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Common and unique transcription factor requirements of human U1 and U6 snRNA genes

Jordi Bernués[2], Kenneth A.Simmen[3], Joe D.Lewis, Samuel I.Gunderson, Maria Polycarpou-Schwarz, Vincent Moncollin[1], Jean-Marc Egly[1] and Iain W.Mattaj

European Molecular Biology Laboratory, Gene Expression Programme, Postfach 10.2209, D-6900 Heidelberg, Germany and [1]Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cédex, France
[2]Present address: Centro de investigación y Desarrollo, CSIC, Jordi Girona Sagaldo 18-26, 08034 Barcelona, Spain
[3]Present address: The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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The human U1 and U6 genes have similar basal promoter structures. A first analysis of the factor requirements for the transcription of a human U1 gene by RNA polymerase II *in vitro* has been undertaken, and these requirements compared with those of human U6 gene transcription by RNA polymerase III in the same extracts. Fractions containing PSE-binding protein (PBP) are shown to be essential for transcription of both genes, and further evidence that PBP itself is required for U1 as well as U6 transcription is presented. On the other hand, the two genes have distinct requirements for TATA-binding protein (TBP). On the basis of chromatographic and functional properties, the TBP, or TBP complex, required for U1 transcription appears to differ from previously described complexes required for RNA polymerase I, II or III transcription. The different TBP requirements of the U1 and U6 promoters are reflected by specific association with either TFIIB or TFIIIB respectively, thus providing a basis for differential RNA polymerase selection.

Key words: human U1 transcription/PSE-binding protein (PBP)/RNA polymerase/small nuclear (sn) RNA genes/ TATA-binding protein (TBP)

Introduction

The spliceosomal U snRNAs U1, U2, U4, U5 and U6 form a functionally related and highly conserved family in eukaryotes. In contrast, the promoters of their genes are quite divergent between species and, in addition, U snRNA promoters in most species display unusual features (Dahlberg and Lund, 1988; Simmen *et al.*, 1992a; Bernués *et al.*, 1992). Vertebrate U snRNA promoters contain a distal sequence element (DSE) located ~250 bp upstream of the initiation site which functions like an enhancer, and a proximal region, including an essential proximal sequence element (PSE) located ~50 bp from the initiation site. The PSE functions in start site positioning and is required for accurate 3′ end formation. A promoter with only these elements, like those of the U1 or U2 genes, is transcribed by RNA polymerase II (pol II). The U6 gene, which is transcribed by RNA polymerase III (pol III), has in addition to the DSE and PSE an essential TATA box located between the PSE and the start site (reviewed by Dahlberg and Lund, 1988; Parry *et al.*, 1989; Bernués *et al.*, 1992).

The finding of an essential TATA element in the U6 and related pol III promoters was of interest since the TATA box had, until then, been considered the main determinant of pol II transcription in a major class of pol II promoters. The first stable interaction between a transcription factor and such a basal pol II promoter is the binding of transcription factor TFIID to the TATA box. All the subsequent interactions required to assemble a functional transcription complex depend on this initial step (Buratowski *et al.*, 1989; Roeder, 1991 and references therein). TBP, the protein directly responsible for binding the TATA box, has been cloned from several organisms. At least in vertebrates and insects TBP has been found to be only one component of one or more multiprotein TFIID complexes (Cavallini *et al.*, 1989; Hahn *et al.*, 1989; Horikoshi *et al.*, 1989; Schmidt *et al.*, 1989; Fikes *et al.*, 1990; Gasch *et al.*, 1990; Hoey *et al.*, 1990; Hoffmann *et al.*, 1990a,b; Kao *et al.*, 1990; Muhich *et al.*, 1990; Peterson *et al.*, 1990; Dynlacht *et al.*, 1991; Pugh and Tjian, 1991; Timmers and Sharp, 1991). In this paper we will follow the nomenclature suggested by Dynlacht *et al.* (1991) who called the cloned protein TBP (for TATA-binding protein) and referred to the complex(es) involved in pol II transcription as TFIID. The work of Timmers and Sharp (1991) suggested that two such complexes exist, called B-TFIID and D-TFIID, which differ in their composition and chromatographic behaviour.

In vivo experiments in which promoter sequences were swapped between vertebrate U6 and U2 genes showed that insertion of a U6 TATA box into a U2 promoter conferred pol III specificity, whereas removal of the U6 TATA box abolished pol III transcription, but allowed pol II transcription from the mutant U6 promoter (Mattaj *et al.*, 1988; Lobo and Hernandez, 1989). More recently, Lescure *et al.* (1991) have shown that insertion of a TATA box into a *Xenopus* U1 promoter conferred pol III specificity, reinforcing the correlation between the presence of a TATA box and the use of pol III for U snRNA genes. This apparent paradox was underlined by the demonstration that transcription of a human U6 gene by pol III *in vitro* required TBP (Lobo *et al.*, 1991; Simmen *et al.*, 1991). The simplest hypothesis to explain polymerase choice in U snRNA promoters at that time was that a PSE-binding factor on its own would select pol II without the involvement of TBP whereas the PSE factor in conjunction with a TATA-bound TBP complex would select pol III.

From other studies, however, it has become clear that TBP plays a general role in transcription. It is a component of TFIIIB, required for the transcription of all pol III promoters, including those of *Saccharomyces cerevisiae* U6 or of tRNA

or 5S ribosomal RNA genes, whose structures are different from those of vertebrate U6 promoters (Margottin *et al.*, 1991; Huet and Sentenac, 1992; Kassavetis *et al.*, 1992; Lobo *et al.*, 1992; Simmen *et al.*, 1992b; Taggart *et al.*, 1992; White *et al.*, 1992; White and Jackson, 1992). TBP is also a component of SL1, which is necessary for transcription from pol I promoters (Comai *et al.*, 1992). In addition, TBP complexes have been shown to function in the pol II transcription of two similar (artificial) TATA-less promoters (Smale *et al.*, 1990; Pugh and Tjian, 1990, 1991; Zhou *et al.*, 1992).

It therefore became likely that TBP would be required for the transcription of the pol II class of U snRNA genes and the simple model of polymerase choice presented above became improbable. In order to gain more insight into the requirements for transcription of the two U snRNA gene classes, we have undertaken an *in vitro* biochemical analysis of U1 and U6 transcription in nuclear extracts of HeLa cells. Our data are consistent with the hypothesis that pol II and pol III U snRNA promoters utilize a common PSE-binding factor but different TBP complexes. This primary difference would then result in selection of alternative basal transcription factors, TFIIB and TFIIIB respectively, and in differential RNA polymerase choice.

Results

Transcription of human U1 requires TBP and at least one other heat-labile factor

The only available large-scale *in vitro* transcription system capable of accurate transcription of pol II U snRNA genes is that described by Gunderson *et al.* (1990). Utilizing the same human U1 promoter G-less cassette fusion construct and slight modifications to the procedure for preparing HeLa cell nuclear extract (see Materials and methods) we have been able to achieve a considerable (>10-fold) increase in U1 transcription efficiency per microgram of nuclear extract (data not shown, Figure 1A, lane 1).

Two RNA products were obtained on transcription of the human U1 template (Figure 1A, lane 1) and their structure was investigated. The major primer extension product in the U1 transcription reaction (Figure 1B, lane 3) corresponded to transcripts initiated at +1, as determined by comparison with both the sequencing reactions run in parallel and by comigration with the primer extension product of transcripts of a template consisting of the promoter of the human transferrin receptor (HTFR) gene fused to a G-less cassette (lane 2). This HTFR construct was designed to initiate at the same nucleotide as accurate human U1 transcripts (Gunderson *et al.*, 1990). The longer extension product was five nucleotides longer, and comigrated with a product derived from an adenovirus major late (AdML) promoter G-less cassette fusion whose transcripts are extended by five bases at the 5′ end by comparison with those from the U1 construct. This is the length expected for read-through products arising from non-specific transcription initiated upstream of the promoter after their cleavage by RNase T1. Thus, the U1 *in vitro* transcription obtained was not only more efficient but also more accurate than that seen by Gunderson *et al.* (1990) since initiation from the U1 promoter template at position −2 was not observed.

To obtain an initial indication of whether TBP was involved in human U1 transcription, recombinant human TBP (2.5 ng) was added to a transcription reaction (Figure 1A, lane 2). A 5-fold increase in transcription was observed, suggesting that TBP might indeed play a role in U1 transcription. Both basal and TBP-stimulated transcription were authentic as judged by sensitivity to 0.2 μg/ml α-amanitin (Figure 1A, lanes 3 and 4) and to point mutations in the PSE (lanes 5 and 6).

An experimentally useful property of TBP complexes that function in pol II and pol III transcription is their heat lability. Heating nuclear extracts to 47°C for 15 min inactivates these TBP complexes while leaving many other transcriptional components intact (Nakajima *et al.*, 1988; Simmen *et al.*, 1991; White *et al.*, 1992). We therefore tested whether U1 transcription required heat-labile components. After incubation of the HeLa nuclear extract at 47°C for 15 min, U1 transcription was abolished. However, TBP addition alone did not restore transcription (see e.g. Figure 1D, lane 1). Simmen *et al.* (1991) had previously observed that, in the case of U6 transcription, at least two heat-labile components were required to restore activity to a heat-treated extract. One of these components bound to phosphocellulose (PC) and was eluted at between 0.35 and 0.6 M KCl. We therefore fractionated some of the HeLa nuclear extract over PC into the conventional (Segall *et al.*, 1980) A, B, C and D fractions and tested the fractions for their ability to restore U1 transcription to a heat-treated extract.

