Photolabels a Limited Number of Proteins in a Transcription Extract—In an effort to identify the contacts made by the CTD during the initial stages of transcription, a derivatized rCTD containing an azide photoprobe was used to cross-link proteins in a transcription extract. The placement of the photoprobe within the CTD was accomplished by phosphorylation of rCTD with casein kinase II in the presence of [35S]$\text{ATP}^\gamma$S and subsequent alkylation of $^{35}$SpCTD with azidophenacyl bromide to generate N$_3$R$_{35}$SpCTD (Fig. 2A). Photolabeling of CTD-interacting proteins was accomplished by incubation of N$_3$R$_{35}$SpCTD with a transcription extract followed by irradiation and then cleavage of cross-linked proteins by PMA (Fig. 2B). Because PMA specifically deaves at the $^{35}$S–P bond, the radioactive label of N$_3$R$_{35}$SpCTD is thus transferred to the protein(s) in contact with the CTD at the time of irradiation.

The DE0.25 transcription extract, though depleted of RNAP II and less crude than a whole cell or nuclear extract, still contains a multitude of proteins of various sizes as shown in a silver-stained gel (Fig. 3A, lanes 1 and 2). This is the same extract used in the transcription experiments shown in Fig. 1. Although the calculated molecular weight of rCTD is approximately 40,000, the M$_r$ is approximately 70,000, due to aberrant mobility on SDS-polyacrylamide gel electrophoresis. A previously reported M$_r$ of 65,000 was obtained for a CTD derived by CNBr cleavage of calf thymus RNAP subunit IIa (Cadena and Dahmus, 1987). The difference in M$_r$ may be due to the presence of additional amino acids on rCTD and/or the variation in the polyacrylamide gel electrophoresis system used. The M$_r$ of N$_3$R$_{35}$SpCTD is the same as that of rCTD (Fig. 3B, lane 1). Both the spectrum of photocross-linked proteins following irradiation...
Identification of CTD Interactions by Photocross-linking

Photolabeled bands to proteinase K (Fig. 3) and not nucleic acids, as demonstrated by the sensitivity of the gel which was enhanced, dried, and exposed to diagnostic x-ray film for several weeks. The positions of N3R35SpCTD and 35SpCTD are indicated.

35SpCTD does not photocross-link or photolabel proteins in the transcription extract. No photolabeled bands except for the 70-kDa band were observed when N3R35SpCTD was incubated with buffer control in the absence of the DE0.25 transcription extract (Fig. 3, lanes 4, 6, and 8). The sizes (× 10–3 kDa) of these markers are indicated on the left.

The specificity of photolabeling was also demonstrated by the following controls. Both the formation of cross-linked and 35S-labeled proteins in the transcription extract are photosensitive. When the reactions were not irradiated, neither photocross-linked nor photolabeled bands were observed (Fig. 3B, lane 4). Cross-linking is also dependent on a reative azide moiety. The addition of diethylthiourea reduces the azide to an unreactive amine, abolishes the ability of N3R35SpCTD to photocross-link to proteins (Fig. 3B, lane 9).

Furthermore, in the absence of the azide moiety (N3R), 35SpCTD does not photocross-link or photolabel proteins in the extract (Fig. 3B, lanes 10–12). The labeled bands are proteins and not nucleic acids, as demonstrated by the sensitivity of the photolabeled bands to proteinase K (Fig. 3B, compare lane 5 with buffer control in lane 6). Finally, the presence of photolabeled proteins is dependent on the presence of the HeLa cell transcription extract. No photolabeled bands except for the 70-kDa band were observed when N3R35SpCTD was incubated in the absence of the DE0.25 transcription extract (Fig. 3B, lanes 7 and 8). As previously mentioned, the 70-kDa band likely corresponds to 35S-labeled CTD derived from intracellular cross-linking of N3R35SpCTD. Both the absence of a photocross-linked band (CTD dimer, etc.) (Fig. 3B, lane 7) and the presence of cross-linked bands (CTD dimer, etc.) (Fig. 3B, lane 2) suggest that the 70-kDa band is a result of intramolecular cross-linking. These experiments establish the specificity of the cross-linking reaction and indicate that N3R35SpCTD can be used to identify proteins that interact with the CTD.

