Analysis of Polymerase II Elongation Complexes by Native Gel Electrophoresis

EVIDENCE FOR A NOVEL CARBOXYL-TERMINAL DOMAIN-MEDIATED TERMINATION MECHANISM*

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Genetic and proteomic approaches have identified numerous proteins that are potentially involved in regulating transcriptional elongation, but the mechanisms of action of these proteins remain largely unknown. We describe an experimental approach using native gel electrophoresis for studying interactions of elongation factors with isolated Pol II elongation complexes. The gel distinguishes Pol IIA and Pol IIB containing complexes. The interaction of DSIF (Spt4/Spt5) with the elongation complexes can be readily detected, and this association is not dependent on the carboxyl-terminal domain of the largest subunit of Pol II. We also report the surprising observation that a monoclonal antibody that binds the carboxyl-terminal domain of Pol II triggers the dissociation of the elongation complex. The action of the antibody could be mimicking the action of cellular factors involved in transcription termination.

Traditional purification techniques have identified numerous proteins that modulate transcriptional elongation by Pol II1 (1). Work during the past few years employing genetic and proteomic approaches has significantly increased this number (2, 3). Many of these factors appear to execute general cellular functions required for the transcription of all protein-encoding genes. Factors such as TFIIIS and elongin act directly on Pol II to counteract the tendency of the enzyme to pause. Others such as FACT and Swi/Snf alter chromatin structure. A significant number of proteins are involved in coupling RNA processing to transcription elongation (4, 5). In contrast to these general elongation factors, DSIF and NELF have been implicated in regulating transcription of specific genes (6–8). Much is not known about how the factors interact with the elongation complex and which can bind simultaneously or in competition with each other.

The unusual carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II has been implicated in mediating the activity of various elongation factors (9). The CTD consists of multiple repeats of 7 amino acids, called heptad repeats, with the consensus Tyr-Ser-Pro-Thr-Ser-Pro-Ser (10). A cycle of phosphorylation and dephosphorylation of serines 2 and 5 of the heptad repeats accompanies transcription initiation, elongation, and termination (11). Pol II appears to associate with a promoter in an unphosphorylated state known as Pol IIA. During initiation or shortly thereafter, serine 5 in the heptads becomes phosphorylated. This could be important for RNA capping as the phosphorylated CTD interacts with guanylyltransferase (12, 13). As Pol II departs from the promoter region, it is phosphorylated on serine 2 (11, 14). Serine 2 phosphorylation has recently been shown to be important for proper 3' end formation (3, 15). The CTD has also been implicated in RNA splicing (16, 17). Both the CTD and the polyadenylation signal in the nascent transcript contribute to transcription termination, although contrary to earlier proposals, it appears that termination does not require cleavage of the nascent transcript (18, 19).

We set out to develop an experimental approach that would allow us to monitor associations of proteins with elongation complexes formed from purified Pol II so we could begin to analyze the interdependencies of these interactions and the potential impact of the CTD. Using native gel electrophoresis, elongation complexes containing Pol IIA have been distinguished from elongation complexes containing Pol IIB, a form of Pol II that lacks the CTD. We show that the binding of the elongation factor DSIF does not require the CTD even though the phosphorylation state of the CTD has been implicated in regulating the action of DSIF (20, 21). We also provide evidence that binding of a protein to the CTD can trigger termination. This could have important implications for transcription termination on protein-encoding genes.

