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A suppressor of mutations in the class III transcription system encodes a component of yeast TFIIIB

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Class III genes depend on TFIIIB for recruitment of RNA polymerase III. Yeast TFIIIB is comprised of three components: TBP, $TFIIIB_{70}$ and a 90 kDa polypeptide contained in the fraction B″. We report the isolation of the yeast gene *TFC7* which, based on genetic and biochemical evidence, encodes the 90 kDa polypeptide. *TFC7* was isolated as a multicopy suppressor of temperature-sensitive mutations in the two largest subunits of TFIIIC. It is an essential gene, encoding a polypeptide of 68 kDa migrating with an apparent size of ~90 kDa. In gel shift assays, recombinant TFC7 protein (rTFC7p) alone did not bind detectably to DNA, or to the TFIIIC–DNA complex even in the presence of TBP or $TFIIIB_{70}$, but it was required to assemble the TFIIIB–TFIIIC–DNA complex. The two-hybrid assay pointed to an interaction between TFC7 protein and τ_{131}, the second largest subunit of TFIIIC (that also interacts with $TFIIIB_{70}$). rTFC7p can replace the B″ component of TFIIIB for synthesis of U6 RNA in a system reconstituted with recombinant TBP and $TFIIIB_{70}$ polypeptides and highly purified RNA polymerase III. Surprisingly, specific transcription of the SUP4 $tRNA^{Tyr}$ gene promoted by rTFC7p was much weaker than with B″. An additional factor activity, provided by the recently identified TFIIIE fraction, was required to restore control levels of transcription.

Keywords: RNA polymerase III/transcription/TFIIIB/TFIIIE

Introduction

The relative simplicity of the RNA polymerase III transcription system has been attractive for exploring the mechanisms of gene activation in eukaryotic cells. This system has been the most thoroughly analyzed in yeast. Two auxiliary transcription factors (TFs), IIIB and IIIC, are required to direct accurate transcription by RNA polymerase III (pol III) on tRNA gene templates (reviewed by Geiduschek and Kassavetis, 1992; White, 1994). The multisubunit factor TFIIIC (or τ) combines the multiple tasks of promoter binding factor (Klemenz *et al.*, 1982; Camier *et al.*, 1985), chromatin antirepressor (Burnol *et al.*, 1993a) and pre-initiation complex assembly factor (Kassavetis *et al.*, 1990). It binds to the A and B blocks of the intragenic promoter via two separate DNA binding domains (Marzouki *et al.*, 1986; Baker *et al.*, 1987; Schultz *et al.*, 1989) and then promotes the binding of TFIIIB to DNA, at a fixed distance upstream of the start site (Kassavetis *et al.*, 1989, 1990). TFIIIB does not bind tDNA detectably by itself. It needs to be assembled on DNA, but the resulting TFIIIB–DNA complex is very tight and withstands stringent conditions that dissociate TFIIIC (Kassavetis *et al.*, 1989, 1990). Hence, it could be shown that TFIIIB–DNA complexes, devoid of TFIIIC, are competent for directing accurate initiation and multiple rounds of transcription by pol III (Kassavetis *et al.*, 1990). TFIIIB–DNA binding is essentially non-specific: no consensus sequence could be derived from TFIIIB binding sites upstream of tRNA, 5S RNA and other class III genes. Furthermore, TFIIIB binding can be redirected by inserting an overlapping site for a sequence-specific protein, and this causes a change in the start site (Léveillard *et al.*, 1993). Thus, TFIIIC positions TFIIIB, which in turn recruits pol III productively. There is a large degree of flexibility in this cascade of interactions. The downstream B block of the SNR6 gene could be replaced by a GAL4 binding site, inserted at various positions, in a chimeric system where the GAL4 binding domain was fused to various components of the pre-initiation complex (Marsolier *et al.*, 1994).

Yeast TFIIIB (yTFIIIB) is a multiprotein factor that comprises at least three components: the TATA binding protein (TBP) (Huet and Sentenac, 1992; Kassavetis *et al.*, 1992; Hernandez, 1993) plus two distinct polypeptides of ~70 kDa and ~90 kDa that were first identified by photocross-linking analysis of TFIIIB–DNA complexes (Bartholomew *et al.*, 1991). yTFIIIB is not a stable molecular entity. It can be resolved into two subfractions, B′, containing TBP and the 70 kDa component ($TFIIIB_{70}$), and B″, containing the 90 kDa polypeptide (B''_{90}) (Kassavetis *et al.*, 1991). Similarly, TBP or $TFIIIB_{70}$ can be dissociated easily from the other TFIIIB components (Huet *et al.*, 1994). TFIIIC-dependent assembly of TFIIIB on the DNA can be reconstituted by the sequential binding of $TFIIIB_{70}$, TBP and B″, a process which is accompanied by noticeable conformational changes of protein–DNA complexes (Kassavetis *et al.*, 1992). B′ ($TFIIIB_{70}$+TBP), but not B″, is capable of assembling onto the TFIIIC–DNA complex. The B″ fraction used in these studies was at a relatively crude stage of purification, but SDS–gel-purified 90 kDa polypeptide(s) from B″ restored all the properties of B″, which strongly indicated that B″ is solely composed of B''_{90} (Kassavetis *et al.*, 1992). Nevertheless, the possibility that B″ could be more complex is not ruled out entirely. Hence, a factor, named TFIIIE, distinct from

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TFIIIB and TFIIIC, and present in B′ and B″ fractions, was found to be required for transcription of tRNA and 5S RNA genes (Dieci *et al.*, 1993). In addition, other factors might be required for optimal transcription.

