TFIIA Is Required for \textit{in Vitro} Transcription of Mammalian U6 Genes by RNA Polymerase III*

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Transcription factor TFIIA, defined by its role in transcription by RNA polymerase II, is also involved in RNA polymerase III transcription of mammalian U6 small nuclear RNA genes. This finding was substantiated by experimental evidence including (i) extensive copurification of an activity required for U6 transcription with TFIIA, (ii) the comparable molecular dimensions of this activity and TFIIA, (iii) the identical heat stability of both activities, and (iv) functional analyses revealing that TFIIA facilitates the interaction of TFIID with the TATA box of the U6 gene. As was shown previously for TFIID, TFIIA is the second basal transcription factor which could be demonstrated to be involved in gene expression by two different RNA polymerases.

Transcription initiation from eukaryotic promoters requires the association of RNA polymerase (pol)† with accessory components, the so called general transcription factors (TF). Basal level transcription by RNA polIII is mediated by a group of general TF including TFIIA, TFIIIB, TFIID, TFIIIE, and TFIIIF (for review see Sawadogo and Sentenac, 1990). Stable binding of TFIID to the TATA box element is the first and prerequisite step for the formation of the preinitiation complex on pol genes. Human TFIID has recently been cloned (Hoffmann et al., 1990; Peterson et al., 1990; Kao et al., 1990). Its C-terminal domain contains both the activities for DNA binding and for interactions with other general TF (Peterson et al., 1990), whereas its N-terminal part is thought to be responsible for the activation by regulatory factors (e.g. Sp1; Peterson et al., 1990; Pugh and Tjian, 1990) either directly or through coactivators. TFIIA is one of the basal polIII transcription factors associated with the TFIID-DNA complex. It was shown to stabilize the TFIID-TATA box interaction, thereby defining the rate-limiting step in transcription preinitiation complex formation on polIII genes (Reinberg et al., 1987; Buratowski et al., 1989). The exact mechanism of TFIIA action is still unclear. Mammalian TFIIA activities were associated with proteins of 13 and 19 kDa from calf thymus (Samuels and Sharp, 1986), with a 43-kDa actin-like protein (Egly et al., 1984) and very recently with a 38-kDa polypeptide from HeLa cells using yeast TFIID affinity chromatography (Usuda et al., 1991).

Mammalian splicesome Usn RNA gene promoters are bipartite in structure. They comprise a proximal promoter and a distal enhancer-like region, which contains an octamer motif (reviewed in Kunkel, 1991). Unlike U1–U5 small nuclear RNA genes, which are transcribed by polII, U6 RNA is transcribed by polIII. The proximal promoter of U6 genes consists mainly of two elements, the proximal sequence element (PSE) located at approximately −60 and a TATA box-like element centered around −27 (Kunkel, 1991). PSE sequences are found in all mammalian Usn RNA promoters and can functionally be interchanged between polII and polIII transcribed U2 and polIII transcribed human U6 genes without altering polymerase specificity (Lobo and Hernandez, 1989). The TATA box-like element, which is absent from polIII transcribed U1–U5 promoters, is essential for vertebrate U6 transcription and acts as a major determinant for polIII specificity of this promoter (Simmen and Mattaj, 1990; Lobo and Hernandez, 1989).

Attempts to isolate and characterize protein components involved in the basal transcription of mammalian U6 genes by RNA polIII have hitherto been impeded by the lack of a suitable \textit{in vitro} transcription system. Very recently we could develop a reconstituted \textit{in vitro} transcription system from HeLa cells which efficiently supports mammalian U6 gene expression (Waldschmidt et al., 1991). Using this system, at least four separate protein components were found to be required in addition to polIII for faithful expression of U6 RNA \textit{in vitro}. These correspond to TFIIIB, TFIIID (Waldschmidt et al., 1991; Simmen et al., 1991; Lobo et al., 1991), a protein specifically binding to the PSE (PBP, PSE binding protein, see also Simmen et al., 1992) and finally an activity in the flow-through after phosphocellulose chromatography (Waldschmidt et al., 1991).

In this report evidence is presented showing that the activity in the latter fraction, which is essential for efficient U6 transcription \textit{in vitro}, could be correlated by its copurification over at least five different chromatographic columns and by the number of its physical and functional properties with the basal polIII transcription factor IIA. TFIIA could be demonstrated to facilitate the interaction of TFIID with the TATA box of the U6 gene.