The AdML promoter was used as a control. As expected (Nakajima *et al.*, 1988) the PC D fraction, which contains D-TFIID, was sufficient to restore transcription to a heat-treated extract, while the A, B and C fractions were inactive (Figure 1C, lanes 9−12). When the same fractions were tested with the human U1 template, the A and B fractions had no effect (Figure 1C, lanes 1 and 2). The D fraction increased read-through transcription, but did not augment authentic U1 transcription (lane 4). The C fraction, in constrast, allowed a low level of U1 transcription (lane 3). When the fractions were tested together with 2.5 ng of recombinant human (h)TBP, a high level of transcription was obtained by combining the C fraction with hTBP (lanes 5−8). Incubation of the C fraction at 47°C for 15 min abolished this effect (data not shown). Thus, as in the case of U6 (Simmen *et al.*, 1991) it appears that efficient U1 transcription requires two heat-labile components, one is TBP and the other is present in the PC C fraction. Note however that, in contrast to the U6 case, the C fraction alone can restore some U1 transcription.

Both U1 and U6 transcription require PSE-binding protein fractions

We wished to investigate this second heat-labile component further and, in particular, to determine whether U1 and U6 require similar or different factors from the C fraction. Initially, the PC C fraction was chromatographed over heparin−Sepharose. Step elution at 100 (FT fraction), 250 and 500 mM KCl was carried out, and the fractions will be referred to as Hep100, Hep250 and Hep500. They were tested for their ability to restore transcription in heat-treated HeLa nuclear extract in the presence of hTBP. Virtually all the activity was found in Hep500 (Figure 1D, lanes 1−5). The transcription observed was sensitive to 0.2 μg/ml α-amanitin (lanes 6−10).

We next turned our attention to U6. In our experiments fractionation of HeLa cell nuclear extracts active in U6

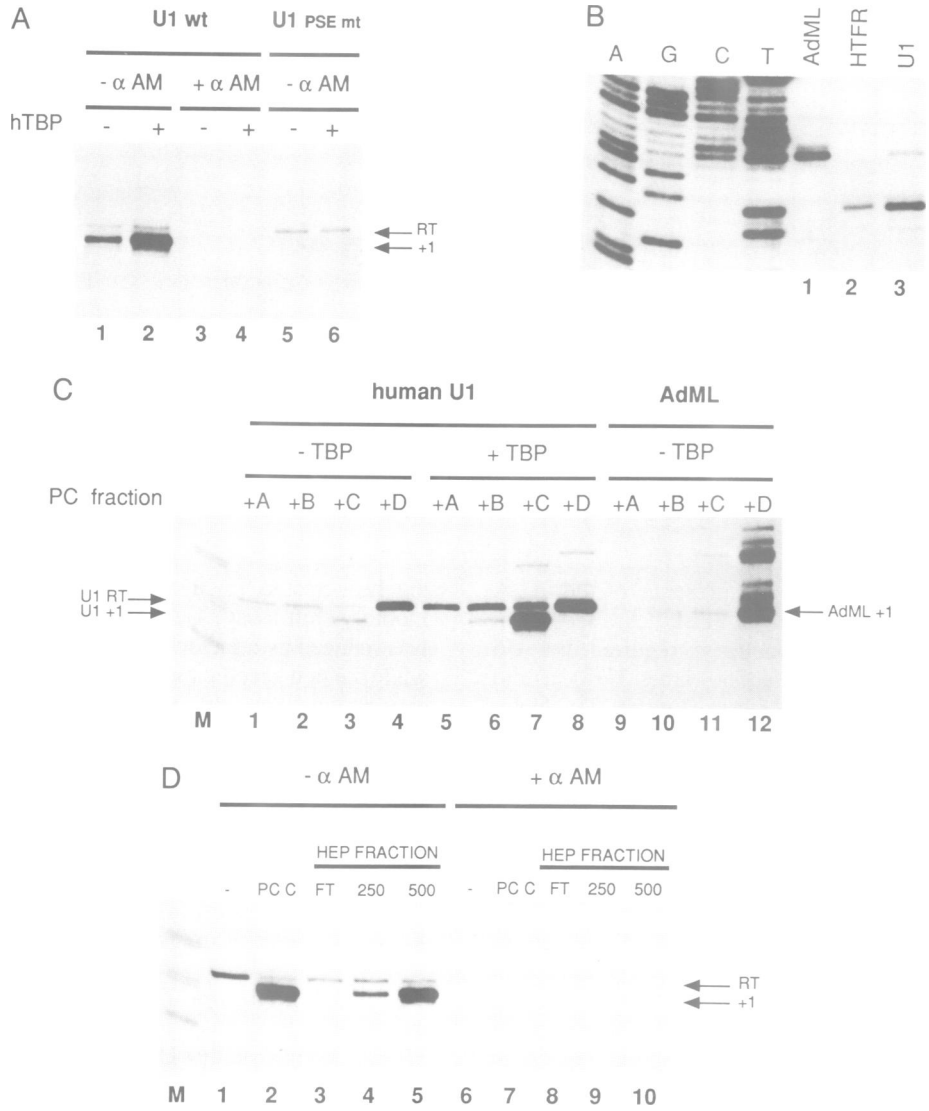

Fig. 1. Heat-labile components required for human U1 transcription *in vitro*. (**A**) *In vitro* transcription of a human U1 promoter G-less cassette template (hU1G−) using HeLa cell nuclear extract. *In vitro* transcription of hU1G− (lanes 1−4) was assayed without (lanes 1 and 3) or with the addition of 2.5 ng of recombinant full-length hTBP (lanes 2 and 4). The assays in lanes 3 and 4 were done in the presence of 0.2 μg/ml of α-amanitin. An hU1 PSE mutant template (containing a CC→TT double point mutation previously shown to render it inactive both *in vivo* and *in vitro*; Gunderson *et al.*, 1990) was assayed on its own (lane 5) or with the addition of 2.5 ng hTBP (lane 6). Correctly initiated (+1) and read-through (RT) transcripts from the hU1 templates are indicated. Note that in this and the other transcription experiments shown in this paper an internal standard for recovery was used, but has been cut from the figures to save space. Only experiments where sample recovery was uniform are shown. (**B**) Primer extension analysis of the transcripts produced by *in vitro* transcription in crude HeLa cell nuclear extracts from the wild-type human U1G− template (lane 3) in comparison with those arising from AdML promoter and human transferrin receptor (HTFR) promoter G-less cassette templates (lanes 1 and 2 respectively) of known structure (Gunderson *et al.*, 1990). DNA sequencing reactions obtained using the same primer with the human U1 template DNA are also shown on the left. (**C**) Heat-treated (47°C for 15 min) HeLa nuclear extract was tested for its ability to support human U1 (lanes 1−8) and AdML transcription (lanes 9−12) in combination with 3 μl of one of four PC fractions (A, B, C and D) as indicated. Additionally, lanes 5−8 received 2.5 ng of recombinant full-length hTBP. The position of hU1 read-through (RT) and correctly initiated transcripts is indicated on the left; correctly initiated AdML transcripts are indicated on the right. M: DNA size markers. (**D**) The ability of the PC C fraction (3 μl, lanes 2 and 7) and the heparin−Sepharose fractions derived from it (3 μl, lanes 3−5 and 8−10) to support hU1 transcription in heat-treated (47°C, 15 min) HeLa cell nuclear extract. All lanes additionally contain 2.5 ng of recombinant full-length hTBP. Lanes 6−10 also contained 0.2 μg/ml of α-amanitin. The position of read-through (RT) and correctly initiated transcripts (+1) is indicated. M: DNA size markers.

transcription reproducibly led to a considerable loss of activity. Recombining all the PC fractions (A, B, C and D) generated very weak transcription independent of the relative amounts of the fractions used. Only the addition of recombinant TBP to this mixture restored activity to the level seen in complete extract (Figure 2A, lanes 1 and 2, and data not shown). This was not due to the loss of enhancer effects, since the same phenomenon was observed using templates from which the DSE had been deleted (data not shown). In the presence of recombinant hTBP, lack of fractions A, B or C caused a significant drop in transcription while lack of fraction D had no effect (Figure 2A, lanes 3−6).

To compare the behaviour of the factor in fraction C required for U6 transcription with that of the U1 factor described above, we examined the ability of the heparin−Sepharose fractions used in Figure 1D to

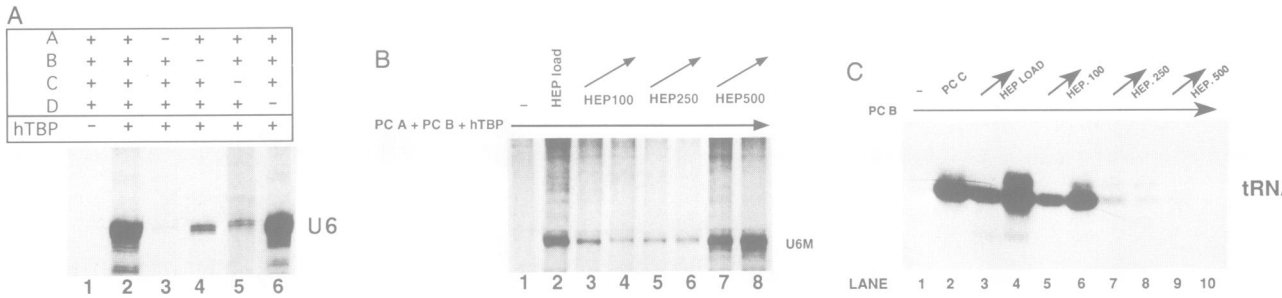

Fig. 2. Requirements of a human U6 gene promoter after extract fractionation. (**A**) *In vitro* transcription reactions were carried out using a human U6 maxigene template and 3 μl of the PC fractions (A−D) indicated by a +. Lanes 2−6 additionally received 2.5 ng of recombinant hTBP; lane 1 received buffer. All reactions were performed in the presence of 2 μg/ml of α-amanitin. The position of U6 transcripts is indicated. (**B**) Reconstitution of human U6 transcription. Reactions containing the PC A and B fractions (3 μl each) and 2.5 ng of recombinant hTBP were complemented either with PC fraction C (2 μl; lane 2) or the heparin−Sepharose fractions derived from it (3 or 5 μl of 100 mM KCl eluate, lanes 3 and 4; 3 or 5 μl of 250 mM KCl eluate, lanes 5 and 6; 3 or 5 μl of 500 mM KCl eluate, lanes 7 and 8). Lane 1 received buffer. The position of U6 maxigene transcripts is indicated. (**C**) The same heparin−Sepharose fractions used in (B) were used, in combination with PC B fraction, to reconstitute tRNA transcription using 100 ng of Mcet 1 template DNA. All other conditions as indicated in (B). The position of tRNA transcripts is indicated.

reconstitute U6 transcription in the presence of the PC A and B fractions and recombinant TBP. As shown in Figure 2B, the bulk of the factor(s) required for U6 transcription fractionated into Hep500 (lanes 3−8), as did the heat-labile U1 transcription factor (Figure 1D). Note, however, that Hep100 supported a low level of U6 (Figure 2B, lanes 3 and 4), but not U1 (Figure 1D, lane 3) transcription.