For a precise description of the process of transcription complex formation, we and others have undertaken the cloning of all the components of the yeast class III transcription system. The genes encoding the three largest subunits of TFIIIC (six subunits) have been cloned and characterized (Swanson *et al.*, 1991; Lefebvre *et al.*, 1992; Parsons and Weil, 1992; Marck *et al.*, 1993; Rameau *et al.*, 1994): τ_{138} interacts with the B block (Gabrielsen *et al.*, 1989b), and a temperature-sensitive (ts) mutation in that subunit affects TFIIIC–DNA interaction (Lefebvre *et al.*, 1994). τ_{95} is thought to bind the A block (Gabrielsen *et al.*, 1989b; Bartholomew *et al.*, 1990). τ_{131}, a protein with 11 tetratricopeptide repeats, interacts with $TFIIIB_{70}$ (Khoo *et al.*, 1994; Chaussivert *et al.*, 1995). τ_{131} is also the only TFIIIC subunit that can be cross-linked to the region upstream of the start site occupied by TFIIIB (Bartholomew *et al.*, 1991). Therefore, it is certainly responsible for initiating TFIIIB assembly on the DNA. The genes encoding two additional TFIIIC subunits (τ_{91} and τ_{55}) have been cloned recently (N.Manaud, R.Arrebola, C.Carles and C.Conesa, unpublished). Their role is still unknown. Most constituents of yeast pol III (16 subunits) have been cloned and mutagenized (Thuriaux and Sentenac, 1992). Interestingly, a pol III-specific subunit, C34, was found to interact with $TFIIIB_{70}$ (Werner *et al.*, 1993; Khoo *et al.*, 1994). C34 belongs to a subgroup of unique subunits of pol III (C34, C31 and C82) that dissociate from a mutant pol III affected in the largest subunit (Werner *et al.*, 1992). Cross-linking (Bartholomew *et al.*, 1993) and mutagenesis studies (Thuillier *et al.*, 1995) further suggest a critical role for C34 and C31 in the initiation complex. Of TFIIIB, two components are well characterized, TBP and $TFIIIB_{70}$. These two proteins interact in the absence of DNA (Huet *et al.*, 1994; Khoo *et al.*, 1994) and $TFIIIB_{70}$ can assemble onto TBP–TATA complexes (Roberts *et al.*, 1995). Several mutations of yeast TBP have been described that interfere specifically with pol III transcription (Cormack and Struhl, 1993; Kim and Roeder, 1994). Analysis of $yTFIIIB_{70}$ (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; López-De-León *et al.*, 1992) and of its human homolog (Wang and Roeder, 1995) revealed a sequence relationship to the pol II factor TFIIB that also binds TBP and recruits polymerase II (Zawel and Reinberg, 1993; Serizawa *et al.*, 1994). Domains of $TFIIIB_{70}$ that interact with TBP, τ_{131} and C34 have been broadly defined (Khoo *et al.*, 1994; Rameau *et al.*, 1994; Chaussivert *et al.*, 1995). At this stage, it became critical to characterize B''_{90} as the last building block of TFIIIB.

Genetic approaches were used successfully to isolate the gene encoding $TFIIIB_{70}$ as a suppressor of TBP (Buratowski and Zhou, 1992; Colbert and Hahn, 1992) or A block down mutations (López-De-León *et al.*, 1992). We followed a similar strategy to identify components of the pol III transcription system. Among multicopy suppressors of a ts mutation in τ_{138}, we found the genes encoding TBP, $TFIIIB_{70}$, two subunits of TFIIIC, τ_{95} and τ_{131}, and one subunit shared by all three RNA polymerases, ABC10α (Lefebvre *et al.*, 1994). Here we report the characterization of another suppressor gene, named *TFC7*. The *TFC7* gene is essential for cell viability. It encodes a component that efficiently restores transcription of a U6 RNA gene in a highly purified system lacking B″. In the case of the SUP4 tRNA gene, full transcriptional activity in the absence of B″ could only be reconstituted in the presence of both TFC7 protein and the previously identified factor TFIIIE (Dieci *et al.*, 1993). Our results indicate that *TFC7* encodes B''_{90}, and that TFIIIB may be more complex than previously thought.

Results

Genetic isolation and characterization of TFC7

In a previous work, we characterized a ts mutation, *tsv115*, that changes one amino acid in τ_{138} and has severe effects on tRNA and 5S RNA synthesis *in vitro*. We isolated a number of genes that partially suppressed the ts phenotype of the mutant cells when expressed on a high copy number plasmid (Lefebvre *et al.*, 1994). Several suppressor genes could be identified readily as known components of the pol III transcription machinery. Unidentified suppressors were tested for suppression of ts mutations affecting other components of the pol III transcription system. One isolated plasmid, pLR204, also suppresses three different mutations in τ_{131}, the second largest subunit of TFIIIC (*tfc4-ΔH1*, *tfc4-ΔTPR8* and *tfc4-Δbasic2*, Chaussivert, 1995; Chaussivert *et al.*, 1995 and data not shown). The plasmid contained a genomic insert of ~4 kb. To identify the encoded polypeptides, a large part of the insert was sequenced. This sequence analysis revealed a putative >63 kDa protein truncated at the C-terminus. The genomic sequence that overlapped the insert had been established within the frame of the European yeast genome sequencing project concerning chromosome XIV. The truncated open reading frame (ORF) was missing the last 53 codons. A 3.9 kb *Xba*I insert encoding the complete gene (hereafter named *TFC7*) retained a suppressor activity similar to that of the original plasmid pLR204. As the *Xba*I fragment harbored only a truncated, promoterless gene upstream of *TFC7*, we ascribed the suppressor activity to *TFC7*.

Figure 1 shows the suppressor activity of the *Xba*I insert on a 2μ plasmid. The level of suppression by *TFC7* of the ts phenotype of *tsv115* was compared with the suppression properties of *BRF1/PCF4/TDS4* (encoding $TFIIIB_{70}$) or *SPT15* (TBP), each gene harbored on high copy vectors. To better visualize the suppressor effect, a series of cell dilutions was plated. As seen in Figure 1, *TFC7* was as good a suppressor as *SPT15* but a weaker suppressor than *BRF1/PCF4/TDS4*.

The *TFC7* gene encodes a 594 amino acid polypeptide with a predicted mass of 68 kDa, and a pI of 6.5 (Figure 2A). The TFC7 protein sequence was compared with the NCBI non-redundant database using the BLAST program server (Altschul *et al.*, 1990). No global sequence similarity was found with other sequences in these databases. However, a 37 amino acid motif, at positions 418–454, was conserved in several ORFs of unknown function [one human and two *Caenorhabditis elegans* genes as well as the rat *Mta1* gene whose expression directly correlated with metastatic potential (Toh *et al.*, 1995; Figure 2B)]. As a tandem repeat of this motif was present in the human ORF Ha0472, we are inclined to think

