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TFIIIC1 Acts through a Downstream Region To Stabilize TFIIIC2 Binding to RNA Polymerase III Promoters

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An in vitro system reconstituted with highly purified RNA polymerase III, TFIIIC2, and TFIIIB has been used to identify two chromatographically distinct human RNA polymerase III transcription factors, TFIIIC1 and TFIIIC1′, which are functionally equivalent to the previously defined TFIIIC1 (S. T. Yoshinaga, P. A. Boulanger, and A. J. Berk, Proc. Natl. Acad. Sci. USA 84:3585–3589, 1987). Interactions between TFIIIC2, TFIIIC1 (or TFIIIC1′), and the VA1 and tRNA$_1^{Met}$ templates have been investigated by DNase I footprint analysis. Homogeneous TFIIIC2 alone shows only a weak footprint over the B-box region of the VA1 and tRNA$_1^{Met}$ templates, whereas TFIIIC1 (or TFIIIC1′) alone shows both a strong interaction over the downstream termination region and a very weak interaction near the A-box region. Importantly, when both factors are present simultaneously, TFIIIC1 (or TFIIIC1′) dramatically enhances the level of TFIIIC2 binding and extends the footprint to a region that includes the A box. The downstream termination region is essential for this cooperative interaction between TFIIIC2 and TFIIIC1 (or TFIIIC1′) on the VA1 and tRNA$_1^{Met}$ templates and plays a role in the overall accuracy and efficiency of RNA polymerase III transcription.

The genes transcribed by RNA polymerase III fall into three subclasses on the basis of their promoter structures and transcription factor requirements (15–17, 51). For subclass 1 and subclass II genes, promoter elements required for transcription initiation are generally found within the coding sequence. The 5S RNA genes (subclass 1) have internal A-box, I-box, and C-box elements. The tRNA, 7SL, and adenovirus VA1 and VA2 RNA genes, as well as *Alu* sequences (subclass II), have a simple internal promoter containing A-box and B-box elements. Subclass 3 genes (U6 and 7SK RNA genes) have upstream promoter elements that include a TATA box element, a conserved proximal sequence element, and a distal sequence element (51). For subclass 1 and 2 genes, internal promoter elements, which are highly conserved between different genes and different species, are generally sufficient for transcription initiation. However, the expression of subclass 1 and 2 genes also can be dramatically influenced both in vivo and in vitro by extragenic flanking sequences, which frequently show little or no conservation and can modulate transcription by influencing either assembly or stability of active transcription complexes (1, 9, 39, 58).

As demonstrated first in vertebrates (2, 14, 28, 43, 44) and subsequently in the yeast *Saccharomyces cerevisiae* (40, 46), RNA polymerase III requires accessory transcription factors which interact with these sequence elements to form a stable preinitiation complex. The core promoter elements (A and B boxes) of subclass III genes are recognized by TFIIIC, which in turn directs the sequential binding of TFIIIB and RNA polymerase III. In the case of 5S RNA genes, the promoter activation process is similar except that TFIIIC recruitment is dependent upon binding of TFIIIA. Consistent with the structure of yeast TFIIIB (4, 7, 30), we previously described a human core TFIIIB containing TATA-binding protein (TBP) and a TFIIB-related 90-kDa polypeptide (50). Unlike yeast TFIIIC, which is composed of six polypeptides and binds both A- and B-box elements in tRNA templates (33, 36, 37, 48), the human TFIIIC has a more complicated and not fully defined structure (26). TFIIIC activity from HeLa cells can be separated into two fractions (TFIIIC1 and TFIIIC2) which are both required for transcription by RNA polymerase III (11, 55) and may be analogous to two partially purified factors (TFIIIC and TFIIID) identified in *Bombyx mori* (58). Highly purified human TFIIIC2 (26, 56) contains five polypeptides (220, 110, 102, 90, and 63 kDa) and binds the B-box region. TFIIIC2 can be isolated in both active (TFIIIC2a) and inactive (TFIIIC2b) forms, and the relative levels of these forms can be influenced by growth conditions and virus infection (19, 20, 45, 54). Although the composition and exact function of TFIIIC1 are still not clear, TFIIIC1 and TFIIIC2 together show weak interactions over both the A-box and B-box elements and flanking regions extending to the 5′ and 3′ ends of the VA1 gene (55).

Using highly purified RNA polymerase III transcription factors from HeLa cells as an assay, we have identified two chromatographically distinct but functionally similar factors, TFIIIC1 and TFIIIC1′, and demonstrated cooperative interactions of these factors with TFIIIC2 on the VA1 and tRNA$_1^{Met}$ templates. These studies further demonstrate novel independent interactions of TFIIIC1 and TFIIIC1′ with a downstream termination region and show that this region plays a role both in cooperative interactions between TFIIIC2 and TFIIIC1 (or TFIIIC1′) and in modulating the efficiency and accuracy of RNA polymerase III transcription.

MATERIALS AND METHODS

Plasmid constructions. pVA1, pXlt$_1^{Met}$, and pH5S8544 were described as before (35). Plasmid pTR1 was constructed by inserting a PCR amplification fragment between −69 and +135 of the tRNA$_1^{Met}$ gene of *Xenopus laevis* into the *Hin*dIII and *Eco*RI sites of pBluescript KS(−) (Stratagene). Plasmids pTR2 (containing tRNA$_1^{Met}$ sequences from −69 to +72), pTR1-4 (containing sequences from −69 to +218), and pTR2-5 (containing sequences from −491 to +135) were constructed as for pTR1.

In vitro transcription and RNA polymerase III assays. The assays were as described previously (50). For measuring single-round transcription, templates and factors were first preincubated for 60 min. Sarkosyl was then added to a final concentration of 0.05%, followed immediately by addition of nucleoside triphosphates. After incubation for 60 min at 30°C, transcription reactions were terminated by the addition of stop mix (25).

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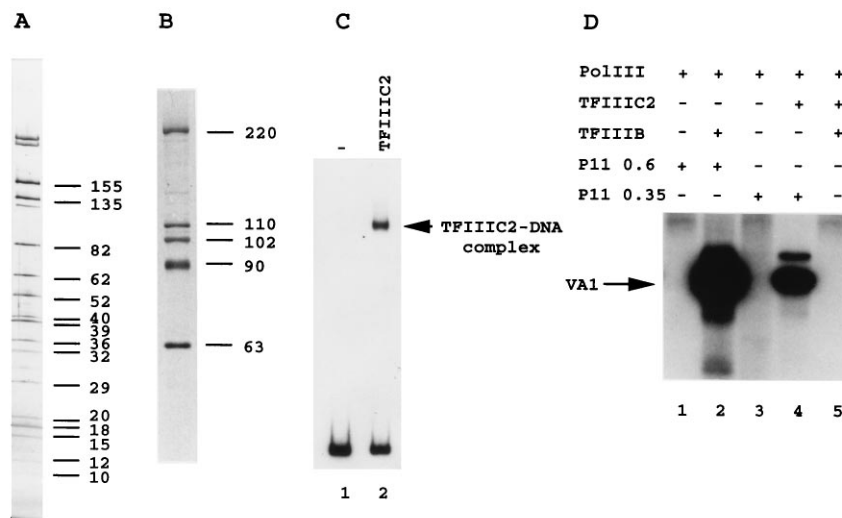