In order to determine whether the factor in the C fraction behaved like TFIIIC, the pol III transcription factor required for tRNA and 5S rRNA gene transcription, we analysed the heparin−Sepharose fractions for their content of TFIIIC. This was achieved by examining their ability to complement the PC B fraction to allow tRNA transcription (Figure 2C). TFIIIC did not bind significantly to the heparin−Sepharose column, but eluted into Hep100 (lanes 3−10). This suggests that TFIIIC is not required for U6 transcription, consistent with the data of Waldschmidt *et al.* (1991) who reached the same conclusion on the basis of experiments in which TFIIIC activity was inhibited by specific oligonucleotide binding.

The same authors (Waldschmidt *et al.*, 1991; Simmen *et al.*, 1992a) provided evidence that a component of the PC C fraction essential for U6 transcription was PBP (PSE-binding protein). PBP can be detected in the C fraction by electrophoretic mobility shift assay using labelled promoter DNA fragments including the mouse U6 PSE sequence as probe [Waldschmidt *et al.*, 1991; Figure 3A, lane 1 (the band marked with an asterisk is not PSE-dependent and the rapidly migrating complex is not reproducibly observed, see below)]. Upon fractionation, PBP was mostly present in Hep500 (Figure 3A, lanes 2−4). The specificity of the complex for intact PSE sequences was demonstrated by competition with unlabelled wild-type and mutant PSE oligonucleotides (Figure 3B, lanes 2−8). When the same oligonucleotides were added to reconstituted U1 transcription reactions, it was found that the wild-type mouse U6 PSE oligonucleotides were efficient competitors of specific U1 transcription (Figure 3C, lanes 1−4) while mutant U6 PSE oligonucleotides were not (lanes 5 and 6) suggesting that PBP is required for U1 as well as for U6 transcription.

Additional evidence for the involvement of PBP in U1 transcription came from an examination of the heat sensitivity of PBP binding to the PSE *in vitro*. The PC C fraction was subjected to mild heat treatment (47°C for 15 min). When the heat-treated fraction was used in an electrophoretic mobility shift assay, the amount of detectable PBP activity was reduced by a factor of 10 (Figure 3B, lanes 11 and 12) while the non-specific binding activity was unaffected. Other experiments (data not shown) confirmed that the activity of the PBP fraction in both U1 and U6 transcription and in PSE binding exhibited similar heat sensitivity. Together with the results described above this implicates PBP in the transcription of human U1 and U6 genes and, furthermore, suggests that PBP is one essential heat-labile factor present in the PC C fraction. Further fractionation will, however, be required to establish whether the U1 and U6 requirements for this fraction are completely identical and confined to PBP.

The requirements for TBP of U1 and U6 are not identical

Having established that U1 and U6 transcription had similar PBP requirements, we next examined their TBP requirements. In a first experiment, we compared the ability of various forms of recombinant TBP, in combination with the PBP-containing Hep500 fraction, to restore U1 or U6 transcription to heat-treated nuclear extract. Full-length recombinant human and yeast TBP and the conserved core region (amino acids 155−335) of human TBP were tested. The three proteins restored AdML transcription to similar levels when tested in the same extracts (data not shown). Neither the Hep500 nor any of the TBP forms could support high levels of U1 or U6 transcription alone, although as before (Figure 1C, lane 3) the PBP fraction did restore some U1 transcription (Figure 4A and B, lanes 1−5). In combination with the PBP fraction, however, all three forms of TBP allowed efficient U6 transcription, although the core fragment also produced a high background smear of transcription (Figure 4A, lanes 6−8). The result with the U1 promoter was different. Here both forms of human TBP had similar activity (Figure 4B, lanes 6 and 7). Yeast TBP, however, did not increase U1 transcription above the level seen with Hep500 alone (Figure 4B, lanes 2 and 8). Thus, transcription of the U1 and U6 genes have different TBP requirements. On their own, all forms of TBP increase read-through transcription to some extent. This increase is suppressed in the presence of specific transcription (Figure 4B, lanes 6 and 7) suggesting that specific and non-specific transcription are competitive processes.

