The yeast protein Xtc1 functions as a direct transcriptional repressor

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Received February 20, 2002; Revised and Accepted April 16, 2002

ABSTRACT

The yeast protein Xtc1 was identified as a protein that binds directly and specifically to the activation domains of acidic activators such as E2F-1, Gal4 and VP16. Additionally, it was shown to co-purify with the RNA polymerase II holoenzyme complex and it was suggested that Xtc1 functions as a regulator of transcription that modulates the response of RNA polymerase II to transcriptional activators. We have further analyzed the transcription function of Xtc1 and show that its fusion to a heterologous DNA binding domain can repress transcription of a reporter gene in vivo in an Srb10/11-dependent manner. We suggest that the presence of Xtc1 in the RNA polymerase II holoenzyme could help to recruit an Srb10-active form of the holoenzyme to target promoters. This same protein has also been implicated in mitochondrial DNA recombination, maintenance and repair. Determination of the subcellular localization using a GFP–Xtc1 fusion shows that it localizes to both the nucleus and the mitochondria in vivo, which is consistent with Xtc1 having a function in both cellular compartments.

INTRODUCTION

Transcription by RNA polymerase II is regulated through the action of activators and repressors of transcription (1). In addition, numerous co-regulatory proteins are found in the Srb/Mediator complex, which is physically associated with RNA polymerase II via the C-terminal domain (CTD) of its largest subunit and, together with the polymerase and a subset of general transcription factors, forms the RNA polymerase II holoenzyme (1).

Classic activators (e.g. Gal4, VP16, E2F-1 and Gcn4) usually contain acidic, negatively charged activation domains and their activation function is exerted through interactions with various components of the transcription machinery, thereby recruiting RNA polymerase II and associated factors to the promoter (2–4). Fusion of a heterologous DNA binding domain to a component of the transcription machinery, such as the TATA-binding protein (TBP) (5–7), TFIIB (8,9), TBP-associated factors (TAFIIs) (4,8,10,11), various Srb proteins and Gal11 (11–14) can activate transcription in the absence of a classic activation domain, proving that recruitment of the transcription machinery is the predominant role of the activation domain.

Transcriptional repressors can be either general or gene specific (15). General repressors usually act through the general transcription machinery and negatively regulate a broad class of functionally unrelated genes (15).

Gene specific repressors regulate the expression of a defined group of genes. Some act by binding to the activation domains of gene specific activators and thereby inhibit their function (reviewed in 20). Other gene specific repressors bind sequences in the upstream regulatory regions of their target genes and recruit other repressor proteins, which then act to repress transcription. For example, the yeast protein Ume6 or the mammalian proteins YY1 and Mad recruit histone deacetylases that deacetylate nucleosomal histones and produce a transcriptionally inactive chromatin structure (21–25). In contrast, the yeast repressors Mig1, α2 or Ctrl recruit the co-repressor complex Tup1/Ssn6 (26–29), which is thought to inhibit gene expression via interactions with components of the transcription apparatus or by modifying chromatin structure (30–32).

Transcriptional repression is also mediated by the kinase–cyclin pair Srb10/11 found in the RNA polymerase II holoenzyme (33). The Srb10 kinase phosphorylates the CTD of RNA polymerase II prior to stable association of the polymerase with promoter DNA and, thus, prevents the formation of a transcriptionally competent initiation complex (34). Srb10 is not a general repressor of transcription. Genome-wide analysis of srb10 mutants has shown that it is required for repression of a defined subset of genes (∼3% of the genome), half of which belong to the group of genes induced by nutrient deprivation (35).

Tethering of repressor proteins to promoter regions by a heterologous DNA binding domain, as in the case of Lex–Tup1, Lex–Ssn6, Lex–Srb10, Lex–Sin3 or Lex–Rpd3, results in repression of transcription (24,26,34,36).

