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Transcription factor IIA stimulates the expression of classical polIII-genes

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ABSTRACT

Protein fractions containing TFIIA, a transcription factor known to be involved in transcription initiation by RNA polymerase II and 5'-regulated polymerase III genes (e.g. U6), were tested for their role in *in vitro* transcription of classical pol III genes. These fractions were shown to stimulate a basal transcription system, reconstituted from highly purified fractions hTFIIIB and hTFIIIC. We demonstrate that this stimulating activity isolated from HeLa cells coelutes over at least six chromatographic steps with hTFIIA. Moreover the native molecular mass and the stability of this activity against heat treatment are comparable to those of hTFIIA. Finally we show that recombinant TFIIA from Saccharomyces cerevisiae can substitute for the human factor in pol III transcription *in vitro* which proves that TFIIA is also involved in the efficient expression of classical pol III genes.

INTRODUCTION

The correct transcription initiation from promoters by purified eukaryotic RNA polymerases requires auxiliary protein components called transcription factors (TF). In the case of RNA polymerase III from mammalian cells three protein fractions were separated by chromatography on phosphocellulose and termed TFIIIA, B and C according to their consecutive elution points from this ion exchanger (1).

The classical target genes for pol III are controlled by different intragenic, discontinuous promoter sequences which can be divided in two subclasses, the AC-type (5S-rRNA) and the AB-type [t-RNA, VA-RNA's, 7SL-RNA, EBER; (2)] which differ in their factor requirement. While the genes of the AB-type promoters were transcribed *in vitro* by the TFIIIB and IIIC fractions, 5S-RNA genes additionally required the TFIIIA fraction (1, 2). Human TFIIIA purified from HeLa cells specifically binds to the ICR of 5S-RNA genes in a primary fashion, while hTFIIIC is able to bind to AB- and AC-type ICR promoters (3). In yeast cells TFIIIA and TFIIIC are considered to represent assembly factors whereas TFIIIB, subsequently incorporated into the preinitiation complex, is thought to be the initiation factor proper for all known pol III genes (4, 5). Our attempts to reconstitute *in vitro* transcription from highly purified factors have proven to be inefficient, indicating that additional components, present in less pure fractions were required to facilitate efficient *in vitro* transcription.

Although classical pol III genes lack a TATA-motif in their promoters, recently published results demonstrate that TBP, a component of TFIID is nevertheless involved in the transcription of t-, VA- and 5S-genes (6).

In contrast to the aforementioned pol III genes, U6 snRNA genes from vertebrates, although transcribed by RNA polymerase III, are controlled by promoter elements resembling those of typical pol II genes. These genes require four different trans-acting protein components in addition to RNA polymerase III for efficient *in vitro* transcription. Besides the general pol III transcription factor TFIIIB (5, 7), the PSE-binding protein PBP (5, 8), and TFIID (5, 9, 10, 11) an additional activity is involved in the expression of the U6-gene (5). Very recently we could demonstrate, that this latter activity is related to pol II transcription factor TFIIA (12). In agreement with its previously described function on TATA-containing polII-promoters (13), TFIIA presumably facilitates the interaction of TBP with the U6 TATA-box (12). TFIIA from HeLa cells has been purified by affinity chromatography and was identified as a 38 kDa polypeptide (14). Very recently it has been shown that TFIIA is also able to dissociate negative components from TBP, thereby possibly acting as an anti-inhibitor (15, 16).

The genes encoding TFIIA from yeast cells could recently be cloned. yTFIIA consists of two subunits termed TOA I and TOA II with a molecular mass of 32.2 and 13.5 kDa, respectively (17). The addition of recombinant TOA I and TOA II to yeast and mammalian cell extracts, which had been selectively depleted from TFIIA activity, could faithfully restore cell free transcription of pol II genes (17).

Here we demonstrate for the first time that TFIIA is also involved in the expression of classical pol III genes (t-RNA, VAI-RNA and 5S-RNA). These results were obtained with hTFIIA purified from HeLa cells as well as with recombinant yTFIIA.