\section*{MATERIALS AND METHODS}

\textbf{Plasmids and Synthetic Double-stranded DNA Oligomers}—The plasmids pUmU6m, and pUmU6s, containing the mouse U6 gene from bp $-150$ to $+190$ or from $-150$ to $+5$ were as described previously (Waldschmidt et al., 1991). The plasmids HLS10 and HLS4 were created by linker-scanning mutagenesis in the background of a human U6 maxigene (Simmen et al. 1991). In the transcriptionally active

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\item †To whom correspondence should be addressed.
\item ‡The abbreviations used are: pol, RNA polymerase; TF, transcription factor; AdMLP, adenovirus major late promoter; HSP70, heat shock protein 70; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PSE, proximal sequence element; PBP, PSE binding protein; bp, base pair(s); WCE, whole cell extract; FPLC, fast liquid protein chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DSE, distant sequence element.
\end{itemize}
RESULTS

The first part of the analyses concentrated on the purification and identification of a human TFIIA (like) activity involved in mammalian U6 transcription in vitro. In the second part, the role of TFIIA in the formation of functional transcription complexes on mammalian U6 genes was studied.

As shown previously (Egly et al., 1984; Samuels and Sharp, 1986) TFIIA, an activity which does not bind to phosphocellulose resins under the experimental conditions tested, plays a central role in addition to TFIID in the basal expression of polII genes from TATA box containing mRNA promoters (Fire et al., 1984). On the basis of the requirement for TFID in the TATA box-dependent expression of the mammalian U6 gene (Waldschmidt et al., 1991; Simmen et al., 1991; Lobo et al., 1991) and in conjunction with our previously reported observation that phosphocellulose flow-through fraction PCAA is required for polII transcription reactions in vitro, TFIIA was a likely candidate for an as yet unidentified U6 transcription factor contained in this fraction.

Following and extending various purification schemes reported previously (Egly et al., 1984; Samuels and Sharp, 1986), fraction PCAA was fractionated through sequential chromatography on a heparin-Sepharose, DEAE-Sephacel, FPLC Mono Q, and hydroxyapatite as described under “Materials and Methods.”

As a first step, TFIIA activity in individual fractions was estimated by employing the established effect of this protein on polII transcription reactions in vitro, and for this purpose expression of the histone H5 gene was investigated. As shown in Fig. 1A, a TFIIA-depleted HeLa WCE (WCEΔ, see “Materials and Methods”) which has only basal activity in vitro run off transcription of the H5 gene (lane 2) could efficiently be stimulated by the addition of phosphocellulose (PCA, lane 3; PCAA, lane 4) and heparin-Sepharose (H5Δ, lane 6) breakthrough fractions, the DAE-SEphacel 0.2 and 0.3 M KCl eluate (DSC0.2, DSC0.3, lanes 8 and 9), the Mono Q 0.4 M KCl
fraction (MQ0.4, lane 13) as well as by the hydroxylapatite 0.3 M potassium phosphate fraction (HyAo.3, lane 14). The fractionation behavior of the TFIIA activity described here could also be demonstrated for the reconstitution of in vitro transcription from the Ad2MLP (data not shown) and exactly corresponds to that reported previously by different investigators (Samuels and Sharp, 1986; Usuda et al., 1991).

As shown in B of Fig. 1, parallel analysis of the column fractions in a reconstituted U6 transcription assay, containing phosphocellulose fractions PCB and PCC, the same protein fractions PCA (lane 3), PCAA (lane 4), HS0.3 (lane 6), DSC0.2,0.3 (lanes 8 and 9), Mono Q0.4 (lane 13) and HyA0.3 (lane 14) could be identified to contain an essential U6 transcription factor. Thus, the U6 TF exactly copurified with TFIIA over at least five different chromatographic resins.

SDS-PAGE Analysis of TFIIA/TFU6 Containing Protein Fractions—Transcriptionally active fractions from each purification step were analyzed by SDS-PAGE. Fig. 2 shows that fraction HyA0.3 (lane 7) obtained after hydroxylapatite contains, among several other proteins including low molecular weight components, a predominant polypeptide which migrated with a denatured molecular weight of approximately 38 kDa, indicating that the chromatographic purification performed here selectively enriched a polypeptide of this molecular mass. Comparable molecular dimensions were recently reported for hTFIIA (Usuda et al., 1991). Although these data suggested that TFIIA is the transcription factor required for U6 transcription in vitro, additional experiments were performed to further strengthen this assumption.

