TFIIF–TAF–RNA polymerase II connection

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RNA polymerase transcription factor IIF (TFIIF) is required for initiation at most, if not all, polymerase II promoters. We report here the cloning and sequencing of genes for a yeast protein that is the homolog of mammalian TFIIF. This yeast protein, previously designated factor g, contains two subunits, Tfg1 and Tfg2, both of which are required for transcription, essential for yeast cell viability, and whose sequences exhibit significant similarity to those of the mammalian factor. The yeast protein also contains a third subunit, Tfg3, which is less tightly associated and at most stimulatory to transcription, dispensable for cell viability, and has no known counterpart in mammalian TFIIF. Remarkably, the *TFG3* gene encodes yeast TAF30, and furthermore, is identical to *ANC1*, a gene implicated in actin cytoskeletal function in vivo (Welch and Drubin 1994). Tfg3 is also a component of the recently described mediator complex (Kim et al. 1994), whose interaction with the carboxy-terminal repeat domain of RNA polymerase II enables transcriptional activation. Deletion of *TFG3* results in diminished transcription in vivo.

[Key Words: RNA polymerase II; TFIIF; TFIID; TAF; transcription; *Saccharomyces cerevisiae*]

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Five purified proteins, termed factors a, b, d, e, and g, are required for promoter-dependent transcription by purified yeast RNA polymerase II (Sayre et al. 1992). Factors a, b, and e are structurally and functionally related to human RNA polymerase II transcription factor (TF) TFIIE, TFIIF, and TFIIB, respectively (Gileadi et al. 1992; Tschochner et al. 1992; Feaver et al. 1994). Factor d can be replaced by recombinant TATA-binding protein (TBP; Flanagan et al. 1990) in support of basal transcription in vitro. A complex fraction containing TBP and associated factors (TAFs; Poon and Weil 1993) enables activated transcription in vitro (D. Poon, Y. Bai, A.M. Campbell, S. Bjorklund, Y.-J. Kim, S. Zhou, R.D. Kornberg, and P.A. Weil, in prep.). Factor g was shown previously to resemble TFIIF in its content of two essential polypeptides and its avid binding to purified RNA polymerase II (Henry et al. 1992). There were, however, notable differences between the yeast and human factors, such as the larger size of the factor-g polypeptides (105 and 54 kD, as compared with 74 and 30 kD for human TFIIF; Sopta et al. 1985) and the presence of a third subunit (30 kD) in the yeast factor that appeared to stimulate transcription but not to be required for the process. We report here the cloning and sequencing of genes for factor-g polypeptides, which clarifies the relationship to TFIIF and opens the way to genetic analysis of the factor in yeast. Our findings further disclose a surprising connection be-

tween basal and regulatory factors, which may shed light on mechanisms of transcriptional activation.

Results

Genes for factor-g subunits

Factor g was purified to homogeneity, and the three subunits were separated by reverse phase chromatography as described previously (Henry et al. 1992). The individual subunits were treated with either trypsin or endoprotease Lys-C, and amino acid sequences were derived from the resulting peptides. For the 30-kD subunit (p30), one peptide sequence of 34 residues (underlined in Fig. 1A) corresponded to open reading frame 2 in the GenBank data base entry for the yeast gene *TBF1* (Brigati et al. 1993). This open reading frame, located downstream of the *TBF1* gene on the noncoding strand, included all 34 residues of the peptide sequence but did not encode the entire protein. Based on the nucleotide sequence in the data base entry, polymerase chain reaction (PCR) primers were designed and a fragment was amplified from genomic DNA that was used to screen a yeast genomic library. A full-length clone was identified, and the coding region was sequenced (Fig. 1A). The occurrence of an intron between codons three and four was apparent from comparison of the deduced amino acid sequence with the amino-terminal sequence of the protein, as well as the presence of canonical yeast splice sites. Splicing at these sites was confirmed by the cloning and sequencing

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Figure 1. (See following page for legend.)

of a full-length cDNA. The gene *TFG3* encodes a protein of 244 amino acids with a molecular mass of 27.4 kD, close to that determined by SDS-PAGE of 30 kD. The amino-terminal sequence and all five peptide sequences determined for p30 were present in the complete amino acid sequence.

For the 54-kD subunit of factor g (p54), degenerate primers were designed on the basis of peptide sequences (underlined in Fig. 1B), and PCR was performed using the "touchdown" method (Don et al. 1991) to limit spurious priming (see Materials and methods). A yeast genomic library was screened with an amplified fragment, two clones were isolated, and the coding region was completely sequenced (Fig. 1B). The gene *TFG2* encodes a protein of 400 amino acids with a predicted molecular mass of 46.6 kD. All peptide sequences determined for p54 were present in the amino acid sequence.

A PCR fragment was obtained for the 105-kD subunit of factor g (p105) in the same manner as for p54, but the screening of a yeast genomic library (Gileadi et al. 1992) was unsuccessful. The PCR fragment was then used to screen a yeast cDNA library, and a single clone was isolated. The coding region was completely sequenced and proved to contain a frameshift mutation, so a 400-bp fragment of this clone was used to screen a yeast genomic library in the λ YES vector (Ramer et al. 1992). Five independent clones were obtained, and the open reading frame of one was completely sequenced (Fig. 1C). The gene *TFG1* encodes a protein of 735 amino acids with a mass of 82.2 kD, somewhat smaller than that from SDS-PAGE of 105 kD. All sequences determined from peptides were present in the amino acid sequence.

Blot hybridization of yeast genomic DNA with fragments of coding regions of *TFG1*, *TFG2*, and *TFG3* (data not shown) indicated that all three genes occur in a single copy in yeast. Blot hybridization of poly(A)⁺ RNA (data not shown) showed that each gene gives rise to a single mRNA in yeast. The deduced amino acid sequences of Tfg1 and Tfg2 proteins contain a high proportion of charged residues, which are broadly distributed rather than concentrated in specific regions, whereas Tfg3 protein is rich in charged residues and threonine. Tfg1 protein contains an acidic region (38% aspartic acid and glutamic acid), located between residues 455 and 580. No zinc finger, kinase, or other motifs were detected in any of the sequences, except for a number of potential phosphorylation sites located throughout each protein.

