Cloning and characterization of a TFIIC2 subunit (TFIIC β) whose presence correlates with activation of RNA polymerase III-mediated transcription by adenovirus E1A expression and serum factors

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TFIIC2 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 TFIIC2a and TFIIC2b. TFIIC2a and TFIIC2b 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 (TFIIC2a). 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 (TFIIC β). Immunoprecipitation studies with anti-TFIIC β antibodies have confirmed that TFIIC β is a bona fide subunit present only in TFIIC2a, that TFIIC2a and the general factor TFIIC1 are associated in unfractionated extracts, and that previously undetected polypeptides (potential TFIIC1 subunits) can be isolated in association with TFIIC2a. 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 TFIIC fraction. Studies with antibodies to TFIIC β have shown that this is strongly correlated with a selective increase in the cellular concentration of the TFIIC β 110-kD subunit and a concomitant rise in the ratio of the active-to-inactive forms of TFIIC2.

[**Key Words:** TFIIC2 subunit; RNA polymerase III; adenovirus E1A; 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 TFIIB and TFIIC 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 TFIIA in addition to TFIIB, TFIIC, and RNA polymerase III. Subclass 3 genes (encoding U6 and 7SK RNAs) have non-transcribed 5' control ele-

ments 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), TFIIB, and RNA polymerase III for efficient *in vitro* transcription.

TFIIC from mammalian cells (Segall et al. 1980) has been purified and fractionated partially into two activities, TFIIC1 and TFIIC2, which are jointly required for activity (Dean and Berk 1987; Yoshinaga et al. 1987, 1989; Z. Wang and R. Roeder, unpubl.). TFIIC2 can be isolated in two forms (now designated TFIIC2a and TFIIC2b) that bind DNA with equal affinity and show equivalent DNase footprint patterns (Hoeffler et al. 1988; Kovelman and Roeder 1992). However, they can be distinguished by (1) the ability of TFIIC2a but not TFIIC2b to restore RNA polymerase III transcription in a heat-treated nuclear extract, (2) the electrophoretic mobilities (TFIIC2b > TFIIC2a) 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 TFIIC2a.

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 (PrIE) (Berger and Folk 1985; Gaynor et al. 1985; Hoeffler 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 TFIIC fraction, variably characterized as an increase in the ratio of TFIIC2a to TFIIC2b (Hoeffler and Roeder 1985; Hoeffler et al. 1988) or a net increase in the concentration of TFIIC2 (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 TFIIC fraction with a similar increase in the ratio of TFIIC2a versus TFIIC2b (Hoeffler 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 TFIIC2 and used this to study regulatory modifications and interactions of TFIIC2.

Results

Cloning of TFIIC β

TFIIC2, 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 TFIIC2 was identified and digested with endoproteinase Lys-C. Microsequence analysis of derived peptides yielded sequences of HLLSELEAAPYL PQEEK, DLRPPYEPINSIK, ADLIPYQDSPEGPDHSSA, and EMTSAEASVEMSLPTPLPGFED. 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 TFIIC β 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 TFIIC β cDNA is shown in Figure 1A. The residues corresponding to those derived from direct protein sequencing are underlined. The cloned TFIIC β 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 TFIIC β was analyzed by computer (GCG, University of Wisconsin, Madison; MacVector, Kodak) and by visual inspection. This re-

vealed 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 TFIIC β 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 TFIIC β 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 TFIIC2, 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 TFIIC β cDNA was then transcribed and translated in a rabbit reticulocyte lysate and the corresponding product was analyzed, along with a partially purified TFIIC2 (P11 fraction), by SDS-PAGE. When the corresponding membrane was probed with anti-TFIIC β antibodies a 110-kD band was detected in both the in vitro translated (Fig. 2A, lane 1) and the natural TFIIC2 (lane 2) fractions. This suggests that the existing clone of TFIIC β contains the complete coding sequence for the presumptive 110-kDa subunit of TFIIC2a.