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MATERIALS AND METHODS

Plasmids

The plasmids pUVAI, $pUht_{met}$, pUh5S containing single copies of the genes coding for VAI-RNA, human t-RNA_{met}^{i} and human 5S-RNA were described previously (3, 18). The plasmid $pUmU6_{0.34}$ containing the mouse U6-gene from bp −150 to +190, was described before (5).

Buffers

buffer 1: 20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 3 mM DTT, 0.2 mM PMSF.

buffer 2: 20 mM Tris−HCl (pH 7.9), 10% (v/v) glycerol, 3 mM DTT, 60 mM KCl, 5 mM $MgCl_2$, 0.2 mM PMSF.

buffer 3: 20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 5 mM $MgCl_2$, 3 mM DTT, 0.2 mM PMSF.

In vitro transcription

The *in vitro* transcription reactions were performed as described previously for classical pol III genes (19) and for the U6-RNA gene (5).

Preparation of cell free extracts

Cytoplasmic cell extract (S100) was prepared as described previously (7).

Isolation of employed transcription factors

To prepare human transcription factors, S100 from HeLa cells was fractionated by phosphocellulose chromatography essentially as described (1). The breakthrough (PC A) was collected and after washing the column with buffer 1 plus 0.1 M KCl, bound proteins were eluted with two consecutive steps of buffer 1 containing 0.35 M KCl (PC B) and 0.6 M KCl (PC C) respectively. Further purification procedures of transcription factors were performed as follows:

hTFIIIA. To obtain highly purified hTFIIIA, the ionic strength of fraction PC A was raised to 0.6 M KCl and it was rechromatographed on a second column of phosphocellulose. Washing with buffer 1 plus 0.6 M KCl (PC AA) was followed by elution with buffer 1 containing 1.0 M KCl (PC AD) as previously described by our group (18).

hTFIIIB. Phosphocellulose fraction B, containing hTFIIIB, was diluted to 175 mM KCl and loaded onto a Heparin-Sepharose column at 5 mg protein/ml bed volume. To remove possible contamination by TFIIA, which does not bind to Heparin-Sepharose (12), the column was extensively washed with buffer 1 plus 175 mM KCl. It was then eluted with buffer 1 + 0.4 M KCl to yield fraction $HS_{0.4}$ (1 mg protein/ml) which was finally dialysed against buffer 2.

hTFIIIC. Phosphocellulose fraction C was dialysed against buffer 3 containing 50 mM $(NH_4)_2SO_4$ and applied to a DEAE Sephadex A25 column at a rate of 5mg protein/ml bed volume. After extensively washing with the same buffer, the column was eluted with buffer 3 plus 0.15 M $(NH_4)_2SO_4$. The resulting fraction $DS_{0.15}$ (0.5mg protein/ml) containing IIIC, TBP and pol III was dialysed against buffer 2.

hTFIIA. Purification of hTFIIA was carried out as published previously by Waldschmidt and Seifart (12).

Phosphocellulose fraction AA (see above) was applied to a Heparin-Sepharose column and eluted with buffer 3 plus 0.1 M KCl yielding fraction $HS_{0.1}$, which was subsequently applied to a DEAE-Sephacel column. After washing with the same buffer, bound protein was eluted with two consecutive steps of buffer 3 containing 0.2 and 0.3 M KCl yielding fractions $DSC_{0.2}$ and $DSC_{0.3}$, respectively. Fraction $DSC_{0.3}$ containing the bulk of TFIIA activity was further purified by chromatography over a Mono Q FPLC column. After extensive washing with buffer 3 plus 0.1 M KCl ($MQ_{0.1}$), bound protein was eluted with buffer 3 containing 0.2, 0.3 and 0.4 M KCl, yielding fractions $MQ_{0.2}$, $MQ_{0.3}$ and $MQ_{0.4}$, respectively. Fraction $MQ_{0.4}$ was subsequently applied to an affinity column with immobilized recombinant hTFIID (see below). TFIIA was eluted with buffer 2 plus 1 M KCl ($IID\text{-}Affi_{1.0}$) as described previously (14, 20).

