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Cloning and Characterization of an Evolutionarily Divergent DNA-Binding Subunit of Mammalian TFIIIC

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Transcription factor IIIC (TFIIIC) is required for the assembly of a preinitiation complex on 5S RNA, tRNA, and adenovirus VA RNA genes and contains two separable components, TFIIIC1 and TFIIIC2. TFIIIC2 binds to the 3′ end of the internal control region of the VA_I RNA gene and contains five polypeptides ranging in size from 63 to 220 kDa; the largest of these directly contacts DNA. Here we describe the cloning of cDNAs encoding all (rat) or part (human) of the 220-kDa subunit (TFIIICα). Surprisingly, TFIIICα has no homology to any of the yeast TFIIIC subunits already cloned, suggesting a significant degree of evolutionary divergence for RNA polymerase III factors. Antibodies raised against the N terminus of recombinant human TFIIICα specifically inhibit binding of natural TFIIIC to DNA. Furthermore, immunodepletion assays indicate that TFIIICα is absolutely required for RNA polymerase III transcription of 5S RNA, tRNA, and VA_I RNA genes but not for the 7SK RNA and U6 small nuclear RNA genes. Transcription from the tRNA and VA_I RNA genes in TFIIIC-depleted nuclear extracts can be restored by addition of purified TFIIIC. In contrast, restoration of 5S RNA gene transcription requires readdition of both TFIIIC and TFIIIA, indicating a promoter-independent interaction between these factors. Immunoprecipitation experiments demonstrate a tight association of all five polypeptides previously identified in the TFIIIC2 fraction, confirming the multisubunit structure of the human factor.

RNA polymerase III transcribes a number of cellular and viral genes encoding small structural RNAs. These class III genes can be divided into three subclasses on the basis of their promoter structures and transcription factor requirements (for review, see references 18, 20, 21, and 22). (i) The tRNA, 7SL, and adenovirus VA_I and VA_{II} genes, as well as *Alu* sequences, have a simple internal promoter containing A-box and B-box elements and require only TFIIIB and TFIIIC as accessory factors for RNA polymerase III. (ii) The 5S RNA genes have more complex internal promoters containing A-box, I-box, and C-box elements and require the gene-specific transcription factor TFIIIA in addition to the common factors TFIIIB and TFIIIC. (iii) The vertebrate U6 and 7SK RNA genes have upstream promoter elements, including a TATA box and a conserved proximal sequence element; these genes require, at a minimum, the TATA-binding protein (TBP), the TFIIIB fraction, and the proximal-sequence-element-binding factor PTF.

In addition to being required as a general initiation factor for transcription from at least two subclasses of class III genes, TFIIIC regulates RNA polymerase III transcription in response to several viral regulatory proteins. For example, the adenovirus E1A and pseudorabies virus immediate-early gene products stimulate RNA polymerase III transcription (3, 19, 24, 55), while infection by poliovirus represses transcription (16). These positive and negative effects can be reproduced in vitro with extracts prepared from infected cells, and in both cases fractions associated with the changes in transcriptional activity copurify with TFIIIC (16, 24, 55). More recent studies suggest that the changes in TFIIIC activity correlate with two different forms of TFIIIC, which can be distinguished by the distinct electrophoretic mobilities of the respective TFIIIC-DNA complexes (9, 23). An upper-band form cofractionates with both the transcriptional and the B-box-binding activities, while a lower-band form binds to the B-box but is less active in transcription. The main physical difference between these two forms seems to result from the absence or modification, in the lower-band form, of the second-largest TFIIIC subunit (29). It has also been suggested that the shift from one form of TFIIIC to the other could involve phosphorylation events (9, 23).

Although the original fractionation studies with vertebrate cell extracts identified only three common components of the RNA polymerase III transcription system (TFIIIB, TFIIIC, and RNA polymerase III) for 5S, tRNA, and VA genes (43, 44), more recent reports have added details to this picture. TFIIIC has been chromatographically separated into two essential activities, TFIIIC1 and TFIIIC2 (12, 52, 54). TFIIIC2 binds with high affinity to the B-box, while TFIIIC1 binds very weakly to the A-box region. In the presence of TFIIIC2, a TFIIIC1-dependent footprint extends to the upstream A-box of the VA RNA intragenic promoter. More recently, work from this laboratory (8) described the isolation of an additional activity from the classical TFIIIB fraction (P11, 0.3 M KCl eluate). This activity, called TFIIIB1, is required only when the other components (notably TFIIIC2) are highly purified and appears to be functionally equivalent to the TFIIIC1 component (52). These observations appear to reconcile the existing data about vertebrate TFIIIC and TFIIIB into a unified picture. Thus, transcription of tRNA and VA genes by RNA polymerase III requires the highly purified TFIIIC component (TFIIIC2) that independently binds the B-box (29, 56), the TBP-containing component (TFIIIB2) of TFIIIB (8, 35, 48, 53), and an additional component derived from either the partially purified TFIIIC fraction (TFIIIC1) or the partially purified TFIIIB fraction (TFIIIB1).

The ability of TFIIIC2 to bind DNA has been used to purify the human factor by affinity chromatography. Fractions eluting

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at high salt concentrations contained five major polypeptides of 220, 110, 102, 90, and 63 kDa, all of which correlated with the TFIIIC2 activity (29, 56). The 220-kDa protein was the only subunit that could be specifically photocross-linked to the VA_I promoter (29, 56). Henceforth, TFIIIC2 will refer to the isolated complex containing these polypeptides and the B-box-binding activity, while TFIIIC will refer to the combined (nonseparated) TFIIIC1 and TFIIIC2 activities as defined in the original complementation assay.

We report here the cloning of cDNAs encoding the 220-kDa polypeptide, which we named TFIIICα, through the use of oligonucleotides based on peptide microsequencing results. An apparently full-length rat cDNA encodes a 2,148-amino-acid (a.a.) protein with the appropriate electrophoretic mobility. Antibodies raised against an overexpressed recombinant protein from partial human cDNAs for TFIIICα have been used to demonstrate the central role of this subunit in DNA binding and transcriptional activation in HeLa cell extracts. Furthermore, immunoprecipitation studies indicate that the five polypeptides in highly purified preparations of TFIIIC2 are in a tightly associated complex and suggest that TFIIIA and TFIIIC may be associated in the cell.

MATERIALS AND METHODS

Purification of TFIIIC2. Affinity purification of TFIIIC2 has been described before (29). The purified polypeptides were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride membrane. The band corresponding to p220 was excised and digested on the membrane with endoproteinase Lys-C, and the products were resolved by reverse-phase high-pressure liquid chromatography and subjected to microsequence analysis at the Rockefeller Protein Sequencing Facility.

Cloning of human and rat TFIIICα cDNAs. A degenerate oligonucleotide was designed on the basis of the sequence of peptide 220a shown in Fig. 2. The sequence of the 53-mer used for the direct screening of a human (Namalwa cell) cDNA library was 5′-TCATAIGT(C/T)TGIGTIAGCATATCICG(C/T)TCAAAIACIATATCIACIGGIAC-3′, where I indicates inosine.

For all the clones, double-stranded DNA sequencing of both strands was performed with synthetic oligonucleotides and the Sequenase DNA sequencing kit (United States Biochemical Corp.).

In vitro translation of rTFIIICα. The full-length rat TFIIICα (rTFIIICα) cDNA was obtained by splicing in vitro the partial clones λR4 and λR13A. Briefly, the plasmid vector pBluescript II SK^- was cut with *Xho*I, filled in with Klenow DNA polymerase, and religated. This modified vector, cut with *Eco*RI and *Sma*I, was ligated to an *Eco*RI-*Sma*I fragment derived from clone λR13A to produce pBSK-*Xho*I^--13A. A *Not*I-*Xho*I fragment of λR4 was finally cloned into the *Not*I- and *Xho*I-cut pBSK-*Xho*I^--13A plasmid to produce p-rTFIIICα.

Transcription and translation of the cDNA in the presence of [^{35}S]methionine were performed with the TNT T7 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions.

