Characterization of cis-elements in the promoter of trz2 encoding Schizosaccharomyces pombe mitochondrial tRNA 3′-end processing enzyme

Jinyu Liu, Linting Huang, Yirong Wang and Ying Huang*

Abstract
The endonuclease tRNase Z is responsible for the 3′-end processing of tRNA precursors, which is one of the essential steps in tRNA maturation. The fission yeast Schizosaccharomyces pombe contains two essential tRNase ZL genes (trz1 and trz2) involved in nuclear and mitochondrial tRNA 3′-end processing, respectively. Our previous studies suggest that trz2 is expressed at a very low level. Here we report characterization of the trz2 promoter. Using lacZ as a reporter, we show that the trz2 promoter contains a HomolD box and a very weak diverged TATA element. The HomolD box is usually found in the promoters of S. pombe ribosomal protein genes. lacZ reporter assays suggest that the HomolD box regulates the expression of both trz2 and the ribosomal protein gene rps2501, which are arranged head-to-head on opposite strands. Overexpression of Rrn7, a candidate HomolD box-binding protein, up-regulates expression of lacZ under the control of the trz2 promoter or the rps2501 promoter. Functional complementation studies suggest that the TATA-like element is essential for trz2 expression, whereas the HomolD box may play a nonessential regulatory role. We also demonstrate that a 57 nt negative regulatory element (NRE) located between the HomolD box and the TATA-like element represses the expression of lacZ under the control of the trz2 promoter. Our results suggest that the low-level trz2 expression may arise from a low level of transcription caused by lack of a strong TATA box and the NRE. Our analysis also suggests that trz2 and rps2501 may be coregulated by the HomolD box.

INTRODUCTION

tRNase Z (also named RNase Z or 3′-tRNase) is a tRNA 3′-endonuclease that participates in tRNA 3′-end maturation. It cleaves tRNA precursors after the first nucleotide following the acceptor stem to generate a tRNA 3′-end suitable for CCA addition (for reviews, see [1–4]). tRNase Z can be classified into a short form (tRNase ZS) with 279–554 amino acids (aa) and a long form (tRNase ZL) with 648–997 aa. tRNase ZS exists primarily in bacteria, archaea, plants and vertebrates, and is localized to the cytosol and the chloroplast [5–7]. Unlike tRNase ZS, tRNase ZL is ubiquitous in eukaryotes, and is primarily localized to the nucleus and the mitochondria [5–7]. Unlike most eukaryotes examined so far, which contain a single tRNase ZL gene encoding both the nuclear and mitochondrial forms of tRNase ZL, four sequenced Schizosaccharomyces species (Schizosaccharomyces octosporus, Schizosaccharomyces cryophilus, Schizosaccharomyces pombe and Schizosaccharomyces japonicus) have two tRNase ZLs (Trz1 and Trz2) encoded by two different genes (trz1 and trz2) [8–10]. We have previously shown that the two essential tRNase ZL genes in S. pombe (designated trz1 and trz2) are required for the 3′-end processing of nuclear and mitochondrial tRNA precursors, respectively [8–10].

Besides its main role in tRNA 3′-end processing, tRNase Z has additional functions. The Bacillus subtilis tRNase ZS is involved in 3′-end maturation of transfer-messenger RNA [11]. In Saccharomyces cerevisiae, tRNase ZS is likely involved in rRNA processing and mitochondrial maintenance [12]. The mouse tRNase ZL is involved in the generation of tRNA-like small cytoplasmic RNA derived from long noncoding RNA MALAT1 [13]. tRNase ZL is also involved in producing the murine γ-herpesvirus-encoded microRNA, which is transcribed as tRNA fusions [14]. The human tRNase ZL (ELAC2) has been proposed to regulate the cell cycle and gene expression [15, 16]. Unlike prokaryotic tRNase ZS, the...
primary substrate for eukaryotic tRNase Z may not be tRNAs. Recently, tRNase Z has been shown to process UbL40 mRNAs and is required for thermosensitive genic male sterility in rice [17].

Unlike nuclear tRNAs, mitochondrial tRNAs whose genes punctuate mitochondrial DNA are generated by endonucleolytic cleavage of polycistronic RNAs [18]. Processing of tRNAs is essential for maturation of other mitochondrial RNAs. Indeed, we and others have shown that the mitochondrial tRNase Z is involved in 5'-end processing of mitochondrial mRNAs and noncoding RNAs other than tRNAs [9, 19].