recombinant hTBP. Human TBP, used for affinity-chromatography, was expressed in *E.coli* and purified as described (12).

recombinant yTFIIA. Clones encoding recombinant yeast TFIIA subunits TOA I and TOA II were kindly provided by Drs J.A.Ranish and S.Hahn and expressed in *E.coli* cells. After denaturation of the recombinant proteins in the presence of 7M urea and renaturation by extensive dialysis against buffer 2, the extract was purified by chromatography on Q-Sepharose as described (17) and the resulting TFIIA fraction was assayed for its function in transcription assays.

SDS polyacrylamide gel electrophoresis. Proteins were electrophoresed on denaturating SDS/polyacrylamide gels containing 17.5% acrylamide/bisacrylamide and visualized by Coomassie brilliant blue R250 staining as previously described (7).

RESULTS

Additional components beside fractions TFIIIB and TFIIIC are needed for efficient pol III transcription

Although RNA polymerase III and its assumed transcription factors could recently be purified (21), total reconstitution from highly purified components was hitherto inefficient and we observed that additional components contained in crude hTFIIIB and hTFIIIC fractions were necessary for efficient *in vitro* transcription. Fig.1 shows, that crude fractions productively

Figure 1. Reconstitution of VAI-transcription with highly purified hTFIIIB and hTFIIIC. *In vitro* transcription assays were performed as described in Materials and Methods. 1 μg pUVAI template DNA was transcribed either by S100 (3 μl; lane 1), PC B and PC C (15 μl each; lane 2), PC B and $DS_{0.15}$ (15 μl each; lane 3), $HS_{0.4}$ and PC C (15 μl each; lane 4), or $HS_{0.4}$ and $DS_{0.15}$ (15 μl each; lane 5).

support the *in vitro* transcription of the VAI gene (lane 2), while the potential of individual factors to reconstitute polIII transcription decreased with progressing purification. This was especially observed for hTFIIIB (lanes 4 and 5).

We would exclude the possibility of a loss of RNA polymerase III as an explanation for the observed phenomenon, since supplementation with purified enzyme did not result in stimulation of *in vitro* transcription under these conditions (data not shown).

TFIIA is involved in the transcription of classical polIII genes

In order to identify the component(s) presumably removed from TFIIIB by progressing chromatography, we analysed the possible involvement of pol II factors TFIIA and TBP in pol III transcription. White *et al.* (6) showed that, beside TFIIIB and TFIIIC, TBP is required for *in vitro* transcription of classical polIII genes, as had already been described for yeast and vertebrate U6 snRNA transcription by RNA polymerase III (5, 9, 10, 11). However, TBP was not a likely candidate to explain the phenomenon observed here because TBP cofractionates with TFIIIB as evidenced by immunoblotting against α-TBP antibodies (our unpublished data and 22). Moreover, addition of recombinant hTBP alone to these purified fractions did not augment transcription (data not shown).

Guided by our previous observation that hTFIIA is required for efficient expression of U6 RNA by RNA polymerase III *in vitro* (12), we investigated the possible role of this protein during transcription of classical pol III genes. As shown in Fig.2 the efficiency of VAI-transcription *in vitro*, catalyzed by highly purified hTFIIIB and hTFIIIC fractions (lane 1; fractions $HS_{0.4}$ and $DS_{0.15}$ respectively) is strongly stimulated by increasing amounts of TFIIA-containing fraction $MQ_{0.4}$ (lanes 2–5). This same fraction was previously documented by our group to be required for the efficient expression of the U6-gene (12). An analogous effect of this fraction was also observed with respect to tRNA- (lanes 6–10) and 5S-RNA synthesis (lanes 11–15).