Photolabeling of the 34- and 74-kDa Proteins by N3R35SpCTD Is Effectively Competed by RNAP IIA and rCTD—The photolabeling results in Fig. 3B indicate that rCTD interacts primarily with two proteins in the DE0.25 transcription extract with Mr of 34,000 and 74,000. To determine whether the 34- and 74-kDa proteins were photolabeled specifically as a result of their interaction with N3R35SpCTD, RNAP IIA, RNAP IIB, and rCTD were added individually to the reactions as potential competitors of these interactions. Reactions contained RNAP II (0.18 pmol or 20 milliunits) and the DHFR template in order to allow preinitiation complex formation. RNAP IIA but not RNAP IIB can form preinitiation complexes on the DHFR promoter (Kang and Dahmus, 1993). In the presence of approximately the same molar amount of N3R35SpCTD (0.22 pmol), RNAP IIA efficiently reduced the photolabeling of the 34- and 74-kDa proteins (Fig. 4, compare lanes 3 and 4 with lanes 5 and 6). However, the presence of RNAP IIB did not diminish the photolabeling of these proteins (Fig. 4, compare lanes 3 and 4 with lanes 7 and 8). The photolabeling of the 34- and 74-kDa proteins was effectively abolished by the presence of a large molar excess of unmodified rCTD (39 pmol) (Fig. 4, lanes 9 and 10). The fact that the 70-kDa band was not affected by either RNAP IIA or rCTD also suggests that it results from intramolecular cross-linking of N3R35SpCTD (compare the 70-kDa band in lanes 4, 6, and 10). Therefore, the 34- and 74-kDa photolabeled bands result from specific interactions with the CTD.

The 34- and 74-kDa Photolabeled Bands Comigrate with TFII E-β and RAP74, Respectively—The observation that rCTD
that rCTD inhibits transcription from class II promoters that suggests that the CTD interacts with an essential TF and that the 34- and 74-kDa photolabeled proteins may correspond to subunits of specific TFs. With this view in mind, photolabeling reactions were carried out with equimolar amounts of each of the recombinant TFs (rTFs) incubated with N3R35SpCTD in the absence of transcription extract. Surprisingly, rTFIIB (33 kDa), α (56 kDa) and β (34 kDa) subunits of rTFIIE and RAP74 (74 kDa) and RAP30 (30 kDa) subunits of rTFIIF were photolabeled when incubated with N3R35SpCTD alone (Fig. 5A, lanes 3–8). Especially noteworthy was the intense photolabeling of RAP74 as compared with RAP30 (Fig. 5A, lane 8). When equimolar amounts of rTFIIB, -IIE, and -IIF were incubated together with N3R35SpCTD, subunits of each of the rTFs were again photolabeled, with RAP74 being photolabeled the most intensely (Fig. 5A, lane 10). Contrary to results from previous studies (Usheva et al., 1992), rTBP (38 kDa) was not efficiently photolabeled under these conditions relative to the other rTFs (Fig. 5A, lanes 11 and 12). An autoradiogram of a longer exposure, however, revealed that rTBP was in fact photolabeled weakly (Fig. 5B, lane 12). In the interpretation of these results it is important to remember that the photoprobe is at the very C terminus of the CTD and therefore may not contact proteins that bind to internal or N-terminal sites on the CTD.