The regulatory mechanism of tRNase Z gene expression has not yet been well studied. Several lines of experimental evidence suggest that tRNase Z gene expression may be tightly controlled. Overexpression of ELAC2 delays cell-cycle progression [15]. In addition, the mRNA level of the Drosophila tRNase Z is induced by juvenile hormone [20]. We have shown that overexpression of trz2 is lethal whereas overexpression of trz1 has no detectable phenotypes [8]. In addition, we have shown that extremely low-level ectopic trz2 expression is required for full suppression of trz2 temperature-sensitive (ts) growth defects [8]. These results suggest that trz2 is normally expressed at very low levels. Consistent with these observations, it has been shown that Trz2 is ranked among the bottom 10% of S. pombe proteins in terms of protein abundance and is ~19-fold less abundant than Trz1 [21].

In this study, using a reporter assay and genetic complementation, we show that the S. pombe trz2 promoter has a HomolD box and a TATA-like element. In addition, we show that a negative regulatory element (NRE) located between the HomolD box and the TATA-like element represses trz2 transcription likely via disruption of interactions between the HomolD box-binding protein and the TATA-like element-binding protein.

METHODS

Strains and media

The strains used in the present study were wild-type yAS56 (h² leu1-32 ura4-D18) and trz2 ts mutant yHD1 (h² trz2-1 leu1-32 ura4-D18) derived from yAS56 as described in Zhang et al. [9]. Strains were grown in rich (YES) or Edinburgh minimal medium (EMM) with appropriate supplements [22]. Standard protocols for genetic manipulation of fission yeast were used as described previously [22].

Plasmid construction

Constructs containing the Escherichia coli β-galactosidase (β-gal) reporter gene (lacZ) under the control of a derivative of the nmt1 (nmt1**) promoter or the trz1 or trz2 promoter were made as follows. The 3075 bp lacZ ORF from E. coli was cloned into the Xhol/Smal sites of pREP82X, which contains a derivative of the nmt1 promoter with a mutated TATA box and the nmt1 terminator [23], generating the nmt1**-lacZ construct (pREP82X-lacZ). A DNA fragment extending from 1.2 kb upstream of the ATG start codon of trz1 was PCR amplified from S. pombe genomic DNA and cloned into the PsiI/Xhol sites of pREP82X-lacZ, generating the trz1 promoter-lacZ construct. Varied 5'-sequences (3000, 2000, 1000, 500, 278, 248 and 170 bp) upstream of the ATG start codon of trz2 were PCR amplified from the S. pombe genomic DNA and cloned into the PsiI/Xhol sites of pREP82X-lacZ to replace the nmt1** promoter, yielding trz2 promoter-lacZ constructs pY1–pY7. To generate the promoterless control vector pU1, a 1.8 kb ura4 gene was PCR amplified from pREP82X, digested with HindIII and XhoI and cloned into HindIII/XhoI-linearized pREP82X-lacZ.

Deletion of the NRE was made by overlap PCR. Briefly, DNA fragments corresponding to the 5'- and 3'-ends of the deletion were amplified by PCR using primer pair trz2-lacZ-JY5 and 110-54-down and primer pair 110-54-up and trz2-lacZ-down, respectively. The inner primers (110-54-down and 110-54-up) contained 20 bp complementary regions. The two fragments were joined by PCR using primers trz2-lacZ-JY5 and trz2-lacZ-down, and the resulting PCR product was digested with PsiI and XhoI and cloned into the PsiI/XhoI sites of pREP82X-lacZ, yielding pY9. Other deletion constructs were prepared similarly by overlap PCR. Replacement of the NRE with a 57 bp random DNA sequence was made by overlap PCR. Briefly, 5'- and 3'-regions flanking the NRE and a 57 bp fragment of the ELAC1 coding sequence were amplified by PCR using primers trz2-lacZ-pY5 and 57E1down and trz2-lacZ-down and 57E1up and 57E2down, respectively. The three fragments were joined by overlap PCR using trz2-lacZ-pY5 and trz2-lacZ-down and cloned into the PsiI/XhoI sites of pREP82X-lacZ, producing pY17.

To express trz2 from its own promoter, a DNA fragment containing 2037 bp of trz2 coding sequence and its flanking sequences (500 bp upstream and 1000 bp downstream) was PCR amplified using primers trz2-for and trz2-down. The PCR product was cloned into the Smal and AatII sites of pWH5 [24], producing the construct pWH5-trz2. To delete the HomolD box from the trz2 promoter, the 5'- and 3'-regions flanking the HomolD box and the TATA-like element were amplified using primer pairs trz2-for and Out-HomolD, and Out-HomolD-fo and trz2-down for HomolD box deletion and primer pairs trz2-for and TATA-out-re, and TATA-out-for and trz2-down for TATA-like element deletion. The PCR fragments were joined by PCR using primers trz2-for and trz2-down and cloned into the sites of pWH5, generating constructs pWH5-DHomolD and pWH5-ΔTATA.