The chromatographic behaviour of the stimulating activity described here (Fig.3) strictly correlates with that of hTFIIA as outlined by Waldschmidt and Seifart (12). Basal activity of VAI-gene transcription was stimulated by the addition of phosphocellulose (PC A, lane 2; PC AA, lane 3), Heparin-Sepharose ($HS_{0.1}$, lane 5) breakthrough fractions, the DEAE-sephacel 0.2 and 0.3 M KCl fractions ($DSC_{0.2}$, lane 7; $DSC_{0.3}$, lane 8) and the Mono Q 0.4 M KCl eluate ($MQ_{0.4}$ lane 12). Finally, the stimulatory activity in fraction $MQ_{0.4}$ was further purified by affinity chromatography on columns containing covalently linked TBP, a method by which TFIIA can specifically be enriched (14, 20). The stimulating activity was found in the 1.0 M KCl fraction of the latter column (lane 14), thereby copurifying with TFIIA over at least five different chromatographic resins.

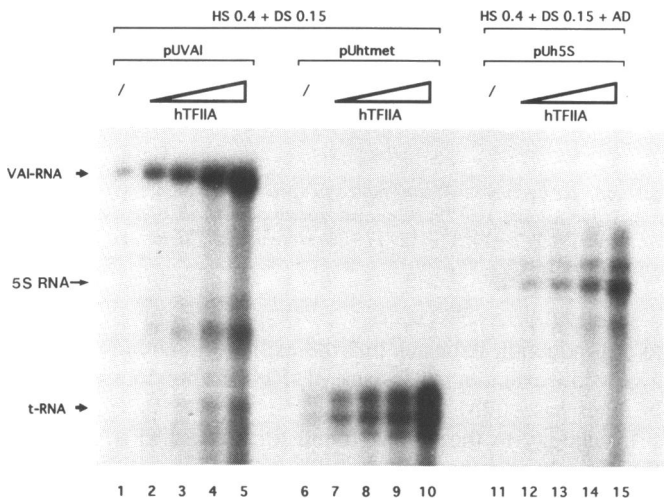

Figure 2. Stimulation of pol III transcription by hTFIIA. *In vitro* transcription reactions were performed as described in Materials and Methods. 1 μg pUVAI (lanes 1–5), pUhtmet (lanes 6–10), pUh5S (lanes 11–15) respectively, were transcribed by reconstitution of TFIIIB containing fraction $HS_{0.4}$, TFIIIC containing fraction $DS_{0.15}$ (15 μl each) and, in the case of h5S-RNA synthesis, TFIIIA containing PC AD (25 μl; lanes 11–15). Stimulation of the basal *in vitro* transcription was obtained by addition of increasing amounts of hTFIIA ($MQ_{0.4}$; 1, 2, 4, 10 μl) as appropriately indicated in the lanes 2–5, 7–10, 12–15.

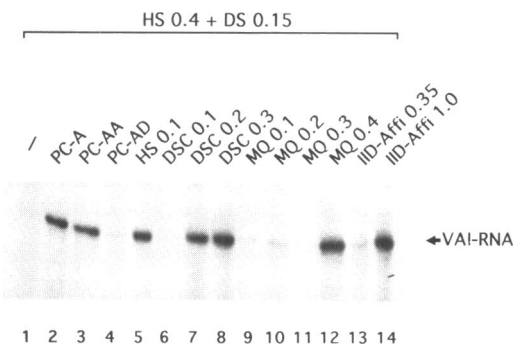

Figure 3. Analysis of protein fractions for TFIIA activity by assaying the stimulation of VAI-RNA transcription *in vitro*. Lanes 1–14: The template pUVAI was transcribed by fractions $HS_{0.4}$ and $DS_{0.15}$ alone (15 μl each; lane 1) or supplemented with PC A (lane 2), PC AA (lane 3), PC AD (lane 4), $HS_{0.1}$ (lane 5), $DSC_{0.1}$, $DSC_{0.2}$, $DSC_{0.3}$ (lanes 6–8), $MQ_{0.1}$, $MQ_{0.2}$, $MQ_{0.3}$, $MQ_{0.4}$ (lanes 9–12), IID-$Affi_{0.35}$ (lane 13) and IID-$Affi_{1.0}$ (lane 14; 20 μl each). The employed protein fractions were prepared as described in Materials and Methods.