EXPERIMENTAL PROCEDURES

Purification of Pol IIA/IIB Mixture—Drosophila nuclear extracts were prepared from 150 to 200 grams of Drosophila embryos as described previously (48) and dialyzed into 150 mM KCl-HGEDPX Buffer (HGEDPX corresponds to 25 mM HEPES, pH 7.6, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, 1 mM sodium bisulfite, 1 mM benzamidine HCl). Extract (15 ml at ~20 mg/ml protein) was loaded onto a 30-ml DEAE-column equilibrated in 150 mM KCl-HGEDPX and washed with 150 ml of 150 mM KCl-HGEDPX and then with 150 ml of 250 mM KCl-HGEDPX. Pol II was eluted with 600 ml of 300 mM KCl-HGEDPX, and fractions with peak activity were pooled to give a total volume of 15 ml. The KCl concentration was adjusted to 150 mM with 10 mM KCl-HGEDPX, and the protein was loaded onto a 15-ml heparin column (POROS 50 HE, Applied Biosystems) previously equilibrated with 150 mM KCl-HGEDPX. The heparin column was washed with 50 ml of 150 mM KCl-HGEDPX and 50 ml of 250 mM KCl-HGEDPX. Pol II was eluted with 500 ml of 300 mM KCl-HGEDPX. Peak fractions of Pol II were pooled and dialyzed into 150 mM KCl-HGEDPX. The Pol II was loaded onto a Mono Q column (Amersham Biosciences, HR5/5). The column was washed with 20 ml of 150 mM KCl-HGEDPX, and bound proteins were eluted in 0.5-ml fractions using a 15-ml gradient from 150 mM KCl-HGEDPX to 400 mM KCl-HGEDPX, followed by a 10-ml gradient from 400 mM KCl-HGEDPX to 600 mM KCl-HGEDPX. Pol II activity was measured in fractions using a promoter independent transcription ass-
sac (28). Peak fractions of activity eluted at ~550 mU, and the protein concentration was 1 mg/ml. Preparation of Pol II—1 ml of Pol II/AIB mixture was treated for 30 min with 0.25 μg/ml chymotrypsin at 30 °C and repurified on the Mono Q column as described above. DNA Template—DNA template was generated by PCR from plasmid containing a G-less cassette. One PCR primer generated a BglIII site at one end and the other primer introduced a biotin tag at the opposite end. The resulting PCR product had a 110-residue G-less cassette and 110 nucleotides of flanking G-containing sequence. The PCR fragment was cut with BglIII, dephosphorylated with shrimp alkaline phosphatase, and finally ligated to a phosphorylated oligonucleotide with the sequence GATCAAAAAAAATTA. The resulting 11-nucleotide overhang sequence was used in vivo for Pol II in the presence of UpG. Reaction mixtures—Reaction mixtures were performed in 15 μl containing 150 mM KCl, 50 mM HEPES, pH 7.6, 1.0 mM MgCl₂, 12% glycerol, 0.5 mM diithiothreitol, 0.5 mM UpG, 20 units of RNasin, 1.0 μl of purified Pol II (1 μg), and 100 ng of tagged G-less template. Pol II was preincubated with the template for 5 min to allow formation of preinitiation complexes. Transcription was initiated by adding a mixture of nucleotides yielding final concentrations of 0.5 mM ATP, 0.5 mM CTP, 0.025 mM UTP, and 1 μCi/reaction of [α-32P]UTP. Each reaction mixture was incubated at 21 °C for 25 min. Analysis of Transcripts—Forty microliters of stop buffer (20 mM EDTA, pH 8.0, 0.2 mM NaCl, 1% SDS, 0.25 mg/ml yeast tRNA, 0.1 mg/ml proteinase K) was added to each transcription reaction. Samples were incubated at 42 °C for 30 min and extracted once with 55 μl of a phenol/chloroform/isoamyl alcohol mixture (25:24:1). The RNA was precipitated with ethanol and analyzed on 8% or 15% denaturing polyacrylamide gels. Radioactive RNA was detected and quantified using a PhosphoImager and ImageQuant software. Analysis of Elongation Complexes by Native Gel Electrophoresis—Electrophoretic mobility shift assays were performed as previously described for TFIIID-DNA complexes (34). Reaction mixtures were loaded directly into the wells without addition of bromphenol blue. Bromphenol blue was run in adjacent lanes to within 1 cm of the bottom of the gel. Gels were transferred to paper, dried, and analyzed with a PhosphoImager. Preparation of Immobilized DNA—Biotinylated DNA templates (0.2 μg) were bound to 20 μl of streptavidin-coated magnetic beads according to the manufacturer’s instructions (Dynal Inc.). Beads were washed once with 150 mM KCl, 50 mM HEPES, pH 7.6, 1.0 mM MgCl₂, 12% glycerol, 0.5 mM diithiothreitol before use in transcription reactions. A 15-μl transcription mixture lacking nucleotides was incubated with the beads for 5 min. Nucleotides were added, and samples were incubated at room temperature for 25 min. The immobilized elongation complexes were washed two times with transcription buffer and finally incubated for 20 min with antibody or buffer in 15 μl of transcription buffer minus nucleotides. RNA was isolated from bound and free fraction and analyzed on a denaturing gel. Preparation of DSIF—Sequences encoding the two subunits of DSIF, sp4 and sp5, were subcloned into the baculovirus expression vector pFastBac1 (Invitrogen). Sp4 was tagged at its COOH terminus with the FLAG epitope (DYKDDDDK). The Spt4 coding region was PCR amplified from the cDNA clone LD44485 (BDGP) with sequences encoding the two subunits outside the region of the CTD (Fig. 2A, lane 2). The resulting fragment was inserted