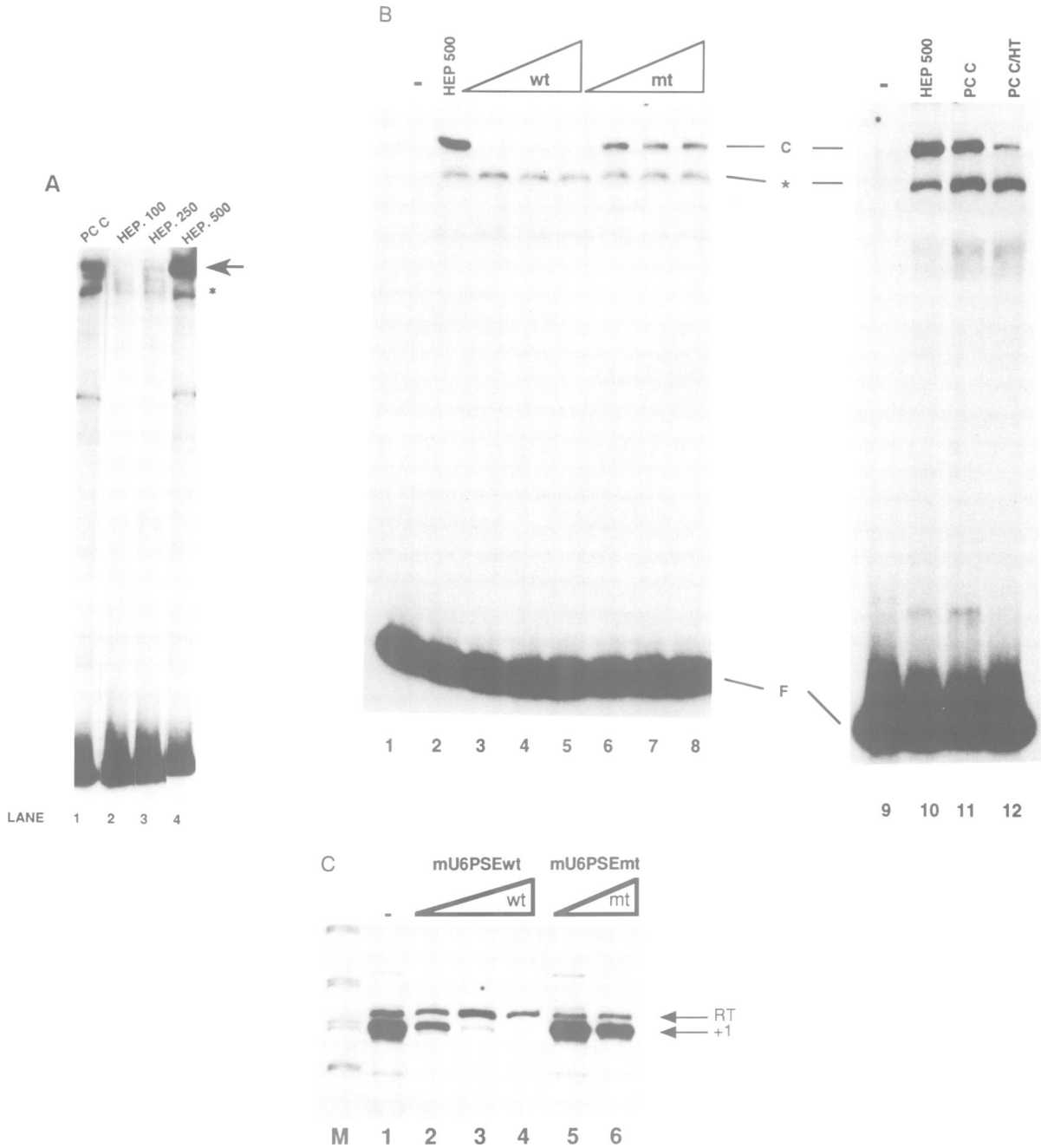

Fig. 3. PSE oligonucleotides inhibit PBP binding and human U1 transcription. (**A**) Electrophoretic mobility shift analysis of PSE-binding activity. The PC C (1 μl, lane 1) and the heparin−Sepharose fractions derived from it (1 μl each, lanes 2−4) were assayed with a promoter fragment containing the mouse U6 PSE (see Materials and methods for details). The PBP−PSE-specific complex is indicated by an arrowhead; a non-specific complex is indicated by an asterisk. (**B**) On the left panel, the specific PBP complex obtained with the heparin−Sepharose 500 mM KCl eluate (using 1 μl of Hep500 per lane, lanes 2−8) was competed with increasing amounts (10, 50 and 100 ng) of oligonucleotides containing either a wild-type (lanes 3−5) or a mutated (lanes 6−8) mouse U6 PSE. Lanes 1 and 2 show the mobility of the free probe and of the uncompeted complex, respectively. On the right panel, the effect of heat-treatment (47°C, 15 min) on the formation of the PBP-specific complex was determined. Lanes 9−11 are controls containing probe alone, and probe plus 1 μl of either 500 mM KCl heparin−Sepharose fraction or untreated PC C fraction, respectively. Lane 12 contains PC C fraction heat-treated at 47°C for 15 min prior to the assay. F: free probe; C: PBP-specific complex; ★: a non-specific complex. (**C**) Human U1 transcription is inhibited *in vitro* by an oligonucleotide containing the mouse U6 PSE. Aliquots (3 μl) the PC C fraction were incubated with different amounts of the appropriate oligonucleotide for 10 min on ice and then 9 μl of heat-treated (47°C, 15 min) HeLa nuclear extract, 2.5 ng of purified hTBP, DNA template, salts and nucleotides were immediately added. In lane 1 the preincubation was with buffer. Lanes 2, 3 and 4 were preincubated with 10, 25 and 50 ng of a wild-type mouse U6 PSE oligonucleotide. Lanes 5 and 6 were preincubated with 10 and 50 ng of a mutant mouse U6 PSE oligonucleotide, respectively. The position of read-through (RT) and correctly initiated (+1) hU1 transcripts is indicated. M: DNA markers. Oligonucleotides were the same as in panel B.

A TBP fraction required for human U1 transcription

The ability of the C and Hep500 fractions to restore low-level human U1 transcription to heat-treated extract (Figures 1C, lane 3, and 4B, lane 2) suggested that these fractions must contain some TBP in addition to PBP. To investigate this, it was necessary to identify components required for U1 transcription and to devise a more direct method of depletion of TBP from these component fractions. To

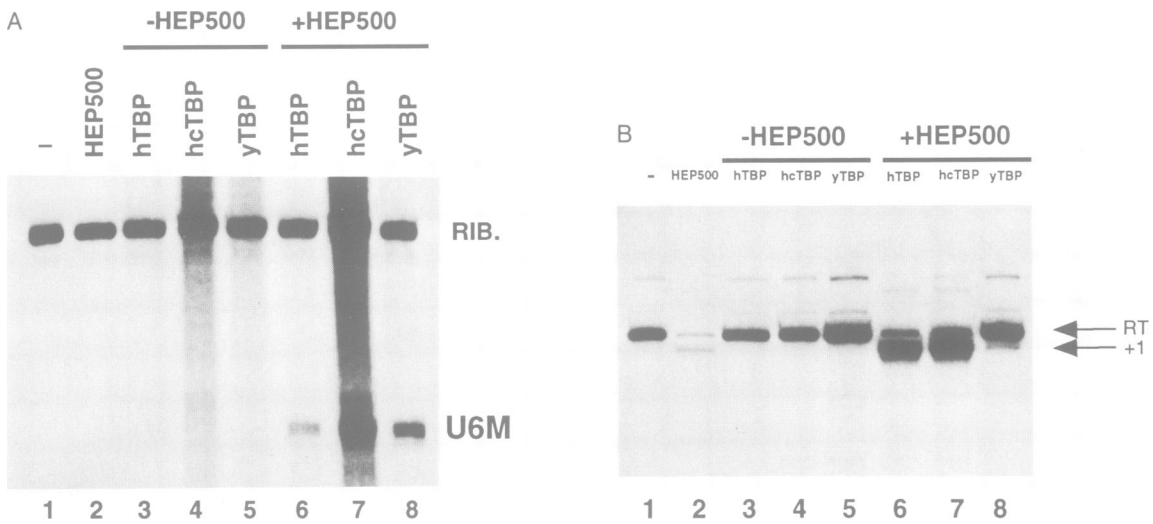

Fig. 4. Different TBP requirements for human U1 and U6 transcription in heat-treated HeLa nuclear extracts. (**A**) Assay of the recombinant TBP activities in human U6 transcription. All lanes contain 6 μl of heat-treated (47°C, 15 min) HeLa cell nuclear extract and additions as indicated: lane 2; 3 μl of the 500 mM KCl heparin−Sepharose fraction; lanes 3−5; recombinant full-length hTBP (hTBP), human core TBP (hcTBP) and full-length yeast TBP (yTBP) respectively (1 μl each, equivalent to roughly 2.5, 2 and 4 ng of hTBP, hcTBP and yTBP respectively); lanes 6−8, as for lanes 3−5 with the addition of 3 μl of 500 mM KCl heparin−Sepharose fraction; lane 1 received buffer. All reactions were performed in the presence of 2 μg/ml α-amanitin. In this figure, the recovery control (RIB) is shown to help evaluate the distortion of apparent signal strength due to the high background observed in the presence of human core TBP. The position of U6 maxigene transcripts (U6M) is indicated. (**B**) Assay of recombinant TBP activities in human U1 transcription. This experiment is analogous to that in (A), except that human U1 transcription was examined and, therefore, no α-amanitin was included in the reactions. The position of read-through (RT) and correctly initiated (+1) transcripts is indicated.

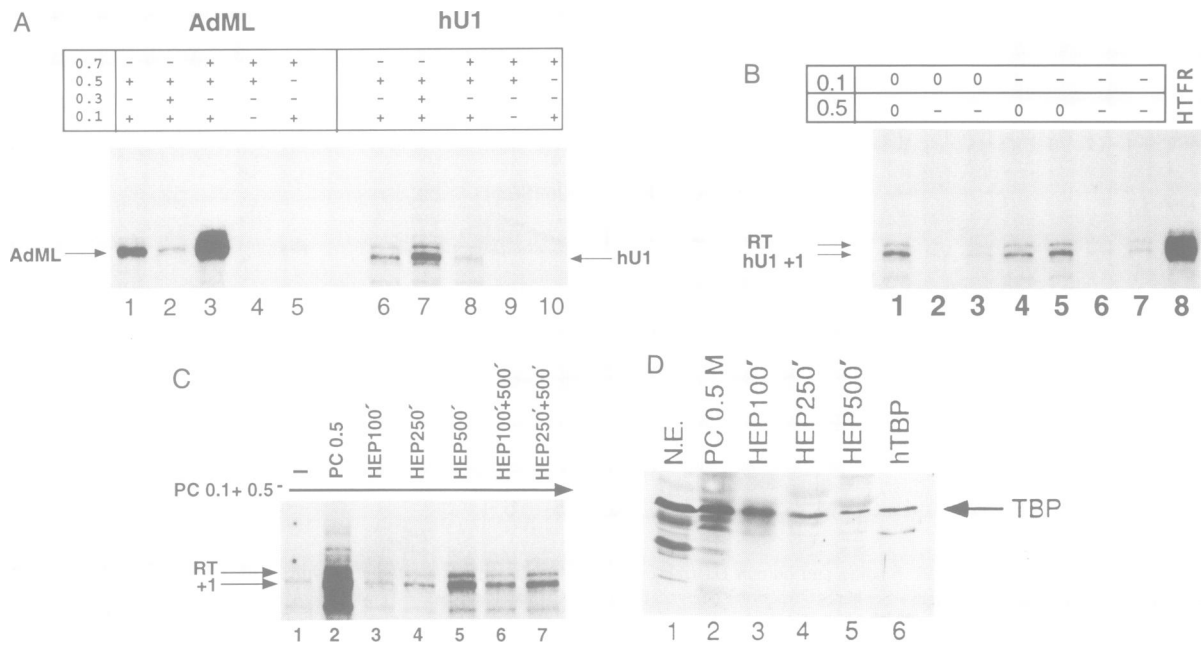

Fig. 5. Reconstitution of human U1 gene transcription after extract fractionation. (**A**) Reconstitution of *in vitro* transcription for AdML (lanes 1−5) and hU1 (lanes 6−10) was performed by combining the PC fractions (2 μl of 0.1 M; 3 μl of 0.3 M and 0.7 M; and 3 μl and 5 μl of 0.5 M for AdML and hU1, respectively) indicated above with a + sign (a − sign indicates that a particular fraction was omitted). Correctly initiated AdML and hU1 transcripts are indicated. (**B**) Human U1 transcription was assayed in a combination of 0.1 M and 0.5 M fractions (lanes 1−7). Mock-depleted fractions are indicated by a zero; TBP-depleted fractions by a − sign. Lanes 1−3 contained 3 μl of mock-depleted 0.1 M fraction together with 10 μl mock-depleted 0.5 M fraction (lane 1) or 8 or 10 μl of TBP-depleted 0.5 M fraction (lanes 2 and 3). Lanes 4−7 contained 2 μl of TBP-depleted 0.1 M fraction together with 8 or 10 μl of mock-depleted 0.5 M fraction (lanes 4 and 5) or of TBP-depleted 0.5 M fraction (lanes 6 and 7). Lane 8 is a control using the HTFR template as a size marker (transcribed with a combination of 0.1, 0.5 and 0.7 M fractions). Correctly initiated transcripts (hU1+1) and read-through transcripts (RT) from the hU1 template are indicated. (**C**) Reconstitution of hU1 transcription *in vitro*. All lanes contained 1 μl of 0.1 M and 5 μl of depleted 0.5 M PC fractions. Additionally, lane 1 received buffer; lanes 2−5 received 6 μl of the undepleted 0.5 M fraction or of the Hep100′, Hep250′ or Hep500′ fractions, respectively. Lane 6 received 3 μl of Hep100′ and 3 μl Hep500′, and lane 7, 3 μl of Hep250′ and 3 μl Hep500′. The position of correctly initiated (+1) and read-through transcripts (RT) is indicated. (**D**) The PC 0.5 M fraction was fractionated over heparin−Sepharose and the TBP content of the different fractions analysed using a polyclonal anti-TBP antiserum. Lanes 1−5 contain 7 μl of unfractionated nuclear extract or 100 μl of PC 0.5 M, Hep100′, Hep250′ and Hep500′, respectively. Lane 6 contains recombinant human TBP as indicated.

identify fractions necessary for U1 transcription, we adopted a chromatographic scheme that allows more efficient separation of the various identified TBP complexes than does the fractionation procedure used above (see e.g. Taggart *et al.*, 1992). This involves step elution from PC with 0.1, 0.3, 0.5, 0.7 and 1.0 M KCl.