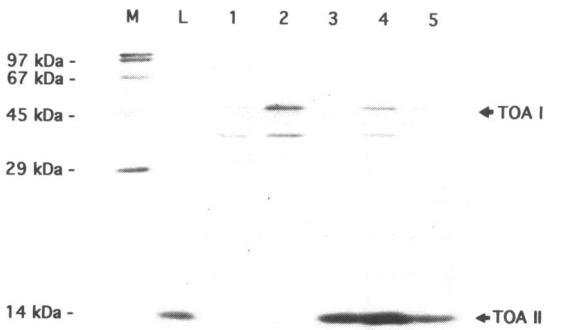

Figure 4. Expression and purification of recombinant yTFIIA. The expression and purification of the two subunits of yTFIIA was carried out as described (17) and they were assayed by 17.5% SDS-PAGE and subsequent staining with coomassie blue. Lane 1: 50 μl lysate of *E.coli* strain BL21 before induction of TOA I expression with 0.4 mM IPTG; Lane 2: 65 μl TOA I; Lane 3: 10 μl TOA II; Lane 4: 7 μl recombinant yTFIIA (after combination of TOA I and TOA II). Lanes 1–4: samples after precipitation with ammonium sulfate. Lane 5: 8 μl recombinant yTFIIA after chromatography on Q Sepharose. M: molecular weight markers. L: Lactalbumin as molecular weight standard (14 kDa). The expressed polypeptides TOA I and TOA II are appropriately indicated.

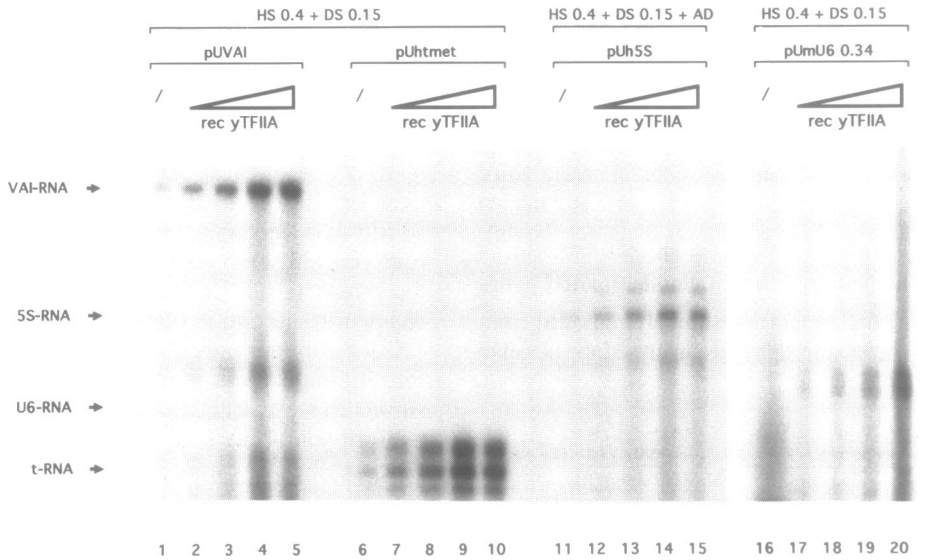

Figure 5. Stimulation of polIII *in vitro* transcription by recombinant TFIIA from Saccharomyces cerevisiae. Reconstituted *in vitro* transcription assays on the VAI (lanes 1–5), htmet (lanes 6–10), h5S (lanes 11–15) and U6 RNA gene (3μg pUmU6$_{0.34}$ DNA lanes 16–20) were analogously performed as described in Fig.1 with the modification that increasing amounts of recombinant yTFIIA (0.1, 0.5, 1, 5 μl; lanes 2–5, 7–10, 12–15, 17–20) were added. To detect the transcribed U6-RNA (lanes 16–20) this part was exposed twice as long (48h) as the part shown in lanes 1–15.