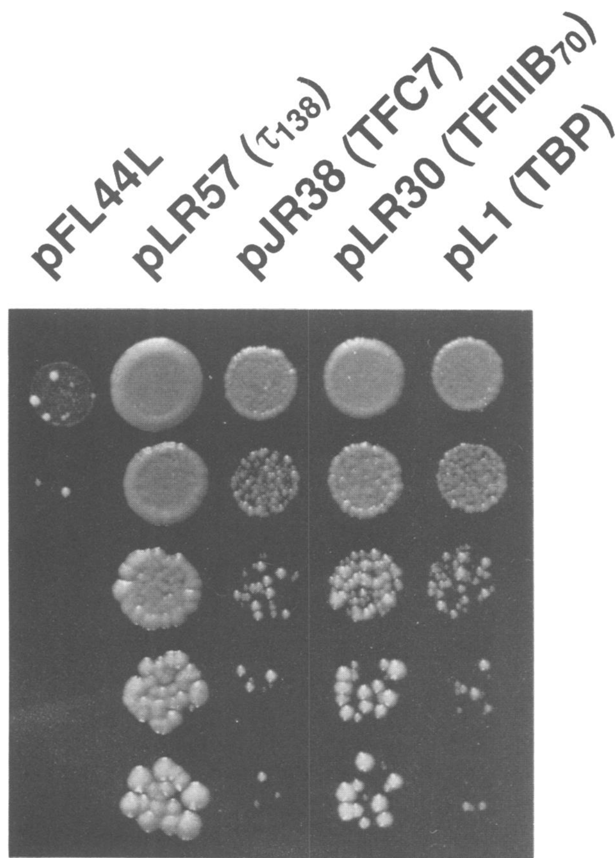

Fig. 1. Extragenic suppression of strain YOL8 carrying a ts mutation in τ_{138}, the largest subunit of TFIIIC (Lefebvre *et al.*, 1994) by *TFC7*, *BRF1/TDS4/PCF4* and *SPT15*. Stationary phase cells harboring different suppressor genes were diluted in water and 3 µl of cell suspension spotted on rich medium plates. From top to bottom, cell suspensions were diluted 10-, 10^2-, 10^3-, 10^4- and 10^5-fold and incubated at 35°C for 3 days. The transformed plasmids and encoded factors are indicated. Plasmid pFL44L corresponds to the vector without insert.

that it corresponds to a novel structural and functional protein domain.

To test whether the suppressor gene was essential for cell viability, a deletion allele was constructed that replaced the region encoding amino acids 203–511 with the *HIS3* gene. The plasmid was linearized, transformed into a diploid strain, and the resulting $His3^+$ cells were sporulated. Of 20 tetrads dissected, all produced two, or occasionally one, viable spores, all of which were auxotrophic for histidine. This result indicates that *TFC7* is essential for cell viability.

TFC7p interacts with components of the pol III pre-initiation complex

The observation that high gene dosage of *TFC7* suppresses several mutations in different components of the pol III system suggested that it might encode an essential transcription component. To characterize the properties of the TFC7 protein, the cloned gene was engineered to add six histidine residues and one T7·Tag epitope at the N-terminus of the protein. The tagged protein was expressed in *Escherichia coli* cells and purified on a Ni^{2+} column. Western analysis of the purified protein fraction was performed using a monoclonal antibody directed against the T7 epitope tag, along with TBP and $TFIIIB_{70}$, each revealed with specific polyclonal antibodies (Figure 3). The T7-tagged TFC7 protein migrated as three main protein bands, the largest of which had an apparent size of ~90 kDa. The histidine and T7 epitope tags (17 amino acids total) could not account for the difference between the predicted (68 kDa) and the apparent size of the protein. This unexpected migration behavior has also been observed in other cases; for example, τ_{95} (apparent size 95 kDa) has a predicted mass of 73.5 kDa (Swanson *et al.*, 1991).

Among the components of the pol III transcription complex that remain to be cloned, there are two polypeptides of ~90 kDa. These are τ_{91} and B''_{90}. The gene for τ_{91} (*TFC6*), recently isolated in our laboratory, encodes a protein of 74 kDa and is distinct from *TFC7* (N.Manaud, R.Arrebola, C.Carles and C.Conesa, unpublished data). Therefore, we explored the possibility that TFC7p might be B''_{90}. Recombinant TFC7p (rTFC7p) was used to reconstitute TFIIIB, in combination with rTBP and $rTFIIIB_{70}$. TFIIIB can be reconstituted in a TFIIIC-dependent pathway and assembled onto a tRNA gene to give a slow migrating TFIIIB–TFIIIC–tDNA complex that can be separated from the TFIIIC–tDNA complex by non-denaturing gel electrophoresis (Kassavetis *et al.*, 1990, 1992). Factor–DNA complexes were formed with the SUP4 $tRNA^{Tyr}$ gene and separated by native gel electrophoresis (Figure 4). The purified rTFC7 protein fraction alone did not interact detectably with the labeled tDNA probe (lane 2), or with the TFIIIC–DNA complex (lane 4), even in the presence of rTBP (lane 5) or $rTFIIIB_{70}$ (lane 6). A weak interaction has been noted previously in gel shift assays between $rTFIIIB_{70}$ and TFIIIC–SUP4 DNA complexes, which was stabilized by TBP (Kassavetis *et al.*, 1992). Under our conditions, these partial complexes were not observed (data not shown). However, the combination of the same amounts of rTBP and $rTFIIIB_{70}$ supplemented with rTFC7p caused ~50% of the TFIIIC–DNA complexes to migrate more slowly (lane 7). Incubation with B″ fraction instead of rTFC7p also caused an upshift of the TFIIIC–DNA complex but gave a more complex pattern of bands (lane 8), suggesting the formation of at least one intermediate in TFIIIB assembly (which may be equivalent to the B′–TFIIIC–DNA complex).

To demonstrate further the interaction of TFC7 with components of the pol III transcription complex, we used the two-hybrid system that detects *in vivo* interactions between two proteins overproduced in yeast cells (Fields and Song, 1989; Chien *et al.*, 1991). The proteins are fused to the DNA binding domain, or to the transcriptional activation domain, of the yeast GAL4 protein. If the two proteins interact, a chimeric GAL4 protein is reconstituted that activates the transcription of a *lacZ* reporter gene. This method previously revealed interactions between $TFIIIB_{70}$ and the pol III subunit C34 (Werner *et al.*, 1993) as well as between $TFIIIB_{70}$ and τ_{131} (Chaussivert *et al.*, 1995). The ORF encoding TFC7 and those encoding other components of the pol III system including TFIIIB ($TFIIIB_{70}$, TBP), TFIIIC (τ_{138}, τ_{131}, τ_{95}) and pol III (C160, C128, C82, C53, AC40, C34, C31, AC19, ABC10α, ABC10β) have been fused to the two GAL4 domains (except for subunits ABC23 and AC19, which have been tested only fused to the GAL4 activation domain) (Lalo