Null mutations

Yeast strains were constructed in which either the entire *TFG2* gene or a portion of *TFG1* or *TFG3* was deleted and replaced with *URA3*. The consequences of the deletions

were determined by sporulation and tetrad dissection (data not shown). In the case of the *TFG1* and *TFG2* deletions, only two spores per tetrad germinated on rich medium and formed colonies, which were *ura*⁻, indicating that both *TFG1* and *TFG2* are essential for cell viability. In the case of the *TFG3* deletion, all four spores of a tetrad were viable on rich medium, two of which were *ura*⁻. The *ura*⁺ colonies were much smaller than the wild-type colonies on rich medium, indicating that although *TFG3* is not essential for cell viability, the null mutant strain grows more slowly, which was confirmed by the measurement of generation times in liquid culture, and is inviable at 37°C.

Homology to TFIIF

Searches of the Swiss-Prot data base (release 28) with the Tfg1 and Tfg2 sequences revealed homologies to TFIIF. Tfg1 exhibits 26.6%, 28.1%, and 27.5% identity and 49.9%, 50.2%, and 50.3% similarity to the proteins for the large subunits of human (Aso et al. 1992; Finkelstein et al. 1992), *Drosophila* (Gong et al. 1993; Kephart et al. 1993), and *Xenopus* (Gong et al. 1992b) TFIIF (RAP74), respectively (Fig. 2A; data not shown). Tfg2 is 30.6%, 30.3%, and 28.8% identical and 50.8%, 51%, and 51.5% similar to the proteins for the small subunits of human (Sopta et al. 1989; Horikoshi et al. 1991), rat (Garrett et al. 1992; Kobayashi et al. 1992), and *Xenopus* (Gong et al. 1992a) TFIIF (RAP30), respectively (Fig. 2B; data not shown). Tfg2 is also 28.6% identical and 50.7% similar to the *Escherichia coli* σ^{70} protein and 22.9% identical and 45.1% similar to *E. coli* σ^{32} (data not shown), both of which were reported previously to have homology to mammalian RAP30 (Garrett et al. 1992). After *TFG3* was cloned and sequenced, it was found to be identical to *ANC1* (EMBL accession number Z26040), a gene implicated in actin cytoskeletal function (Welch and Drubin 1994).

Recombinant factor g

To demonstrate that the cloned genes encode functional subunits of factor g, *TFG2* and *TFG3* were expressed in bacteria, with six-histidine tags fused to the amino termini of the proteins to facilitate purification. *TFG1* could not be subcloned into an expression vector, perhaps because it is toxic to *E. coli*. Purified Tfg3 protein was nearly homogeneous, whereas purified Tfg2 protein had one major contaminant, a 30-kD degradation product (Fig. 3A). Both bacterially expressed proteins were slightly larger than the corresponding subunits of factor g purified from yeast (Fig. 3A), probably reflecting either the addition of the six-histidine tag or proteolysis during

Figure 1. Nucleotide and deduced amino acid sequences of the genes for factor g. (A) Genomic sequence of *TFG3* (GenBank accession number U13017) that encodes p30. The intron is from nucleotides 124 to 228, located between amino acids 3 and 4. Amino acid sequence is shown below the nucleotide sequence. Residue numbers are on the right. Restriction sites used for creation of the null mutants are indicated in italics. Peptide sequences used for cloning are underlined. (B) *TFG2*, gene for p54 (GenBank accession number U13016); notation as in A. (C) *TFG1*, gene for p105 (GenBank accession number U13015); notation as in A.

A

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81 ADAGRSNVKVKDEDPNEYEPFLRAIPKEDLENMRTHLLKFSKKKINPV Y
   | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
2 AALGPSSQNV.....TEYVVR.VPKN..TTKYNIMAFNAADKVN.. H
131 TDFHLPVRLHRRKDTNQLPQLTRAIVQRQKEISEYKKAAEQERSTPNSG Y
   | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
39 ..F.....ATWN.QARLER.....DLSN..KKIYQEEEMFESSG H
181 GNNKSGTSLNNTVKDGSQTPPTVDSVTIKNTANGVNSIPTVTGSSVPPA Y
   . . . . . | . | . | . | . | . | . | . | . | . | . | . |
67 ....AGSEFNRKLRREARRKKYIGVLKEFRPED.QFWLLRVNGSKGRKF H
231 SPTTVSAIESNG.....LSNGSTSAANGLDGNA..STANLANGRLVTKLE Y
   . . . . . | . | . | . | . | . | . | . | . | . | . | . |
111 KGIKKGGVTENTSYITTCQPDGAPEAFVPHNWNPTFLARHRTLTA..E H
275 DAGFA.EDPTKVMGVKYDGKVTNEPEPEEGTMDPLADVAPDGGGRAKRG Y
   : : : : : | . | . | . | . | . | . | . | . | . | . | . |
159 EAESEWERRRNKV.LNHF....SIMQRRLLKQDQD.....EDESEKRRG H
324 NLRKTRQLKVLNDENAKKLRFEFPYVPMWDFDGYNTWVGSYEAAGNSDY Y
   | | | . | . | . | . | . | . | . | . | . | . | . | . |
199 ..RKASBLRHD.....LE.....DDLE.....MSS..DASDA.. H
374 VLLSVEDDGSPTMIPADKYKFTARNKYATLTIDEAKRMDKKSSEVPRW Y
   . | . | . | . | . | . | . | . | . | . | . | . | . | . |
224 ..SGSEGG...RIFKAK.....KKAPLA..KGRKKKKKKG.... H
424 LMKHLDNIGTTTTRYDRTRKRLKAVADQAMDEDDRDENSEVELDYDEP Y
   . | . | . | . | . | . | . | . | . | . | . | . | . | . |
253 .....SDDEAFEDSDGDPEGGQVDY.... H
474 ADDEEAPIIDNGEQENKESQRIKEMLOANAMOLRDEEAPSENEDEL F Y
   . | . | . | . | . | . | . | . | . | . | . | . | . | . |
274 .....MSDSSSSQSEEPESKAKAPQEGKGVDEQSDSESESEKRP H
524 GEKKIDEDGERI....KKALQKTELAALYSSDENEINPYLSESDIENKE Y
   . | . | . | . | . | . | . | . | . | . | . | . | . | . |
317 PEEDKSESEKKAAPTQSEKKRRKDSSESDSESDIDSEASSALFMAKK H
569 NESPVKKEEDSDTLSEKSRSSPKKQKATNAHVHKEPTLRVKSIRKNCVI Y
   . | . | . | . | . | . | . | . | . | . | . | . | . | . |
367 KTFPKREKRPSSG...SSRGNRSGPTSAEGG..STSTLRAAASK.... H
619 ILKGDKKILKSPFEGEWNPQTTKAVDSNNASNTVPSPIKQREGLNSTVA Y
   | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
408 .LEQGRV.SEMPAAXRLRLDTGPGSLGKSTPQPPSGKTTFNSGDVQVT H
669 E....REESTPAPTITERDIIIEAGDGKVNKEFGKPIRRKYPGAENKLM Y
   | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
456 EDAYRRLYLRKP.MTTKDLLKKFQTKKTGL.....SSEQTVNV L H
715 FAIVKKLC..RKVGNHMLK.KE Y
   . | . | . | . | . | . | . | . | . | . | . | . | . | . |
494 AQILKRLNPERKMINDRMGHPSLKE H

