A Nonproteolytic Function of the 19S Regulatory Subunit of the 26S Proteasome Is Required for Efficient Activated Transcription by Human RNA Polymerase II†

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ABSTRACT: We recently reported that the 19S regulatory subunit of the yeast 26S proteasome stimulates transcription elongation by RNA polymerase II. However, because of basic differences between yeast and mammals in the components and cellular location of the proteasome, it is crucial to assess whether this is a general phenomenon. Here we address this question and demonstrate that (1) the nonproteolytic activity of the 19S (PA700) complex of the proteasome is required for efficient activated transcription in the mammalian in vitro system, (2) this requirement applies to both natural and artificial activators, and (3) highly purified PA700 can provide this activity. In vitro transcription assays using HeLa cell nuclear extracts reveal that antibodies against human Trip1p/Rpt6 (mammalian Sug1p), one of the six ATPases in the PA700, significantly inhibit activated transcription. Similarly, immunodepletion of the PA700 from the extract also significantly reduces activated, but not basal, transcription and add-back of the highly purified mammalian PA700 restores the activity. Finally, inhibitors of the proteasome's peptidase activities do not affect transcription although the peptidase activity is almost completely inhibited. These findings indicate that the requirement for a nonproteolytic activity of the 19S complex in transcription is general in eukaryotes.

Expression of protein-encoding eukaryotic genes is most commonly regulated at the level of transcription. Recently, there has been increased interest in the elongation phase of the transcription cycle as a point of regulation (*1*−*4*). It has been known for some time that the expression of many genes (*5*−*11*) as well as transactivation by several activator proteins (*3*, *12*−*15*) are regulated at the level of elongation. Work from several laboratories has identified a number of factors that positively or negatively affect RNA polymerase II (pol II)[1] elongation, including P-TEFb (positive transcription elongation factor) (*16*), DSIF (DRB-sensitivity-inducing factor) (*3*), NELF (negative elongation factor) (*17*) and FACT (facilitates chromatin transcription) (*18*, *19*). A surprising recent addition to this list of elongation factors is the 19S regulatory particle of the yeast 26S proteasome (*1*). Here we investigate the role of the 19S complex in mammalian transcription.

The 19S complex is an assembly of approximately 18 proteins that caps both ends of the barrel-like 20S core of the proteasome. The 19S binds with proteins marked for degradation, usually by polyubiquitination, and feeds them to the protease sites in the interior of the 20S complex. This activity involves unwinding of the protein substrate by six ATPases (Sug-like proteins, Rpt1−6) that exist in the base of the 19S complex (*20*, *21*). We demonstrated that in a crude yeast extract, RNA pol II elongation was crippled by the addition of antibodies raised against the Sug1 protein (Rpt6) or by heating an extract made from a temperature-sensitive *sug1*−*20* strain. Elongation in the heat-inactivated extract could be reconstituted by addition of immunopurified wild-type 19S complex. Experiments using temperature-sensitive mutations in 20S subunits and highly specific chemical inhibitors demonstrated that this effect was not due to proteasome-mediated proteolysis but, rather, to a nonproteolytic activity of the 19S complex. In vivo experiments revealed that certain *sug1* alleles are unusually sensitive to inhibitors of elongation, consistent with the idea that the observed biochemical activities of the 19S complex reflect a biologically relevant role in transcription in vivo. Furthermore, the 19S complex was found to co-immunoprecipitate with FACT, consistent with its association with elongation complexes (*1*). There had been several biochemical and genetic hints of a role for Sug1p and the 19S complex in transcription, but these had previously been ascribed to indirect effects of proteasome-mediated proteolysis. This work for the first time attributed a direct, nonproteolytic role for the proteasome in transcription, at least in yeast.

Given the fundamental importance of this new finding in transcription elongation enzymology, it was of great interest to ask if this phenomenon is common to all eukaryotes or somehow specific to *Saccharomyces cerevisiae*. This is

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[1] Abbreviations: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DSIF, DRB-sensitivity-inducing factor; GTF, general transcription factor; NE, nuclear extract; PIC, preinitiation complex; pol II, polymerase II; Z-L3VS, peptide vinyl sulfone carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone.

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particularly relevant since the proteasome in yeast has different components (*22*) and patterns of cellular localization (*23*−*26*) than the mammalian form. In this study, we show that antibodies against Trip1/Rpt6, the mammalian analogue of Sug1, (also known as p45/S8) largely abolish activated, but not basal, transcription in a HeLa nuclear extract (NE). In addition, immunodepletion of the 19S complex (also called PA700) from a crude NE significantly reduces activated but not basal transcription. Addition of highly purified bovine PA700 significantly restores activated transcription. Experiments using chemical inhibitors of proteasome-mediated proteolysis suggest that the 19S requirement in transcription does not reflect a critical proteolytic event. These findings present a compelling argument that the PA700/19S complex plays a critical, nonproteolytic, role in RNA pol II transcription in all eukaryotic cells.