Bacterial expression of glutathione-*S*-transferase–hTFIIICα fusion protein. A 1.17-kb *Dra*I-*Eco*RI fragment derived from the human cDNA clone λ19BJA/B was subcloned into the expression vector pGEX-3X (Pharmacia) linearized with *Sma*I and *Eco*RI. Bacteria containing the resulting plasmid were grown at 26°C to maximize the production of soluble protein. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG), cells were harvested by centrifugation and then lysed by sonication in buffer L (phosphate-buffered saline [PBS] with the addition of 1 mM disodium EDTA and 0.1 mg of lysozyme [Sigma] per ml). After centrifugation at 10,000 × *g* for 15 min, the supernatant was incubated with glutathione-agarose matrix (Sigma) and washed extensively with PBS containing 1 mM disodium EDTA. The matrix-bound protein was denatured at 100°C in 2% SDS buffer and loaded onto a preparative SDS-polyacrylamide gel. The purified protein was electroeluted before being injected into rabbits.

Antiserum preparation. New Zealand rabbits were initially bled to obtain preimmune serum and then injected intradermally with 100 μg of antigen emulsified with an equal volume of complete Freund's adjuvant. Rabbits were boosted subcutaneously every 4 weeks with 50 μg of antigen emulsified with an equal volume of Freund's incomplete adjuvant. Blood was collected from dorsal ear veins 10 days after each boost. For immunoblotting, crude serum was purified by 50% ammonium sulfate precipitation.

Immunoblot analysis. Proteins (50 to 100 μg of nuclear extract) were separated on an 8% polyacrylamide–SDS gel and transferred to a nitrocellulose membrane (BA83; Schleicher & Schuell). After blocking with 5% nonfat dry milk (Carnation) in PBS, the membrane was incubated for 2 h at room temperature in 1,000-fold-diluted rabbit anti-TFIIICα serum. Antibodies were diluted in buffer W (PBS containing 0.6% nonfat dry milk and 0.2% Tween 20). Signals were detected by the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions.

Gel mobility shift analysis. The conditions used for the TFIIIC gel mobility shift assay have been described before (29). Briefly, the binding reaction mixes contained 8% (vol/vol) glycerol, 3.5 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 5 mM dithiothreitol, 0.04 mM disodium EDTA, and 70 mM KCl. Each reaction mix (25 μl) generally contained 3 μg of poly(dI · dC)-poly(dI · dC) and 0.5 μg of pBluescript II SK^- as nonspecific DNA competitors, 1 to 4 μg of TFIIIC fraction, and 20 fmol of VA_I probe (prepared as described previously [23]). Antibodies were added to the mixture in the amounts noted in the figure legends. Reactions were initiated by the addition of protein and incubation for 30 min at 30°C. The protein-DNA complexes were resolved on a 4% nondenaturing polyacrylamide (acrylamide-bisacrylamide, 37.5:1) gel in 0.25× TBE (22.5 mM Tris-borate, 0.5 mM disodium EDTA). Electrophoresis was done at 125 V for 2 h at room temperature.

Immunodepletion of TFIIIC from HeLa nuclear extract. To deplete TFIIIC from the HeLa cell nuclear extract (13), 600 μl of anti-TFIIICα antiserum was incubated with 200 μl of protein A-Sepharose (Sigma) for 1 h at 4°C with constant mixing. After being washed four times with buffer BC100 (buffer BC [20 mM HEPES-KOH (pH 7.9), 20% (vol/vol) glycerol, 0.2 mM disodium EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride] with the addition of 100 mM KCl) and once with buffer BC300 (buffer BC with 300 mM KCl), the resin was incubated with 500 μl of HeLa nuclear extract for 12 h at 4°C with nutation. After centrifugation, the supernatant was dialyzed against buffer BC100, and aliquots were quickly frozen in liquid nitrogen. In some experiments, the HeLa nuclear extract was absorbed to a DE52 (DEAE-cellulose) matrix (Whatman) in the presence of 0.5 M KCl. The flowthrough was then used for the immunodepletion.

In vitro transcription assays. Transcription reactions were performed essentially as described before (8). The templates

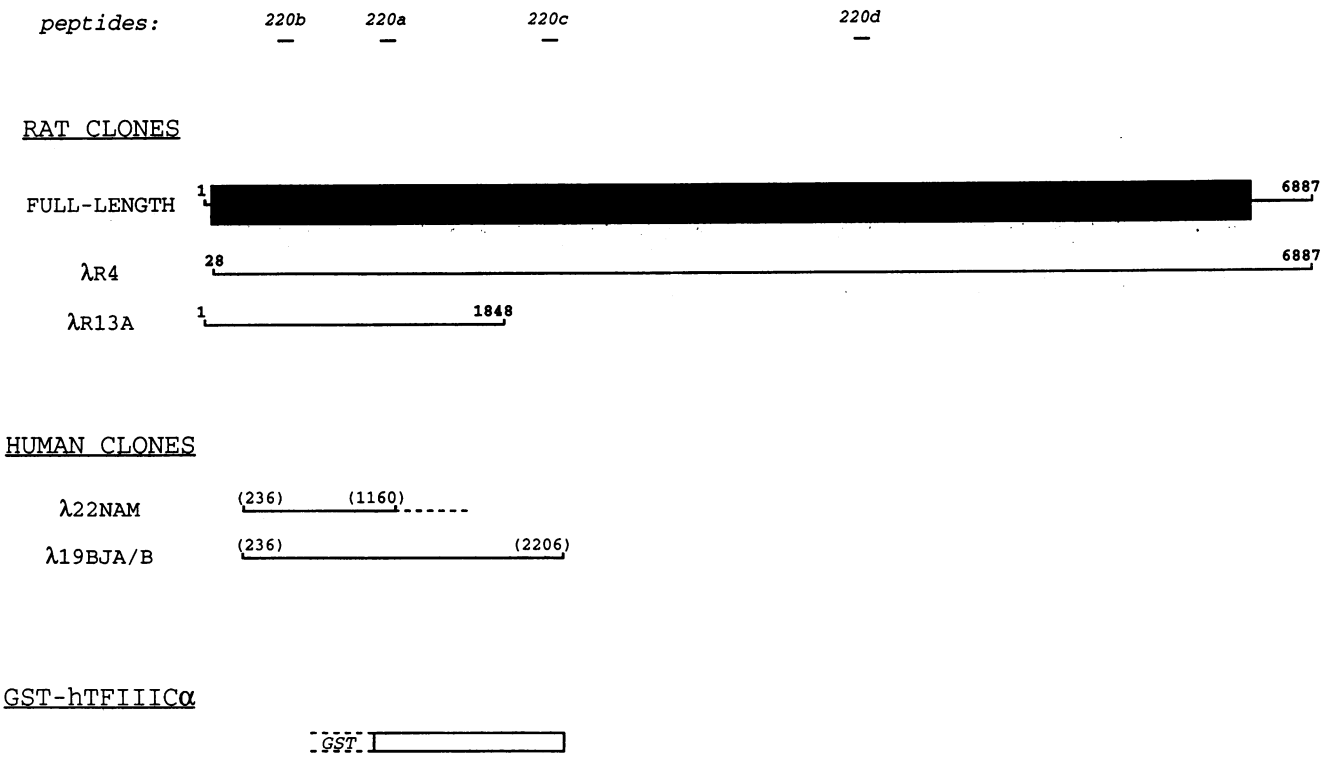

FIG. 1. Schematic diagram of the cDNA clones. The rat full-length clone was generated in vitro by combining clones λR4 and λR13A. The shaded box represents the coding region. The first and last nucleotides of the cDNA clones are numbered according to the numbering of the full-length sequence (for the human clones, the nucleotide positions shown in parentheses were assigned on the basis of sequence similarity). The dashed line at the 3′ end of clone λ19BJA/B indicates an intronic sequence. The positions of the peptide sequences and of the region of hTFIIICα used to express a glutathione-*S*-transferase (GST) fusion protein are also indicated.

used (at a concentration of 20 μg/ml) were pVA_I (8), ptRNA (p*Xl*tmet$_1$ [15]), p5S (pH5S8544 [37]), p7SK (O^+ P^+ [39]), and pU6 (mU6 −315/+286 [11]).

