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Human transcription factors $IIIC_2$, $IIIC_1$ and a novel component $IIIC_0$ fulfil different aspects of DNA binding to various pol III genes

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ABSTRACT

Human transcription factor $IIIC_2$ interacts with the TFIIIA–5S DNA complex and forms a ternary TFIIIA/$IIIC_2$–5S DNA complex. Formation of this complex does not preclude simultaneous binding of $TFIIIC_2$ to the B-box sequence of a second template. This suggests that the domain(s) or subunit(s) required for indirect recognition of the 5S promoter by $TFIIIC_2$ are different from those necessary for direct binding of $TFIIIC_2$ to B-box-containing pol III promoters. Whereas $TFIIIC_2$ is only required for transcription of the 'classical' pol III genes, $TFIIIC_1$ is generally required for transcription of all pol III genes, including that of the U6 gene. The activity of $TFIIIC_1$ strongly enhances specific binding of basal pol III factors TFIIIA, $TFIIIC_2$ and the PSE binding protein (PBP) to their cognate promoter elements and it acts independently of the corresponding termination regions. Moreover, we characterize an activity, $TFIIIC_0$, purified from phosphocellulose fraction C, which shows strong DNase I protection of the terminaton region of several pol III genes and which is functionally and chromatographically distinct from $TFIIIC_1$ and $TFIIIC_2$.

INTRODUCTION

In higher eukaryotic cells, three general types of pol III promoters can be distinguished (1). The type 1 promoter (5S rRNA genes) comprises gene internally located A- and C-boxes and an intermediate element. Transcription from this promoter requires TFIIIA, TFIIIC, TFIIIB and pol III. Type 2 promoters (VA and tRNA genes, Alu sequences) are characterized by gene internal A- and B-boxes. Expression of these genes needs TFIIIC, TFIIIB and pol III. Type 3 promoters are located 5′ of the transcription initiation site and are composed of a TATA-like box, a proximal sequence element (PSE) and a distal sequence element (DSE). Specific initiation of transcription on these promoters depends on the TATA binding protein TBP, the PSE binding protein PBP (2,3), also designated PTF (4–6) or SNAPc (7,8), TFIIIB and pol III.

Characterization of pol III transcription factors is most advanced in yeast cells. yTFIIIA has been cloned and represents a single polypeptide of 50 kDa (9). yTFIIIC, which consists of six polypeptides, recognizes A- and B-boxes and incorporates yTFIIIB into the preinitiation complex (10,11). yTFIIIB activity is known to consist of yTBP and two associated proteins of 70 (BRF1) and 90 kDa (TFIIIB″) (12).

In mammalian cells the situation is more complex. It has recently been shown that human transcription factor IIIB can be separated into two functionally different isoforms, TFIIIB-α and TFIIIB-β. hTFIIIB-β is predominantly active on genes with intragenic promoters and contains TBP and associated proteins, whereas TFIIIB-α is devoid of TBP and shows strong preference for *in vitro* transcription of U6 snRNA (13).

Human TFIIIC activity can also be resolved into two functional components ($TFIIIC_1$ and $TFIIIC_2$) by Mono Q (14) or sequence-specific DNA affinity chromatography (15). $TFIIIC_2$ binds preferentially to the B-box region of type 2 genes with high affinity (14,16) and consists of at least five polypeptides, the largest of which directly contacts the DNA (17,18). $TFIIIC_1$ also represents a multisubunit complex consisting of several polypeptides. Both $TFIIIC_1$ and $TFIIIC_2$ are required for optimal transcription of 5S rRNA (19), tRNA and VAI genes (14,16), but only $TFIIIC_1$ and not $TFIIIC_2$ is necessary for 7SK transcription (5).

The genes encoding TFIIIA have been cloned from *Xenopus laevis* (20) and human cells (21,22). Although considerable interspecies variation has been observed in the sequence of the protein, it always represents a single, nine zinc finger polypeptide which binds to the ICR of 5S rRNA genes. It has been reported that human and *Xenopus* oocyte TFIIIA bind with higher affinity to their respective homologous than to heterologous 5S genes (23). In addition to TFIIIA-mediated recruitment of TFIIIC, an interaction of human TFIIIC with the 5′ flanking region has been reported for the *Xenopus* 5S rRNA gene in the absence of TFIIIA (19,24). Since *in vitro* transcription mediated by a TFIIIC-depleted nuclear extract required readdition of both TFIIIC and TFIIIA, a promoter-independent interaction of human TFIIIC with human TFIIIA was postulated (18).

Here we show that incorporation of $hTFIIIC_2$ into the 5S rRNA transcription complex is mediated by TFIIIA and involves other polypeptides or domains of $TFIIIC_2$ than those required for interaction with the B-box sequence. In addition, we present data showing that $TFIIIC_1$ is required for transcription of all types of pol III promoters and that this factor enhances the binding of TFIIIA, $TFIIIC_2$ and PBP. Moreover, we characterize an additional activity, $TFIIIC_0$, which binds to the termination region of several pol III genes and is distinct from $TFIIIC_1$ and $TFIIIC_2$.

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MATERIALS AND METHODS

Buffer 1: 20 mM HEPES, pH 7.9, 10% (v/v) glycerol, 3 mM DTT, 0.2 mM PMSF. Buffer 2: 20 mM Tris–HCl, pH 7.9, 10% (v/v) glycerol, 5 mM $MgCl_2$, 3 mM DTT, 0.2 mM PMSF.

Plasmids

ph5S and pBh5S contained the gene for human synthetic or genomic 5S rRNA respectively (23,25). ph5Sn was generated by subcloning the *Sal*I–*Bam*HI fragment of ph5S (23) into pUC19. pUVAI, $pUht_i^{met}$ and $pUmU6_{0.34}$ contained the genes coding for VAI RNA, human methionine initiator tRNA and mouse U6 snRNA respectively (2,24). $pUht^{met}$ was constructed by subcloning the *Bam*HI–*Hin*fI fragment of $pUht_i^{met}$ (24) into the *Sma*I site of pUC18. $mU6_{0.155}$ contained the TATA and PSE sequences of the U6 gene (2). For competition analysis mutants of the *Escherichia coli* phenylalanine tRNA gene were employed, which either lacked the 5′ sequence (tΔ-5′), the B-box (tB-del) or contained the wild-type B-box and a mutated A-box (tA-mut) (26,27). The 'irrelevant' 31 bp synthetic double-stranded oligonucleotide contained the TATA motif of the Ad2Ml promoter.

In vitro transcription

In vitro transcription reactions were performed as previously described for 'classical' pol III genes (28) and the mU6 gene (2,13).

