A novel TBP-associated factor of SL1 functions in RNA polymerase I transcription

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In mammalian RNA polymerase I transcription, SL1, an assembly of TBP and associated factors (TAFs), is essential for preinitiation complex formation at ribosomal RNA gene promoters *in vitro*. We provide evidence for a novel component of SL1, TAF_I41 (*MGC5306*), which functions in Pol I transcription. TAF_I41 resides at the rDNA promoter in the nucleolus and co-purifies and co-immunoprecipitates with SL1. TAF_I41 immunodepletion from nuclear extracts dramatically reduces Pol I transcription; addition of SL1 restores the ability of these extracts to support Pol I transcription. In cells, siRNA-mediated decreased expression of TAF_I41 leads to loss of SL1 from the rDNA promoter *in vivo*, with concomitant loss of Pol I from the rDNA and reduced synthesis of the pre-rRNA. Extracts from these cells support reduced levels of Pol I transcription; addition of SL1 to the extracts raises the level of Pol I transcription. These data suggest that TAF_I41 is integral to transcriptionally active SL1 and imply a role for SL1, including the TAF_I41 subunit, in Pol I recruitment and, therefore, preinitiation complex formation *in vivo*.

Introduction

Accurate initiation of transcription by the three eukaryotic RNA polymerases, with few exceptions, requires a complex of TBP (TATA-box-binding protein) and TBP-associated factors (TAFs), which perform distinct roles in mediating a specific interaction between the polymerases and their target promoters (Hernandez, 1993; Goodrich and Tjian, 1994; Hahn, 1998). The complement of TAFs in each complex is distinct to each class (Pol I, II or III) of genes and their cognate promoters.

There are substantial similarities in Pol I transcription of rDNA genes between organisms as diverse as yeast and mammals (reviewed in: Hannan *et al*, 1998; Grummt, 1999; Reeder, 1999; Nomura, 2001; Moss and Stefanovsky, 2002; Grummt, 2003; Moss, 2004; Russell and Zomerdijk, 2005). In mammalian Pol I transcription *in vitro*, TBP–TAF complex SL1 (selectivity factor 1), known as TIF-IB in mouse, is essential (Comai *et al*, 1992, 1994; Zomerdijk *et al*, 1994; Heix *et al*, 1997; Friedrich *et al*, 2005) and upstream binding factor UBF functions as an activator of transcription (Bell *et al*, 1988; Jantzen *et al*, 1990; Hisatake *et al*, 1991; O'Mahony and Rothblum, 1991; Jantzen *et al*, 1992; Kuhn and Grummt, 1992). UBF can influence rRNA gene expression at multiple levels, functioning as an activator at promoter escape by Pol I *in vitro* (Panov *et al*, 2006), as a regulator (repressor) of elongation (Stefanovsky *et al*, 2006) and as an antirepressor (Kuhn and Grummt, 1992; Brou *et al*, 1993; Pelletier *et al*, 2000) in the context of chromatin, and has been implicated in large-scale chromatin decondensation *in vivo* (Chen *et al*, 2004; Mais *et al*, 2005).

The human SL1 complex of ~ 300 kDa includes TBP and the three TAFs TAF_I48, TAF_I63 and TAF_I110 (Comai *et al*, 1992, 1994; Zomerdijk *et al*, 1994). SL1 from human and rodents can bind the promoter independently and direct specific initiation of transcription in the absence of UBF (Smith *et al*, 1990, 1993; Schnapp and Grummt, 1991; Friedrich *et al*, 2005). Core promoter binding is mediated by the TAFs in SL1 (Rudloff *et al*, 1994; Beckmann *et al*, 1995). Another important role for the TAFs in SL1 inferred from *in vitro* analyses is in the recruitment of a functional Pol I complex to the start site via its interaction with human RRN3 (murine TIF-IA), a component of initiation-competent Pol Iβ (Miller *et al*, 2001; Yuan *et al*, 2002; Friedrich *et al*, 2005). Furthermore, at least two subunits of SL1 interact with UBF (Kwon and Green, 1994; Beckmann *et al*, 1995), and these interactions prevent the otherwise rapid dissociation of UBF from the rDNA promoter (Friedrich *et al*, 2005). Taken together, the *in vitro* data imply that SL1 drives or nucleates preinitiation complex (PIC) formation at the rDNA promoter, leading to productive initiation of transcription by Pol I. Reconstitution of transcription *in vitro* using recombinant SL1 comprised of the four previously characterized components has been difficult and inefficient, suggesting that additional components and/or specific modifications might be required to assemble a fully active SL1 (Heix *et al*, 1997). Here, we describe our identification of a novel TAF component of SL1 (TAF_I41) and present evidence that TAF_I41 plays an important role in Pol I transcription. In the course of these studies, we also uncovered evidence to suggest that SL1, including the TAF_I41 subunit, is required for Pol I recruitment and, thus, PIC formation in Pol I transcription *in vivo*.

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Results and discussion

Human nucleolar protein TAF$_I$41 co-purifies with the SL1 protein complex and activity

Silver staining of TBP-antibody affinity purified SL1 revealed only four major protein bands corresponding to TBP, and TAF$_I$s 48, 63 and 110 (Comai *et al*, 1992). However, amino-acid sequence analyses of tryptic peptides derived from the TBP and TAF$_I$48 region of the gel (Comai *et al*, 1994) revealed a peptide (KQMNVGEDLENEDFD) with no homology to any of the previously identified TAFs or TBP, matching amino-acid sequences within several human ESTs and encoded by a gene (*JOSD3* (Josephine domain containing protein 3) or *MGC5306*; accession number NM_024116) located on human chromosome 11q21. Other peptide sequences with homology to the protein encoded by this gene (KAIFERFK and KLAGDSFIVSSEFPVRLSVY) were derived from the same region of the gel, mixed with peptide sequences from TAF$_I$48 (KTSANISALIK and KGPVTDDEEV, respectively), confirming the presence of this protein in TBP-antibody affinity purified SL1. The encoded protein of 278 amino acids (Supplementary Figure S1) has a calculated molecular weight of 32 kDa, but an apparent molecular weight of ∼41 kDa. (Intriguingly, a faint band of ∼41 kDa is sometimes visible on protein gels following silver staining of TBP-antibody affinity purified SL1 (Supplementary Figure S2A).) This protein has been reported to interact in a yeast two-hybrid assay with DNA polymerase β and a possible function in base excision repair was therefore suggested (Wang *et al*, 2004). We provide evidence that this protein is also an SL1-specific TBP-associated factor (TAF$_I$) involved in Pol I transcription and refer to it hereafter as TAF$_I$41.

TAF$_I$41 contains a basic stretch of amino acids including a putative NLS (83–108), a serine-rich region in the N-terminal quarter of TAF$_I$41 and two acidic regions (184–192 and 200–216),

and these features are conserved in a putative mouse homologue, mTAF$_I$41 (accession number: NM_029248), of 322 amino acids and a calculated molecular weight of 37 kDa (Supplementary Figure S1) (variants NM_026541 and NM_027261 of 288 and 290 amino acids, respectively, and calculated molecular weight 33 kDa, are truncated in the C-terminus). mTAF$_I$41 shows 56% identity and 63% similarity to human TAF$_I$41 in the first 278 amino acids (Supplementary Figure S1).