FIG. 1. Structural and functional analysis of RNA polymerase III factors. (A) SDS-polyacrylamide gel electrophoresis of highly purified RNA polymerase III. Two hundred nanograms of RNA polymerase III (sucrose gradient fraction) was separated on an SDS–5 to 18% gradient gel and visualized by silver staining. Polypeptides coeluting with RNA polymerase III activity are indicated at the right in kilodaltons. (B) SDS-polyacrylamide gel electrophoresis of highly purified TFIIIC2. Four hundred nanograms of TFIIIC2 (oligonucleotide affinity column fraction) was separated on an SDS–6% gel and stained with silver. Subunits ascribed to TFIIIC2 are indicated to the right. (C) Gel shift analysis of the TFIIIC2 preparation. Lane 1, probe alone; lane 2, probe plus 10 ng of purified TFIIIC2. (D) VA1 transcription activity. Transcription reactions were reconstituted with 5 ng of highly purified RNA polymerase III (PolIII) and the following combinations of TFIIIB and TFIIIC preparations: lane 1, 2.8 μg of the crude TFIIIC (P11 0.6 M KCl) fraction alone; lane 2, 300 ng of highly purified TFIIIB plus 2.8 μg of the crude TFIIIC fraction; lane 3, 7 μg of the crude TFIIIB (P11 0.35 M KCl) fraction alone; lane 4, 5 ng of highly purified TFIIIC2 plus 7 μg of the crude TFIIIB fraction; lane 5, 300 ng of highly purified TFIIIB plus 5 ng of highly purified TFIIIC2.

Purification of transcription factors and RNA polymerase III. HeLa cell nuclear extract and S100 were prepared as described by Dignam et al. (13). Two hundred milliliters of HeLa cell S100 (16 mg of protein per ml) was loaded onto a P11 column (5 by 16 cm), and the column was step eluted with BC350 and BC600 as described elsewhere (50). The P11 BC350 (P11 0.35 M KCl) fraction was used to further purify TFIIIB and RNA polymerase III. The BC350 fraction was dialyzed against buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-NaOH, [pH 7.9], 5% glycerol, 1 mM EDTA, 3 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl and then loaded onto a fast protein liquid chromatography (FPLC) Mono Q column (HR10/10; Pharmacia). The column was eluted with a 200-ml linear gradient from 100 to 600 mM KCl in buffer A. The TFIIIB activity was eluted at 280 mM KCl, while the RNA polymerase III activity was eluted at 350 mM KCl. The fractions containing TFIIIB or RNA polymerase III were pooled separately, dialyzed against buffer B (20 mM HEPES-NaOH [pH 7.9], 10% glycerol, 1 mM EDTA, 3 mM DTT) containing 100 mM KCl, and loaded onto a high-pressure liquid chromatography (HPLC) SP-5PW column (75 by 7.5 mm; Bio-Rad). The column was eluted with a 60-ml linear gradient from 100 to 600 mM KCl in buffer B. TFIIIB activity was eluted at 130 mM KCl and used as the partially purified TFIIIB for transcription and footprint analysis. The preparation used here had a protein concentration of 15 ng/μl. The HPLC SP-5PW column fractions containing RNA polymerase III activity were pooled, dialyzed against buffer C (25 mM Tris-HCl [pH 7.9], 25% glycerol, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) containing 50 mM ammonium sulfate, and loaded onto a DEAE-Sephadex A25 column (1 by 4 cm). The column was eluted with a 20-ml linear gradient from 50 to 500 mM ammonium sulfate in buffer C. The RNA polymerase III activity was eluted at 320 mM ammonium sulfate and further purified by sucrose gradient centrifugation. Then 0.2 ml of the RNA polymerase III preparation (DEAE-Sephadex A25 fraction) was loaded onto 4.0-ml, 5 to 20% sucrose gradients containing 0.25 M ammonium sulfate in buffer A and centrifuged for 18 h at 56,000 rpm and 4°C in an SW60 rotor (Beckman). Fractions (0.2 ml) were collected from the bottom and assayed for RNA polymerase III activity by random transcription assay. The fraction used here had a protein concentration of 20 ng/μl.

To purify TFIIIC2, 200 ml of HeLa nuclear extract (12 mg of protein per ml) was fractionated on a P11 column (5 by 12 cm) as described above. The P11 BC600 (P11 0.6 M KCl) fraction was dialyzed against buffer A containing 100 mM KCl and purified through an FPLC Mono Q column (HR10/10) as described above. The TFIIIC2-containing fractions were pooled, dialyzed against buffer A containing 100 mM KCl, and loaded onto an FPLC Mono S column (HR5/5). The column was eluted with a 25-ml linear gradient from 100 to 600 mM KCl in buffer A. TFIIIC2 was further purified through HPLC SP-5PW and B-box oligonucleotide affinity columns as described previously (26). The preparation used here contained 5 ng of protein per μl.

To purify TFIIIC1 and TFIIIC1′, 20 ml of the HeLa nuclear extract was loaded onto a P11 column (1.5 by 6.5 cm), and the column was eluted with a 100-ml linear gradient from 100 to 1,000 mM KCl in buffer A. TFIIIC1 and TFIIIC1′ were eluted at 280 and 390 mM KCl, respectively. The TFIIIC1- and TFIIIC1′-containing fractions were pooled separately, dialyzed against buffer A containing 100 mM KCl, and purified on an FPLC Mono Q column (HR10/10) as described above. TFIIIC1′ eluted at 210 mM KCl, while TFIIIC1 eluted at 230 mM KCl, The fractions used here had protein concentrations of 0.5 μg/μl.

The P11 column flowthrough fraction (5 μg of protein per μl) of HeLa nuclear extract was used as a source of TFIIIA for 5S gene transcription.

Gel mobility shift assay. Probe was prepared by end labeling of the 129-bp *Xba*I-*Bst*EII fragment of plasmid pVA1 with [α-^{32}P]GTP and the Klenow fragment. Twenty-five-microliter reaction mixtures contained 12% glycerol, 75 mM KCl, 6 mM $MgCl_2$, 20 mM HEPES-NaOH (pH 7.9), 3 mM DTT, 0.4 mM EDTA, 100 ng of poly(dI-dC), 5 μg of bovine serum albumin, and 5 fmol of the labeled probe. Reaction mixtures were incubated at 30°C for 30 min, loaded onto a 4% polyacrylamide gel (acrylamide/bisacrylamide, 37.5:1) in 0.25× Tris-borate-EDTA, and electrophoresed at 125 V for 2 h at room temperature. The gel was dried and exposed to X-ray film.