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B

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46 ENNETKVYEESLDLERSNRQVWLRLPMLAEKWRDRNNLHGQELGKI Y
   | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
3 ERGE.....LDLTGAKQNTGVWLKVPKYLSSQW.AKASGRG.EVGKL H
96 RINKDGSK..ITLLNENDNDSIPHEYDLELTKKVVENSVYVTEQNLKKY Y
   | | . | . : : : | | | . | . | . | . | . | . | . | . |
44 RIAKTQGRTEVSPTLNE.....DLA.....NIHDI H
144 QQRKKELEADPEKQRAYLKKQEREELKKKQKQKRRNRKKNHRVMT Y
   . . . . | . | . | . | . | . | . | . | . | . | . | . |
69 GGPASVSAPRE.....HPFVL H
194 DRDGRDRYIPYVKTIPKKAIVGTVCH..EQVMPSEMDPHYHKI.VEQR Y
   . | . : : . . . . . | . : : | . : : | . : : | . : : |
86 QSVGGQTLTVPTESDDKLSLEGIVVQRAECRPAASENTMRLLKRLQIES H
241 RNIVKLNNKERITLDELTVGVTMSHTGMSMRSDNSNLFVGREKAKSNIK Y
   . | . | . . | . | . | . | . | . | . | . | . | . | . |
136 SKPVRLSQQ....LDK..VVTNYKPVANHQYNIET.....KKKEDGK H
291 SIRMPKKEILDYLFKLFDEYDYWSLKLKERTROPEAHLKCELDKVAIV Y
   . | . | . | . | . | . | . | . | . | . | . | . | . | . |
175 RARADQHVLDMLFSAPEKKHQYTNLKDLDVITKQPVVYLKELIKVQGN H
341 KKGYPAPKYTLRPEYKLLKEBERKA Y
   | | . . . | . | . | . | . | . | . | . | . | . | . |
225 VKGIHKNTELKPEYRHYQGEKSD H

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Figure 2. Sequence similarity between yeast factor g and human TFIIF. (A) Alignment of Tfg1 and human RAP74 sequences using the BESTFIT computer alignment program (see text). Identical amino acids (vertical line), amino acids whose comparison value is ≥ 0.5 (colon), and amino acids whose comparison values is ≥ 0.1 (dot) are indicated. Sequences are identified as yeast (y) and human (h) on the right, and residue numbers are indicated on the left. (B) Alignment of Tfg2 and human RAP30 sequences. Notations are as in A.

purification from yeast. When tested in a reconstituted transcription assay (Fig. 3B), neither Tfg2 nor Tfg3 was able to function alone in the absence of other factor-g subunits. Tfg2 was active, however, in the presence of p105, resolved from the other subunits of factor g in the denatured state, and renatured for transcriptional analysis. The further addition of Tfg3 was slightly stimulatory. These findings were consistent with those obtained previously with the renatured subunits of factor g, in which the combination of p54 and p105 yielded transcription activity that was slightly enhanced by the addition of p30 (Henry et al. 1992).

In the absence of function of either Tfg1 or Tfg3 in transcription in vitro, evidence for the identity of p105 with Tfg1 and p30 with Tfg3 was sought by raising antibodies against both bacterially expressed Tfg1-glutathione *S*-transferase (GST) fusion protein and Tfg3. The anti-Tfg1 antibodies reacted with p105, and the anti-Tfg3 antibodies reacted with both p30 and the bacterially expressed Tfg3, whereas the preimmune sera failed to react with any of the proteins (Fig. 3C). We conclude that p105 and p30 are the products of the *TFG1* and *TFG3* genes, respectively.

Association of Tfg3 with TFIIF

Because Tfg3 had little effect on factor g function in tran-

scription and, furthermore, because it could be partially resolved from the two larger subunits by gel filtration or glycerol gradient sedimentation (Henry et al. 1992), the question arose whether Tfg3 is associated with the other subunits or whether it merely comigrates during factor-g purification. Two findings establish that Tfg3 is truly a component of factor g. First, Tfg3 could be immunoprecipitated from whole cell extracts by antibodies against Tfg1, and conversely, Tfg1 was immunoprecipitated by antibodies against Tfg3 (data not shown). Immunoprecipitation of all three subunits of factor g by anti-Tfg3 antibody was demonstrated with a more purified fraction (data not shown). Second, 35 S-labeled Tfg3 interacted specifically with Tfg1, both in solution (Fig. 3D) and in blots (Fig. 3E). Binding in solution was assessed with Tfg1 as renatured p105 or as p105/p54 complex isolated from the *tfg3* null strain. 35 S-Labeled Tfg3 was coimmunoprecipitated by anti-Tfg1 antibodies in the presence of either form of Tfg1 but not in its absence.