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A      |   10       |   20       |   30       |   40       |   50
  1 MSSIVNKSGT RFAPKVRQRR AATGGTPTPK PRTPQLFIPE SKEIEEDNSD
    NDKGVDENET AIVEKPSLVG ERSLEGFTLT GTNGHDNEIG DEGPIDASTQ
101 NPKADVIEDN VTLKPAPLQT HRDQKVPRSS RLASLSKDNE SRPSFKPSFL
    DSSSNSNGTA RRLSTISNKL PKKIRLGSIT ENDMNLKTFK RHRVLGKPSS
201 AKKPAGAHRI SIVSKISPPT AMTDSLDRNE FSSETSTSRE ADENENYVIS
       ▼
    KVKDIPKKVR DGESAKYFID EENFTMAELC KPNFPIGQIS ENFEKSKMAK
301 KAKLEKRRHL RELRMRARQE FKPLHSLTKE EQEEEEEKRK EERDKLLNAD
    IPESDRKAHT AIQLKLNPDG TMAIDEETMV VDRHKNASIE NEYKEKVDEN
401 PFANLYNYGS YGRGSYTDPW TVEEMIKFYK ALSMWGTDFN LISQLYPYRS
    RKQVKAKFVN EEKKRPILIE LALRSKLPPN FDEYCCEIKK NIGTVADFNE
               ▼
501 KLIELQNEHK HHMKEIEEAK NTAKEEDQTA QRLNDANLNK KGsggimtnd
    lkvyrktevv lgtiddlkrk klkernnddn ednegseeep eidq
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B              Start  1                 10                  20                  30               38  End
TFC7p           418   D P W T V E E M I K F Y K A L S M W G T D F N L I S Q L Y - P Y R S R K Q V   454
Ha0472 box1     104   D E W T V E D K V L F E Q A F S F H G K T F H R I Q Q M L - P D K S I A S L   140
Ha0472 box2     295   A R W T T E E Q L L A V Q A I R K Y G R D F Q A I S D V I - G N K S V V Q V   331
C14B9.6         216   E E W S P E E R S L F K S R Q A D H V K I F H G L T E F F - V D K T A S D L   252
Mta1            286   E E W S A S E A N L F E E A L E K Y G K D F T D I Q Q D F L P W K S L T S I   323
CO4A2.2          97   D N M T Q D D A K K F A K G I K Q L G K N F S R I H R E L L P H H S R E Q L   134

Consensus :           D E W T   E E     L F   A         G K D F   I   Q       P   K S     Q L
                      E     S   D                                                            V
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Fig. 2. Sequence analysis of *TFC7*. (**A**) Deduced amino acid sequence of *TFC7*. Upper case letters indicate the part of the protein encoded by the insert that was originally found in the suppressor screen. Arrows (▼) mark the corresponding region deleted by gene disruption. The region with a conserved sequence motif is underlined. The accession number of the sequence is U38415. (**B**) The amino acid sequence of TFC7p is aligned with the following protein sequences: human Ha0724, rat Mta1, *C.elegans* C14B9.6 and CO4A2.2 (GenBank accession Nos D31888, U02522, L15188 and U23448, respectively). Identical amino acids are boxed, conserved substitutions are shaded and grouped as follows: [S, T, P, A, G], [N, D, E, Q, H], [R, K], [M, I, L, V] and [F, Y, W]. The amino acid position of the first and last residue of each sequence is indicated, as well as the deduced consensus sequence; amino acids conserved between at least five sequences are underlined.

et al., 1993; Werner *et al.*, 1993; Chaussivert *et al.*, 1995). All combinations of fusion proteins with GAL4 DNA binding or activation domains were assayed against the complementary TFC7 fusions. Most combinations of hybrid proteins gave background levels of β-galactosidase activity. However, when TFC7 was fused to the GAL4 DNA binding domain and τ_{131} to the GAL4 activation domain, significant levels of β-galactosidase activity were detected, suggesting that TFC7 and τ_{131} interacted directly or indirectly. We also tested several deletion mutants of τ_{131}, as some of them were found previously to interact more strongly with $TFIIIB_{70}$ than the whole protein (Chaussivert *et al.*, 1995). As shown in Figure 5, one deletion mutant (ΔTPR2, lacking 34 amino acids from position 162 to 195) induced a 20-fold higher level of β-galactosidase activity than the complete fusion protein. Most of the other fusions were inactive, or weakly active, like intact τ_{131}. None of the constructs harboring fusion proteins with τ_{131}, τ_{131} mutants or TFC7, activated *lacZ* transcription when tested alone (i.e. in the absence of the complementary hybrid protein). These two-hybrid experiments confirmed that TFC7 was a component of the pre-initiation complex TFIIIB–TFIIIC–DNA.

TFC7 is required for RNA pol III transcription

A critical functional assay was to test the ability of rTFC7p to replace B″ for transcription of different class III genes. In the experiment shown in Figure 6A, the SUP4 tRNA gene was transcribed in a reconstituted system containing pol III and TFIIIC (both purified to near homogeneity) rTBP, rTFIIIB70 and B″ fraction (lane 1). The addition of B″ was absolutely required for transcription (lane 2). Replacement of B″ by recombinant TFC7 protein (rTFC7p, lane 3) restored a specific but weak transcription activity. Varying the concentration of rTFC7p over a large range, in search of an optimal stoichiometry, did not improve the transcription signal. In addition, a mixing experiment showed that the purified rTFC7p fraction did not contain an inhibitor that would interfere with B″-dependent transcription or degrade the transcript (data not shown).

Figure 6B shows a similar experiment using the U6 RNA gene as a template. The promoter of the *SNR6* gene is particular in having a distant B block, downstream of the termination signal, a weak intragenic A block and a TATA box at –30 (Burnol *et al.*, 1993b; eschenlauer *et al.*, 1993). The presence of the TATA box allows specific transcription in the absence of B block or of TFIIIC (Burnol *et al.*, 1993b; Eschenlauer *et al.*, 1993), but the same components of TFIIIB (TBP, $TFIIIB_{70}$ and B″) are required for gene activation (Joazeiro *et al.*, 1994). As shown in Figure 6B, the reconstituted system requires B″ for *SNR6* transcription (lanes 1 and 2). Remarkably, with this template, B″ could be replaced effectively by rTFC7 protein: at saturating rTFC7p concentrations, the amount of transcript reached about half the control level (lane 3 and data not shown). In view of the size of rTFC7p, its role in pre-initiation complex formation and its transcription factor activity, we conclude that *TFC7* encodes B''_{90}.