DNase I footprint. For the footprint assay, 25 μl of reaction mixture containing 2 fmol of the ^{32}P-labeled DNA fragment, 50 ng of poly(dI-dC), 5 μg of bovine serum albumin, 10 mM HEPES-NaOH (pH 7.9), 70 mM KCl, 5 mM DTT, 0.2 mM EDTA, 6 mM $MgCl_2$, 12% glycerol, and appropriate amounts of the purified factors was incubated at 30°C for 45 min. Twenty-five microliters of the Ca^{2+}-Mg^{2+} solution (5 mM $CaCl_2$, 10 mM $MgCl_2$) and 0.5 to 6 μl of DNase I (0.4 ng/μl) were added. The reaction mixture was incubated at room temperature for 2 min. The digestion was terminated by adding 25 μl of stop solution (0.2 M NaCl, 30 mM EDTA, 1% sodium dodecyl sulfate [SDS], 100 μg of yeast tRNA per ml). After phenol-chloroform extraction, ethanol precipitation, and resuspension in 5 μl of gel loading buffer (86% formamide, 0.02% each bromophenol blue and xylene cyanol, 1× Tris-borate-EDTA), samples were heated for 3 min at 90°C and loaded onto an 8% polyacrylamide–7 M urea gel. DNA bands were visualized by autoradiography.

RESULTS

Purification of RNA polymerase III, TFIIIC2, and TFIIIB. RNA polymerase III activity was purified to the point at which it contained no detectable TFIIIB, TFIIIC, or TBP, as monitored by transcription and Western blot (immunoblot) assays (data not shown). Upon sucrose gradient centrifugation, 15 polypeptides coeluted with RNA polymerase III activity (Fig. 1A). This highly purified RNA polymerase III supports gene-

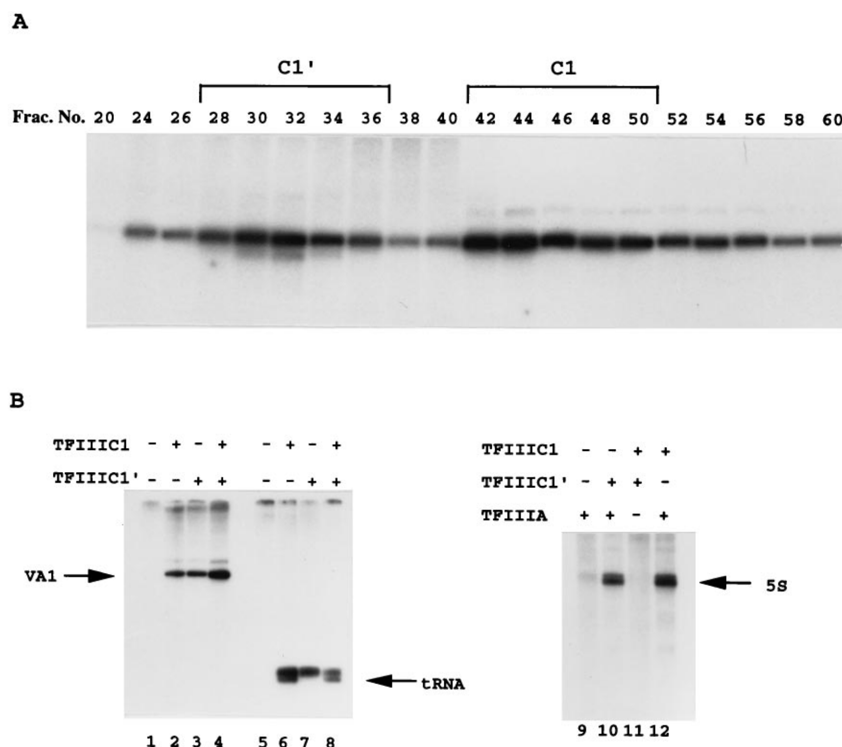

FIG. 2. Chromatographic resolution and functional analysis of TFIIIC1 and TFIIIC1′. (A) Two P11 column fractions, TFIIIC1 (fractions 42 to 50) and TFIIIC1′ (fractions 28 to 36), restore VA1 gene transcription when reconstituted with highly purified TFIIIB (300 ng), TFIIIC2 (5 ng), and RNA polymerase III (5 ng). Two microliters of each column fraction was assayed. (B) One microgram of TFIIIC1 (lanes 2, 6, and 12) or TFIIIC1′ Mono Q fraction (lanes 3, 7, and 10) can direct RNA polymerase III transcription of the VA1, tRNA, and 5S RNA genes in a system reconstituted with highly purified RNA polymerase III, TFIIIC2, and TFIIIB. Ten micrograms of the TFIIIA fraction was added to 5S RNA gene reactions as indicated.

specific transcription in the presence of TFIIIB and TFIIIC (Fig. 1D, lanes 2 and 4). This analysis revealed more subunits than the nine subunits previously reported by Jaehning et al. (22), probably because the larger amounts analyzed allowed visualization of smaller subunits. According to the present results, the polypeptide composition of the human RNA polymerase III is comparable to that of the well-characterized yeast RNA polymerase III that contains 16 subunits (15). At present, only one cloned human RNA polymerase III-specific subunit has been reported, and this subunit shows a low sequence similarity to the equivalent subunit of the yeast RNA polymerase III (21).

During the purification of TFIIIC2, activity was monitored by transcription in a reconstituted system containing a crude (P11 0.35 M KCl) TFIIIB fraction and the above-described RNA polymerase III and by gel shift assay with the VA1 template. FPLC Mono Q and Mono S columns were used instead of the heparin-Sepharose column used in our previous procedure (26). We have now purified TFIIIC2 to near homogeneity and provided stronger evidence that human TFIIIC2 is a heteromeric complex of five polypeptides (220, 110, 102, 90, and 63 kDa) (Fig. 1B). The purified five-subunit TFIIIC2 binds to the VA1 template in mobility shift assays (Fig. 1C, lane 2) and supports RNA polymerase III transcription on the VA1 template in conjunction with crude TFIIIB (P11 0.35 M KCl) and purified RNA polymerase III (Fig. 1D, lane 4). The peptide composition and activity of this more highly purified TFIIIC2 are consistent with those reported previously (26, 27, 29, 45, 56).

TFIIIB was purified by monitoring transcription activity in a reconstituted system containing crude TFIIIC (P11 0.6 M KCl) and purified RNA polymerase III. TFIIIB was completely separated from B-TFIID (49), RNA polymerase III, TFIIIC1, and TFIIIC1′ (see below) after passage through the HPLC SP-5PW column. The TFIIIB fraction contains TBP (monitored by Western blot analysis), supports transcription of the VA1 template in association with TFIIIC and the highly purified RNA polymerase III (Fig. 1D, lane 2), and can functionally substitute for the immunopurified FLAG-tagged TFIIIB (6) and the recombinant core TFIIIB (50) in transcription assays.