Electrophoretic mobility shift analyses (EMSA)

Fragments were gel purified and labelled at their 3′-termini with [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. DNA binding assays of protein fractions were performed as described before (2,24), with the following modifications. After incubation with non-specific competitor DNA [1 μg pUC9 DNA and 2 μg poly(dI·dC)] the labelled DNA fragments were added and incubation was prolonged at 30°C for 45 (for the 5S and U6 genes) or 90 min (for the VAI and ht^{met} genes).

DNase I footprint analyses

Incubation conditions were as described for EMSA. Samples were cleaved with 40 ng DNase I for 1 min at room temperature and further processed as described before (23). An end-labelled *Msp*I digest of pBR322 was used as a size marker to assign the protected regions.

Preparation of cellular and nuclear extracts and isolation of transcription factors

Cytoplasmic (S100) or nuclear (P100) extracts from HEK (human embryonic kidney) cells were prepared from several batches of 30 l suspension cultures with an index of 7×10^5 cells/ml as described previously (28,29).

To purify human transcription factors, the extracts were fractionated by phosphocellulose chromatography (Whatman P11) essentially as described (28). Further purification procedures were performed as follows.

hTFIIIA. The PCA fraction (from S100) was rechromatographed on a second phosphocellulose column as described (30). Fraction AD contained TFIIIA (0.05 mg protein/ml).

hTFIIIB. The PCB fraction (from S100), containing hTFIIIB and RNA pol III, was dialysed against buffer 2 including 60 mM KCl and applied to an EMD DEAE Fractogel column (Merck, Darmstadt, Germany). The column was washed with the same buffer and bound proteins were eluted with a linear gradient of 60–500 mM KCl as described (13).

hTFIIIB-α. Fractions eluting with 110–220 mM KCl from the EDF gradient were dialysed against buffer 1 including 100 mM NaCl and loaded onto a Mono S column (Pharmacia) at 10 mg protein/ml bed volume. After washing the column, bound proteins were eluted stepwise with 220 and 500 mM NaCl (Teichmann, unpublished results). Fraction $MS_{0.5}$ contained hTFIIIB-α (1.5 mg protein/ml).

hTFIIIB-β. Fractions eluting with 250–310 mM KCl from the EDF gradient were dialysed against buffer 2 including 60 mM KCl and loaded onto an EMD SO^{3-} Fractogel column (Merck). After washing the column, bound proteins were eluted with a linear gradient of 60–400 mM KCl (Teichmann, unpublished results). Fractions eluting at 270–350 mM KCl contained hTFIIIB-β (0.3 mg protein/ml) and were free of $TFIIIC_0$, $TFIIIC_1$, $TFIIIC_2$ and pol III.

RNA pol III. Fractions eluting with 320–450 mM KCl from the EDF gradient (loaded with PCB) were applied to an EMD SO^{3-} Fractogel column, which was equilibrated with buffer 1 including 360 mM KCl. After washing the column, bound proteins were eluted with a linear gradient of 360–700 mM KCl (Meißner, unpublished results). Fractions eluting at 500–600 mM KCl contained pol III (0.6 mg protein/ml) and were dialysed against buffer 1 including 60 mM KCl and 50% (v/v) glycerol. These fractions were devoid of TFIIIB-α and TFIIIB-β as well as $TFIIIC_0$, $TFIIIC_1$ and $TFIIIC_2$ and were used for all *in vitro* transcription assays described in this paper.

hTFIIIC. All operations to prepare hTFIIIC activity were conducted in the presence of 7.5 mM DTT. The PCC fraction (from P100) was dialyzed against buffer 2 including 60 mM KCl and loaded onto a Mono Q column (Pharmacia) at 10 mg protein/ml bed volume. A linear gradient of 60–400 mM KCl in buffer 2 eluted $TFIIIC_0$ at 150–190 mM ($MQ_{0.2}$; 0.25 mg protein/ml), $TFIIIC_1$ at 250–290 mM ($MQ_{0.27}$; 0.3 mg protein/ml) and $TFIIIC_2$ at 350–380 mM KCl ($MQ_{0.5}$; 0.4 mg protein/ml).

Alternatively, fraction PCC was loaded onto a Q Sepharose column (Pharmacia) at 10 mg protein/ml bed volume. After washing with buffer 2 including 60 mM KCl, bound proteins were eluted stepwise with 200 ($QS_{0.2}$ containing $TFIIIC_0$), 270 ($QS_{0.27}$ containing $TFIIIC_1$) and 500 mM KCl ($QS_{0.5}$ containing $TFIIIC_2$). Fraction $QS_{0.2}$ was dialysed against buffer 2 including 60 mM KCl and further applied to a Hi Trap™ SP column (Pharmacia) at 15 mg protein/ml bed volume. After washing, the column was eluted with 200 and 400 mM KCl. Fraction $SP_{0.4}$, containing the bulk of $TFIIIC_0$ (0.7 mg protein/ml) activity, was used for footprint analyses on the h5S rRNA gene.

PBP. PBP activity was purified as described previously (2). Alternatively, PBP activity was enriched by Cibacron blue (CB; Sigma) gradient chromatography (1.9–2.5 M NaCl, 30–40% v/v ethylene glycol) analogous to the purification of SNAPc described by Henry *et al.* (8).

Recombinant hTBP. Human TBP was expressed in *E.coli* and purified as described (31).

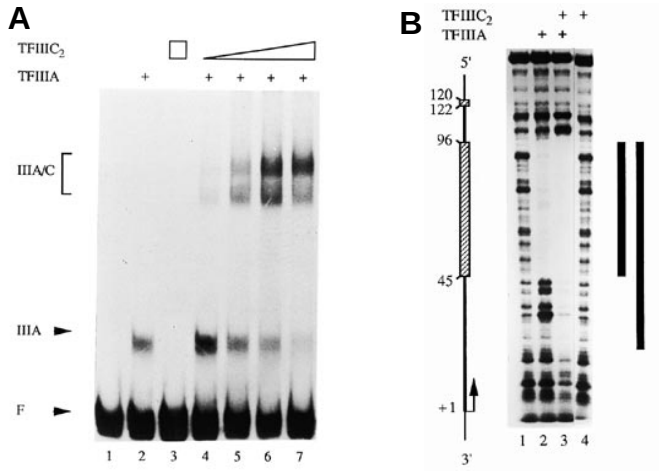

Figure 1. Interaction of $TFIIIC_2$ with the hTFIIIA–5S complex. (**A**) 10, 20, 30 or 40 µl $hTFIIIC_2$ (fraction $MQ_{0.5}$, lanes 4–7) and 5 µl hTFIIIA (fraction AD) were incubated with the end-labelled h5S gene fragment as described in Materials and Methods. Reactions shown in lanes 1–3 contain either no protein (lane 1), 5 µl fraction AD (lane 2) or 40 µl fraction $MQ_{0.5}$ (lane 3). Arrows depict free DNA (F) and DNA–protein complexes (IIIA and IIIA/C). (**B**) A comparative footprint analysis on the coding strand of the h5S gene (170 bp *Eco*RI–*Hin*dIII fragment) conducted as described in Materials and Methods. Lane 1, no protein; lane 2, 20 µl fraction AD; lane 3, 20 µl fraction AD and 30 µl fraction $MQ_{0.5}$; lane 4, 30 µl fraction $MQ_{0.5}$. The positions of the protected regions are appropriately indicated relative to the 5S gene.