Immunoprecipitation of TFIIIC2. Anti-TFIIICα rabbit antibodies were bound to protein A-Sepharose resin (Sigma), washed in 0.2 M sodium borate (pH 9.0), and cross-linked in the presence of 20 mM dimethyl pimelimidate. After 30 min, the residual active groups were blocked by incubating the resin in 0.2 M ethanolamine. The non-cross-linked immunoglobulins were removed by a 0.2 M glycine (pH 2.5) wash. Then, 250 μl of a TFIIIC fraction (P11, 0.6 M KCl eluate) was incubated with 20 μl of antibody-coupled resin in buffer BC300 plus 0.1% (vol/vol) Nonidet P-40 for 3 h at 4°C. The precipitated beads were then washed five times in 50 volumes of buffer BC300–0.1% Nonidet P-40, and the bound proteins were denatured in SDS and separated by SDS-PAGE. Silver staining was performed with Rapid Ag Stain (ICN).

Nucleotide sequence accession number. The GenBank accession number of the rTFIIICα sequence is L28801.

RESULTS

Cloning and sequence analysis of TFIIICα. Transfer of affinity-purified TFIIIC2 to a membrane and subsequent microsequencing analysis yielded four peptide sequences. After screening a human (Namalwa cell) random-primed λZAP II library with a degenerate 53-mer designed from peptide 220a, a cDNA (λ22NAM) containing 1 kb of coding sequence was isolated. λ22NAM was subsequently used as a probe for screening several other human libraries. The longest cDNA obtained, λ19BJA/B, contained a 1,970-bp open reading frame encoding three of the proteolytic peptides generated from purified TFIIICα (Fig. 1). Further screenings of human cDNA libraries did not yield larger clones.

Because of the availability of a rat cDNA library with very long inserts (47) and of the high degree of structural and functional conservation of many of the eukaryotic transcription factors already cloned, we undertook to isolate the rat homolog of TFIIICα. A λgt10 library derived from rat BRL cells was screened with the insert of λ19BJA/B. We determined the complete DNA sequence of the longest positive clone, λR4 (6,860 nucleotides); an additional stretch of 27 bases at the 5′ end was subsequently found in a shorter cDNA insert (λR13A) (Fig. 1). The composite sequence reveals an open reading frame of 2,148 residues (Fig. 2), with a calculated molecular mass of 242,631 Da and a predicted isoelectric point of 6.9. Four peptide sequences (220a, 220b, 220c, and 220d) derived from hTFIIICα, three of which had already been identified in the human clone λ19BJA/B, were present in an identical or highly conserved form in the deduced amino acid sequence, confirming the identity of the gene as rTFIIICα (Fig. 2).

Alignment of the partial primary structure of hTFIIICα (hTFIIICα-PT) with the rat sequence revealed a high degree of similarity (89%) (Fig. 3 and 4). The aligned regions could be subdivided into two highly conserved domains present at the N and C termini (92 and 93% identity, respectively) and separated by a less conserved stretch of 80 a.a. (56% identity). Interestingly, the predicted secondary structure of the 80-a.a. stretch indicated the presence of turns in both the human and the rat proteins (Fig. 4), suggesting a potential role as a

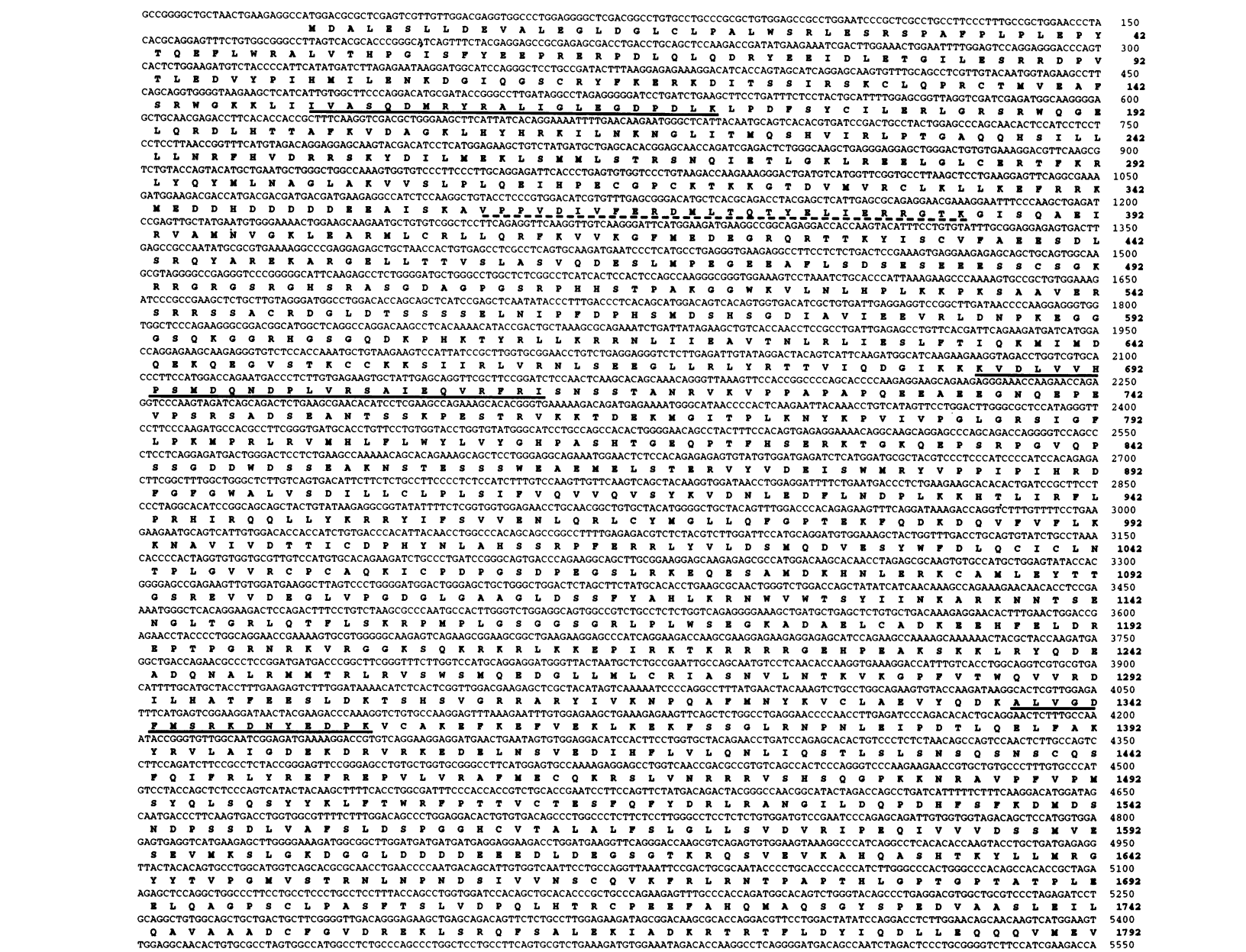

FIG. 2. Nucleotide and predicted amino acid sequence of rTFIIICα. The numbers at the right indicate the nucleotide (lightface) and the amino acid (boldface) positions. The underlined regions correspond to the sequences of peptides 220b, 220a, 220c, and 220d (from top to bottom). Peptide 220a (dashed underline) was used to synthesize an oligonucleotide "guessmer" for screening the cDNA libraries.

relatively unstructured loop connecting two conserved domains of TFIIICα.

During the review of the manuscript for this article, the sequence of a full-length clone encoding hTFIIICα (hTFIIICα-FL) was deposited in the gene data base (33). The sequence of hTFIIICα-FL matches closely that of rTFIIICα, and the sequence of our hTFIIICα-PT is nearly identical to that of the corresponding region of hTFIIICα-FL (Fig. 3).