An additional factor is required for transcription with highly purified TFIIIB, TFIIIC2, and RNA polymerase III. In contrast to the results observed with less purified factors (see above), highly purified preparations of TFIIIB, TFIIIC2, and RNA polymerase III failed to support transcription of VA1, $tRNA_1^{Met}$, and 5S RNA genes (Fig. 1D, lane 5; Fig. 2B, lanes 1, 5, and 9). This result indicates that an additional transcription factor(s) is present in both the crude TFIIIB fraction (P11 0.35 M KCl) and the crude TFIIIC fraction (P11 0.6 M KCl), each of which effects transcription in conjunction with the complementary purified components (Fig. 1D, lanes 2 and 4). Indeed, when assayed in a system reconstituted with highly purified TFIIIB, TFIIIC2, and RNA polymerase III, two such components were detected in the gradient-eluted P11 column fractions of HeLa nuclear extract (Fig. 2A). One component, independently detected in the crude TFIIIB fraction (P11 0.35 M KCl), is designated TFIIIC1′ (initially called TFIIIB1 [6]) and eluted at 280 mM KCl (Fig. 2A, fractions 28 to 36). The other component, independently detected in the crude TFIIIC fraction (P11 0.6 M KCl), is designated TFIIIC1 and eluted at 390 mM KCl from the P11 column (Fig. 2A, fractions 42 to 50). Each component was further purified through an FPLC Mono

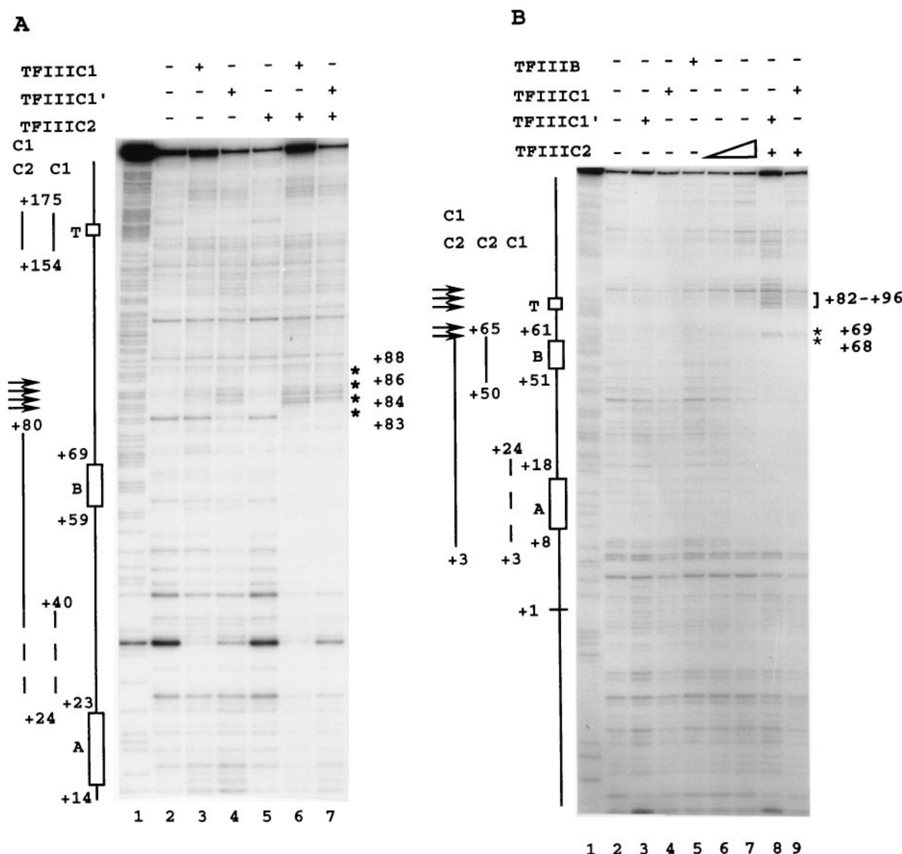

FIG. 3. TFIIIC1 or TFIIIC1′ increases TFIIIC2 binding to the VA1 and tRNA genes. (A) DNase I footprint on the 248-bp *Xba*I-*Eco*RI fragment of the VA1 gene labeled at the 3′ end of the transcribed strand. (B) DNase I footprint on the 210-bp *Hin*dIII-*Eco*RI fragment of the $tRNA_1^{Met}$ gene labeled at the 3′ end of the transcribed strand. Labeled fragments were digested with DNase I after incubation with TFIIIC1 (1 μg), TFIIIC1′ (1 μg), or TFIIIC2 (100 ng in all cases except panel B, lane 7, which contained 300 ng) as indicated. The hypersensitive sites (base pair positions) are indicated at right, and the footprint regions are displayed schematically at left. Protected regions were located by alignment with a Maxam-Gilbert G+A reaction (lane 1).

Q column, from which TFIIIC1′ and TFIIIC1 eluted at 210 and 230 mM KCl, respectively. After passage through the Mono Q column, neither fraction contained detectable TFIIIB, TFIIIC2, RNA polymerase III, or TBP (data not shown). Either TFIIIC1 or TFIIIC1′ alone can restore the transcription activity of VA1, $tRNA_1^{Met}$, and 5S RNA genes in a reconstituted system containing the highly purified TFIIIB, TFIIIC2, and RNA polymerase III (Fig. 2B, lanes 2, 3, 6, 7, 10, and 12). TFIIIC1 appears to be chromatographically and functionally similar to TFIIIC1 characterized by Yoshinaga et al. (55).

Although TFIIIC1′ and TFIIIC1 were functionally equivalent in the transcription of the VA1 and 5S RNA genes (Fig. 2B, lanes 2, 3, 10, and 12), slight differences were observed in transcription of the $tRNA_1^{Met}$ gene. The overall level of transcription from the $tRNA_1^{Met}$ gene was twofold lower with TFIIIC1′ than with TFIIIC1 (Fig. 2B, lane 7 versus lane 6). In addition, when reactions contained both TFIIIC1′ and TFIIIC1, transcription levels from the VA1 gene increased about twofold, while those from the $tRNA_1^{Met}$ gene decreased twofold relative to the levels observed with only TFIIIC1 (Fig. 2B, lanes 4 and 8 versus lanes 2 and 6). One possible explanation for the latter observation is that the less active TFIIIC1′ titrates other RNA polymerase III transcription factors or forms less productive preinitiation complexes on the $tRNA_1^{Met}$ gene. In addition to TFIIIC1′ or TFIIIC1, all of the other components (TFIIIB, TFIIIC2, and RNA polymerase III) are required for transcription of the VA1 and $tRNA_1^{Met}$ RNA genes (data not shown). The existence of two functionally equivalent isoforms of TFIIIC1 in the P11 0.35 and P11 0.6 fractions could explain our previous failure (26) to reproduce the earlier observation (55) that the TFIIIC activity from HeLa cells could be separated into two fractions (TFIIIC1 and TFIIIC2).

TFIIIC1 and TFIIIC1′ increase TFIIIC2 binding to the VA1 and $tRNA_1^{Met}$ templates through interactions with the termination region. DNase I footprint analyses were conducted to determine the specific DNA binding activity of TFIIIC1′, TFIIIC1, and TFIIIC2 on the VA1 (Fig. 3A and 4A) and $tRNA_1^{Met}$ (Fig. 3B and 4B) templates. In agreement with previous observations (10, 19, 56), purified TFIIIC2 alone showed interactions that were restricted to the B-box region of the VA1 and $tRNA_1^{Met}$ templates. However, a clear footprint was visible only when as much as 300 ng of the highly purified factor was used (Fig. 3B, lane 7 versus lane 2; Fig. 4A, lane 10 versus lane 2), with almost no interactions evident at 100 ng of factor (Fig. 3A, lane 5; Fig. 3B, lane 6; Fig. 4A, lane 9; Fig. 4B, lane 5). In contrast, in the presence of either TFIIIC1′ or TFIIIC1, strong protections on the B-box of the full-length VA1 and $tRNA_1^{Met}$ templates were clearly seen with just 100 ng of TFIIIC2 (Fig. 3A, lanes 6 and 7 versus lane 5; Fig. 3B, lanes 8 and 9 versus lane 6). The protected regions (summarized on the left in Fig. 3A, 3B, 5A, and 5B) were also expanded compared to those observed with TFIIIC2 alone; the +52 to +70 footprint on the VA1 gene was expanded to +40 to +80, while

