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Biochemistry

TFC3: Gene encoding the B-block binding subunit of the yeast transcription factor IIIC

(RNA polymerase III/tRNA/intron/epitope tagging)

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ABSTRACT **Yeast transcription factor IIIC (TFIIIC) is a multisubunit protein complex that interacts with two control elements of class III promoters called the A and B blocks. Here we describe the gene encoding the 138-kDa subunit (τ_{138}), which is involved in B-block binding. From the DNA sequence, the open reading frame, interrupted by an intron with an unusual 3′ splice acceptor site, is in agreement with all the microsequencing data for peptides within τ_{138}. *TFC3* is a single-copy gene located on chromosome I; it is essential for cell viability as shown by a gene disruption experiment. Epitope-tagging of the *TFC3* gene product and DNA binding experiments are consistent with the presence of one copy of τ_{138} in TFIIIC–DNA complexes.**

Transcription of the DNA encoding tRNA (tDNA) and 5S rRNA (5S rDNA) requires the participation of multiple factors (reviewed in refs. 1 and 2): the two general class III transcription factors, TFIIIB and TFIIIC, the 5S rRNA gene-specific factor TFIIIA (3), and the general factor TBP, the TATA box-binding protein component of TFIID (4–6). TFIIIC interacts with multiple DNA and protein target sites. This complex protein recognizes the two intragenic promoter elements of tRNA genes, called the A and B blocks, as the first step of gene activation. Then it recruits an upstream factor TFIIIB, a component that by itself does not detectably bind DNA (7). The B′ component of TFIIIB is capable of assembling onto the TFIIIC–DNA complex (8) and contains a 70-kDa polypeptide that can be crosslinked to DNA close to the start site (9). Therefore, that component (as well as TBP?) is likely to interact with TFIIIC. In 5S rRNA genes, TFIIIC binds to the preformed TFIIIA–5S rDNA complex, suggesting that there are protein–protein contacts between TFIIIA and TFIIIC. TFIIIB–DNA complexes, artificially deprived of TFIIIC or TFIIIC+TFIIIA, can direct multiple rounds of transcription by RNA polymerase III or C (pol III). Therefore, it is not clear whether pol III needs to contact TFIIIC during transcription, although this seems rather likely in view of the size and gene-internal position of the factor.

Yeast TFIIIC, also called τ, is a large multisubunit complex (550–650 kDa) formed of two domains, τ_A and τ_B (10), of about 300 kDa each, which can be visualized by scanning transmission electron microscopy (11). At least four polypeptides were clearly identified in purified TFIIIC–DNA complexes (138, 131, 95, and 60 kDa) (12). The 138- and 95-kDa subunits (τ_{138} and τ_{95}) can be specifically crosslinked to tDNA by UV-irradiation (12). A protease-resistant form of the τ_B domain that binds the B block is recognized by antibodies directed to τ_{138} (12). Elegant photocrosslinking experiments further mapped τ_{138} over the B block, while τ_{95} was found at the level of the A block (13). Altogether, these results strongly suggested that τ_{95} and τ_{138} are responsible for the recognition of the A and B blocks, respectively. On the other hand, the fact that τ_{131} could be crosslinked upstream of the transcription start site makes it the best candidate for assembling TFIIIB (9, 13).

We have undertaken the cloning of TFIIIC subunits to unravel their role in transcription complex assembly. Others have achieved the cloning of genes for τ_{95} (14, 15) and τ_{131} (C. Marck, personal communication); here we describe the gene encoding τ_{138}.§