To explore the association between TAF$_I$41 and human SL1 from cells, SL1 was extensively purified from HeLa nuclear extracts as described in Figure 1A and B. Fractions from salt-gradient elution of the POROS Heparin column were analysed for the presence of TAF$_I$41 by immunoblotting, using an antibody generated against three TAF$_I$41 peptides (TAF$_I$41-peptides antibody), and for the ability to support SL1-dependent rDNA promoter-specific transcription. TAF$_I$41 co-purified with TAF$_I$110 and TAF$_I$63 subunits of SL1 and with SL1 activity (Figure 1B; POROS Heparin). The desalted 0.7 M POROS Heparin fraction of SL1 was applied to an SP-Sepharose column, step-eluted and applied to a TBP-antibody affinity column, yielding extensively purified SL1 (Supplementary Figure S2A, shows a silver-stain gel of SL1 similarly purified by Heparin Agarose, SP-Sepharose and TBP-antibody affinity chromatography). TAF$_I$41 co-purified with SL1 eluted from the TBP antibody column (Figure 1B, TBP-antibody affinity column, lane 5). The peptide-eluted SL1 was passed over a Mono S column. Significantly, this extensively purified SL1 preparation demonstrated TAF$_I$41 in the fractions that contained SL1 activity (Figure 1B; Mono S).

Immunoblotting with TAF$_I$41-peptides antibody demonstrated that TAF$_I$41 co-migrates with TBP at ∼40 kDa (Figure 1C, compare lanes 4 and 5); separation was achieved in 2D gels owing to the difference in the pIs of these proteins (Figure 1D). The electrophoretic mobility of TAF$_I$41 shifted upon dephosphorylation (data not shown), indicating that

Figure 1 Human nucleolar protein TAF$_I$41 co-purifies with TBP-antibody affinity-purified SL1 and is found at the rDNA promoter in human cells. (**A**) Human SL1 purification scheme. For analytical purification of SL1, HeLa cell nuclear extract was applied to POROS Heparin Agarose and SP-Sepharose columns, as previously described (Comai *et al*, 1992; Friedrich *et al*, 2005), then salt gradient eluted from a POROS Heparin column. The SL1 fractions were pooled, desalted on G25 Sepharose, step-eluted from SP-Sepharose and applied to a TBP-antibody affinity column. SL1 complexes were eluted with an excess of TBP (3G3) peptide and this eluate was applied to a Mono S column, which was developed by a linear salt gradient (KCl at concentrations (M) as indicated). (**B**) TAF$_I$41 co-purifies with SL1 protein and activity from HeLa cell nuclear extracts. Fractions obtained by salt gradient elution from the POROS Heparin column (B) were analysed for SL1 protein by immunoblotting, with antibodies specific for SL1 subunits TAF$_I$110 and TAF$_I$63, and an antibody generated against three peptides of TAF$_I$41 (see Materials and methods). The bulk of TAF$_I$41 eluted with the other SL1 subunits at 0.7 M KCl (fractions 21 and 22). Fraction 22 supported specific SL1-dependent reconstituted Pol I transcription. The 0.7 M KCl fraction was desalted to 0.2 M KCl, passed over SP-Sepharose and a salt-step elution was performed. The bulk of SL1 including TAF$_I$41 eluted at 0.65 M KCl and supported specific SL1-dependent transcription. This SL1 fraction was loaded onto a TBP-antibody affinity column (input, lane 1), some of which flowed through (FT, lane 4). The column was washed at 0.5 and 1.0 M KCl (lanes 2 and 3). Bound proteins from the washed column were eluted with excess TBP-epitope peptide 3G3 (1 mg/ml) (lane 5) and fractions were immunoblotted for SL1 with antibodies specific for TAF$_I$110 and TAF$_I$63, and the TAF$_I$41-peptides antibody. SL1 was concentrated and purified away from the 3G3-peptide on a Mono S column, developed with a linear salt gradient. The Mono-S purified SL1 fractions 19–25 (fractions 19, 21, 23 and 25 are shown in lanes 7–10) were pooled and tested for the ability to support SL1-dependent transcription with UBF and Pol I (lanes 1–3, arrowhead). (**C**) TBP and TAF$_I$41 display similar apparent molecular weights. SL1 (the 0.7 M fraction from the POROS-Heparin column, see A, B) was immunoblotted with antibodies specific for TAF$_I$110, TAF$_I$63, TAF$_I$48 and TBP (top panel, lanes 1, 2, 3 and 5, respectively) and the TAF$_I$41-peptides antibody (lane 4). A band of similar mobility to those of TBP and TAF$_I$41, detected by TAF$_I$48 antibodies, is either a degradation product of TAF$_I$48 or could represent a protein encoded by a splice variant 2 of TAF$_I$48 (top panel, lane 3). (**D**) TBP and TAF$_I$41 are separable by two-dimensional gel electrophoresis. SL1 (the 0.7 M fraction from the POROS-Heparin column, see A and B) was subject to first dimension isoelectric focusing (pH 3–10), then to second dimension separation (4–12% Bis–Tris gradient gel, Invitrogen). SL1 proteins were immunoblotted, probed first with TAF$_I$41-peptides antibody (upper panel) and then with antibodies specific for TBP (middle panel). The lower panel shows an overlay of the signals from these immunoblots. (**E**) Overexpressed TAF$_I$41 accumulates in nucleoli. HeLa cells were transfected with expression vectors pEGFP-TAF$_I$41. After 24 h, the cells were fixed and viewed by confocal microscopy. DNA was stained with DAPI. Overlay is a composite of the DAPI (blue) and EGFP-TAF$_I$41 (green) signals. Scale bar, 10 nm. (**F**) ChIP analysis indicates that TAF$_I$41 is present at the promoter of the rRNA genes. ChIP analysis (from HeLa cells) used TAF$_I$41-peptides antibody (and as a control rabbit preimmune serum) or a TBP mouse monoclonal antibody (and as a control mouse IgG), followed by quantitative real-time PCR with primers specific for the promoter region and the internal transcribed spacer (ITS-2) of the human rDNA (see Materials and methods). The relative levels of rDNA associated with TAF$_I$41 and TBP at these sites are expressed as percentage of input chromatin and are from two independent experiments.

TAF$_I$41 is a phosphoprotein. Collectively, these data provide evidence to suggest that TAF$_I$41 is a previously overlooked subunit of human SL1. The comigration of TAF$_I$41 with TBP may explain why it had remained elusive for more than 10 years following the initial identification of SL1 subunits (Comai *et al*, 1994; Zomerdijk *et al*, 1994). Consistent with a role for TAF$_I$41 as a component of SL1 in Pol I transcription, overexpressed EGFP-TAF$_I$41 accumulated in the nucleoli, the site of Pol I transcription, (Figure 1E) and chromatin immunoprecipitation (ChIP) assays detected TAF$_I$41 and TBP at the rDNA promoter in cells (Figure 1F).

TAF$_I$41 co-immunoprecipitates with SL1 from human cells

Co-precipitation of EGFP-TAF$_I$41 with the endogenous SL1 subunits TAF$_I$110, TAF$_I$63 and TBP (Figure 2A) was detected following immunoprecipitation, using GFP-specific antibodies, from human cells expressing EGFP-TAF$_I$41. In a similar experiment with Flag-tagged TAF$_I$41, we also demonstrated co-precipitation of TAF$_I$41 with the SL1-subunits (Figure 2B, lane 7). Conversely, endogenous TAF$_I$41 co-precipitated with Flag-TAF$_I$63 following immunoprecipitation, using Flag-specific antibodies, from human cells expressing Flag-tagged TAF$_I$63 (Figure 2B, lane 5).