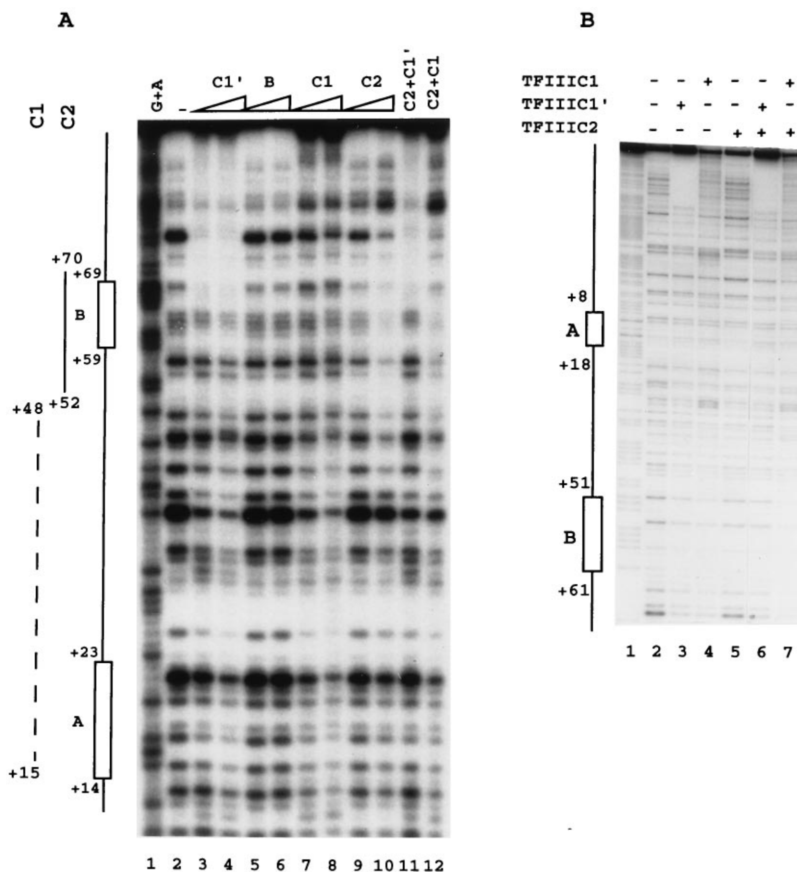

FIG. 4. Downstream sequences are essential for cooperative binding of TFIIIC2 and TFIIIC1 (or TFIIIC1′) to the VA1 and tRNA templates. (A) DNase I footprint pattern on the 129-bp *Xba*I-*Bst*EII fragment of the VA1-containing A and B boxes and a radioactive label at the 3′ end of the transcribed strand. Probe was incubated with 1 μg (lanes 3, 5, 7, 11, and 12) or 2 μg (lanes 4, 6, and 8) of TFIIIC1 or TFIIIC1′ as indicated and 100 ng (lanes 9, 11, and 12) or 300 ng (lane 10) of TFIIIC2. (B) DNase I footprint on the 147-bp *Hin*dIII-*Eco*RI fragment of the $tRNA_1^{Met}$ gene containing A and B boxes and a radioactive label at the 3′ end of the transcribed strand. The probe was incubated with 1 μg of TFIIIC1 or TFIIIC1′ and 100 ng of TFIIIC2 in the combinations indicated. Protected regions (base pair positions) are displayed schematically at the left. Lane 1 is a Maxam-Gilbert G+A reaction.

the +50 to +65 footprint on the $tRNA_1^{Met}$ gene was expanded to +3 to +65. In addition, hypersensitive sites (+83, +84, +86, and +88 for VA1; +68 and +69 for $tRNA_1^{Met}$) appeared between the B box and the termination regions on both VA1 and $tRNA_1^{Met}$ genes (Fig. 3A, lanes 6 and 7 versus lane 5; Fig. 3B, lanes 8 and 9 versus lane 6 [summarized on the right]). These sites may be indicative of a bend between the B box and the termination region.

Interestingly, independent footprint analysis with TFIIIC1′ and TFIIIC1 fractions on shorter DNA probes showed that either could bind strongly to the termination region of the VA1 template (Fig. 5, lanes 3 to 6). Similar interactions of TFIIIC1 or TFIIIC1′ with the termination region of the $tRNA_1^{Met}$ were also observed but were weaker than those on the VA1 template (data not shown). This may reflect the fact that the VA1 termination sequence is a strong terminator while the $tRNA_1^{Met}$ termination sequence resembles very weak terminators (3). Significantly, interactions over the termination regions were also observed in the presence of both TFIIIC2 and TFIIIC1, although the length of the probes used for these analyses precluded high resolution in the termination region. Protection was more evident for the VA1 gene, in the presence (Fig. 3A, lane 7 versus lane 5) or absence (Fig. 3A, lane 3 versus lane 2) of TFIIIC2. In the case of the $tRNA_1^{Met}$ gene, interactions were barely visible on the long probe in the absence of TFIIIC2 (Fig. 3B, lanes 4 and 3 versus lane 2) and were further obscured in the presence of TFIIIC2 because of the proximity of the induced hypersensitive sites (Fig. 3B, lanes 8 and 9 versus lane 6). To investigate the possible relevance of the interaction between TFIIIC1′ or TFIIIC1 and the downstream termination region, a 129-bp VA1 gene fragment containing A-box and B-box regions, but lacking the termination region, was used to examine cooperative binding between TFIIIC1 (or TFIIIC1′) and TFIIIC2 at a low (100-ng) level of TFIIIC2. In this case, the footprint of TFIIIC2 over the B box of the VA1 template was not dramatically enhanced or extended in the presence of either TFIIIC1′ or TFIIIC1 (Fig. 4A, lanes 11 and 12 versus lane 9), in contrast to what was observed on a VA1 fragment containing the termination region in addition to A- and B-box regions (Fig. 3A, lanes 6 and 7 versus lane 5). Similar results were observed when a 147-bp $tRNA_1^{Met}$ gene fragment containing A-box and B-box regions, but no termination region, was used in the footprint analysis (Fig. 4B, lanes 6 and 7 versus lane 5; compare with Fig. 3B, lanes 8 and 9 versus lane 6, where the termination region was present). These results indicate that 3′ downstream sequences play a role in the cooperative interaction between TFIIIC1 (or TFIIIC1′) and TFIIIC2 on intact templates. It should be noted that the strong protection elicited by the TFIIIC1′ fraction at the ends of the DNA probes in the analysis of Fig. 4A (lanes 3,

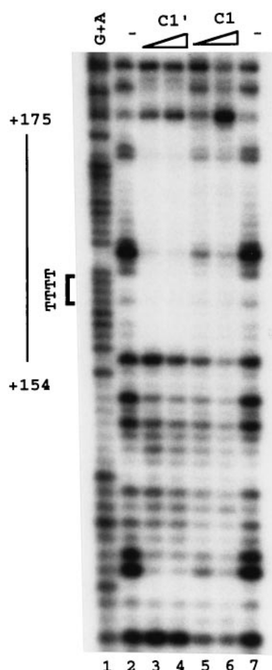

FIG. 5. TFIIIC1 and TFIIIC1′ interactions over the termination sequence region. The 119-bp *Bst* EII-*Eco*RI fragment of the VA1 gene labeled at the 3′ end of the transcribed strand was incubated with 1 μg (lanes 3 and 5) or 2 μg (lanes 4 and 6) of TFIIIC1 or TFIIIC1′ as indicated and digested with DNase I. The protected regions (base pair positions) and the termination sequences are indicated at the left. Lane 1 is a Maxam-Gilbert G+A reaction.

