Cloning and characterization of a TFIIIC2 subunit (TFIIICβ) whose presence correlates with activation of RNA polymerase III-mediated transcription by adenovirus E1A expression and serum factors

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TFIIIC2 is a general factor essential for transcription of 5S RNA, tRNA, and VA RNA genes by mammalian RNA polymerase III and consists of two forms designated TFIIIC2a and TFIIIC2b. TFIIIC2a and TFIIIC2b share common subunits of 220, 102, 90, and 63 kD but differ with respect to transcription activity and the presence of a presumptive 110-kD subunit in the active form (TFIIIC2a). Because both forms can bind the promoter directly, a selective role for the 110-kD subunit in the regulation of RNA polymerase III activity has been suggested. To investigate this possibility, we have cloned and expressed a cDNA encoding the 110-kD subunit (TFIIICβ). Immunoprecipitation studies with anti-TFIIICβ antibodies have confirmed that TFIIICβ is a bona fide subunit present only in TFIIIC2a, that TFIIIC2a and the general factor TFIIIC1 are associated in unfractionated extracts, and that previously undetected polypeptides (potential TFIIIC1 subunits) can be isolated in association with TFIIIC2a. Previous studies have shown that increases in RNA polymerase III activity during infection of cells by adenovirus [with concomitant E1A expression] or during cell growth at high serum concentration results from an increased activity in the TFIIIC fraction. Studies with antibodies to TFIIICβ have shown that this is strongly correlated with a selective increase in the cellular concentration of the TFIIICβ 110-kD subunit and a concomitant rise in the ratio of the active-to-inactive forms of TFIIIC2.

[Key Words: TFIIIC2 subunit; RNA polymerase III; adenovirus EIA; cloning; characterization]

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RNA polymerase III is responsible for the transcription of a number of genes [class III] encoding small nuclear and cytoplasmic RNAs of both cellular and viral origin [for review, see Geiduschek and Tocchini-Valentini 1988; Gabrielsen and Sentenac 1991, Geiduschek and Kassavetis 1992; Hernandez 1993]. These genes have been classified broadly on the basis of promoter structure and accessory factor requirements. Both subclass 1 and subclass 2 genes have promoter elements [internal control regions [ICRs]] that are located within transcribed DNA. Subclass 2 genes [encoding tRNA, 7SL, and adenoviral VA RNAs, as well as Alu sequences] have two ICRs [A box and B box] and require factors TFIIIB and TFIIIC in addition to RNA polymerase III for in vitro transcription. Subclass 1 genes [encoding 5S RNAs] have three ICRs [A box, I box, and C box] and require the gene-specific factor TFIIIA in addition to TFIIIB, TFIIIC, and RNA polymerase III. Subclass 3 genes [encoding U6 and 7SK RNAs] have non-transcribed 5' control elements that include a TATA box and a proximal sequence element [PSE] and appear to require the TATA-binding protein [TBP], PSE-binding transcription factor [PTF], TFIIIB, and RNA polymerase III for efficient in vitro transcription.

TFIIIC from mammalian cells [Segall et al. 1980] has been purified and fractionated partially into two activities, TFIIIC1 and TFIIIC2, which are jointly required for activity [Dean and Berk 1987; Yoshinaga et al. 1987, 1989; Z. Wang and R. Roeder, unpubl.]. TFIIIC2 can be isolated in two forms [now designated TFIIIC2a and TFIIIC2b] that bind DNA with equal affinity and show equivalent DNase footprint patterns [Hoeflir et al. 1988; Kovelman and Roeder 1992]. However, they can be distinguished by (1) the ability of TFIIIC2a but not TFIIIC2b to restore RNA polymerase III transcription in a heat-treated nuclear extract, (2) the electrophoretic mobilities [TFIIIC2b > TFIIIC2a] of corresponding DNA-protein complexes, and (3) their subunit complexities; they share common subunits of 220, 102, 90, and 63 kD but appear to differ with respect to other polypeptides, most notably the presence of a 110-kD polypeptide unique to TFIIIC2a.

The cellular potential for transcription of class III

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genes by RNA polymerase III in vitro and in vivo has been reported to be enhanced during viral infection by the expression of immediate early proteins of adenovirus [E1A and/or E1B] and pseudorabies virus [PrfE] [Berger and Folk 1985; Gaynor et al. 1985; Hoeflter and Roeder 1985; Yoshinaga et al. 1986; Sollerbrant et al. 1993] and to be suppressed by poliovirus infection [Fradkin et al. 1987]. Fractionation of extracts from cells infected with wild-type and E1A-defective adenoviruses has indicated that the E1A-dependent transcription reflects a change in the TFIIIC fraction, variably characterized as an increase in the ratio of TFIIIC2a to TFIIIC2b [Hoeflter and Roeder 1985; Hoeflter et al. 1988] or a net increase in the concentration of TFIIIC2 [Yoshinaga et al. 1986]. In related studies it was also found that extracts from cells grown at high serum [5%] concentration were more active in transcription by RNA polymerase III than extracts from cells grown at low serum (0.5%). The serum-induced change was also localized to the TFIIIC fraction with a similar increase in the ratio of TFIIIC2a versus TFIIIC2b [Hoeflter et al. 1988].

To analyze further the regulation of RNA polymerase III-mediated transcription we have cloned and characterized a cDNA encoding the 110-kD subunit of TFIIIC2 and used this to study regulatory modifications and interactions of TFIIIC2.