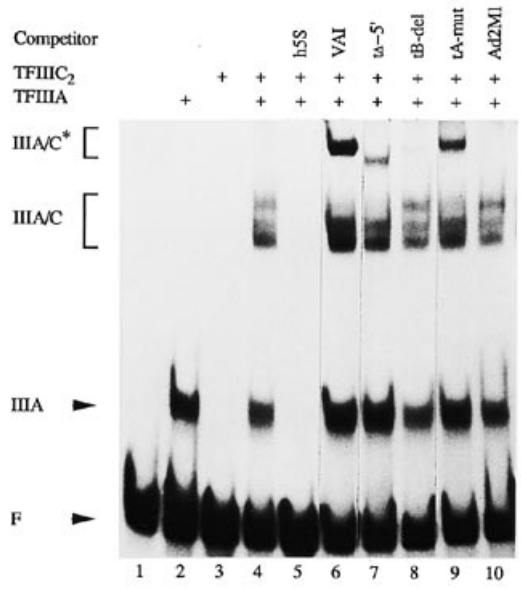

Figure 2. Competition analysis of the 'TFIIIA/C_2–5S DNA complexes'. The end-labelled h5S fragment was incubated without protein (lane 1), with 3 µl fraction AD (lane 2), with 30 µl fraction $MQ_{0.5}$ (lane 3) or with 3 µl fraction AD and 30 µl fraction $MQ_{0.5}$ (lane 4). For competition analysis the samples (lanes 5–10) containing 3 µl fraction AD and 30 µl fraction $MQ_{0.5}$ were preincubated for 30 min at 30°C with 150 ng of the following competitors before adding the labelled fragment: *Eco*RI–*Hin*dIII fragments of ph5S (lane 5, 170 bp), pUVAI (lane 6, 281 bp) and mutants of the gene for *E.coli* $tRNA^{Phe}$ lacking the 5′ sequence (tΔ-5′, lane 7, 242 bp), lacking the B-box (tB-del, lane 8, 300 bp) or containing a wild-type B-box and a mutated A-box (tA-mut, lane 9, 309 bp) as described in Materials and Methods or an 'irrelevant' control oligo containing the TATA box of the Ad2Ml promoter (Ad2Ml, lane 10, 31 bp). Arrows depict free (F) and complexed DNA (IIIA, IIIA/C and IIIA/C*).

Recombinant hTFIIIA. The coding sequence of hTFIIIA (21) was cloned into a pGEX-2T expression vector (Pharmacia). Human TFIIIA was expressed in *E.coli* XL-1 Blue cells, transformed with pGEX-2T/hTFIIIA constructs and affinity purified on a glutathione–agarose matrix (Sigma) as described (32).

RESULTS

$hTFIIIC_2$ interacts with the hTFIIIA–5S gene complex

Human TFIIIA specifically binds to the ICR of the human 5S gene (IIIA, Fig. 1A, lane 2, and B, lane 2), whereas purified $hTFIIIC_2$ alone does not (Fig. 1A, lane 3, and B, lane 4). However, incubation of increasing quantities of $hTFIIIC_2$ in the presence of a constant amount of hTFIIIA resulted in formation of three slower migrating protein–DNA complexes (Fig. 1A, lanes 4–7, designated IIIA/IIIC), representing an association of TFIIIA, $TFIIIC_2$ and 5S DNA, as will subsequently be shown. The same complexes were also observed when fraction AD was replaced by recombinant human (rh) or *Xenopus laevis* (*Xl*o) TFIIIA. However, interaction of $hTFIIIC_2$ with the homologous hTFIIIA–5S DNA complex was much stronger than with heterologous *Xl*oTFIIIA on the same fragment (data not shown).

TFIIIA-mediated association of $TFIIIC_2$ with the 5S promoter sequence is conclusively supported by DNase I footprinting. As shown in Figure 1B, addition of $TFIIIC_2$ to a saturating amount of hTFIIIA led to enlargement of the TFIIIA footprint (lane 2), extending DNase I protection on the coding strand of the 5S gene up to position +20 (Fig. 1B, compare lanes 2 and 3).

The specific B-box binding activity of $TFIIIC_2$ is maintained after formation of a TFIIIA/$TFIIIC_2$–5S gene complex

The multiprotein complex $TFIIIC_2$, essentially required for transcription of the tRNA and 5S rRNA genes, shows a strong DNase I protection preferentially over the B-box of type 2 promoter genes (Fig. 6, lanes 2 and 7) and is incorporated into the 5S transcription complex via an interaction with TFIIIA (Fig. 1A, lanes 4–7, and B, lane 3). It was hence of importance to investigate whether the same or different domains of $TFIIIC_2$ were required for incorporation of this factor into a tRNA or 5S RNA transcription complex. Therefore, complex formation on the 5S gene was challenged with an excess of unlabelled 5S (containing TFIIIA binding sequence), VAI or *E.coli* tRNA genes (both containing high affinity $TFIIIC_2$ binding sites).

Surprisingly, incubation of the TFIIIA/$TFIIIC_2$–5S DNA complexes with B-box-containing fragments specifically resulted in additional complexes migrating even more slowly (Fig. 2, lanes 6, 7 and 9). The mobility of these 'supercomplexes' (designated IIIA/C*), which were also observed when the TFIIIA fraction was replaced by rhTFIIIA (data not shown), correlated with the size of the competitor fragments used (lane 6, 281 bp VAI fragment; lane 7, 242 bp tΔ-5′ fragment; lane 9, 309 bp tA-mut fragment). It is hence likely that they comprise the factors TFIIIA and $TFIIIC_2$ on the 5S gene and additionally contain the respective B-box fragments. In order to determine the sequence requirements for 'supercomplex' formation, the TFIIIA/$TFIIIC_2$–5S DNA complexes were incubated with an excess of two individual $tDNA^{Phe}$ mutants. As shown in Figure 2, B-box deletion (tB-del) prevented 'supercomplex' formation (lane 8), whereas an A-box mutant (tA-mut) was still able to supershift the TFIIIA/$IIIC_2$–5S DNA complex (lane 9). An additional control was conducted to check the composition of the IIIA/IIIC* 'supercomplexes'