The TFIIC β amino acid sequence was compared with sequences from the 220-kD human (L'Etoile et al. 1994) and rat (Lagna et al. 1994) TFIIC α 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 TFIIC and no significant sequence similarities were found. Consistent with this analysis anti-TFIIC β antibody failed to react on a Western blot with any polypeptide of partially purified yeast TFIIC (data not shown). The lack of similarity between TFIIC β and yeast TFIIC subunits is not surprising considering the degree of evolutionary divergence between yeast and vertebrate TFIIC (see Discussion).

TFIIC β is associated only with the low mobility transcriptionally active form (TFIIC2a) of TFIIC2

As discussed in the introductory section our previous studies have demonstrated two distinct and chromato-

Figure 2. (A) In vitro translation of TFIIC β cDNA. Immunoblot of rabbit reticulocyte lysate translated TFIIC β and purified TFIIC2 (nuclear extract, P11 0.6 fraction) probed with anti-TFIIC β antibody. The bands detected have a migration rate corresponding to 110 kD. (B,C) Immunoblot of TFIIC2a and TFIIC2b. Purified TFIIC2a and TFIIC2b fractions (4500-fold) were loaded on 7% SDS-PAGE and analyzed by immunoblot with anti-TFIIC β antibodies (B) or by silver stain (C). The polypeptides in the 140- to 200-kD range in the TFIIC2b preparation are not regarded as likely subunits as they do not copurify strictly (during gradient elution) with the TFIIC2b activity and the other polypeptides. Moreover, the fact that TFIIC2b has a faster migration rate under gel mobility-shift assays than does TFIIC2a makes it unlikely that these high molecular mass proteins are part of the TFIIC2b promoter complex.

110-kD polypeptide in TFIIC2a but no cross-reacting polypeptide in TFIIC2b. 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 TFIIC2a, whereas the 77-kD polypeptide in TFIIC2b may be either a contaminant or a reversibly associated component that could play a regulatory role.

Given the proposed role of TFIIC β in effecting changes in the levels of TFIIC2a in response to certain stimuli, its presence in various subcellular and chromatographic fractions was monitored by immunoblot analysis. TFIIC β was detected only in the nuclear extract-derived P11 fraction (0.6 M KCl eluate) that contains TFIIC and not in other P11 fractions (0.1, 0.3, and 1.0 M KCl eluates). TFIIC β also was not detected in current S100 and nuclear pellet fractions, consistent with our previous experience that TFIIC2 activity was undetectable in the same fractions (data not shown). During the further purification of TFIIC2a through four chromatographic steps (see Material and methods) the

TFIIC β subunit was detected only in fractions that contained TFIIC2a activity. Thus, the protein (TFIIC β) encoded by the isolated cDNA copurifies, as expected, only with TFIIC2a and does not appear to exist in a subcellular compartment independently of TFIIC2a.

TFIIC β remains bound in a TFIIC2a-promoter complex

To gain additional information on the subunit status of TFIIC β and to determine whether it might associate reversibly with the common TFIIC2 subunits, especially as TFIIC2a and TFIIC2b can each form a promoter complex (Hoeffler et al. 1988), it was important to determine whether TFIIC β remains associated with binding of TFIIC2a to the promoter. Analysis of TFIIC2a by electrophoretic mobility-shift assay (EMSA) with a VA1 promoter fragment (Fig. 3) revealed a characteristic TFIIC2a-VA1 complex that was dependent on the presence of TFIIC2a (cf. lane 2 with lane 1) and completely supershifted by anti-TFIIC β antibody (lane 4) but not by preimmune serum (lane 3). These results indicate that indeed TFIIC β remains associated with binding of TFIIC2a to the promoter, providing strong support for its role as an integral subunit. The interesting possibility that the TFIIC β interaction within TFIIC2a might be

Figure 3. Retardation of TFIIC2a promoter complexes by anti-TFIIC β 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 TFIIC2a; (lane 3) probe, TFIIC2a, and preimmune serum (final antibody concentration, 50 μ g/ml); (lane 4) probe, TFIIC2a, and anti-TFIIC β antibodies (final antibody concentration, 50 μ g/ml). Arrow 2 points to the TFIIC2a-promoter complex; arrow 1 points to the TFIIC2a-promoter complex supershifted by anti-TFIIC β antibodies.