4, and 11) and Fig. 4B (lanes 3 and 6) represents a nonspecific DNA end-binding protein.

These studies represent the first demonstration that TFIIIC1 can interact directly with downstream sequences near the termination site, that TFIIIC2 and TFIIIC1 can bind cooperatively, and that the downstream sequences are necessary for the observed cooperativity. We also confirmed a previous report by Yoshinaga et al. (55) that TFIIIC1 can qualitatively alter the TFIIIC2 footprint on the VA1 gene by both 5′ and 3′ extensions. However, they reported more extended footprints (including 5′ sequences over the A box and further upstream and 3′ sequences downstream of the B box and at the end of the gene) that were not evident here. This may reflect differences in the purity and concentration of the various factors used. Related studies of Drosophila and silkworm tRNA genes have shown a role for 3′ sequences in the assembly or stabilization of active preinitiation complexes and in cooperative binding of partially purified factors (41, 58).

Although effects of TFIIIB and RNA polymerase III on TFIIIC footprints have been clearly shown for yeast (24), effects of human TFIIIB and RNA polymerase III, alone or in combination with TFIIIC2 and TFIIIC1, have not yet been demonstrated on VA1 and $tRNA_1^{Met}$ templates.

Downstream sequences containing the termination region affect transcription of VA1 and $tRNA_1^{Met}$ genes. Since a downstream region was necessary for the cooperative binding of TFIIIC1 (or TFIIIC1′) and TFIIIC2, its function in transcription was tested in a system reconstituted with purified factors and linearized templates that either contained or lacked the termination region. In the case of the VA1 gene, a linear template with downstream sequences intact supported a transcription level about threefold lower than that observed with the corresponding supercoiled template, with either TFIIIC1, TFIIIC1′, or both together (Fig. 6A, lanes 6 to 8 versus lanes 2 to 4). Removal of the termination region from the linearized template reduced transcription a further three- to fivefold, and the effect was greater for TFIIIC′-mediated transcription (fivefold reduction) than for TFIIIC1-mediated transcription (threefold reduction) (Fig. 6A, lanes 10 to 12 versus lanes 6 to 8). These results indicate a role for the termination region in the overall efficiency of transcription. In addition, while supercoiled and linearized pVA1 templates containing the downstream termination region gave the normal transcription products, the linearized template lacking the downstream region yielded several discrete transcripts (Fig. 6A, lane 10 to 12). Primer extension analysis of the latter indicated transcripts initiated at +9, +10, +11, and +12 in addition to the transcripts started at the normal position (data not shown). This apparent utilization of multiple initiation sites may reflect the formation of unstable preinitiation complexes in the absence of the termination region, consistent with the termination region-dependent effect of TFIIIC1 on TFIIIC2 binding.

A similar analysis was performed with the $tRNA_1^{Met}$ template in the reconstituted system (Fig. 6C). All supercoiled templates (pTR1, pTR1-4, and pTR2-5) analyzed gave roughly the same level of transcription activity in the reconstituted system (Fig. 6C, lanes 2 to 4 and 8 to 10), indicating that 5′ sequences preceding position −69 and sequences more than 63 bp past the termination site of the $tRNA_1^{Met}$ template do not affect transcription in this reconstituted system. Similar to the situation observed for the VA1 gene, linearization of the intact $tRNA_1^{Met}$ template reduced transcription about threefold (Fig. 6C, lanes 5 and 11 versus lanes 2 and 8). These levels were further reduced by removal of the termination region from the linearized template, and again the effects were greater for TFIIIC′-mediated transcription (15-fold) than for TFIIIC1-mediated transcription (3-fold) (Fig. 6C, lane 6 versus lane 5 and lane 12 versus lane 11). Recent studies with the yeast system showed that the termination region is involved in RNA polymerase III recycling, through multiple rounds of initiation, on the same template (12). To determine whether a similar phenomenon could account for the repressive effect of deleting the downstream sequences in the present system, we took advantage of the ability of 0.05% Sarkosyl to selectively block transcription reinitiation (on performed preinitiation complexes) to assay single-round transcription (25). The transcription signal decrease as a result of loss of the downstream region was as great under single-round conditions (Fig. 6E, lane 6 versus lane 5) as under multiple-round conditions (lane 3 versus lane 2). Figure 6E also shows an effect of template conformation on multiple-round transcription (greater with the supercoiled template) (lane 2 versus lane 1) but not on single round transcription (lane 5 versus lane 6).

These results indicate that 3′ downstream sequences can affect both the overall efficiency (VA1 and $tRNA_1^{Met}$ genes) and the accuracy (VA1 gene) of RNA polymerase III-mediated transcription by affecting assembly of the preinitiation complex. This finding is consistent with the observation that these downstream sequences can have both quantitative and qualitative effects on interactions of TFIIIC1 and TFIIIC2 with the template. It may be significant that deletion of the downstream sequence has a greater effect on transcription mediated by TFIIIC1′ (5- to 15-fold) than on that mediated by TFIIIC1 (3-fold). Although this could reflect selective interference by the end-binding activity present in TFIIIC1′, but not TFIIIC1, the fact that the end-binding activity binds distal to the B box on the tRNA gene (Fig. 4B, lanes 3 and 6) makes this less likely.

