Yeast BTF3 protein is encoded by duplicated genes and inhibits the expression of some genes *in vivo*

Guo-Zhen Hu and Hans Ronne*

Ludwig Institute for Cancer Research, Uppsala Branch, Uppsala Biomedical Center, Box 595, S-751 24 Uppsala, Sweden

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ABSTRACT

BTF3 is a human protein that is thought to be involved in transcription by RNA polymerase II [Zheng *et al.*, Cell 50, 361–368, 1987]. A yeast homologue of BTF3, Egd1p, has been identified by its ability to enhance DNA binding of the Gal4p activator [Parthun *et al.*, Mol. Cell. Biol. 12, 5683–5689, 1992]. We have cloned a second yeast gene, *BTT1*, which also encodes a BTF3 homologue. Btt1p and Egd1p are highly similar in sequence, which suggests that they are duplicated proteins with similar functions. Gene disruptions were used to investigate the function of the two proteins. Consistent with published results, we found that loss of *EGD1* causes a minor defect in *GAL* gene induction. Loss of *BTT1* has little if any effect. Surprisingly, we found that cells which lack both genes instead express the *GAL1* and *GAL10* mRNAs at much higher levels than wild type cells. This suggests that BTF3 really plays a negative role in *GAL* gene expression. Further experiments revealed that expression of the *ACT1* and *SSO1* genes also is elevated in cells that lack *EGD1* and *BTT1*. In contrast, expression of rRNA and tRNA was not affected. We conclude that Btt1p and Egd1p have redundant functions *in vivo*, and that they exert a negative effect on the expression of several genes that are transcribed by RNA polymerase II.

INTRODUCTION

Initiation of transcription by RNA polymerase II (RNA polymerase B) requires a number of accessory factors that bind to the polymerase in a multiprotein complex (1–3). These proteins include the TATA-binding protein TBP (BTF1) and associated factors (TAFs) within the TFIID complex (4, 5), but also several other proteins such as TFIIA (STF), TFIIB, TFIIE (BTF2), TFIIIF, TFIIIG, TFIH, and TFIIJ. An essential role in transcription has been demonstrated for most, but not all, of these proteins.

One protein whose function remains unclear is BTF3 (RNA polymerase B Transcription Factor 3). BTF3 was first identified as a factor in HeLa cells that is involved in transcription from the adenovirus major late promoter (6). It was reported that BTF3

is required for transcription from several class II promoters, irrespective of the presence of a TATA box, and that it binds to RNA polymerase II *in vitro* (6). BTF3 was therefore proposed to be a general transcription factor for class II promoters. However, a subsequent study reported that BTF3 is not an essential component of the class II preinitiation complex (7). Two isoforms of BTF3, BTF3a and BTF3b, are generated by differential splicing of the same mRNA (8). BTF3b lacks the 44 N-terminal residues. Both isoforms bind to RNA polymerase II, but only BTF3a is thought to be active.

A possible clue to the function of BTF3 was obtained by work in the yeast *Saccharomyces cerevisiae* (9). Thus, it was shown that stable DNA binding by the Gal4p activator requires a small accessory protein known as Egd1p (Enhanced Gal4 DNA binding). This protein was sequenced, and was found to be a yeast homologue of BTF3 (9). It was inferred that BTF3 could function as a co-activator which stabilizes the binding of specific activators to DNA. However, a disruption of *EGD1* does not cause any significant effects, except for a small defect in *GAL10* induction when cells are shifted from glucose to galactose (9). The absence of a clear disruption phenotype in yeast is frequently due to gene duplications (10, 11). It was therefore proposed that a second gene may exist which duplicates the function of *EGD1* (9).

Consistent with this, we have found a new yeast gene, *BTT1* (for BTF Three), which encodes a second BTF3 homologue. The function of BTF3 in yeast was investigated by disrupting both the *EGD1* and *BTT1* genes. Surprisingly, this does not cause a transcriptional defect. Instead, it causes a significant increase in the expression of several genes that are transcribed by RNA polymerase II.

MATERIALS AND METHODS

Yeast strains and plasmids

The cloning of plasmid pPAM1 containing the *PAM1* and *BTT1* genes has been described (12). The *EGD1* gene was amplified from W303-1A genomic DNA using oligonucleotide primers derived from the published *EGD1* and *PMA2* sequences (9, 13). The *BTT1* gene was disrupted by cloning the *HIS3* *Bam*HI fragment between the two *Bsg*I sites, and the *EGD1* gene by

*To whom correspondence should be addressed

cloning the *LEU2* *HpaI*–*SalI* fragment into the unique *EspI* site. *LEU2* was inserted in the reverse orientation from *EGD1*, since the forward orientation generates an in-frame fusion between the gene 3' to *LEU2* and *EGD1*. The resulting constructions were used for one-step gene disruptions (14) in the W303-1A background (15).