Association of Tfg3 with other transcription protein assemblies

The *TFG3* sequence proved to encode three peptides derived from yeast TAF30: IKTQQHILPEVPPV (residues 11–24), KIPHDNLNLFQES (residues 100–111), and

Figure 3. Analysis of recombinant factor-g polypeptides. (A) SDS-10% polyacrylamide gel of 1 μ g of bacterially expressed Tfg3 (lane 1), 4 μ g yeast factor g (lane 2), and 4 μ g of bacterially expressed Tfg2 (lane 3). Molecular mass markers (sizes in kD) are indicated at left. (B) Transcription assay of factor-g polypeptides. Reactions contained 150 ng of yeast factor g, 1.5 μ l of renatured p105 subunit (fxn 45), and 25 ng each of bacterially expressed Tfg2 and Tfg3, as indicated above the lanes. (C) Western blot of factor-g polypeptides. (Lanes 1,3) 100 ng of bacterially expressed Tfg3; (lanes 2,4-6) 400 ng of yeast factor g. The blots were incubated with preimmune (for Tfg3), anti-Tfg3, preimmune (for Tfg1), or anti-Tfg1 serum as indicated. (D) Immunoprecipitation demonstrating association of Tfg3 with factor g and direct interaction with p105. (Lane L) 35 S-labeled Tfg3. All reactions contained labeled Tfg3 and anti-Tfg1 antibody. (Lane 1) No factor g; (lane 2) p105/p54; (lane 3) renatured p105. (E) Far Western blot of yeast factor g showing direct interaction between p105 and 35 S-labeled Tfg3. Molecular mass markers (in kD) are indicated at left; the position of p105 is shown at right.

SLWDYVK (residues 234–240). The identity of Tfg3 with yTAF30 was shown further by the following. First, the two proteins comigrated in SDS-PAGE (Fig. 4A). Second, affinity-purified anti-Tfg3 antibodies reacted specifically with yTAF30 in blots of yeast TAF preparations (Fig. 4B). Third, Tfg3 could be immunoprecipitated from whole-cell extracts with anti-TBP antibodies, and conversely, TBP could be immunoprecipitated with anti-Tfg3 antibodies (Fig. 4C). Neither TBP nor Tfg3 was immunoprecipitated from whole-cell extract in control reactions containing either preimmune serum or an unrelated monoclonal antibody (data not shown). Finally, Tfg3 was found to be associated with yTAF90 and yTAF130, but not with yTAF170, as revealed in both immunoblots and stained gels (Fig. 4D,E), consistent with previous evidence for the occurrence of these proteins in distinctive complexes with TBP (Poon et al. 1994; D. Poon, Y. Bai, A.M. Campbell, S. Bjorklund, Y.-J. Kim, S. Zhou, R.D. Kornberg, and P.A. Weil, in prep.).

Tfg3/TAF30 is also regarded as a component of the multiprotein mediator of transcriptional activation, as both factor-g activity and polypeptides of the sizes expected for the three subunits of factor g were detected in mediator (Kim et al. 1994). The presence of Tfg3 in mediator and in the complex of mediator with core RNA polymerase II, termed holo-RNA polymerase II (Kim et al. 1994), was confirmed by immunoblotting (Fig. 4B). Direct binding of factor g to core polymerase has been demonstrated (Henry et al. 1992), but no Tfg3 could be detected in purified core RNA polymerase preparations by immunoblotting (data not shown).

Tfg3 is involved in transcription in vivo

Transcriptional activation in a fully defined system in vitro requires either TAFs (D. Poon, Y. Bai, A.M. Campbell, S. Bjorklund, Y.-J. Kim, S. Zhou, R.D. Kornberg, and P.A. Weil, in prep.) or mediator (Kim et al. 1994). These apparently disparate mechanisms are now related through the common occurrence of Tfg3/TAF30 in both TFIID and mediator. Whether activated transcription actually depends in any way on Tfg3/TAF30 was investigated with the use of a *CYC1-lacZ* reporter gene in wild-type and *tfg3* null mutant strains in vivo. Both galactose-inducible (Gal4 protein-binding) and constitutive (thymidine-rich) upstream activating sequences (UASs) remained effective in the null mutant strain, but the overall level of transcription was reduced five to sixfold (Table 1). In the absence of a UAS, the level of transcription fell below the level of detection. This significant but not absolute dependence on Tfg3/TAF30 is consistent with the diminished growth rate, but nonetheless viability, of the null mutant strain.

Discussion

The evidence presented here, together with that from previous work (Henry et al. 1992), identifies general transcription factor g as the counterpart in yeast of TFIIF in human cells: Both the yeast and the human protein contain two subunits essential for transcription in vitro; both proteins bind tightly and specifically to purified RNA polymerase II (Sopta et al. 1985; Flores et al. 1989);

Figure 4. Tfg3 is a component of yeast TFIID. (A) Yeast factor-g subunit p30 (lane 1) comigrates with yTAF30 (lane 2) in a SDS-10% polyacrylamide gel. Molecular mass markers (in kD) are at left; the 30-kD protein bands are indicated at right. (B) Affinity-purified anti-Tfg3 antibodies react with 30-kD polypeptides in holo-RNA polymerase II (lane 1), mediator (lane 2), factor g (lane 3), and TFIID (lane 4). Holo-RNA polymerase II and mediator were purified as in Kim et al. (1994), and factor g and TFIID were as in A. Molecular mass markers (in kD) are at left. (C) Tfg3 is associated with TBP in whole-cell extracts. Extracts were immunoprecipitated with antibodies against either Tfg3 (lanes 1,3) or TBP (lanes 2,4) and immunoblotted with these antibodies as indicated. (D) Tfg3 is associated with RNA polymerase II-specific yeast TAFs. Whole-cell extracts prepared from yeast strains expressing the indicated HA-tagged proteins were immunoprecipitated with monoclonal antibody 12CA5 and immunoblotted with antibodies against Tfg3. (E) Yeast TAFs can be immunoprecipitated by antibodies against Tfg3. Antibody against Tfg3 (lane 1) or mAb 12CA5 (lane 2) was used to immunoprecipitate yeast TAFs from a complex fraction. The precipitates were electrophoresed in a SDS-10% polyacrylamide gel. Molecular mass markers (in kD) are at left.

and the yeast proteins, Tfg1 and Tfg2, exhibit ~50% sequence similarity to the human proteins, RAP74 and RAP30, respectively. Tfg2, like RAP30 [McCracken and Greenblatt 1991; Garrett et al. 1992], is also ~50% similar in sequence to *E. coli* σ^{70} .

