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TFIIH Plays an Essential Role in RNA Polymerase I Transcription

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Summary

TFIIH is a multisubunit protein complex that plays an essential role in nucleotide excision repair and transcription of protein-coding genes. Here, we report that TFIIH is also required for ribosomal RNA synthesis in vivo and in vitro. In yeast, pre-rRNA synthesis is impaired in TFIIH *ts* strains. In a mouse, part of cellular TFIIH is localized within the nucleolus and is associated with subpopulations of both RNA polymerase I and the basal factor TIF-IB. Transcription systems lacking TFIIH are inactive and exogenous TFIIH restores transcriptional activity. TFIIH is required for productive but not abortive rDNA transcription, implying a postinitiation role in transcription. The results provide a molecular link between RNA polymerase I transcription and transcription-coupled repair of active ribosomal RNA genes.

Introduction

All three classes of nuclear RNA polymerases utilize auxiliary transcription factors to recognize gene promoters and specifically initiate transcription. Transcription complexes are assembled at promoters, either through the recruitment of the respective RNA polymerases and initiation factors or by the binding of large preassembled complexes that contain general transcription initiation factors and many other polypeptides. These megadalton sized complexes, referred to as "holoenzymes," contain RNA polymerase, general transcription factors, and coactivators as well as proteins that play a role in protein modification, RNA chain elongation, RNA processing, and DNA repair. With regard to RNA polymerase I (Pol I), approximately 10% of the enzyme is contained within the holoenzyme, a multiprotein complex with an apparent molecular mass of >2000 kDa (Saez-Vasquez and Pikaard, 1997; Seither et al., 1998; Albert et al., 1999; Hannan et al., 1999). The Pol I holoenzyme has been purified from *Brassica oleacera, Xenopus laevis,* mouse, and rat, and has been shown to contain all or most of the factors required for transcription initiation.

In mammals, promoter selectivity is brought about by the selectivity factor TIF-IB/SL1, a complex containing TBP and three Pol I-specific TAFs (Comai et al., 1992; Eberhard et al., 1993). TIF-IB/SL1 cooperates with UBF, a member of the HMG box family of architectural factors, in preinitiation complex formation. The complex of TIF-IB/SL1 and UBF catalyzes the recruitment and binding of Pol I together with associated auxiliary factors, e.g., TIF-IA and TIF-IC, to the ribosomal gene promoter (for review, see Grummt, 1999). In addition, protein-modifying enzymes, i.e., casein kinase II, histone acetyltransferase, and certain components of the DNA repair/replication machinery, such as Ku70/80, topoisomerase I, and PCNA, have been shown to be associated with the Pol I holoenzyme. In an attempt to characterize the polypeptides that are contained in the Pol I holoenzyme complex, we found that TFIIH, a basal Pol II transcription initiation factor, is an integral component of the Pol I holoenzyme. Human TFIIH is a multisubunit complex composed of nine subunits ranging in size from 34 to 89 kDa (for review, see Coin and Egly, 1998). In addition to its role in transcription of protein- coding genes, TFIIH is involved in nucleotide excision repair. TFIIH is recruited into preinitiation complexes at promoters by interaction with TFIIE and Pol II. Preinitiation complex formation is the first step in transcription initiation, which is followed by promoter opening, synthesis of the first phosphodiester bond, and promoter clearance. TFIIH possesses DNA-dependent ATPase, DNA helicase, as well as protein kinase activity capable of phosphorylating the C-terminal domain (CTD) of the largest Pol II subunit (Serizawa et al., 1993; Lu et al., 1992; Schaeffer et al., 1993). The two largest TFIIH subunits, XPB and XPD, are ATP-dependent helicases of opposite polarity. Genetic studies revealed that the helicase activity of the RAD3 subunit, the yeast homolog of XPD, is dispensable for Pol II transcription but not for the repair function of TFIIH (Feaver et al., 1993). The multiple roles of TFIIH might explain why mutations within defined subunits give rise to genetic disorders such as *Xeroderma pig-*

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mentosum, Cockayne syndrome, and trichothiodystrophy (Lehmann, 2001).

In this study, we present experimental evidence that, besides its established roles in Pol II transcription and nucleotide excision repair, TFIIH serves a function in ribosomal gene transcription. We show that part of cellular TFIIH is localized within the nucleolus at sites of active rDNA transcription. Significantly, Pol I transcription declines with similar kinetics in yeast strains harboring temperature-sensitive mutations of either TFIIH or Pol I. Biochemical fractionation and immunoprecipitation experiments reveal that TFIIH is associated with two components of the Pol I transcription apparatus, e.g., TIF-IB and Pol I. Consistent with an essential role in rRNA synthesis, purified TFIIH stimulates Pol I transcription in a reconstituted system, and depletion of TFIIH from in vitro assays abrogates Pol I transcription. These observations suggest an important function of TFIIH on the cells' overall metabolic activity and may explain some of the clinical features that are associated with inherited TFIIH defects.

Results

TFIIH Is Associated with a Subpopulation of Pol I and TIF-IB

Mammalian Pol I is heterogeneous with regard to chromatographic properties, electrophoretic mobility, and ability to support specific transcription of rDNA (reviewed in Grummt, 1999). The initiation-competent form of Pol I is associated with accessory proteins that contribute to ribosome biosynthesis. Unexpectedly, when the Pol I holoenzyme was examined for immunoreactivity toward different subunits of TFIIH, all subunits of TFIIH were consistently found (data not shown). To examine whether TFIIH is physically associated with Pol I, a partially purified Pol I preparation was further fractionated on a MonoQ-FPLC column (Figure 1A, MonoQ1). While this chromatographic step separated the majority of TFIIH from Pol I, a significant portion ($\sim$5%) of TFIIH coeluted with Pol I. Western blots with antibodies against subunits of TFIIH, e.g., XPB and p62, revealed that rechromatography of TFIIH-containing Pol I fractions (#17-22) did not remove TFIIH (MonoQ2).

To examine whether Pol I associated with TFIIH is the transcription-competent enzyme moiety, we precipitated Pol I from TFIIH-containing fractions with antibodies against Pol I (α-RPA53) and TFIIH (α-XPB), respectively, and monitored coprecipitation of Pol I and TFIIH on Western blots (Figure 1B). Consistent with TFIIH being associated with Pol I, comparable amounts of Pol I and TFIIH were coimmunoprecipitated with either antibody. In parallel, the immunoprecipitates were assayed for Pol I activity in a reconstituted system. No transcripts were synthesized in the presence of control beads (Figure 1C). In contrast, both immunoprecipitates efficiently promoted rDNA transcription, indicating that TFIIH is associated with the transcriptionally competent form of Pol I.