Furthermore, intact and functional SL1 was immunoprecipitated, with Flag-specific antibodies, from human cells expressing Flag-TAF$_I$41; the (Flag-peptide-eluted) immunocomplexes were capable of directing SL1-dependent accurate transcription initiation in reactions reconstituted with Pol Iβ and the rDNA promoter (Figure 2C, lanes 6, 7 and 8). SL1-dependent transcription was also observed with
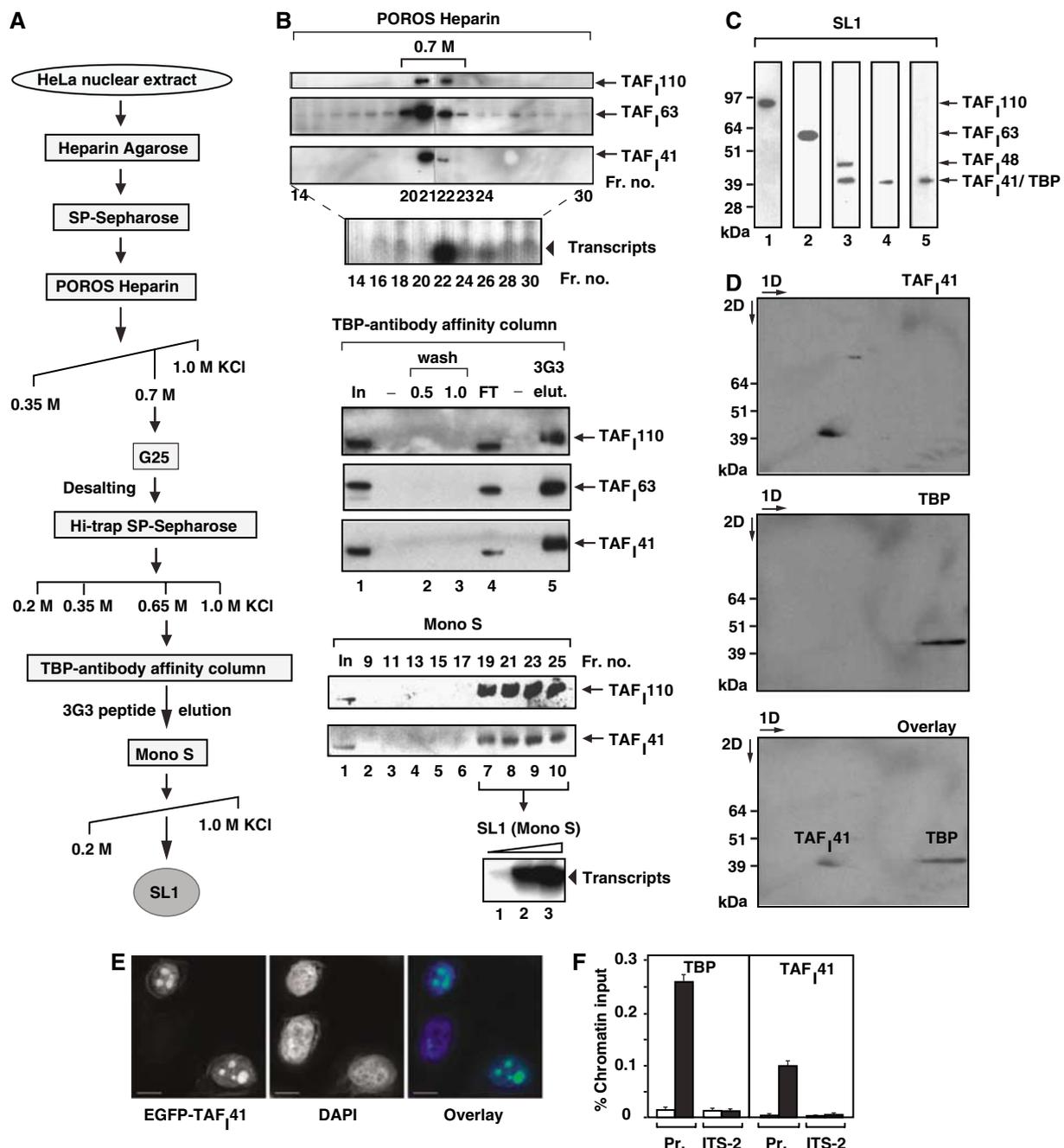
Figure 1 For caption see page 1561.
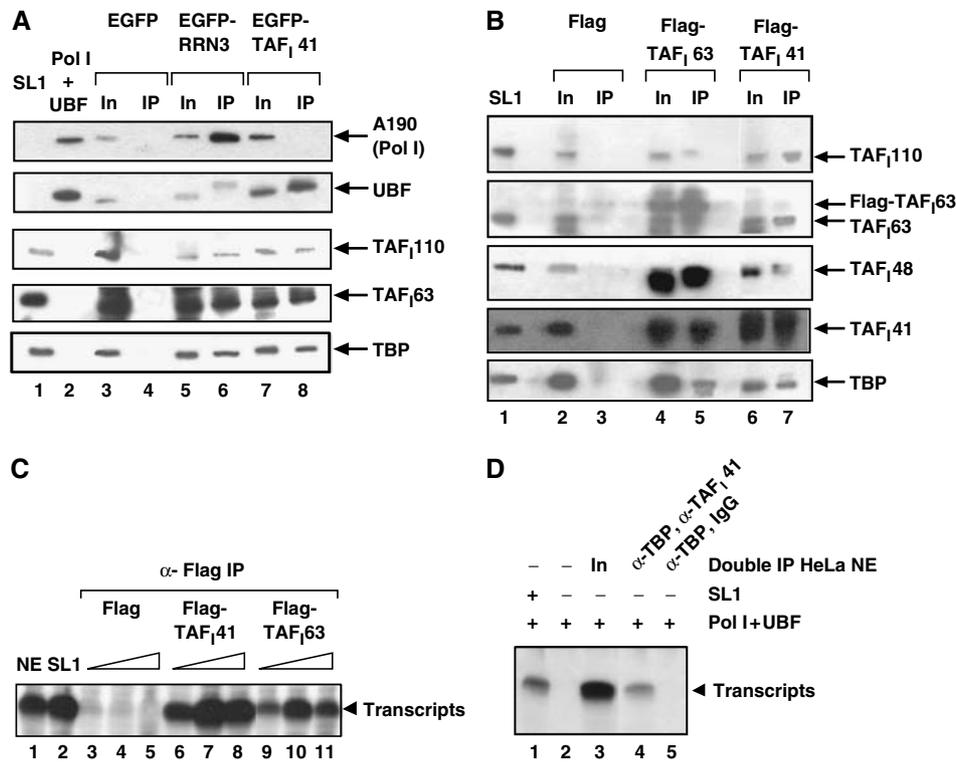
Figure 2 TAF$_I$41 is a component of transcriptionally active SL1. (**A**) EGFP-TAF$_I$41 co-immunoprecipitates with SL1 and UBF from human cells. Immunoprecipitation (IP) with GFP-specific antibodies was from HEK293 whole-cell extracts 48 h post-transfection of the cells with pEGFP-TAF$_I$41 (lane 8), empty expression vector (lane 4) or pEGFP-RRN3 (lane 6). Immunoblotting used antibodies specific for the Pol I largest subunit A190, UBF and SL1 subunits TAF$_I$110, TAF$_I$63 and TBP. Input (In), 10% of extract used for IP (lanes 3, 5 and 7). SL1 (lane 1), and Pol I and UBF (lane 2) were loaded as markers. (**B**) Endogenous TAF$_I$41 and Flag-TAF$_I$41 co-immunoprecipitate with SL1 from human cells. IP with Flag-specific antibodies was from HEK293 whole-cell extracts 48 h post-transfection of the cells with pcDNA-Flag-TAF$_I$41 (lane 7), empty expression vector (lane 3) or pcDNA-Flag-TAF$_I$63 (lane 5). Immunoblotting used antibodies specific for SL1 subunits TAF$_I$110, TAF$_I$63, TAF$_I$48, TBP and TAF$_I$41-peptides antibody. Input (In), 80% of extract used for IP (lanes 2, 4 and 6). Purified SL1 (lane 1) was loaded as a marker. (**C**) TAF$_I$41-associated proteins from cells support SL1-dependent Pol I transcription. Immunoprecipitated complexes of 40 (lanes 3, 6 and 9), 80 (lanes 4, 7 and 10) and 160 ng (lanes 5, 8 and 11) prepared in B, from HEK293 cells transfected with pcDNA-Flag-TAF$_I$41 (lanes 6–8), empty vector (lanes 3–5) or pcDNA-Flag-TAF$_I$63 (lanes 9–11), were tested for SL1-dependent activity in a specific transcription assay with Pol I, UBF and the rDNA promoter. Controls are transcription in a HeLa nuclear extract (lane 1) and from a partially purified SL1 fraction (the 0.7 M fraction from the POROS-Heparin column, see A, B; lane 2) added to the reconstituted transcription assay. (**D**) Endogenous TAF$_I$41 co-immunoprecipitates with TBP-containing complexes from human cells and the TAF$_I$41-TBP-containing complexes support rDNA promoter-specific Pol I transcription. TBP-containing complexes immunoprecipitated from HeLa cell nuclear extracts using TBP-specific monoclonal antibody (3G3) beads were eluted from the beads using an excess of epitope peptide, then incubated with TAF$_I$41-peptides antibody in a second IP. The SL1-dependent transcription activity of the TBP-TAF$_I$41-containing complexes was determined in an in vitro transcription assay with Pol I and UBF (lane 4). In the control sample, IgG was used in the second immunoprecipitation reaction in place of the TAF$_I$41-peptides antibody (lane 5). The SL1 activity of the input Hela nuclear extracts is shown in lane 3. Lanes 1 and 2 contain control reconstituted transcription reactions with Pol I and UBF, with and without SL1, respectively.

