Modulation of RNA Polymerase II Elongation Efficiency by C-terminal Heptapeptide Repeat Domain Kinase I*

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Hyperphosphorylation of the C-terminal heptapeptide repeat domain (CTD) of the RNA polymerase II largest subunit has been suggested to play a key role in regulating transcription initiation and elongation. To facilitate investigating functional consequences of CTD phosphorylation we developed new templates, the double G-less cassettes, which make it possible to assay simultaneously the level of initiation and the efficiency of elongation. Using these templates, we examined the effects of yeast CTD kinase I or CTD kinase inhibitors on transcription and CTD phosphorylation in HeLa nuclear extracts. Our results showed that polymerase II elongation efficiency and CTD phosphorylation are greatly reduced by CTD kinase inhibitors, whereas both are greatly increased by CTD kinase I; in contrast, transcription initiation is much less affected. These results demonstrate that CTD kinase I modulates the elongation efficiency of RNA polymerase II and are consistent with the idea that one function of CTD phosphorylation is to promote effective production of long transcripts by stimulating the elongation efficiency of RNA polymerase II.

The process of eukaryotic transcription by RNA polymerase II is regulated at multiple steps during initiation and elongation. A structural feature of RNA polymerase II (pol II)1 implicated in several aspects of regulation is the unique C-terminal domain (CTD) of the largest subunit; this domain is composed of multiple repeats of a YSPTSPS consensus heptamer. Finding that the CTD is hyperphosphorylated in vivo suggested that this modification directs some important roles of pol II during transcription and has stimulated efforts to isolate and characterize CTD kinases as one approach to revealing the functional significance of CTD phosphorylation (1).

Extensive studies have revealed some roles of the CTD and its phosphorylation, but many details remain unknown (2–10). This is mainly due to the extreme complexity of the transcription process and apparatus and to incomplete characterization of the multiple components necessary to effect and regulate transcription initiation and elongation. A convincing finding from both in vivo and in vitro experiments is that the CTD is unphosphorylated in initiation complexes (subunit form IIa), but hyperphosphorylated in elongation complexes (form IIo), suggesting that CTD phosphorylation is an event occurring during or after the transition from initiation to elongation (11–17). Evidence also continues to accumulate supporting the importance of regulating the elongation phase of transcription (14–17). It has been suggested that CTD phosphorylation is coupled to the control of elongation (13), and recently a Droso phila factor that stimulates elongation in the presence of a number of other components has been found to display CTD kinase activity (18).

In this paper, we report in vitro evidence that the yeast CTD kinase I we previously purified, characterized, and cloned (19–21) promotes efficient elongation by RNA polymerase II in HeLa nuclear extracts. We also report new templates that facilitate measurements of elongation efficiency in nuclear extracts; these double G-less cassette constructs carry a promoter-proximal G-less region and a different length distal G-less region in the same transcription unit.

EXPERIMENTAL PROCEDURES

Materials—DRB, creatine, and creatine kinase were obtained from Sigma and H8 from Seikagaku. [α-32P]UTP was purchased from Du Pont NEN. RNase T1 and Protease K were from Boehringer Mannheim. Nucleotides and the polymers poly(C) and poly(dI-dC) were from Pharmacia Biotech Inc.

Construction of the Double G-less Cassette Templates—These templates were constructed by subcloning the adenovirus major late promoter SacI fragment (−405 to +1575 with respect to the transcription initiation site) into the SacI site of the plasmid p(C2AT)19 (22), followed by insertion of a synthetic 85-base pair G-less sequence between PvuII (+32) and HindIII (+194) sites for the plasmid pSLG402 and between PvuII (±32) and BgII (+1215) sites for the plasmid pSLG407, respectively.

Yeast CTD Kinase I Purification—Yeast CTD kinase I (CTDK-I)1 was purified essentially as described (19) from a CTDK-I overproducing strain, made by transformation with two different high copy plasmids carrying the three genes encoding CTDK-I. CTK1 was carried by YEp24 (23) while CTK2 and CTK3 were carried by pRS426 (24). This yeast strain produces about 10-fold more kinase than the untransformed strain.

In Vitro Transcription Reactions—HeLa nuclear extracts were prepared as described (25). In vitro transcription reactions were performed in a 25-μl reaction mixture containing 10 mM Hepes pH 7.9, 10 mM MgCl2, 12% glycerol, 60 mM KCl, 5 mM creatine phosphate, 5 μg of creatine kinase, 10 μg/ml poly(I-C), 10 μg/ml poly(dI-dC), 10 μg/ml template DNA, and HeLa nuclear extract (50 μg of protein). Reactions were started by adding nucleotides to 60 μM GTP, ATP, CTP, and 20 μM [α-32P]UTP (5 μCi). α-Aminanit was added to 5 μg/ml where indicated. After incubation at 30 °C for 90 min, the reactions were treated sequentially with RNase T1 and protease K; then, were phenol-extracted and ethanol-precipitated as described (26). The products were analyzed on a 6% polyacrylamide-urea gel. Radioactivity incorporated into the fragments was quantitated using a PhosphorImager, and the molar ratios of products was calculated by taking into account the number of uridines in each fragment (24 for the 85-nt cassette and 138 for the 377-nt cassette). DRB (Sigma) stock was made in 70% ethanol and stored at 20 °C, and

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1 The abbreviations used are: pol II, polymerase II; CTD, C-terminal heptapeptide repeat domain; CTDK, CTD kinase; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; nt, nucleotide(s); HIV, human immunodeficiency virus.