The fractions obtained were first tested with an AdML template. In the presence of the 0.1 and 0.5 M fractions, weak transcription was observed (Figure 5A, lane 1). The 0.3 M fraction was not required for AdML transcription, and, in fact, had an inhibitory effect (lane 2). Both the 0.1 and 0.5 M fractions were essential (lanes 4 and 5). The D-TFIID-containing 0.7 M fraction stimulated transcription 5-fold (lane 3) but was not absolutely required. The 1.0 M fraction behaved similarly to the 0.7 M fraction (data not shown).

In the case of human U1 transcription, combination of the 0.1 and 0.5 M fractions was again sufficient for transcription (Figure 5A, lane 6), and both these fractions were also essential (lanes 9 and 10). However, the effects of the 0.3 and 0.7 M fractions were different than with the AdML promoter. The 0.7 M fraction had a reproducible inhibitory effect (lane 8 and data not shown) while the 0.3 M fraction was stimulatory (lane 7). The 1.0 M fraction had a modest inhibitory effect on human U1 transcription, similar to that of the 0.7 M fraction, when combined with the 0.1 and 0.5 M fractions (data not shown).

To determine which of the two essential fractions contained the TBP necessary for human U1 transcription, depletion experiments were carried out with a polyclonal antiserum raised against human TBP (Simmen *et al.*, 1992b). The 0.1 and 0.5 M fractions were either mock-depleted (designated 0) or TBP-depleted (−) and their transcription activity tested. Removal of TBP from the fractions was confirmed by Western blot analysis (data not shown). Depletion of the 0.5 M fraction severely reduced transcription (Figure 5B, lanes 1, 2, 3, 6 and 7) while depletion of the 0.1 M fraction had no effect (lanes 1 and 4−7). Thus, the 0.5 M fraction contained the TBP required for U1 transcription.

Much of the TBP in the 0.5 M fraction has been reported to be in complexes that may be functionally different from the active D-TFIID in the 0.7 M fraction, although their composition is related to that of D-TFIID (Taggart *et al.*, 1992). To examine further the state of the TBP required for U1 transcription, we fractionated the 0.5 M PC fraction over heparin−Sepharose, to produce fractions analogous to those obtained previously from the PC C fraction (Figures 1−3). These fractions were tested for their ability to reconstitute U1 transcription together with the 0.1 M PC fraction and the TBP-depleted 0.5 M fraction. To distinguish these fractions from those derived from the C fraction, we denote them Hep100′, Hep 250′ and Hep500′.

The activity recovered was mainly in Hep500′ (Figure 5C, lanes 1−5), although this fraction only contained a minority of the total TBP from the 0.5 M fraction, as judged by Western blotting (Figure 5D, lanes 1−5). Combination of Hep500′ with either Hep250′ or Hep100′ failed to stimulate transcription further (Figure 5C, lanes 5−7). The decreased transcription level in lanes 6 and 7 was due to the fact that they contained half as much Hep500′ as did lane 5. We conclude that the TBP (or TBP complex) required for U1 transcription is a minor fraction of the total TBP present in the 0.5 M fraction and that it cofractionates with PBP on heparin−Sepharose. U1 transcription activity was reproducibly lost when the 0.5 M fraction was passed over heparin−Sepharose (e.g. Figure 5C, lanes 2 and 5). In contrast, the Hep500 fraction had a higher specific activity than the C fraction when tested, in combination with hTBP and the A and B PC fractions, in U6 transcription assays (J.Lewis, unpublished data). This suggests that PBP is probably stable to heparin−Sepharose fractionation but that another component required for U1 transcription, perhaps the TBP complex, is partially inactivated at this stage.

The similar heat sensitivity of TBP and PBP (see above) suggested that the two factors might be present as a single complex. We have, however, found no direct evidence to support this hypothesis. In fact, TBP depletion from these fractions or from the PC C fraction had no significant effect on the quantity of PBP detectable by electrophoretic mobility shift assay and addition of recombinant hTBP to heat-treated C fraction did not restore PBP complex formation (data not shown).

It remained possible that the TBP (complex) required for U1 transcription was identical to one of the complexes previously described as being competent to support pol II transcription (D-TFIID and B-TFIID/TFIIIB, Timmers and Sharp, 1991; Simmen *et al.*, 1992b) fortuitously present in the 0.5 M PC fraction as a contaminant from either the 0.7 M or the 0.3 M fraction. If this were the case, either purified D-TFIID or B-TFIID/TFIIIB should be able to reconstitute U1 transcription together with the TBP-depleted 0.5 M and the 0.1 M PC fractions.

To test the activity of the purified TBP complexes and to compare them with recombinant TBP and the Hep500 fraction, we first used the AdML promoter. The amount of D-TFIID and B-TFIID/TFIIIB utilized was quantified as follows. Recombinant human TBP and D-TFIID were equalized for their ability to reconstitute AdML transcription in a heat-treated extract. Then amounts of D-TFIID and B-TFIID/TFIIIB (Mono Q fractions, see Simmen *et al.*, 1992b) that contained the same amount of TBP, as determined by Western blotting, were used. Since comparatively little TBP was present in Hep500, we could only add as much of this fraction as possible within the volume constraints of the assays.

The combination of 0.1 M and TBP-depleted 0.5 M fraction allowed background levels of AdML and U1 transcription (Figure 6A, lanes 1 and 6). Either recombinant human TBP or purified D-TFIID strongly stimulated AdML transcription while the B-TFIID/TFIIIB fraction had weak activity (lanes 2, 3 and 5). The Hep500′ fraction had no effect on AdML transcription (lane 4) demonstrating that it contained no detectable free TBP. In contrast, Hep500′ was the only purified fraction that could stimulate U1 transcription (lanes 8−10) although to a significantly lesser extent than recombinant TBP (lane 7). Note, however, that there is a considerable (~100-fold) difference in the amount of TBP added in these two reactions. Both D-TFIID and the B-TFIID/TFIIIB fractions reduced human U1 transcription to a slight extent (lanes 8 and 10), and this inhibition increased in proportion to the amount of these fractions added to the assay (data not shown), ruling out the possibility that either is the essential TBP-containing complex required for U1 transcription or that the activity of the 0.5 M fraction is due to contamination with either of these TBP complexes. Note that the inhibition by purified B-TFIID/TFIIIB contrasts

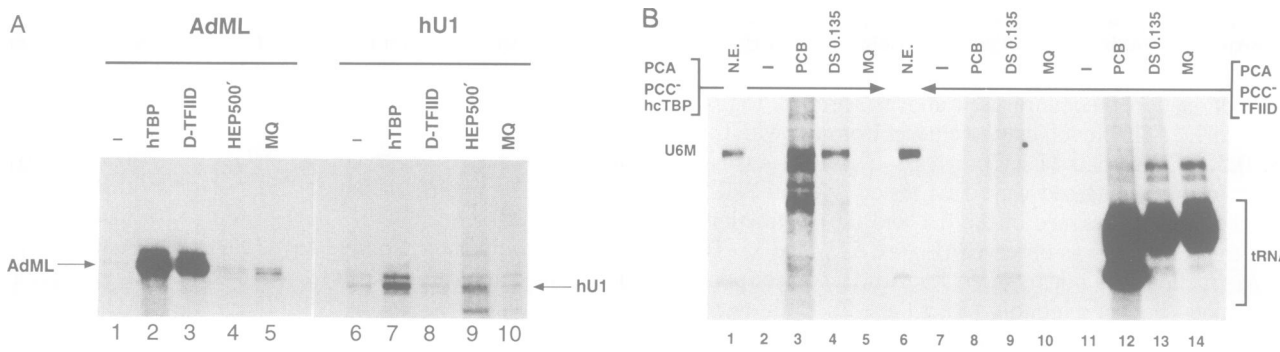

Fig. 6. Reconstitution of human U1 and U6 transcription in fractionated extracts with various purified TBP complexes. (**A**) Lanes 1–5 assayed TBP requirements for AdML and lanes 6–10 those for hU1. All lanes received 2 μl of mock-depleted PC 0.1 M fraction and 6 μl of TBP-depleted PC 0.5 M fraction. Additionally, lanes 1 and 6 received buffer; lanes 2 and 7 received 2.5 ng hTBP; lanes 3 and 8 received 4 μl of highly purified D-TFIID; lanes 4 and 9 received 4 μl of Hep500′; and lanes 5 and 10 received 4 μl of a Mono Q fraction containing B-TFIID/TFIIIB activity. Correctly initiated AdML and hU1 transcripts are indicated. (**B**) Complementation assay for human U6 and tRNA genes. Lanes 1–10 contain the hU6 maxigene (100 ng) and lanes 11–14 the Mcet1 $tRNA^{Pro}$ gene (100 ng). Except for lanes 1 and 6, all other lanes received 3 μl of PC A and 3 μl of TBP-depleted PC C (PCC^-) fractions; lanes 2–5 received 1 μl of hcTBP, and lanes 7–14 2 μl of highly purified D-TFIID. Additionally, 4μl of buffer (lanes 2, 7 and 11), PC B fraction (lanes 3, 8 and 12), DS 0.135 fraction (lanes 4, 9 and 13) and Mono Q fraction (lanes 5, 10 and 14) were added. Lanes 1 and 6 received 10 μl of unfractionated HeLa nuclear extract. The position of hU6 maxigene and tRNA transcripts is indicated.

with the stimulation observed with the PC 0.3 M fraction from which it is purified, indicating that a different component of the 0.3 M fraction is stimulatory.