Flag immunocomplexes from extracts of cells expressing Flag-TAF$_I$63, a previously established subunit of SL1 (the difference in transcription levels perhaps reflecting differences in the specific activities and/or yields of functional SL1 complexes incorporating Flag-TAF$_I$41 or Flag-TAF$_I$63). Moreover, SL1 activity was detectable in immunocomplexes precipitated with TBP-specific antibodies, eluted with TBP-epitope peptide, and then re-precipitated with TAF$_I$41-specific antibody but not with IgG antibodies (Figure 2D, lanes 4 and 5, respectively). Therefore, TAF$_I$41 appears to be an integral component of at least some portion of transcriptionally active SL1 complexes.

SL1 components TAF$_I$48, TAF$_I$63 and TAF$_I$110 have previously been shown to interact with each other and with TBP in GST pull-down experiments and in binding assays with in vitro translated or baculovirus-Sf9 cell-expressed proteins (Comai et al, 1994). TAF$_I$41 interacts detectably with the other TAFs of SL1 and, weakly, with TBP and RRN3 in such

assays and with TAF$_I$110 in Far Western analyses (see Supplementary Figure S3). Although it is not known whether such interactions occur in the context of the SL1 complex or indeed the PIC, such interactions would be consistent with TAF$_I$41 being a subunit of the stable multiprotein complex SL1, with a potential role in the assembly of productive Pol I PICs.

The majority of the transcriptionally active SL1 complexes contain TAF$_I$41

We next asked whether immunodepletion of TAF$_I$41 from nuclear extracts would deplete a significant amount of SL1 activity. A TAF$_I$41 antibody generated against amino acids 98–204 of TAF$_I$41 (antibody D) selectively immunoprecipitated SL1 subunits TAF$_I$110, TBP and TAF$_I$41 from HeLa nuclear extract (Figure 3A, lane 2 compared to lane 4) and, therefore, partially immunodepleted these SL1 subunits from the extract (the partial immunodepletion of TAF$_I$41 and TBP is detectable

A α-TAF$_I$41D IgG

B

α-TAF$_I$41D IgG

C

IgG α-TAF$_I$41D

D Mouse rDNA promoter

α-
IgG TAF$_I$41D
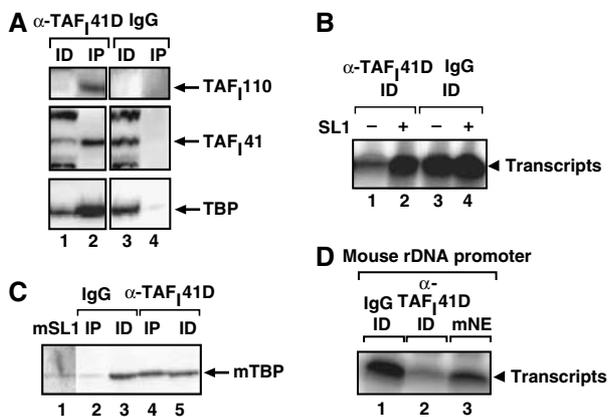
Figure 3 Human TAF$_I$41 antibodies deplete SL1 activity from human and mouse nuclear extracts. (**A**) Human TAF$_I$41 antibodies deplete SL1 subunits from HeLa cell nuclear extract. HeLa nuclear extracts were incubated with hTAF$_I$41 antibody D (raised against a recombinant human TAF$_I$41 deletion mutant, see Materials and methods; lanes 1 and 2) or control (IgG; lanes 3 and 4) antibodies overnight at 4°C. Immunoprecipitates (IP, lanes 2 and 4) and 10% of the immunodepleted extracts (ID, lanes 1 and 3) were immuno-blotted with antibodies specific for TAF$_I$110, TBP and TAF$_I$41-peptides antibody. The signal in the ID for TAF$_I$110 (lanes 1 and 3) was too high and this resulted in a 'white-out' following chemiluminescence. (**B**) Human TAF$_I$41 antibodies deplete SL1 transcriptional activity from HeLa cell nuclear extract. Extracts from (A), immunodepleted (ID) using hTAF$_I$41 antibody D (lanes 1 and 2) or control (IgG, lanes 3 and 4), were analysed in a specific transcription assay with the human rDNA promoter, in the absence (lanes 1 and 3) or presence of added purified SL1 (Superose 6 fraction; 1 μl, lanes 2 and 4). Transcripts were analysed by S1 nuclease protection. (**C**) Human TAF$_I$41 antibodies immunoprecipitate mouse TBP from mouse 3T3 nuclear extract. Mouse 3T3 nuclear extract (25 μl) was incubated with hTAF$_I$41 antibody D (lanes 4 and 5) or control (IgG, lanes 2 and 3) overnight at 4°C. Immunoprecipitated complex (IP) and 10% of the immunodepleted extract (ID) were immunoblotted with antibodies specific for TBP. Partially purified mouse SL1 (TIF-IB) (lane 1) was loaded as a marker. (**D**) Human TAF$_I$41 antibodies partially deplete Pol I transcription activity from mouse cell nuclear extract. In all, 15% of the extracts immunodepleted by hTAF$_I$41 antibody D (lane 2) or control (lane 1) antibodies were analysed in a specific transcription assay using the mouse rDNA promoter. Transcripts were analysed by S1 nuclease protection with a 5′-end labelled oligonucleotide complementary to the first 40 nt of the mouse pre-rRNA. Lane 3 contains the control transcription reaction with mouse nuclear extract (mNE).

in Figure 3A, lane 1 compared to lane 3). The TAF$_I$41 antibody D-depleted extract supported a reduced level of Pol I transcription (Figure 3B, lane 1 compared to lane 3). Addition of SL1 to the TAF$_I$41 antibody D-depleted extract restored the ability of the extract to support Pol I transcription (Figure 3B, lane 2). These results suggest that most transcriptionally active SL1 complexes contain TAF$_I$41.