2 J. M. Lee, unpublished results.
kinase inhibitor, inhibits pol II transcription in vivo not only on initiation events but also on the elongation process, presented about 0.65 of the shorter one. These results, showing a 0.35 molar ratio compared with the shorter transcript (3 and nuclear extracts (18, 27, 28). We tested this compound for been found to reduce elongation efficiency of pol II in whole cell and elongation (promoter-distal transcription), respectively, Radioactivity incorporated into the fragments was quantitated using a PhosphorImager, and the molar ratios were calculated as described. M, size markers, length in nucleotides indicated at right.

H8 (Sigma) stock in 100% ethanol and stored at 4 °C. These inhibitors were added to the reactions as 0.1 total volume in 10% ethanol.

Western Blotting—Aliquots were taken from the transcription reactions during incubation (50 min), run on a 5% SDS-polyacrylamide gel electrophoresis mini-gel, and transferred to nitrocellulose. Western blotting was performed to detect the subunit form IIo (arrow) using the affinity-purified antibodies to the linker sequence of the 85-nucleotide fragment the G-less sequence begins at the promoter-distal transcription, the ratio of polymerases elongating past position 1888 or 1888 for pSLG402 and pSLG407, respectively. Radioactivity incorporated into the fragments was quantitated using a PhosphorImager, and the molar ratios were calculated as described. M, size markers, length in nucleotides indicated at right.

RESULTS AND DISCUSSION

To compare easily initiation levels and elongation efficiency in nuclear extracts we devised new templates carrying two G-less segments, modified from the original G-less cassette template (22). One G-less sequence was placed at a promoter-proximal position and another at a promoter-distal position, as shown in Fig. 1a. These double G-less cassette templates produce short and long RNA T1-resistant products which represent measures of “initiation” (promoter-proximal transcription) and elongation (promoter-distal transcription), respectively, from the same promoter. Quantitation of the distal and proximal G-less products yields a ratio that is a measure of elongation efficiency. Using these templates and HeLa nuclear extracts we analyzed transcription from the adenovirus major late promoter (Fig. 1b). Transcription of template pSLG402 produced the longer RNA (whose 3' end is at position +1888) in a 0.35 molar ratio compared with the shorter transcript (3' end at +124), determined as described in the legend to Fig. 1. With template pSLG407 the longer RNA (3' end at +867) represented about 0.65 of the shorter one. These results, showing that elongation efficiency was less than unity, demonstrate that transcript production in HeLa nuclear extracts depends not only on initiation events but also on the elongation process, as seen previously (27).

The nucleotide analog DRB, now known to be a protein kinase inhibitor, inhibits pol II transcription in vivo and has been found to reduce elongation efficiency of pol II in whole cell and nuclear extracts (18, 27, 28). We tested this compound for its inhibitory effects on initiation and elongation using the double G-less cassette templates. In good agreement with previous studies using HeLa nuclear extracts (27), DRB inhibited production of long transcripts more than production of the short transcript. In the presence of 100 μM DRB, transcription past position +1888 was inhibited approximately 3-fold whereas transcription past +124 was inhibited only 30% (Fig. 2, a and b). Another protein kinase inhibitor, H8, also inhibited elongation but had virtually no effect on the short transcript (Fig. 2, c and b). This observation suggests that H8 inhibits a subset of DRB-sensitive kinases, namely those affecting elongation. These data imply that some protein kinases sensitive to DRB and H8 in nuclear extracts play roles in transcription, especially facilitating the elongation process.

DRB and H8 inhibit CTD phosphorylation in vivo (29, 30) and in vitro (2, 31) and have been suggested to be inhibitors of transcription factor TFIIH (however see Ref. 18). The functionally significant targets in vivo are not actually known, however, as metazoan nuclei contain more than one DRB-sensitive CTD kinase activity, not all of which are well characterized. We examined CTD phosphorylation patterns in HeLa extracts under transcription conditions in the presence of these inhibitors using an antibody specific for the phosphorylated CTD (subunit IIo (20)). The results (Fig. 2c) showed that CTD phosphorylation is indeed decreased by DRB and H8 in a pattern paralleling the inhibition of long transcript synthesis, consistent with the suggestion that inhibition of CTD phosphorylation contributes to decreased elongation efficiency. This suggestion is also consistent with results from a Drosophila-derived system (18).