Moreover, all preparations of TIF-IB, the Pol I-specific murine TBP-TAF complex, contained significant levels of TFIIH. To find out whether TFIIH is physically associated with TIF-IB, a TIF-IB-containing fraction was incubated with antibodies against TFIIH, and TIF-IB was visualized on immunoblots with antibodies against $TAF_{I}95$. As demonstrated in Figure 1D, a significant portion of TIF-IB coprecipitated with TFIIH (lanes 3 and 4). Significantly, the TFIIH/TIF-IB complex was not dissociated by 0.7 M KCl (lane 4), demonstrating the tight association of TFIIH with TIF-IB.

TFIIH Localizes to Sites of Active rRNA Synthesis

If a subfraction of TFIIH was involved in Pol I transcription, one would expect that it localizes to the nucleolus. To evaluate the nuclear distribution of TFIIH in living cells, human fibroblasts that have a mutated *XPB* gene were transfected with an expression vector encoding a GFP-XPB fusion protein. Cell lines were selected that stably express the fusion protein at physiological levels. Western blot analysis and immunoprecipitation experiments revealed that GFP-XPB was incorporated into the TFIIH complex and corrects the UV sensitivity of the parental cell line (data not shown). Confocal microscopic analysis of fibroblasts that stably express XPB-GFP revealed a homogenous green fluorescence signal throughout the nucleus (Figure 2A). In addition, virtually all cells displayed localization of tagged TFIIH within the nucleoli. Similar patterns were observed in SV40 transformed wild-type fibroblasts that were immunostained with antibodies against other subunits of TFIIH (data not shown). Thus, TFIIH is localized to both the nucleoplasm and nucleoli.

To examine whether endogenous TFIIH localizes to sites of active rDNA transcription, the intranucleolar distribution of TFIIH was analyzed by electron microscopy and immunogold labeling (Figure 2B). Staining with antibodies against XPD revealed a higher density of TFIIH in nucleoli than in the nucleoplasm. Significantly, immunogold particles were enriched in the dense fibrillar component of nucleoli, the sites of active rDNA transcription (Hozák et al., 1994), suggesting a role of TFIIH in rRNA synthesis.

TFIIH Is Essential for rDNA Transcription In Vivo

The structural and functional conservation of basal transcription machineries between yeast and mammals prompted us to investigate whether TFIIH is involved in rDNA transcription in vivo. We used yeast strains that carry temperature-sensitive mutations of TFIIH subunits, i.e., Tfb1 and Kin28, the yeast homologs of p62 and Cdk7, and measured the amount of newly synthesized 35S pre-rRNA versus stable 25S rRNA at various times after shifting to the nonpermissive temperature. As demonstrated in Figure 3, pre-rRNA synthesis was greatly reduced in TFIIH mutant strains (*tfb1-ts* and *kin28-ts*). At the restrictive temperature, *rpa43-ts*, a gene encoding an essential subunit of Pol I, pre-rRNA synthesis dropped with similar kinetics and to the same extent as in *tfb1-ts* and *kin28-ts*. Thus, the phenotype of the TFIIH mutants resembles that of *rpa43-ts*. Pol I transcription in wild-type strains remained essentially unaffected under the same experimental conditions. To rule out that impaired rDNA transcription in the TFIIH mutants was due to inhibition of Pol II transcription, we performed control experiments with the yeast strain *rpb1-1* that carries a temperature-sensitive mutation in

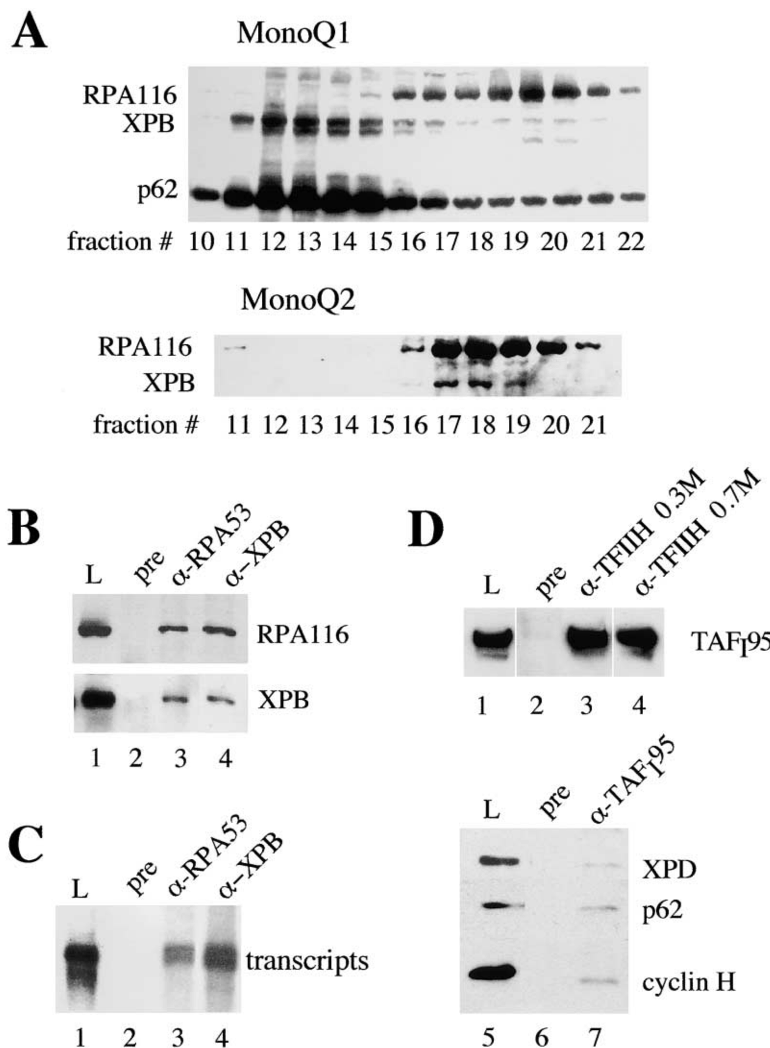

Figure 1. TFIIH Is Associated with Transcriptionally Active RNA Polymerase I

(A) Copurification of TFIIH with RNA polymerase I. Pol I was chromatographed on MonoQ, and RPA116 and TFIIH subunits were visualized on Western blots. Fractions #20–23 were rechromatographed and coelution of Pol I and TFIIH was analyzed.