To compare the TBP requirement of U1 as defined above with that of U6, we carried out a similar experiment with fractions required for U6 transcription. The PC A fraction was combined with TBP-depleted PC C fraction and recombinant TBP. No transcription was observed (Figure 6B, lane 2), demonstrating that factors other than TBP in the B fraction were required for U6 transcription. The PC B fraction, and the DEAE-Sephadex (DS 0.135) and Mono Q fractions derived from it (Simmen *et al.*, 1992b), were shown to contain similar amounts of TFIIIB activity by their ability to complement the TBP-depleted C fraction to reconstitute tRNA transcription (Figure 6B, lanes 11–14). These fractions were then tested for U6 activity. In combination with recombinant human TBP the TFIIIB fractions were able to generate U6 transcription in the reconstitution experiment (Figure 6B, lanes 2–5) suggesting that TFIIIB is required for U6 transcription, consistent with previous work (Waldschmidt *et al.*, 1991). The Mono Q fraction appeared significantly less active in U6 than in tRNA transcription when compared with DS 0.135, although reconstitution of U6 transcription with the Mono Q fraction was readily detectable after longer autoradiographic exposure (data not shown). Attempts to reconstitute U6 transcription activity by the addition of other Mono Q fractions have thus far not been successful (our unpublished data).

We wished to determine whether D-TFIID could substitute for recombinant TBP in U6 transcription. Although the D-TFIID fraction was highly active when tested with the AdML promoter (Figure 6A, lane 3) it was unable, either alone or in combination with the TFIIIB fractions, to reconstitute U6 transcription (Figure 6B, lanes 6–10). This was not attributable to a non-specific inhibitory effect of the D-TFIID fraction on pol III transcription, since it did not affect tRNA transcription (Figure 6B, lanes 11–14 and data not shown).

U1, but not U6, requires TFIIB

The discovery that a subunit of TFIIIB is highly related to the pol II basal factor TFIIB (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; López De León *et al.*, 1992) has led to the hypothesis that polymerase choice might occur in two steps. First a particular complex containing both TBP and a member of the TFIIB family would form on a promoter, and the nature of this complex would then specify the RNA polymerase chosen [see Rigby (1993) for a review]. Since the above results suggested that U6, but not U1, required TFIIIB we wished to test whether the converse would be true for TFIIB.

To determine whether TFIIB was required for U1 transcription we compared the sensitivity of U1 transcription with that of AdML transcription, which is known to require TFIIB, to inhibition by monoclonal anti-TFIIB antibodies. Transcription from both promoters was similarly inhibited (Figure 7A, lanes 1, 2, 5 and 6). Furthermore, addition of recombinant TFIIB in similar amounts was sufficient to restore both AdML and U1 transcription (lanes 3, 4, 7 and 8). The experiment in Figure 7A also included the addition of 2.5 ng TBP to each reaction. Similar results were obtained without TBP addition if 3- to 5-fold more recombinant TFIIB was added. This demonstrates that human U1 transcription requires TFIIB.

Addition of similar quantities of anti-TFIIB antibodies to U6 transcription reactions had no effect (data not shown) suggesting that TFIIB was not required by U6. However, when ~50-fold more antibody was added, significant inhibition was seen (Figure 7B, lanes 1 and 2). Two facts led us to believe that this effect was not due to TFIIB depletion. The first was the simultaneous reduction observed in tRNA transcription, which is thought not to require TFIIB (lane 2). The second was the failure to restore either U6 or tRNA transcription with recombinant TFIIB (lane 3). After examining the ability of various fractions to overcome the inhibitory effect, we discovered that addition of recombinant TBP, either in combination with TFIIB (lane 4) or alone (lane 5) could fully restore both U6 and tRNA transcription. Although we do not understand the basis of TBP depletion by the anti-TFIIB antibodies, these results strongly suggest that TFIIB is not required for human U6 transcription.

Discussion

We have begun a biochemical investigation of factors required for the *in vitro* transcription of human U1 snRNA

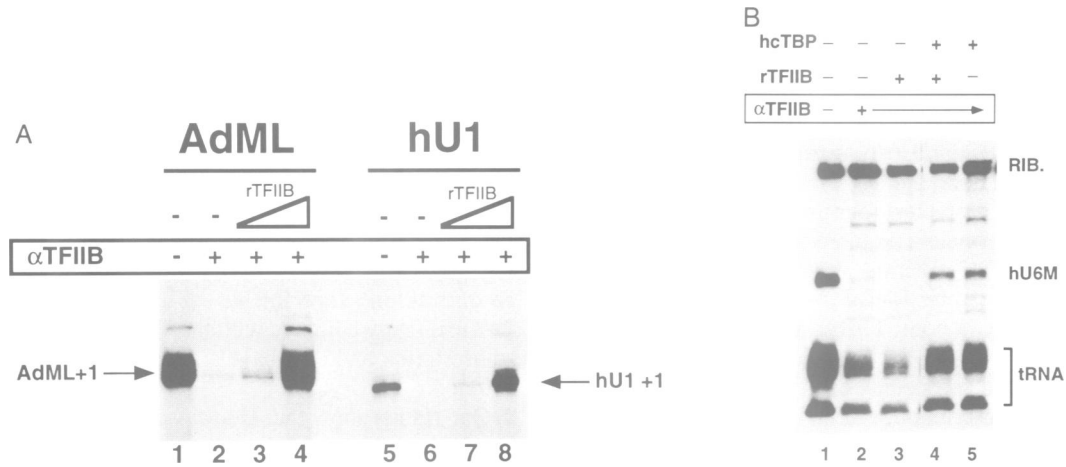

Fig. 7. TFIIB is required for human U1 but not for human U6 transcription. (**A**) TFIIB is required for hU1 transcription *in vitro*. All lanes contain 9 μl of nuclear extract preincubated on ice for 10 min with either 1 μl of a 1:40 dilution of the 2G8 anti-TFIIB monoclonal antibody (lanes 2–4 and 6–8) or buffer (lanes 1 and 5). Recombinant TFIIB [either 2 μl (lanes 3 and 7) or 4 μl (lanes 4 and 8) salts], nucleotides and templates [either 200 ng of AdML template (lanes 1–4) or 1 μg hU1G− (lanes 5–8)] were added. *In vitro* transcription was as described in Materials and methods. All lanes received, in addition, 5 ng of recombinant human TBP. The position of correctly initiated transcripts for AdML and hU1 are indicated. (**B**) TFIIB is not required for transcription of a human U6 maxigene *in vitro*. *In vitro* transcription reactions were performed essentially as described in Materials and methods, using 100 ng of a human maxigene and 7.5 ng Mcet1 $tRNA^{Pro}$ as templates. Before the addition of template, nucleotides and salts, the nuclear extract (6 μl) was preincubated on ice for 10 min in the absence (lane 1) or presence (lanes 2–5) of 1 μl of undiluted monoclonal antibodies (2G8) raised against recombinant human TFIIB. Recombinant human TFIIB (rTFIIB) or human core TBP (hcTBP) were added (0.1 μl each; as indicated by +) just prior to the addition of template, nucleotides and salts in lanes 3–5. Sample recovery (RIB), tRNA (tRNA) and U6 maxigene (hU6M) transcripts are indicated.

genes. Given the similarity of promoter structure exhibited by members of the vertebrate U snRNA gene family transcribed by pol II (e.g. U1) and by pol III (e.g. U6), it was of particular interest to search for similarities and differences in their factor requirements. Both classes of promoter appeared to utilize the same PBP, but differences were seen in the requirements of U1 and U6 for TBP and for TFIIB and TFIIIB. While previous studies have emphasized similarities in factor utilization by the two promoter classes, these are the first reported differences which help to explain the basis of alternative polymerase choice by the two promoters.

PSE-binding protein and U snRNA transcription

A fraction derived from HeLa cell extract required for the transcription of a mouse U6 snRNA gene was characterized by Waldschmidt *et al.* (1991). This fraction contained a factor, named PBP, which specifically bound to the PSE of the mouse gene. Subsequently, a correlation between the ability of various U6 PSE sequences to activate transcription *in vitro* and *in vivo* and to bind PBP *in vitro* was established (Simmen *et al.*, 1992a), supporting the hypothesis that PBP was involved in U6 transcription. Parallel studies of transcription of another human snRNA gene, that encoding 7SK RNA, led to the identification of a factor, named PSE transcription factor (PTF), whose properties suggest that it is likely to be identical to PBP (Murphy *et al.*, 1992). PTF, like PBP, was shown to bind to the PSEs of a number of mammalian U snRNA genes *in vitro*, including those with diverged sequences like that present in the human U1 gene. Like PBP, PTF showed highest affinity for the mouse U6 PSE (Murphy *et al.*, 1992; Simmen *et al.*, 1992a). Interestingly, two members of the octamer transcription factor family, Oct 1 and Oct 2, were shown to potentiate PTF binding to PSE sequences and the stimulation of 7SK transcription by PTF (Murphy *et al.*, 1992). This will be discussed further below. For the rest of this report we will use the PBP nomenclature.