into EcoRI/XhoI cut pFastBac1. The resulting plasmid was called pFB-Spt5-FLAG. Viral particles encoding Spt5-FLAG and Spt4-HA were produced according to the Bac-to-Bac Baculovirus Expression System (Invitrogen). To express the two subunits, 500 ml of SF9 cells grown in SF9 serum-free medium (Invitrogen) were infected with each recombinant virus at a multiplicity of infection of 3. Three days post-infection, the cells were collected and washed once with phosphate-buffered saline. Cells were suspended in 30 ml of TBS buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, and 1× complete protease inhibitor cocktail [Roche Applied Science] and sonicated 3 times for 8 s intervals. The cell lysates were cleared by centrifugation at 8,000 × g for 10 min at 4 °C. The cell lysate was mixed with a 1:ml packed volume of anti-FLAG M2 affinity gel (Sigma) and gently agitated at 4 °C for 2 h. The suspension was packed into a column, and the fluid was allowed to drain. The packed resin was washed with 10 ml of TBS buffer. dDSIF was eluted with five consecutive 1-ml aliquots of TBS buffer supplemented with 100 μg/ml FLAG peptide. Peak fractions were stored at −80 °C. Greater than 80% of the dDSIF could be immunoprecipitated with antibody against the HA tag located on Spt4 indicating that the majority of the Spt5 was associated with Spt4. Antibodies—8WG16 antibody (ascites fluid) was obtained from Covance. RNA-3 antibody (ascites fluid) was obtained from Research Diagnostics Inc. One microliter of antibody was used per transcription reaction. RESULTS Formation of Stalled Elongation Complexes on a Tailed Template—Pol II normally requires several general transcription factors to initiate transcription (22). We sought to develop a facile way for analyzing elongation complexes that could be set up with purified Drosophila Pol II. It was previously shown that purified yeast Pol II would initiate transcription at a 3′-tail similar to the one illustrated in Fig. 1A when transcription was initiated with the UpG dinucleotide (23). We attached the 3′-tail to a G-less cassette so that elongation complexes stalled at the end of the cassette when GTP was omitted from the transcription reaction. Fig. 1B (lane 1) shows that one major transcript of the expected size was produced when the tailed template was incubated with purified Drosophila Pol II, UpG, ATP, CTP, and radiolabeled UTP. Transcription was dependent on UpG indicating that the Pol II was initiating from the 3′-tail (Fig 1B, lane 5). As expected for Pol II, transcription was inhibited by α-amanitin (Fig. 1B, lane 4). Sometimes transcription of tailed templates occurs anomalously. The nascent transcript hybridizes along its entire length to the transcribed strand of DNA, displacing the non-transcribed strand of DNA (24). The RNase sensitivity of the transcripts indicated that Drosophila Pol II was transcribing our tailed template without extensive DNA strand displacement. The nascent transcript was degraded by RNase A indicating that the transcript was single-stranded (Fig. 1B, lane 3). The nascent transcript was not degraded by RNase H confirming that it had not hybridized to the transcribed strand of DNA (Fig. 1B, lane 2). We verified that the RNase H was active by showing that an oligonucleotide complementary to a specific region of the nascent transcript could target cutting by RNase H (data not shown). If the elongation complexes stalled at the end of the G-less cassette were to be a suitable model for an elongation complex, they should resume elongation when provide GTP. Fig. 1C (lane 1) shows the nascent transcript produced in the absence of GTP. Fig. 1C (lane 3) shows that all stalled complexes resumed elongation following addition of GTP to produce a run-off transcript. The weak band marked with an asterisk in lane 1 appeared to be formed by a low level of Pol II reading through the four Gs located at the end of the G-less cassette, since formation of this product was inhibited by the chain terminator, 3′-methoxy-GTP (lane 2). Most likely, one of the nucleotides was contaminated with a low level of GTP. Detection of Pol II and Pol IIB Elongation Complexes by Native Gel Electrophoresis—Native gel electrophoresis has been widely used to analyze protein-nucleic acid complexes. However, there have been relatively few reports using this to analyze Pol II-containing elongation complexes. When we subjected our stalled elongation complexes to electrophoresis on a native gel, two distinct complexes were observed (Fig. 2A, lane 2). To detect these complexes, radiation nucleotides were incorporated into the nascent transcript prior to loading the complexes on the gel. Both complexes were determined to contain Pol II because they shifted mobility when incubated with a monoclonal antibody called ARNA-3 (25) that bound to the largest subunit outside the region of the CTD (Fig. 2A, lane 3).
The absence of GTP. The next point: a read-through product formed by contaminating RNase H. Lane 2 represents a read-through product formed by contaminating GTP. Lane 1 elongation when supplied GTP.