(B) Coimmunoprecipitation of TFIIH and Pol I. Immobilized preimmune IgGs (lane 2), α-RPA53 (lane 3), or α-XPB/p62 antibodies (lane 4) were incubated with pooled fractions #17–21 from the MonoQ2 column. The input (L) and coprecipitated Pol I (RPA116) and TFIIH (XPB) were analyzed on immunoblots.

(C) TFIIH antibodies precipitate initiation-competent Pol I. The immunoprecipitates from above were assayed in a reconstituted transcription system.

(D) Coimmunoprecipitation of TIF-IB and TFIIH. Partially purified TIF-IB (L, lanes 1 and 5) was incubated with bead bound preimmune IgGs (lanes 2 and 6), α-TFIIH antibodies (lanes 3 and 4), or α-TAF$_I$95 antibodies (lane 7). Beads were washed with AM-300 or AM-700 (lanes 3 and 4). We analyzed on immunoblots 25% of the load and 50% of bead bound proteins.

Rpb1p, the largest subunit of RNA polymerase II (Nonet et al., 1987). At nonpermissive conditions, pre-rRNA synthesis in the *rpb1-ts* strain was also reduced; however, the decrease in Pol I transcription was less pronounced and occurred more slowly than in the TFIIH temperature-sensitive mutants.

TFIIH Is Required for rDNA Transcription In Vitro

Given that two major components of the transcription apparatus, e.g., Pol I and TIF-IB, are associated with TFIIH, we sought to compare the transcriptional activity of purified murine Pol I and TIF-IB that either contain or lack TFIIH. Following the purification scheme depicted in Figure 4A, fractions of both Pol I and TIF-IB were obtained that differ in their content of TFIIH. Transcriptional activity was assayed in a reconstituted system that contained Pol I and TIF-IB that either lack or contain endogenous TFIIH. As shown in Figure 4B, transcriptional activity strongly depends on the source of Pol I and TIF-IB. TFIIH-containing Pol I (Pol I^{IIH}) synthesized the same amounts of transcripts, regardless of whether TFIIH-deficient or TFIIH-containing TIF-IB (lane 3) was used. In contrast, Pol I lacking TFIIH failed to transcribe the rDNA template in the presence of TFIIH-deficient TIF-IB. When assayed in the presence of TIF-IB^{IIH}, however, transcriptional activity of TFIIH-deficient Pol I compared to that of Pol I^{IIH}, indicating that specific Pol I transcription requires the presence of TFIIH.

As a second approach to compare the activity of TFIIH-deficient and TFIIH-containing TIF-IB, we purified TIF-IB by immunoprecipitation with α-TBP and α-p62 antibodies, respectively. After precipitation with anti-TBP antibodies and stringent washing, TIF-IB was virtually free of TFIIH (Figure 4C). Precipitation with α-p62 antibodies, on the other hand, selectively enriched TFIIH/TIF-IB complexes. The rationale of this experimental approach was to use equal amounts of TIF-IB and TIF-IB^{IIH} derived from the same fraction to rule out the possibility that transcription activation with TIF-IB^{IIH} was due to positive cofactors that may be present in the fractions used. In the absence of TIF-IB, no specific transcripts were synthesized. Addition of TFIIH-free TIF-IB did not promote specific transcription. Significantly, the same amounts of TIF-IB^{IIH} stimulated transcription more than 30-fold. When tested in the presence of Pol I^{IIH}, both TIF-IB and TIF-IB^{IIH} exhibited the same transcriptional activity. The tight association of TFIIH with

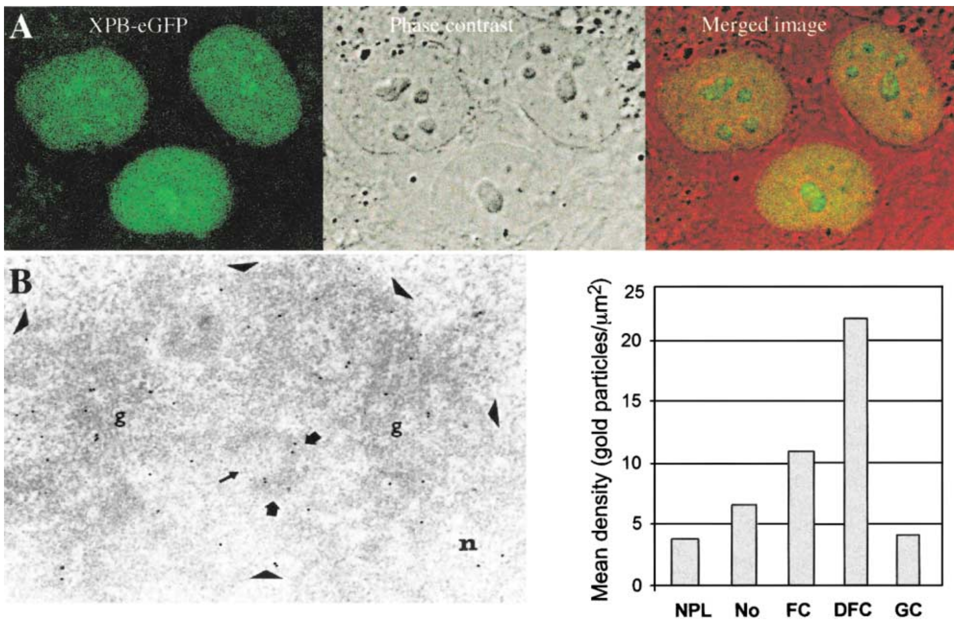

Figure 2. Nucleolar Localization of TFIIH

(A) Localization of XPB-eGFP in living cells. A confocal image of XPCS2BA-SV fibroblasts which stably express the XPB-eGFP fusion protein is shown next to the phase contrast, the merged image of XPB-eGFP (green), and transmitted light image of the same cells (red).

(B) Ultrastructural localization of TFIIH. Ultrathin sections of HeLa cells were stained with α-XPD antibodies. The nucleolar region (arrowheads) is labeled by gold particles, marking the locations of TFIIH. Thin arrows mark fibrillar centers and thicker arrows mark the dense fibrillar component. The nucleolar granular component (g) and the nucleoplasm (n) are marked. The histogram shows the density of gold particles (grains/μm^2) in the nucleoplasm (NPL), nucleoli (No), the fibrillar center (FC), dense fibrillar component (DFC), and granular component (GC).

subfractions of both Pol I and TIF-IB provides an explanation why the requirement of TFIIH in Pol I transcription has not been noted before. In the reconstituted system used, Pol I and TIF-IB were purified from selected fractions that exhibit high specific transcriptional activity. These fractions turned out to correspond to Pol I^{IIH} and TIF-IB^{IIH}, and therefore, the system did not require exogenous TFIIH.