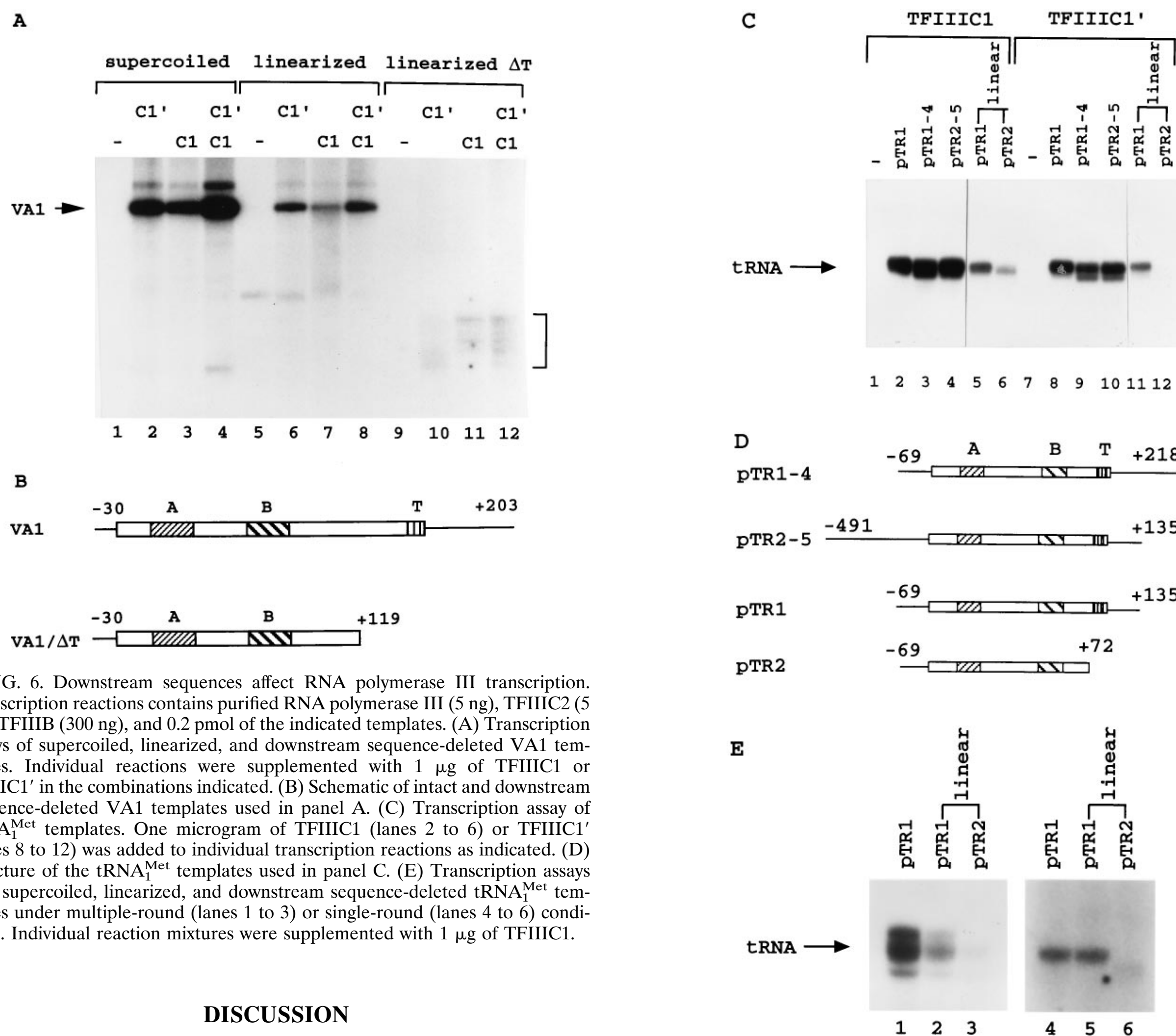

FIG. 6. Downstream sequences affect RNA polymerase III transcription. Transcription reactions contains purified RNA polymerase III (5 ng), TFIIIC2 (5 ng), TFIIIB (300 ng), and 0.2 pmol of the indicated templates. (A) Transcription assays of supercoiled, linearized, and downstream sequence-deleted VA1 templates. Individual reactions were supplemented with 1 μg of TFIIIC1 or TFIIIC1′ in the combinations indicated. (B) Schematic of intact and downstream sequence-deleted VA1 templates used in panel A. (C) Transcription assay of $tRNA_1^{Met}$ templates. One microgram of TFIIIC1 (lanes 2 to 6) or TFIIIC1′ (lanes 8 to 12) was added to individual transcription reactions as indicated. (D) Structure of the $tRNA_1^{Met}$ templates used in panel C. (E) Transcription assays with supercoiled, linearized, and downstream sequence-deleted $tRNA_1^{Met}$ templates under multiple-round (lanes 1 to 3) or single-round (lanes 4 to 6) conditions. Individual reaction mixtures were supplemented with 1 μg of TFIIIC1.

DISCUSSION

This study confirms the previous observation that human TFIIIC can be split into two components, TFIIIC2 and TFIIIC1, which are both required for transcription by RNA polymerase III. It further documents the presence of a second chromatographically distinct form of TFIIIC1, designated TFIIIC1′, and novel DNA interactions of TFIIIC1 and TFIIIC1′ that have qualitative and quantitative effects both on TFIIIC2 binding and on transcription of tRNA and VA1 RNA genes. These results both clarify and extend previous observations on factor requirements and mechanisms for RNA polymerase III-mediated transcription.

The complexity and function of human TFIIIC. Using near homogeneous preparations of a circa 15-subunit RNA polymerase III and a 5-subunit TFIIIC2, as well as a highly purified preparation of TFIIIB, we have documented the presence of two chromatographically distinct but functionally similar forms (TFIIIC1 and TFIIIC1′) of another factor that is jointly required for transcription of class III genes. The TFIIIC1 described here appears chromatographically and functionally the same as that reported by Berk and colleagues (11, 55). The fact that TFIIIC1 and TFIIIC1′ are present, respectively, in crude TFIIIC and TFIIIB preparations provides an explanation for previous discrepancies regarding RNA polymerase III factor requirements (26) and necessitates that this fact be considered in any studies with partially purified factors. At present we do not know the structural basis for the distinct chromatographic properties of TFIIIC1 and TFIIIC1′ and whether they reflect naturally distinct complexes or partial disruption of a single complex during extract preparation and fractionation.

In agreement with previous studies on the VA1 gene (10, 19, 56), our DNase I footprint analyses have shown that the near homogeneous five-subunit TFIIIC2 interacts exclusively with the B-box region of VA1 and tRNA genes in the absence of the other components and that TFIIIC1 can extend the observed footprint to upstream regions that extend to or beyond the A box and to downstream sequences that include the termination region. These analyses have also shown, for the first time, that components in the TFIIIC1 preparation can bind independently to the termination region and that TFIIIC1 can significantly enhance the level of TFIIIC2 binding. These latter observations may have been missed previously (55) because of the use of a less purified preparation of TFIIIC1 and a saturating level of TFIIIC2. Because of the independent TFIIIC1 binding activity, it is likely that interactions downstream of the B-box region in the presence of TFIIIC2 and TFIIIC1 are due, at least in part, to direct TFIIIC1 contacts. But whether the TFIIIC2- and TFIIIC1-dependent upstream interactions result from direct interactions of TFIIIC1 or from TFIIIC1-induced interactions of TFIIIC2, or both, remains to be determined. Information relevant to this point comes from cloning and

sequence analysis of human TFIIIC2 subunits. The largest subunit, which directly contacts the B-box region (26), shows no homology to the B-box-binding subunit of yeast TFIIIC (27, 29). However, recent studies (20a) have revealed human TFIIIC2 subunits homologous both to the yeast TFIIIC subunit which contacts the A box (37, 48) and to an interacting yeast subunit which contacts TFIIIB (33). Hence, it is likely that the 5′ part of the TFIIIC2-TFIIIC1 footprint reflects, at least in part, induced TFIIIC2 interactions.