We wondered if the existence of two complexes might be due to the presence of different forms of Pol II. The elongation complexes were supplied GTP. Radioactive transcripts were detected in the reactions. We performed on stalled elongation complexes, and RNasin was omitted from the reactions.

In the absence of GTP, RNA polymerase stalls when it encounters four Gs located at the end of the G-less cassette. The underlined AC in the 3′ tail attached adjacent to a G-less cassette.

Further analysis of the Pol II preparation indicated that the complex did indeed contain Pol IIB (Fig. 2, lane 2 and lane 2). The mobility of this complex was altered by ARNA-3 but not by 8WG16 indicating that the complex did indeed contain Pol IIB (Fig. 2A, lanes 4 and 6).

DSIF Associates with Both Pol IIA and Pol IIB-containing Elongation Complexes—Our motivation for developing the native gel assay was to investigate the interactions of proteins with elongation complexes. DSIF has been implicated in both stimulating and inhibiting transcriptional elongation (8, 29, 30). Its inhibitory action depends on another protein called NELF (20). Since the CTD has also been implicated in regulating the action of DSIF, we were interested in determining whether direct interactions between DSIF and our elongation complexes could be detected by native gel electrophoresis. As shown in Fig. 3 (lanes 1 and 2), DSIF associated with both Pol IIA and Pol IIB elongation complexes.

In addition to Pol II, elongation complexes contain both DNA and RNA. Hence, it was possible that the shift in mobility was due solely to binding between DSIF and nucleic acid. If binding...
transcripts produced by the mixture of Pol IIA and Pol IIB (Fig. 4, lanes 1 and 2). Since SWG16 did not cause Pol IIB to release transcript (Fig. 4, lanes 7 and 8), we conclude that the antibody was causing Pol IIA complexes to release transcript. It is likely that Pol IIA has dissociated from the immobilized template because the presence of an 8–10-nucleotide RNA-DNA heteroduplex located at the active site is essential for the stable association of Pol II with DNA (33).