To overexpress rnr7 in S. pombe, rnr7 was obtained by PCR using genomic DNA as a template. The PCR product was cloned into the XhoI and Smal sites of pREP3X containing the nmt1 promoter, creating pREP3X-rnr7.

To integrate the trz2 gene under control of its own promoter into the chromosome, a DNA fragment containing 2037 bp of trz2 coding sequence and its flanking sequences
(500 bp upstream and 1000 bp downstream) was PCR amplified from plasmid pWH5-trz2 using primers trz2-for and trz2-sacI-down. The PCR product was cloned into the SmaI and SacI sites of the integration vector pJK148 [25] to give pJK148-trz2. Plasmids for integrating the trz2 gene under control of mutant versions of its own promoter lacking either the HomolD box or the TATA box were constructed similarly. These plasmids were linearized with NruI and transformed into trz2 ts mutant yHD1.

To generate two point mutations in the HomolD box, the trz2 promoter from pY10 was amplified by PCR as two overlapping pieces using inner primers (D-motif-mut-re and D-motif-mut-for) that contain two substitution mutations that convert TGTGACTG to TGT

lacZ, creating pJY19. Four point mutations in the TATA-like element of the trz2 promoter were generated similarly by overlap PCR. All constructs were verified by DNA sequencing. The sequences of the primers used in this study are listed in Table S1 (available in the online Supplementary Material).

5'-Rapid amplification of cDNA ends and quantitative real-time PCR

Total RNA from S. pombe cells was isolated by the E.Z.N.A. Yeast RNA kit (Omega). The 5'-rapid amplification of cDNA ends (RACE) was performed according to the 5'-RACE System for Rapid Amplification of cDNA Ends kit (Invitrogen). Sequencing was performed with a reverse primer RC86-R2 [for determination of the transcription start site (TSS) of trz2 with the wild-type promoter] or lacZ-nested GSP (for determination of the TSS of lacZ with the mutant trz2 promoter lacking the NRE). Real-time quantitative reverse transcription PCR (RT-qPCR) was performed after the DNA contamination was removed with DNase I (Omega). Reverse transcription for RT-qPCR was performed with the iScript cDNA Synthesis kit (Bio-Rad). RT-qPCR was performed in triplicate on a StepOne Real-Time PCR System (Applied Biosystems). PCR was then performed with AmpliTaq Gold for real time (SYBRSelect Master Mix; Applied Biosystems). The relative fold changes in the levels of mRNAs in wild-type cells were calculated using the 2^{-ΔΔCt} method with normalization to act1 mRNA levels. All of the graphs were plotted using Origin version 8.5 (OriginLab).

In vitro β-gal assay

β-gal assays were performed with S. pombe cells grown to mid-exponential phase in EMM lacking uracil. Protein extracts of S. pombe cells were prepared using the glass bead method as described previously [26]. The protein concentration of the samples was determined using a BCA Protein Quantification kit (Beyotime) and BSA as a standard. The absorbance was measured using a SpectraMax M2 Multi-Mode Microplate Reader. β-gal activity was measured by a spectrophotometric assay [27]. Briefly, 100 µl of samples was mixed with 900 µl of Z buffer. The reaction was initiated by addition of 0.2 ml ONPG, incubated at 28 °C until a pale yellow colour occurred and stopped by addition of 0.5 ml of 1 M Na2CO3. The released o-nitrophenol was measured at 420 nm using a SmartSpec 3000 spectrophotometer. The specific activity was represented in nanomoles per minute per milligram protein. The protein concentration and β-gal activity were determined in triplicate.

Bioinformatic analysis

DNA sequence analysis was performed by searching the PomBase (www.pombase.org/), the Ensembl Fungi database (http://fungi.ensembl.org/index.html) and the NCBI database (www.ncbi.nlm.nih.gov). Promoter and cis-elements were identified using the GPMiner software program (http://gpminer.mbc.nctu.edu.tw/index.php). The sigscan program (www-bimas.cit.nih.gov/molbio/signal/) was used to search for the random DNA sequences to replace the NRE.

Statistical analysis

All determinations were performed in triplicate and repeated three times. Results are reported as the mean±SD. The P-value is calculated using independent samples t-test in the SPSS version 16.0 software package (SPSS). P<0.05 was considered as statistically significant.