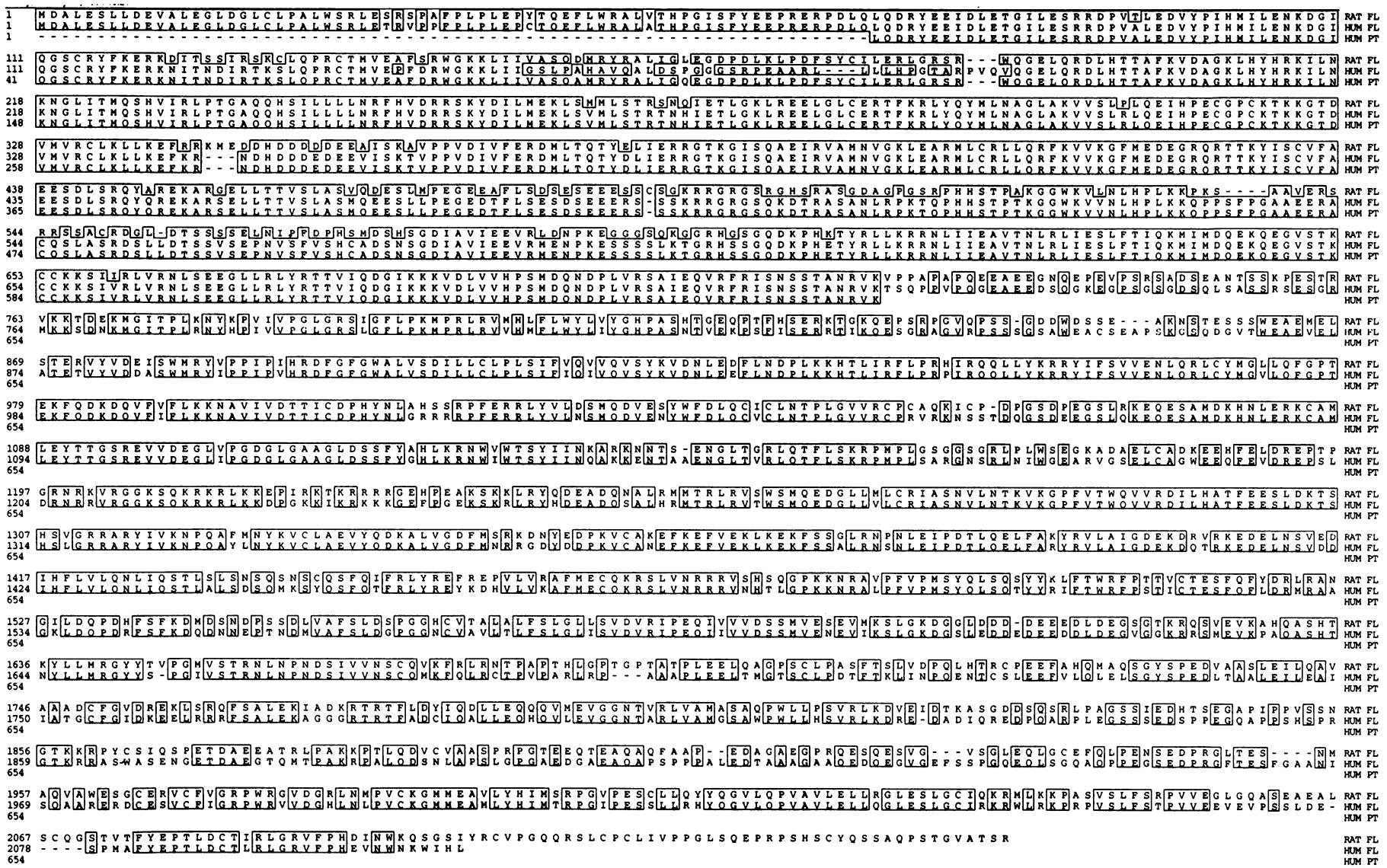

FIG. 3. Sequence alignment of full-length (FL) rat, full-length human (33), and partial (PT) human (this study) TFIIICα. Identical regions are boxed. The multiple alignment program used was MegAlign (DNA*).

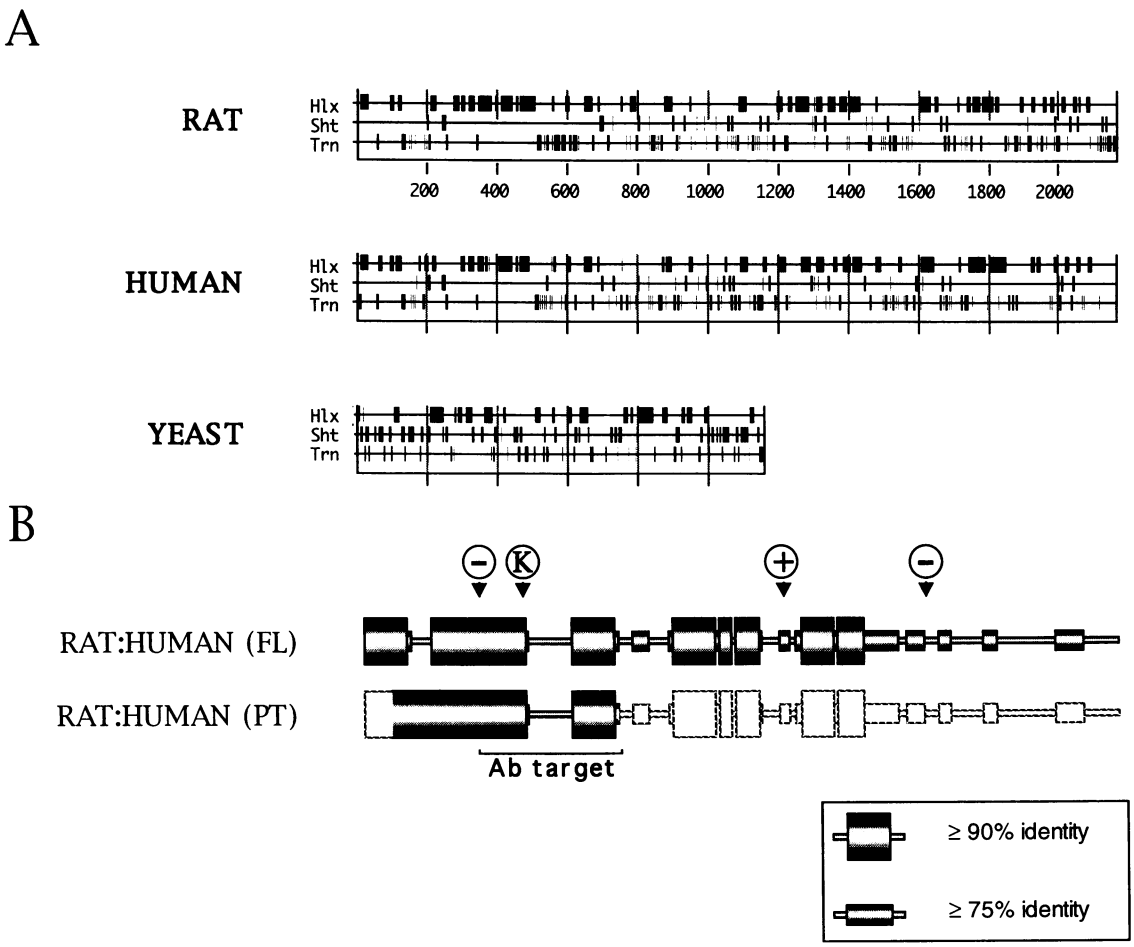

FIG. 4. Structural conservation of TFIIICα from different species. (A) Schematic representation of secondary-structure predictions for the polypeptides encoded by the rat and the full-length human (33) TFIIICα clones and the yeast homolog *TFC3* gene (32). The predictions were obtained with the MacVector program (IBI); the graphic displays only the regions where the results obtained by both the Chou-Fasman and Robson-Garnier methods agree. (B) Scheme of the conserved regions between rat and human full-length (FL) (33) or partial (PT) (this study) TFIIICα. The region of the human protein used to raise antibodies in rabbits is indicated (Ab target). Circled symbols show the positions of the charged stretches detailed in Fig. 5: −, acidic; +, basic; K, casein kinase II site.