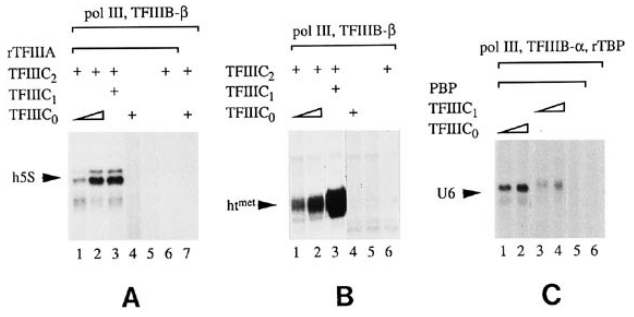

Figure 3. Requirement for $TFIIIC_1$ and $TFIIIC_0$ for expression of different pol III genes. Transcription reactions contained the factors indicated above each lane and either 1 µg pBh5S (A), 1 µg $pUht_i^{met}$ (B) or 2 µg $pUmU6_{0.34}$ (C) as template DNA and were conducted as described in Materials and Methods. (**A** and **B**) Pol III, 3 µl fraction SO^{3-} (500–600 mM KCl); TFIIIB-β, 4 µl fraction SO^{3-} (270–350 mM KCl); TFIIIA, 1 µl rhTFIIIA; $TFIIIC_2$, 5 µl fraction $MQ_{0.5}$; $TFIIIC_0$, 5 (lane 1) and 10 µl (lane 2) fraction $MQ_{0.2}$; $TFIIIC_1$, 7 µl fraction $MQ_{0.27}$. (**C**) Pol III, 3 µl fraction SO^{3-} (500–600 mM KCl); TFIIIB-α, 20 µl fraction $MS_{0.5}$; PBP, 20 µl fraction CB (1.9–2.5 M NaCl, 30–40% EG); $TFIIIC_0$, 10 (lane 1) and 20 µl (lane 2) fraction $MQ_{0.2}$; $TFIIIC_1$, 10 (lane 3) and 20 µl (lane 4) fraction $MQ_{0.27}$. All samples in (C) were supplemented with 0.2 µl rhTBP and 2.5 µl fraction PCA.

observed in Figure 2. Incubation of $TFIIIC_2$ and TFIIIA with unlabelled h5S gene fragment and subsequent competition with a labelled fragment containing the VAI gene likewise resulted in formation of 'supercomplexes' (data not shown).

Collectively, our data clearly indicate that $TFIIIC_2$ is able to interact with the TFIIIA–5S DNA complex and a B-box sequence simultaneously. Therefore, the domain of $TFIIIC_2$ which interacts with the TFIIIA–5S gene complex clearly differs from the domain of $TFIIIC_2$ responsible for specific binding of the B-box.

Competition with fragments containing the VAI gene (lane 6) or an *E.coli* $tRNA^{Phe}$ gene (tΔ-5′; lane 7) led to disappearance of only the slowest of the three bands of the TFIIIA/$TFIIIC_2$–5S complexes. Therefore, the two faster migrating complexes may either not contain the B-box binding subunits of $TFIIIC_2$ or the relevant DNA binding region of a subpopulation of $TFIIIC_2$ is not accessible in the presence of TFIIIA, preventing B-box-specific competition. A non-specific control oligo containing the TATA box of the adeno 2 major late promoter did not prevent formation of any of the three IIIA/$IIIC_2$–5S complexes (lane 10).

Factors specifically required for transcription of the human 5S gene *in vitro*

It has previously been demonstrated that both human $TFIIIC_1$ and $TFIIIC_2$ are required in addition to *Xlo*TFIIIA and a comparatively crude fraction of hTFIIIB for *in vitro* transcription of the *Xenopus* 5S gene (19). In order to check which factors are essentially required in a homologous transcription system, we analysed $hTFIIIC_1$ and $hTFIIIC_2$ for their ability to reconstitute h5S rRNA transcription *in vitro* in the presence of highly purified TFIIIB-β (13), pol III and rhTFIIIA. In agreement with the findings recently presented by Wang and Roeder (33) and confirming the results of Fradkin *et al.* (19), the data presented in Figure 3 clearly show that besides pol III, TFIIIB-β and rhTFIIIA, both $TFIIIC_1$ and $TFIIIC_2$ were required for synthesis of human 5S rRNA (Fig. 3A, lane 3), ht_i^{met} (Fig. 3B, lane 3) or VAI (data not shown).

Moreover, starting from a nuclear PCC fraction we functionally characterized an additional activity eluting at 150–190 mM KCl from Mono Q ($MQ_{0.2}$) or Q Sepharose ($QS_{0.2}$). This activity was able to partially substitute for $TFIIIC_1$ activity in *in vitro* transcription of 'classical' pol III genes (Fig. 3A, 5S rRNA, and 3B, ht_i^{met}, lanes 1 and 2). However, this fraction not only differed in its chromatographic properties, but was also functionally distinct from $TFIIIC_1$ fractions hitherto described, as will subsequently be dealt with in more detail. To discriminate between $TFIIIC_1$ activity and this functionally related fraction, we refer to this activity as $TFIIIC_0$.

In contrast to 'classical' pol III genes, the mouse U6 gene was efficiently transcribed in the absence of $TFIIIC_2$. Transcription of this gene alternatively required $TFIIIC_1$ (Fig. 3C, lanes 3 and 4) or the $TFIIIC_0$ fraction (lanes 1 and 2) when reconstituted in an *in vitro* transcription assay containing rhTBP, highly purified human pol III, TFIIIB-α (13) and PBP (2). However, it should be pointed out that maximal U6 snRNA synthesis in this transcription system was 3-fold higher in the presence of $TFIIIC_0$ than in the presence of $TFIIIC_1$ (Fig. 3C, compare lanes 1 and 2 with 3 and 4). In contrast, for transcription of the ht_i^{met} gene (Fig. 3B, compare lane 3 with lanes 1 and 2) or VAI gene (data not shown) $TFIIIC_1$ led to a higher rate of RNA synthesis than observed with $TFIIIC_0$. For transcription of the h5S gene only minor differences were observed when $TFIIIC_1$ was replaced by $TFIIIC_0$ (Fig. 3A, compare lane 3 with lanes 1 and 2). Reactions containing both $TFIIIC_1$ and $TFIIIC_0$ resulted in a higher transcription level from the ht_i^{met} and VAI genes than with either factor alone, while 5S rRNA or U6 snRNA synthesis were only slightly increased (data not shown). Taken together, minimal transcription of the VAI, ht_i^{met} and h5S genes can be supplemented more efficiently by $TFIIIC_1$ than by $TFIIIC_0$, whereas $TFIIIC_0$ is clearly more effective than $TFIIIC_1$ in the case of the U6 snRNA gene.