The 34- and 74-kDa proteins photolabeled prominently by N3R35SpCTD in the DE0.25 extract comigrate with the β subunit of TFIIIE and the RAP74 subunit of TFIIIF, respectively (Fig. 5A, compare lane 2 with lanes 6, 8, and 10). rTFIIB migrates faster than the 34-kDa photolabeled band and produced a doublet of photolabeled bands (as in Fig. 5A, lane 10) when reactions containing the DE0.25 extract were supplemented with rTFIIB (data not shown). In addition, a more careful analysis of a longer exposure of the same gel (Fig. 5B) reveals that some of the weaker photolabeled bands comigrate with RAP30, the α subunit of TFIIIE, and TBP (Fig. 5A and B, lanes 2).

**DISCUSSION**

The CTD is an essential but enigmatic structure found in the largest subunit of RNAP II. The objective of the studies presented here is to determine the interactions between the CTD and proteins present in a transcription extract using a cleavable, radioactive azide-based photoprobe. The demonstration inhibits transcription from class II promoters suggests that the CTD interacts with an essential TF and that the 34- and 74-kDa photolabeled proteins may correspond to subunits of specific TFs. With this view in mind, photolabeling reactions were carried out with equimolar amounts of each of the recombinant TFs (rTFs) incubated with N3R35SpCTD in the absence of transcription extract. Surprisingly, rTFIIB (33 kDa), α (56 kDa) and β (34 kDa) subunits of rTFIIE and RAP74 (74 kDa) and RAP30 (30 kDa) subunits of rTFIIF were photolabeled when incubated with N3R35SpCTD alone (Fig. 5A, lanes 3–8). Especially noteworthy was the intense photolabeling of RAP74 as compared with RAP30 (Fig. 5A, lane 8). When equimolar amounts of rTFIIB, -IIE, and -IIF were incubated together with N3R35SpCTD, subunits of each of the rTFs were again photolabeled, with RAP74 being photolabeled the most intensely (Fig. 5A, lane 10). Contrary to results from previous studies (Usheva et al., 1992), rTBP (38 kDa) was not efficiently photolabeled under these conditions relative to the other rTFs (Fig. 5A, lanes 11 and 12). An autoradiogram of a longer exposure, however, revealed that rTBP was in fact photolabeled weakly (Fig. 5B, lane 12). In the interpretation of these results it is important to remember that the photoprobe is at the very C terminus of the CTD and therefore may not contact proteins that bind to internal or N-terminal sites on the CTD.

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The CTD is an essential but enigmatic structure found in the largest subunit of RNAP II. The objective of the studies presented here is to determine the interactions between the CTD and proteins present in a transcription extract using a cleavable, radioactive azide-based photoprobe. The demonstration
azido-modified $^{35}$SpCTD (N$_3$R$^{35}$SpCTD) was added to a transcription extract and irradiated with ultraviolet light to covalently cross-link the derivatized CTD with proteins that contact the CTD. The novelty of this cross-linking approach is that the photoprobe is positioned within the CTD by phosphorylation with protein kinase in the presence of $[^{35}$S]ATP $\gamma$S and the subsequent alkylation of the $^{35}$S with the photoprobe. Cleavage of the cross-linked proteins results in the transfer of $^{35}$S from the CTD to the protein in contact with the CTD at the time of irradiation. Accordingly, the specificity of the cross-linking reaction is determined by the specificity of the protein kinase that positions the $^{35}$S within the CTD. Since casein kinase II phosphorlylates the most C-terminal serine, these studies specifically identify proteins that contact the C terminus of the CTD.

Despite the myriad of proteins present in the transcription extract, only a few proteins were prominently photolabeled by N$_3$R$^{35}$SpCTD. The two most intense photolabeled bands have a Mr of 34,000 and 74,000. Photoaffinity labeling was specific in that cross-linking was dependent on (a) ultraviolet irradiation, (b) a reactive aryl azide moiety, and (c) the transcription extract. Furthermore, PMA cleavage resulted in the transfer of $^{35}$S to proteins, as indicated by proteinase K sensitivity.