The fact that rTFC7p could not fully restore the transcription factor activity of the B″ fraction on a tRNA gene was intriguing. One possibility could be that B″ was more complex than previously thought, and that recombinant B''_{90} (rB''_{90}) was necessary, but not sufficient, for optimal activity. A novel factor of small size, named TFIIIE, has been proposed to be involved in transcription of tRNA and 5S RNA genes at a stage after gene commitment (i.e. past TFIIIB assembly). TFIIIE activity was found to be present in the B′ as well as B″ fractions (Dieci *et al.*,

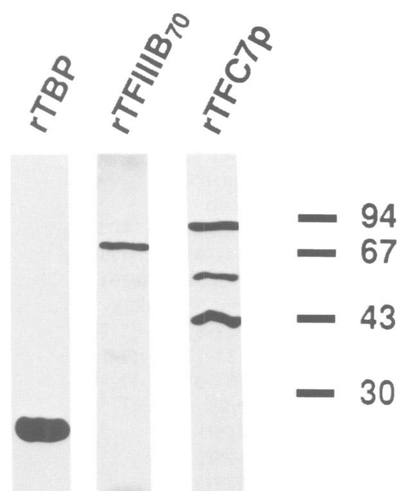

Fig. 3. Purification of rTBP, rTFIIIB$_{70}$ and rTFC7p. Recombinant TFIIIB$_{70}$ and TFC7 proteins were expressed as hexahistidine fusions and purified from *E.coli* cells under native conditions. Eluted polypeptides (1–2 μg; 60 ng of rTBP, purified as described in Burton *et al.*, 1991) were subjected to SDS–PAGE, transferred onto a membrane, and incubated with an anti-TBP or anti-TFIIIB$_{70}$ antiserum or with antibodies directed against the T7·Tag™ epitope. Immune complexes were visualized using the ECL chemoluminiscence kit (Amersham). Molecular weight marker bands are indicated in kDa.

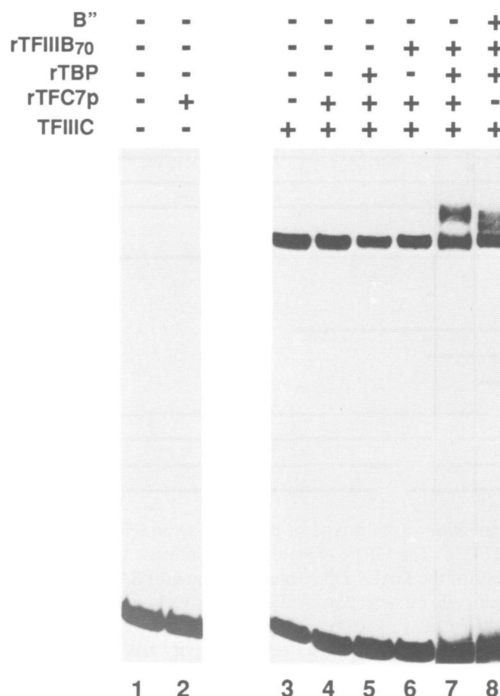

Fig. 4. Role of rTFC7p in factor–DNA complex formation. Binding studies, performed with a 280 bp ^{32}P-labeled fragment harboring the yeast SUP4 tRNA^{Tyr} gene as probe. Lanes 1 and 2 show free tDNA and tDNA pre-incubated with rTFC7p, respectively. In lanes 3–8, affinity-purified TFIIIC has been pre-incubated with the tDNA, and recombinant TFIIIB components or B″ fraction were added subsequently (see Materials and methods) as indicated. Protein–DNA complexes were analyzed by gel retardation electrophoresis and revealed by autoradiography.

1993, 1995). Therefore, we attempted to restore full transcription activity by supplementing the TFIIIB recombinant system (rTBP, rTFIIIB$_{70}$, rB″$_{90}$) with the same TFIIIE fraction previously isolated by Dieci *et al.* (1993). As shown in Figure 7, we confirmed that TFIIIE alone cannot replace B″ activity (lane 4) in SUP4 tRNA^{Tyr} gene transcription; rTFC7p (rB″$_{90}$) generated only a low transcription signal (lane 1); but the combination of rTFC7p + TFIIIE restored efficient transcription (lane 2) comparable with the signal obtained with B″ (lane 3). In the case of U6 RNA gene transcription, addition of TFIIIE to the recombinant TFIIIB system also led to full or even higher transcription efficiency compared with the control reaction containing fraction B″ (lanes 5–8). These results support the idea that TFIIIE is an additional transcription factor. The mode of action of this factor is under investigation.

Discussion

The demonstration that the *TFC7* gene encodes B″$_{90}$, the 90 kDa subunit of TFIIIB, is based on both genetic and biochemical evidence. *TFC7* was found among multicopy suppressors of mutations affecting different components of the class III transcription system. The recombinant TFC7 protein displayed physical and functional properties consistent with those of B″$_{90}$: apparent size of ~90 kDa, role in TFIIIB–TFIIIC–DNA complex formation and transcription factor activity. While yTFIIIB is believed to be comprised of three components, TBP, TFIIIB$_{70}$ and B″$_{90}$, some of our results support the need for at least one additional component.

The *TFC7* gene was isolated as a high copy suppressor of a ts mutation within the B block binding subunit of TFIIIC. The same mutation was suppressed by overexpression of TFIIIB$_{70}$ and, to a lesser extent, of TBP (Lefebvre *et al.*, 1994), suggesting that increasing the concentration of these TFIIIB components increased the rate of B′ assembly (TFIIIB$_{70}$–TBP complex). There is evidence that TFIIIB$_{70}$ is limiting *in vivo* (López-De-León *et al.*, 1992). Our suppression studies further indicate that B″$_{90}$ levels are also suboptimal *in vivo*, and support the idea of a stepwise assembly of TFIIIB components onto TFIIIC–DNA complexes. TFIIIB is not a stable molecular entity (Huet *et al.*, 1994) and TFIIIC-dependent TFIIIB assembly on the DNA can be achieved by sequential binding of TFIIIB$_{70}$, TBP and B″ (Kassavetis *et al.*, 1992). If this multistep assembly occurs *in vivo* (see López-De-León *et al.*, 1992), regulation of pol III genes may be exerted by modulating the nuclear concentration of TFIIIB$_{70}$ and B″$_{90}$.