MATERIALS AND METHODS

Construction of DNA Templates. Plasmids pTF3-3E4T-550 C_2AT and pG5E4T-550 C_2AT with a 380 base pair (bp) G-less cassette (C_2AT) fused 550 bp downstream of the transcription initiation site from adenovirus early region 4 (E4) core promoter and containing three and five binding sites for ATF/E4TF3 and Gal4, respectively, were constructed for transcription analysis. Plasmids pTF3-6 C_2AT (*3*) and p(C_2AT)19 (*17*) were kindly provided by Prof. H. Handa, and pTF3-3E4T-629 was provided by Prof. T. Akaike of Tokyo Institute of Technology, Japan, respectively. pTF3-3E4T-629 containing three ATF/E4TF3 binding sites upstream of the E4 promoter TATA box and a 629 bp *Hinc* II-*Hind* III fragment of pBR322 downstream of transcription initiation site was derived from pTF3-6C_2AT. A detailed protocol for pTF3-3E4T-629 composition will be supplied on request. Plasmid pΔtet C_2AT was constructed as follows: p(C_2AT)19 was cut with *Sac* I, blunt ended, and then cleaved with *Eco* RI to clone an *Eco* RI−*Nru* I fragment from pBR322. pΔtet C_2AT was cleaved with *Pst* I, blunt ended, and then cut with *Sph* I. The resulting DNA fragment was cloned into the *Sph* I and *Eco* RV sites of pTF3-3E4T-629 to construct pTF3-3E4T-550 C_2AT. ATF/E4TF3 sites were replaced with five tandemly repeated Gal4 binding sites to construct pG5E4T-550 C_2AT or the plasmid pTF3-3E4T-550 C_2AT was cut with *Bam* H1, purified after gel electrophoresis, and then ligated to construct pE4T-550 C_2AT, which lacks the ATF/E4TF3 binding sites.

Construction of Trip1 Expression Plasmid. An expression vector encoding a His6-tagged version of Trip1 protein was constructed by inserting a PCR-generated fragment that contains *PSMC5* cDNA sequences from 1 to 243 nucleotides into pQE-30 (Qiagen) between *Bam* HI and *Hind* III sites. The PCR-amplified cDNA was confirmed by DNA sequencing.

Purification of Mammalian PA700. Mammalian PA700, purified from bovine red blood cells (*27*) was a kind gift of Prof. George DeMartino of UT-Southwestern Medical Center.

Expression and Purification of Recombinant Proteins. Expression and purification of glutathione-S-transferase (GST), Gal4 (1−141aa)-VP16 fused to GST and His6-tagged N-terminal Trip1 proteins were carried out as described (*1*). Purified His6-tagged Trip1 was subjected to 12.5% SDS-polyacrylamide gel electrophoresis (PAGE), and proteins were recovered from the gel. After acetone precipitation, the protein was denatured and renatured as described (*28*). Protein concentration was determined by SDS-PAGE using bovine serum albumin as a standard.

Immunodepletion of Trip1p. Mouse ascites (25 μL) for monoclonal anti-human Trip1/Rpt6 antibodies (*29*), a generous gift of Prof. Klavs B. Hendil (University of Copenhagen, Denmark), were added to 50 μL of protein G-Sepharose (Roch) and incubated overnight at 4 °C. After 1 h blocking of the isolated resin with bacterial extract at 4 °C, the resin was extensively washed with transcription (TXN) buffer (20 mM HEPES−NaOH, pH 7.9, 10% Glycerol, 6 mM $MgCl_2$, 60 mM KCl, 0.5 mM EDTA) containing 0.1% NP-40. The washed resin was then incubated with 80 μL of HeLa NE (3 mg/mL) (Promega) in TXN buffer without NP-40 and incubated for 2.5 h at 4 °C. Mouse ascites for anti-human CD3 antibodies was used as an internal control. The unbound fraction was collected and used for Western and transcription analyses.

Antibodies and Western Blotting. Rabbit anti-Trip1 antibodies raised against the N-terminal 100 amino acids (aa) of human Trip1p/Rpt6 fused to GST and anti-human 20S antibodies were a gift from Prof. Rick Young (Massachusetts Institute of Technology) and Prof. George DeMartino, respectively. Sera from GST-Trip1p immunized rabbits was purified first on a Sepharose A column as described (*1*) and then loaded on a GST column. The unbound antibodies were concentrated and dialyzed, and the concentration was determined (*1*). Antibodies against the components of human PA700/19S complex and transcription factors were from Affinity Bioreagents and Santa Cruze, respectively. Antibody against Gal6 has been described (*30*, *31*). Rabbit anti-DSIFp160 (*32*) and mouse anti-CTD (8WG16), recognizing the C-terminal domain of the largest subunit of RNA pol II, were generous gifts from Prof. Richard Gaynor (UT-Southwestern Medical Center) and Dr. Caroline Kane (University of California at Berkeley), respectively. Mouse anti-TAF_{II} 250 antibodies against the C-terminal 100 aa (1698aa-1798aa) of human TAF_{II} 250 were generated by genetic immunization as described (*33*). Western blotting was conducted as described (*25*).