However, these latter sequences differ in a 38-a.a. region (between positions 152 and 189 of hTFIIICα-FL) and in the codon for the amino acid at position 161 (Fig. 3). This region of hTFIIICα-PT is highly conserved in rTFIIICα (Fig. 3 and 4) and corresponds to the sequence of proteolytic peptide 220b (Fig. 2). It is likely, therefore, that the full-length hTFIIICα sequence corresponds to an alternatively spliced form of the same gene.

rTFIIICα and hTFIIICα-FL show an overall high degree of conservation (75.2% similarity and 73.6% identity) (Fig. 3). The aligned hTFIIICα-FL and rTFIIICα sequences can be subdivided into a highly conserved N-terminal domain, spanning approximately two-thirds of the proteins, and a less conserved C-terminal domain (Fig. 3 and 4B). The predicted secondary structure of rat and human TFIIICα is also highly conserved (Fig. 4A), with stretches rich in turns often corresponding to the less conserved regions (Fig. 4, compare panels A and B). Regions with unusual charge distributions are also conserved (Fig. 4 and 5). Two acidic stretches (10 E or D residues among 11 a.a.) are present in rTFIIICα, starting from amino acid positions 344 and 1606. Another negatively charged region (10 E or D residues among 21 a.a., beginning at position 466) contains a consensus sequence for the phosphorylation site of casein kinase II. An extended basic region occupies a central position in the protein (18 R or K residues among 29 a.a., beginning at position 1198). Considering the frequency of unusually long charged regions in RNA polymerase II transcription factors (27), these findings might indicate a function in transcription activation and/or DNA binding. Finally, the presence of seven sequences corresponding to the consensus nuclear localization signal K(RK)X(RK) (7) is also noteworthy.

Protein data base searches did not score extensive similarity of TFIIICα to other known genes. Surprisingly, *TFC3*, the gene encoding the B-box-binding subunit of yeast TFIIIC, has only 10% similarity with rTFIIICα (see Discussion). Furthermore, the divergence between these two proteins appears to extend to their predicted secondary structures (Fig. 4A, compare rat and yeast sequences).

Expression of recombinant TFIIICα and verification of the clone. To begin an analysis of the functional properties of TFIIIC, a 383-a.a. fragment of hTFIIICα fused to the glutathione-*S*-transferase protein was expressed in bacteria (Fig. 1 and 4). Antibodies raised against this recombinant polypeptide were then used in the immunoassay shown in Fig. 6. As expected from the sequence homology, anti-hTFIIICα anti-

acidic stretches:

344- **E D D** H **D D D D D E E**

1606- **D D D D E E E D** L **D E**

casein kinase II site:

466- **D E** S L M P **E** G **E E** A F L S **D** S **E** S **E E E**

basic region:

1198- **R** N **R K** V **R** G G **K** S Q **K R K R** L **K K E** P I **R K** T **K R R R R**

FIG. 5. Summary of the charged regions and sites of rTFIIICα. The number at left indicates the position of the first amino acid in the sequence. Negatively charged amino acids are shown in boldface, positively charged amino acids are shown in outline letters, and putative phosphorylation sites are underlined.

bodies reacted efficiently with a protein of approximately 220 kDa in both human and rat nuclear extracts (Fig. 6, lanes 1 and 2). A polypeptide with the same electrophoretic mobility could be detected by immunoblot in several chromatographic fractions containing the TFIIIC activity but not in those containing the partially purified TFIIIB or RNA polymerase III (data not shown).

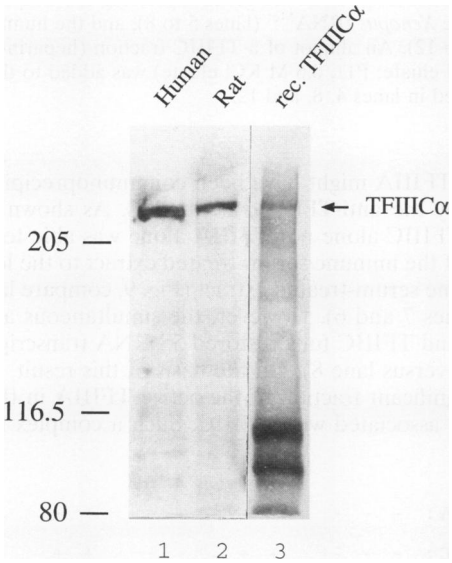

FIG. 6. Immunoblot of human and rat TFIIICα and in vitro transcription-translation of the rat cDNA clone. Lanes 1 and 2, human (HeLa cell; lane 1) and rat (liver; lane 2) nuclear extracts were analyzed by SDS-PAGE and immunoblotting with anti-hTFIIICα antibodies. The bands were detected by chemiluminescence and exposure to an autoradiographic film. Lane 3, in vitro transcription-translation of the recombinant (rec.) rTFIIICα cDNA. The full-length cDNA was transcribed and translated in vitro in the presence of [^{35}S]methionine. The translation products were analyzed by SDS-PAGE, transferred to a nitrocellulose membrane, and autoradiographed directly. Lanes 1 to 3 were all run in the same polyacrylamide gel and transferred at the same time to a nitrocellulose membrane. Lane 3 was directly exposed to a film, while lanes 1 and 2 were first subjected to a standard immunoblot procedure and then exposed. Molecular size markers are indicated on the left (in kilodaltons). The largest translation product (arrow) comigrates with rTFIIICα.

Since the predicted molecular mass of rTFIIICα is slightly higher then the apparent size of the immunoreactive protein in the rat nuclear extract, we investigated the possibility of posttranslational modifications by comparing the migration of natural and recombinant rTFIIICα polypeptides in the same SDS gel. The cDNA encoding rTFIIICα was transcribed and translated in vitro, and the autoradiograph of the labeled products is shown in Fig. 6 (lane 3). Since the mobility of the largest labeled polypeptide is indistinguishable from that of the endogenous protein detected by immunoblot, we conclude that our cDNA encodes the full-length rTFIIICα and that the protein is not extensively modified in vivo.

The DNA-binding domain of TFIIICα may lie near the N-terminal end. It is known that TFIIIC2 binds to the B-box located in the internal control region of cellular tRNA and adenovirus VA genes. Two forms of the TFIIIC2-DNA complex, upper band (U) and lower band (L), can be distinguished by their different electrophoretic mobilities in a gel shift assay (23). Since photo-cross-linking experiments indicated that TFIIICα is the only subunit of TFIIIC2 which makes close contacts with bases located in the promoter of the VA_I RNA gene (29, 56), we investigated the effect of the anti-hTFIIICα antibodies on the formation of hTFIIIC2-VA_I promoter complexes monitored by a gel shift assay (Fig. 7). A high concentration of immune serum (0.4 mg/ml) completely abolished the binding of both the U and L forms of TFIIIC2 to DNA (Fig. 7, compare lanes 7 and 2). A lower concentration (0.04 mg/ml) also produced substantial inhibition of binding but allowed detection of a residual supershifted complex (Fig. 7, lane 6, between the dots). We interpret this result as an indication that the anti-hTFIIICα antiserum can recognize TFIIICα in solution and in the context of the other TFIIIC subunits. Furthermore, the N-terminal domain of TFIIICα might be involved in the interaction with DNA, since the dominant effect of the polyclonal anti-N-terminal antibody is to inhibit DNA binding rather than to supershift the TFIIIC2-DNA complex. The results of immunoprecipitation experiments (see Fig. 11) seem to exclude the alternative possibility that the immune serum disrupts the quaternary structure of TFIIIC2 and thus indirectly inhibits DNA recognition.