A mouse TAF$_I$41 orthologue in mSL1 (TIF-IB)

The discovery of a potential mouse orthologue in the sequence database prompted us to try and determine whether a functional TAF$_I$41 exists in mouse cells. There are no bands detectable at or around the calculated molecular weight of 37 kDa for the mouse homologue in TBP-affinity purified mouse SL1 (TIF-IB) (Rudloff *et al*, 1994), although reduced silver staining and/or co-migration with, for example, mTAF$_I$48 could not be excluded. The human TAF$_I$41-peptides antibody did not detect a potential mouse homologue follow

ing immunoblotting of mouse nuclear extracts (data not shown). Nonetheless, human TAF$_I$41 antibody D immunoprecipitated TBP from mouse nuclear extracts (Figure 3C, lane 4 compared to 2) and reduced Pol I transcription in these extracts (Figure 3D, lane 2 compared to 1), suggesting that mouse TAF$_I$41 might be a component of transcriptionally active mSL1 (TIF-IB) complexes. SL1 is a major determinant in the species specificity of Pol I transcription (Heix and Grummt, 1995). Overexpression of human TAF$_I$41 in mouse cells did not reprogramme these cells to transcribe a human rDNA promoter (data not shown).

siRNA-mediated downregulation of TAF$_I$41 expression in cells decreases Pol I transcription

A positive role for TAF$_I$41 in Pol I transcription is suggested by the findings that TAF$_I$41 is a component of active SL1 complexes. To determine whether or not TAF$_I$41 is required for Pol I transcription in cells, we downregulated its expression using siRNAs. Human embryonic kidney 293 (HEK293) cells transfected with TAF$_I$41-siRNA showed reduced levels of TAF$_I$41 mRNA (data not shown) and protein (to less than 25%; Figure 4A, lanes 1–3 compared to lanes 4–6). Concomitant with downregulated TAF$_I$41 expression, a decreased level of 47S pre-rRNA was detected in these cells (Figure 4B, lanes 1–3 compared to lanes 4–6, and bar graph). TAF$_I$41-siRNA also mediated downregulation of TAF$_I$41 expression (Figure 4C) and decreased pre-rRNA levels (Figure 4D, lane 1) in SJSA cells, in contrast to the control siRNA (Figure 4C and D, lane 4). siRNA-mediated downregulation of the expression of SL1 subunit TAF$_I$48 or essential Pol I-associated factor RRN3 (Figure 4C) similarly reduced Pol I transcription (Figure 4D, lanes 2 and 3, respectively). Consistent with these findings, nuclear extracts from TAF$_I$41-siRNA-treated cells supported a reduced level of Pol I transcription (Figure 4E, lane 1 compared to 4) and an increased level of transcription was detectable in these extracts following addition of SL1 (Figure 4E, lanes 2 and 3). Taken together, these results suggest that siRNA-mediated downregulation of TAF$_I$41 expression in cells specifically impairs the function of SL1, implying a role for TAF$_I$41 in SL1 function.

Downregulation of TAF$_I$41 expression decreases the rDNA promoter occupancy of SL1 and Pol I in cells

The observed reductions in Pol I transcription in cells transfected with siRNAs for the TAF$_I$48 and TAF$_I$41 subunits of SL1 (Figure 4) support an *in vivo* role for SL1 in Pol I transcription of the rRNA genes. To provide further evidence for this and examine the role of SL1 and TAF$_I$41 in Pol I transcription *in vivo*, we downregulated TAF$_I$41 expression in cells and analysed the consequences for SL1 and Pol I occupancy at the rDNA repeat by ChIP. siRNA-mediated downregulation of TAF$_I$41 expression not only impaired SL1 function (Figure 4) but also drastically decreased the rDNA promoter occupancy of SL1 subunit TAF$_I$110 and Pol I subunit A135 (Figure 5). SL1 can direct PIC formation *in vitro* (Smith *et al*, 1990, 1993; Schnapp and Grummt, 1991; Friedrich *et al*, 2005), but a role for SL1 in this process *in vivo* had not been demonstrated. Our finding that the presence of Pol I at the rDNA promoter is dependent on the presence of SL1 provides evidence to suggest that SL1, including TAF$_I$41, is required for Pol I recruitment and, hence, PIC formation
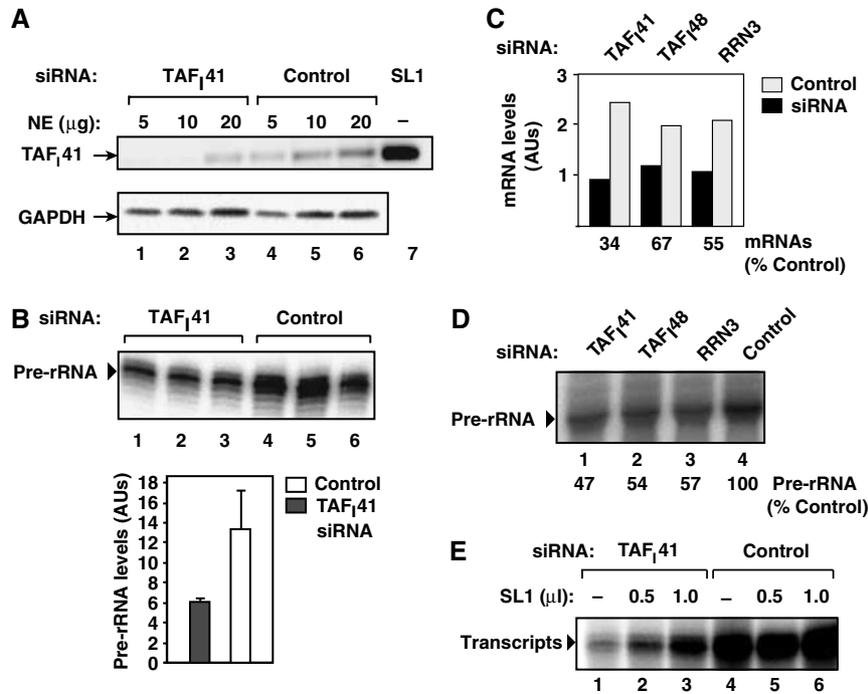
Figure 4 siRNA-mediated downregulation of TAF$_I$41 expression in cells leads to a decrease in Pol I transcription levels. (**A**) TAF$_I$41-specific siRNA reduces TAF$_I$41 protein levels in cells. Nuclear extracts (5, 10 and 20 μg, respectively) of HEK293 cells transfected with TAF$_I$41-specific siRNA (lanes 1–3) or scrambled siRNA (control; lanes 4–6) were immunoblotted using TAF$_I$41-peptides antibody and GAPDH-specific antibody. Lane 7 contains SL1 as a marker. (**B**) TAF$_I$41-specific siRNA reduces Pol I transcription levels in cells. Pre-rRNA levels in total RNA extracted from HEK293 cells transfected, in triplicate, with TAF$_I$41-specific siRNA (lanes 1–3) or scrambled siRNA (control; lanes 4–6) were analysed by S1 nuclease protection with an oligonucleotide complementary to the first 40 nt of the 47 S pre-rRNA. Combined data are represented in the graph. (**C**) siRNA-mediated reduction of hTAF$_I$41 TAF$_I$48 and RRN3 mRNA levels. Messenger RNA levels in total RNA from SJSA cells transfected with TAF$_I$41-, TAF$_I$48- and RRN3-specific or scramble (control) siRNAs were determined by RT–PCR and normalized to β-actin mRNA levels. (**D**) siRNA-mediated reduction of TAF$_I$41, TAF$_I$48 or RRN3 gene expression reduces pre-rRNA synthesis in cells. Pre-rRNA levels in total RNA extracted from the siRNA-treated SJSA cells (see C) were analysed by S1 nuclease protection as in (B). (**E**) Pol I transcription in extracts of TAF$_I$41-specific siRNA-treated cells can be partially restored by addition of SL1. Nuclear extracts (30 μg) of HEK293 cells transfected with siRNAs specific for TAF$_I$41 (lanes 1–3), or scrambled (control; lanes 4–6) were analysed for specific transcription activity in the absence (lanes 1 and 4) or presence of purified SL1 (Superose 6 fraction; 0.5 μl, lanes 2 and 5, or 1 μl, lanes 3 and 6).