TFIIH Complements Transcriptional Activity in a TFIIH-Deficient System

To demonstrate the requirement of TFIIH in rDNA transcription, immunodepletion experiments were performed. A fraction containing all proteins required for Pol I transcription (DEAE-280; Schnapp and Grummt, 1996) was depleted from TFIIH by incubation with antibodies against cdk7 before the transcriptional activity of the fraction was assayed. Significantly, depletion of TFIIH by α-cdk7 severely reduced Pol I transcription, whereas depletion of Pol II by α-RPB1 did not affect rDNA transcription (Figure 5A).

Next, we used a TFIIH-deficient reconstituted transcription system to examine whether exogenous TFIIH would complement transcriptional activity. Initially, we used partially purified TFIIH and found that addition of this fraction to transcription assays lacking TFIIH re-

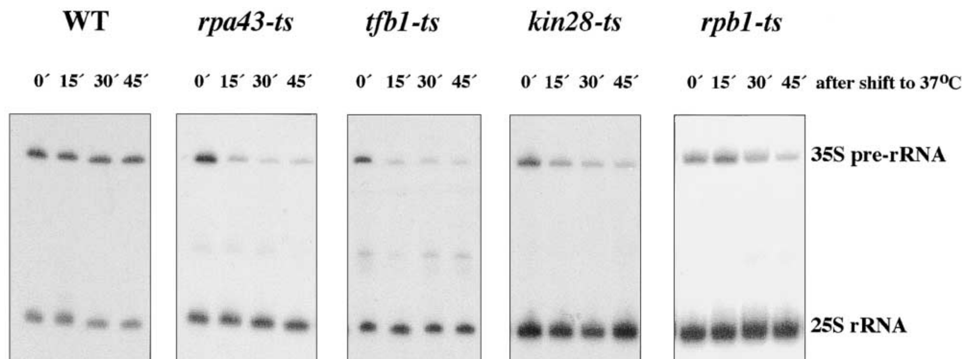

Figure 3. Temperature-Sensitive TFIIH Mutants Impair Yeast Pol I Transcription

Yeast cultures containing temperature-sensitive mutants of the indicated genes or the wild-type strain YSB207 (wt) were shifted to 37°C, and the levels of 35S pre-rRNA and 25S rRNA were determined by primer extension analysis at the indicated times after the temperature shift.

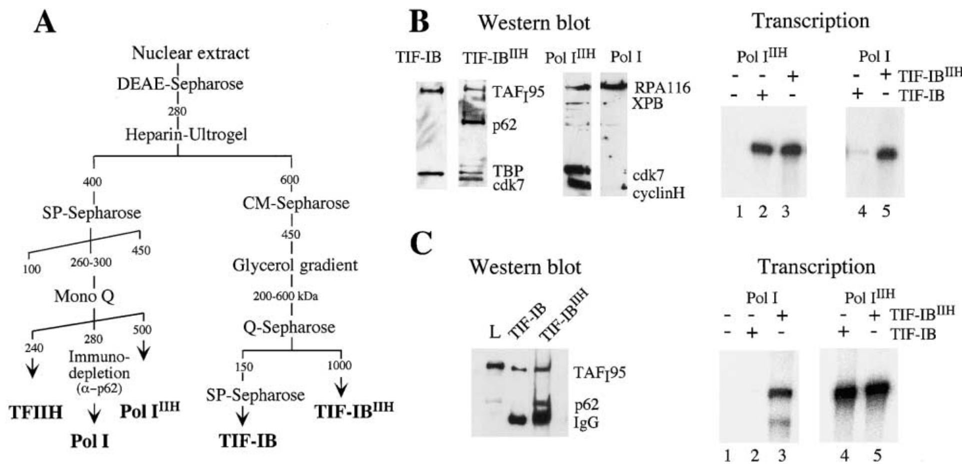

Figure 4. TFIIH Is Required for Pol I Transcription In Vitro

(A) Diagram showing the chromatographic steps used to purify Pol I, Pol I^{IIH}, TIF-IB, and TIF-IB^{IIH}. The numbers indicate the KCl concentrations (mM) at which the respective proteins elute from the columns.

(B) Pol I transcription in a reconstituted system containing endogenous TFIIH. A Western blot showing the absence or presence of TFIIH in Pol I, Pol I^{IIH}, TIF-IB, and TIF-IB^{IIH} is shown at the left. The individual fractions containing or lacking TFIIH, respectively, were assayed for their ability to reconstitute transcription in the presence of purified TIF-IA, TIF-IC, and recombinant UBF.

(C) TIF-IB immunopurified with α-p62, but not α-TBP antibodies reconstitute transcription in a TFIIH-free transcription system. The left panel shows a Western blot demonstrating the absence of TFIIH in TIF-IB$^{\alpha\text{-TBP}}$ and presence of TFIIH in TIF-IB$^{\alpha\text{-p62}}$. The right panel shows in vitro transcriptions containing TIF-IA, TIF-IC, recombinant UBF, TFIIH-free Pol I (MQII), and equal amounts of TIF-IB$^{\alpha\text{-TBP}}$ (lane 2) or TIF-IB$^{\alpha\text{-p62}}$ (lane 3). In lanes 4 and 5, TIF-IB$^{\alpha\text{-TBP}}$ and TIF-IB$^{\alpha\text{-p62}}$ were assayed in the presence of TFIIH-containing Pol I (Pol I^{IIH}).

stored transcription in a concentration-dependent manner (Figure 5B). To exclude the possibility that transcriptional activation was due to traces of essential Pol I transcription factors that may be present in the TFIIH preparation used, individual components required for Pol I transcription, i.e., TIF-IA, TIF-IC, and TIF-IB, were omitted from the assays. Clearly, TFIIH did not substitute for any of these basal transcription factors, indicating