Gene specificity of TFIIIC. Electrophoretic mobility shift experiments (discussed above) demonstrate that anti-hTFIIICα antibodies interact with native TFIIIC. This observation provided us with the means to rigorously assess the role of TFIIIC, in particular the p220 subunit, in transcription of

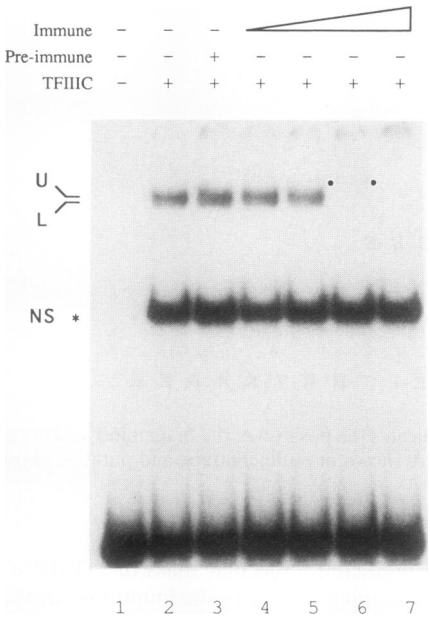

FIG. 7. TFIIICα participates in the binding of TFIIIC2 to the VA_I promoter. A TFIIIC fraction (P11, 0.6 M KCl eluate) was allowed to bind to a 129-bp DNA fragment containing the internal control region of the VA_I gene. At the end of the preincubation period, the TFIIIC2-VA_I complex was challenged with preimmune serum (lane 3, 0.4 mg/ml) or with increasing amounts of anti-TFIIICα antibodies (immune; lane 4, 0.4 μg/ml; lane 5, 4 μg/ml; lane 6, 40 μg/ml; lane 7, 0.4 mg/ml). NS, nonspecific band. U, upper-band form of TFIIIC2; L, lower-band form of TFIIIC2. The two dots flank the weak supershifted complex in lane 6.

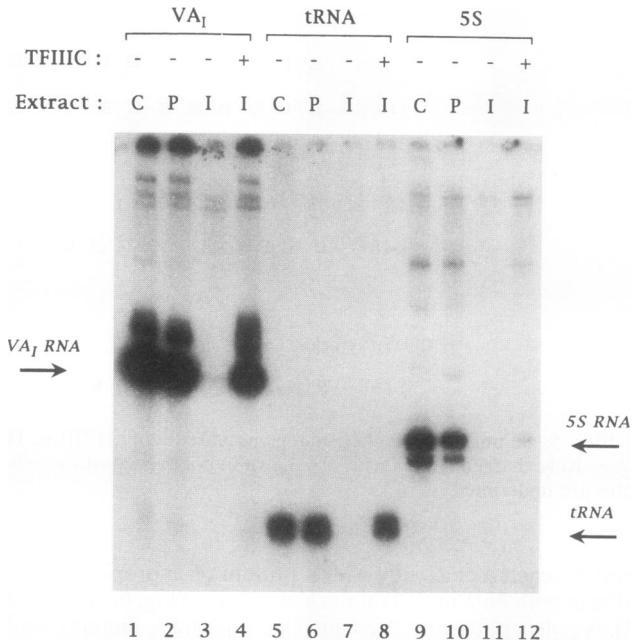

FIG. 8. A TFIIICα-depleted extract is unable to support RNA polymerase III transcription of genes of subclasses 1 and 2. The anti-hTFIIICα antiserum was purified on protein A-agarose beads. HeLa nuclear extract (control extract, lanes C) was incubated with the protein A-agarose pre-incubated with preimmune serum (lanes P) or the antibody-coupled matrix (lanes I). The supernatants were assayed by in vitro transcription of the genes expressing the VA_I RNA (lanes 1 to 4), the *Xenopus* $tRNA^{Met}$ (lanes 5 to 8), and the human 5S RNA (lanes 9 to 12). An aliquot of a TFIIIC fraction (heparin-Sepharose, 0.6 M KCl eluate; P11, 0.6 M KCl eluate) was added to the depleted extract used in lanes 4, 8, and 12.

different subclasses of class III genes. Although original template competition experiments (30) established that TFIIIC was required for the formation of stable preinitiation complexes on 5S RNA, tRNA, and VA RNA genes, the possibility of functionally different forms of TFIIIC with overlapping subsets of subunits could not be excluded. This hypothesis is particularly significant in light of the discrepancy among reports describing the purification of TFIIIC (10, 29, 41, 42, 56) (see Discussion). We therefore sought to determine the requirement for TFIIICα and associated subunits in the transcription of VA_I, tRNA, and 5S RNA genes by using TFIIICα-depleted HeLa nuclear extracts. As shown in Fig. 8, transcription of all the templates tested was dramatically reduced after immunodepletion of TFIIICα (compare lanes 3 and 1, lanes 7 and 5, and lanes 11 and 9) but was unaffected by treatment with preimmune serum (lanes 2, 6, and 10). This decrease was consistent with the ~100-fold reduction in the amount of hTFIIICα detected by immunoblot (data not shown). Furthermore, addition of a TFIIIC fraction (heparin-Sepharose, 0.6 M KCl eluate; P11, 0.6 M KCl eluate) to the depleted extract fully restored the transcription activity of the VA_I and the tRNA templates (compare lanes 4 and 2 and lanes 8 and 6). This restoration was specific for TFIIIC, since partially purified TFIIIB and RNA polymerase III fractions were ineffective (data not shown). Thus, we conclude that TFIIICα is necessary for the activity of genes encoding VA_I, tRNA, and 5S RNAs.

Surprisingly, transcription of the 5S RNA gene in immunodepleted extracts was only marginally rescued by the addition of TFIIIC (Fig. 8, lane 12 versus lane 10). Since TFIIIA is the only factor known to be required specifically for 5S RNA transcription but not for tRNA or VA_I transcription, we tested whether TFIIIA might have been coimmunoprecipitated with TFIIIC by the anti-TFIIICα antibodies. As shown in Fig. 9, neither TFIIIC alone nor TFIIIA alone was able to bring the activity of the immune serum-treated extract to the level of the preimmune serum-treated extract (Fig. 9, compare lanes 5 and 4 and lanes 7 and 6). However, the simultaneous addition of TFIIIA and TFIIIC fully restored 5S RNA transcription (Fig. 9, lane 9 versus lane 8). On the basis of this result, it appears that a significant fraction of the active TFIIIA in the nuclear extract is associated with TFIIIC. Such a complex resists salt

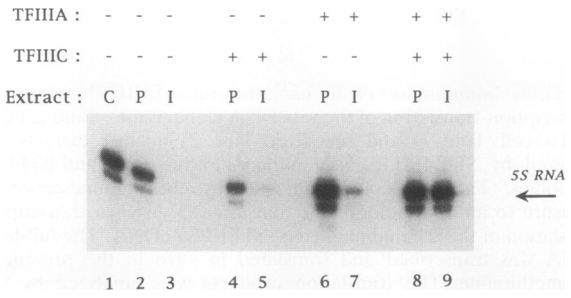

FIG. 9. TFIIIC and TFIIIA are codepleted from the nuclear extract. Shown in in vitro transcription of the 5S RNA template. Lanes C, control untreated extract; lanes P, preimmune serum-depleted extract; lanes I, TFIIICα-depleted extract. Where indicated, a TFIIIC fraction (heparin-Sepharose, 0.6 M KCl eluate; P11, 0.6 M KCl eluate) and a TFIIIA fraction (oligonucleotide affinity purified [37]) were added.

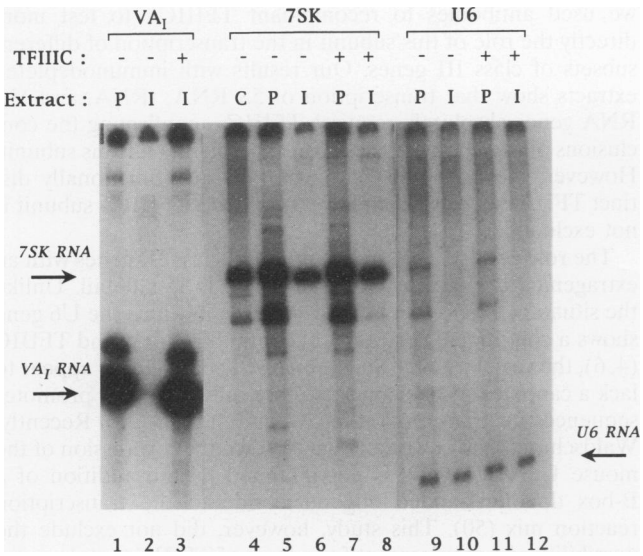

FIG. 10. A TFIIICα-depleted extract can support RNA polymerase III in vitro transcription of subclass 3 genes. The arrows indicate the transcripts of the correct length. Lanes 1 to 3, VA$_I$ RNA; lanes 4 to 8, human 7SK RNA; lanes 9 to 12, mouse U6 small nuclear RNA. For the symbols, see the legend to Fig. 9.