The photolabeling of the 34- and 74-kDa proteins by N$_3$R$^{35}$SpCTD was a direct result of their interactions with CTD as indicated by the observation that RNAP II and the unmodified rCTD reduced and abolished, respectively, the photolabeling of these proteins. In contrast, photolabeling of the 34- and 74-kDa proteins in the transcription extract may be the TFs sequestered by 74-kDa proteins was unaffected by the presence of the CTD. In addition, there was no evidence for the assembly of a complex between the 74-kDa protein and any other TF in the extract. Since photolabeling of the 34- and 74-kDa proteins by N$_3$R$^{35}$SpCTD results in efficient photolabeling, the interactions between the CTD and rTFs alone. The differential labeling may be a consequence of the positioning of the photoprobe at the C terminus of the CTD and the precise organization of proteins within a putative macromolecular complex that interacts with the CTD. According to this interpretation, many of the general TFs have an affinity for the CTD and can contact the CTD at the location of the photoprobe when incubated alone. In the transcription extract, however, the TFs may assemble into a specific complex, in which case only TFIIE-$\beta$ and RAP74 are accessible to the photoprobe. This interpretation is supported by the observation that when TFIIE-$\beta$ and -IIF were incubated together with N$_3$R$^{35}$SpCTD, the photolabeling of RTFIB diminished significantly as compared with when it was present alone (Fig. 5A, compare lanes 4 and 10). Another determinant that has not been addressed is the relative amounts of each of the general TFs present in the DE0.25 extract. When the rTF was incubated alone with N$_3$R$^{35}$SpCTD, 8 pmol of each rTF was added to the reaction. Conceivably, the relative molar ratios of each of the general TFs may influence the pattern of photolabeling. However, since the extract is optimal for transcription from class II promoters, the relative amount of each factor present reflects what is necessary for in vitro transcription and should also reflect its relative concentration in vivo.

The results presented in this report are consistent with the recent finding that yeast RNAPII holoenzyme is comprised of core RNAPII in addition to several of the general TFs, the SRBs, and other proteins (Koleske and Young, 1994; Kim et al., 1994). The formation of the holoenzyme appears to involve a direct interaction of the RNAPII core with a mediator complex comprised of some 20 polypeptides (Kim et al., 1994). The CTD may play a direct role in the mediator-core interaction. One attractive interpretation is that mammalian cells also contain a mediator-like complex that interacts directly with the CTD and that the different intensities of photolabeling of the general TFs in the transcription extract reflect the various positioning of these factors around the CTD as well as around each other. In other words, TFIIIE-$\beta$ and RAP74 may be positioned near the end of the CTD molecule, where the photoprobe has facile access to them. The other factors may be present in a more interior location of the CTD, which makes their accessibility to the photoprobe less likely. TBP appears to be the least accessible to the photoprobe. Thus, positioning the photoprobe in a different location within the CTD may yield a different pattern of photolabeling intensities.

An alternative interpretation to the variable photolabeling intensities of the general TFs in the extract is that the intensities truly reflect the frequency with which the CTD interacts with each factor. This would mean that TFIIIE-$\beta$ and RAP74 interact with the CTD to a greater extent than any other TF, whereas TBP interacts very weakly with CTD relative to the other TFs. As previously noted, a strong interaction between TBP and the CTD was demonstrated by protein affinity studies in which a column containing one heptamer repeat sufficiently depleted a transcription extract of TBP activity such that transcription was compromised (Usheva et al., 1992).

The placement of the azido-based photoprobe on rCTD is the first step in the determination of CTD interactions. Advantages to this photoaffinity labeling method are (a) the probe can be positioned at known sites within the CTD, (b) the aryl azide is highly reactive upon irradiation, and (c) the cross-linked proteins can be identified following efficient cleavage and transfer of the radioactive label. The long term goal is to place the photoprobe on the CTD of RNAP II itself so that the contacts made by the CTD can be examined as RNAP II progresses through transcription.
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