**Results**

**Cloning of TFIIICβ**

TFIIIC2, purified as described previously [Kovelman and Roeder 1992], was subjected to preparative SDS-PAGE and transferred to an Immobilon-P [Millipore] membrane. The 110-kD subunit of TFIIIC2 was identified and digested with endoproteinase Lys-C. Microsequence analysis of derived peptides yielded sequences of HILSELEAPYPQEEK, DLRRPYEPINSIK, ADLIPYQDSPEGPDHSSA, and EMTSAEASVEMLSPTLPFGED. Oligonucleotides corresponding to these peptide sequences were synthesized and used to probe a human cDNA library. The largest cDNA in the derived clones contained 1.5 kb of coding sequence and 1 kb of 3' untranslated sequence, but still lacked ~1 kb of 5' coding sequence. Although subsequent screening of a number of libraries failed to retrieve more 5' sequences, the complete 5'-coding sequence was finally obtained using 5' rapid amplification of cDNA ends [RACE] [see also Materials and methods]. The RNA size of TFIIICβ was determined using the complete coding cDNA sequence as a probe on an RNA blot. A single band at 4.5 kb was detected [data not shown].

The complete sequence of the TFIIICβ cDNA is shown in Figure 1A. The residues corresponding to those derived from direct protein sequencing are underlined. The cloned TFIIICβ cDNA encodes a protein of 911 amino acids with a predicted mass of 100.7 kD and an estimated pI of 7.0. The protein sequence of TFIIICβ was analyzed by computer [GCG, University of Wisconsin, Madison, MacVector, Kodak] and by visual inspection. This revealed stretches of basic [residues 90–113, 12/24 basic; residues 141–148, 6/8 basic; Fig. 1A,B] and acidic [residues 237–261, 12/25 acidic; Fig. 1A,B] regions, with a potential basic helix [residues 141–158, Fig. 1A, curved underline] next to a potential acidic helix [residues 244–260; Fig. 1A, curved underline] at the amino terminus. Five putative WD40 repeats [residues 499–513, 571–585, 585–599, 629–643, 852–866; Fig. 1B,C] of potential importance for protein–protein interaction were also found [see Discussion].

A search of the nonredundant data bases with the BLAST network service [Altschul et al. 1990; National Center for Biotechnology Information] was performed to determine possible sequence similarities [protein and DNA] between TFIIICβ and other entries. A DNA sequence encoding a protein showing a perfect match [100%] to our cloned sequence was found [N. Nomura, N. Miyajima, Y. Kawarabayashi, S. Tabata, unpubl.]. This gene was a sequenced cDNA. No other information beside the nucleic acid sequence was found. Outside of this sequence no other significant similarity was found between TFIIICβ and other proteins in the data bases [including entries in the protein library as well as translations in all six frames of available DNA sequences].

To determine whether the cloned cDNA contained complete coding sequences for the 110-kDa polypeptide in TFIIIC2, a fragment corresponding to the carboxy-terminal 595 residues was expressed in bacterial, purified to homogeneity, and used to prepare antibodies [see Materials and methods]. The putative TFIIICβ cDNA was then transcribed and translated in a rabbit reticulocyte lysate and the corresponding product was analyzed, along with a partially purified TFIIIC2 [P11 fraction], by SDS-PAGE. When the corresponding membrane was probed with anti-TFIIICβ antibodies a 110-kD band was detected in both the in vitro translated [Fig. 2A, lane 1] and the natural TFIIIC2 [lane 2] fractions. This suggests that the existing clone of TFIIICβ contains the complete coding sequence for the presumptive 110-kDa subunit of TFIIIC2a.

The TFIIICβ amino acid sequence was compared with sequences from the 220-kD human [L'Etoile et al. 1994] and rat [Lagna et al. 1994] TFIIICα subunit and with the 95-kD [TFC1: Swanson et al. 1991, Parson and Weil 1992], 131-kD [TFC4: Marck et al. 1993], and 138-kD [TFC3: Lefebvre et al. 1992] subunits of yeast TFIIIC and no significant sequence similarities were found. Consistent with this analysis anti-TFIIICβ antibody failed to react on a Western blot with any polypeptide of partially purified yeast TFIIIC [data not shown]. The lack of similarity between TFIIICβ and yeast TFIIIC subunits is not surprising considering the degree of evolutionary divergence between yeast and vertebrate TFIIIC [see Discussion].

TFIIICβ is associated only with the low mobility transcriptionally active form (TFIIIC2a) of TFIIIC2

As discussed in the introductory section our previous studies have demonstrated two distinct and chromato-
graphically separable forms of TFIIIC2 in nuclear extracts: one (now designated TFIIIC2a) that forms a promoter complex with a lower electrophoretic mobility and is transcriptionally active in heat-treated nuclear extracts that have preferentially lost TFIIIC activity and a second (now designated TFIIIC2b) that forms a promoter complex with a higher mobility and is transcriptionally inactive in the heat-treated nuclear extract.