$TFIIIC_1$ strengthens the specific binding of all primary DNA binding basal pol III factors, TFIIIA, $TFIIIC_2$ and PBP

To further investigate the molecular mechanism by which $TFIIIC_1$ acts, we studied what effect the $TFIIIC_1$ fraction exerted on binding of hTFIIIA to the human 5S gene.

As demonstrated in Figure 4A, the amount of specific TFIIIA–5S DNA complex (IIIA, lane 2) strongly increased upon addition of $TFIIIC_1$ (lane 3). $TFIIIC_1$ alone showed no detectable interaction with the 5S DNA fragment (lane 4). These results were confirmed by DNase I footprint analyses: addition of $TFIIIC_1$ to non-saturating amounts of TFIIIA strengthened the DNase I protection without extending the protected region (data not shown).

The increase in complex formation upon addition of $TFIIIC_1$ activity was not restricted to binding of TFIIIA to the 5S gene. EMSA performed with non-saturating amounts of $TFIIIC_2$ on an end-labelled VAI gene likewise resulted in a strong enhancement of $TFIIIC_2$–VAI complex formation and/or stability upon addition of $TFIIIC_1$ (IIIC, Fig. 4A, compare lanes 6 and 7). Moreover, addition of $TFIIIC_1$ to non-saturating amounts of PBP binding to an end-labelled mU6 gene including the upstream promoter sequence also resulted in increased PBP–U6 complex formation and/or stability (PBP, Fig. 4A, compare lanes 10 and 11).

$TFIIIC_1$ in our hands did not bind to DNA by itself (Fig. 4A and B, lanes 4, 8 and 12). Although incubation of $TFIIIC_1$ with labelled VAI fragments in the absence of $TFIIIC_2$ led to formation of a retarded complex (Fig. 4A and B, lane 8), this complex was

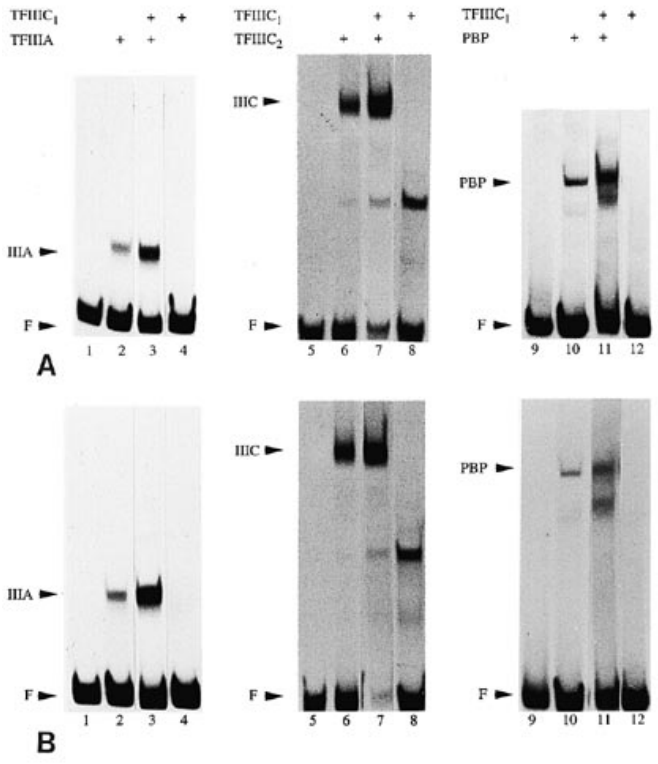

Figure 4. $TFIIIC_1$ strongly enhances specific binding of basal pol III factors. Binding reactions for EMSA, containing the fractions indicated above each lane, were incubated with end-labelled fragments containing the h5S gene from +1 to +122 (**A**, lanes 1–4), the VAI gene from –33 to +185 (A, lanes 5–8) or the mU6 gene from –150 to +190 (A, lanes 9–12) or with end-labelled terminator-less fragments (**B**) containing the h5S gene from +1 to +106, the VAI gene from –33 to +150 or the promoter of the mU6 gene from –150 to +5. Arrows indicate complexed (IIIA, IIIC and PBP) or free (F) DNA. TFIIIA, 2 µl fraction AD; $TFIIIC_1$, 5 µl fraction $MQ_{0.27}$; $TFIIIC_2$, 4 µl fraction $MQ_{0.5}$; PBP, 7 µl fraction $DS_{0.25}$.

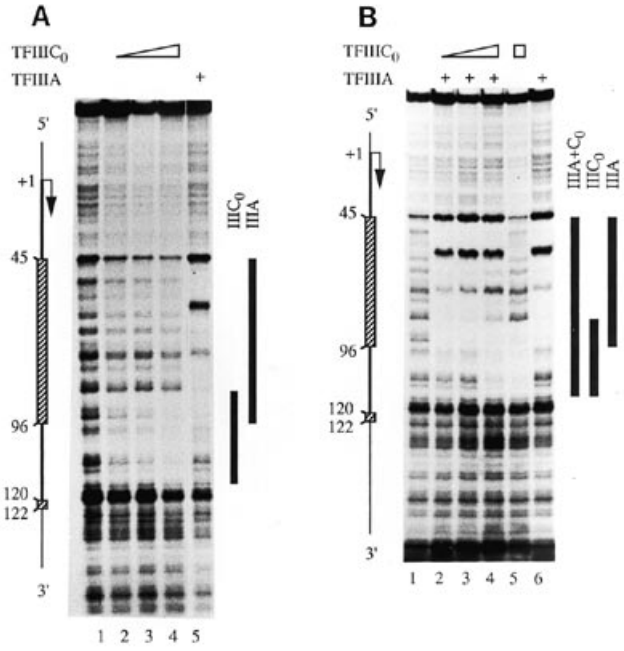

Figure 5. DNase I protection of the termination region of the h5S gene by the $TFIIIC_0$ fraction. (**A**) 7, 15 or 30 µl $TFIIIC_0$ (fraction $SP_{0.4}$, lanes 2–4) were incubated with the end-labelled *Eco*RI–*Hin*dIII 223 bp fragment of the h5S gene (non-coding strand) for footprint analysis as described in Materials and Methods. The h5S fragment was incubated without protein (lane 1) and with 20 µl fraction AD (lane 5). (**B**) Standard binding reactions contained 20 µl fraction AD in the presence of 7, 15 or 30 µl fraction $TFIIIC_0$ (fraction $SP_{0.4}$, lanes 2–4) and the 223 bp h5S fragment. Reactions shown in lanes 1, 5 and 6 contain either no protein (lane 1), 30 µl $TFIIIC_0$ (fraction $SP_{0.4}$, lane 5) or 20 µl fraction AD (lane 6). The positions of the protected regions are appropriately indicated relative to the 5S gene.

not competed for by B-box sequences. Moreover, footprint experiments performed with fractions enriched in $TFIIIC_1$ did not show any DNase I protection pattern on either the $htRNA^{met}$ (Fig. 6, lanes 10 and 11) or the VAI gene fragment (data not shown).