In addition, the characteristic heat stability of the hTFIIA protein was used to characterize the stimulating component. The stimulatory activity was found to be stable up to 60°C for 10 minutes. Since these data are identical to those observed for hTFIIA previously (12, 23), they have not been shown here. From these results we exclude the possibility that stimulation by the $MQ_{0.4}$ fraction is caused by cross-contamination with hTFIIIB, TBP or pol III, because these proteins are much more susceptible to elevated temperatures (cited in 12).

Furthermore, the molecular mass of the stimulatory activity contained in the fraction $MQ_{0.4}$ was analysed by glycerol gradient centrifugation. The activity sediments with a native molecular mass of 38 +/− 10 kDa (data not shown), which is in good agreement with the values previously reported for TFIIA (12, 14, 23).

The chromatographic behaviour, the heat sensitivity and the native molecular mass of the stimulating activity strongly suggest the involvement of the RNA polymerase II transcription factor IIA in the expression of classical pol III genes.

Yeast TFIIA can substitute for the human factor in pol III transcription *in vitro*

Final identification of the stimulating activity as TFIIA was achieved with the recombinant transcription factor from yeast. Since cloned human TFIIA is not yet available, we used the yeast analogue, which can functionally substitute for the human factor in pol II transcription *in vitro* (17, 24). For this purpose the two subunits (TOA I: 32.2 kDa; TOA II: 13.5 kDa) of the protein were separately expressed in *E.coli* and purified by chromatography on Q Sepharose as described (17).

Fig.4 shows the SDS-PAGE analysis of TOA I (lane 2), TOA II (lane 3) and the renatured yTFIIA before (lane 4) and after purification (lane 5). TOA I reveals an apparent molecular mass of 43 kDa as previously described (24). The presence of small amounts of the expressed polypeptide in the *E.coli* lysate prior to the induction indicates that the expression already occurs to a very low extent in the absence of IPTG, as previously observed in other cases (25).

In analogy to the results found for HeLa hTFIIA fractions (Fig.2), a basal transcription system with highly purified hTFIIIB ($HS_{0.4}$) and hTFIIIC ($DS_{0.15}$) was clearly stimulated by the addition of recombinant yTFIIA (Fig.5). This was observed for VAI- (lanes 1–5), t- (lanes 6–10), h5S- (lanes 11–15) and U6-RNA synthesis (lanes 16–20). The latter result confirms our previous finding (12) that TFIIA is required for efficient expression of U6-RNA and it shows that its function can be substituted in a HeLa transcription system by the recombinant yeast protein. Collectively, these results clearly demonstrate that TFIIA is not only involved in efficient expression of many pol II genes (26) but also plays a role in pol III transcription.

DISCUSSION

The protein components required for the basal expression of classical pol III genes were previously identified as TFIIIB and TFIIIC for the AB-type promoters, with TFIIIA being additionally required for transcription of the gene for ribosomal 5S RNA (AC-type). Although these results were obtained with highly purified fractions of TFIIIB (7) and TFIIIC (3, 27), they do not exclude the participation of other contaminating or comigrating proteins.