The sequence homologies noted here are found particularly in regions near the amino and carboxyl termini of the proteins, as well as in an acidic region in the middle of Tfg1 and RAP74 [Aso et al. 1992]. For Tfg2 and RAP30, the best sequence alignment was between a subterminal region (ending at residue 365 out of a total of 400) of the yeast protein and the extreme carboxyl terminus of the human protein (Fig. 2B). However, the extreme carboxyl

termini of both proteins (residues 366–400 of Tfg2) exhibited greatest homology with region 4.2 of bacterial σ factors (Fig. 5; for homology to rat RAP30, see Garrett et al. 1992). It may well be that the extreme carboxyl terminus, rather than the subterminal region of Tfg2, is the functional homolog of the carboxyl terminal region of RAP30.

Although *TFG3* showed no significant sequence homology to genes encoding transcription factors, it proved to be identical to a known gene, *ANC1*, implicated in cytoskeletal function. *ANC1* was identified in a screen for mutants failing to complement temperature-sensi-

Table 1. Effect of *tfg3* deletion on transcription in vivo

Plasmid	UAS	– Galactose		+ Galactose	
		wild type	<i>tfg3</i>	wild type	<i>tfg3</i>
pCZA	None	2.0	<1	2.3	<1
pCZGAL	<i>GAL10</i>	4.0	<1	40.7	7.4
pCZ(DED48) ²	(dA : dT) ²	42.6	6.9	49.8	12.0

Single-copy reporter plasmids containing *CYC1-lacZ* fusions with the indicated UAS elements were introduced into either wild-type or *tfg3* null strains and grown in the presence or absence of galactose.

	Region 4.2	
Tfg2	TLGELADEQ TGSAGD NAQGD AEA . . DLEDEIEMEDVV	400
σ^{32}	TLQELADRYGVSAERVRQLEKNAMKKLR AAE	283
σ^{70}	TLEEVGKQFDVTRERIRQIEAKALRKL RHP . SRSEVL	607
σ^K	TQREIAKELGISRSYSVSRIEKRALMKMPHEFYRAEK	131

Figure 5. Sequence homology between Tfg2 and region 4.2 of bacterial σ factors. Alignment of the carboxy-terminal residues of Tfg2 with the carboxy-terminal residues of *E. coli* σ^{70} and σ^{32} , and *Bacillus subtilis* σ^K (*spoIIIC* gene). Boldface type indicates similar and identical amino acids. The residue number is indicated at right. The following sets of amino acids are chemically similar: S and T; A and G; F, W, and Y; I, L, M, and V; D, E, N, and Q; H, K, and R.

tive mutations in actin (Welch et al. 1993). Mutations in the *ANC1* gene also cause osmosensitivity and defects in actin organization. A data base search identified two human proteins, ENL (Tkachuk et al. 1992; Yamamoto et al. 1993) and AF-9 (Nakamura et al. 1993), as having possible amino- and carboxy-terminal sequence homology to Tfg3/TAF30 (22.7% and 26.2% identity, 49.4% and 47.8% similarity to ENL and AF-9 proteins, respectively; Fig. 6). Both the *ENL* and *AF-9* genes were revealed by chromosomal translocations in acute leukemias causing their fusion to the trithorax gene. Both genes contain regions rich in serine and charged residues, similar to those found in some transcriptional activators, and it has been proposed that fusion of ENL to trithorax, which contains DNA-binding motifs, results in a rogue activator protein (Tkachuk et al. 1992). Our findings raise the alternative possibility that fusion of ENL and AF-9 to trithorax brings about persistent recruitment of TFIID and RNA polymerase II holoenzyme to polymerase II promoters, causing inappropriate transcriptional activation.

Whatever the significance of the homology between Tfg3 and both ENL and AF-9, two recent biochemical studies raise the possibility of factor-g/TFIIF function in activated as well as basal transcription. In the first study, low amounts of TFIIF were sufficient for basal transcription in vitro, whereas additional TFIIF enabled activation by serum response factor (SRF) and GAL4-VP16, but not Sp1 (Zhu et al. 1994). TFIIF also relieved squelching by SRF, and RAP74 bound to DNA interacted with either SRF or GAL4-VP16. The second study identified TFIIF as a stoichiometric component of a multiprotein mediator complex that enables a response to both GAL4-VP16 and GCN4 activator proteins in transcription in vitro (Kim et al. 1994). To these lines of evidence must now be added the equivalence of the Tfg3 subunit of TFIIF to TAF30, whose association with TBP in TFIID may be instrumental in activated transcription. Tfg3/TAF30 protein seems unlikely to mediate a physical

linkage between TFIIF and TFIID, inasmuch as the two complexes are discrete and both contain a stoichiometric amount of the protein. Rather, the existence of a common subunit points to common functional requirements, such as interaction with other components of the transcription apparatus or coupling to regulatory processes.

Materials and methods

Purification and amino acid sequencing of factor-g subunits

Factor g was purified to homogeneity, and the subunits were separated by reverse-phase chromatography as described previously (Henry et al. 1992). Amino-terminal sequencing, tryptic digestion, and peptide sequencing were performed by J. Kenny (PAN Facility, Stanford University, CA), and endoproteinase Lys-C digestion and peptide sequencing were performed by William S. Lane (Harvard Microchemistry Facility, Cambridge, MA).

Cloning of TFG3

A single long peptide sequence, LNEDDLGVVQMVTDNKT-PEMNVNTNVEEGXFII, obtained from Lys-C digestion of the protein, is located in open reading frame number 2 of the GenBank data base entry for yeast TBF1 (accession number X69394; Brigati et al. 1993). Based on this sequence, two primers were designed, 5'-ATATCTGCAGAGAATTGAGGAACAAGGTT-3' and 5'-TTTGGTTCTGCAGTTGTGTT-3' (restriction sites are underlined), to amplify the fragment corresponding to nucleotides 441-701 (Fig. 1A) from yeast genomic DNA by PCR. This 251-bp fragment was used to screen a yeast genomic library in the plasmid pBluescript II KS (Stratagene) transformed into the *E. coli* XL1 strain (Stratagene) (Gileadi et al. 1992). A total of 10⁴ colonies were plated, transferred to Hybond-N filters (Amersham), and probed with the fragment labeled by random priming. Hybridization was performed at 55°C in 6× SSC (3 M sodium chloride, and 0.3 M sodium citrate), 1% SDS, and 0.25% dry milk (Johnson et al. 1984). After washing four times at 50°C with 0.1× SSC, and 0.5% SDS, the filters were exposed to XAR-5 film (Kodak) at -80°C for 16 hr. Following rescreening, three independent clones were obtained (plasmids designated pPL2, pPL3, and pPL4). The entire coding region was sequenced on both strands using the dideoxynucleotide chain termination method with a T7 Sequenase kit (U.S. Biochemical). The gene was found to contain an intron, so the same PCR fragment was used to screen a yeast cDNA library constructed in λYES (Elledge et al. 1991). A total of 90,000 plaques were screened, and four independent clones were obtained, one of which contained the entire spliced gene (plasmid pPL5).