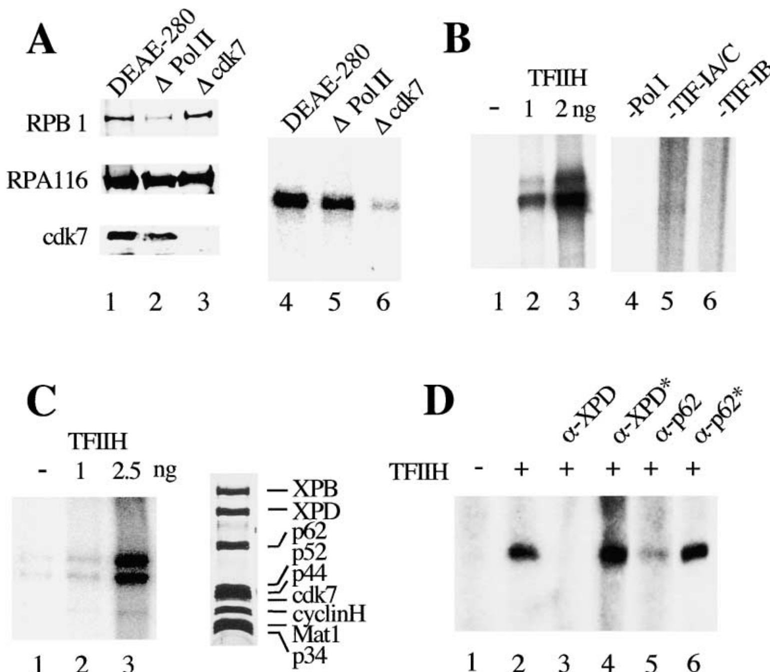

Figure 5. Purified TFIIH Reconstitutes Pol I Transcription

(A) Depletion of TFIIH from cellular fractions impairs Pol I transcription. We incubated 100 μl of a DEAE-280 fraction (Schnapp and Grummt, 1996) with 5 μg of polyclonal antibodies as indicated. The immunoprecipitates were bound to protein G-Sepharose and TFIIH was monitored on immunoblots (lanes 1–3). The transcriptional activity of the DEAE-280 fraction before and after immunodepletion was assayed by in vitro transcription (lanes 4–6).

(B) Transcriptional activation with partially purified TFIIH. Reactions contained 30 ng circular template DNA (pMr170-BH), 2 μl of affinity-purified TIF-IB (TIF-IB$^{\alpha\text{-TBP}}$), 1 μl of Pol I (MonoQ2), 1 ng of recombinant UBF, 3 μl of TIF-IA/TIF-IC (Poly-lysine-650 fraction), 30 ng TTFΔN185, and 1 or 2 μl of TFIIH (MonoQ1, lanes 2–4). Proteins were incubated for 20 min at 30°C before adding nucleotides and DNA. In lanes 5–7, the indicated factors were omitted from the reactions.

(C) Reconstitution of transcriptional activity by purified TFIIH. Immunopurified TFIIH was preincubated with Pol I and TIF-IA/TIF-IC for 20 min at 30°C before adding to transcription reactions. Silver-stained subunits of TFIIH are shown on the right.

(D) TFIIH antibodies prevent TFIIH-mediated activation of Pol I transcription. Immunopurified TFIIH was incubated with α-XPD (lane 3) or α-p62 (lane 5) for 20 min at 30°C, then for 20 min at 30°C with Pol I and TIF-IA/TIF-IC before addition to a reconstituted transcription system lacking TFIIH (lane 1). TFIIH-mediated activation of Pol I transcription is shown in lane 2. In reactions 4 and 6, the antibodies were inactivated by boiling for 5 min.

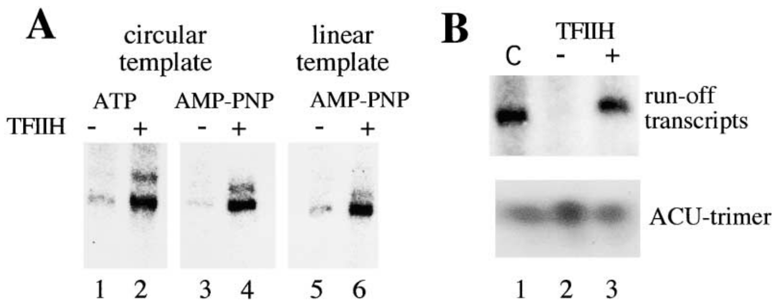

Figure 6. Mechanism of TFIIH-Mediated Pol I Transcription Activation

(A) TFIIH activates Pol I transcription without hydrolysis of the β-γ bond of ATP. Transcription was performed on circular (lanes 1–4) or linear (lanes 5 and 6) templates in the presence of either ATP/GTP or AMP-PNP/GMP-PNP as indicated.

(B) TFIIH plays a role in a step subsequent to transcription initiation. TFIIH was preincubated with Pol I, transcription factors, and template (pMr170-BH) in reactions containing either all four nucleotides to synthesize run-off transcripts (upper panel) or ATP and CTP (lower panel). After incubation for another 15 min, the synthesis of abortive transcripts (pppApCpU) was started by adding [α-^{32}P]UTP. In lane 1, a positive control with TIF-IB^{IIH} is shown. In lanes 2 and 3, the reactions contained TIF-IB devoid of TFIIH (TIF-IB $^{\alpha\text{-TBP}}$) and no exogenous TFIIH (lane 2) or 10 ng of purified TFIIH (lane 3).

that activation by TFIIH was not due to contaminating Pol I transcription factors in the TFIIH preparation. To exclude the possibility that a hitherto unknown activity present in the partially purified TFIIH preparation was responsible for transcriptional activation, we purified TFIIH to molecular homogeneity by biochemical fractionation followed by immunopurification with anti-p44 antibodies. This highly purified TFIIH reconstituted transcriptional activity in the TFIIH-deficient transcription system, too (Figure 5C). To substantiate the specificity of transcriptional activation by TFIIH, the purified TFIIH complex was incubated with antibodies against XPD and p62 before adding to the transcription reactions (Figure 5D). Consistent with TFIIH being required for Pol I transcription, both antibodies inhibited transcription. If the antibodies were inactivated by heat treatment, they did not counteract TFIIH-mediated reconstitution of Pol I transcription.