Earlier structural studies indicated that these two species contained common polypeptides of 220, 102, 90, and 63 kD, but differed with respect to the presence of a 110-kD polypeptide in TFIIIC2a, and the apparent absence of 95- and 77-kD polypeptides in TFIIIC2b [Kovelman and Roeder 1992]. More recently a modification in the purification protocol has yielded larger amounts of TFIIIC2 and allowed more definitive structural analyses. An analysis of this TFIIIC2 by SDS-PAGE failed to reveal the presence of the 95-kD polypeptide in the latter (Fig. 2C; see also figure legend). To investigate further the presence of the 95-kD polypeptide, and a possible relationship to other polypeptides in the two species, anti-TFIIIC8 antibodies were used to probe an immunoblot of TFIIIC2a and TFIIIC2b. The analysis in Fig. 2B shows the presence of an intact immunoreactive

**Figure 1.** Primary structure of TFIIIC8. (A) DNA and protein sequence of TFIIIC8. The numbers at left denote nucleotide sequence, and the underlined numbers denote residue number. The four straight underlined areas indicate regions corresponding to peptide sequences from purified protein. The regions with the wavy underlines are basic helix–loop–basic motif sequences from purified protein. The regions with the numbered underlines are WD40 repeats. The numbers at right denote the amino acid sequence. The characters represent residues that match the consensus sequence of WD40 repeats. The characters represent residues that are in 10% of all WD40 sequences surveyed (van der Voorn and Ploegh 1992).
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TFIIIC\(\beta\) subunit was detected only in fractions that contained TFIIIC2a activity. Thus, the protein [TFIIIC\(\beta\)] encoded by the isolated cDNA copurifies, as expected, only with TFIIIC2a and does not appear to exist in a subcellular compartment independently of TFIIIC2a.

**TFIIIC\(\beta\) remains bound in a TFIIIC2a—promoter complex**

To gain additional information on the subunit status of TFIIIC\(\beta\) and to determine whether it might associate reversibly with the common TFIIIC2 subunits, especially as TFIIIC2a and TFIIIC2b can each form a promoter complex [Hoeffer et al. 1988], it was important to determine whether TFIIIC\(\beta\) remains associated with binding of TFIIIC2a to the promoter. Analysis of TFIIIC2a by electrophoretic mobility-shift assay [EMSA] with a VA1 promoter fragment [Fig. 3] revealed a characteristic TFIIIC2a—VA1 complex that was dependent on the presence of TFIIIC2a (cf. lane 2 with lane 1) and completely supershifted by anti-TFIIIC\(\beta\) antibody (lane 4) but not by preimmune serum (lane 3). These results indicate that indeed TFIIIC\(\beta\) remains associated with TFIIIC2a with promoter recognition, providing strong support for its role as an integral subunit. The interesting possibility that the TFIIIC\(\beta\) interaction within TFIIIC2a might be 110-kD polypeptide in TFIIIC2a but no cross-reacting polypeptide in TFIIIC2b. These results eliminate the possibility that the 77-kD species might represent a modified or derived form of the 110-kD polypeptide. However, they are consistent with the view [substantiated below] that the 110-kD polypeptide is a bona fide subunit that is unique to TFIIIC2a, whereas the 77-kD polypeptide in TFIIIC2b may be either a contaminant or a reversibly associated component that could play a regulatory role.

Given the proposed role of TFIIIC\(\beta\) in effecting changes in the levels of TFIIIC2a in response to certain stimuli, its presence in various subcellular and chromatographic fractions was monitored by immunoblot analysis. TFIIIC\(\beta\) was detected only in the nuclear extract-derived 0.6 M KCl eluate that contains TFIIIC and not in other P11 fractions [0.1, 0.3, and 1.0 M KCl eluates]. TFIIIC\(\beta\) also was not detected in current S100 and nuclear pellet fractions, consistent with our previous experience that TFIIIC2 activity was undetectable in the same fractions [data not shown]. During the further purification of TFIIIC2a through four chromatographic steps [see Material and methods] the

![Figure 2.](image-url) **Figure 2.** [A] In vitro translation of TFIIIC\(\beta\) cDNA. Immunoblot of rabbit reticulocyte lysate translated TFIIIC\(\beta\) and purified TFIIIC2 [nuclear extract, P11 0.6 fraction] probed with anti-TFIIIC\(\beta\) antibody. The bands detected have a migration rate corresponding to 110 kD. [B,C] Immunoblot of TFIIIC2a and TFIIIC2b. Purified TFIIIC2a and TFIIIC2b fractions (4500-fold) were loaded on 7% SDS-PAGE and analyzed by immunoblot with anti-TFIIIC\(\beta\) antibodies [B] or by silver stain [C]. The polypeptides in the 140- to 200-kD range in the TFIIIC2b preparation are not regarded as likely subunits as they do not copurify strictly (during gradient elution) with the TFIIIC2b activity and the other polypeptides. Moreover, the fact that TFIIIC2b has a faster migration rate under gel mobility-shift assays than does TFIIIC2a makes it unlikely that these high molecular mass proteins are part of the TFIIIC2b promoter complex.

![Figure 3.](image-url) **Figure 3.** Retardation of TFIIIC2a promoter complexes by anti-TFIIIC\(\beta\) antibody. EMSA using the labeled VA1 promoter of adenovirus. The free probe was run off the gel. [Lane 1] probe alone; [lane 2] probe and TFIIIC2a; [lane 3] probe, TFIIIC2a, and preimmune serum [final antibody concentration, 50 \(\mu\)g/ml]; [lane 4] probe, TFIIIC2a, and anti-TFIIIC\(\beta\) antibodies [final antibody concentration, 50 \(\mu\)g/ml]. Arrow 2 points to the TFIIIC2a—promoter complex; arrow 1 points to the TFIIIC2a—promoter complex supershifted by anti-TFIIIC\(\beta\) antibodies.
Figure 4. Immunodepletion of nuclear extract using anti-TFIIC\(\beta\) antibody. In vitro transcription from the VA1 promoter with preimmune-depleted nuclear extract [lane 1] or with anti-TFIIC\(\beta\) antibody-immunodepleted nuclear extract with no addition [lane 2], with TFIIC1 added [lane 3], with TFIIC2a added [lane 4], with TFIIB added [lane 5], with RNA polymerase III added [lane 6], or with TFIIC1 plus TFIIC2a [lane 7].