Measurement of Peptidase Activity. The proteasome peptidase activity was measured as described (*34*), except that a peptide-AMC derivative (50 μM) of Suc-LLVY-AMC (Subs. Y), Z-LLE-AMC (Subs. E), and Boc-LRR-AMC (Subs. R) (Bachem) was used to analyze the trypsin-like (Tryp-L), peptidyl-glutamyl peptide hydrolyzing (PGPH) and chymotrypsin-like (CT-L) proteosomal peptidase activities, respectively (*35*, *36*).

Kinetically Synchronized Transcription Assays. Transcription reactions were carried out essentially as before (*1*), with minor modifications. Reactions (16 μL) in TXN buffer containing 125 ng of supercoiled templates and 36 μg of 19S/PA700-depleted or undepleted HeLa NE with or without Gal4-VP16 were assembled on ice. Reactions were incubated for 40 min at 30 °C and then 9 μLof TXN buffer containing 5 μCi of α-^{32}P−UTP (3000 Ci/mmol, Amersham) and the ribonucleoside triphosphates (NTPs) mixture (final concentration: 50 μM of each ATP, GTP, and CTP and 1 μM of UTP) was added. After an additional 10 min incubation at 30 °C, the reactions were terminated and G-less transcripts

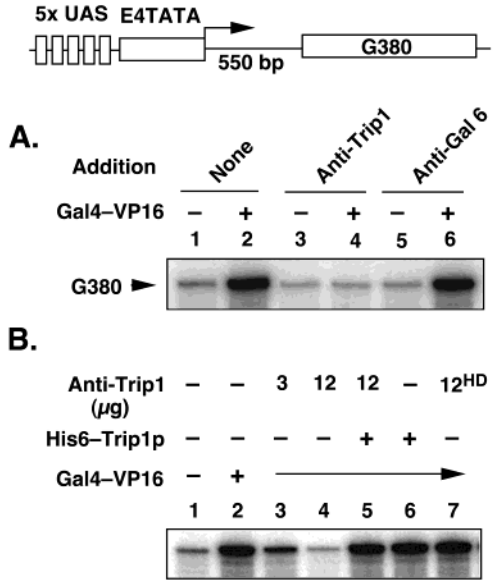

FIGURE 1: Antibodies against Trip1p/Rpt6 inhibit transactivation by Gal4-VP16. (A) Antibodies against Trip1p/Rpt6 inhibit transcription in vitro. The template (shown above the gel) and an aliquot of HeLa NE (36 μg) were preincubated for 40 min at 30 °C in the presence (+) or absence (−) of Gal4-VP16 (75 ng) and the indicated antibodies (12 μg). After 10 min of nucleotide (A/G/C/UTP) addition, the reactions were terminated and the 380-mer G-less transcripts (G380) were purified and analyzed as described (*1*). (B) Prior heat inactivation or incubation of the anti-Trip1p antibodies with recombinant Trip1p alleviates antibody inhibition of transcription. Transcription reactions were carried out as described in panel A, except that before being added into transcription reaction, the anti-Trip1 antibodies were incubated for 30 min at room temperature in the presence (lane 5) or absence (lane 4) of the 3 μg recombinant Trip1p (His6-Trip1p). In lanes 3 and 6, 3 μg of anti-Trip1 antibodies and His6-Trip1p alone was added, respectively. In lane 7, heat-denatured (HD) antibodies (incubated for 10 min at 90 °C) were added.