Northern blots

Northern blot analysis was performed as previously described (17, 18), but with a different hybridization protocol (19) being used for the oligonucleotide probe. The probes used were a 1.0 kb *SacI*–*EagI* fragment of *GAL1* (20), a 2.0 kb *EcoRI*–*EcoRV* fragment carrying the *SUP4* tRNA gene (23). To detect yeast 18S rRNA, we used a synthetic oligonucleotide with the sequence 5'-GCTTTTAACTGCAACAACCTT-3', which is complementary to a single-stranded region within the rRNA (24). The amounts of hybridizing probe in each lane were quantified in a Molecular Dynamics 400S Phosphorimager.

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Other methods

The methods used for yeast genetics and molecular cloning have been described (16, 17). Yeast cells were grown in rich YP media (1% yeast extract, 2% peptone) containing 2% glucose or 2% galactose as a carbon source. Galactose fermentation was assayed in the presence of 20 µg/ml ethidium bromide to prevent non-specific aerobic growth (25).

RESULTS

Yeast *BTT1* gene encodes a new BTF3 homologue

We have previously cloned a yeast gene, *PAM1*, which in high copy number can suppress the need for protein phosphatase 2A (12). During the sequencing of *PAM1*, we found a 3' adjacent open reading frame (*BTT1* for *BTF* Three), which predicts a 16.7 kDa protein. A TFasta search (26) of the EMBL Data Base revealed that the protein encoded by the *BTT1* gene is related to the human transcription factor BTF3 (8), and also to Egd1p, a recently described BTF3 homologue in yeast (9).

The sequence of the *BTT1* promoter was analyzed for the presence of known target sites for DNA-binding proteins in yeast (27). The only sequence motif found was an $\alpha 2$ operator half-site at position –155. The significance of this is unclear, since functional $\alpha 2$ operators usually contain two half-sites that are separated by 17 nucleotides (28). We also compared the *BTT1* and *EGD1* promoters in an attempt to identify common regulatory elements. However, we found no significant similarities, except for the fact that neither gene has a TATA box. Instead, both genes have AT-rich stretches in their 5' untranslated regions. Such stretches are frequently found in constitutive yeast promoters, where they are thought to functionally replace the TATA box (29).

An alignment of Btt1p, Egd1p and BTF3 revealed that the two yeast proteins are much more similar to each other than to the human protein. This suggests that the two yeast genes arose by gene duplication long after the separation of metazoa from fungi. Curiously, the similarity between Btt1p and the published Egd1p sequence is restricted to the N-terminal halves of the two proteins. In contrast, the similarity between Btt1p and BTF3 extends also

into the C-terminal region, even though these two proteins are less closely related to each other. An inspection of the *EGD1* sequence revealed that the 3' half of the gene could encode a sequence that is related to Btt1p and BTF3 if it is translated out of frame. This observation prompted us to check the sequence of *EGD1*. We found that our sequence differs from the published sequence in two positions. One of these differences is a frameshift which causes the 3' half of *EGD1* to be read in the same frame as *BTT1* and the human BTF3 gene. An alignment of Btt1p, our Egd1p sequence, and human BTF3b is shown in Figure 1.

Finally, it should be noted that neither Egd1p nor Btt1p has an N-terminal extension similar to that in BTF3a. Instead, the N-termini of the two yeast proteins coincide with that of the alternatively spliced BTF3b gene product. It suggests that BTF3b could be the most ancient form of the mammalian molecule. This is intriguing, since it is BTF3a and not BTF3b which is active in the *in vitro* assay (8). However, the *in vivo* activities of BTF3a and BTF3b still remain to be elucidated.

Cells that lack both *BTT1* and *EGD1* are viable

The function of Btt1p was investigated by disrupting the *BTT1* gene (14). We found that the *BTT1* deficient cells are viable and can grow well on different carbon sources, including galactose. This is similar to the phenotype of *EGD1* deficient cells (9), which we could confirm in our genetic background. We proceeded to investigate the effect of disrupting both genes in the same cell. To achieve this, we crossed two haploid strains that had been disrupted for either gene. The resulting diploid was sporulated, and tetrads were dissected. We found that spores which lack both *BTT1* and *EGD1* still are viable, and can grow on different carbon sources, including galactose. This suggests that BTF3 is a non-essential protein in yeast, or possibly that a third related gene exists which duplicates the function of *EGD1* and *BTT1*. However, we saw no evidence of a third gene in Southern blots. Furthermore, a disruption of both *EGD1* and *BTT1* causes significant transcriptional effects (see below). We conclude that if a third BTF3 gene exists in yeast, it can only in part provide the same function(s) as *EGD1* and *BTT1*.