Mechanism of TFIIH-Mediated Activation of Pol I Transcription

The helicase of TFIIH hydrolyzes the β-γ bond of ATP to catalyze open complex formation at Pol II promoters. ATP hydrolysis is also required by a step subsequent to transcription initiation, most likely promoter clearance (Goodrich and Tjian, 1994; Dvir et al., 1996). On the other hand, ATP is not required under conditions where TFIIH is dispensable for initiation. These conditions include transcription from promoters containing a short stretch of mismatched base pairs surrounding the transcriptional start site as well as on negatively supercoiled templates (Timmers, 1994). As Pol I transcription on linear templates requires neither ATP hydrolysis nor protein phosphorylation (Schnapp and Grummt, 1991; Lofquist et al., 1993), we examined whether transcription activation by TFIIH depends on ATP hydrolysis and whether TFIIH is dispensable for transcription on circular rDNA templates. Transcriptional activation by TFIIH was assayed on linear and circular templates either in the presence of ATP and GTP or the nonhydrolyzable nucleotide analogs AMP-PNP and GMP-PNP (Figure 6A). The template used is a minigene representing a fusion of a 5′-terminal rDNA promoter fragment and a 3′-terminal fragment containing two terminator elements. In the presence of the transcription termination factor TTF-I, correctly terminated transcripts were produced. Significantly, TFIIH-mediated transcription activation occurred on both linear and circular DNA, and the same level of activation was observed regardless of whether the assays contained ATP or AMP-PNP. This demonstrates that, in contrast to Pol II, the topology of the template does not affect the requirement for TFIIH and neither the ATPase, helicase, nor protein kinase activity of TFIIH are required for Pol I transcription activation.

In an attempt to pinpoint the step(s) at which TFIIH affects Pol I transcription, we have adopted the abortive initiation assay. This assay takes advantage of the fact that functional initiation complexes can cycle short RNA products specific to the transcriptional start site when provided with limited nucleotide substrates (McClure et al., 1978). In these experiments, TFIIH-deficient Pol I and transcription factors were incubated with the DNA template in the presence of ATP and CTP (the first two nucleotides of mouse pre-rRNA) to form initiated complexes. In the experiment in Figure 6B, the reactions contained either TIF-IB^{IIH} (lane 1) or TFIIH-free TIF-IB in the absence or presence of TFIIH (lanes 2 and 3). The reaction was divided into two parts. One half was supplemented with GTP and [α-^{32}P]UTP, and incubated for 60 min to synthesize RNA. The other half was supplemented with [α-^{32}P]UTP alone. In the latter reactions, Pol I does not progress into the elongation phase, because the fourth nucleotide of pre-rRNA (GTP) is not present. The trinucleotide pppApCpU is released from the ternary complex and transcription begins again. Significantly, the synthesis of full-length transcripts required the presence of TFIIH, whereas TFIIH was dispensable for abortive product formation. This result indicates that TFIIH serves a function in a step subsequent to transcription initiation, i.e., promoter clearance, transcription elongation, or reinitiation.

Discussion

The most interesting finding that emerges from this study is that TFIIH, besides its well-established role in transcription of protein-coding genes and DNA repair, is also required for rRNA synthesis. Several lines of experimental evidence support this finding. First, fluorescent labeling and electron microscopy reveals that part

of cellular TFIIH is localized within the nucleoli at sites of active rDNA transcription. Second, TFIIH is contained in the Pol I holoenzyme and is associated with 5%–10% of Pol I and TIF-IB, the Pol I-specific TBP-TAF complex that binds to the murine rDNA promoter and nucleates initiation complex formation. Third, reconstituted mammalian transcription systems that are depleted of TFIIH are transcriptionally inactive but can be complemented by TFIIH. Finally, a rapid decline in yeast rRNA synthesis occurs in a *tfb-ts* mutant upon shift to the nonpermissive temperature. A similar drop in the synthesis of 35S pre-rRNA and the accumulation of fully processed 25S and 18S rRNA has been observed in *rad3-ts* and *rad25-ts* mutants, yeast homologs of mammalian ERCC2/XPD and ERCC3/XPB (Qiu et al., 1993; Guzder et al., 1994). However, these previous studies could not rule out that the reduction in rRNA synthesis in the ts mutants was due to a secondary effect of the stringent response rather than to direct involvement of RAD3 and RAD25 in Pol I transcription. By comparing pre-rRNA synthesis in mutants carrying temperature-sensitive mutations in the *RPB1*, *RPA43*, or *TFB* gene, we observed a nearly identical effect of the *tfb-ts* allele on the synthesis of pre-rRNA as that of the *rpa43-ts* allele. The degree and kinetics of inhibition was different from that observed in the cells harboring mutations in a subunit of Pol II. This suggests an intimate link between TFIIH function and Pol I transcription.

Using human cell lines that stably express EGFP-tagged XPB, we have studied the spatial distribution of TFIIH by confocal microscopy. As expected, XPB-GFP was homogenously distributed in the nucleoplasm. However, virtually all cells also contained TFIIH in the nucleoli. Previous studies localized TFIIH subunits also in Cajal bodies (Grande et al., 1997; Jordan et al., 1997). Cajal bodies contain a marker protein of unknown function, p80-coilin, and many components involved in transcription and processing of nuclear RNAs (Raska et al., 1990; Meier and Blobel, 1992), suggesting a role in the assembly of transcription complexes. It has been postulated that the three eukaryotic nuclear RNA polymerases associate with their respective transcription and processing factors in Cajal bodies, from which they are transported as preassembled complexes to the actual sites of transcription (Gall, 2000). In support of dynamic interactions between Cajal bodies and the nucleolus, Cajal bodies are frequently found in direct contact with nucleoli or even move into nucleoli (Bohmann et al., 1995; Lyon et al., 1997; Malatesta et al., 1994). The presence of TFIIH within Cajal bodies suggests that TFIIH is targeted to these organelles to assemble with both the Pol II and Pol I transcription machinery prior to appearance in the nucleolus or nucleoplasm.

Electron microscopy and immunogold labeling with antibodies against XPD revealed the localization of nucleolar TFIIH at sites of active rDNA transcription. Transcription of rDNA generates two nucleolar structures, the dense fibrillar component (DFC) and the granular component (GC), the sites of pre-rRNA synthesis and preribosome maturation, respectively. The fact that TFIIH was enriched in the dense fibrillar component is consistent with a role of TFIIH in rRNA synthesis. In support of TFIIH serving a function in Pol I transcription, immunoprecipitation experiments and biochemical fractionations demonstrated a tight association of TFIIH with a subfraction of Pol I and TIF-IB. It was recognized early that functionally distinct subpopulations of Pol I exist that differ both in their chromatographic behavior and transcriptional properties. The majority of Pol I, the bulk enzyme, is not capable to form productive initiation complexes, whereas a minor fraction, presumably the part of Pol I contained in the holoenzyme, is the initiation-competent form of Pol I (reviewed by Grummt, 1999). Significantly, TFIIH copurifies with the initiation-competent Pol I moiety, and TFIIH antibodies immunoprecipitate a subfraction of Pol I that is capable of interacting with Pol I-specific transcription initiation factors and auxiliary proteins.