Figure 4. Immunodepletion of nuclear extract using anti-TFIIIC β antibody. In vitro transcription from the VA1 promoter with preimmune-depleted nuclear extract (lane 1) or with anti-TFIIIC β antibody-immunodepleted nuclear extract with no addition (lane 2), with TFIIIC1 added (lane 3), with TFIIIC2a added (lane 4), with TFIIIB added (lane 5), with RNA polymerase III added (lane 6), or with TFIIIC1 plus TFIIIC2a (lane 7).

dynamic and only transiently required for transcription initiation remains to be tested.

TFIIIC β is involved in transcription by RNA polymerase III and potentially in interactions of TFIIIC2a with TFIIIC1

To determine whether TFIIIC β is essential for transcription by RNA polymerase III, antigen-purified antibodies raised against the bacterially expressed carboxy-terminal fragment of TFIIIC β were used to immunodeplete a HeLa extract (Fig. 4). As judged by immunoblot this treatment removed >90% of TFIIIC β , 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-TFIIIC β 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 TFIIIC1, TFIIIC2a, TFIIIB, 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 TFIIIC1 and TFIIIC2a 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 TFIIIC2a removes

TFIIIC1 as well as TFIIIC2a activity quantitatively. These results indicate that TFIIIC β is an essential component of TFIIIC2a and further suggest that some or all TFIIIC1 components are tightly associated with TFIIIC2a in nuclear extracts.

In an attempt to identify possible TFIIIC2a-associated polypeptides the anti-TFIIIC β antiserum was used to immunoprecipitate TFIIIC2a. For this purpose a conventional nuclear extract was adjusted to 300 mM KCl and applied either to an anti-TFIIIC β antibody–protein A column or to a control preimmune antibody–protein A column. The columns were washed with a BC buffer containing 1 M 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 TFIIIC2a 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 TFIIIC1.

Effects of adenovirus infection and cellular growth state on the cellular level of TFIIIC β

It was reported previously that RNA polymerase III transcription initiation activity is increased both by the viral

Figure 5. Immunoprecipitation of the TFIIIC2a complex and associated polypeptides using anti-TFIIIC β antibody. HeLa nuclear extract was incubated for 1 hr with anti-TFIIIC β antibody (lanes 2,4) or preimmune antibody (lanes 1,3) cross-linked to protein A–Sepharose 4B beads. The beads were washed with BC buffer containing 1 M KCl and eluted with 0.2 M glycine (pH 2.5). The eluate was analyzed by SDS-PAGE with 5% (lanes 1,2) and 4–20% (lanes 3,4) polyacrylamide and stained with silver. The estimated sizes of the subunits are indicated to the right of each panel.

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 TFIIC activity, it has not been clear whether the increase in TFIIC activity resulted from an increase in specific activity (Hoeffler and Roeder 1985; Hoeffler et al. 1988) or from an increase in the amount of TFIIC (Yoshinaga et al. 1986).

To investigate further the effects of adenovirus infection (including E1A expression) on TFIIC 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 TFIIC fractions (P11 0.6) from the two extracts were subjected to SDS-PAGE and blotted onto membranes that were probed simultaneously with anti-TFIIC β and anti-TFIIC α antibodies. Because the 220-kD TFIIC α subunit was shown previously to be a component of both TFIIC2a and TFIIC2b (Kovelman and Roeder 1992), the signal from the corresponding antibody should reflect the total level of TFIIC2. As shown in Figure 6B, the levels of TFIIC α 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 TFIIC2 in the cell. In contrast, the level of TFIIC β , which is only associated with the low mobility form of TFIIC2, 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 TFIIC β and the corresponding active form of TFIIC2. Because TFIIC β 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 TFIIC β . More recently, preliminary analysis of total cellular RNA levels has indicated a significant effect of E1A expression on the level of TFIIC β 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 TFIIC β and TFIIC α showed comparable levels of TFIIC α in both extracts but lower levels of TFIIC β 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 TFIIC2 are