TFIIC\(\beta\) is involved in transcription by RNA polymerase III and potentially in interactions of TFIIC2a with TFIIC1

To determine whether TFIIC\(\beta\) is essential for transcription by RNA polymerase III, antigen-purified antibodies raised against the bacterially expressed carboxy-terminal fragment of TFIIC\(\beta\) were used to immunodeplete a HeLa extract (Fig. 4). As judged by immunoblot this treatment removed >90% of TFIIC\(\beta\), whereas preimmune serum had no effect (data not shown). Similarly, whereas the preimmune antibodies had only a minimal (<20%) effect on transcription levels (data not shown), anti-TFIIC\(\beta\) antibodies reduced transcription to undetectable levels [lane 2] relative to the high level of transcription observed in extracts treated with preimmune antibodies [lane 1]. Independent additions of highly purified TFIIC1, TFIIC2a, TFIIB, and RNA polymerase III [lane 3, 4, 5, and 6, respectively] to the immunodepleted extract had no effect, with basal transcription remaining undetectable. In contrast, the simultaneous addition of TFIIC1 and TFIIC2a restored transcription to levels similar to that observed with the preimmune-treated control [lane 1]. Hence, immunodepletion with antibodies against a single unique subunit of TFIIC2a removes TFIIC1 as well as TFIIC2a activity quantitatively. These results indicate that TFIIC\(\beta\) is an essential component of TFIIC2a and further suggest that some or all TFIIC1 components are tightly associated with TFIIC2a in nuclear extracts.

In an attempt to identify possible TFIIC2a-associated polypeptides the anti-TFIIC\(\beta\) antisera was used to immunoprecipitate TFIIC2a. For this purpose a conventional nuclear extract was adjusted to 300 mM KCl and applied either to an anti-TFIIC\(\beta\) antibody–protein A column or to a control preimmune antibody–protein A column. The columns were washed with a BC buffer containing 1 mM KCl and eluted with a low pH buffer. The eluates were immediately adjusted to pH 6.8 and analyzed by SDS-PAGE on either 5% [Fig. 5, lanes 1, 2] or 4–20% [Fig. 5, lanes 3, 4] acrylamide gels. Along with the previously defined polypeptide bands of 220, 110, 102, 90, and 63 kD [lanes 2, 4], associated polypeptides of ~58, 42, and 33 kD were visible in potentially stoichiometric amounts [lane 4]. The 58-, 42-, and 33-kD polypeptides were not detected in purified TFIIC2a when analyzed on similar gradient gels [data not shown]. Although the function of these polypeptides remains to be established, they are candidates for subunits of TFIIC1.

Effects of adenovirus infection and cellular growth state on the cellular level of TFIIC\(\beta\)

It was reported previously that RNA polymerase III transcription initiation activity is increased both by the viral
E1A gene product and by high serum growth conditions (Berger and Folk 1985; Gaynor et al. 1985; Hoeffler and Roeder 1985; Hoeffler et al. 1988). Although the enhanced RNA polymerase III initiation activity was localized to a fraction containing TFIIIC activity, it has not been clear whether the increase in TFIIIC activity resulted from an increase in specific activity (Hoeffler and Roeder 1985; Hoeffler et al. 1988) or from an increase in the amount of TFIIIC (Yoshinaga et al. 1986).

To investigate further the effects of adenovirus infection (including E1A expression) on TFIIIC we infected HeLa cells with wild-type adenovirus (Ad2) and a mutant adenovirus with the E1A gene deleted (dl312). Extracts were produced from cells 6 hr after infection and tested in an in vitro transcription assay with adenovirus VA1 and cellular 5S and tRNA genes. Extracts from cells infected with wild-type adenovirus showed a 10-fold higher level of activity than did extracts from cells infected with the E1A-defective adenovirus (Fig. 6A). To determine the basis for the increased activity crude TFIIIC fractions (P11 0.6) from the two extracts were subjected to SDS-PAGE and blotted onto membranes that were probed simultaneously with anti-TFIIICβ and anti-TFIIICα antibodies. Because the 220-kD TFIIICα subunit was shown previously to be a component of both TFIIIC2α and TFIIIC2β (Kovelman and Roeder 1992), the signal from the corresponding antibody should reflect the total level of TFIIIC2. As shown in Figure 6B, the levels of TFIIICα were similar for the wild-type [E1A+] and mutant [E1A−] cell extracts, suggesting that the E1A-dependent viral gene expression has no effect on the level of total TFIIIC2 in the cell. In contrast, the level of TFIIICβ, which is only associated with the low mobility form of TFIIIC2, was increased ~10-fold in the wild-type [E1A+] extract relative to the mutant [E1A−] extract. On the basis of these results we conclude that expression of the E1A gene product resulted, directly or indirectly, in an increase in the level of TFIIICβ and the corresponding active form of TFIIIC2. Because TFIIICβ was not detected in other phosphocellulose fractions (P11 0.1, 0.35, 1.0), nuclear pellet, and S100 fractions, either before or after adenovirus infection (data not shown), it appears that E1A expression results either in an increased rate of synthesis or in the stabilization of TFIIICβ. More recently, preliminary analysis of total cellular RNA levels has indicated a significant effect of E1A expression on the level of TFIIICβ mRNA (E. Sinn and R.G. Roeder, unpubl.).