Several lines of evidence suggest that transcription of U1, as well as that of U6, requires PBP. First, the same PBP-containing fraction is required for transcription of both genes. Second, the transcriptional activity of this fraction is very heat-labile, as is PBP binding to PSE-containing oligonucleotides *in vitro*. Third, oligonucleotides containing the mouse U6 PSE are effective competitors of both PBP binding and of human U1 gene transcription *in vitro*, while oligonucleotides containing mutant PSE sequences are not. Fourth, when either the human or mouse U6 PSE is introduced into the U1 promoter, or the U1 PSE into the U6 promoter, there is no difference in polymerase specificity or transcription efficiency between the hybrids and the original wild-type promoters. (J.Bernués and S.Gunderson, unpublished data). In combination, these results lead to the conclusion that transcription of human U1 snRNA genes, like that of U6 genes, requires PBP, although further work will be required to determine whether the two gene classes need additional (identical or different) components of the PBP fraction.

Other common requirements

PBP may not be the only factor required for both U1 and U6 transcription. It is interesting that both promoters show an absolute requirement for the 0.1 M KCl (or A) PC fraction. Several factors present in this fraction could provide a plausible explanation for this. First is TFIIA. It has recently been shown that U6 transcription *in vitro* requires TFIIA or a factor with extremely similar properties (Waldschmidt and Seifart, 1992; see also Reddy, 1988). U6 is the first pol III gene whose transcription has been shown to require this factor. It is, on the other hand, very plausible that pol II transcription of U1 would, in common with many other pol II genes (see Roeder, 1991, for a review), require TFIIA.

A second component of this fraction which may be required for both U1 and U6 transcription is Oct 1. As mentioned above, Oct 1 potentiates PBP binding to PSE-containing oligonucleotides *in vitro*, and the ability of PBP to stimulate 7SK transcription (Murphy *et al.*, 1992). Although neither U1 nor U6 transcription in fractionated extract is dependent upon the Oct-1-binding sites present in the DSEs of the two promoters (our unpublished data) it may be that Oct-1 is nevertheless required for their transcription. When the 0.1 M PC fraction was subjected to further chromatographic steps, U6 transcription activity was found to co-fractionate with both TFIIA and Oct 1 (J.Lewis, unpublished data).

In contrast, a third component of the 0.1 M KCl fraction implicated in U1 transcription, PSE-1/Ku (Gunderson *et al.*, 1990; Knuth *et al.*, 1990), did not cofractionate with U6 transcription activity. In fact, the most highly purified components derived from the A, B and C fractions that had U6 transcription activity contained little or no PSE-1/Ku as determined by Western blotting (S.Gunderson, J.Lewis, K.Simmen and R.Waldschmidt, unpublished data). This, coupled with the strong evidence implicating PBP in snRNA gene transcription (Waldschmidt *et al.*, 1991; Murphy *et al.*, 1992; Simmen *et al.*, 1992a; this paper), makes it unlikely that PSE-1/Ku is an essential, generally required, PSE-binding factor. Our results, however, do not rule out the possibility that PSE-1/Ku may play a role in U1 transcription since the most purified fractions sufficient for U1 transcription still contain considerable amounts of PSE-1/Ku (unpublished data). Indeed, a general role for PSE-1/Ku in pol II transcription has recently been proposed (Dvir *et al.*, 1992; Gottlieb and Jackson, 1993).

Differential TBP requirements for U1 and U6 transcription

The data presented here, together with previous studies (Lobo *et al.*, 1991; Simmen *et al.*, 1991; Waldschmidt *et al.*, 1991) demonstrate that, as for all other eukaryotic genes thus far examined (see Introduction) transcription of vertebrate U snRNA genes of both classes requires TBP. Our results suggest, however, that both have unusual TBP requirements. The TBP (or TBP complex) required for U1 snRNA transcription fractionates differently from the previously identified TBP complexes involved in pol I, II and III transcription in HeLa cell extracts [see Rigby (1993) for a review and references] and cannot be functionally substituted for by them (Figure 6). While TBP complexes have previously been observed in the same PC fraction as the U1 activity (Taggart *et al.*, 1992), our further fractionation on heparin–Sepharose (Figure 5) led to the conclusion that only a minority of the TBP in the 0.5 M PC fraction is active in U1 transcription (although we cannot rule out the possibility that the heparin–Sepharose chromatography resulted in a partial disruption of TBP complexes in this fraction). The low abundance has thus far hampered further characterization of the state of the TBP in the active fraction, but larger scale experiments should resolve this point. The nature of this complex is of particular interest since human U1 is the first genuine TATA-less pol II promoter whose transcription factor requirements have been examined, previous experiments with TATA-less promoters all having been carried out with artificial constructs that, unlike U1, require D-TFIID-containing fractions for their transcription (Smale *et al.*, 1990; Pugh and Tjian, 1990, 1991; Zhou *et al.*, 1992). With regard to this point it is of interest that the changes made to the Dignam *et al.* (1983) protocol that led to increased efficiency of U1 gene transcription (see Materials and methods) led to a drop in the efficiency of AdML transcription (J.Bernués, unpublished data). In parallel, we noted that comparatively little TFIID activity or TBP protein was present in the D fraction of the extracts optimized for U1 activity. This, together with the inhibitory effect of D-TFIID in the reconstitution experiments (Figure 6A), suggests that D-TFIID may compete with the TBP complex active in U1 transcription and thus have a negative effect on human U1 promoter activity.

Our results show that U6 transcription has different TBP requirements from U1. First, yeast TBP is capable of restoring U6 transcription activity to heat-treated HeLa cell extracts, but is incapable of restoring U1 transcription, suggesting that TBP has to interact with different components of the extract when involved in transcription from the two promoters. (Note that these experiments are all carried out in conditions where deletion of the enhancer-like DSE from the U1 or U6 promoter has no effect on transcription efficiency, and thus basal promoter activity was measured.) The second line of evidence is that U6 and U1 transcription *in vitro* require different TBP-containing fractions.

U6 transcription requires TFIIIB-containing fractions (Waldschmidt *et al.*, 1991; Figure 6B), as well as, apparently, a second component of the PC B fraction not required for tRNA transcription. This can be deduced from the differential loss of U6 and tRNA transcriptional activity upon Mono Q fractionation of TFIIIB activity (Figure 6). TFIIIB is a TBP-containing complex (Huet and Sentenac, 1992; Kassavetis *et al.*, 1992; Lobo *et al.*, 1992; Simmen *et al.*, 1992; Taggart *et al.*, 1992; White and Jackson, 1992). However, TFIIIB fractions alone are insufficient to fulfil the TBP requirement of the U6 gene. After fractionation, TBP addition was essential to obtain efficient transcription, suggesting that fractionation had disrupted or destroyed an essential component of the U6 transcription machinery which could be substituted by recombinant TBP. This alone would suggest that the U6 TBP requirement is different from that of other previously studied genes, since other promoters that show such behaviour have not been described. Together with the TFIIIB requirement, it furthermore suggests that U6 transcription involves more than one TBP molecule, and possibly more than a single TBP complex. It has previously been reported that D-TFIID-containing fractions can substitute for TBP in restoring U6 transcription in fractionated systems, albeit with very low efficiency (Waldschmidt *et al.*, 1991; Lobo *et al.*, 1992). However, highly purified D-TFIID does not have this ability (Figure 6B). We would suggest that the previous results may be explained by the presence of small quantities of free TBP in the crude D-TFIID fractions, and conclude that either free TBP or an unstable TBP complex is involved in U6 transcription.

Polymerase choice

Previous work has suggested that U6 transcription requires TFIIA (Waldschmidt and Seifart, 1992). The differential requirements of U1 and U6 transcription for TFIIB and TFIIIB described here are the first examples of dissimilar

requirements for basal factors in the transcription of these genes. We propose the following model for the differential ability of the promoters to attract TBP complexes. The only DNA sequence absolutely required for basal vertebrate pol II U snRNA gene transcription is the PSE (reviewed by Dahlberg and Lund, 1988; Parry *et al.*, 1989; Bernués *et al.*, 1992). PBP bound to the PSE, perhaps in combination with TFIIA and/or Oct 1 or other members of the Oct family (Murphy *et al.*, 1992) would bind to the form of TBP identified here as being necessary for U1 transcription, possibly via a tethering factor or TFII-I (see Pugh and Tjian, 1990, 1991; Roeder, 1991). This complex would then attract TFIIB and, from that point, pol II transcription complex assembly could follow its conventional route (reviewed by Roeder, 1991). In contrast, we would propose that PBP and either free TBP or an unstable TBP complex would interact with the PSE and TATA sequences of the U6 promoter respectively. The combination of these factors, again probably in conjunction with Oct 1 and TFIIA, would act as assembly factors for the pol III transcription factor, TFIIIB. These factors would thus be functionally analogous to TFIIIC in tRNA, or to TFIIIA plus TFIIIC in 5S rRNA transcription (Kassavetis *et al.*, 1990). In this model, the ability of the two classes of U snRNA promoter to select different polymerases would be explicable by their interaction with alternative TBP complexes, just as has been previously proposed for other pol I, II and III promoters (see Rigby, 1993, for review) whose structures are much more obviously different from one another than are those of the human U1 and U6 genes.

Materials and methods

Nuclear extract preparation and fractionation

HeLa cell nuclear extracts were prepared as described by Dignam *et al.* (1983) except that a reduced volume of buffer C was used in the extraction step. The amount of buffer C used corresponded to one-third of the packed cell volume. For pol III transcription studies the extracts were fractionated over PC (Whatman P11) into four fractions according to Segall *et al.* (1980). The protein concentration of the fractions used here were PC A (100 mM flowthrough, 4 mg/ml protein), PC B (at 0.35 M KCl, 2 mg/ml protein), PC C (at 0.6 M KCl, 0.9 mg/ml protein) and PC D (at 1 M KCl, 0.7 mg/ml protein). Similar concentrations were obtained from repeated fractionations.