In this context the recently presented finding by Wang and Roeder (33) of a termination region-dependent increase in $TFIIIC_2$ binding upon addition of $TFIIIC_1$ or $TFIIIC_1'$ is relevant. These authors found a strong protection over the termination region of the VAI and *Xl*otRNA^{met} genes with either $TFIIIC_1$ or $TFIIIC_1'$. Regarding these results and taking into account that we do not observe termination binding in our $TFIIIC_1$ fractions (Fig. 6), we analysed whether the improved binding properties of the primary DNA binding factors observed upon addition of $TFIIIC_1$ depended on the termination region of these genes. As depicted in Figure 4B, incubation of $TFIIIC_1$ and the different primary DNA binding proteins (TFIIIA, lanes 2 and 3; $TFIIIC_2$, lanes 6 and 7; PBP, lanes 10 and 11) with fragments containing the specific promoter sequences but lacking the termination region increased the specific protein–DNA interactions up to the same level as was observed with fragments containing the corresponding termination regions (Fig. 4A). These findings strongly suggest that the enhanced binding of TFIIIA, $TFIIIC_2$ and PBP to their cognate promoter sequences upon addition of $TFIIIC_1$ is independent of an interaction of $TFIIIC_1$ with the termination region of these genes. We exclude a non-specific stabilization of the TFIIIA–5S DNA complex, because equimolar amounts of BSA had no effect. In addition, $TFIIIC_1$ neither influenced the formation of non-specific protein–DNA complexes nor the binding of TBP to the histone H5 TATA box (data not shown).

Binding of $TFIIIC_0$ to the termination region of class III genes

Footprint analyses of fractions enriched in $TFIIIC_0$ with the h5S gene fragment revealed a strong DNase I protection over the 3′ border of the C-box extending to the vicinity of the transcription termination site on the non-coding (Fig. 5A, lanes 2–4) or coding strand (data not shown) of the h5S gene. This protection was independent of hTFIIIA. Moreover, $TFIIIC_0$ protection over a region from position +93 to +115 (Fig. 5A, lanes 2–4, and B, lane 5) and the typical IIIA protection over the ICR from position +45 to +99 (Fig. 5A, lane 5, and B, lane 6) did not exclude each other, but led to an extended protection from position +45 to +115 in the presence of both activities (Fig. 5B, compare lanes 2–4 with lanes 5 or 6).

Since $TFIIIC_1$ influenced binding of $TFIIIC_2$ to type 2 promoters (Fig. 4) and $TFIIIC_0$ is partially able to substitute for $TFIIIC_1$ activity in the *in vitro* transcription of these genes (Fig. 3B), we additionally performed footprint experiments on the human $tRNA^{met}$ gene to analyse the molecular mechanism by which $TFIIIC_1$ and $TFIIIC_0$ act. Addition of fractions enriched in

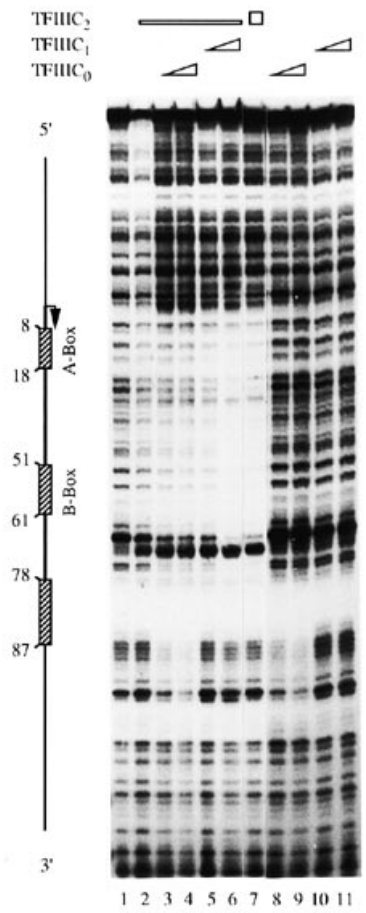

Figure 6. Footprint analysis of $TFIIIC_0$, $TFIIIC_1$ and $TFIIIC_2$ on the human $tRNA^{met}$ gene. The end-labelled 315 bp ht^{met} fragment was incubated with saturating (40 µl fraction $MQ_{0.5}$, lane 7) or non-saturating amounts of $TFIIIC_2$ (10 µl fraction $MQ_{0.5}$, lanes 2–6) without (lane 2) or in the presence of $TFIIIC_0$ (15 or 30 µl fraction $MQ_{0.2}$, lanes 3 and 4) or $TFIIIC_1$ (15 or 30 µl fraction $MQ_{0.27}$, lanes 5 and 6). Reactions shown in lanes 8–11 contained either $TFIIIC_0$ (15 or 30 µl fraction $MQ_{0.2}$, lanes 8 and 9) or $TFIIIC_1$ (15 or 30 µl fraction $MQ_{0.27}$, lanes 10 and 11) in the absence of $TFIIIC_2$. Lane 1 reflects a control reaction without protein.

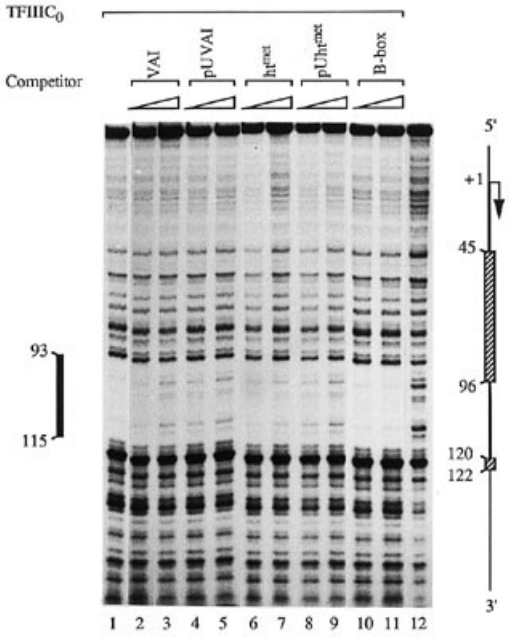

Figure 7. Competition analysis of the DNase I protection of $TFIIIC_0$. After preincubation of 30 µl $TFIIIC_0$ (fraction $SP_{0.4}$, lanes 1–11) with 1 µg pUC9 DNA and 2 µg poly(dI·dC) as non-specific competitor, the samples in lanes 2–11 were preincubated for an additional 30 min at 30°C with either 150 or 300 ng fragment (lanes 2, 3 and 6, 7) or 250 or 500 ng plasmid (lanes 4, 5 and 8, 9) of the genes for VAI (lanes 2–5) or ht^{met} (lanes 6–9) or 150 or 300 ng fragment containing the B-box sequence (lanes 10 and 11), indicated at the top of the figure, before adding the end-labelled h5S fragment. Lane 12 serves as a control reaction without protein.