The gel shift and the two-hybrid assays contributed to show that TFC7p/B″$_{90}$ is a component of TFIIIB. The recombinant protein did not bind detectably to the SUP4 tRNA gene by itself. The same is true for TFIIIB$_{70}$, even in the presence of TBP (Kassavetis *et al.*, 1991) [at least on TATA-less genes, otherwise TFIIIB$_{70}$ can bind to TBP–TATA complexes (Roberts *et al.*, 1995)]. This observation strongly suggested that B″$_{90}$, as is probably the case for TFIIIB$_{70}$ (see Kassavetis *et al.*, 1992), has a cryptic DNA binding domain that is revealed during the TFIIIC-dependent assembly process. The potential DNA binding properties of B″$_{90}$ deserve to be examined to shed light on the exceptional stability of TFIIIB–DNA complexes. B″$_{90}$ did not bind directly to TFIIIC–DNA complexes,

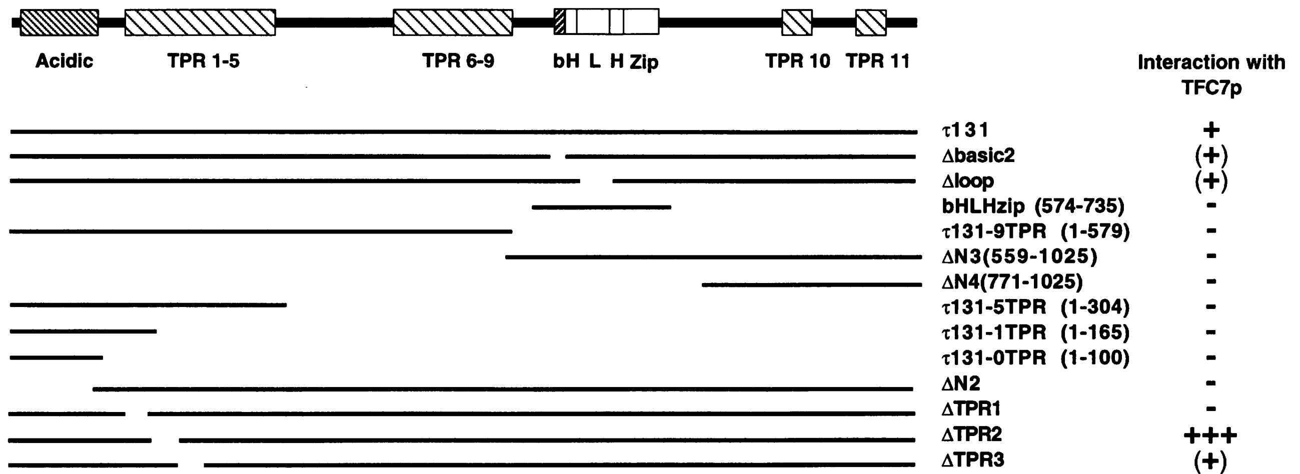

Fig. 5. Interaction of wild-type or mutant τ_{131} proteins with TFC7p. The two-hybrid system was used to study protein–protein interactions between τ_{131} and TFC7p. The ORFs of wild-type or mutant τ_{131} proteins were fused in-frame with the GAL4 activation domain (GAL4 768–881). TFC7p was fused with the GAL4 DNA binding domain (GAL4 1–147). Transcription activation of the *lacZ* reporter gene was assayed by growing the transformed cells on selective medium and overlaying with X-Gal agar. Arbitrary values are given for white (–) and for different degrees of blue coloration [(+), +, +++] of cell patches on X-Gal plates (representation modified after Chaussivert *et al.*, 1995). In the case of τ_{131} and the deletion mutant ΔTPR2, β-galactosidase activity has been determined as 7 and 210 U, respectively (nanomoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per min and per mg of protein).

even in the presence of rTBP or $rTFIIIB_{70}$. The three components rTBP, $TFIIIB_{70}$ and B''_{90}, were necessary and sufficient to form the slow migrating supercomplex TFIIIB–TFIIIC–DNA. B''_{90} thus behaved like the B″ fraction that does not interact with the TFIIIC–DNA complex but alters the footprint of the B′–TFIIIC–DNA intermediate complex and locks into place TBP, $TFIIIB_{70}$ and the 90 kDa polypeptide (Kassavetis *et al.*, 1991).

The stepwise assembly of TFIIIB involves conformational changes of the τ_{131} subunit that probably unmask an interaction domain required for docking B''_{90} (Kassavetis *et al.*, 1992). This hypothesis may explain our observation from the two-hybrid experiments that a deletion mutant of τ_{131}, ΔTPR2, interacted more strongly with B''_{90} than the entire protein. Remarkably, the *PCF1-1* dominant mutation that suppresses a tRNA gene A block down mutation *in vivo* lies in the same TPR sequence of the τ_{131} subunit (Rameau *et al.*, 1994). This mutation was found to increase the amount of TFIIIB and B″ activity in mutant cell extracts, and the rate of formation of pre-initiation complexes (Willis *et al.*, 1992; Rameau *et al.*, 1994). On the basis of our two-hybrid interaction results, the second TPR sequence appears to play a negative regulatory role in the interaction of τ_{131} with B''_{90}. *PCF1-1* mutation probably suppresses the negative function of TPR2 and facilitates the assembly of B''_{90} into the pre-initiation complex by τ_{131}.

We show that rB''_{90}, in association with rTBP and $rTFIIIB_{70}$, directs specific transcription of the U6 RNA gene by highly purified pol III. In the absence of TFIIIC, and of the downstream B block, this assay constitutes a minimal transcription system that retains the basal properties needed for the specific recruitment of RNA polymerase III. This well characterized transcription assay, which demonstrated the transcription factor activity of rB''_{90}, should be useful for further analysis of the process of RNA polymerase recruitment. Transcription of the SUP4 tRNA gene is more complex since it requires the TFIIIC-dependent assembly of TFIIIB and transcription through the TFIIIC–DNA complex. The above minimal system, supplemented with highly purified TFIIIC, transcribed the SUP4 tRNA gene specifically but inefficiently as compared with the control transcription assay containing B″. One may invoke several causes for this low level of transcription. As some simple explanations such as the presence of inhibitors in the recombinant protein fraction or an inappropriate protein stoichiometry were ruled out by titration or mixing experiments, we explored the intriguing possibility that this highly purified system could be missing an important component. Indeed, addition of TFIIIE restored control transcription levels. TFIIIE previously was isolated from the rest of the pol III system of yeast nuclear extracts by a simple adsorption step on DEAE–Sephadex (Dieci *et al.*,