Cloning of TFG2

Four sequences were obtained by tryptic digestion, and two sequences were obtained by Lys-C digestion of the 54-kD subunit. Based on the two peptides indicated in Figure 1B, the following degenerate oligonucleotides were synthesized: (1) 5'-ATATGAATTCTAGAATGAGYACACNGGNATG-3'; (2) 5'-ATATGAATTCTAGAATGCNACACNGGNATG-3'; (3) 5'-ATATGGATCCTCGAGYTTTAYTCNCGNCG-3'; and (4) 5'-ATATGGATCCTCGAGYTTTAYTCNCGGYCT-3' (R is A or G; Y is C or T; N is A, C, T, or G; underlined nucleotides represent restriction sites). Sense primers 1 and 2 were combined, as were antisense primers 3 and 4. PCR was performed by the "touch-

Tfg3	1	MVATVKRTIRIKTQQHI-LPEVPPVENFVRQMSIEIVLLDDEGKEIPATIPDKVVIYHLH
AF-9	1	MASSCSVQVKLELGHRAQVRKKPTVEGFT-HDW---MVVRGPEHSNIQHFVEKVVVFLH
ENL	1	MDNQCTVQVRLELGHRAQLRKKPTTEGFT-HDW---MVVRGPEQCDIQHFVEKVVVFLH
		* *
Tfg3	60	PTFANPNRTFTDPPFRIEEQGMGFFLDISVFLLEKAGERKJPHDLNF---LQESYEVEH
AF-9	58	ESFPRKRVCKDPPYKVEESGYAGFILPIEVYFNKEEPRKVRFDYDLFLHLEGHPPVNH
ENL	58	DSFPKRRVCKEPPYKVEESGYAGFILPIEVHFNKEEPRKVCFTDYDLFLNLEGNPPVNH
		* *
Tfg3	117	VI--QIPLNKP
AF-9	118	LRCEKLTENN
ENL	118	LRCEKLTENN
		* *
Tfg3	214	NVEEGEIIIDLYSLPEGLKSLWDYVKKN--TE
AF-9	529	HTNTTFDFDLCSLDKTTVRKLSQSYLETSGTS-
ENL	537	NVTNTTFDFDLFSLDKTTVRKLSQSCLEA--VAT
		* *

Figure 6. Sequence similarity between Tfg3 and the amino and carboxyl termini of two human proteins, AF-9 and ENL, involved in t(11;19) chromosomal translocations in acute leukemia. Protein names and amino acid residues are indicated at left. Identical (*) and similar (.) residues are identified below. Alignments were determined using the CLUSTAL V computer application (Higgins et al 1992).

down" method (Don et al. 1991) using 35 cycles of 1 min at 94°C, 2 min at a variable temperature, and 3 min at 72°C, with 1 µg of each set of primers and 0.1 µg of yeast genomic DNA. The temperature of the second step was 65°C for the first two cycles, was decreased one degree every other cycle to a temperature of 55°C, and was 55°C for the final 15 cycles. A PCR product of 294 bp was used to screen the yeast genomic library (Gileadi et al. 1992) as described above. Two independent clones were obtained, one of which contained the complete *TFG2* gene (on pPL16). The entire coding region was sequenced on both strands as described above, using custom primers, exonuclease III deletions, and subcloning techniques.

Cloning of TFG1

Tryptic digestion of the 105-kD subunit yielded a single peptide sequence that overlapped one of the three peptide sequences obtained by Lys-C digestion. Based on these sequences (Fig. 1C), the following primers were synthesized: 5'-ATATGGATCCTCGAGCKYTGNACDATYTC-3' and 5'-ATATGAATTC-TAGAAARAAARATHAAYCCNGT-3' (K is G or T; D is G, T, or A; H is C, T, or A; underlined nucleotides represent restriction sites). PCR was performed as described above, yielding a 101-bp fragment. The yeast genomic library (Gileadi et al. 1992) was screened unsuccessfully, so the fragment was used to screen the λYES cDNA library as described previously. A single positive clone was obtained, and the coding region of the gene was completely sequenced on both strands as before. The 418-bp *Bam*HI-*Hind*III fragment, which corresponds to base pairs 1184-1601 in Figure 1C, was used to screen the λYES yeast genomic library (Ramer et al. 1992) as described for the cDNA library. Five independent genomic clones were obtained, one of which contained the complete coding region for *TFG1* (plasmid pPL39). The complete clone was subcloned into the *Xho*I site of pBluescript II SK (plasmid pPL41), and the complete coding region was sequenced on both strands, as described.

Null mutations

All three factor-g genes were disrupted with the use of a *Hind*III fragment from YEp24 bearing the *URA3* gene, which was end-filled with Klenow enzyme in the presence of deoxynucleoside triphosphates. For the disruption of *TFG3*, pPL2 was digested with *Nde*I and *Pst*I to remove a 536-bp fragment from the coding region, blunted, and ligated with the *URA3* fragment to create pPL13. For disruption of *TFG2*, pPL16 was digested with *Nde*I and blunted. The 1578-bp fragment that was removed included the entire coding region and some flanking sequence. Ligation with the *URA3* fragment created pPL29. Disruption of *TFG1* was accomplished by digestion of pPL41 with *Xba*I and *Sma*I to remove a portion of the polylinker, followed by digestion of the resulting plasmid (pPL65) with *Stu*I and *Bam*HI, end-filling with Klenow enzyme, and insertion of the *URA3* fragment to create pPL66.