$TFIIIC_1$ (Fig. 6, lanes 5 and 6) to a non-saturating amount of the B-box binding fraction $TFIIIC_2$ intensified the protection observed with this fraction alone (lane 2). Addition of $TFIIIC_0$ (lanes 3 and 4) only resulted in a slight increase, whereas hypersensitive sites appeared on the initiation site and between the B-box and the termination region of this gene (lanes 2–7). More importantly, $TFIIIC_0$ (lanes 8 and 9), but not $TFIIIC_1$, activity (lanes 10 and 11) correlated with a strong protection over the termination region of the $htRNA^{met}$ gene from position +86 to +104. This protection was not influenced by addition of $TFIIIC_2$ (lanes 3 and 4). Comparative footprint experiments using an end-labelled VAI gene fragment also showed a specific DNase I protection over the termination region from position +154 to +182 of this gene mediated by $TFIIIC_0$ fractions (data not shown).

Competition experiments proved that the termination regions of the three genes are in fact bound by the same component in the $TFIIIC_0$ fraction. As shown in Figure 7, the strong DNase I protection from position +93 to +115 of the h5S gene (lane 1) was alleviated upon competition with unlabelled fragment or plasmid DNA of the VAI (lanes 2–5) or ht^{met} (lanes 6–9) genes, whereas the specific $TFIIIC_2$ binding sequence did not influence $TFIIIC_0$-dependent protection (B-box, lanes 10 and 11). $TFIIIC_0$ binding was competed for more efficiently by plasmid (lanes 4, 5, 8 and 9) than by fragment DNA (lanes 2, 3, 6 and 7). We can exclude a non-specific competition by vector DNA, because all samples were preincubated with an excess of pUC9 DNA.

It should be emphasized that the strong enhancement of complex formation of different primary DNA binding factors observed upon addition of $TFIIIC_1$ (Figs 4 and 6) was not obtained with fractions enriched in $TFIIIC_0$ on either the tRNA gene (Fig. 6, lanes 3 and 4), the 5S gene (Fig. 5B, lanes 2–4) or the U6 gene (data not shown). Therefore, the component $TFIIIC_0$ reported here is clearly distinct from $TFIIIC_1$.

DISCUSSION

Purification of hTFIIIC activity resulted in the separation of three multiprotein activities, $TFIIIC_0$, $TFIIIC_1$ and $TFIIIC_2$. Whereas $TFIIIC_2$ is essentially required for transcription of genes with an internal promoter, $TFIIIC_1$ is required to reconstitute transcription of all pol III genes. The novel component, $TFIIIC_0$, binds to the termination region of pol III genes and is distinct from $TFIIIC_1$ and $TFIIIC_2$.

Interaction of $hTFIIIC_2$ with the hTFIIIA–5S gene complex

Preinitiation complex assembly on 5S rRNA genes requires TFIIIA in addition to TFIIIC, TFIIIB and pol III. This is conceivably due to the low affinity of $TFIIIC_2$ for direct recognition of the 5S promoter, lacking a B-box motif. Hence, we and others have not been able to

detect any direct interaction of $TFIIIC_2$ with the 5S gene (Fig. 1A and B; 34,35). However, it was also reported that high concentrations of $TFIIIC_2$ are able to directly contact the 5S promoter (19,24). This could possibly be due to a different purification procedure for TFIIIC, since it has also been reported that hTFIIIA can interact with $TFIIIC_2$ in the absence of promoter elements (18). Therefore, these preparations could conceivably contain low amounts of TFIIIA and direct recognition of the 5S promoter by $TFIIIC_2$ could be supported by these traces of TFIIIA possibly present in the fractions used.

In our hands, $hTFIIIC_2$ is able to bind to the TFIIIA–5S gene complex, thereby generating three additional complexes of lower mobility in EMSA (IIIA/IIIC, Figs 1A and 2). Incubation of $hTFIIIC_2$ with recombinant instead of purified hTFIIIA or recombinant *Xlo*TFIIIA also resulted in formation of three complexes of lower mobility (data not shown), providing definitive proof that all of these complexes contain TFIIIA. In contrast to these findings, previous studies reported that the TFIIIA–5S DNA complex is not altered by the addition of TFIIIC (34,36,37). In agreement with our results, however, Keller *et al.* (35) showed that binding of *Xlo*TFIIIC to the homologous TFIIIA–5S gene complex led to the appearance of three distinct TFIIIA/TFIIIC–5S DNA complexes with different gel mobilities. It is likely that the differences described in the above-mentioned studies, concerning formation of the TFIIIA/TFIIIC–5S DNA complexes, could result from different DNA templates and proteins used, as well as from varying incubation and EMSA conditions.

It should be pointed out that the appearance of the TFIIIA/TFIIIC–5S DNA complexes presented here coincided with extended protection of the 5S gene promoter against DNase I in footprint experiments, indicating that the TFIIIA/TFIIIC complex was not due to non-specific protein–DNA interactions. The window generated by hTFIIIA alone, ranging from +45 to +99 of the human 5S gene and thus covering the A- and C-boxes, is extended toward the initiation site up to position +20 by addition of $hTFIIIC_2$ (Fig. 1B). This protection against DNase I is identical to that of the *Xlo*5S gene observed after addition of TFIIIC, in which case the TFIIIA footprint was likewise extended to position +20 (38). Moreover, we found that addition of $hTFIIIC_2$ to the hTFIIIA–5S gene complex resulted in disappearance of the typical hypersensitive site at position +62 of the non-coding strand (data not shown). This finding indicates that $hTFIIIC_2$ is incorporated into the TFIIIA–5S DNA complex from the opposite side of the double helix.

$hTFIIIC_2$ has the potential to interact with the TFIIIA–5S gene complex and the B-box sequence simultaneously

For further characterization of the three hTFIIIA/$TFIIIC_2$–5S gene complexes, competition experiments were conducted, showing that the complex with the slowest mobility can be specifically competed for by B-box sequences, leading to the appearence of 'supercomplexes' (IIIA/IIIC*, Fig. 2, lanes 6, 7 and 9). Incubation with non-specific fragments did not result in the generation of a 'supercomplex' (Fig. 2, lanes 8 and 10). Therefore, $TFIIIC_2$ in this TFIIIA/$TFIIIC_2$–5S gene complex must be able to simultaneously contact the TFIIIA–5S DNA complex and the B-box sequences. Thus $TFIIIC_2$ is either incorporated into the TFIIIA–5S DNA complex solely by protein–protein interactions or it makes use of DNA binding surfaces other than those needed for B-box recognition.