MATERIALS AND METHODS

Amino Acid Sequence Determination. The polypeptide components of affinity-purified τ preparation (100–150 pmol) (14) were separated by preparative electrophoresis on a SDS/6% polyacrylamide slab gel, fixed for 30 min in 45% methanol/10% acetic acid/45% water (vol/vol), lightly stained with Coomassie blue, and extensively destained with the fixation solution. A band of gel containing the 138-kDa protein to be sequenced was excised, washed with 7 ml of 5% methanol three times for 15 min to remove the SDS, and then equilibrated twice for 15 min with 7 ml of digestion buffer (0.1 M ammonium acetate, pH 8.2). The gel piece was transferred to a 2.2-ml Eppendorf tube and partially dried in a Speed-Vac evaporator for ≈20 min at 60°C. The gel was then manually crushed with a micropestle (Eppendorf) and mixed with 1 ml of digestion buffer containing trypsin (Merck) to give an enzyme/protein ratio of 1/50 (wt/wt). After overnight digestion at 37°C, the tryptic peptides were recovered from the crushed gel by centrifugation on a Spin-X tube (Costar); the gel was washed with 750 μl of 10 mM ammonium acetate (pH 8.2); and the two filtrates were pooled, filtered by centrifugation in a Spin-X tube, and partly concentrated to 200 μl in the Speed-Vac evaporator. The tryptic peptides were purified by reverse-phase HPLC on a chromatograph 130-A (Applied Biosystems) with first an Aquapore RP-300 column (100 × 2.1 mm, Brownlee Lab), which was developed at a flow rate of 200 μl/min with a 9-ml linear gradient from 0 to 60% acetonitrile in 25 mM ammonium acetate (pH 6.5). Fractions with absorption peaks at 214 nm were collected, diluted with H_2O to ≈200 μl, and directly applied onto a microbore Aquapore RP-300 column (250 × 1 mm). Peptides were eluted with a 4.5-ml linear gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid at 100 μl/min. All chromatographic separations were performed at 40°C. Each absorption peak was collected separately and spotted onto Polybrene-

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Abbreviations: TFIID, TFIIIA–C, transcription factors IID, IIIA–C, respectively; tDNA and rDNA, DNA encoding tRNA and rRNA; mAb, monoclonal antibody; HMG, high mobility group.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M98261).

pretreated filter disks. Sequence analysis was performed on a model 477-A protein sequencer (Applied Biosystems).

Amplification of a *TFC3* Gene Fragment. Degenerate oligodeoxynucleotides were designed from the amino acid sequence data of two peptides: a 23-mer oligonucleotide, 5'-GCNCARGTNACNGGNCARMGNCC-3', encoding Ala-Gln-Val-Thr-Gly-Gln-Arg-Pro (the arginine assignment used to design this oligonucleotide proved to be incorrect; see Fig. 1) and a 20-mer oligonucleotide, 5'-GCNACRAARTTRT-CYTCRTT-3', corresponding to Asn-Glu-Asp-Asn-Phe-Val-Ala (N stands for A, C, G, T; R for A or G, Y for T or C; and M for A or C). The two oligonucleotides were used in the polymerase chain reaction (PCR) to amplify a yeast genomic DNA fragment as described (14). A 910-base-pair (bp) genomic DNA fragment was amplified and ligated into the *Eco*RV site of the pBluescript KS plasmid (Stratagene).

Localization of the Intron. Total yeast RNA was extracted from yeast strain SC63 [*Matα, rpc82::HIS3, ura3-52, trp1-Δ1, his3-Δ200, ade2-101, lys2-801, leu2-Δ*; pEMBLYCp32-(URA3)-RPC82] by a miniprep version of RNA isolation with phenol and SDS (16) and was reverse-transcribed by using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) with oligonucleotide 2 (5'-CTCCTGATTTG-GCAACTTCGAGAAGTAG-3'), which is complementary to the *TFC3* transcript (position 427 in Fig. 1; see Fig. 2*B*). The cDNA thus generated was used as template in a PCR with oligonucleotide 2 and oligonucleotide 1 (5'-AGAGAGAA-GAGGACGTTTGG-3'), which corresponds to a *TFC3* sequence localized upstream of the putative 5' splice junction (position −45 in Fig. 1; see Fig. 2*B*) as primers (GeneAmp DNA amplification reagent kit, Perkin–Elmer/Cetus). The resulting 499- and 409-bp DNA fragments corresponding to the unspliced and spliced mRNA were ligated into the *Eco*RV site of the pBluescript KS plasmid to give plasmids pOL41 and pOL43, respectively.