in vivo. This is consistent with the observed dramatic decrease in Pol I A135 occupancy in transcribed regions of the rDNA repeat (Figure 5).

TAF$_I$41 interacts with UBF and might function as a coactivator in UBF-activated transcription

TAF$_I$41 overexpressed in cells co-immunoprecipitated with UBF from cell extracts (Figure 2A, lane 8). Interaction between TAF$_I$41 and UBF was observed in affinity chromatography with Ni-NTA-immobilized His-TAF$_I$41 (from recombinant baculovirus-infected insect cells) using *in vitro* translated UBF (Figure 6A, lane 2) and in GST-TAF$_I$41 pull downs of baculovirus-Sf9 cell-expressed recombinant UBF (Figure 6B, lane 2). Moreover, Far-western analysis also demonstrated that *in vitro* translated ^{35}S-labelled TAF$_I$41 interacted with renatured recombinant UBF (Figure 6C, lane 2).

To analyse the functional significance of the UBF–TAF$_I$41 interaction, we tested the effect of TAF$_I$41 upon UBF-activated transcription in a reconstituted transcription reaction. Preinitiation complexes of SL1 and Pol I were assembled on an immobilized rDNA promoter template. Addition of recombinant TAF$_I$41 did not affect basal transcription from these templates (Figure 6D, lane 9 compared to lanes 1 and 5). However, UBF-activated transcription was blocked by addition of recombinant TAF$_I$41 (Figure 6D, lanes 10–12). This squelching of UBF activation is likely to be specific, because

GST alone (Figure 6D, lanes 6–8) did not produce the effect. These data suggest a potential coactivator function for TAF$_I$41, but we do not yet have direct evidence for this. We are currently working to improve the efficiency and efficacy of reconstitution of Pol I transcription using SL1 assembled from its individual subunits TBP and TAF$_I$s, 110, 63, 48 and 41, to enable further study of the individual roles of TAF$_I$41 and the other SL1 subunits.

In conclusion, we have demonstrated that TAF$_I$41 is a novel component of transcriptionally active SL1, important in Pol I transcription. Interestingly, the introns of the *TAF$_I$41* gene (*MGC5306*) encode six H/ACA box snoRNAs and two C/D box snoRNAs (Kiss *et al*, 2004), providing a link between transcription of the 47S pre-rRNA by Pol I and its processing and modification to produce the 18S, 5.8S and 28S rRNAs. We have observed a correlation between TAF$_I$41 expression levels and Pol I transcription activity in cells. Furthermore, our data implicate the SL1 complex, including TAF$_I$41, in recruitment of the Pol I complex to the rDNA promoter *in vivo*, perhaps through an interaction with RRN3/TIF-IA. We speculate that the TAF$_I$41 subunit of SL1 might also be required to upregulate Pol I transcription for sustained hyperproliferation of cells, particularly given the observed overexpression of TAF$_I$41 (MGC5306) in certain tumours ((Wang *et al*, 2004); Pathak and Zomerdijk, unpublished results). The importance of TAF$_I$41 with respect to cell fate is also suggested by the
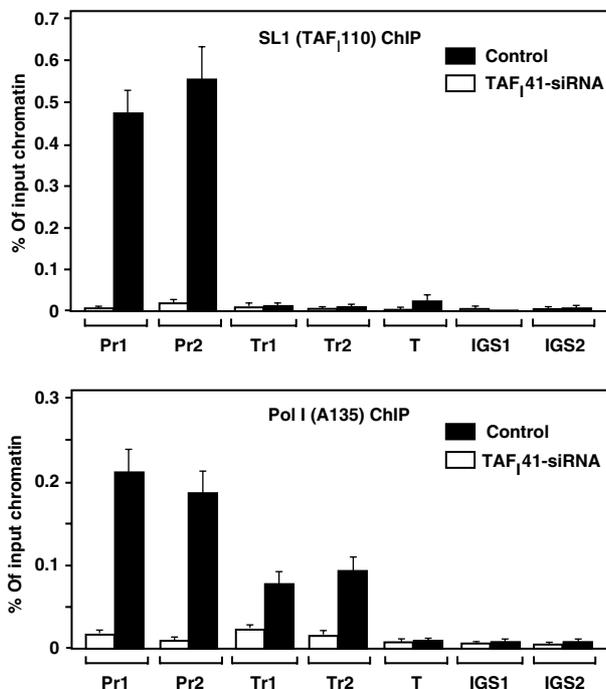
Figure 5 Reduced occupancy of SL1 and Pol I at the rDNA in TAF$_I$41-siRNA-treated cells. ChIP analysis of control or TAF$_I$41-siRNA-treated HEK293 cells (72 h post-transfection) with antibodies specific for TAF$_I$110 (SL1) or A135 (second largest subunit of Pol I) and with an array of primers sets for the human rDNA repeat (Pr1 and Pr2: UCE and core promoter sequences; Tr1 and Tr2, the transcribed 18S and 28S rRNA gene sequences; T, sequences downstream of terminator; IGS1 and IGS1, sequences in pseudo-gene CDC27 and p53-binding moiety in the intergenic spacer of the rDNA repeat; see Materials and methods). The levels of rDNA associated with TAF$_I$110 (SL1) and A135 (Pol I) at each site (relative to control antibodies) are expressed as percentage of input chromatin.

finding that decreased expression of TAF$_I$41 activates the p53 response pathway (Pathak and Zomerdijk, unpublished results), induces apoptosis and reduces cell proliferation (Wang *et al*, 2004) most likely as a consequence of the downregulation of Pol I transcription.