Figure 6. (A) Adenovirus E1A gene product and serum enhance levels of transcription by RNA polymerase III. *In vitro* transcription using nuclear extracts prepared from cells infected with wild-type adenovirus (Ad2) (lanes 1,5,9), from cells infected with E1A-defective adenovirus (dl312) (lanes 2,6,10), from cells grown in 10% serum (lanes 3,7,11), and from cells grown in 0.5% serum (lanes 4,8,12). The same amount of nuclear extract protein was used in each *in vitro* transcription reaction. (B) TFIIC β and TFIIC α levels in wild-type virus (E1A⁺), mutant virus (E1A⁻), high-serum and low-serum extracts. Immunoblot analysis of the TFIIC fractions (nuclear extract, P11 0.6) from cells infected with wild-type adenovirus (lane 1) vs. E1A-defective adenovirus (lane 2) or from cells grown in 10% serum (lane 3) vs. 0.5% serum (lane 4). TFIIC fractions correspond to the 0.6 M KCl eluates obtained by fractionation of nuclear extracts (the same as those used in A) on phosphocellulose and were subjected to SDS-PAGE and immunoblot with a mixture of anti-TFIIC α and anti-TFIIC β antibodies.

largely responsible for the difference in TFIIC2 activity under different growth conditions.

Discussion

TFIIC 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 (TFIIC β) of one of the two forms (TFIIC2a) of the derived TFIIC2. This cDNA has been used to investigate the primary sequence of TFIIC β , regulatory modifications of TFIIC2 involving TFIIC β , and interactions of TFIIC2 with TFIIC1.

Cloning and sequence analysis of TFIIC β

Amino acid sequence information from the 110-kD polypeptide present in purified TFIIC2a was used to obtain a cDNA encoding the corresponding protein. That the encoded protein is a bona fide subunit of TFIIC2a is indicated by the ability of anti-TFIIC β antibodies (raised against recombinant protein) to inhibit TFIIC function in transcription assays and to supershift TFIIC2a-VA promoter complexes, thus indicating that TFIIC β is an integral part of the TFIIC2a complex that initially binds DNA.

The cloned TFIIC β 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; Tzamaras and Struhl 1994) and transcriptional activation (TAF_{II}80) (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; Goebel and Yanagida 1991), and in the case of TUP1 a direct role for the WD40 repeats in protein-protein interactions has been demonstrated (Tzamaras and Struhl 1994; Komachi et al. 1994). This leads to speculation that the WD40 motifs may be involved in reversible interactions of TFIIC β with other factors such as TFIIC1 or TFIIB. It is interesting in this regard that the 131-kD subunit of yeast TFIIC contains TPR repeats (Marck et al. 1993), although no WD40 repeats have yet been found in the published sequences of other yeast TFIIC subunits (E. Sinn, unpubl.).

Given that the five-subunit yeast TFIIC and human TFIIC (TFIIC2 plus TFIIC1) 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 TFIIC α and the large subunit of yeast TFIIC (both of which contact B-box elements) show no significant sequence similarity (Lagna et al. 1994). Following this trend, TFIIC β also shows no significant sequence similarity to any of the three cloned subunits (138, 131, and 95 kD) of yeast TFIIC. Consistent with these findings, anti-TFIIC β 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 TFIIC subunits. However, as discussed below, it is likely that human counterparts to some of the yeast TFIIC components most likely reside in the as yet uncharacterized TFIIC1 subunits, and sequences for some of the yeast TFIIC subunits have not yet been reported.

Subunit structures of variant forms of TFIIC2

We reported previously the resolution of two forms of the B box-binding component of TFIIC, one (now designated TFIIC2a) that formed a lower mobility promoter complex and showed transcriptional activity in a heat-treated nuclear extract and one (now designated TFIIC2b) 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 TFIIC2a and candidate subunits of 220, 102, 95, 90, 77, and 63 kD for TFIIC2b (Kovelman and Roeder 1992). The use of antibodies against TFIIC β has verified that the 110-kD subunit is uniquely present in TFIIC2a 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 TFIIC2b because it has not been detected in more recent larger scale and higher yield preparations of TFIIC2b, either by direct SDS-PAGE analysis (Fig. 2C; Z. Wang and R.G. Roeder, unpubl.) or by SDS-PAGE analysis after immunoprecipitation with anti-TFIIC α antibodies (L'Etoile et al. 1994; Lagna et al. 1994). In contrast, the 77-kD polypeptide has copurified consistently with TFIIC2b, although it has not been detected by immunoprecipitation with anti-TFIIC α 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 TFIIC2a contains subunits of 220, 110, 102, 90, and 63 kD, whereas TFIIC2b differs with respect to the absence of the 110-kD subunit and the possible presence of a substitute 77-kD subunit.