***In Vitro* Synthesis of the 138-kDa Subunit.** A *TFC3* gene without the intron was constructed by replacing the 185-bp *Xcm* I/*Pvu* II restriction fragment of pOL45 containing the intron by the 95-bp *Xcm* I/*Pvu* II restriction fragment of pOL43. The *TFC3* open reading frame without the intron was cloned under the control of the phage T7 promoter of pBluescript KS (plasmid pOL55). The plasmid was linearized with *Pst* I and transcribed with T7 RNA polymerase; 2 μg of RNA was used for translation in a nuclease-treated rabbit reticulocyte lysate (Promega).

Disruption of the *TFC3* Gene. The *TFC3* gene was inactivated by replacing the 1.6-kilobase (kb) *Cla* I/*Msc* I fragment of pOL45 by the 1.7-kb *Cla* I/*Bam*HI fragment of pSZ63 (17) that contains the yeast *HIS3* gene (see Fig. 2*A*). The resulting plasmid, pOL48, was cleaved with *Eco*RI and *Sal* I to release the disrupted gene (4.8 kb) from the pBluescript KS polylinker, and DNA was used to transform a *his3/his3* diploid yeast strain CMY214 (18). HIS$^+$ transformants were analyzed by Southern blot hybridization of their genomic DNA digested by *Eco*RI by using a ^{32}P-labeled 3.4-kb *Xcm* I/*Bst*NI *TFC3* fragment (see Fig. 2*A*).

Epitope Tagging. The epitope derived from the influenza hemagglutinin protein Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala was added after the initiation codon by oligonucleotide-mediated, site-directed mutagenesis. The oligonucleotide used for mutagenesis (5'-GATAAATCGTCAGTACcgcggcgtaatc-tggaacatcgtatgggtaCATTTTTCTTGTGGC-3') contains the epitope coding sequence followed by an alanine codon, which creates a *Sac* II restriction site, and by nucleotides that are complementary to *TFC3* DNA on each side of the insertion site (in uppercase letters). Mutagenesis was performed as described by Kolodziej and Young (19) with the Muta-Gene kit (Bio-Rad). Wild-type and modified copies of the *TFC3* gene on centromeric plasmids were tested for the ability to complement a deleted *TFC3* allele. These genes were cloned in the yeast centromeric vector pUN75 (20) to give pOL49 (wild-type) and pOL61 (Tag-*TFC3*) and were introduced into the diploid yeast strain yOL3 containing a disrupted chromosomal copy of *TFC3*. After sporulation, two haploid strains were selected that contained the chromosomal disrupted *TFC3* allele and either the tagged or the wild-type *TFC3* gene on a centromeric plasmid. These strains were used to affinity-purify factor τ (14). TFIIIC–DNA complexes were analyzed by gel retardation with a 200-bp ^{32}P-labeled DNA fragment harboring the $tRNA_3^{Glu}$ gene as described (12).

RESULTS

Isolation of the *TFC3* Gene. Affinity-purified factor τ was used to isolate the τ_{138} polypeptide by preparative SDS/PAGE (12, 14). The 138-kDa polypeptide was digested by trypsin, and several peptides were partially sequenced. Oligonucleotide probes were designed from five peptide sequences, and different combinations of probes were used as primers to amplify a yeast genomic DNA fragment by PCR. A 910-bp yeast genomic DNA fragment obtained by PCR with one primer combination was cloned and sequenced. Its sequence was found to encode another one of the tryptic peptides (Fig. 1). To clone the whole gene, a λEMBL3a library of *Saccharomyces cerevisiae* S288C genomic DNA was screened by hybridization with the 910-bp DNA fragment. One of the recombinant clones isolated contained a unique *Nar* I/*Hgi*AI restriction fragment of 4.7 kb hybridizing to the probe (Fig. 2*A*). This fragment was cloned into th · *Cla* I/*Eco*RV site of pBluescript KS vector to give pOL45. A DNA restriction fragment derived from the 4.7-kb fragment hybridized to a mRNA species of 3.7 kb (result not shown) as expected for the RNA that would encode a 138-kDa polypeptide.