Similar effects also were seen with extracts from cells grown at high [10%] versus low [0.5%] serum concentrations. Thus, extracts from high serum-grown cells showed 10-fold higher levels of transcription from the VA, 5S, and tRNA genes than did extracts from low serum grown cells [Fig. 6A]. Immunoblot analysis using antibodies to TFIIICβ and TFIIICα showed comparable levels of TFIIICα in both extracts but lower levels of TFIIICβ in the low serum cellular extracts [Fig. 6B]. These results are consistent with those from the adenovirus infection [E1A expression] experiments and indicate that alterations in specific activity of TFIIIC2 are largely responsible for the difference in TFIIIC2 activity under different growth conditions.
Discussion

TFIIIC plays a primary role in promoter recognition and preinitiation complex formation on a subset of genes transcribed by RNA polymerase III and has been implicated in the regulation of these genes by viral immediately early proteins and altered growth conditions [see introductory section]. Toward a further analysis of these problems we report the cloning and characterization of a cDNA encoding the second largest subunit (TFIIICβ) of one of the two forms [TFIIIC2a] of the derived TFIIIC2. This cDNA has been used to investigate the primary sequence of TFIIICβ, regulatory modifications of TFIIIC2 involving TFIIICβ, and interactions of TFIIIC2 with TFIIIC1.

Cloning and sequence analysis of TFIIICβ

Amino acid sequence information from the 110-kD polypeptide present in purified TFIIIC2a was used to obtain a cDNA encoding the corresponding protein. That the encoded protein is a bona fide subunit of TFIIIC2a is indicated by the ability of anti-TFIIICβ antibodies [raised against recombinant protein] to inhibit TFIIIC function in transcription assays and to supershift TFIIIC2a–VA promoter complexes, thus indicating that TFIIICβ is an integral part of the TFIIIC2a complex that initially binds DNA.

The cloned TFIIICβ cDNA encodes a protein of 911 amino acids with a predicted molecular mass of 100.7 kD and an estimated pI of 7.0. The amino terminus contains short stretches, rich in acidic or basic residues, with the potential to form α-helices. Also present are five regions that conform to the consensus sequence of the conserved B-region of WD40 repeats [for review, see Gilman 1987; van der Voorn and Ploegh 1992]. This motif has been found in a variety of proteins implicated in diverse cellular processes that include signal transduction, cell cycle progression, RNA splicing, and both transcriptional repression [TUP1] [Keleher et al. 1992; Tzamarias and Struhl 1994] and transcriptional activation [TAF180] [Dynlacht et al. 1993; Kokubo et al. 1993]. Genetic studies [Keleher et al. 1992] have suggested interactions of WD40 family proteins with proteins containing a tetratricopeptide [TPR] repeat [for review, see Boguski et al. 1990; Goeb and Yanagida 1991], and in the case of TUP1 a direct role for the WD40 repeats in protein-protein interactions has been demonstrated [Tzamarias and Struhl 1994; Komachi et al. 1994]. This leads to speculation that the WD40 motifs may be involved in reversible interactions of TFIIICβ with other factors such as TFIIIC1 or TFIIIB. It is interesting in this regard that the 131-kD subunit of yeast TFIIIC contains TPR repeats [Marck et al. 1993], although no WD40 repeats have yet been found in the published sequences of other yeast TFIIIC subunits [E. Sinn, unpubl.].

Given that the five-subunit yeast TFIIIC and human TFIIIC (TFIIIC2 plus TFIIIC1) recognize similar promoter sequences [B box and A box] in various class III genes, and that at least some other general transcription components are conserved between yeast and human [Young 1991; Pinto et al. 1992; Hernandez 1993], it was surprising to find that human TFIIICα and the large subunit of yeast TFIIIC (both of which contact B-box elements) show no significant sequence similarity [Lagna et al. 1994]. Following this trend, TFIIICβ also shows no significant sequence similarity to any of the three cloned subunits [138, 131, and 95 kD] of yeast TFIIIC. Consistent with these findings, anti-TFIIICβ antibodies failed to detect any immunologically related proteins in yeast extracts. Hence, although at least some components [TBP and RNA polymerase subunits] of the RNA polymerase III transcription machinery show substantial sequence conservation between yeast and human [Hernandez 1993; Ittmann et al. 1993] the present and past [L’Etoile et al. 1994; Lagna et al. 1994] observations point either to a functional convergence or to a low sequence conservation in TFIIIC subunits. However, as discussed below, it is likely that human counterparts to some of the yeast TFIIIC components most likely reside in the as yet uncharacterized TFIIIC1 subunits, and sequences for some of the yeast TFIIIC subunits have not yet been reported.

Subunit structures of variant forms of TFIIIC2

We reported previously the resolution of two forms of the B box-binding component of TFIIIC, one [now designated TFIIIC2a] that formed a lower mobility promoter complex and showed transcriptional activity in a heat-treated nuclear extract and one [now designated TFIIIC2b] that formed a higher mobility promoter complex and failed to show transcriptional activity in the same assay. SDS-PAGE analysis of purified components revealed candidate subunits of 220, 110, 102, 90, and 63 kD for TFIIIC2a and candidate subunits of 220, 102, 95, 90, 77, and 63 kD for TFIIIC2b [Kovelman and Roeder 1992]. The use of antibodies against TFIIICβ has verified that the 110-kD subunit is uniquely present in TFIIIC2a and that it is tightly associated in this complex with the 220-, 102-, 90-, and 63-kD polypeptides. On the other hand the 95-kD polypeptide now appears not to be a component of TFIIIC2b because it has not been detected in more recent larger scale and higher yield preparations of TFIIIC2b, either by direct SDS-PAGE analysis [Fig. 2C; Z. Wang and R.G. Roeder, unpubl.] or by SDS-PAGE analysis after immunoprecipitation with anti-TFIIICα antibodies [L’Etoile et al. 1994; Lagna et al. 1994]. In contrast, the 77-kD polypeptide has copurified consistently with TFIIIC2b, although it has not been detected by immunoprecipitation with anti-TFIIICα antibodies [which might have resulted in its dissociation] [L’Etoile et al. 1994; Lagna et al. 1994]. On the basis of these analyses, we conclude that TFIIIC2a contains subunits of 220, 110, 102, 90, and 63 kD, whereas TFIIIC2b differs with respect to the absence of the 110-kD subunit and the possible presence of a substitute 77-kD subunit.