Btt1p and Egd1p together inhibit *GAL1* and *GAL10* expression

It was reported that a disruption of *EGD1* causes a 2–3 fold reduction in the rate of induction of the *GAL10* gene (9).

Figure 1. Alignment of the Btt1p, Egd1p and human BTF3b sequences. The predicted sequence of Egd1p is from the present investigation, and differs from the published sequence (9) in its C-terminal half. Identical residues are enclosed within boxes.

However, this effect was only seen when the cells were grown on a repressing carbon source prior to induction (9). To elucidate the role of *EGD1* and *BTT1* in *GAL* gene expression, we prepared mRNA at different time points from glucose repressed cells that were shifted to galactose. Northern blot analysis was used to monitor the induction of the *GAL1* and *GAL10* genes. We found that *GAL* gene expression is reduced 2-fold in *EGD1* disrupted cells, as previously described (9). In contrast, a disruption of *BTT1* has little if any effect (Figure 2). Surprisingly, cells that lack both *EGD1* and *BTT1* instead have a 2–3 fold higher level of *GAL1* and *GAL10* expression than wild type cells (Figure 2). Thus, while loss of Btt1p has little effect in wild type cells, it causes a 5–7 fold derepression of *GAL1* and *GAL10* in cells that lack Egd1p. Similarly, loss of Egd1p in cells that already lack Btt1p causes a 2–3 fold derepression of *GAL1* and *GAL10*. This unexpected finding suggests that Egd1p and Btt1p have redundant functions *in vivo*, and that they act to inhibit rather than to enhance *GAL* gene expression.

Btt1p and Egd1p also inhibit *ACT1* and *SSO1* expression

In an attempt to normalize the mRNA levels, we hybridized our filters to a probe from the constitutively expressed *ACT1* gene encoding actin (20). Surprisingly, we found that a disruption of both *EGD1* and *BTT1* causes a significant increase in the *ACT1* mRNA level (Figure 3), which was confirmed by normalizing against ribosomal RNA (see below). This suggests that expression of *ACT1* also is under negative control by Egd1p and Btt1p.

We proceeded to examine the effects on two other constitutively expressed genes that are transcribed by RNA polymerase II.

Thus, we used probes from the *PPH21* gene, which encodes the catalytic subunit of protein phosphatase 2A (11), and the *SSO1* gene, which encodes syntaxin, a membrane protein involved in vesicular transport (22). We found that the *SSO1* mRNA is expressed at a higher level in cells that lack both Btt1p and Egd1p. However, the effect was not as pronounced as with the *GAL* and *ACT1* genes. For *PPH21*, there was no significant effect (Figure 3). It should be noted that the *ACT1* and *GAL* genes are more highly expressed than the *PPH21* and *SSO1* genes. This suggests that the effect of BTF3 may be dependent on the level of expression.

To investigate the effect of BTF3 on RNAs that are transcribed by RNA polymerases I and III, we probed our filters with an oligonucleotide that is complementary to the yeast 18S ribosomal RNA, and with a fragment containing the *SUP4* gene, which encodes a tyrosine-inserting tRNA. We found that a disruption of either *BTT1*, *EGD1*, or both genes together had no significant effect on the amounts of rRNA or tRNA (Figure 3). We conclude that the inhibitory role of BTF3 does not extend to genes that are transcribed by RNA polymerases I and III.

DISCUSSION

We have cloned and characterized the *BTT1* gene which encodes a second yeast homologue of the mammalian transcription factor BTF3. The Btt1p protein sequence is 28% identical to human BTF3 (Figure 1), and 48% identical to Egd1p, the previously known BTF3 homologue in yeast (9).

Egd1p was originally identified by its ability to enhance DNA binding of the Gal4p activator, and was proposed to play a positive role in *GAL* gene expression. Consistent with this, a small reduction in the rate of *GAL10* induction occurs in *EGD1* deficient cells (9). However, this effect is transient, and it is only seen in when cells are grown in glucose before induction. The *GAL4* gene is repressed during growth in glucose (30, 31). The observed effect therefore suggests that Egd1p may be required to enhance Gal4p function when the protein is present in small amounts. In contrast, derepressed cells with adequate amounts of Gal4p do not seem to require Egd1p for *GAL10* expression.

To explain the small effect of disrupting *EGD1*, it was proposed that a second BTF3 homologue could exist in yeast which in part duplicates Egd1p function (9). Our finding that a second BTF3 homologue is encoded by the *BTT1* gene confirms this prediction.