Fradkin *et al.* (19) reported a TFIIIA-independent interaction of $hTFIIIC_2$ with the *Xenopus borealis* 5S rRNA gene and concluded from competition experiments that $hTFIIIC_2$ interacts with *Xb*5S gene and tRNA-type genes through separate binding domains or polypeptides. Extending these observations, our data directly show the potential for the simultaneous interaction of $hTFIIIC_2$ with the tRNA-type gene and with the hTFIIIA–5S DNA complex (Fig. 2).

The other two TFIIIA/$TFIIIC_2$–5S gene ternary complexes cannot be affected in their mobility by B-box sequences (Fig. 2). This raises the possibility that $TFIIIC_2$ exhibits different conformations upon binding to the TFIIIA–5S DNA complex, only one of which is accessible to B-box sequences. Further experiments have to check whether these three bands correspond to different transcriptional activities of the TFIIIA/IIIC complex and whether they are related to the two different TFIIIC–VAI complexes in EMSA observed by Kovelman and Roeder (39). These authors found that only the slower migrating form ($TFIIIC_2$a) correlates with TFIIIC transcriptional activity. UV crosslinking experiments are currently underway to examine the composition of the three distinct complexes.

$hTFIIIC_1$ potentiates binding of pol III factors to DNA without involving the corresponding termination region

As demonstrated in EMSA and DNase I footprinting experiments, $hTFIIIC_1$ not only strongly enhanced binding of $hTFIIIC_2$ to type 2 promoters (Figs 4A and 6), but also improved binding of TFIIIA to the ICR of the h5S rRNA gene and of the PSE binding protein PBP to the U6 promoter (Fig. 4A). In all these cases, enhancement was independent of the presence or absence of the corresponding termination region of these genes (Fig. 4B). Therefore, we conclude that $hTFIIIC_1$ can generally strengthen the specific association of primary DNA binding pol III transcription factors and that this potentiation is independent of an interaction with the termination region. This observation could represent an important function of this activity for transcription of pol III genes (Fig. 3). However, the main role of $TFIIIC_1$ activity cannot be explained merely by improving DNA binding, because high amounts of the primary binding factors are not sufficient to fully complement transcription (data not shown).

Very importantly, we also show that $TFIIIC_1$ can be chromatographically separated from an additional activity ($TFIIIC_0$), stemming from phosphocellulose fraction C and displaying a strong protection of the termination region of class III genes, whereas $TFIIIC_1$ does not contact these sequences (Fig. 6). In agreement with our results, $TFIIIC_1$ fractions purified by Yoshinaga *et al.* (14) also displayed no termination binding of type 2 promoter genes. In contrast, Wang and Roeder (33) recently reported the existence of two functionally equivalent isoforms of $hTFIIIC_1$ activity, which were purified either from phosphocellulose fraction B ($TFIIIC_1'$) or C ($TFIIIC_1$). Both activities correlated with strong binding activity to the termination region and weak A-box binding activity on VAI and tRNA genes and both enhanced binding of $TFIIIC_2$ to type 2 promoters in a manner dependent on the termination region (33). While we cannot reconcile these discrepancies, it is conceivable that $TFIIIC_1$ and $TFIIIC_1'$ purified by Wang and Roeder (33) both contain $TFIIIC_0$ in addition to $TFIIIC_1$.

$hTFIIIC_0$, but not $hTFIIIC_1$, binds to the termination region of type 1 and type 2 promoter genes

In contrast to Wang and Roeder (33), we could separate the termination binding activity from $TFIIIC_1$ and we designated this

fraction $TFIIIC_0$ (Figs 5 and 6). This activity specifically bound to the termination region of the $htRNA^{met}$ (Fig. 6), the VAI (data not shown) and the h5S gene (Fig. 5A). The protected regions around the termination site of these three genes are enriched in GC stretches, but show no significant sequence homology among themselves. Competition experiments, in which DNase I protection of the termination region was challenged with various DNA sequences, confirmed that these genes all bind the same component of the $TFIIIC_0$ fraction (Fig. 7). It should be noted that $TFIIIC_0$ binding was competed for more efficiently by plasmid than by fragment DNA. This finding may suggest a topology-dependent interaction of the $TFIIIC_0$ fraction with the respective DNA and helps to explain why a significantly higher amount of fraction $TFIIIC_0$ is necessary to protect the termination region in footprint experiments (Figs 5–7) than is required for reconstituted *in vitro* transcription (Fig. 3). Whereas binding of $TFIIIC_2$ to a type 2 promoter commits this gene and excludes transcription of a second gene added subsequently (40), the interaction of $TFIIIC_0$ with AB or AC promoter genes is not stable enough to preclude transcription of a second template subsequently added together with the remaining components of the pol III apparatus (data not shown).

Apart from the documented differences in their DNA binding properties, the functional diversity of $TFIIIC_0$ and $TFIIIC_1$ is supported by their differential effect on U6 (Fig. 3C) and tRNA (Fig. 3B) transcription. In addition, whereas $TFIIIC_1$ significantly enhanced binding of $TFIIIC_2$ and formation of the $TFIIIC_2$–VAI complex also occured more rapidly in the presence of this factor, $TFIIIC_0$ only slightly increased, but did not accelerate, formation of $TFIIIC_2$–VAI complexes (data not shown). Moreover, $TFIIIC_0$ also seems to differ from both $TFIIIC_1$ and $TFIIIC_1'$ described by Wang and Roeder (33), who reported similar effects of these two isoforms on VAI transcription. While these differences are subtle, we find that VAI RNA synthesis was much less efficient in the presence of $TFIIIC_0$ than in the presence of $TFIIIC_1$ (data not shown), whereas the observed difference was less pronounced for the ht_i^{met} gene (Fig. 3B).

Collectively, our results clearly show that three individual components can be chromatographically separated from phosphocellulose fraction C which fulfil different aspects of DNA binding to pol III genes. Whereas $TFIIIC_2$ binds to the B-box of type 2 promoters, $TFIIIC_1$ enhances association of pol III factors primarily binding to DNA ($TFIIIC_2$, TFIIIA and PBP), acting in a way which is independent of the termination signal. $TFIIIC_0$, in contrast, strongly binds to the termination region of various pol III genes and activates transcription by a mechanism which is not entirely clear at present, but could be related to the finding (41) that the termination region of pol III genes is required for multiple reinitiation cycles.

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