Sequence Analysis. The sequence of the entire 4708-bp *Nar* I/*Hgi*AI DNA fragment contained one large open reading frame of 3410 bp that encompassed all of the peptides that were microsequenced (Fig. 1) (21). Surprisingly, no methionyl residue was found within the open reading frame upstream of the most N-terminal tryptic peptide. This observation suggested that the gene contained an intron. Indeed, a yeast internal splice signal sequence 5'-TACTAAC-3' was found upstream of the open reading frame, preceded by a potential 5' splice junction donor sequence (5'-GTATGT-3') (22). To confirm the presence of an intron, the gene transcripts were amplified by reverse PCR (23). Total yeast RNA was reverse-transcribed by using an antisense primer hybridizing downstream of the putative intron (oligonucleotide 2 in Fig. 2*B*), and the resulting single-stranded cDNA was amplified by PCR by using complementary oligonucleotide primers that flanked the putative intron (oligonucleotides 1 and 2 in Fig. 2*B*). Surprisingly, two PCR products of about 400 and 500 bp were amplified to the same level (not shown). These two fragments were cloned and sequenced. The sequence of one of them was identical to that determined on the cloned 4708-bp fragment from yeast genomic DNA. The other fragment was lacking 90 bp and therefore corresponded to the spliced mRNA. The intron boundaries inferred from this internal deletion are mapped to positions immediately upstream of the GTATGT sequence and just downstream of an AAG sequence (Figs. 2*B* and 1).

The *TFC3* gene is therefore composed of two exons of 70 and 3410 bp and of an intron of 90 bp that contains two stop codons in phase with the start codon (Fig. 1). The calculated molecular weight of the 1160-amino acid protein encoded by the spliced mRNA (132,026) is close to the M_r 138,000 estimated by SDS/PAGE. Nevertheless, to investigate possible posttranslational modifications, *in vitro* transcription/translation of the *TFC3* gene was performed. A synthetic *TFC3* gene without intron was constructed and placed under the control of a T7 RNA polymerase promoter. Fig. 3 shows that the largest labeled polypeptide comigrated with the τ_{138}