Previously, we purified a protein kinase from yeast that specifically and efficiently hyperphosphorylates the CTD of yeast and mammalian pol II (19). Yeast CTD kinase I (CTDK-I) is a member of the cyclin-dependent kinase subfamily (20, 21) and distinct from factor TFIIH or other known kinases harboring CTD phosphorylating activity (3, 18, 32, 33). We found that yeast CTDK-I is resistant to DRB (20) but sensitive to H8 (under standard kinase assay conditions (20), 100 μM H8 inhibited CTDK-I activity by more than 95%). This finding prompted us to test the hypothesis that the yeast CTD kinase would increase transcription elongation efficiency in HeLa nuclear extracts by phosphorylating the CTD in the presence of DRB. As seen in Fig. 3a, CTD kinase I increased production of the long (distal) transcript much more than it affected the short (proximal) one, restoring DRB-inhibited transcription completely. These results support the idea that DRB inhibits elongation through inhibiting CTD phosphorylation and showed that the DRB-resistant yeast CTD kinase can restore the DRB-inhibited elongation. In contrast, little stimulation of elongation by the kinase was observed in the presence of H8, indicating that the stimulation in the presence of DRB is truly dependent on the catalytic activity of the H8-sensitive, DRB-resistant yeast kinase (The small increase of transcription elicited by CTD kinase in the presence of H8 is probably due to incomplete inhibition of the added kinase.)

When CTD kinase I was added to transcription reactions without inhibitors, elongation was also increased, suggesting that CTD kinase activities in the HeLa extracts are present in amounts limiting transcription elongation. After CTDK-I addition, the ratio of polymerases elongating past position +1888 relative to those elongating past position +124 was 0.75, whereas the comparable ratio for elongation past position +867 was 0.95 (Fig. 3b and Table I). Hence the amount of kinase added was sufficient to render nearly all transcribing polymerases capable of elongating almost 1 kilobase with an efficiency close to 1. Examination of the phosphorylation state of the CTD in the transcription reactions showed that adding yeast kinase increased CTD phosphorylation levels (Fig. 3c) as...
expected. That CTD phosphorylation did not quantitatively correlate with the stimulation of transcription elongation may be because not all polymerase molecules in the extract participate in transcription.

Our results show that adding yeast CTD kinase I to HeLa nuclear extracts results in stimulation of both elongation and CTD phosphorylation in a DRB-resistant, H8-sensitive manner. This observation is consistent with the idea that CTD phosphorylation is responsible for the observed stimulation of elongation, as has been reported by Price and colleagues (18) using a Drosophila system. On the other hand, we cannot exclude the possibility that CTDK-I phosphorylates other proteins in the crude system and that these modifications then result in the increase of elongation efficiency. Clarifying this situation will ultimately require reconstituting elongation control using purified components.

Our data in the crude system correlate with the result that H8 did not inhibit synthesis of short transcripts in a purified system, although it inhibited CTD phosphorylation (2). We also want to point out that our results are consistent with the suggestion that HeLa cells have several CTD kinases, some of which are resistant to DRB and H8, because these inhibitors did not completely block CTD phosphorylation or in vitro elongation; it may be that both inhibitor-resistant and inhibitor-sensitive kinases participate in CTD phosphorylation during elongation and/or the transition from initiation to elongation. Alternatively, in vitro elongation may be partially independent of CTD phosphorylation. It is also possible that there might be promoter specificity to CTD phosphorylation effects on elongation and that distinct CTD kinases function at different promoters, as suggested by previous results (12). Along these lines, it was reported that elongation from the Tat-transactivated HIV long terminal repeat promoter is more sensitive to DRB than elongation from the un-transactivated HIV promoter (27); in addition, different viral transactivators appear to be associated with distinct CTD kinase activities (34). Furthermore, on Chironomus chromosomes in living cells CTD phosphorylation (and effective transcript production) is sensitive to DRB for Balbiani ring 2 genes but not for a heat-shock gene (30), reminiscent of differential DRB sensitivity for CTD phosphorylation under stress and non-stress conditions in mammalian cells (35). In this context we point out that yeast CTDK-I differs from Drosophila p-TEFb in subunit composition and DRB sen-

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3 E. Egyhazi, A. Ossoinak, A. Pigon, J. M. Lee, and A. L. Greenleaf, submitted for publication.
A number of important questions regarding CTD kinase and CTD phosphorylation remain to be answered. For example, the mechanisms that might couple CTD phosphorylation to enhanced elongation efficiency have yet to be determined. A number of possible mechanisms exist that are not necessarily mutually exclusive (and are potentially gene-class specific). One possibility is that the hyperphosphorylated CTD counteracts inhibitory components bound to the template chromatin. On the other hand, it is becoming clear that CTD phosphorylation modulates interactions between pol II and other proteins (e.g. Refs. 36–42). Thus, CTD phosphorylation may influence either the composition or activity of the elongation complex; it might effect loss or inactivation of inhibitory components or recruitment of activation of stimulatory factors. The availability of purified CTD kinases provides tools and techniques that will help to answer these outstanding questions.

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