The CTD Antibody Inhibits Transcription at Low Nucleotide Concentrations—We were puzzled by a previous report showing that SWG16 did not inhibit transcription elongation by purified Pol IIA (26). Pol II pauses transiently when transcribing most DNA, so we anticipated that there would be transient opportunities for the antibody to terminate elongation. To address this issue, the effect of SWG16 during transcription under different nucleotide concentrations was analyzed. In the presence of 500 μM ATP, 500 μM CTP, and 25 μM UTP, the antibody had no effect on the amount of transcript produced (Fig. 5, lanes 1 and 2, and C, column 1). In contrast, lowering nucleotide concentrations to 25 μM ATP, 25 μM CTP, and 10 μM UTP renders transcription sensitive to SWG16. SWG16 inhibited transcription by the Pol IIA/IB mixture by 2-fold (Fig. 5, A, lanes 3 and 4, B, lanes 3 and 4, and C, column 2). The residual transcription that occurred in the presence of antibody could be ascribed to Pol IIB, since Pol IIB transcription was insensitive to the antibody (Fig. 5, B, lanes 1 and 2, and C, column 3). Low nucleotide concentrations increase the frequency and duration of pauses. We conclude that elongation complexes were only susceptible to termination by SWG16 when the lifetime of the pause was of sufficient duration.

Evidence That Binding of a Single Antibody Molecule to the CTD Is Sufficient to Trigger Termination—The CTD is often described as an array of heptad repeats related to the consensus YSPTSPS (19). The CTD of human and yeast contain, respectively, 21 and 19 heptads that exactly match the consensus. As shown in Fig. 6, however, Drosophila Pol II only has two heptads that exactly match the consensus. This raises the possibility that the SWG16 antibody terminates transcription by binding a single site rather than multiple sites in the CTD. To investigate this possibility, we analyzed what effect limiting amounts of SWG16 had on the elongation complexes. If binding were to occur without dissociation of the elongation complex, these associations should shift the mobility of the complex. As shown in Fig. 6, there was no evidence that SWG16 shifts the mobility of the complex even at levels that are insufficient to disrupt all of the complexes. Two possibilities could explain these results. Binding of the antibody anywhere in the CTD could trigger termination. Alternatively, the antibody binds most readily to a single site on the CTD and this is sufficient to trigger termination. We favor the latter possibility because of the paucity of heptad repeats in the Drosophila CTD that exactly match the consensus.
FIG. 5. Subsaturating levels of 8WG16 do not result in shifted Pol IIA elongation complexes. The left portion of the figure shows the effect of varying the amount of 8WG16 used to disrupt the elongation complexes. Pol IIA and Pol IIB elongation complexes (EC) were formed, and then complexes were incubated with varying amounts (0, 0.1, 0.3, 0.5 μl) of 8WG16 antibody for 10 min. Complexes were analyzed by native gel electrophoresis.}

**DISCUSSION**

**Analysis of Elongation Complexes by Native Gel Electrophoresis**—We were pleased to find that Pol IIA and Pol IIB-containing elongation complexes could be distinguished by native gel electrophoresis. This could be very useful in deciphering the functions of the CTD in elongation. We tested several gel systems, but only the system we used previously to analyze TFIID-DNA complexes proved effective (34, 35). Most attempts to monitor physical interactions between Pol II and other proteins are done when Pol II is not transcriptionally engaged on a template. The structure of Pol II engaged on DNA is significantly different from free Pol II (32, 36). These differences in conformation from the free enzyme could present unique surfaces for interaction with elongation factors that might be best observed in the context of a bona fide elongation complex.

Any protein that associates solely with nucleic acid has the potential to shift the mobility of the elongation complex. We have found a simple way to test for contact with Pol II. Treatment of the elongation complexes with proteinase K damages the Pol II surface without disrupting all of the ternary complexes. A protein binding solely to a nucleic acid component of the elongation complex should still shift the mobility of the damaged complex, whereas a protein that interacts with the Pol II is less likely to. Equally effective controls might be generated with bacterial or phage polymerases that should generate elongation complexes that are unlikely to interact with Pol II-specific elongation factors.