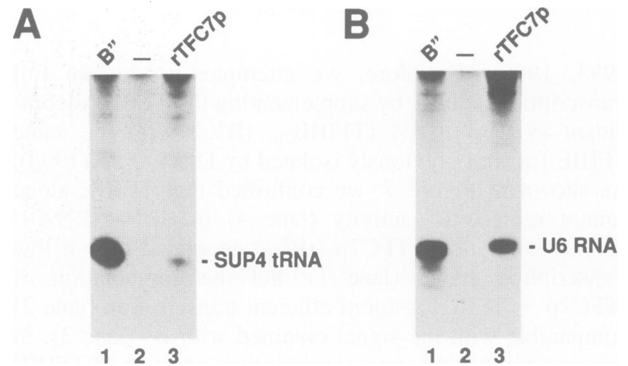

Fig. 6. *In vitro* transcriptional activity of recombinant TFC7 protein. (**A**) tRNA gene transcription. *In vitro* transcription of the SUP4 tRNA gene was performed as described in Materials and methods, in the presence (lane 1) or absence (lanes 2 and 3) of fraction B″. Recombinant TFC7 protein was added to the reaction in lane 3. (**B**) U6 RNA gene transcription. The experiment was the same as in (A), except that transcription reactions were programmed with the truncated form of the yeast U6 RNA gene (Brow and Guthrie, 1988) in the absence of TFIIIC (Moenne *et al.*, 1990).

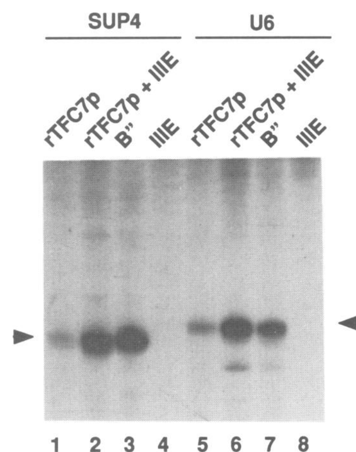

Fig. 7. Reconstitution of B″ activity by rTFC7p and TFIIIE. *In vitro* transcription of the SUP4 tRNA gene and *SNR6* was carried out as described in Materials and methods, in the presence of fraction B″ (lanes 3 and 7), or of rTFC7p alone (lanes 1 and 5), rTFC7p + fraction TFIIIE (lanes 2 and 6), or fraction TFIIIE alone (lanes 4 and 8).

1993). TFIIIE factor activity was found to be required for transcription of tRNA and 5S RNA genes, but it is not functionally equivalent to TFIIIB, TBP, $TFIIIB_{70}$ or B″ (Dieci *et al.*, 1993, 1995). In our recombinant system, TFIIIE could not replace B''_{90}, but stimulated transcription of the SUP4 tRNA as well as the SNR6 genes. TFIIIE also stimulated 3- to 4-fold a transcription system reconstituted with TBP and TFIIIB* (depleted of TBP by Q-Sepharose chromatography; J.Huet, unpublished data). The mode of action of TFIIIE is still unclear. The factor may increase the rate of assembly of TFIIIB, or stabilize the complex throughout multiple rounds of transcription; it could act as a chaperone to favor the unmasking of the DNA binding domain(s) postulated in $TFIIIB_{70}$ and B''_{90}. It is intriguing that fully recombinant TFIIIB needed to be supplemented with TFIIIE, whereas a similar system containing gel-purified B''_{90} apparently did not (Joazeiro *et al.*, 1994). A simple explanation would be that the B″ fraction used in these reconstitution experiments had a low content of TFIIIE. In that case, gel-purified B''_{90} should provide the same basal level of transcription activity as the control reaction containing B″. Alternatively, in keeping with the chaperone hypothesis, it could be that the gel-isolated polypeptide had adopted the active conformation state spontaneously through the renaturation procedure. In our recombinant system, $TFIIIB_{70}$ and B''_{90} were never subjected to a denaturation–renaturation step: Ni^{2+} adsorption chromatography was performed on soluble proteins from the bacterial extract.

The comparison of the yeast TFIIIC components and their higher eukaryotic counterparts first suggested an important evolutionary divergence. The two largest subunits of human TFIIIC are unrelated to the known subunits of yeast TFIIIC (L'Etoile *et al.*, 1994; Sinn *et al.*, 1995). The recent isolation of the human homolog of $yTFIIIB_{70}$, however, suggests that the basic mechanisms of TFIIIB assembly, promoted by TFIIIC, have been conserved (Wang and Roeder, 1995). At variance with yTFIIIB, the minimal human TFIIIB factor for *in vitro* transcription of 5S RNA, tRNA or VA1 RNA genes is composed of only two polypeptides, hTBP and $hTFIIIB_{90}$ ($yTFIIIB_{70}$ homolog). As this minimal factor does not represent the complete TFIIIB for transcription of other class III genes like U6 and 7SK RNA genes, the existence of a mammalian homolog to B''_{90} is a likely possibility. While this paper was being reviewed, the same gene encoding B''_{90} was published by Kassavetis *et al.* (1995) and named *TFC5*. We will from now on adopt this gene name.

Materials and methods

Yeast strains and methods

YOL8 (Lefebvre *et al.*, 1994) was used for suppression studies; YNN281, YNN282 (Heinemeyer *et al.*, 1993) for gene disruption, and YJR3 (MATa, *ade2-101, lys2-801, trp1-Δ1, his3-Δ200, tfc7::HIS3*, pJR38-*TFC7*) for complementation experiments.

Lithium acetate transformation was performed as described by Ito *et al.* (1983) and Gietz and Schiestl (1991). Preparation of media, tetrad dissection and other yeast methods were performed by standard techniques (Ausubel *et al.*, 1994).

Genetic isolation of TFC7

Multicopy suppressors of the strain YOL8, carrying a ts mutation in *TFC3* have been isolated as described in Lefebvre *et al.* (1994) and partially sequenced. One of the isolated plasmids (pLR204) carried an insert of 4.2 kb harboring an ORF which lacked its 3′ end, thus coding for a protein of 64 kDa (an in-frame stop codon was found 60 bases downstream of the insertion site). The nucleotide sequence of the region of this gene (including the complete ORF N2682 of 1785 bp) was determined in the course of the European Union BIOTECH program to sequence the entire chromosome XIV and isolated as a 3.9 kb *Xba*I fragment of cosmid C14-17 on pBS-C14-17/X1 harboring ORF N2682. A *Sac*I–*Bam*HI digestion of pBS-C14-17/X1 inserted into the corresponding sites of YEp352 (Hill *et al.*, 1986) gave pJR38.