Glycerol Gradient Centrifugation Analyses Revealed Similar Molecular Dimensions of hTFIIA and the TFIIA-like U6 TF—Analytical glycerol gradient rate zonal centrifugation of fraction HyA0.3 was performed as described under "Materials and Methods." Fractions of the gradient were functionally identified by their ability to reconstitute in vitro transcription of the histone H5 (or Ad2MLP, data not shown) or the U6 gene. As shown in Fig. 3 the activity, functionally identified on both genes, sedimented as a single peak with a native molecular mass of 38 ± 10 kDa, supporting the findings obtained in Fig. 2. This result is in good agreement with the molecular dimensions of hTFIIA obtained after SDS-PAGE (Usuda et al., 1991) and glycerol gradient centrifugation performed by other investigators (Samuels and Sharp, 1986; Usuda et al., 1991). The low transcriptional activity observed for H5 and U6 expression in the bottom of the gradient (Fig. 3, fraction 12) was repeatedly observed and could be due to protein aggregation possibly caused by self-assembly of the 38-kDa polypeptide as was also discussed by Egly et al. (1984) and Usuda et al. (1991).

Heat Inactivation of Fraction HyA0.3—Transcription factor activity in fraction HyA0.3 was relatively stable at elevated incubation temperatures. As shown in Fig. 4, incubation for 10 min at 60 °C yielded 50% transcriptional activity in both histone H5 and U6 transcription. Heating at 80 °C, however, completely inactivated transcription factor activity. Comparable or slightly higher heat stabilities were reported previously for hTFIIA fractions depending on their state of purification (Samuels et al., 1982; Samuels and Sharp, 1986).

Functional Analysis of TFIIA in the U6 in Vitro Transcription: Fraction TFIIA does Not Influence Unspecific RNA Polymerase II or III Activity—Fraction HyA0.3 containing TFIIA, was analyzed for its ability to stimulate RNA polymerase II or III activity in a nonspecific ribonucleotide incorporation assay. As demonstrated in Fig. 5, fraction HyA0.3 free of any detectable RNA polymerase II or III activity (bars E), was not able to stimulate either polII (left part of Fig. 5, bars B–D) or polIII (right part of Fig. 5, bars B–D)–dependent transcription.

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RNA synthesis from unspecific calf thymus or pUC (data not shown) DNA. These results clearly demonstrate that transcriptional activation of H5 and U6 genes evoked by fraction HyAo.3 was not due to a nonspecific effect.

Effect of TFIIA on TFIIID-DNA Binding—The effect of TFIIA on TFIIID binding to the TATA box element of the histone H5 and the U6 gene was examined by mobility shift assays using partially purified recombinant human TFIIID and probes containing the H5 TATA box or the U6 TATA box. As shown in Fig. 6A, a weak complex of TFIIID and the U6 probe could be obtained (lanes 2–4, IID). The addition of purified TFIIA fraction HyAo.3 led to stabilization of the TFIIID-DNA interaction resulting in the formation of an additional complex of lower mobility (lanes 6–8, IID-IIIA). The observed DNA-protein interaction is specific for the U6 TATA box, since competition with plasmid DNA containing a U6 (HLS10, lane 9; hU625, lane 11) or classical polII TATA box (AdMLP, lane 13; HSP70, lane 14) completely abolished complex formation. DNA containing a transcriptionally inactive U6 gene lacking the TATA box (HLS4, lane 10) or containing the U5 promoter upstream of the TATA box (hU6despse, lane 12) did not serve as a competitor.

Comparable results were also obtained using the TATA box of the H5 gene as a probe (Fig. 6B). It could be demonstrated that TFIIA also stabilizes the sequence-specific interaction of TFIIID with this polII TATA box promoter element as already reported by other investigators for different polII genes (Buratowski et al., 1989; Usuda et al., 1991).