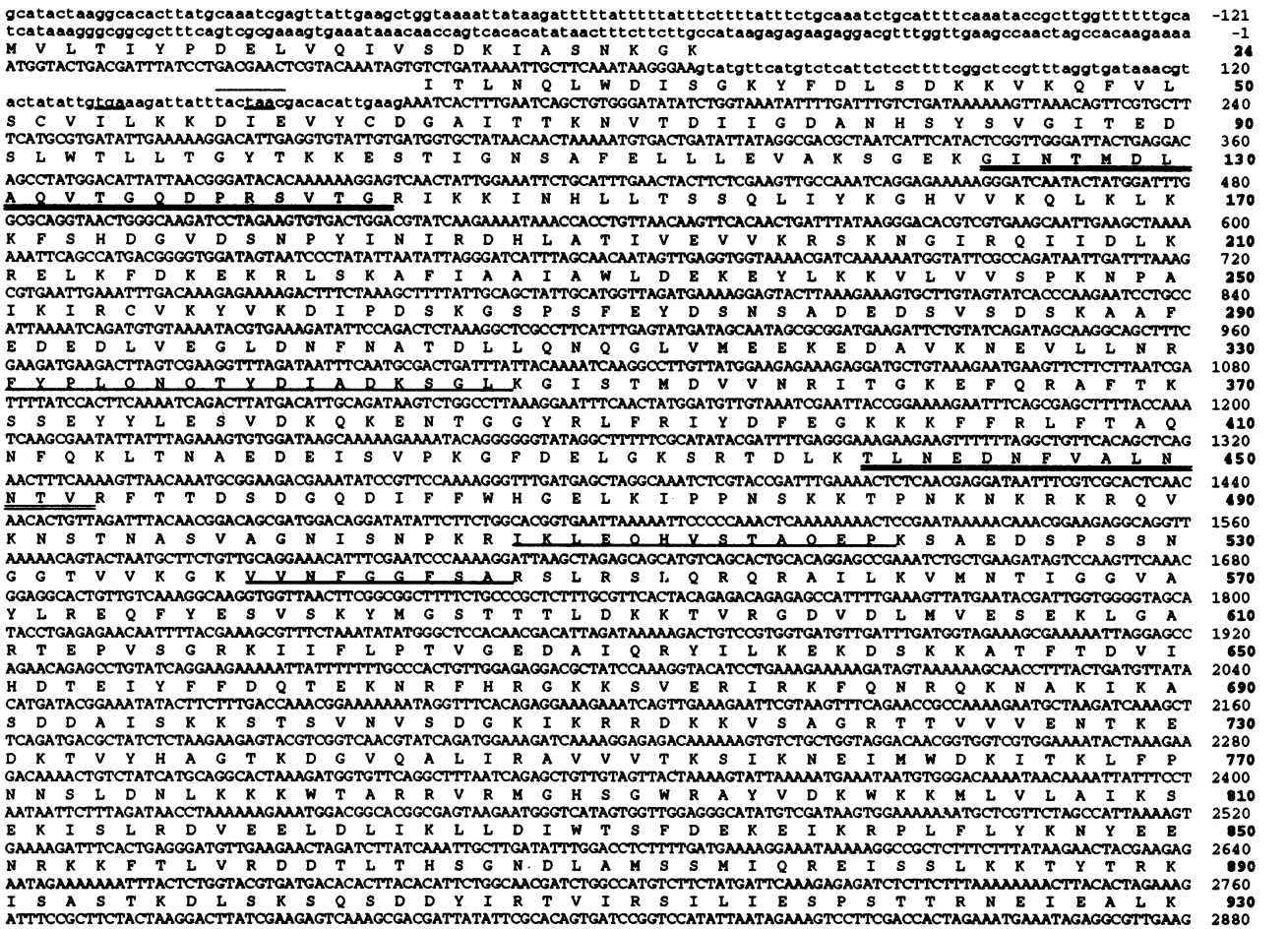

FIG. 1. Sequence of *TFC3*. The sequence of the *Nar* I/*Hgi*AI DNA fragment was determined on both strands by the dideoxy method with the ^{T7}Sequencing kit (Pharmacia). The first nucleotide of the start codon is designated +1. The amino acid sequence (given in the one-letter code) is shown above the DNA sequence. The peptides determined by microsequencing are underlined, and the two peptides used to deduce primer oligonucleotides for the PCR are underlined twice. The splice signal sequence 5′-TACTAAC-3′ is overlined, and the two stop codons of the intron in phase with the start codon are underlined.

subunit by SDS/PAGE (Fig. 3, lanes 1 and 2). Smaller polypeptides probably resulted from abortive translation or partial proteolysis. This result indicated that the isolated DNA fragment contained the entire *TFC3* gene and that the protein is not extensively modified *in vivo*. The *TFC3* gene product is a very basic protein with a calculated pI of 10.16. Comparison of its amino acid sequence with protein sequences in data bases failed to show any extensive sequence similarity with other proteins (GenBank release 72, EMBL release 31, and Swiss-Prot release 22). The protein contained four sequences that correspond to a consensus nuclear localization signal of the form [Lys(Lys or Arg)Xaa(Lys or Arg)] (24). Two regions of the polypeptide encoded by *TFC3* show similarity to HMG boxes (29) (C. Marck, personal communication). These regions extend from amino acid 1 to 68 and 1037 to 1110, respectively (see Fig. 1 and black boxes in Fig. 2). The sequence did not contain any other structural motif known to interact with DNA. Attempts to observe a direct interaction of the ^{35}S-labeled translation products with tDNA in gel-shift assays were unsuccessful, as in the case of τ_{95} (14).