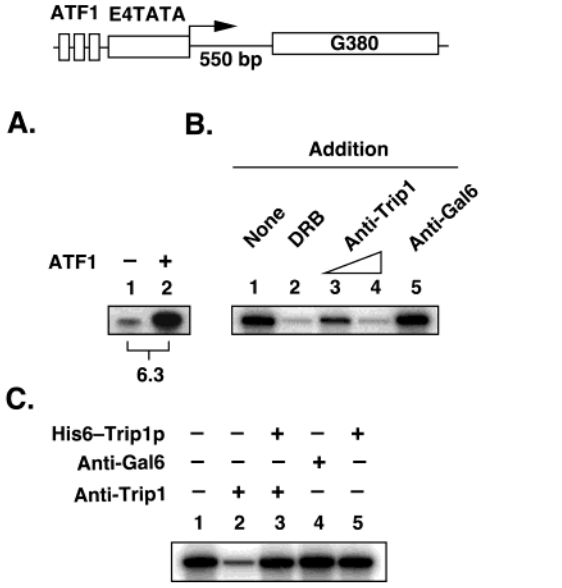

FIGURE 2: Activated transcription by the natural activator ATF/E4TF3 is inhibited by anti-Trip1/Rpt6 antibodies. (A) Transcription activation by ATF/E4TF3 in vitro. Transcription reactions were carried out as described in Figure 1, except that templates with (+) pTF3-3E4T-550 C_2AT (shown above the gel) or without (−) pE4T-550 C_2AT (see Materials and Methods) three ATF/E4TF3 (ATF1) binding sites were used. Fold activation of transcription by endogenous ATF/E4TF3 (6.3-fold) is shown under the gel. (B) Inhibition of ATF/E4TF3 activated transcription by DRB and antibodies against Trip1p/Rpt6. Transcription reactions were carried in the presence and absence of the indicated antibodies as described in Figure 1, except that a template with three ATF/E4TF3 binding sites was used. DRB (50 μM) was added along with NTPs. (C) Prior incubation of the anti-Trip1/Rpt6 antibodies with the His6-Trip1p alleviates antibody inhibition of transactivation by ATF/E4TF3. Transcription reactions were carried out as described in panel B of Figure 1.

were purified and quantified as described (*1*). In some reactions, GTP was omitted from NTP mix and 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazol (DRB) or the highly purified mammalian PA700 was added after 40 min incubation of extract with template.

Transcription reactions in which antibody inhibition was analyzed were carried out as before (*1*), except that prior to addition into the transcription reactions, purified rabbit anti-Trip1/Rpt6 antibodies were either incubated for 30 min at room temperature with or without recombinant Trip1p or heat-treated for 10 min at 90 °C. The effect of proteasome inhibitors on transcription was analyzed as described (*1*).

RESULTS

Antibodies against Trip1p Inhibit Transcription in Vitro. To test whether mammalian 19S complex is involved in transcription, experiments in a HeLa NE were carried out. The DNA template shown at the top of Figure 1 (also see Materials and Methods) was mixed with HeLa NE in the presence or absence of Gal4-VP16. The solutions were incubated for 40 min at 30 °C to synchronize preinitiation complex (PIC) formation. This was followed by an initiation/elongation step for 10 min in the presence of all four ribonucleoside triphosphates, a time sufficient to produce mostly 380-mer full-length G-less transcripts (*1*). As expected, the presence of Gal4-VP16 stimulated transcription significantly (Figure 1, lanes 1 and 2) and full-length G-less products was hard to detect if GTP was excluded from the NTP mix (data not shown).

With this basic characterization of the system complete, the effect of antibodies raised against Trip1p/Rpt6 (the human analogue of yeast Sug1p) on transcription was assessed. As evident in Figure 1A, these anti-Trip1 antibodies largely inhibited activated, but not the basal, transcription (lanes 1−4). Addition of control antibodies (anti-Gal6) did not inhibit transcription (compare lanes 1 and 2 with 5 and 6). Although basal transcription level was slightly reduced in the presence of anti-Trip1 antibodies (Figure 1A, lanes 1 and 3), this does not reflect a specific effect of the antibodies since similar effect is also observed in the presence of control (anti-Gal6) antibodies (compare lane 1 with 3 and 5). On the other hand, this slight effect of the control antibodies was insignificant and/or undetectable for activated transcription (Figure 1 and also see Figure 2). Therefore, comparing lanes in Figure 1A without (1, 3, and 5) or with (2, 4, and 6) activator strongly suggests that the anti-Trip1 antibody largely abolishes activated, but not basal, transcription. When the anti-Trip1 antibodies were preincubated with recombinant Trip1p (His6-Trip1) (Figure 1B, lanes 5 and 6) for 30 min at room temperature or the antibodies were heat-treated for 10 min at 90 °C (lane7), inhibition was abolished, indicating that the inhibition observed is a specific effect of the antibody binding to Trip1p.