The effect of TFIIA on the binding of TFIIID to the U6 gene promoter sequence was further analyzed by DNase I footprinting. As shown in Fig. 7, increasing amounts of a protein fraction, containing purified TFIIID expressed in E. coli, protected the coding strand of the U6 probe from bp –32 to –18 (indicated by an open bar) encompassing the U6 TATA box (i.e. bp –31 to –26, solid bar) and generated hypersensi-
It has been shown previously that efficient expression of mammalian U6 genes in vitro requires at least four separate protein components in addition to RNA polymerase III. They correspond to TFIIB, TFIID, PBP, and a component in the flow-through upon chromatography on phosphocellulose (Waldschmidt et al., 1991). In the present report, the activity contained in the latter fraction was partially purified and further characterized as a polypeptide resembling human TFIIB, which stabilizes the interaction of TFIID with the TATA box sequence of mammalian U6 genes.

U6 Transcription in Vitro Requires TFIIB—The activity required for U6 transcription in vitro, which is contained in the purified protein fraction HyA0.9, has been designated as TFIIB on the basis of the following experimental observations.

First, chromatographic behavior of the U6 transcription factor activity on phosphocellulose, heparin-Sepharose, DEAE-Sepharose, DEAE-Sephacel, FPLC Mono Q, and hydroxylapatite columns was identical to that observed for TFIIB and agrees with previous reports dealing with the purification of mammalian TFIIB (Egly et al., 1984; Samuels and Sharp, 1986; Usuda et al., 1991). Due to its different chromatographic behavior, it could also be ruled out that the factor required for U6 transcription is identical to TFIIF, shown previously to be functionally related to TFIIB (Sumimoto et al., 1990).

Second, the native and denatured molecular dimensions of the activity required for U6 transcription (approximately 38 ± 10 kDa) corresponded to that reported for mammalian TFIIB (Egly et al., 1984; Samuels and Sharp, 1986; Usuda et al., 1991). Third, functional analyses concerning the stability of the U6 transcription factor against heat treatment demonstrated similar sensitivities of both TF activities at temperatures already reported for TFIIB inactivation (Samuels et al., 1982; Samuels and Sharp, 1986).

Additional evidence for the correlation between the factor required for U6 transcription and TFIIB can also be derived from preliminary template commitment assays, showing that the TF activity contained in the most purified protein fraction (HyA0.9) is incorporated into functional transcription complexes formed on polII (H5 or AdMLP) genes and the U6 gene in vitro when nonlimiting amounts of TFIID were used during the preincubation (data not shown).

Moreover, recent data suggest that transcription factor IIA may also be involved in the expression of “classical” polIII genes governed by internal promoters. The exact mechanism by which this occurs is currently under investigation and will be published elsewhere. Final proof that one form of TFIIB (like activity) is involved in transcription by both RNA polymerases II and III from internal and external promoter sequences can only be rendered by the reconstitution of appropriate in vitro transcription systems with recombinant human TFIIB which is, however, not yet available.

Functional Role of TFIIB during U6 Gene Expression—The general transcription factor TFIIB is known to stimulate basal polII gene expression from TATA box containing promoters by facilitating or stabilizing the binding of TFIID to its target sequence (Reinberg et al., 1987; Buratowski et al., 1989). On the basis of the requirement for TFIIB in the TATA box-dependent mammalian U6 gene expression (Simmen et al., 1991; Waldschmidt et al., 1991; Lobo et al., 1991) in conjunction with the identification of TFIIB as a transcription factor required for U6 transcription, the question of how TFIIB acts in the transcription of the U6 gene by polIII was investigated.

From the results presented in Fig. 5, it can be excluded that TFIIB activates faithful H5 and U6 in vitro transcription.

**DISCUSSION**

Fig. 7. DNase I protection of the U6 promoter by TFIID and TFIIB. The protein fractions IID and HyA0.9 were incubated with the 210-bp EcoRI/HindIII fragment of pUmU6.1ss as described previously (Waldschmidt et al., 1991). Treatment with DNase I digestion are protected from DNAse I digestion are contained 5'-12' that could be observed, but the amount of TFIID required for protection was at least 2-fold reduced (Fig. 7).

The PSE (bp -68 to -49) and TATA (bp -31 to -26) sequences of the U6 promoter are indicated by solid bars. Sequences protected from DNase I digestion are boxed by open bars and correspond to bp -32 to -18 for TFIID-DNA interactions. M, radioactively labeled pBR322 HpaII digest served as a size marker. Lower part, schematic representation of sequences protected from DNase I digestion (thick lines) by PBP (bp -79 to -37 on the noncoding and bp -78 to -42 on the coding strand; from Waldschmidt et al., 1991) and TFIID (bp -33 to -19 on the noncoding and bp -32 to -18 on the coding strand) in the 5'-flanking region of the mouse U6 promoter. The PSE and TATA consensus sequences are indicated by thin lines.
simply by stimulation of general RNA polymerase II or III activity.