Figure 2. Northern blot analysis with mRNA from wild type, *btt1*, *egd1*, and *egd1 btt1* strains. The cells were grown overnight in YPD (1% yeast extract, 2% peptone, 2% glucose), spun down and resuspended in 1% yeast extract, 2% peptone, to which 2% galactose was added at time zero (9). Samples were removed after various times and used for RNA preparation. The hybridizing mRNA was quantified in a phosphorimager. The amount of mRNA is expressed in percent of the fully induced and derepressed wild type level (12 h after induction).

Figure 3. Expression of *ACT1*, *SSO1*, *PPH21*, *SUP4* tRNA, and 18S rRNA in wild type, *btt1*, *egd1*, and *btt1 egd1* strains. The cells used for RNA preparation are the same as time point zero in Figure 2, which were grown overnight in YPD. The hybridizing RNA was quantified in a phosphorimager.

To elucidate the function of BTF3 in yeast, we monitored the expression of different genes in strains disrupted for either *BTT1*, *EGD1*, or both genes. Surprisingly, our results suggest that BTF3 plays a negative rather than a positive role in *GAL* gene expression (Figure 2). Similarly, the constitutively expressed *ACT1* gene also seems to be under negative control by BTF3. However, the *SSO1* gene was not as strongly affected, and expression of *PPH21*, rRNA, and tRNA did not change significantly in the BTF3 deficient strain (Figure 3). We conclude that BTF3 inhibits RNA polymerase II-dependent gene expression *in vivo*, an effect which is most clearly seen with strongly expressed genes such as *ACT1* and the *GAL* genes.

These results are surprising since human BTF3 is thought to be a positive co-factor in transcription (6, 8). However, this was questioned in a later study, and the function of human BTF3 therefore remains unclear (7). Our results are also surprising in view of the fact that loss of *EGD1* alone has a small negative effect on *GAL* gene induction (9). If BTF3 inhibits *GAL* gene expression, one would rather expect to see a small increase in transcription when one of the two BTF3 genes is lost. The negative effect of disrupting *EGD1* alone was seen also in our experiments (Figure 2), so it is not due to differences in genetic backgrounds or experimental conditions. Thus, while complete loss of BTF3 enhances the expression of several genes including the *GAL* genes, Egd1p seems to have a small positive effect on the *GAL* genes when the amount of Gal4p is limited. The reason for this paradox remains to be determined.

The mechanism by which yeast BTF3 inhibits the expression of the *GAL* and *ACT1* genes also remains to be elucidated. It cannot be excluded that BTF3 exerts its effect post-transcriptionally. However, the fact that Egd1p stabilizes DNA binding by Gal4p (9) suggests that it is a nuclear protein which is associated with DNA and therefore is likely to affect transcription. Similarly, the fact that human BTF3 binds to RNA polymerase II also suggests that an effect on transcription is most likely (6). It should be noted that the *GAL* genes are regulated normally in the BTF3 deficient strain (Figure 2). The only difference is that the levels of expression are higher. We therefore think it is unlikely that BTF3 is a transcription factor which is directly involved in regulating gene expression. Similarly, the fact that cells that lack both Egd1p and Btt1p are viable argues against BTF3 being a crucial component of the basal transcription complex.

Conceivably, BTF3 could be an accessory chromatin-associated protein. Expression from strong promoters is known to require nucleosome dissociation (32). Thus, histone H4 mutations can increase transcription *in vivo* by promoting nucleosome dissociation (33), and transcription can also be stimulated *in vitro* by removal of histones (34). It is possible that loss of a chromatin-associated protein similarly could facilitate transcription.

If BTF3 is a chromatin-associated protein, this could also account for the fact that Egd1p facilitates DNA binding by Gal4p and has a positive effect on *GAL* gene expression when Gal4p is limited (9). Gal4p is likely to recognize DNA in a chromatin context, where interactions with other proteins could be important for stabilizing DNA binding. It is not inconceivable that Gal4p may have developed a specific interaction with one of the two duplicated BTF3 homologues in order to facilitate its initial DNA recognition.

A similar argument could also explain why human BTF3 binds to RNA polymerase II and stimulates transcription *in vitro* (6,

8). The initial binding of RNA polymerase II to the promoter could be facilitated by specific interactions with other proteins, including BTF3. This does not rule out that a complete loss of BTF3 could facilitate mammalian gene expression *in vivo*, as it does in yeast. The corresponding experiment, a targeted disruption of the BTF3 gene in a mammalian cell line, remains to be done.

In conclusion, we have found that the role of BTF3 in gene expression is much more complex than previously thought. It seems to comprise both a general inhibitory effect on genes that are transcribed by RNA polymerase II, but also some positive effects. Clearly, further experiments will be necessary to elucidate the function of BTF3 in yeast as well as in human cells.

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