RESULTS

The S. pombe trz2 gene likely has a very weak promoter

Our previous results and findings by others suggest that Trz2 is present in very low abundance in S. pombe cells [8, 9, 21]. The low abundance of Trz2 is most likely due to weak transcription. To determine the strength of the trz2 promoter, we chose the region between the ATG start codon and 1.0 kb upstream as the promoter fragment of trz2 and fused it to the lacZ ORF of E. coli (pJY3). As controls, we also fused the lacZ ORF with the 1.2 kb upstream of trz1 and the 1.2 kb DNA fragment containing a derivative of nmt1 promoter (nmt1**) with a mutation in the TATA box, which dramatically decreases the promoter activity [23]. We measured β-gal activity in whole-cell extracts prepared from S. pombe strain yAS56 carrying these lacZ constructs and a control reporter construct without a promoter. β-gal activities from the promoterless control vector pU1, the trz2 promoter-lacZ, trz1 promoter-lacZ and nmt1** promoter-lacZ constructs were 0.23±0.06, 0.31±0.04, 12.7±1.4 and 54.5±6.9, respectively (Fig. 1). β-gal activity produced by the trz2 promoter-lacZ construct was approximately at the background level. These results suggest that trz2 is driven by a very weak promoter.

Identification of a negative regulatory element in the trz2 promoter

To map the TSS of trz2, we performed 5'-RACE. This experiment revealed that trz2 has a TSS located 60 bp upstream of the ATG start codon (Fig. 2b). Recently, Li et al. [28] reported that the TSS of trz2, as determined using
a deep sequencing-based approach, maps to 72 bp upstream of the ATG start codon. It is possible that the difference between our result and that of Li et al. [28] may reflect different methods used in determination of the TSS.

DNA sequence analysis revealed the presence of a TATA-like element and a HomolD box (CAGTCACA or in its inverted form TGTGACTCT) located between −35 and −27 (with respect to the TSS of trz2) and −118 and −111 in the 5′-flanking region of trz2, respectively. Because β-gal activity of the trz2 promoter- lacZ is so low that it is almost unmeasurable, their functional importance cannot be examined directly by deletion of these cis-elements. Therefore, we first set out to identify negative regulatory elements within the proximal promoter region of the trz2 gene that are responsible for low expression of trz2. To do so, we cloned 5′-sequences with various sizes (3000, 2000, 1000, 500, 278, 248 and 170 bp) upstream of the ATG start codon of trz2 into a lacZ reporter vector, generating a series of progressive 5′-deletion constructs (pY1–pY7). The resulted constructs were introduced into yAS56 cells. As shown in Fig. 2(a), these constructs showed near-background levels. Because deletions up to position −111 had no significant effect on β-gal activity, it was likely that no negative regulatory element is present in the region between −2941 and −111.

We serendipitously found that deletion from −110 to −54 in the 278 or 500 bp trz2 promoter fragment (constructs pY9 and pY10) resulted in a significant increase in β-gal activity, suggesting the presence of an NRE between nucleotides −110 and −54. Deletion of the NRE did not affect the TSS of trz2, as determined by 5′-RACE (Fig. 2b). To determine the fine structure of the NRE region, we divided the NRE into two regions: NRE1 (−110 to −83) and NRE2 (−83 to −54). When the NRE1 or NRE2 alone was removed individually from the promoter, the deletions had no significant effect in expression (Fig. 2a, pY14 and pY15). Only the combination of the NRE1 and NRE2 deletions had a remarkable effect which was significantly higher than background [constructs pY9 and pY10 in Fig. 2(a)].

The downstream boundary of the NRE was determined by using deletions (constructs pY20–pY23) that remove progressively more DNA downstream or upstream from the downstream end (position −54). The effects of these deletions were determined as described above. Removing additional DNA downstream from this position reduced β-gal activity to nearly background levels (Fig. 2a).

The data above suggest that the NRE (−110 to −54) plays a repressive role in trz2 transcription. To determine whether the repressor activity of the NRE was sequence specific, we generated construct pY17 in which the NRE was replaced with a 57 bp random DNA sequence from ELAC1, which encodes human tRNase Z*. This sequence identified by the SPLICSCAN program does not show any sequence similarity to the NRE sequence and would not introduce new cis-elements into the promoter. As shown in Fig. 2(a), β-gal activity produced by pY17 was close to background level, suggesting that the replacement of the NRE did not abolish the repression. Moreover, a β-gal reporter gene construct (pY24) containing a deletion identical in size to the NRE but extending from −93 to −36 produced a similar level of β-gal activity to pY10 lacking the NRE (Fig. 2a). These results suggest that transcriptional repression by the NRE is not likely mediated by binding site(s) for sequence-specific repressor(s) at NRE.