In the original yeast study (*1*) and the work above, the artificial activator, Gal4-VP16, was used. Therefore, it was important to determine the effect of the anti-Trip1 antibody on a reaction stimulated by a native transcription factor, ATF/E4TF3 (also called ATF1) (*3*, *28*). First, transcription activation by the native activator ATF/E4TF3 was confirmed by performing transcription assays using templates, pE4T-550 C_2AT containing only the core promoter (TATA) sequence and pTF3-3E4T-550 C_2AT with three ATF/E4TF3 binding sites (top of Figure 2, also see Materials and Methods). As shown in Figure 2A, the endogenous ATF/E4TF3 protein in the extract activated transcription more than 6-fold over basal level (lanes 1 and 2), consistent with the previous observation of adding purified ATF/E4TF3 in an ATF/E4TF3-depleted transcription system (*28*). We also found that in the absence of Gal4-VP16, transcription level from the template used in Figure 1 (Lane 1) was essentially equal to one that contains only the core promoter sequence, and this transcription level, which is designated as basal transcription, is not significantly affected by anti-Trip1 antibodies (Figure 1A and data not shown). Therefore, we analyzed the effect of anti-Trip1 antibody on activated transcription by ATF/E4TF3. As reported before (*3*, *37*), a transcription elongation inhibitor, DRB, strongly inhibited the production of the full-length product when added along with the NTPs (Figure 2B, lane 2). As shown in Figure 2B, addition of the anti-Trip1p antibody also inhibited transcription significantly, while the control anti-Gal6p antibody did not (compare lanes 1, 3, 4, and 5). In Figure 2C, the specificity of this inhibition was confirmed by demonstrating that preincubation of the antibody with recombinant Trip1p abolished inhibition.

On the basis of the data shown in Figures 1 and 2, we conclude that the activity of Trip1 protein is essential for activated transcription of both artificial and natural activators in the mammalian system.

Immunodepletion of 19S Reduces Activated Transcription. To probe the role of the 19S complex in transcription in another way, an immuno-depletion experiment was carried out using an anti-Trip1 monoclonal antibody (*29*) coupled to protein G resin or, as a control, resin coupled with an anti-CD3 antibody. The HeLa NE depleted with anti-Trip1p-affinity resin and control resin will hereafter be designated 19S-dNE and the mock-dNE, respectively. Western blotting revealed that Trip1p was depleted by the anti-Trip1p-affinity resin to <2% of the native level (Figure 3, panel 1, lanes 4 and 5), while the depletion of the extract with control resin (mock-dNE, panel 1, lanes 2 and 3) had no effect. Rpn7/S10, another PA700 component, was also essentially absent in the immuno-depleted extract (panel 2, lanes 4 and 5), arguing that the anti-Trip1 antibody retained the entire 19S complex and not simply Trip1p alone (Figure 3). The level of MSS1/Rpt1, another of the ATPases in the base region of the 19S complex, was also analyzed. MSS1 has been reported to also reside in another complex distinct from the 19S complex (*26*). This is consistent with only partial depletion of this protein from the extract by anti-Trip1 antibody (Figure 3, panel 3, lanes 4 and 5). Western blotting using anti-20S antibodies revealed that the 20S core proteasome was depleted ~50% by the anti-Trip1 antibody. This indicates that either as much as one-half of the 20S core proteasome in extracts is not associated with the 19S complex, that the antibody and the 20S complex at least partially competes for Trip1p or that the 26S proteasome is unstable under the conditions used.

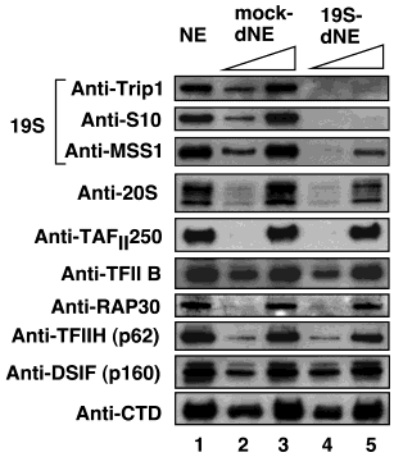

FIGURE 3: Characterization of Immunodepletion of the 19S complex from crude NE. Depletion of the 19S complex from crude HeLa NE by anti-Trip1/Rpt6 antibodies was described in details under "Materials and Methods". 19S-depleted NE (19S-dNE) and mock-depleted NE (mock-dNE) were generated by incubating the HeLa NE with protein G-Sepharose previously mixed with ascites from Trip1p/Rpt6 or CD3 immune mice, respectively. Untreated NE (9 μg) (lane1) and 3 μg (lanes 2 and 4) and 9 μg (lanes 3 and 5) of the indicated depleted extract were resolved on a 5% or 10% SDS-PAGE gel and analyzed by Western blotting using the specific antibodies raised against the indicated 26S proteasome (19S and 20S), transcription factors and RNA Pol II.