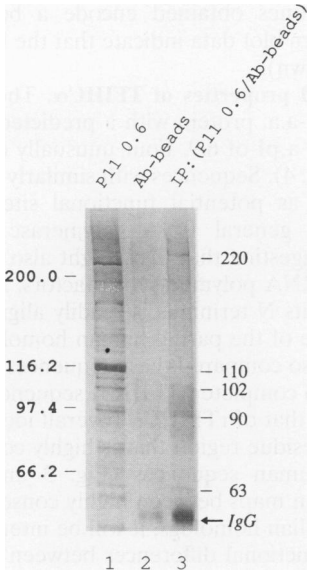

FIG. 11. TFIIIC2 is composed of five subunits. The anti-hTFIIICα antiserum, cross-linked to a protein A-agarose matrix, was incubated with a TFIIIC fraction (P11, 0.6 M KCl eluate). The matrix was then extensively washed, resuspended in SDS gel loading buffer, and analyzed by SDS-PAGE. The bands were visualized by silver staining. Lane 1, untreated P11 fraction. Lane 2, antibody-coupled matrix. Lane 3, immunoprecipitated proteins. The positions of protein size markers are shown on the left (in kilodaltons). The apparent sizes of the immunoprecipitated polypeptides are shown on the right. IgG, immunoglobulin G heavy chain.

concentrations of up to at least 0.5 M KCl, suggesting a relatively stable interaction. Furthermore, TFIIIC and TFIIIA remain associated after chromatographic removal of nucleic acids from the nuclear extract (on a DE52 matrix at 0.5 M KCl) (data not shown), making the hypothesis of a DNA-mediated interaction unlikely.

Vertebrate U6 and human 7SK RNA genes lack a canonical B-box and require only upstream promoter sequences for transcription in vivo and in vitro (reviewed in reference 38). Furthermore, U6 RNA synthesis cannot be inhibited by an excess of a double-stranded synthetic B-box oligonucleotide (50). It is not clear, however, whether TFIIIC could nonetheless be involved in U6 and 7SK RNA transcription by a mechanism that does not require its binding to a B-box element. To address this point, we tested the transcription of mouse U6 and human 7SK genes in the TFIIICα-depleted nuclear extract (Fig. 10). Unlike VA$_I$ RNA expression (Fig. 10, lanes 1 to 3), neither 7SK nor U6 RNA synthesis was inhibited in the absence of TFIIICα (compare lanes 6 and 5 and lanes 10 and 9). Similarly, addition of purified TFIIIC to the immune serum-treated and preimmune serum-treated extracts did not alter the transcription activity (Fig. 10, lanes 7, 8, 11, and 12). Thus, we conclude that TFIIICα and its associated polypeptides are not required for the in vitro expression of mouse U6 and human 7SK RNAs.

TFIIIC2 is composed of five subunits. We (29) and others (56) have described the copurification of five polypeptides of 220, 110, 102, 90, and 63 kDa in fractions associated with the TFIIIC2 activity. To determine whether these proteins are all associated in a stable complex in solution, we cross-linked the anti-hTFIIICα antibodies to protein A-agarose beads and incubated the resin with a partially purified TFIIIC fraction (P11, 0.6 M KCl eluate). The bound proteins were then washed, separated on an SDS gel, and silver stained. As shown in Fig. 11, at least four polypeptides coprecipitated with TFIIICα (p220): their estimated molecular weights correspond exactly to the values expected for the proteins previously shown to copurify with the TFIIIC2 activity. Interestingly, the TFIIIC-antibody complex was not disrupted by high ionic strength (up to 1 M NaCl) or heat (10 min at 45°C) (unpublished observation). Therefore, we conclude that p220, p110, p102, p90, and p63 are all TFIIIC2 subunits which form a very stable complex in the absence of the target DNA.

DISCUSSION

Earlier studies of RNA polymerase III and its accessory factors provided the first insights into the mechanism and regulation of transcription initiation in vertebrates. Unfortunately, progress in this field has been hindered by the complexity of the factors involved and by difficulties in their biochemical characterization. TFIIIC, which plays a primary role in promoter recognition and formation of stable preinitiation complexes, has exemplified this problem (for a review, see reference 20). For a more detailed understanding of the central role of vertebrate TFIIIC, we document the cloning of a cDNA encoding the largest subunit of the derived TFIIIC2 component (see the introduction). This cDNA has been used to study the primary structure, DNA-binding domain, promoter specificity, and evolutionary conservation of TFIIIC as well as the mechanism of action of an interacting gene-specific activator (TFIIIA).

Isolation of cDNA clones encoding the largest subunit of TFIIIC2. TFIIICα (or p220) copurifies with the TFIIIC2 activity and is the only polypeptide that can be specifically photo-cross-linked to the VA$_I$ DNA (29, 56). We isolated partial human and apparently complete rat cDNAs encoding TFIIICα by using the amino acid sequence information obtained from the purified human protein. Polyclonal antibodies raised against the N terminus of recombinant hTFIIICα inhibit DNA binding and transcription activation by TFIIIC, confirm-

ing that the clones obtained encode a bona fide TFIIIC subunit. Southern blot data indicate that the hTFIIICα gene is unique (not shown).

Structure and properties of TFIIICα. The rTFIIICα gene encodes a 2,148-a.a. protein with a predicted molecular mass of 242 kDa and a pI of 6.9. Four unusually charged stretches are present (Fig. 4). Sequences with similarly high charge have been indicated as potential functional sites both in gene-specific and in general RNA polymerase II transcription factors (27), suggesting that they might also be important for the activity of RNA polymerase III factors. A 654-a.a. region located toward its N terminus is readily aligned to the highly similar sequence of the partial human homolog (89% similarity). We have also compared these sequences with the recently determined (33) complete hTFIIICα sequence. This sequence closely matches that of rTFIIICα (overall identity, 73.6%) but differs in a 38-residue region that is highly conserved between our rat and human sequences (Fig. 3 and 4). Since the 38-residue region maps between highly conserved stretches of the two mammalian homologs, it will be interesting to analyze the potential functional differences between the recombinant proteins.

A surprising feature of the TFIIICα sequence, despite the cross-linking data indicating direct DNA contacts, is the apparent lack of a known DNA-binding domain. A search of several data bases failed to identify significant homologies to known DNA-binding proteins. Therefore, it is possible that TFIIICα contains either a novel or a divergent form of a known DNA-binding domain. Alternatively, the lack of a consensus DNA-binding domain may reflect a significant contribution of another subunit to the DNA-binding ability of TFIIIC2—for example, by generalized DNA interactions in the B-box region which stabilize the specific TFIIICα-promoter contacts that are detected by UV cross-linking. A relevant example illustrating this possibility is the stabilization of site-specific TBP binding to the TATA box by TFIIA (5). The observation that in vitro-translated rTFIIICα is unable to bind DNA (not shown) is consistent with this notion. In any case, the inhibition of TFIIIC2 binding to DNA by antibodies raised against the N terminus of TFIIICα points to a possible role of this region in DNA recognition, although steric effects of the antibodies on other subunits cannot be completely ruled out. Definitive proof of the putative role of the N-terminal region in DNA binding must await mutagenesis and more detailed immunological studies.