Interactions of TFIIC2a with TFIIC1

Immunoprecipitation studies reported here showed that

anti-TFIIC β antibodies, but not preimmune serum, can remove quantitatively both TFIIC2a and TFIIC1 activities from unfractionated nuclear extracts. These results indicate that at least one of the components that comprise the TFIIC1 activity is in stable association with TFIIC2a (either directly with TFIIC β or with another subunit) and present in an equal or substoichiometric ratio relative to TFIIC2a. Analysis of the immunoprecipitates revealed polypeptides of 58, 42, and 33 kD, in addition to those normally observed in highly purified TFIIC2a. Consistent with the possibility that these new polypeptides might represent TFIIC1 subunits, the observed interactions were resistant to washing with 1 M KCl. Although there are reports that TFIIC2 can interact with human TFIIA (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 (DeJong and Roeder 1993) failed to show cross-reactivity with any of the newly detected polypeptides. In further support of the possibility that these are TFIIC1 subunits, they are equivalent in size to a subset of polypeptides present in our most highly purified preparations of TFIIC1 (Z. Wang and R.G. Roeder, unpubl.). The failure to detect these novel polypeptides in previous preparations of chromatographically purified TFIIC2a (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-TFIIC α immunoprecipitates (L'Etoile et al. 1994; Lagna et al. 1994) may have resulted from their dissociation from TFIIC2a by the antibodies used. In the present analysis the quantitative removal of TFIIC1 activity from nuclear extracts with an antibody (anti-TFIIC β) specific for TFIIC2a further suggests that TFIIC1 is not associated with the inactive form (TFIIC2b) of TFIIC2 and that TFIIC β could play a direct role in stabilizing interactions of TFIIC2a with TFIIC1. Alternatively, TFIIC1 could be so tightly bound to TFIIC2b that it cannot readily exchange to TFIIC2a. In the former case any increase in the level of TFIIC2a (through TFIIC β association with TFIIC2b) would necessitate a corresponding increased intranuclear concentration of TFIIC1; this could involve recruitment from the cytoplasm, consistent with the presence of high level of TFIIC1 in the S100 fraction (Z. Wang and R.G. Roeder, unpubl.).

Previous studies have shown that the five-subunit yeast TFIIC binds to both B and A boxes of promoters, whereas chromatographically purified human TFIIC2a and TFIIC1 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 TFIIC2a plus TFIIC1 with yeast TFIIC is supported by the finding of an apparently strong physical interaction in unfractionated nuclear extract between TFIIC1 and TFIIC2a. It is apparent, however, that there is a greater structural complexity in the human TFIIC (TFIIC2 plus

TFIIC1) than in yeast TFIIC, 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 TFIIC (p95; Bartholomew et al. 1990) to be found in human TFIIC1 and not in TFIIC2.

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, TFIIC 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 TFIIC2. 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 TFIIC2 (Hoeffler and Roeder 1985; Hoeffler et al. 1988; Kovelman and Roeder 1990). Here we have used antibodies to the common TFIIC2 subunit (TFIIC α) and to the TFIIC2a-specific subunit (TFIIC β) to verify that the E1A-mediated effect indeed correlates with an increased TFIIC2 specific activity that results from an increased TFIIC2a:TFIIC2b ratio. At present it is not clear whether the effect of E1A on TFIIC2 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 anti-TFIIC α and anti-TFIIC β antibodies further established that the effects of serum on TFIIC2 activity result from an increased TFIIC2 specific activity, with an increase in the ratio of TFIIC2a:TFIIC2b. Thus, the proposed mechanism used by adenovirus to enhance the cellular capacity for transcription by RNA polymerase III appears to be the same as that normally used by the cell during serum-stimulated growth.