***TFC3* Is a Single Gene, Essential for Cell Viability, and Located on Chromosome I.** To determine whether *TFC3* is present in single copy in the yeast genome, an internal *Xcm* I/*Bst*NI fragment (Fig. 2*A*) was used to probe Southern blots of yeast genomic DNA that had been singly digested by *Bam*HI, *Cla* I, *Hin*dIII, or *Eco*RI. In each case, the size and number of labeled restriction fragments indicated that *TFC3* is unique in the haploid genome (results not shown). *TFC3* was localized on chromosome I by probing a Southern blot of chromosomal DNA separated by pulse-field electrophoresis with the *Xcm* I/*Bst*NI probe for *TFC3* (results not shown). To investigate whether *TFC3* is essential for growth, a strain was constructed in which one of the chromosomal copies of the *TFC3* gene was partially deleted and disrupted by the wild-type *HIS3* gene (see Fig. 2*A*). Southern blot analysis of genomic DNA of the transformants confirmed that one of the

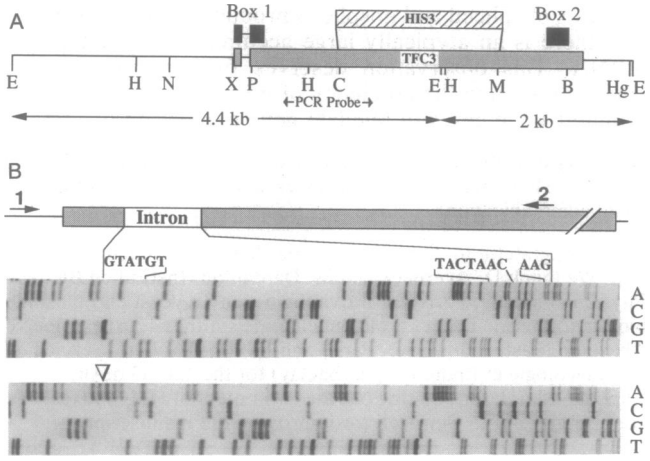

FIG. 2. (*A*) Restriction map of the *TFC3* chromosomal locus and replacement of the *Cla* I/*Msc* I internal fragment by the *HIS3* gene. The *TFC3* coding region is represented by the interrupted stippled box. The putative high mobility group (HMG) sequences (29) are shown as black boxes. The PCR probe and the size of relevant restriction fragments are shown. B, *Bst*NI; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; Hg, *Hgi*AI; M, *Msc* I; N, *Nar* I; P, *Pvu* II; X, *Xcm* I. (*B*) Location of the intron. The arrows 1 and 2 indicate the position of the oligonucleotides 1 and 2 used for PCR analysis of TFC3 mRNA. The autoradiograms of the sequencing gel of the unspliced (upper gel) and spliced cDNA (lower gel) are shown. The intron consensus splicing sequences are indicated, and the white triangle points out the junction between the two exons.

two chromosomal copies of the *TFC3* gene had been replaced by the *HIS3* disrupted construct (Fig. 4*A*). Upon sporulation, all asci analyzed (of 22) gave rise to only two viable spores (Fig. 4*B*). As expected, the two resulting colonies from each tetrad were always His$^-$, showing that the *TFC3* gene is essential. As observed previously in the case of the *TFC1* gene (14), spores containing a disrupted *TFC3* gene could germinate and grow for five or six generations before ceasing to divide.