Transcription by Pol II requires TFIIH-mediated ATP hydrolysis (Bunick et al., 1982) that is required both for open complex formation and promoter clearance (Goodrich and Tjian, 1994; Jiang and Gralla, 1995; Dvir et al., 1997). Promoter clearance results in the transition of an initiation complex to an elongation complex. During this process, numerous protein-protein and protein-DNA interactions that were established during initiation must be disrupted while the melted region of the DNA begins to move away from the start site and transcripts are synthesized. In reactions lacking TFIIH, the transcription complex encounters a block to elongation proximal to the promoter (Kumar et al., 1998). TFIIH needs to be incorporated into the transcription complex prior to the formation of the promoter-proximal stalled complex, and pausing decreases in the presence of an activator and TFIIH. The accumulation of stalled complexes is reduced in the presence of TFIIH, suggesting that TFIIH is required for promoter clearance. With regard to Pol I transcription, our data also suggest a function of TFIIH in a step subsequent to transcription initiation. Using an abortive initiation assay, we uncovered that in TFIIH-deficient reactions the same amounts of abortive transcripts were generated as in TFIIH-containing, transcriptionally active reactions. This suggests that for Pol I transcription, TFIIH is not required for open complex formation and synthesis of the first phosphodiester bonds, but serves a role in a postinitiation step, presumably transcription elongation. Indeed, in TFIIH-deficient transcription systems, we frequently observe the synthesis of $\sim$70 nucleotides transcripts which seem to resemble Pol I molecules that have paused downstream of the transcription start site. The synthesis of such paused transcripts occurs also in the absence of TIF-IC, a basal Pol I-associated factor that is required both for initiation and elongation of Pol I transcription (Schnapp et al., 1994). Pulse-chase experiments revealed that TIF-IC converts stalled transcription complexes into elongation-competent complexes. In contrast, exogenous TFIIH fails to relieve this premature arrest, i.e., the paused complex could not be chased to full-length transcripts by the addition of TFIIH (data not shown). The results suggest that TFIIH needs to be recruited into the initiation complex at the rDNA promoter by association with a subfraction of Pol I and/or TIF-IB. Once a stable transcription elongation complex has been formed, TFIIH does not affect Pol I transcription. This observation is reminiscent of similar experiments with Pol II demonstrating that TFIIH was unable to re-

sume elongation of promoter-proximal stalled transcription complexes (Kumar et al., 1998).

One major difference of TFIIH function in Pol I and Pol II transcription is the requirement of ATP. Transcription by Pol II requires the hydrolysis of the β-γ bond of ATP both for the formation of a stable open complex and promoter clearance (Dvir et al., 1996: Kumar et al., 1998; Holstege et al., 1997). In contrast, neither bacterial RNA polymerase nor mammalian Pol I or III require ATP as an energy donor for transcription initiation (Bunick et al., 1982; Lofquist et al., 1993). This suggests significant differences between the mechanism of open complex formation and transcription initiation by different RNA polymerases. Unregulated transcription initiation at many prokaryotic promoters requires an RNA polymerase holoenzyme, consisting of four core subunits, $\alpha_2\beta\beta'$, and a dissociable factor, σ^{70}. Structural information on the σ^{70} subunit supports the hypothesis that binding of the bacterial holoenzyme to the nontemplate strand of the −10 consensus element results in stabilization of the single-stranded conformation of the promoter. This nonenzymatic mode of open complex formation explains the strong temperature dependence of promoter opening in the bacterial system (von Hippel et al., 1984). In contrast, chemical footprinting and topological analyses revealed that eukaryotic Pol I does not catalyze strand separation of the template until transcription initiation has taken place (Lofquist et al., 1993). The available experimental evidence suggests that transcription initiation is accompanied by conformational changes in the RNA polymerase-DNA complex that lead to the conversion of a closed to an open complex. The finding that TFIIH activates Pol I transcription in the absence of hydrolyzable ATP is consistent with this interpretation and indicates that neither the helicase nor the protein kinase activity of TFIIH is required for rDNA transcription.

Admittedly, we are still ignorant about the mechanism underlying activation of Pol I transcription by TFIIH. Nevertheless our findings have strong implications not only with regard to understanding the role of TFIIH in transcription of class I and II genes but also in transcription-coupled DNA repair (TCR). Until recently, it was anticipated that only genes transcribed by Pol II are subject to transcription-coupled repair (Balajee et al., 1999). However, in a recent study, Conconi et al. (2002) have separated active from inactive yeast ribosomal genes by restriction enzyme digestion and differential psoralen crosslinking, and theyexamined whether transcribed genes are more efficiently repaired than inactive ones. They demonstrate that strand-specific DNA repair occurs in transcriptionally active rDNA while being absent in the inactive rDNA fraction. Thus, proteins involved in nucleotide excision repair have preferred access to the active, nonnucleosomal rDNA chromatin. The fact that transcription-coupled repair is not restricted to Pol II but also occurs in genes transcribed by Pol I lends further support to the biological significance of TFIIH in Pol I transcription. The localization of TFIIH within the nucleolus and its association with the transcriptionally active fraction of mammalian Pol I suggests that transcription-coupled repair of ribosomal genes is not restricted to yeast but also plays a role in mammalian cells.

Experimental Procedures

Purification of Transcription Factors

The purification of Pol I, TIF-IA, TIF-IB, and TIF-IC from nuclear extracts of Ehrlich ascites cells has been described (Schnapp and Grummt, 1996). To purify Pol I^{IIH} and Pol I, a MonoS fraction containing Pol I and TFIIH was fractionated on a MonoQ HR 10/10 column (Pharmacia) using a linear salt gradient (150–600 mM KCl). Fractions eluting with 280–400 mM KCl were pooled, dialyzed, and rechromatographed on MonoQ HR 5/5. Residual TFIIH was removed by immunodepletion with α-p62 monoclonal antibodies. TIF-IB/TIF-IB^{IIH} was purified either from CM-400 or S-700 fractions by glycerol-gradient centrifugation, chromatography on Q-Sepharose, and concentration on SP-Sepharose. To immunopurify TFIIH-deficient and TFIIH-containing TIF-IB, 7 μl of partially purified, concentrated TIF-IB fractions (MonoS-700) were incubated for 3 hr with 4 μg of α-TBP (3G3) or α-p62 (Santa Cruz) antibodies coupled to 40 μl Dynabeads. Immunoprecipitates were washed successively with buffer AM (20 mM Tris-HCl [pH 7.9], 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, and 0.5 mM DTT) containing 1000, 700, 500, and 100 mM KCl, respectively, plus 0.1% NP-40. TIF-IB^{IIH} was precipitated with anti-p62 antibodies and washed with buffer AM containing 400, 150, and 100 mM KCl plus 0.1% NP-40. TFIIH was purified according to the scheme depicted in Figure 4A. TFIIH eluting at 240 mM from the MonoQ resin was concentrated by precipitation with ammonium sulfate and immunopurified with anti-p44 antibodies (1H5) as described (Coin et al., 1999).