**Transcription of rps2501 and trz2 is likely coregulated by the HomolD box**

In *S. pombe*, trz2 and the *S. pombe* ribosomal protein S25 gene 1 (*rps2501*) are arranged in a head-to-head orientation on opposite strands with a distance of 235 bp between the two initiation codons. Analysis of the core promoter sequence of these two genes revealed the presence of a bidirectional HomolD box shared between the promoters of *rps2501* and trz2 (Fig. 3). This HomolD box is located 57 and 170 bp upstream of *rps2501* and trz2, respectively. The HomolD box has been shown to be required for transcription of the ribosomal protein in *S. pombe* [29]. We speculated that the HomolD box mediates the transcription of both *rps2501* and trz2. In *S. pombe*, there are two *rps25* genes (*rps2501* and *rps2502*) and the two proteins share 94.4 % amino acid identity overall. It is unknown why two *rps25* genes exist in *S. pombe* and other species.
Fig. 2. Deletion analysis of the trz2 promoter. (a) Nucleotide positions are assigned relative to the TSS (designated +1) of trz2. The lengths of 5′-sequences upstream of the ATG start codon of trz2 are indicated in bp to the right. The 5′- and 3′-positions for the endpoints of each deletion are shown. A black box represents the HomolD box, a white box represents the NRE and an ellipse represents the TATA-like element. A 57 bp random DNA sequence is represented by a dark grey box. β-galactosidase (β-gal) activities from extracts of S. pombe cells expressing the indicated constructs were measured as described in Fig. 1. Data shown are the mean±SEM of three or more independent experiments. The background β-gal activity of the promoterless control is 0.23±0.06 (mean±SEM). (b) Deletion of the NRE does not alter the TSS of trz2. The partial sequences of the 5′-RACE products used to determine the TSSs are shown. 5′-RACE was performed using total RNA isolated from wild-type S. pombe cells without a reporter (for determination of the TSS of trz2) or carrying the trz2 promoter-lacZ construct lacking the NRE (construct pJY10). Only sequences of sense strands are shown. The downward arrow indicates the TSS. The horizontal arrow pointing to the right indicates the translation start site (ATG).
We determined whether the HomolD box was required for transcription of \textit{rps2501} by using the \textit{lacZ} reporter gene assay. We constructed \textit{rps2501} promoter-\textit{lacZ} constructs containing 2000, 1000 or 500 bp of the 5′-upstream sequence of \textit{rps2501}. Since \textit{S. pombe} strains carrying these three constructs produced similar levels of β-gal (Fig. 4), we arbitrarily chose 500 bp of the 5′-upstream sequence of \textit{rps2501} for further deletion analysis. As expected, deletion of the HomolD box led to a dramatic reduction (~20-fold) in β-gal activity (Fig. 4), suggesting that the HomolD box plays a key role in regulation of \textit{rps2501} transcription.

To examine whether the HomolD box is also involved in transcription of \textit{trz2}, we generated deletions and point mutations in the HomolD box of \textit{trz2} promoter-\textit{lacZ} constructs (p\textit{JY9} and p\textit{JY10}). These two constructs lacking the NRE were used because they produced β-gal activity significantly above background. As shown previously, deletion of the NRE increased β-gal activity. However, further deletion of the HomolD box in either 500 bp (construct p\textit{JY12}) or 278 bp \textit{trz2} promoter fragments (p\textit{JY13}) reduced β-gal activity to a near-background level (Fig. 5a). In addition, we used overlapping PCR to introduce two point mutations into the HomolD box (where the fourth G and the seventh T were replaced by C and A, respectively). These two nucleotides are nearly invariant and have been shown to be critical for the HomolD box activity [30]. The mutation of the HomolD box abolished β-gal activity (Fig. 5b, p\textit{JY19}). These results suggest that transcription of \textit{rps2501} and \textit{trz2} may be coregulated by the HomolD box.

![Fig. 3. Schematic diagram of the intergenic region between the two ATG start codons of \textit{rps2501} and \textit{trz2}. The sequences of the 235 bp intergenic region between the two start codons of \textit{rps2501} and \textit{trz2} are shown below the schematic diagram. The TATA-like element and HomolD box are shown boxed. The NRE sequence is underlined. The TSSs and direction of transcription are indicated by arrows. The TSS position of \textit{trz2} is determined by 5′-RACE analysis and the TSS position of \textit{rps2501} is assigned according to PomBase. The TSSs of \textit{trz2} and \textit{rps2501} are indicated with large and small solid arrows, respectively. The ATG start codon is denoted by the lowercase bold letters. CDS stands for protein-coding sequence.](image-url)