TFC3 Gene Product Is Part of the TFIIIC–tDNA Complex. To demonstrate the presence of *TFC3* gene product in specific factor–DNA complexes, the amino terminus of the TFC3 protein product was tagged with an epitope recognized

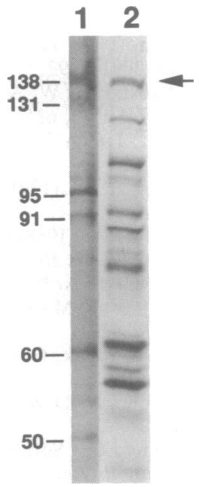

FIG. 3. *In vitro* transcription/translation of the *TFC3* gene. *TFC3* gene was transcribed and translated *in vitro* in the presence of [^{35}S]methionine. Translation products (lane 2) and the affinity-purified τ fraction (lane 1) were analyzed by electrophoresis on SDS/6% polyacrylamide gel and revealed by autoradiography. Protein components of the τ-factor preparation were silver-stained and identified by their apparent molecular weight × 10^{-3} (lane 1). The largest translation product comigrated with the τ_{138} subunit (arrow).

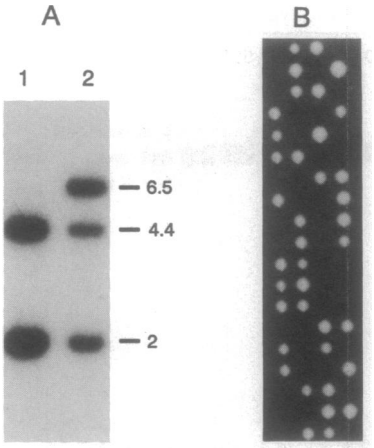

FIG. 4. The *TFC3* gene is unique and essential. (*A*) Southern analysis of *TFC3* gene disruption. The diploid strain CMY214 (His$^-$) was transformed with a 4.8-kb DNA fragment containing the *TFC3* gene disrupted by the *HIS3* marker. Insertion of *HIS3* removes the unique *Eco*RI site in the *TFC3* gene and generates a 6.5-kb *Eco*RI fragment (see Fig. 2). The genomic DNA of several HIS$^+$ transformants was isolated, digested with *Eco*RI, and subjected to Southern analysis by hybridization with a 3.4-kb *Xcm* I–*Bst*NI *TFC3* fragment overlapping the intragenic *Eco*RI site (see Fig. 2). Lanes: 1, CMY214 strain; 2, analysis of a HIS$^+$ transformant. (*B*) Tetrad analysis from the HIS$^+$ diploid transformant yOL3 containing one wild-type and one disrupted copy of *TFC3*.

by a monoclonal antibody (an epitope derived from influenza hemagglutinin protein) (19). If the tagged protein is present in TFIIIC–DNA complexes, antibody binding should affect the electrophoretic migration of the complex in the mobility-shift assay (12). The epitope-coding sequence was joined in phase to the *TFC3* open reading frame just after the initiation codon by oligonucleotide-mediated site-directed mutagenesis. Haploid cells could grow normally with the tagged gene harbored on pOL61. Factor τ was affinity-purified from the strain expressing the tagged protein (14) and used in the gel retardation assay (12). TFIIIC–DNA complexes were formed with the $tRNA_3^{Glu}$ gene and then incubated with increasing concentrations of monoclonal antibody (mAb) 12CA5 prior to electrophoresis. 12CA5 mAb directed against the epitope converted the TFIIIC–DNA complex into a slowly migrating complex, the proportion of which increased with increasing mAb concentration (Fig. 5, lanes corresponding to the Tag-*TFC3*). In contrast, the TFIIIC–DNA complex made with τ factor from cells in which the *TFC3* gene was untagged was not shifted by anti-Tag antibodies (Fig. 5, lanes corresponding to wild type). These results indicated that the product of the *TFC3* gene is part of the TFIIIC–DNA complex and therefore corresponds to the τ_{138} subunit. The formation of only one single slowly migrating complex band even in the presence of an excess of mAb is consistent with the presence of only one combining site for the anti-Tag antibody per complex.

DISCUSSION

This report describes the isolation of the *TFC3* gene encoding τ_{138}, the largest subunit of the yeast transcription factor TFIIIC. This polypeptide comigrates with τ–tDNA complexes on native polyacrylamide gels, can be crosslinked specifically to tDNA, and is likely to be involved in B-block recognition (12, 13). By an epitope-tagging method, we have confirmed the presence of *TFC3* gene product in TFIIIC–DNA complexes.