Interactions of TFIIIC2a with TFIIIC1

Immunoprecipitation studies reported here showed that
anti-TFIIICβ antibodies, but not preimmune serum, can remove quantitatively both TFIIIC2a and TFIIIC1 activities from unfraccionated nuclear extracts. These results indicate that at least one of the components that comprise the TFIIIC1 activity is in stable association with TFIIIC2a (either directly with TFIIICβ or with another subunit) and present in an equal or substoichiometric ratio relative to TFIIIC2a. Analysis of the immunoprecipitates revealed polypeptides of 58, 42, and 33 kD, in addition to those normally observed in highly purified TFIIIC2a. Consistent with the possibility that these new polypeptides might represent TFIIIC1 subunits, the observed interactions were resistant to washing with 1 M KCl. Although there are reports that TFIIIC2 can interact with human TFIIIA (Lagna et al. 1994) and that human TFIIA may be involved in transcription by RNA polymerase III (Meissner et al. 1993) immunoblots with antibodies to the recombinant 38-kD human TFIIA (B. Moorefield, E. Sinn, and R. Roeder, unpubl.) and to the 19- and 35-kD subunits of human TFIIA (Delong and Roeder 1993) failed to show cross-reactivity with any of the newly detected polypeptides. In further support of the possibility that these are TFIIIC1 subunits, they are equivalent in size to a subset of polypeptides present in our most highly purified preparations of TFIIIC1 (Z. Wang and R.G. Roeder, unpubl.). The failure to detect these novel polypeptides in previous preparations of chromatographically purified TFIIIC2a (Yoshinaga et al. 1989, Kovelman and Roeder 1992) most likely reflects their dissociation and separation using these purification procedures, and their apparent absence in anti-TFIIICα immunoprecipitates (L’Etoile et al. 1994; Lagna et al. 1994) may have resulted from their dissociation from TFIIIC2a by the antibodies used. In the present analysis the quantitative removal of TFIIIC1 activity from nuclear extracts with an antibody (anti-TFIIICβ) specific for TFIIIC2a further suggests that TFIIIC1 is not associated with the inactive form (TFIIIC2b) of TFIIIC2 and that TFIIICβ could play a direct role in stabilizing interactions of TFIIIC2a with TFIIIC1. Alternatively, TFIIIC1 could be so tightly bound to TFIIIC2b that it cannot readily exchange to TFIIIC2a. In the former case any increase in the level of TFIIIC2a (through TFIIICβ association with TFIIIC2b) would necessitate a corresponding increased intranuclear concentration of TFIIIC1; this could involve recruitment from the cytoplasm, consistent with the presence of high level of TFIIIC1 in the S100 fraction (Z. Wang and R.G. Roeder, unpubl.).

Previous studies have shown that the five-subunit yeast TFIIIC binds to both B and A boxes of promoters, whereas chromatographically purified human TFIIIC2a and TFIIIC1 bind, respectively, to regions at or near the B box and A box (Yoshinaga et al. 1989; Kovelman and Roeder 1992; Z. Wang and R.G. Roeder, unpubl.). This suggestion of a functional equivalence of human TFIIIC2a plus TFIIIC1 with yeast TFIIIC is supported by the finding of an apparently strong physical interaction in unfraccionated nuclear extract between TFIIIC1 and TFIIIC2a. It is apparent, however, that there is a greater structural complexity in the human TFIIIC (TFIIIC2 plus TFIIIC1) than in yeast TFIIIC, unless essential components remain to be elucidated for the latter factor. Furthermore, from the present data one might expect any homology to the A box-binding subunit of yeast TFIIIC (p95; Bartholomew et al. 1990) to be found in human TFIIIC1 and not in TFIIIC2.

Mechanism of transcriptional stimulation by E1A and altered growth conditions

The adenovirus E1A protein stimulates transcription by RNA polymerases II and III of a number of genes of both cellular and viral origins (for review, see in Berk 1986). In the case of class III genes, TFIIIC was shown to be one of the targets of the E1A-dependent effects on transcription during adenovirus infection (Hoeffler and Roeder 1985). On the basis of template commitment assays, Yoshinaga et al. (1986) reported that this reflected an increased concentration of what current studies indicate must have been TFIIIC2. However, our own studies (using template commitment, mobility-shift, and single round transcription assays) indicated an effect on the specific activity of a preexisting population of TFIIIC2 (Hoeffler and Roeder 1985; Hoeffler et al. 1988; Kovelman and Roeder 1990). Here we have used antibodies to the common TFIIIC2 subunit (TFIIICα) and to the TFIIIC2a-specific subunit (TFIIICβ) to verify that the E1A-mediated effect indeed correlates with an increased TFIIIC2 specific activity that results from an increased TFIIIC2a:TFIIIC2b ratio. At present it is not clear whether the effect of E1A on TFIIIC2 is direct or indirect. Related, given the use of wild-type versus E1A-defective viruses in our analysis, the possible involvement of other virus-coded proteins (such as E1B; see Sollerbrant et al. 1993) in conjunction with E1A cannot be excluded.