All three plasmids were digested with restriction enzymes that cut in the polylinker but not in the cloned gene and transformed into the *S. cerevisiae* strain CRY3 (*MATa/α ade2-1 can1-100 his3-11 15 leu2-3 112 trp1-1 ura3-1*; Kean et al. 1993). Yeast genomic DNA was isolated, and Southern blot analysis was performed to verify that the *URA3* gene had recombined correctly into each of the factor-g genes. The strains harboring the null mutations were induced to undergo meiosis. Tetrads were dissected by micromanipulation on YPD agar (Sherman and Hicks 1991) and replica plated to synthetic medium lacking uracil.

Identification of homologous proteins

The protein data base Swiss-Prot (release 28) was searched with the amino acid sequences of Tfg1, Tfg2, and Tfg3 using the BLAZE program (IntelliGenetics, Inc.). To determine the degree of homology to individual proteins, percentiles were calculated by the BESTFIT program (Genetics Computer Group, Madison, WI; Devereux et al. 1984) comparing the amino acid sequences of the proteins to the corresponding homologous proteins of other organisms. The parameters were gap weight of 2.0 and gap length weight of 0.1. For sequence analysis to identify potential protein sequence motifs, the PROSITE data base (Bairoch 1991) was searched using QUEST (IntelliGenetics).

Recombinant Tfg2 and Tfg3

To express the proteins in bacteria, the coding regions of the genes were first amplified by PCR and cloned into the expression plasmid. The primers used for Tfg3 were 5'-ATATCATATGCATCACCATCATCACCACGTAGCGACAGTAAAAACA-ACCAT-3' and 5'-ATATGGATCCTTACACGGTATTTTTC-TT-3'. The first oligonucleotide contained an *Nde*I site (underlined) followed by six histidine codons, and the second oligonucleotide contained a *Bam*HI site (underlined). The PCR product was cloned into the pET11a expression plasmid (Novagen) to create pPL9. The primers for Tfg2 were 5'-ATATCATGGCTCACCATCACCACCATCATAGCAGTGGTTCA-GCAGGGGCA-3' and 5'-ATATGGATCCCTAAACGACATC-TTCCATTT-3'. The first oligonucleotide contained an *Nco*I site (underlined) followed by an inserted valine codon and six histidine codons, whereas the second oligonucleotide contained a *Bam*HI site (underlined). The PCR fragment was cloned into pET11d (Novagen) to create pPL28.

The expression plasmids were transformed into *E. coli* BL21 (DE3) and grown in 2 liters of Luria broth (LB) supplemented with 200 µg/ml of ampicillin (Sigma) at 30°C. Cells were grown to an OD₆₀₀ of 0.6, and the cells were induced with 0.4 mM IPTG (Calbiochem) and grown for an additional 2 hr. The cells were harvested by centrifugation and suspended in lysis buffer [0.05 M Tris at pH 8.0, 0.5 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol, 2 mM imidazole, protease inhibitors (Sayre et al. 1992), and 0.02% Tween-20]. After brief sonication, cellular debris was removed by centrifugation at 10,000 rpm for 20 min in a Beckman JA-20 rotor.

The soluble fraction containing Tfg3 protein (39 ml, 5.8 mg/ml) was incubated for 2 hr with 6 ml of Ni²⁺-nitrilotriacetic acid-agarose (Ni-NTA) resin (Qiagen) equilibrated in lysis buffer. The slurry was washed in a column at 21 ml/hr with 6 ml of lysis buffer, followed by 6 ml of 0.5 M NaCl, 50 mM PIPES (pH 6.3), 20% glycerol, 2 mM imidazole, 0.01% NP-40, 5 mM β-mercaptoethanol, and protease inhibitors and then 6 ml of the same buffer except with 0.1 M NaCl rather than 0.5 M NaCl. The column was developed with a linear gradient (100 ml) of 0–0.15 M imidazole in 50 mM HEPES (pH 7.6), 0.1 M NaCl, 20% glycerol, 0.01% NP-40, 5 mM β-mercaptoethanol, and protease inhibitors, and the protein eluted in a broad peak starting at 25 mM imidazole. Fractions containing protein were pooled (19 ml, 0.5 mg/ml) and applied at 8.4 ml/hr to a 4-ml Bio-Gel HTP hydroxylapatite (Bio-Rad) column equilibrated in buffer P-0.01 (0.1 M NaCl, 20% glycerol, 5 mM β-mercaptoethanol, and protease inhibitors, containing 0.01 M potassium phosphate at pH 7.7). The column was washed with 10 ml of buffer P-0.01 and developed with a linear gradient to buffer P-0.3 (same as P-0.01, except with 0.3 M potassium phosphate). Protein eluted in a sharp peak at 0.02 M potassium phosphate.

Tfg2 was purified by essentially the same procedure as Tfg3.

The protein eluted from Ni-NTA agarose in a broad peak at 75 mM imidazole, and from hydroxylapatite at 0.15 M potassium phosphate.

Transcription assays

The transcription assay for factor g contained (in 25 μ l) 250 ng of plasmid DNA template, 120 ng of homogeneous bacterially expressed yeast TFIIB, 36 ng of homogeneous bacterially expressed yeast TBP (Kelleher et al. 1992), 40 ng of homogeneous bacterially expressed yeast TFIIE (Feaver et al. 1994), 1.5 μ l yeast factor b (Mono S fraction, Sayre et al. 1992), and 180 ng of homogeneous RNA polymerase II (Sayre et al. 1992). The factor-g fraction was purified to apparent homogeneity from yeast as described previously (Henry et al. 1992). Renatured Tfg1 was the same as that used in Figure 4 of Henry et al. (1992). The fractions containing Tfg2 and Tfg3 were those shown in Figure 3A, lanes 1,3. The DNA template contained the yeast *CYC1* promoter fused to a G-less cassette (Lue et al. 1989). Assays were performed as described previously (Sayre et al. 1992) except that the reactions contained 110 mM potassium acetate.