An important characteristic of our elongation complexes is that they do not exhibit the anomalous strand displacement often associated with elongation complexes formed on tailed templates (24). We chose the specific tail and dinucleotide to initiate transcription because Lang et al. (23) had reported previously that strand displacement did not occur with yeast Pol I, Pol II, or Pol III. The reformation of the DNA duplex on the upstream side of the transcription bubble and the resulting presentation of the nascent transcript will likely be important for analyzing elongation processes involving the nascent transcript.

**Association of DSIF Is Independent of the CTD**—Since our system could distinguish Pol IIA and Pol IIB elongation complexes, we took the opportunity to determine whether the CTD impacted on binding of DSIF. The issue interested us because the CTD has been implicated in regulating the action of DSIF (20). In collaboration with another factor called NELF, DSIF acts as an inhibitor of elongation. NELF and DSIF were observed to inhibit transcription elongation by Pol IIA and Pol IIB but not by Pol IIO. This is a conundrum because Pol IIB lacks the CTD, while the difference between Pol IIA and Pol IIO suggests that the CTD regulates the action of NELF and DSIF. Our native gel electrophoresis result clearly shows that the CTD is not required for binding of DSIF. Future work will employ this assay to determine how phosphorylation of NELF, DSIF, and Pol II affect the interactions of these components in the context of an elongation complex.

**A Protein Binding the CTD Triggers Termination**—Three independent types of experiments provided evidence that the
26 heptads that exactly match the consensus (10). In contrast, of human and yeast Pol II have, respectively, 21 of 52 and 19 of heptad repeats deviating from the consensus (26, 45). The CTD of Pol II deduced from x-ray crystal data reveals that 10 of the subunits form a functioning unit (32, 40). Two additional subunits, rpβ4 and rpβ7, dock on the surface (41, 42). The CTD is not visible in the x-ray structure indicating that it is unstructured or mobile (36). The linker region that connects the CTD to the rest of the enzyme projects out from the body of the enzyme suggesting that the CTD is not intimately associated with the rest of the protein. Thus, the situation appears to be one in which an antibody binding to a domain of the enzyme distinct from the domain involved in catalysis triggers disruption of an extremely stable nucleoprotein complex.

Understanding how the antibody causes termination of paused complexes requires much more investigation. Learning more about the structure of the CTD within the context of an elongation complex should be enlightening. We are intrigued by reports that the CTD interacts with DNA (43, 44). If the association of the CTD with DNA is slow, this association might only occur during long pauses, which seems to be a prerequisite for 8WG16-mediated termination. It will also be important to learn where 8WG16 binds the CTD. The binding site could be highly localized in the Drosophila CTD. The CTD of human and yeast Pol II have, respectively, 21 of 52 and 19 of 26 heptads that exactly match the consensus (10). In contrast, the CTD of Drosophila Pol II only has two heptads that exactly match the consensus (Fig. 6). 8WG16 has been shown to recognize a synthetic peptide composed of three consensus heptads, but no information is available concerning the recognition of heptad repeats deviating from the consensus (26, 45).

Possible Implications for Transcription Termination on Protein-encoding Genes—Previous studies have implicated the CTD in transcription termination in humans (16). Truncation of the CTD caused the resulting Pol II expressed in human cells to ignore a termination signal. Mutations in specific polyadenylation factors impair termination (46), and two of these factors, Pcf11 and Rna14, associate with the CTD (18). Termination appears to depend on the polyadenylation signal located in the nascent transcript, but recent data indicate that cleavage of the nascent transcript, which is a prerequisite for polyadenylation, is not required for termination (18, 47). Termination appears to be stochastic once the Pol II has transcribed the polyadenylation signal. Perhaps the polyadenylation signal in the nascent transcript causes a factor bound to the CTD to cue termination by a mechanism that is fortuitously mimicked by the 8WG16 monoclonal antibody.
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