As reported (*26*, *38*, *39*, and Sun, L., Johnston, S. A., and Kodadek, T. (2002) Physical association of the APIS complex and general transcription factors, *Biochem. Biophys. Res. Comm. 296*, 991−999.), a small amount of several general transcription factors (GTFs) associate physically with the 19S complex in crude extracts. This raised the possibility that depletion of the 19S from extract may also deplete some GTFs to the point of depressing transcription activity. To probe this question, Western blots were employed to analyze the levels of several of these proteins in the 19S-dNE. As seen in Figure 3, the levels of TAF_{II} 250 (a subunit of TFIID), TFIIB, RAP30 (a subunit of TFIIF), and p62 (a subunit of TFIIH) were similar in the mock-dNE and the 19S-dNE, respectively. In addition, the levels of RNA Pol II and DSIFp160 (large subunit of DSIF) were also similar in the mock-dNE and the 19S-dNE. Thus, the levels of the GTFs tested and RNA pol II did not changed significantly in the 19S-depleted extract.

Next, the transcription activities of the 19S-depleted and mock-depleted extracts were evaluated to explore the effects of the 19S in transcription. Efficient transactivation by Gal4-VP16 (~8-fold over basal) was observed in both untreated extract and mock-dNE (Figure 4A, lanes 1−4). In contrast, the 19S-dNE supported only an approximately 2-fold increase in transcription over basal levels (compare lanes 1 and 2 with 5 and 6). Despite this significant decrease of activated transcription (lanes 2, 4, and 6), the basal transcription level was largely unaffected (compare lanes 1, 3, and 5). This is consistent with the data in Figure 3 that the reduction in activated transcription cannot be ascribed to an indirect depletion of the GTFs and RNA pol II. Transcription

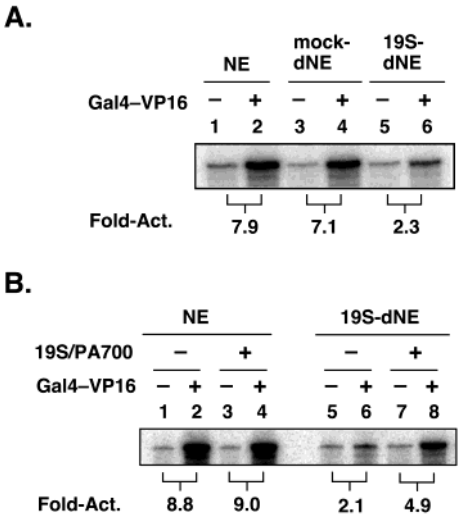

FIGURE 4: 19S complex is required for efficient transcription activation. (A) Immunodepletion of the 19S complex abolishes activated transcription. Equal amounts (36 μg) of indicated extract were used for transcription assays in the presence (+) or absence (−) of Gal4-VP16 as described in Figure 1. Full-length G-less transcripts were analyzed and quantified as before (*1*), and fold activation (Fold-Act.) of transcription is shown under the gel. (B) Add-back of highly purified PA700/19S complex in 19S-dNE restores activated transcription. Transcription reactions were carried out as described in panel A, except that Gal4-VP16 was added (+) or not (−) during the 40 min incubation of template and extract. The purified mammalian 19S/PA700 (30 ng) was added after 40 min incubation as indicated. Fold activation (Fold-Act.) of transcription is shown under the gel.

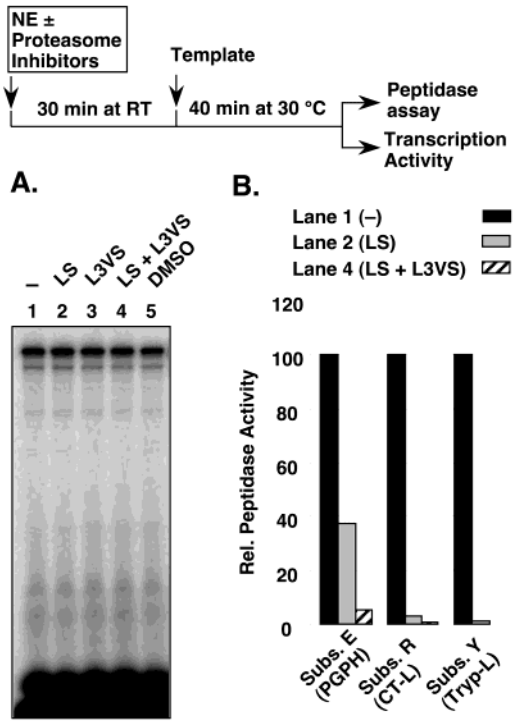

FIGURE 5: Involvement of the 19S in transcription is independent of proteasome-mediated proteolysis. (A) Proteasome inhibitors have no effect on transcription. The experimental protocol to analyze the effect of proteasome inhibitors on transcription is shown above the gel. One aliquot of NE and template (pTF3-6 C_2AT) mixture was removed after 40 min incubation at 30 °C to analyze the transcription level in the presence (+) or absence (−) of 100 μM of proteasome inihibitors, lactacystin (LS), and Z-L3VS (L3VS). The control solvent DMSO, used to prepare the stock solution of Z-L3VS, was also added in transcription reaction (lane 5). (B) Proteasome inhibitors significantly inhibited the proteasomal peptidase activity. After 40 min incubation of NE and template, an aliquot of the transcription reaction corresponding to lanes 1, 2, and 4 was employed to measure the proteasomal peptidase activity. Peptidase activity was measured as described (*34*), except that suitable peptide substrates were used to measure the indicated peptidase activity. Values shown are the mean of three independent experiments.

activation by the natural activator, ATF/E4TF3, was also significantly decreased in the 19S-depleted extract (data not shown).