![Fig. 4. The HomolD box is required for \textit{rps2501} transcription. The \textit{rps2501} promoter fragments varying in length from 2000, 1000 and 500 bp were fused to the \textit{lacZ} reporter. The HomolD box was deleted from the 500 bp \textit{rps2501} promoter fragment (p\textit{JY30}). β-gal activity was measured as described in Fig. 1.](image-url)
trz2 promoter has a TATA-like element

The TATA box is involved in determining the TSS of a gene [31]. In S. pombe, the TATA box is usually located within a region −25 to −37 upstream of the TSS [28]. We identified a TATA-like element (TAAAGTAAA) located 27–35 bp upstream of the TSS of trz2 by inspection. Since deviation of the TATA sequences from the consensus sequence often weakens the promoter, we tested whether replacement of the TATA-like element in the trz2 promoter with a TATA promoter led to a significant increase in β-gal activity (Fig. 6, pLT4). Deletion of the NRE further increased β-gal activity by −57-fold (Fig. 6, pJY33). These results suggest that lack of a strong TATA box contributes to the low level of trz2 transcription.

HomoID box and TATA-like element are required for transcription of trz2

To provide in vivo evidence that the HomoID box and the TATA-like element are required for transcription of trz2, which is essential for the viability of S. pombe, we used advantage of a ts allele of trz2, trz2-1. We constructed vectors that contain trz2 under the control of its own promoter or mutant versions in which either the HomoID box or the TATA-like element was deleted. Expression of trz2 under the control of its own promoter (pWH5-trz2) could fully rescue the trz2-1 ts lethal phenotype. In contrast, expression of trz2 under the control of mutant versions of the trz2 promoter lacking either the HomoID box or the TATA-like element could not fully suppress the ts phenotype of trz2-1 at 37°C (Fig. 7a; pWH5-ΔHomoID and pWH5-ΔTATA).

To avoid possible artefacts caused by overexpression, we tested whether expression of a single-copy trz2 gene from a promoter lacking either the HomoID box or the TATA-like element could complete the trz2-1 ts lethal phenotype. To do so, we integrated the trz2 gene under the control of the wild-type or mutant versions of the trz2 promoter into the chromosome of the trz2-1 mutant. As shown in Fig. 7(b), lack of the TATA-like element abolished the suppression of the ts phenotype of trz2-1 at 37°C, whereas lack of the HomoID box resulted in a partial suppression of the ts phenotype.

Rrn7 activates HomoID box-directed transcription in vivo

The RNA polymerase I transcription factor Rrn7 has been shown to bind to the HomoID box of the S. pombe ribosomal protein gene promoter [32]. In addition, it has been shown that S. pombe C/D small nucleolar RNA (snoRNA) and ribosomal protein genes are regulated by the HomoID box and that their expression is increased when rrn7 is overexpressed [33]. Thus, Rrn7 is likely a candidate for binding in the HomoID box in the trz2 promoter. To determine whether Rrn7 is involved in trz2 transcription, we examined the effect of rrn7 overexpression on expression of a lacZ construct carrying a 500 bp trz2 promoter fragment (Ptrz2).
The results showed that the lacZ mRNA level and β-gal activity expressed under the control of P\textsubscript{trz2} were increased 3.2- and 2.9-fold, respectively, when \textit{rrn7} was overexpressed on plasmid pREP3\textsubscript{x}-rrn7 (Fig. 8a, b). These data suggest that Rrn7 may be involved in transcription of \textit{trz2}. Because \textit{rps2501} and \textit{trz2} are localized head-to-head on opposite strands and share the HomolD box, we tested whether \textit{rrn7} overexpression also had an effect on the \textit{rps2501} expression.

### Table

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>β-gal activity</th>
<th>Length of 5′-sequence (bp)</th>
<th>Deletion endpoints 5′ 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJY4</td>
<td>&lt;0.35</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>pLT4</td>
<td>5.2±0.5</td>
<td>500</td>
<td>-110 -54</td>
</tr>
<tr>
<td>pJY10</td>
<td>6.3±0.8</td>
<td>500</td>
<td>-110 -54</td>
</tr>
<tr>
<td>pJY33</td>
<td>296±16</td>
<td>500</td>
<td>-110 -54</td>
</tr>
</tbody>
</table>

### Fig. 6
Replacement of the \textit{trz2} TATA-like element with the \textit{nmt1} TATA box increases \textit{trz2} transcription. The 500 bp \textit{trz2} promoter fragments in the presence of the NRE (pJY4) or absence of the NRE (pJY10) were fused to the \textit{lacZ} gene, and the \textit{trz2} TATA-like element was replaced with the \textit{nmt1} TATA box (pLT4 and pJY33). The mutated nucleotides are indicated by stars. β-gal activity was measured as described in Fig. 1.