Materials and methods

TAF$_I$41 antibodies

TAF$_I$41 peptides (MDKSGIDSLDH; ENEKNAPWRKIL; KAKNTGQ RGLKM), coupled to KLH, were used to generate rabbit polyclonal antibodies. This antibody is referred to as TAF$_I$41-peptides antibody and is used throughout this study primarily for probing immunoblots. The characterization of the TAF$_I$41-peptides antibody specificity is described (Supplementary Figure S2B and C).

Human TAF$_I$41 protein D (amino acids 98–204) was cloned in pGEX-4T vector and expressed in *Escherichia coli* BL21(DE3). GST-TAF$_I$41 (98–204) protein was affinity purified on glutathione–Sepharose and after thrombin cleavage to release the TAF$_I$41 protein portion from GST, purified TAF$_I$41 protein was used to generate polyclonal sheep antibodies (Scottish National Blood Transfusion Service), which were affinity purified on HiTRAP NHS-activated HP coupled to purified hTAF$_I$41 protein D according to the manufacturer's instructions (Amersham Biosciences). This antibody was used in the immunoprecipitation and depletion experiments of Figure 3.

Pol I transcription machinery components

Human SL1 was purified from HeLa cell nuclear extracts for analytical purposes as described in Figure 1A and B (and based in
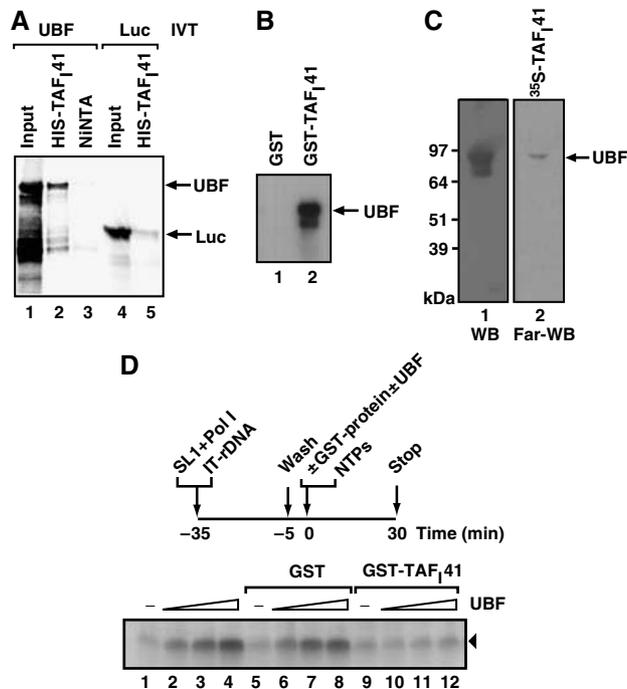
Figure 6 TAF$_I$41 interacts with UBF and can squelch UBF-dependent activation of Pol I transcription. (**A**) Recombinant His-TAF$_I$41 interacts directly with *in vitro* translated UBF. His-TAF$_I$41 (lanes 2 and 5), baculovirus-expressed in insect cells and purified on NiNTA beads, was incubated with *in vitro* translated (IVT) ^{35}S-radio-labelled UBF (lanes 1–3) or luciferase (negative control protein; lanes 4 and 5). Empty NiNTA beads were included as a control (lane 3). Beads were washed and bound proteins analysed by autoradiography. (**B**) GST-TAF$_I$41 interacts directly with recombinant UBF. GST-TAF$_I$41 (lane 2) or GST (lane 1) proteins on beads were incubated with purified recombinant baculovirus-Sf9 cell-expressed human UBF1. Beads were washed and bound proteins analysed by immunoblotting with UBF-specific antibodies. (**C**) A direct interaction between TAF$_I$41 and UBF is detectable by Far-Western analysis. *In vitro* translated ^{35}S-radiolabelled TAF$_I$41 was incubated with membrane-bound, then denatured and renatured UBF protein (Far-WB; lane 2). Binding was analysed by autoradiography. In control lane 1, denatured–renatured UBF was detected by UBF-specific antibodies following Western blotting (WB). (**D**) TAF$_I$41 squelches UBF-activated but not basal SL1-directed Pol I transcription. Preinitiation complexes (PICs) of SL1 (Superose 6 fraction) and Pol I were assembled on an immobilized human rDNA promoter template (IT-rDNA, Fr4; Panov *et al*, 2001) for 30 min on ice. The PICs were washed in TM10/0.075 M KCl, then GST-TAF$_I$41 (50 ng) was added concomitantly with 0, 1, 10 and 20 ng of UBF (lanes 9, 10, 11 and 12, respectively) and NTPs and transcription was analysed by S1 nuclease protection. In the control samples, in place of GST-TAF$_I$41, no protein (lanes 1–4) or GST (50 ng; lanes 5–8) was used.

part on protocols described by Friedrich *et al*, 2005). For preparative scale purification, we purified SL1 through the following columns: Heparin Agarose, SP-Sepharose, POROS-Heparin, Mono S and Superose 6, as detailed previously (Miller *et al*, 2001; Friedrich *et al*, 2005); this purified SL1 is referred to as Superose 6 fraction SL1. UBF and Pol I were purified as described previously (Miller *et al*, 2001; Panov *et al*, 2006). Hela cell nuclei were purchased from the National Cell Culture Center (Minneapolis, MN, USA).

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitations used antibodies for human TAF$_I$41 (TAF$_I$41-peptides antibody, used in Westerns and IPs or TAFI41-antibody D, used in IPs; see above), TAF$_I$110, TAF$_I$63, TAF$_I$48, RRN3, TBP (Comai *et al*, 1994; Zomerdijk *et al*, 1994; Heix *et al*, 1997; Miller *et al*, 2001), GAPDH (clone 6C5, Research Diagnostics Inc.), Flag (Sigma) and GFP (Roche). For immunoprecipitation of Flag- or EGFP-tagged proteins, HeLa cells were

transfected (Effectene, Qiagen) with pcDNA-Flag-TAF₁41, pcDNA-Flag-TAF₁63 (Flag peptide coding sequence and full-length cDNA of human TAF₁41 or TAF₁63 subcloned into pcDNA3.2/V5-DEST vector, Invitrogen) or pEGFP-TAF₁41, pEGFP-RRN3 expression vectors (full-length cDNA of human TAF₁41 or RRN3 subcloned into pEGFP, Invitrogen), and 'empty' expression vectors were used as controls these transfections. Precleared nuclear extract (0.4 mg) from these transfected cells was incubated with Flag-specific or GFP-specific antibody beads in TM10/0.2 M KCl (50 mM Tris–HCl pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 1 mM sodium-metabisulphite, 1 mM DTT, 0.015% NP40) for 2 h at 4°C, beads were washed and Flag precipitants were eluted by Flag peptide as described (Panova *et al*, 2006). For immunoprecipitation of endogenous proteins, 0.4 mg of precleared nuclear extract was incubated with antibody on Protein A Dynabeads in TM10/0.2 M KCl overnight at 4°C; beads were washed extensively in TM10/0.2.