It has recently been shown (6) that the TATA-box binding component of TFIID (TBP), previously identified to be required for the transcription of U6 RNA by RNA polymerase III (5, 9, 10, 11), is also involved in the expression of classical pol III as well as pol I genes (6, 28, 29, 30). Moreover, attempts to fully reconstitute pol III transcription with highly purified components (TFIIIB, IIIC, TBP + pol III) turned out to be inefficient. We have consistently observed that rigorous purification, especially of TFIIIB from mammalian cells, is

accompanied by a considerable loss of activity. These results, which are also born out in Fig.1, suggest the involvement of (an) additional factor(s) beside IIIB and IIIC—and IIIA in the case of 5S rRNA. This effect cannot merely be explained by a conceivable depletion of TBP, because transcription of the classical pol III genes requires only a very low amount of TBP (6; and our own unpublished results), the presence of which could be verified by western-blot analysis of hTFIIIB and hTFIIIC containing fractions used for the assay (our unpublished results). Moreover, addition of the recombinant human TBP protein alone did not stimulate transcription activity. This could indicate that distinct TBP-associated factors (TAFs) exist in a complex specifically directing polIII transcription as was very recently reported by Taggart et al (31).

Our attempts to characterize additional components involved in the expression of classical pol III genes led to the detection of an activity in the flowthrough fraction of phosphocellulose, which significantly stimulated the expression of t-RNA,VA-RNA, 5S-RNA and U6-RNA and which was finally identified as TFIIA on the basis of the following experimental evidence:

Purification of this activity from HeLa cells over at least four additional chromatographic steps revealed co-purification with mammalian TFIIA (12, 14, 23, 32). Secondly, the stability of the protein up to 60°C (data not shown) agrees with values reported for hTFIIA (12, 23). This observation also excludes cross contamination of the hTFIIA fractions with TBP, TFIIIB and/or TFIIIC, which are inactivated at this temperature (5, 23, 33). Finally, the stimulating activity has a native molecular mass of approximately 38 +/− 10 kDa which is in agreement with the value reported in the literature (12, 14, 23).

Definitive proof that the activity in question is related to TFIIA was obtained from experiments with recombinant yeast TFIIA expressed in *E.coli*. The functional activity of this protein from yeast in mammalian and other vertebrate cell free transcription systems for pol II genes has previously been demonstrated (17, 20). The fact that *in vitro* transcription reactions, reconstituted with purified human TFIIIB, IIIC, TBP (and IIIA in the case of 5S-rRNA) and pol III could be significantly stimulated by the addition of recombinant yeast TFIIA shows that this transcription factor is also involved in the expression of classical pol III genes (Fig.5). Moreover, the results obtained with yTFIIA confirm our recently published report concerning the requirement of TFIIA for the efficient transcription of the mammalian U6 gene (12).

The mechanism by which TFIIA influences intitiation on TATA-less pol III genes is unclear at present. From our unpublished data we conclude that TBP does not directly bind to internal pol III promoters (or to cryptic TATA-sequences in the plasmid-vector). Hence the stabilization of TBP-binding to the U6-TATA box, previously suggested as a possible function of TFIIA (12), cannot explain the role of TFIIA reported here. It should also be pointed out that the transcription reactions of ribosomal 5S RNA were performed with a synthetic 5S gene (34) lacking external control regions. Therefore the action of TFIIA during 5S RNA transcription does not depend on presumptive 5′-regulating sequences.

It has recently been shown that the TATA-binding protein TBP stabilizes a transcription complex on the VAI gene (35) and participates in assembling the yeast multisubunit transcription factor IIIB on tRNA genes (36, 37) presumably by locking the TFIIIB-TBP-TFIIIC-DNA-complex (36). It is likewise conceivable that TFIIA is incorporated into the transcription complex by protein-protein interactions involving TBP and/or the established pol III transcription factors IIIA, IIIB and IIIC. This is presently being investigated and will be published elsewhere. It should be kept in mind that TFIIA could also act as an anti-inhibitor either by inducing a conformational change of TBP (16) or by competing with negative components binding to TBP (15).

To answer the question whether TFIIA is essentially required or merely involved in potentiating transcription initiation on classical pol III promoters it will be necessary to selectively deplete TFIIA from these fractions by specific antibodies against hTFIIA, the generation of which must, however, await the future cloning and expression of the gene for human TFIIA. In spite of this limitation, the results presented here show that TFIIA is the second pol II transcription factor involved in the expression of classical pol III genes.

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