Although the structure of TFIIIC1 is not yet clear, size analysis (55) and ongoing purification studies (50a) suggest that it is multisubunit in nature. Given that human TFIIIC2 has a complexity close to that of yeast TFIIIC, it is apparent either that the human RNA polymerase III factors are more complex or that additional factors remain to be identified in yeast. The complexity of human TFIIIC may reflect the requirement for interactions (as in yeast) with split promoter elements separated by variable distances, the differential function of TFIIIC in transcription of class III genes with a variety of internal and external promoter elements (see the introduction), or a diversity of critical checkpoints for modulating transcription by RNA polymerase III. In relation to the latter point, modulation of TFIIIC activity in response to virus infection or changes in cell growth have been correlated with changes in the level of a single TFIIIC2 subunit (45). Thus far, TFIIIC1 appears to be essential for transcription of all subclasses of class III genes, whereas TFIIIC2 appears not to be necessary for the U6 and 7SK genes (53).

Downstream sequences near the termination site play a role in the cooperative binding of TFIIIC2 and TFIIIC1 and in modulating transcription by RNA polymerase III. Studies to date have described several types of downstream sequences and corresponding functions. The best defined is a consensus sequence, comprised of a cluster of four or more T residues surrounded by GC-rich sequences, that acts as a signal for termination by RNA polymerase III (3). Although purified RNA polymerase III alone can terminate at such sequences (8), other factors could enhance termination efficiency through effects on RNA polymerase III transcript release from the template. La antigen, which binds the 3′ poly(U) end of nascent RNA polymerase III transcripts, has been reported to have such effects in human cell-free systems (18, 31) and to simultaneously enhance recycling (reinitiation) by RNA polymerase III (32). Consistent with a direct role for termination elements in transcription initiation efficiency, a human terminator element has been shown to affect human *Alu* template activity in vivo (9), while in vitro studies have implicated a yeast termination region in RNA polymerase III reinitiation on the same template (12).

Other studies have shown various effects of 3′ flanking sequences that have not been related to termination sequences per se. Thus, the 3′ flanking region of the yeast *SUP4-o* gene was reported to modulate transcription both in vivo and in vitro (1), while stable complex formation on Drosophila $tRNA^{Arg}$ (41) and silkworm $tRNA_2^{Ala}$ (52, 57, 58) genes was shown to be dramatically enhanced by 3′ downstream sequences. In the silkworm studies, these sequences were essential for cooperative binding of silkworm TFIIIC and TFIIID, probably equivalent to human TFIIIC1 and TFIIIC2, but had little effect on transcription; moreover, these studies revealed no interactions over the termination region and may have reflected a dependence on additional B-box elements just beyond the termination site (58). More recent studies have identified an activity that binds to a 45-bp region at the end of a *Xenopus* 5S RNA gene (38), although this activity appears not to be involved in TFIIIC binding (47).

In this study, we have shown novel interactions of TFIIIC1 and TFIIIC1′ with a circa 20-bp region centered on the termination sequences of the VA1 gene and, further, that the region containing these sequences is essential for cooperative binding of TFIIIC2 and TFIIIC1 (or TFIIIC1′). Deletion of these sequences also had moderate to large effects (2- to 15-fold) on transcription, being greater for TFIIIC1′ than for TFIIIC1. These results extend those reported by Yoshinaga et al. (55), who demonstrated that TFIIIC1 extends the TFIIIC2 footprint in both 5′ and 3′ directions (as confirmed here) but failed to show any interactions with, or functions in transcription initiation of, the termination region. This may have reflected the use of more purified or concentrated TFIIIC1 preparations in our analyses. Because TFIIIC1 is not totally purified, it is not yet certain that the protein(s) in our TFIIIC1 preparation which binds directly to the termination region is the same as that which binds cooperatively with TFIIIC2 to the various promoter elements or whether it could have a second function (e.g., in terminator-dependent recycling of RNA polymerase III [see above]). However, our observations that the 3′ flanking region is essential for cooperative TFIIIC2-TFIIIC1 binding and that the termination region interactions persist in the TFIIIC1-TFIIIC2 promoter complex suggest that the same factor is involved. Altogether, therefore, our results suggest that 3′ downstream/termination sequences play a role in the cooperative binding of essential transcription factors and in the overall efficiency and accuracy of transcription initiation. Exactly how this relates to the reported effects of the yeast terminator (above) and the human La antigen on RNA polymerase III (re) initiation remains to be determined. Although La antigen is present in both TFIIIC1 and TFIIIC1′ preparations (by immunoblot analysis), immunodepletion analyses with anti-La antibodies have failed to show any relationship between La and TFIIIC1 or TFIIIC1′ activity (data not shown).

Mechanism of RNA polymerase III transcription. Early template commitment studies with unfractionated extract revealed the formation of complexes that were highly stable during transcription and that could account in part for the high efficiency of transcription of class III genes (3, 42). Studies with isolated factors (TFIIIA, TFIIIB, and TFIIIC) confirmed and extended these observations by describing factor interaction sites and assembly pathways, as well as variable factor requirements for stable complexes on different genes (2, 14, 28, 40, 41, 44, 46). This provided a basis for understanding the mechanism by which RNA polymerase III could transcribe through the internal promoter without dissociation of the internally bound factors (TFIIIC or TFIIIA and TFIIIC). Early models proposed alternative (cyclic) release and rebinding of TFIIIA domains bound to the 5′ and 3′ regions of the 45-bp internal control region of the 5S gene (34) or of TFIIIC domains bound to the A and B elements of tRNA promoters (5). Although internally bound yeast factors are only transiently required for the in vitro formation of active preinitiation complexes (containing only promoter-bound TFIIIB and RNA polymerase III) (23), this does not preclude the persistence of complete complexes in the natural situation and as demonstrated for the vertebrate system.

On the basis of the present data showing stable factor interactions not only over the A- and B-box regions but also over the downstream termination region, the general model can be extended by invoking the termination region as an additional template anchor point for the complete TFIIIC complex, generating a nucleoprotein structure on which TFIIIB and RNA polymerase III assemble to initiate transcription. The presence of a large structure with at least three primary sequence-specific recognition sites could thus allow prolonged transcription

with a lower probability of factor loss (from the complex) during transient dissociation from a given site. This flexible locking model integrates initiation, elongation, and termination processes and factors into a single complex that also may help account for the high efficiency of accurate transcription by RNA polymerase III. In support of this model, deletion of the termination sequences in the present study was correlated with a lower transcription efficiency and less accurate initiation, as well as loss of cooperative binding of TFIIIC2 and TFIIIC1 on the DNA. The model is further supported by our prior demonstration (45) that TFIIIC2 and TFIIIC1 can stably interact in the absence of DNA. As mentioned above, recent studies have also show that a yeast termination region can effect RNA polymerase III recycling on the same template (12). While a similar recycling by human RNA polymerase III remain to be demonstrated, this a reasonable possibility in view of the present results and our previous demonstration of differential Sarkosyl sensitivities for primary versus secondary initiation events on the VA1 gene (25). However, in contrast to the observation in yeast that loss of termination sequences eliminates recycling by RNA polymerase III (12), our results showed that deletion of the termination region reduced proportionately both single- and multiple-round transcription events. In conjunction with the demonstrated role of this region in stabilization of the preinitiation complex, these results indicate that sequences in the termination region also can influence RNA polymerase III transcription through effects on preinitiation complex assembly. Our present results also suggest potential functional differences between TFIIIC1 and TFIIIC1′ in their action through termination sequences, but whether this reflects differential effects on the stability of preinitiation complexes or possible RNA polymerase III recycling remains to be determined.

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