To more definitively probe the issue of whether the loss of activity observed in the 19S-dNE was indeed due to loss of the 19S complex alone and not to some indirect effect, an add-back assay was performed using highly purified mammalian PA700/19S complex (*27*). The PA700/19S complex employed was completely devoid of detectable 20S and any known general transcription factors, as determined by mass spectrometry (data not shown). For the add-back assay, an equal amount of untreated and 19S-depleted extract was incubated for 40 min in the presence or absence of Gal4-VP16. The purified PA700/19S complex was then added along with NTPs, and the reactions were terminated after 10 min. G-less transcripts were extracted and analyzed. As anticipated, addition of the purified PA700/19S complex to the untreated extract had no detectable effect on transcription levels (Figure 4B, lanes 1−4). However, addition of the purified PA700/19S complex to the 19S-dNE restored >50% of the activity relative to the control extract (compare lanes 6 and 8 and lanes 4 and 8). A further increase in transcription was not observed if the 19S/PA700 complex was added to the 19S-dNE during PIC formation (data not shown), suggesting that at least under these conditions, the 19S complex exerts its effects downstream of PIC formation, consistent with our previous observation in yeast system (*1*).

Requirement for PA700/19S Complex Does Not Reflect a Proteolytic Event. Having established a role of the PA700/19S complex in RNA pol II transcription, the next step was to begun to address the nature of its role. If the addition of anti-Trip1 antibodies or depletion of the 19S inhibits proteolysis and so reduces transcription activity, the most obvious model is that the 19S complex in the context of the 26S proteasome helps to mediate some proteolytic event critical for efficient transcription. To test this model, the effect of the potent and selective proteasome inhibitors, lactacystin (*40*) and peptide vinyl sulfone carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L3VS) (*41*), on transcription was examined. HeLa NE was incubated in the presence or absence of proteasome inhibitors for 30 min at room temperature. Template was then added and incubated for another 40 min at 30 °C to allow PIC formation. The sample was then split and employed in peptidase and transcription assays. In the former, peptide substrates suitable to address the level of the three different peptidase activities of the 20S core, CT-L, Tryp-L, and PGPH activities (*35*, *36*) were employed. The results, shown in Figure 5A, reveal that transcription was unaffected whether 100 μM of the indicated proteasome inhibitors was added alone (lanes 1−3) or in combination (lane 4). In contrast, measurement of the proteasomal peptidase activity of the samples correspond to lanes 1, 2, and 4 of transcription assay reveals that the vast majority of all three hydrolytic activities of the proteasome

were inhibited (Figure 5B). It is important to note that in the presence of both lactacystine and Z-L3VS (Figure 5A, lane 4), both CT-L and Tryp-L activities were almost undetectable. We conclude that it is unlikely that the requirement for the 19S in mammalian transcription is due to its role in proteolysis.

DISCUSSION

Recently, we reported that in yeast at least the 19S regulatory particle of the 26S proteasome was critical for efficient elongation of RNA pol II and that this event did not reflect a proteolytic event (*1*). This result was surprising because it had previously been thought that the major, and possibly only, biochemical activity of the 19S complex was to facilitate proteolysis by the 20S proteasome core subunit (*42*). Therefore, it became important to establish whether this is a general phenomenon of eukaryotic transcription or something peculiar to *S. cerevisiae*. We demonstrate here that mammalian transcription seems to have the same requirement for 19S function in transcription. Addition of antibodies against the Sug1 homologue Trip1 (Figures 1 and 2) or immunodepletion of the 19S complex from HeLa NE (Figures 3 and 4) significantly inhibits activated, but not basal, transcription of both an artificial and native activator. Importantly, this effect is largely reversed by addition of a highly purified form of the mammalian PA700/19S (Figure 4). As is the case in yeast, the addition of inhibitors of the 20S proteolytic activity has no measurable effect on transcription although the proteasome's peptidase activities was almost completely inhibited (Figure 5).