Antibodies

To raise antibodies against Tfg3, a portion of the peak hydroxylapatite fraction (Fig. 3A, lane 1) was injected into a rabbit (Berkeley Antibody Co., Richmond, CA) and polyclonal serum was collected 10–14 days postimmunization. For antibodies against Tfg1, a 121 amino acid fragment of the protein fused to GST was expressed in bacteria as follows. The carboxy-terminal portion of the *TFG1* gene (encoding residues 466–735) was amplified by PCR, with a *Bam*HI site introduced by the sense primer and an *Eco*RI site by the antisense primer. The amplified fragment was cloned into pGEX-3X (Pharmacia), cut with *Bam*HI and *Eco*RI to give pPL71, which was then digested with *Bgl*III and *Eco*RI, blunted with Klenow fragment, and religated to create pPL206, which expresses residues 466–587 of Tfg1 fused to GST. *E. coli* SURE strain (Stratagene) was transformed with pPL206, grown in 4 liters of LB supplemented with 200 μ g/ml of ampicillin at 30°C to an OD₆₀₀ of 0.3, and induced with 0.4 mM IPTG for an additional 2 hr. The cells were harvested by centrifugation and suspended in 80 ml of GST buffer (0.15 M NaCl, 10% glycerol, 1% Triton X-100, 50 mM HEPES at pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors). After brief sonication, cellular debris was removed by centrifugation. The soluble fraction (70 ml, 9.9 mg/ml) was incubated overnight at 4°C with 2 ml of glutathione–Sepharose 4B (Pharmacia) equilibrated in GST buffer. The slurry was washed in a column with 15 ml of GST buffer, and eluted with 0.5 M NaCl, 0.1 M HEPES (pH 7.6), 5 mM glutathione, 10% glycerol, and 1 mM EDTA. The peak fractions, which contained full-length and degradation products of the fusion protein and a single contaminating *E. coli* protein, were pooled and injected into a rabbit (Berkeley Antibody Co.). Polyclonal serum was collected 10–14 days after immunization.

Tfg3 protein, Tfg1–GST fusion protein, and GST were also coupled individually to cyanogen bromide-activated Sepharose (Sigma). Anti-Tfg3 and anti-Tfg1 polyclonal sera were affinity-purified by binding the antibodies to the Tfg3 Sepharose and Tfg1–GST Sepharose columns, respectively, washing, and eluting with 100 mM glycine (pH 2.5) as described (Harlow and Lane 1988). The Tfg1–GST eluate was then passed through the GST column and concentrated on a protein A–Sepharose (Bio-Rad) column (Harlow and Lane 1988).

Western blots were performed according to Chasman and Kornberg (1990). Dilutions were 1/200 and 1/500 for the anti-Tfg3

and anti-Tfg1 antibodies, respectively. The secondary antibody was goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad), used at a dilution of 1/1000.

Amino acid sequencing of TAF30

Yeast TAF30 was purified and transferred to nitrocellulose as described (D. Poon, Y. Bai, A.M. Campbell, S. Bjorklund, Y.-J. Kim, S. Zhou, R.D. Kornberg, and P.A. Weil, in prep.). The protein was digested with trypsin and microsequenced by R. Tjian (University of California, Berkeley).

Immunoprecipitations

To study the interaction between Tfg3 and the rest of yeast TFIIF, proteins were combined in 200 μ l of immunoprecipitation buffer (20 mM HEPES at pH 7.6, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 10% glycerol, and 0.05% NP-40) and incubated on ice for 1 hr. All reactions contained 5 μ l of ³⁵S-labeled Tfg3. Reaction 2 also contained ~200 ng of p54/p105, isolated from a *tfg3* null strain (see below), and reaction 3 contained 5 μ l of renatured p105 (Henry et al. 1992). After the incubation, 50 ng of affinity-purified Tfg1 antibody was added and the immunoprecipitations were performed as described (Bardwell et al. 1992). The beads were washed three times in immunoprecipitation buffer and eluted with 0.1 M glycine (pH 2.5). The elutions were subjected to electrophoresis in a SDS–10% polyacrylamide gel.

Two-subunit factor g (p54/p105) was purified from the *tfg3* null strain by the same purification scheme (Henry et al. 1992) as wild-type factor g, except with a Mono Q HR5/5 FPLC column (Pharmacia) in place of the DEAE-5-PW HPLC column (Bio-Rad), and with an SP-5-PW HPLC column (Bio-Rad) added at the end of the purification. The two-subunit factor g behaved similarly to the wild-type protein on all columns.

To study the association of Tfg3 with yeast TFIID, yeast whole-cell extracts were prepared and immunoprecipitated as described (Poon et al. 1994). The immunoprecipitates were analyzed by 4–15% gradient SDS-PAGE, transferred to PVDF filters, blotted with the indicated antibodies, and visualized using chemiluminescence. Extract was also prepared from the strain expressing hemagglutinin (HA)-tagged yeast TAF90 (D. Poon, Y. Bai, A.M. Campbell, S. Bjorklund, Y.-J. Kim, S. Zhou, R.D. Kornberg, and P.A. Weil, in prep.) and purified using Bio-Rex70 as described (Poon and Weil 1993). The Bio-Rex 1000 mM potassium acetate fraction was applied to a P11 column (Whatman) and developed with a linear gradient from 200–1200 mM potassium acetate. The fraction containing the peak of both Tfg3 and yeast TAF90, which did not contain factor g, was immunoprecipitated and analyzed by SDS-PAGE.

Far Western blotting

For the Far Western blot, 1 μ g of factor g was electrophoresed in a 4–20% gradient gel and transferred to nitrocellulose. Denaturation and renaturation were performed as described (Vinson et al. 1988). The blot was incubated overnight at 4°C in hybridization buffer (20 mM HEPES at pH 7.6, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 1 mM DTT, 0.05% NP-40, 1% dry milk) with 5 μ l of ³⁵S-labeled Tfg3 (shown in Fig. 3D), washed with hybridization buffer, and exposed to XAR film.

β -Galactosidase assays

Wild-type CRY3 and *tfg3* null strains were transformed with the indicated plasmids (Kelleher et al. 1990). Single transformants

were grown on minimal medium (0.67% yeast nitrogen base, 2% raffinose with or without 2% galactose as indicated) supplemented with all required amino acids except uracil and grown at 30°C to an OD₆₀₀ of 0.3. CRY3 strains were grown for a total of 8 hr, whereas *tfg3* null strains were grown for 24 hr. Whole-cell extracts were made and β -galactosidase activities (expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein) were determined as described (Kelleher et al. 1990).

Other methods

Growth of yeast and *E. coli*, DNA and RNA isolation, and standard DNA manipulations were as described (Sambrook et al. 1989; Guthrie and Fink 1991). SDS-PAGE was done according to Laemmli (1970), and proteins were visualized with Coomassie blue R-250.

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