TFC3 encodes a very basic protein (pI 10.16) of 1160 amino acids. Two putative HMG boxes were found by sequence comparison, consistent with the DNA binding property of τ_{138}. As the B-block DNA sequence structure is slightly

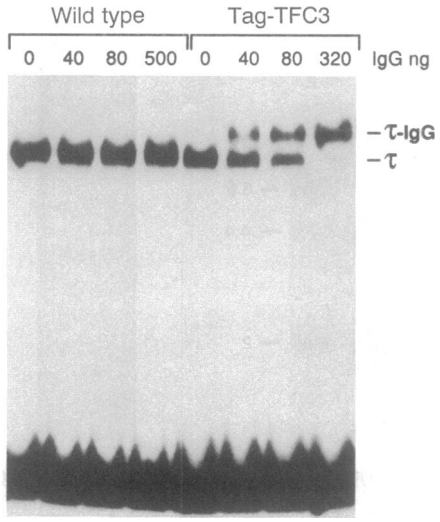

FIG. 5. *TFC3* encodes a subunit of factor τ. Factor τ was purified from a strain containing an epitope-tagged *TFC3* gene (lanes Tag-TFC3) or a wild-type *TFC3* gene (lanes Wild type). τ–DNA complexes were preformed with a ^{32}P-labeled DNA fragment containing a $tRNA_3^{Glu}$ gene for 10 min at 25°C, then treated with various amounts of a mAb directed against the Tag-epitope, as indicated, for 1 hr at 25°C. Protein–DNA complexes were analyzed by gel-retardation assay and revealed by autoradiography. τ shows the position of the τ–DNA complexes; τ-IgG shows the position of τ–DNA complexes bound by the anti-Tag antibodies.

palindromic (1), each HMG box may interact with one side of the palindrome. The B-block binding domain of factor τ, called τ_B, was visualized by scanning transmission electron microscopy as an entity corresponding in mass to a molecular size of 300 kDa. While τ_{138} is known to comprise part of τ_B (12, 13), this large size implies either that several copies of τ_{138} make up τ_B or that other components are present. We explored the stoichiometry of τ_{138} in the bound τ particle, using the epitope-tagged subunit. The finding of only one supershifted band of complex due to antibody binding supports the idea that only one τ_{138} subunit is present in TFIIIC. Therefore, to account for the mass of τ_B, additional polypeptides must be present. These may either be the 91- and/or 50-kDa components found in the affinity-purified factor (12, 13). The observation that a zinc chelator like 1,10-phenanthroline inhibits B-block binding (12) also points to the presence of another polypeptide in τ_B, since τ_{138} does not contain potential metal-binding domains.

The genes encoding three of the proteins comprising τ are now cloned: τ_{95} (14, 15), τ_{138} (this work), and τ_{131} (C. Marck, personal communication). In view of the multiple interactions that this multisubunit factor undergoes with several DNA control elements and transcription components, it will be of great interest to define the functional domains of the protein by mutagenesis of the cloned genes. Human factor TFIIIC also appears to be a multisubunit protein (25), the study of which may benefit from the isolation and analysis of the yeast genes.

TFC3 contains two exons. Sequence analysis of the PCR-amplified cDNA corresponding to the unspliced and the spliced forms of TFC3 mRNA indicated that the splice acceptor junction is unusual. Instead of the expected (C or T)AG sequence which is the general case (22), the gene has a 5′-AAG-3′ splice acceptor junction. This might conceivably influence the efficiency of the splicing reaction (26). Indeed, PCR amplification of total yeast RNA generated similar amounts of spliced and unspliced products, which suggested that there is an atypically large accumulation of unspliced mRNA. This observation deserves further investigation, since variation in the efficiency of mRNA splicing has been found in some cases to regulate gene expression (27, 28).

Note Added in Proof. *TFC3* lies next to *CEN1* and corresponds to *FUN24/TSV115* (ref. 30; D.B. Kaback and B.F.F. Ouellette, personal communication).

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