Specificity of TFIIIC function for different class III genes. Classic template commitment experiments (30) have indicated that the 5S RNA, tRNA, and VA RNA genes require the common factor TFIIIC for the formation of stable preinitiation complexes. Nevertheless, the reagents available at the time and the nature of the assay itself did not allow a clear assessment of the possibility that functionally different forms of TFIIIC might be involved in the transcription of each set of genes. Results identical to those described therein might have been obtained if there existed specific isoforms of TFIIIC with shared but not identical sets of subunits in either of the derived components (TFIIIC1 or TFIIIC2 [see the introduction]). In one model, a given isoform might stably bind to each of the 5S, tRNA, and VA RNA gene promoters but activate transcription of only a specific subset. In a second model, subunits with distinct binding specificities might simply sequester a common limiting subunit, such as one required for interaction with TFIIIB. In the context of TFIIIC heterogeneity, it is interesting to note the discrepancy among several reports describing the purification of this factor (10, 29, 41, 42, 56), consistent with the possibility of different TFIIIC forms. In order to study this, we used antibodies to recombinant TFIIICα to test more directly the role of this subunit in the transcription of different subsets of class III genes. Our results with immunodepleted extracts show that transcription of 5S RNA, tRNA, and VA RNA genes absolutely requires TFIIICα, confirming the conclusions of the template exclusion experiments for this subunit. However, the possibility of structurally and functionally distinct TFIIIC species which have a common TFIIICα subunit is not excluded.

The role of TFIIIC in transcription of class III genes with an extragenic promoter has been analyzed in less detail. Unlike the situation in *Saccharomyces cerevisiae*, in which the U6 gene shows a conditional requirement for both a B-box and TFIIIC (4, 6), the vertebrate U6 and human 7SK RNA genes appear to lack a canonical B-box and to require only upstream promoter sequences for transcription in vivo and in vitro (38). Recently, Waldschmidt and coworkers have shown that expression of the mouse U6 RNA gene is not inhibited by the addition of a B-box double-stranded oligonucleotide to the transcription reaction mix (50). This study, however, did not exclude the possibility of a requirement for a form of TFIIIC that does not bind to the B-box. A relevant parallel can be drawn with RNA polymerase II-transcribed promoters that lack TATA elements but nonetheless require the TATA-binding factor TFIID (40) and fail to show inhibition by TATA oligonucleotides (36). However, when tested for the TFIIIC requirement, transcription of U6 and 7SK RNA genes by RNA polymerase III did not depend on the presence of TFIIICα in the extract. Thus, TFIIIC (or at least the TFIIIC2 component) appears to be a factor that is specific for class III genes that contain internal transcribed promoters and is not required for genes displaying an extragenic promoter.

TFIIIC interacts with TFIIIA in nuclear extracts. In immunodepleted extracts lacking TFIIIC activity, transcription of tRNA and VA RNA genes was restored by addition of exogenous TFIIIC, whereas restoration of 5S RNA transcription required TFIIIA addition as well. These results indicate the existence of a stable DNA-independent TFIIIC-TFIIIA complex whose formation has important implications for the mechanism of action of the gene-specific activator TFIIIA. TFIIIA interacts with the 5S promoter in the absence of other factors (15), but the resulting complex is unstable, as revealed by a template challenge assay. TFIIIC does not independently bind the 5S promoter because of the lack of a canonical B-box but, along with TFIIIA, forms a highly stable promoter complex that is resistant to challenge by other 5S or by tRNA or VA RNA promoters (30). It is likely that the recognition site of TFIIIC consists of a combination of DNA contacts and protein contacts (with TFIIIA). It also has been assumed that TFIIIC is recruited to the promoter by recognition (and stabilization) of the TFIIIA-promoter complex. However, the prior formation of a TFIIIC-TFIIIA complex could allow the direct formation of a stable TFIIIC-TFIIIA-promoter complex via a concerted reaction, bypassing the metastable stage resulting from the formation of a simple TFIIIA-DNA complex (30). Whether the TFIIIA interaction with TFIIIC occurs via TFIIIC1 or TFIIIC2 remains to be determined.

Interactions between gene-specific transcription factors (like TFIIIA) and general factors (like TFIIIC for intragenic class III genes) in the absence of DNA are not restricted to RNA polymerase III factors. For example, several investigators have observed interactions between TBP, the DNA-binding subunit of the general factor TFIID, and a variety of activators, such as herpes simplex virus VP16 (26, 46), Epstein-Barr virus Zta (34), adenovirus 13S E1A (25, 31), and the cellular proteins p53 (49) and c-Rel (28). Therefore, specific protein-protein

interactions may provide a general mechanism by which one transcription factor facilitates the recruitment of another factor to the promoter, with concomitant stabilization of the resulting complex by protein-protein (and protein-DNA) interactions. The cloning of human TFIIIA (37) and the availability of antibodies directed against it will soon allow us to analyze such interactions (with TFIIIC) on the 5S promoter in greater detail.

TFIIIC2 is a multisubunit complex. Antibodies raised against human TFIIICα can immunoprecipitate the five polypeptides previously shown to copurify with the TFIIIC2 activity (29, 56). Thus, p220 appears to be a component of a stable multisubunit complex containing at least four other proteins. However, the limits of resolution of the assay might not allow detection of substoichiometric components, such as TFIIIA or a less abundant subset of TFIIIC subunits (such as those in TFIIIC1). Clearly, the further characterization of vertebrate TFIIIC now awaits the cloning of the remaining subunits from both TFIIIC2 and TFIIIC1. Ultimately, this will allow the coexpression of their cDNAs in order to reconstitute the activity of this complex transcription factor from its individual components.

Structural and functional relationships of class III factors in different species. A more detailed characterization of TFIIIC structure has been carried out for yeast TFIIIC. At least four polypeptides cofractionate with the TFIIIC (or τ) activity (138, 131, 95, and 60 kDa) (17). Cross-linking studies identified τ_{138} and τ_{95} as the DNA-binding subunits (17) and mapped their recognition sites over the B-box and the A-box, respectively (2). *TFC3*, the gene encoding τ_{138}, has been cloned recently (32). Despite several similarities between yeast and human TFIIIC (conservation of the B-box consensus sequence, multisubunit structure, and interaction of the largest subunit with the B-box), the primary structures of the B-box-binding subunits τ_{138} and mammalian TFIIICα have a similarity of only 10%, well below the score attributable to chance. Furthermore, the identical residues are spread throughout the amino acid sequence and are separated by long gaps. Together with the striking difference in size (τ_{138} is approximately half the size of rTFIIICα), these observations seem to point either to a functional convergence of distinct genes in humans and *S. cerevisiae* or to an extremely low conservation of related genes. It is also noteworthy that the overall similarity between human and rat TFIIICα (75.2%) is below the average for homologous nuclear genes from these two organisms.

Although surprising, this finding is consistent with previous data suggesting a certain degree of species specificity in RNA polymerase III transcription. For instance, the human initiator methionine tRNA gene and the *Xenopus laevis* 5S RNA gene cannot be transcribed in vitro in an *S. cerevisiae* extract, whereas several yeast tRNA and 5S RNA genes are accurately transcribed. Template commitment experiments also indicate that the human $tRNA_i^{Met}$ is unable to form stable transcription complexes with yeast transcription factors. Accordingly, the human $tRNA_i^{Met}$ gene is also not expressed in vivo in yeast cells (14). In contrast, human cell extracts are less selective in terms of species specificity and can accurately transcribe and process yeast tRNA genes (14, 45).

Intriguingly, the sequences of yeast and vertebrate TFIIIA have also been shown to be poorly conserved (1). Although they both contain nine zinc fingers, *S. cerevisiae* and *X. laevis* TFIIIA are only ~20% identical; if the seven consensus amino acids of the zinc finger motif are excluded, the identity decreases to ~8%. Perhaps not surprisingly, *Xenopus* TFIIIA binds 100- to about 1,000-fold less efficiently to the yeast 5S RNA gene than does yeast TFIIIA (51).

Taken together, these findings suggest that at least some of the factors involved in RNA polymerase III transcription have undergone a significant degree of evolutionary divergence, perhaps to the point that the products of unrelated genes have assumed analogous functions during the evolution of TFIIIC from fungi to vertebrates.

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