**TFIIA Facilitates TFIIID Binding on Both U6 and PolII TATA Box Promoter Sequences**—Mobility shift analyses revealed only a weak binding of TFIIA alone to DNA fragments containing the U6 or H5 TATA box, respectively. This could conceivably be due to the low affinity of TFIIA to the TATA sequences tested and/or to the stability (e.g. the off rate) of the TFIIA-DNA complex in the mobility shift assay, in which case the protein-DNA interaction must remain stable over the duration of electrophoretic separation. Under the mobility shift conditions used (low concentration of MgCl₂), detection of TFIIID-TATA complexes have previously been described to be critical (Buratowski et al., 1989; Usuda et al., 1991), but this experimental approach nevertheless provides an ideal system for the detection of stabilizing effects of TFIIA on TFIIID-DNA interactions.

Purified TFIIA fractions did not form a detectable protein-DNA complex, suggesting that TFIIA does not bind to U6 or H5 promoter sequences around the TATA box in a primary fashion, an effect already reported for this factor on various polII genes (Usuda et al., 1991). However, addition of TFIIA clearly enhanced the binding of TFIIID to both TATA box elements and resulted in a new complex of lower mobility, probably containing both polypeptides. Competition experiments using either various U6 promoter mutants or typical polII TATA elements, unequivocally show that the TFIIID-TFIIA-DNA complex forms very specifically and stably on both tested TATA box sequences. Interestingly, competition analyses further demonstrated functional similarities of the tested TATA box-containing fragments, indicating that both the U6 and the polII TATA elements could possibly be interchangeable in their ability to stably incorporate the TFIIID polypeptides. After completion of this manuscript, this effect was further supported by experiments, where polII TATA elements could functionally replace the U6 TATA box in the expression of U6 genes (Lobo et al., 1991). As demonstrated by the appearance of the TFIIID-TFIIA-DNA complex, TFIIA remains stably associated after binding of TFIIID to the U6 TATA box, thus possibly resembling TF assembly during polII transcription complex formation (Buratowski et al., 1989).

By the footprint analyses presented, it could be shown for the first time that TFIIID binds to the TATA box of the U6 gene. Recombinant TFIIID binds specifically to the -32 to -18 region of the coding and to bp -33 to -19 of the noncoding strand of the mouse U6 gene. Although identical with respect to the boundaries of the TATA box protection, a clear difference in the footprint pattern between recombinant human TFIIID and the purified TFIIA fraction from HeLa cells could be observed. A hypersensitive site near the initiation region could only be detected when purified HeLa TFIIA was used, possibly reflecting an additional or modified protein component associated with the HeLa TFIIA activity (data not shown). By addition of TFIIA to the binding reaction, enhanced binding of TFIIID (at least 2-fold) could be observed. The effect of TFIIA on TFIIID-DNA interaction in the footprint assay was less pronounced than that observed in the mobility shift analyses (compare Figs. 6 and 7). This could be due to the fact that the function of TFIIA is to stabilize TFIIID-DNA complexes rather than to modify the intrinsic DNA binding properties (i.e. affinity or specificity) of TFIIID. As observed by Buratowski et al. (1989) and Usuda et al. (1991) addition of TFIIA evokes an extension from 0 to 4 bp on both strands of the TATA box footprint depending on the polII gene that was analyzed. Addition of TFIIA to the TFIIID-U6 TATA box binding reaction does not result in a detectable extension of the footprint boundaries. This observation could conceivably be due to the specific architecture of the U6 transcription complex which also requires the binding of the PBP in close proximity of the TATA box (Waldschmidt et al., 1991; see also the lower part of Fig. 7). Moreover, the employed footprinting assay would possibly not resolve slight extensions of the TFIIID footprint caused by TFIIA addition, because no DNAse I cleavage sites could be obtained in the region of interest.

To clarify the actual function of TFIIA in the mechanism of polII transcription preinitiation complex formation on the basal mammalian U6 gene promoter, additional experiments employing highly purified, or recombinant transcription components (i.e. TFIIA, TFIIIB, PBP, polII) will be necessary. The identification and characterization of TFIIA as a component required for efficient U6 gene expression in vitro, facilitating TFIIID binding to the U6 TATA box, is another important step toward this goal.

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