### Fig. 7
Both the HomolD box and the TATA-like element are required for expression of \textit{trz2}. (a) \textit{trz2} overexpressed from the promoter lacking either the HomolD box or the TATA-like element cannot fully suppress a ts phenotype of \textit{trz2}-1. The empty vector (pWH5) and plasmids expressing \textit{trz2} from its own promoter (pWH5-trz2) or the \textit{trz2} promoter lacking either the HomolD box (pWH5-ΔHomolD) or the TATA-like element (pWH5-ΔTATA) were transformed into a \textit{trz2} ts mutant (\textit{trz2}-1). pWH5 was also transformed into the wild-type strain as a control (WT/pWH5). Tenfold serial dilutions of the indicated cells were spotted on EMM plates lacking leucine and incubated at 26 or 37 °C for 5–6 days. (b) Lack of the TATA-like element abolishes the suppression of a ts phenotype of \textit{trz2}-1. Plasmid expression of the \textit{trz2} gene under the control of the wild-type promoter (pJK148-trz2) or mutant promoters lacking the HomolD box (pJK148-ΔHomolD) or the TATA-like element (pJK148-ΔTATA) was integrated into \textit{trz2}-1. As controls, the empty plasmid pJK148 was integrated into the wild-type (WT/pJK148) and the \textit{trz2}-1 mutant (\textit{trz2}-1/pJK148). Tenfold serial dilutions of the indicated cells were spotted on EMM plates lacking leucine and incubated at 26 or 37 °C for 6–7 days.
We found that rrn7 overexpression increased β-gal activity that is expressed under the control of the rps2501 promoter (P_{rps2501}) by ~2.2-fold (Fig. 8c). As a positive control, we found that rrn7 overexpression increased β-gal activity expressed under the control of the rps2202 promoter (P_{rps2202}) by ~2.1-fold (Fig. 8d), which is consistent with a previous finding that rrn7 overexpression increases the expression level of rps2202 by ~3.0-fold. As negative controls, overexpression of rrn7 did not affect β-gal activity expressed under the control of the trz1 promoter (P_{trz1}) and the rps2502 promoter (P_{rps2502}), both of which lack the HomolD box (Fig. 8e, f).

**DISCUSSION**

In our previous studies, we observed that overexpression of trz2 but not trz1 caused a dosage-dependent growth defect in S. pombe cells, abnormalities in cell-cycle progression and apoptotic cell death, suggesting that trz2 expression is under a precise control to avoid overexpression of the gene ([8] and unpublished results). Consistent with this, we were unable to detect trz2 transcripts by Northern blotting, which was most likely due to their low abundance. These previous findings and others also suggest that a low level of Trz2 is sufficient for mitochondrial tRNA 3'–end maturation [8, 21]. Here we characterize the trz2 promoter. Using the trz2 promoter-lacZ constructs, we find that the lacZ gene under the control of the trz2 promoter produced a background level of β-gal activity, suggesting that the trz2 promoter is a very weak promoter. Our results suggest that a very low level of trz2 expression may be due in part to the existence of a very weak diverged TATA element and an NRE sequence, although other mechanisms such as post-transcriptional regulation may also contribute to weak expression of trz2. In this regard, an additional AUG codon was found eight nucleotides upstream of the AUG translation initiating codon of the trz2 gene.

![Figure 8](image-url)

**Fig. 8.** Overexpression of Rrn7 increases both β-gal transcripts and activity from the trz2 promoter-lacZ construct. (a) Overexpression of rrn7 increases β-gal transcripts produced from the trz2 promoter-lacZ construct. Total RNA was isolated from yAS56 carrying two plasmids pJY4 (a lacZ construct) and pREP3X (empty vector), or pJY4 and pREP3X-rrn7 overexpressing rrn7. The lacZ mRNA levels were determined by RT-qPCR and values were normalized to the S. pombe actin gene act1. The values represent the means ± SD of three independent experiments. (b–f) Overexpression of rrn7 increases β-gal activity from the trz2 promoter-lacZ construct (b), the rps2501 promoter-lacZ construct (c) and the rps2202 promoter-lacZ construct (d), but does not increase β-gal activity from the trz1 promoter-lacZ construct (e) and the rps2502 promoter-lacZ construct (f). β-gal activity was determined as described in Methods. See the legend of Fig. 1 for detail. Error bars represent the so of triplicates. Asterisks indicate significant differences (**P<0.001). P-value is calculated based on two-sample t-test.
This upstream AUG is not in frame with the trz2 ORF, and may negatively impact on the translation efficiency of trz2.