Localization of XPB-eGFP in Living Cells

The cDNA encoding GFP (eGFP, CLONTECH) was cloned in frame to the 3′ end of the XPB gene. The fusion construct was transfected into XPCS2BA-SV fibroblasts and subsequently selected for G418- and UV-resistance. Cells expressing the fusion protein at physiological levels were isolated by FACS.

In Vitro Transcription Assays

Templates used were pMrWT containing mouse rDNA sequences from −170 to +155, or pMr170-BH, a minigene representing a fusion between a murine 5′-proximal rDNA fragment (−170/+292) and a 3′-terminal rDNA fragment containing two transcription terminator elements (Budde and Grummt, 1998). Transcription reactions (25 μl) contained 10–40 ng of template DNA, 2.5 μl of TIF-IA/TIF-IC (MonoQ-fraction), 10 ng of FLAG-UBF, 1–3 μl of TIF-IB, and 3–5 μl Pol I in 12 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.12 mM EDTA, 85 mM KCl, 12% (v/v) glycerol, 0.66 mM each ATP, CTP, and GTP, 12.5 μM UTP, and 1 μCi of [α-^{32}P]UTP (800 Ci/mmol). If pMr170-BH was used as template, the assays were supplemented with 30 ng recombinant TTFΔN184. After incubation for 60 min at 30°C, RNA was isolated and analyzed on 4.5% polyacrylamide gels.

Abortive Initiation Assay

TFIIH was preincubated for 20 min at 30°C with Pol I and transcription factors before the template and ATP and CTP were added. After 20 min, 1 μCi [α-^{32}P]UTP was added and reactions were incubated for 45 min. After treatment with 1.5 units of alkaline phosphatase for 15 min, the reactions were stopped by adding 0.4% SDS. The ACU trimers were extracted with phenol/chloroform, lyophilized, and analyzed on a 25% acrylamide sequencing gel.

Coimmunoprecipitation Experiments

RNA polymerase I holoenzyme was isolated by gel filtration of a DEAE-280 fraction in buffer AM-100 on Sepharose CL2B. A volume of 250–300 μl of the fractions containing >2 MDa protein complexes were incubated with 5 μg of specific antibodies or preimmune IgGs that were bound to 50 μl sheep-anti-rabbit or sheep-anti-mouse Dynabeads, blocked for 30 min with BSA, insulin, and phosphatidylcholin (2 mg/ml) in the presence of insulin (1 mg/ml) and protease inhibitors. After rotation for 3 hr, the beads were collected by magnetic attraction and washed five times with buffers AM-100/0.4%NP-40, AM-150/0.1%NP40, and AM-100. Proteins were separated by 9% SDS-PAGE and analyzed on Western blots.

Immunoelectron Microscopy

HeLa cells were fixed for 40 min on ice in SB-buffer (0.1 M Na/K-phosphate [pH 7.3]) containing 3% paraformaldehyde and 0.1% glutaraldehyde. After washing in SB buffer, the cells were centrifuged into 1% agarose, incubated for 20 min in 20 mM glycine in SB, and washed and dehydrated by ethanol washes (Hozák et al., 1994). Ethanol was replaced by LR White resin (Polysciences), and the blocks were polymerized by exposure to UV light for 24 hr at 4°C. Sections (80 nm) were blocked by 10% goat serum in PBS supplemented with 0.1% Tween 20 and 1% BSA and then incubated for 45 min with anti-XPD antibodies (5 μg/ml). After three washes with PBS supplemented with 0.005% Tween 20, the sections were incubated for 30 min with 10 nm gold conjugated with goat anti-rabbit IgG antibodies (1:50; British BioCell International, Ltd.). Sections were contrasted with uranyl acetate and visualized in a Philips CM 100 electron microscope. Control samples were incubated as above except that the primary antibody was omitted. For statistical analysis, the number of gold particles in the nucleoplasm, fibrillar centers, dense fibrillar component, and granular component was measured in digital images of 20 cells using image-processing software (Laboratory Imaging Ltd., Prague).

Yeast rRNA Analysis

The following yeast strains were used. YSB207(WT): *MAT a, his3Δ200, leu2-3,112, ura3-52, tfb1Δ::LEU2, (pRS316-TFB1)*;YSB260 *(tfb1-ts): Mat a, his3Δ200, leu2-3,112, ura3-52, tfb1Δ:LEU2, (pRS313-tfb1−Δ101)* (Matsui et al., 1995); GPY11-6*(rpa43-ts)*: *Mat a, ade2-101, ura3-52, lys2-801, trp1-Δ63, his3-Δ200, leu2-Δ1, Δrpa43:LEU2 (pGP5-6)* (Peyroche et al., 2000); kin28-ts16 (*kin28-ts*) (Cismovski et al., 1995), *rpb1-1(rpb1-ts):Mat α;ura3-52, rpb1-1* (Nonet et al., 1987). Exponentially growing cells were shifted to 37°C and 75 ml aliquots were used for RNA preparation. Pelleted cells were suspended in 0.4 ml of a buffer containing 50 mM sodium acetate (pH 5.3), 10 mM EDTA, and 1% SDS, and incubated for 4 min at 65°C in the presence of 50% phenol. Isolated RNA (6 μg) of were analyzed by primer extension (Keener et al., 1998) using 0.2 pmoles of a primer that hybridizes 130 nt downstream of the Pol I start site (5′-ACACGC TGTATAGAGACTAGGC-3′) and 0.02 pmoles of a primer that hybridizes 80 nt downstream of the 5′-end of 25S rRNA (5′-ACTAAGGCAA TCCCGGTTGGTTTC-3′). Radiolabeled cDNA was analyzed on 8% SDS-polyacrylamide gels.

Antibodies

α-RPA116, α-RPA53, α-TAF$_I$95 (Seither et al., 1998), α-XPB(1B3), α-XPD(2F6), α-p62(3C9), α-p44(1H5), and α-cdk7(2F8) (Coin et al., 1999) have been described. α-XPB, α-p62, α-cdk7, α-cyclin H, α-Ku70, and α-Rb were obtained from Santa Cruz Biotechnology, Inc.

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