Analogous to the situation observed during adenovirus infection, the growth of HeLa cells at high serum concentration (10%) was shown to enhance markedly the capacity for transcription by RNA polymerase III (Hoeffler et al. 1988; Fig. 4). A similar analysis of extracts from high (10%) versus low (0.5%) serum grown cells with E1A cannot be excluded.

Modification of basic transcription factors as a global regulatory mechanism for large classes of genes

To the extent that TFIIIC2 is required for transcription of class III genes they could be broadly regulated by a regulatory modification that changes the ratio of active/inactive forms. Although TFIIIC appears to be essential for transcription of most class III genes (including those for tRNA, VA RNA, and 5S RNA), it has been reported
not to be involved in the transcription of 7SK and U6 genes in vertebrate cell extracts (Waldschmidt et al. 1991; Lagna et al. 1994) and to be conditionally required for U6 genes in yeast (Brow and Guthrie 1990; Moenne et al. 1990; Burmøl et al. 1993). Although the earlier vertebrate studies did not exclude the requirement for a form of TFIIIC2 that does not bind the B box (for review, see Lagna et al. 1994), more definitive studies with antibodies to TFIIICα have eliminated a role for TFIIIC2 in 7SK and U6 transcription in cell extracts (Lagna et al. 1994). Thus, effects of TFIIIC2 regulatory modifications might well be restricted to a subset of class III genes. This model of regulation may have relevance to the broad class of genes transcribed by RNA polymerase II, as recent studies indicate that different class II genes may have differential requirements for some general initiation factors (Parvin et al. 1994).

Regarding the actual regulatory mechanism our past and present studies argue for a model involving two different structural forms of the multi-subunit TFIIIC2, an active form [TFIIIC2a] that contains the 110-kD polypeptide and an inactive form [TFIIIC2b] that lacks this polypeptide (but may contain another distinct polypeptide; see above). Although both forms may bind to internal promoter elements of 5S, tRNA, and VA RNA genes, only the active form leads to a productive preinitiation complex (with TFIIIC1, TFIIIB, and RNA polymerase III) in nuclear extracts. The ability to remove TFIIIC1 activity quantitatively from nuclear extracts with a TFIIIC2a-specific antibody suggests that the 110-kD polypeptide may influence directly or indirectly interactions of TFIIIC2 with TFIIIC1, and thus control overall function. Hence, we postulate that TFIIICβ (the 110-kD polypeptide) is a central controlling subunit for transcription by RNA polymerase III for TFIIIC2-dependent promoters. This is consistent with the suggestion that TFIIICβ is the limiting component in RNA polymerase III-mediated transcription in cellular extracts and that the level of TFIIICβ (and TFIIIC2a) can be influenced by growth conditions and by virus infection. At the same time we cannot exclude the possibility that these conditions effect other modifications [e.g., post-translational] that may also affect the assembly or stability of active TFIIIC2a or TFIIIC2a–TFIIIC1 complexes.

Materials and methods

Purification and protein sequencing

For microsequence analysis of TFIIICβ, TFIIIC2a was purified as described previously (Kovelman and Roeder 1992). Microsequence analysis was performed as described [Lagna et al. 1994] and yielded peptide sequences of EMTSAESVEMSPT-PLPGFED, HILSELEAAPYLPQEEK, DLRRRYPETINSIK, and ADIPYQDSPEGPDHSSA. For subsequent studies TFIIIC2a and TFIIIC2b were purified from HeLa nuclear extract as described previously [Kovelman and Roeder 1992] with the following modifications. Heparin–Sepharose 4B and DNA cellulose columns were used. Fast protein liquid chromatography (FPLC) Mono Q [HR5/5, Pharmacia Biotech] and FPLC Mono S [HR5/5, Pharmacia Biotech] steps were added after the P11 col- umn. Both FPLC columns were 1 ml in size and were eluted with 20-ml gradients [100–600 mm KCl] in BC buffer [20 mm Tris [pH 7.9], 1 mm EDTA, 0.5 mm PMSF, 1 mm DTT, 10% glycerol].

Cloning of TFIIICβ

Oligonucleotide probes corresponding to the partial protein sequences (see text) of TFIIICβ were synthesized. Inosine [I] was used in positions of fourfold degeneracy. The oligonucleotides were A, GAICTGARGCCICCCITAYCTICTICARGARGA-RAA; B, CCITATGARCCIAITAAYWSIATIA; C, CGIGATCTI- ATICTAYCARGATWSICCGARGICCIGATCA; and D, G- ARGITCTCCGTTARATGGCTCTCCICCICTICCGGITT.

The oligonucleotides were kinase labeled and used in an equal molar mixture to screen 1 x 10^6 plaques from a Bjab library. Fifteen positive plaques were identified and two of the clones contained sequences encoding a protein that included three of the four partial protein sequences. The 5′ sequences were isolated by 5′ RACE according to the manufacturer instructions (5′ RACE kit, Gibco/BRL). Primers used were gene specific primer 1, 5′-TGGCTCAGTGGGTGTCTCATTC-3′; gene-specific primer 2, 5′-GGCACCGGTGACTAGTACCGCCGTAAGAAGGACACATC-3′; and anchor primer, 5′-CUCGUACUAUGGACAGGGCTGCACTAGTACCAGGGIIGGIGG-CCIGG-3′.