The trz2 promoter appears to contain a very weak divergent TATA element at the TATA-box expected location because replacing this TATA-like element with a strong one results in a significant increase in β-gal activity. Complementary studies with the trz2 gene under the control of mutant trz2 promoters indicate that the TATA-like element is essential for expression of trz2 and is very likely to be a functional TATA box which recruits the TATA box-binding factor TFIID to initiate transcription.

The NRE sequence identified in the promoter of trz2 appears to be a typical negative regulatory element. It is unlikely that binding sites for sequence-specific repressors at the NRE mediate repression for the following reasons. First, as shown in Fig. 2, a lacZ construct (pPY24) carrying a deletion which is of the same size as the NRE but is shifted 18 bp upstream from the original site produces β-gal activity at a level comparable to that produced from the lacZ construct carrying a deletion of the NRE sequence (constructs pPY9 and pPY10). Second, replacement of the NRE sequence with a random DNA sequence does not abolish the repressive function of the NRE. Third, we have failed in our attempts through DNA affinity purification to identify repressive factor(s) that bind to the NRE. The exact mechanism by which the NRE represses trz2 transcription remains unknown. One possible explanation is that deletion of the NRE brings the HomolD box and the TATA-like element closer together, which may facilitate their interaction. Another possible explanation is that deletion of the 57 bp NRE sequence may generate optimal spacing between the HomolD box position and the TSS. A typical HomolD box is present at 39–55 bp upstream of the TSS [29]. In contrast, the HomolD box position in trz2 is 110 bp upstream of the TSS, which does not appear to be an optimal position for achieving high transcription activity.

The HomolD box was initially found in the TATA-less promoters of S. pombe ribosomal protein genes and has been shown to regulate the expression of ribosomal protein genes [29]. The bioinformatic analysis of the cis-elements in 17 fully sequenced fungal species suggests that an ancient HomolD box plays a key role in coexpression of fungal ribosomal protein genes [34]. The bioinformatics and phylogenetic studies also suggest that there is a gradual switch from a HomolD-based mechanism to a RAP1-controlled mechanism for coregulation of ribosomal protein genes during evolution [34]. The HomolD box is also found in the TATA-containing promoters of S. pombe C/D snoRNA genes and is required for their transcription [33, 35]. The HomolD box may function to coordinate the transcription of the ribosomal protein genes and C/D snoRNA genes in S. pombe [33].

Our analysis reveals the existence of the HomolD box in the trz2 promoter. The identification and characterization of the HomolD box in the polymerase II promoter other than ribosomal protein gene promoters have not been previously reported. Several lines of evidence suggest that the HomolD box is likely to play a regulatory role in trz2 expression. First, mutations or deletion of the HomolD box reduces β-gal activity in the absence of the NRE. Second, trz2 expressed from its own promoter is able to fully complement the temperature sensitivity of the Δtrz2 mutant strain, whereas trz2 expressed from its native promoter but lacking the HomolD box cannot. Third, overexpression of the HomolD box-binding protein Rrn7 increases both lacZ mRNA level and β-gal activity compared to vector alone. However, further work is required to address how the HomolD box functions in trz2 transcription.

In S. pombe, trz2 and rps2501 are arranged on opposite strands in a head-to-head orientation. Our data suggest that transcription of trz2 and rps2501 is potentially coregulated by a bidirectional HomolD box, which may provide a layer of control for cytosol protein synthesis and mitochondrial gene expression.

Like S. pombe, other fission yeasts including S. octosporus, S. japonicus and S. cryophilus contain two tRNase Z’s [7]. Interestingly, the HomolD box is also found in the promoter regions of mitochondrial trz2 genes (Fig. S1). Moreover, the trz2 and rps2501 genes in these fission yeasts have a similar arrangement to what is found in S. pombe (Fig. S1), suggesting that transcriptional coregulation of trz2 and rps2501 is evolutionarily conserved in the Schizosaccharomyces groups.

In addition, sequence analysis reveals that the promoters of mitochondrial trz2 genes from a few other fungal species such as Ustilago maydis and Pleurotus ostreatus and Paracoccidioides brasilensis contain the HomolD box, but no nearby rps25 gene was found in the same strand or in the opposite strand of the DNA (Fig. S1). It is likely that the HomolD box may also regulate trz2 transcription in fungal species other than S. pombe.

Our analysis reveals that transcription of trz2 may be dependent on two elements, the HomolD box and the TATA-like element, suggesting the integration of two Pol II promoter systems in the trz2 promoter. A similar observation has been reported for transcription of U3 snoRNA, which requires both the HomolD and TATA boxes [35].


Edited by: H. Sychrova and V. J. Cid

Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.