In vitro transcription
In vitro Pol I transcription assays with human rDNA promoter (Fr 4, −193 to +239) or mouse rDNA promoter (−182 to +23) fragments were performed as described (Miller *et al*, 2001; Panov *et al*, 2001). The SL1 used for add-back into *in vitro* transcription reactions (Figures 3 and 4) was purified over the following columns: Heparin Agarose, SP-Sepharose, POROS-Heparin, Mono S and Superose 6, as detailed previously (Miller *et al*, 2001; Friedrich *et al*, 2005). This SL1 (Superose 6 fraction) is free of UBF and Pol I.

siRNA-mediated repression of hTAF₁41, TAF₁48 and RRN3 expression
HeLa, HEK293 or human astrocytoma SJSA cells (cultured in DMEM with 10% FCS) were transfected (Oligofectamine, Invitrogen) with synthetic siRNA (100 nM; Dharmacon) for hTAF₁41 (target sequence 5′-AAGTGATTCATCAAGTGACTC), TAF₁48 (5′-AAGAGGTACTCACC AATTATG), RRN3 (5′-AAATATGCGTGCATTAGAGAA) or Control (5′-AACAGTCGCGTTTGCGACTGG). After 48 h, nuclear extracts were prepared, and analysed by immunoblotting (with GAPDH serving as a reference) and/or used in *in vitro* transcription reactions; total RNA was isolated from the siRNA-treated cells to assess pre-rRNA levels by S1 nuclease protection as described (James and Zomerdijk, 2004). Messenger RNA levels were analysed by quantitative real-time PCR as outlined. Three siRNAs for TAF₁41 were tested; the TAF₁41-siRNA that caused the most effective repression of TAF₁41 gene expression in cells is used throughout this study.

mRNA extraction and quantification by real-time PCR
Total RNA from siRNA-treated cell cultures was isolated using the RNeasy mini-kit (Qiagen), including an on-column DNase I treatment step. RNA (200 ng) was subsequently reverse-transcribed into cDNA with Superscript II (Invitrogen) using random hexamers. Real-time PCR (Taqman, Applied Biosystems) was carried out with an ABI Prism 7900 sequence detector using the following protocol: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. Primers used were: for TAF₁48: forward (f) primer 5′-TGCTCAGTGGTGCAGGAATG and reverse (r) 5′-CCTCCAATGGC CACAGTTTC, and Taqman probe: 6-FAM-ATTTTCCTTGGCTT CAAACATACG-TAMRA; for RRN3: (f) primer 5′-AGAACCAGCTGTT AGATCCAGACATA, and (r) 5′-TCAAGTACATGATAGAAGAACGGAA TTC and Taqman probe 6-FAM-AGGATGACCAGATCATCAACTGGCT-TAMRA.

TAF₁41 mRNA was first reverse transcribed and then the cDNA was amplified using SYBR Green PCR master mix (Applied Biosystems) and specific primers in an ABI Prism 7900 system (Applied Biosystems). The primers used were: (f) 5′-CATGTGA CATCTGATGCTGTGGAAC and (r) 5′-CGTGAACACTTTCAGGTGTAC GAAC. PCR conditions used were: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 1 min. Primers and Taqman probe for β-actin were purchased from Applied Biosystems. Relative quantification of gene expression was performed using the comparative threshold (C_T) method. Changes in mRNA expression levels were calculated following normalization to β-actin mRNA levels.

In vitro binding assays
Interactions of TAF₁41 with UBF or luciferase were analysed using recombinant baculovirus-Sf9 cell-expressed His-TAF₁41 (purified on Ni-NTA beads according to the manufacturer's instructions (Qiagen)) and ^{35}S-labeled *in vitro* translated (TNT-coupled reticulocyte lysate system (Promega)) UBF or luciferase in *in vitro* binding assays.

In an alternative protein–protein binding assay, GST or GST-TAF₁41 (expressed in *E. coli* and purified on glutathione–Sepharose according to the manufacturer (Amersham Biosciences)) was mixed with baculovirus-Sf9 cell-expressed and purified UBF (Panov *et al*, 2006).

In vitro binding assays were performed in 75 mM KCl in TM10 buffer plus 0.015% NP-40 for 30 min at 0°C. After binding, beads were extensively washed with 0.1 M KCl in TM10 and bound protein were analysed by autoradiography.

Far-Western analyses involved SDS–PAGE of recombinant purified UBF, transfer to membrane, denaturation and renaturation of membrane-bound UBF, incubation of the membrane with ^{35}S-radiolabelled *in vitro* translated TAF₁41 (using subclone pcDNA3.2/V5-DEST(Invitrogen)-TAF₁41) and analysis of bound proteins by autoradiography as described (Miller *et al*, 2001).

Nucleolar chromatin immunoprecipitations
Nucleolar chromatin immunoprecipitations (ChIPs) were performed essentially as described by O'Sullivan *et al* (2002) with some modifications. HeLa or HEK293 cells were cultured to approximately 70% confluency, and the medium was refreshed 1 h before crosslinking. Crosslinking was performed for 10 min at 37°C by the addition of freshly made paraformaldehyde to a final concentration of 0.25–1%. Cells were collected into cold PBS supplemented with 1 mM AEBSF, pelleted, washed with PBS, and resuspended in high-magnesium buffer (10 mM HEPES pH 7.5, 0.88 M sucrose, 12 mM MgCl₂, 1 mM DTT, Complete protease inhibitors (Roche)). Nucleoli were released by sonication, pelleted, resuspended in low-magnesium buffer (10 mM HEPES pH 7.5, 0.88 M sucrose, 1 mM MgCl₂, 1 mM DTT, Complete protease inhibitors (Roche)), further sonicated and pelleted. The nucleoli were then resuspended in 1% SDS, 10 mM EDTA (pH 8), 50 mM Tris–HCl (pH 8), Complete protease inhibitor, and further SDS was added to a final concentration of 2%. After the lysis of the nucleoli (37°C for 15 min), the solution was diluted five-fold with TE buffer containing Complete protease inhibitors, sonicated, the resulting sheared nucleolar chromatin was centrifuged (15 000 g for 1 min), and the supernatant was used in nucleolar ChIP assays after quantification of input total DNA and determining the size of resulting sheared nucleolar DNA (fragments in the 250–300 bp size range).

Nucleolar ChIPs were performed using TBP-antibody (mouse monoclonal 3G3, kind gift from Dr L Tora), TAF₁41-peptides rabbit polyclonal antibody, TAF₁110 rabbit polyclonal antibody (Zomerdijk *et al*, 1994), A135 (Pol I second largest subunit) goat polyclonal antibody (Santa Cruz Biotech, Inc.) or the appropriate control IgG (Sigma) or preimmune serum immobilized on Protein G para-magnetic beads (Dynal). QPCR analysis was performed using SYBR Green PCR Master Mix and ABI7000 (both Applied Biosystems). For the ChIP analysis in Figure 1, we used the following primer pairs in the human rDNA repeat (positions according to GenBank Acc No U13369): 42 918–42 936 and 7–33 (core promoter); 7064–7090 and 7266–7291 (ITS-2). For ChIP analysis in Figure 6, the following primer pairs were used: 42 787–42 829 and 42 832–42 881 (Pr1: promoter UCE); 42 896–42 940 and 42 949–42 993 (Pr2: core promoter); 4501–4545 and 4656–4700 (Tr1: 18S rRNA gene); 12 100–12 144 and 12 260–12 304 (Tr2: 28S rRNA gene); 13 487–13 531 and 13 680–13 724 (just downstream of terminator, T); 35 822–35 866 and 35 987–36 031 (intergenic spacer IGS1: CDC27 pseudo-gene); 39 325–39 369 and 39 478–39 522 (IGS2: p53-binding moiety).

Supplementary data
Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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