These results mirror those obtained in the yeast system, though some minor quantitative differences are noted. For example, whereas addition of immunopurified yeast 19S complex restored >90% activity in a heat-treated, temperature-sensitive *sug1*−*20* extract (*1*), addition of the highly purified mammalian 19S/PA700 to the 19S-dNE rescued only ∼55% of the original activity (Figure 4B). This was not a limitation of the amount of complex added, since more 19S/PA700 did not increase transcription further (data not shown). One possibility is that this less than quantitative rescue is due to the fact that bovine PA700/19S was added to a human transcription system. Insufficient human 19S was available to carry out an analogous experiment. Another possibility is that the PA700 preparation employed could have lost a stimulatory factor during the purification that is loosely associated with the complex in extracts and is immunodepleted by the anti-Trip1 antibody. Of note is that the yeast 19S used in the add-back experiments was immunopurified and probably had more accessory factors associated with it than the highly biochemically purified PA700 used in this study. For example, the yeast 19S had Rad23 and Cdc68 associated with it (*1*). It may be possible to probe this point by testing earlier fractions of the 19S preparation for their ability to stimulate transcription in the 19S-dNE.

A second important feature of this study is that a native cellular activator, ATF/E4TF3 (*3*, *28*), was employed (Figure 2) in addition to the artificial activator Gal4-VP16. ATF/E4TF3 present as endogenous protein in the extract activated transcription more than 6-fold (Figure 2A) and transcription activation mediated by this protein was clearly dependent on PA700 activity (Figure 2 and data not shown). It is interesting in this regard that ATF/E4TF3-activated transcription is known to require DSIF (*3*). Since the level of DSIF in the 19S-dNE was the same as that of untreated extract (Figure 3), this argues that the PA700/19S complex and DSIF may facilitate ATF1-mediated transactivation by different mechanisms.

In both this study and that of the role of the 19S complex in yeast transcription (*1*), inactivation or depletion of the 19S complex mainly affected activated, rather than basal, transcription. This implies that the 19S complex is involved in the transcription cycle at a point(s) specifically mediated by activators and that in the absence of an activator, it does not evince an effect. It has been reported that transcription activation by both Gal4-VP16 (*14*) and ATF/E4TF3 (*3*) is regulated, in part, at the elongation phase of transcription. Since the 380 bp G-less cassette was inserted into the templates used in this study at a site ca. 550 nucleotides downstream of the transcription initiation site, the RNase T1-resistant transcripts must represent elongation products, suggesting that both the yeast and mammalian 19S affect the activated transcription in elongation phase and/or a phase of transcription cycle that precedes elongation. Given previous reports of direct contacts between 19S proteins, particularly Sug1p/Trip1p/Rpt6, Sug2p/Rpt4, and MSS1/Rpt1 and activation domains (*38*, *43*−*46*), one attractive possibility is that the19S or a subcomplex of the 19S is recruited by activators to the transcribed gene. Indeed, recently we report that Gal4 activation domain recruits a subcomplex of the yeast 19S, APIS, to the activated *GAL1* promoter (*47*). Although no method for mammalian APIS purification is yet available, it will be interesting to analyze whether like 19S, add-back of the mammalian APIS can restore activated transcription in the 19S-dNE.

Finally, whereas the 19S complex is clearly required for efficient transcription in the HeLa extract, this does not appear to involve a proteasome-mediated proteolytic event. Inhibition of the vast majority of all three peptidase activities of the proteasome by lactacystin and ZL3VS (Figure 5) had no effect on transcription. Again, this mirrors the results obtained in the yeast system (*1*). These results, combined with the finding of Russell et al. of a nonproteolytic role of the 19S complex in yeast nucleotide excision repair (*34*), make clear that the 19S complex has other important roles in cellular metabolism in addition to facilitating proteasome-mediated proteolysis. The precise nature of the nonproteolytic process that the 19S complex mediates in RNA pol II transcription is not yet clear. Certainly, one reasonable hypothesis, given the known chaperonin activity of the complex (*48*, *49*), is that it acts to remodel transcription and/or stalled elongation complexes (*50*). The 19S complex can clearly remodel macromolecular complexes independent of the 20S core proteasome, as demonstrated by its ability to dissociate the Cdc2-cyclin B complexes in a proteolysis-independent manner (*51*). Further precedent for this type of mechanism is provided by the role of bacterial ClpX in phage Mu transposition. ClpX is a hexameric chaperonin that probably is a bacterial homologue of the six ATPases present in the base of the 19S complex. ClpX plays an important role in ClpP-mediated proteolysis, analogous to the role that the 19S complex plays in mediating core proteasome-based protein degradation. However, ClpX acts independently of ClpP in disrupting the otherwise hyperstable phage Mu

protein A-DNA tetramer, a critical event in the phage life cycle (*52*, *53*). Nonetheless, it is important to point out that biochemical studies of the 19S complex are in their infancy and its role in nucleic acid metabolism has been appreciated only recently. Therefore, it is possible that entirely novel activities of this complex remain to be discovered and that these will be critical for efficient RNA pol II transcription.

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