PC C fraction (containing PBP activity) was further fractionated over heparin−Sepharose and fractions were collected at 100, 250 and 500 mM KCl. The protein concentration of the heparin−Sepharose fractions used were 0.25, 0.4 and 0.9 mg/ml respectively. PC B fraction was further fractionated over DEAE-Sephadex and subsequently over Mono Q as recently described (Simmen *et al.*, 1992b). All fractions were extensively dialysed against buffer D (100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 20 mM HEPES, pH 7.9, 0.1 mM PMSF) and stored at −80°C until use.

For pol II transcription studies nuclear extracts were fractionated over PC (Whatman P11) essentially as described by Reinberg and Roeder (1987). The fractions utilized in the experiments shown were collected at 0.1 M (4 mg/ml protein), 0.3 M (2.9 mg/ml protein), 0.5 M (2.3 mg/ml protein), 0.7 M (0.83 mg/ml protein) and 1.0 M KCl (0.29 mg/ml protein). Similar concentrations were obtained in several fractionations. Fraction PC 0.5 M was further fractionated over heparin−Sepharose exactly as described above for the PC C fraction. All fractions were extensively dialysed against buffer D (100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, 20 mM HEPES, pH 7.9, 0.1 mM PMSF) and stored at −80°C until use.

DNA templates and expression constructs

For pol II transcription the templates used were: AdML404[180] G− and AdML50[180] G− (interchangeably used as AdML; Simmen *et al.*, 1991), HTFR G− and hU1 G− (Gunderson *et al.*, 1990).

For pol III transcription the templates were a human U6 maxigene (hU6RVM, Simmen *et al.*, 1991) and a nematode $tRNA^{Pro}$ gene (Mcet1, Ciliberto *et al.*, 1982).

The expression plasmid containing the full-length human TBP cDNA 6×His-tagged at the N-terminus was provided by A.Hoffmann and R.Roeder (The Rockefeller University, New York). The human TBP core plasmid was obtained by subcloning a 6×His-tagged C-terminal fragment coding for amino acids 155−335 into the pET vector (provided by A.Berkenstam and H.Stunnenberg, EMBL, Heidelberg). The yeast TBP expression construct used was described by Burton *et al.* (1991): The human TFIIB expression plasmid (Ha *et al.*, 1991) was a gift of D.Reinberg.

In vitro transcription

Pol II reactions were in a final volume of 25 μl and contained 3.5 mM $MgCl_2$, 1.2 mM 3′-*O*-methyl-GTP (Pharmacia), 0.4 mM ATP, 0.4 mM CTP, 2 units RNase T1, 1.25 mM DTT, 2% polyethylene glycol 8000 (Sigma), 0.2 mM EDTA, 20 μCi [α-^{32}P]UTP (800 Ci/mmol), 33 mM HEPES, pH 7.9 and nuclear extract equivalent to 150−170 μg of protein. For *in vitro* reconstitution experiments fractions were used as described in each case. $MgCl_2$ concentration was always adjusted to 5 mM for AdML transcription.

DNA templates were used at 1 μg (hU1 G−), 0.2 μg (AdML) and 0.35 μg (HTFR G−) per reaction. Transcription was allowed to proceed for 60−90 min at 30°C and samples were processed as described by Gunderson *et al.* (1990). In all cases an unrelated riboprobe was added after the transcription reaction was completed as a recovery control. Transcripts were analysed on 8% denaturing poylacrylamide gels, dried and exposed to X-ray sensitive film (Kodak) at −80°C with intensifying screens.

Mapping of start sites by primer extension used an oligonucleotide complementary to sequences between +59 and +41 in the human U1 and HTFR G− cassettes. The same oligonucleotide was complementary to +64 to +46 of the AdML G− cassette used. Conditions for primer extension were exactly as in Gunderson *et al.* (1990). Pol III transcription conditions were as described by Simmen *et al.* (1991) except that PC A, B and C fractions prepared as described above were used.

TBP-immunodepletion of PC fractions and immunoblot analysis

The fractions, as described in the text, were TBP-immunodepleted with a rabbit polyclonal anti-hTBP antibody prepared and used as described in Simmen *et al.* (1992b). Mock-depletion was performed in exactly the same way but using preimmune serum. The presence of TBP in fractions was assayed using 100 μl of the indicated fractions with the ECL system (Amersham) for Western blotting (Simmen *et al.*, 1992b).

Gel retardation assays

Gel retardation assays were performed using conditions modified from those described by Fried and Crothers (1981). The protein fractions were pre-incubated with 600 ng poly[d(I·C)] in 1 × binding buffer (5% Ficoll, 100 mM KCl, 20 mM Tris−HCl, pH 8, 1 mM DTT) at 30°C for 30 min. Then 20 000 c.p.m. of a kinased probe was added and incubation continued for a further 30 min. DNA−protein complexes were then resolved on a 6% non-denaturing polyacrylamide gel in 0.5 × TBE. The probe contained the mouse U6 gene promoter sequences from −80 to −4 (Ohshima *et al.*, 1981) and was excised from a PCR-generated derivative of the mouse U6 gene. The sequences of the two oligonucleotides used for the competition experiments are given below.

WT:
ACAAAAGGAAACTCACCCTAACTGTAAAGTAATTGTGTGTT
TGTTTTCCTTTGAGTGGGATTGACATTTCATTAACACACAA

MT:
ACAAAAGGAAACTAAGATCTGCTGTAAAGTAATTGTGTGTT
TGTTTTCCTTTGATTCTAGACGACATTTCATTAACACACAA

TBP overexpression and purification

Overexpression of the full-length and core human TBP proteins was as follows: *Escherichia coli* BL21 (LysE) transformants were grown in Superbroth, supplemented with 4% glucose, 25 μg/ml chloramphenicol and 100 μg/ml ampicillin, at 30°C until the OD_{600} was ~0.7. At that point IPTG was added to 0.5 mM and cultures were grown for another 2 h at 30°C. Cells were then harvested by centrifugation, washed and lysed with 30 ml of lysis buffer (0.5 M NaCl, 20% glycerol, 1 mM EDTA, 20 mM 2-mercaptoethanol, 20 mM HEPES, pH 7.9, 0.1% NP40, 1 mM PMSF, 20 μg/ml pepstatin A, 20 μg/ml leupeptin, 1% aprotinin) per litre of culture by extensive sonication on ice. Cell debris were pelleted by ultracentrifugation (100 000 *g*, 4°C, 1 h) and the supernatant was further clarified by passing through a DEAE-Sephadex A-25 column equilibrated in the same buffer. The flow-through was collected and applied to a 0.5−1 ml Ni^{2+}-NTA

column (Diagen) equilibrated in lysis buffer. After loading, the resin was extensively washed, first with lysis buffer followed by D buffer and finally eluted with 3–5 vol of D buffer containing 100 mM imidazole. Active fractions were pooled, aliquoted, frozen in liquid N_2 and stored at −80°C. Recombinant yeast TBP was expressed in *E.coli* and purified as described in Burton *et al.* (1991).

TFIIB

Recombinant TFIIB was expressed in *E.coli* and purified as described (Ha *et al.*, 1991). The 2G8 monoclonal anti-TFIIB antibody (V.Moncollin, unpublished data) was purified as follows. 1 ml ascites fluid was diluted 1:10 with phosphate-buffered saline (PBS) and passed over a protein A–Sepharose column. The column was washed with 100 ml of PBS then eluted with 0.05 M sodium acetate, pH 4. Fractions (1.5 ml) were neutralized with 750 μl 1 M Tris–HCl pH 8.0 and dialysed against buffer C (see D-TFIID purification section) before use.

Endogenous D-TFIID purification

D-TFIID was purified from HeLa whole cell extract by sequential chromatography on Heparin-Ultrogel and DEAE-5PW (Moncollin *et al.*, 1986) followed by hydrophobic and DNA-affinity chromatography as described below. The DEAE 0.25 M KCl active fractions were pooled, adjusted to 0.9 M $(NH_4)_2SO_4$ with solid ammonium sulphate and loaded onto a TSK-Phenyl-5PW column (2.15 cm × 15 cm, flow-rate 2.5 ml/min) equilibrated with buffer A [59 mM Tris–HCl, pH 7.9, 50 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 8.7% glycerol and 0.9 M $(NH_4)_2$ SO_4]. After washing with buffer A, the proteins were eluted with a 250 ml linear gradient from 0.9 to 0 M $(NH_4)_2SO_4$ in buffer A. The active fractions, eluted at 0.2 M $(NH_4)_2SO_4$, were pooled (50 ml) and dialysed against buffer B (50 mM Tris–HCl, pH 7.9, 50 mM KCl, 17.5% glycerol, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM DTT). This fraction was then incubated at 4°C for 15 min with 100 μg/ml of poly(dG-dC) and 5 mM $MgCl_2$. 2 ml of TATA-containing DNA affinity resin (Moncollin *et al.*, 1990) were added and the incubation continued for 15 min. The resin was then packed and washed with 10 column volumes of buffer B. Proteins were eluted in fractions of 1 ml with buffer B containing increasing concentrations of KCl (in increments of 0.1 M). The D-TFIID-containing fractions (eluted at 0.4–0.5 M KCl) were dialysed against buffer C (50 mM Tris–HCl, pH 7.9, 50 mM KCl, 17.5% glycerol, 0.1 mM EDTA, 0.5 mM DTT) and stored at −80°C.

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