Sequence analysis

DNA sequencing was performed as part of the framework of the European Union BIOTECH program to sequence the entire chromosome XIV. Overlapping subclones in plasmid cloning vectors were generated from regions of cosmid 14-17 by the nested deletion method (Henikoff, 1984). Double-stranded template DNA from deletion subclones was purified using the QIAwell 96 Purification System (QIAGEN). Cycle sequencing reactions were carried out using *Taq* DNA polymerase, fluorescent terminators and primers, and run on an ABI 373A Stretch Sequencer (Applied Biosystems). Contig assembly and DNA sequence editing was done using the DNASTAR software package (Lasergene).

Homology between human, mouse and *C.elegans* sequences and TFC7 was revealed by comparison of complete and partial amino acid sequences using the BLAST program (Altschul *et al.*, 1990).

Disruption of TFC7

A *TFC7* disruption vector (pJR36) was constructed by replacing the *Eco*RV–*Nde*I fragment of *TFC7* with a *Bam*HI fragment of *HIS3* (surrounded by stop codon modules) of the plasmid YDpH (Berben *et al.*, 1991). An *Xma*I disruption fragment of pJR36 was used to transform a *his3/his3* diploid strain constructed by crossing strains YNN281×YNN282. The structures of His^+ disruptants were verified by Southern analysis. One of these disruptants was transformed with pJR38. After sporulation and dissection, a His^+ spore (harboring also pJR38) was chosen to give YJR3.

Expression of TFC7 in E.coli

For construction of *TFC7* expression vectors, restriction sites were introduced upstream (*Xma*I–*Not*I) and downstream (*Eco*RI) of the *TFC7* reading frame by PCR-mediated mutagenesis, followed by ligation of the PCR product into the vector pGEM-T (Promega). In order to test functionality of the PCR product, the gene was isolated from pGEM-T-*TFC7*, fused to a GAL1 promoter on a high copy vector and transformed into YOL8, where suppressor activity on galactose media could be confirmed. The same construct, transformed into YJR3, was able to replace the plasmid pJR38 harboring *TFC7*.

The *E.coli* expression vector pJR46a was constructed by introducing the *TFC7* gene on a *Not*I fragment isolated from pGEM-T-*TFC7* into pET-28b (Novagen). This construct was transformed into the *E.coli*

strain BL21(LysS) for expression of TFC7p fused at its N-terminus to six histidines and the T7·Tag. An overnight culture of BL21(LysS) transformed with pJR46a was diluted 250-fold in 200 ml of fresh medium and grown for ~2.5 h at 37°C before adding isopropyl-β-thiogalactoside (IPTG) to 0.55 mM. After a further 2 h of growth at 30°C, the cells were harvested and proteins were purified by a Ni^{2+} batch column, essentially as described in Chaussivert *et al.* (1995). Western analysis was performed to monitor rTFC7 purification by T7·Tag™ antibodies.

DNA binding assays

Transcription factor–tDNA interaction was monitored by gel retardation assay, essentially as described (Kassavetis *et al.*, 1990; Huet and Sentenac, 1992). TFIIIC (50 ng, affinity-purified) was incubated with a 280 bp ^{32}P-labeled *Bam*HI–*Cla*I DNA fragment from the pRS316-SUP4 plasmid carrying the yeast SUP4 $tRNA^{Tyr}$ gene (3–10 fmol; ~3000–10 000 c.p.m.) and 100 ng of competitor DNA in 15 μl of binding buffer containing 20 mM Tris–HCl (pH 8), 1 mM EDTA (pH 8), 125 mM KCl, 2.5 mg/ml bovine serum albumin, 10% glycerol. After 10 min incubation at 25°C, 5 μl of mixtures containing proteins and carrier DNA in an ~1:1 (w/w) ratio were added and the final reaction (adjusted to a concentration of 150 mM KCl) was further incubated for 15 min at 25°C. Mixtures contained combinations of 130 ng of $rTFIIIB_{70}$, 40 ng of rTBP, 0.6 mg of B″ fraction or 50 ng of rTFC7p as indicated. Complexes were analyzed by non-denaturing gel electrophoresis in 5% polyacrylamide gels (Gabrielsen *et al.*, 1989b).

In vitro transcription assay

Plasmid DNAs used for *in vitro* transcription reactions were pRS316-SUP4, harboring the yeast SUP4 tRNA gene, and pTaq6, harboring a truncated form of the yeast U6 RNA gene lacking the extragenic B box (Brow and Guthrie, 1988). Transcription reactions were carried out in a final volume of 50 μl and contained 20 mM Tris–HCl pH 7.9, 90 mM KCl, 5 mM $MgCl_2$, 10% glycerol (v/v), 0.1 mM EDTA, 0.5 mM each of ATP, CTP and GTP, 30 μM UTP and 10 μCi of [α-^{32}P]UTP, 250 ng of plasmid DNA, 50 ng of affinity-purified TFIIIC (Gabrielsen *et al.*, 1989a), 40 ng of rTBP (Burton *et al.*, 1991), 50 ng of $rTFIIIB_{70}$ (Colbert and Hahn, 1992), 0.4 mg of B″ fraction (Kassavetis *et al.*, 1992) or 30 ng of rTFC7p, 0.5 μg of TFIIIE fraction (Dieci *et al.*, 1993) and 50 ng of RNA polymerase III. Reactions programed with the U6 gene did not contain TFIIIC. The template was first pre-incubated (15 min at 22°C) with TFIIIC and the TFIIIB components (or with TFIIIB components only, in the case of U6 RNA gene) to allow for the formation of stable pre-initiation complexes. Pol III and NTPs were then added and transcription was allowed to proceed for 30 min at 22°C. Synthesized RNAs were visualized after electrophoretic separation in 8 M urea, 6% polyacrylamide gels and autoradiography.

GAL1–lacZ activation assay

Two-hybrid system vectors carrying *TFC7* have been constructed by digestion of pGEM-T-*TFC7* by *Xma*I, filling-in by Klenow polymerase and subsequent digestion by *Sal*I. The isolated fragments were cloned into pAS2 and pACT2 (Harper *et al.*, 1993). Correct in-frame fusion of the gene was confirmed by sequencing and Western analysis.

Nine independent transformants for each combination of plasmids were grown as patches for 2 days at 30°C on SC solid medium containing 2% raffinose as carbon source. β-Galactosidase activity was revealed by overlaying the cells with X-gal agar and incubating the plates for 24 h at 37°C. β-Galactosidase activity was measured in yeast extracts as described previously (Werner *et al.*, 1993).

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