Bacterial expression of TFIIICβ

A fragment encoding the carboxy-terminal 595 residues of TFII- Cβ was cloned into the pRSETC bacterial expression vector (Invitrogen) to generate a fusion protein with six histidines at the amino terminus. The resultant plasmid [pHisIIIC3C17-911] was transformed into BL21-DE3 cells [Novagen] and grown in Luria broth. Expression was induced when OD_{600}=0.6 by the addition of IPTG to 1 mM. At 3 hr after induction the cells were harvested by centrifugation at 2000g for 30 min and resuspended in one-tenth culture volume of guanidinium buffer [6 M guanidine hydrochloride, 20 mm sodium phosphate [pH 7.8], 500 mm NaCl]. The extract was sonicated [Branson Sonifier 450] for 3 min at a setting of 5 at 100% duty cycle. After centrifugation at 2000g for 30 min the supernatant was saved and the pellet was discarded. Batch binding to NTA-nickel [Novagen] resin [Hemdan et al. 1989; Janknecht et al. 1991] was performed at room temperature at a ratio of 1 ml of resin per liter of culture. NTA–nickel resin was washed with 20 column volumes of denaturing buffer 7.8 [20 mm sodium phosphate [pH 7.8], 500 mm NaCl, 8 M urea], followed by 20 column volumes of denaturing buffer 6.0 [20 mM sodium phosphate [pH 6.0], 500 mm NaCl, 8 M urea], and eluted with 2 column volumes of denaturing elution buffer 4.0 [20 mM sodium phosphate [pH 4.0], 500 mm NaCl, 8 M urea]. The denaturing elution buffer 4.0 eluate was purified by preparative SDS-PAGE. The band corresponding to the expressed protein was excised and electroeluted using standard conditions.

Antibody production and immunoprecipitation

Antibody production in New Zealand White rabbits and immunoprecipitation were done as described [Harlow and Lane 1988] and briefly summarized here. Three hundred micrograms of bacterially expressed TFIIICβ was used in the initial injection and booster injections were made every month with 50 μg of protein. Blood was withdrawn 10 days after every antigen boost and antibodies were purified by protein A–Sepharose 4B column and antigen–Sepharose 4B [Sigma] column using standard pro-
c edures [Harlow and Lane 1988]. The final volume of antibodies was identical to the starting volume but with a protein concentration of 1.2 mg/ml. Purified antibodies were cross-linked directly to protein A-Sepharose 4B resin (2 mg protein per milliliter of resin) by dimethylpimelimidate [procedure in Harlow and Lane 1988]. Samples to be immunoprecipitated were incubated with antibody resin at 4°C for 1 hr in a rotator, washed with 100 column volumes of BC1000 [20 mM Tris (pH 7.9), 20% glycerol, 0.2 mM EDTA, 1 mM KCl] and eluted with one column volume of 0.2 M glycine [pH 2.5]. The eluate was neutralized with one-tenth volume 1 M Tris [pH 6.8], and immediately mixed with SDS-PAGE loading buffer.

For immunodepletion of nuclear extracts, antigen purified anti-TFIICB antibodies [or preimmune antibodies as a negative control] were cross-linked to protein A-Sepharose 4B beads by dimethylpimelimidate. After extensive washing with 300 mM KCl (in BC buffer) the beads were incubated (1:1 ratio) with HeLa nuclear extract in a spin reaction column. The mixture was rotated at 4°C for 4 hr, and the depleted extract was separated from the protein A-Sepharose 4B by centrifugation.

**Virus stock and infection**

Adenovirus was propagated and titered using techniques described previously [Hoeffler and Roeder 1985]. Wild-type adenovirus type 2 was titered on HeLa monolayer cells and the Ad5 mutant d1312 [Gaynor and Berk 1983] was titered on 293 cells. Cells to be infected were concentrated 10-fold by centrifugation and resuspension in one-tenth volume serum-free media. Infected with Ad2, infected with d1312, high serum, low serum) and transcription assays

**Extract preparation, general factor purification, and transcription assays**

Nuclear extract was prepared from HeLa cells [uninfected, infected with Ad2, infected with d312, high serum, low serum] [Hoeffler et al. 1988] by the procedure of Dignam et al. [1983], except that the nuclear extraction was performed with 0.5 mM KCl rather than 0.42 mM KCl in the extraction buffer. Transcriptions were performed as described previously [Kovelman and Roeder 1992]. Templates used were covalently closed circular DNAs at concentrations of 2.5 mg/ml for all promoters [VA1 promoter, pVA1; tRNA, ptRNA; 5S RNA, p5S (Lagna et al. 1994)]. For assays in the reconstituted system, RNA polymerase III was purified as described by Kovelman and Roeder [1992]. TFIIIB [Mono Q fraction] and TFIIIC1 [Mono Q fraction] were purified as described by Chiang et al. [1993] except that TFIIIC1 was purified from S100 P11 0.6 M KCl fraction.

**EMSA immunoblot assays**

The labeled adenovirus VA1 gene probe encompassed a region [from -30 to +99] containing both the A block and the B block consensus sequence and was prepared as described [Hoeffler et al. 1988]. Binding conditions were 8% glycerol [vol/vol], 3.5 mM MgCl2, 20 mM HEPES [pH 7.9], 1 mm DTT, 0.04 mM EDTA, 70 mM KCl, and 1-5 ng of purified TFIIIC2a in a total reaction volume of 25 μl. After incubation at 30°C for 15 min the samples were loaded directly into 3.5% PAGE [acylamide/ bis-acryl amide 37:5:1] in 0.25 x TBE [22 mM Tris, 22 mM boric acid, 0.6 mM Na4EDTA] and electrophoresed at 100 V for 5 hr at 4°C. Gels were dried and exposed to X-ray film.

Immunoblots were performed as described by the manufacturer [ECL system, Amersham]. Antigen-purified anti-TFIICB antibodies had a protein concentration of 1.2 mg/ml and were typically used at 1:5000 dilution.

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