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

To the extent that TFIIC2 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 TFIIC 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; Burnol et al. 1993). Although the earlier vertebrate studies did not exclude the requirement for a form of TFIIC2 that does not bind the B box (for review, see Lagna et al. 1994), more definitive studies with antibodies to TFIIC α have eliminated a role for TFIIC2 in 7SK and U6 transcription in cell extracts (Lagna et al. 1994). Thus, effects of TFIIC2 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 TFIIC2, an active form (TFIIC2a) that contains the 110-kD polypeptide and an inactive form (TFIIC2b) 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 TFIIC1, TFIIB, and RNA polymerase III) in nuclear extracts. The ability to remove TFIIC1 activity quantitatively from nuclear extracts with a TFIIC2a-specific antibody suggests that the 110-kD polypeptide may influence directly or indirectly interactions of TFIIC2 with TFIIC1, and thus control overall function. Hence, we postulate that TFIIC β (the 110-kD polypeptide) is a central controlling subunit for transcription by RNA polymerase III for TFIIC2-dependent promoters. This is consistent with the suggestion that TFIIC β is the limiting component in RNA polymerase III-mediated transcription in cellular extracts and that the level of TFIIC β (and TFIIC2a) 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 TFIIC2a or TFIIC2a-TFIIC1 complexes.

Materials and methods

Purification and protein sequencing

For microsequence analysis of TFIIC β , TFIIC2a was purified as described previously (Kovelman and Roeder 1992). Microsequence analysis was performed as described (Lagna et al. 1994) and yielded peptide sequences of EMTSAEASVMSLPT-PLPGFED, HLLSELEAAPYPQEEK, DLRPPYEPINSIK, and ADLIPYQDSPEGPDHSSA. For subsequent studies TFIIC2a and TFIIC2b 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 omitted. 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 TFIIC β

Oligonucleotide probes corresponding to the partial protein sequences (see text) of TFIIC β were synthesized. Inosine (I) was used in positions of fourfold degeneracy. The oligonucleotides were A, GAICTIGARGCIGCICCITAYCTICCICARGARGA-RAA; B, CCITATGARCCIIATIAAYWSIATIAA; C, GCIGATCTI-ATICCITAYCARGATWSICCGARGGICCGATCA; and D, G-ARGCITCCGTIGARATGTCCCTICCIACICCICTICCGITT. The oligonucleotides were kinase labeled and used in an equal molar mixture to screen 1×10^6 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'-TGGCTCAGTGGGTGTGTCTCATTC-3'; gene-specific primer 2, 5'-GGCCACGCGTCGACTAGTACCCCG-TGAAGAAGGACACATC-3'; and anchor primer, 5'-CUACU-ACUACUAGGCCACGCGTCGACTAGTACGGGIIIGGGIIIG-GGIIG-3'.

Bacterial expression of TFIIC β

A fragment encoding the carboxy-terminal 595 residues of TFIIC β was cloned into the pRSETC bacterial expression vector (Invitrogen) to generate a fusion protein with six histidines at the amino terminus. The resultant plasmid (pHisIIC317-911) 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 TFIIC β was used in the initial injection and booster injections were made every month with 50 μ g 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-

cedures (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 M 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-TFIIC β 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 dl312 (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. Infection was for 1 hr at a multiplicity of infection of 20 to 1. Fresh medium with 5% serum was added after infection to original volume. Cells were harvested 6 hr after infection for analysis and extract production.

Extract preparation, general factor purification, and transcription assays

Nuclear extract was prepared from HeLa cells (uninfected, infected with Ad2, infected with dl312, 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 M KCl rather than 0.42 M 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). TFIIB (Mono Q fraction) and TFIIC1 (Mono Q fraction) were purified as described by Chiang et al. (1993) except that TFIIC1 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 TFIIC2a in a total reaction volume of 25 μ l. After incubation at 30°C for 15 min the samples were loaded directly into 3.5% PAGE (acrylamide/bis-acrylamide 37.5:1) in 0.25 $\times$ TBE (22 mM Tris, 22 mM boric acid, 0.6 mM Na₂EDTA) 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 manufac-

turer (ECL system, Amersham). Antigen-purified anti-TFIIC β antibodies had a protein concentration of 1.2 mg/ml and were typically used at 1:5000 dilution.

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