Recently, a novel yeast protein, Xtc1, was identified using an in vitro crosslinking technique (37). Xtc1 could be covalently crosslinked in a promoter-dependent manner to the activation domain of a photoactive derivative of Lex–E2F-1 in an in vitro transcription system. It was also shown to bind directly and specifically to the activation domains of two other acidic activators, Gal4 and VP16, and to co-purify with the RNA polymerase II holoenzyme (37). The interaction of Xtc1 with
acids. Acidic activation domains positively correlated with their ability to activate transcription. Emili et al. suggested that Xtc1 could act to regulate the response of RNA polymerase II to transcriptional activators (37).

Interestingly, this same protein has also been implicated to function in mitochondrial DNA (mtDNA) recombination and maintenance in yeast cells (38), and to be involved in repair of oxidative damage to the mitochondrial genome (39). Thus, two different functions have been proposed for Xtc1: one is regulation of RNA polymerase II-dependent transcription and the other is regulation of mtDNA recombination, maintenance and repair.

In this report we have further analyzed the function of Xtc1 in transcription regulation by RNA polymerase II and its subcellular localization in yeast cells in vivo.

MATERIALS AND METHODS

Yeast strains and plasmids

The reporters for monitoring β-galactosidase activity are shown in Figure 1 and were integrated at the URA3 locus of Saccharomyces cerevisiae strains GGY1H (gal4 gal80 tyr1 ade leu2 his3 ura3 trp1::HIS3), NLY2 (Δgal4 Δgal80 ura3 his3 leu2 trp1 lys2), and Δsrb10 or Δsrb11 derivatives of NLY2 (30). The reporters GG9, SS36 and RY171 were integrated in the URA3 locus of GGY1H to give strains GGY1H::GG9, GGY1H::RY171 and GGY1H::SS36, whereas SS38-3 was integrated in the same locus of wild-type, Δsrb10 and Δsrb11 derivatives of NLY2, to give NLY2::SS38-3, NLY2Δsrb10::SS38-3 and NLY2Δsrb11::SS38-3.

The reporter JK1621 (2μ, URA3), containing a LexA operator upstream of the CYC1 promoter, was transformed into strain FT5 (ura3, trp1, his3, leu2).

All Gal4 (1–147 amino acids) fusion constructs were constructed using the vector pAS2 (Clontech). DNA fragments coding for Xtc1 or deletion constructs were amplified by PCR using appropriate primers and cloned between the NdeI and BamHI sites of pAS2. The fusion of Xtc1 to the LexA DNA binding domain was constructed by inserting the gene coding for Xtc1 between the EcoRI and BamHI sites in the multicloning site of vector pBTM116 (2μ, TRP1). For construction of vectors expressing fusions of Xtc1 to the green fluorescent protein (GFP), the XTC1 gene was PCR amplified using primers with the appropriate restriction enzyme sites (Xhol and XbaI for the N-terminal fusion, and XbaI and BamHI for the C-terminal fusion) and cloned into the multicloning sites of pGFP-N-FUS or pGFP-C-FUS, respectively (CEN/ARS, MET25) (40). For fluorescence microscopy, pGFP-N-Xtc1 or pGFP-C-Xtc1 were transformed into the strain W3031B (ade2 trp1 leu2 his3 ura3).

β-galactosidase assays

For assaying β-galactosidase activities, yeast strains were transformed with the Gal4 or LexA fusion constructs and grown in selective 2% glucose media to mid-log phase. Specific β-galactosidase activities were determined from yeast cultures by the method of Adams et al. (41). Each assay was performed in triplicate from at least three independent transformants. The standard error was between 10 and 20%. The β-galactosidase activities obtained with the fusion constructs are expressed as percentages of the activity of the Gal4 or LexA DNA binding domain, except for the reporter SS36, which has no Gal4 binding sites and for which the reference activity was that of the reporter alone.

Protein extracts and western blotting

Protein extracts were prepared as described previously (41) except that the breaking buffer contained 1 M NaCl. For western blotting, 500 μg of proteins from each extract preparation were resolved on a 12.5% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with a 1:400 dilution of anti-Gal4 DNA binding domain monoclonal antibody (Santa Cruz Biotechnology), followed by a 1:3000 dilution of goat anti-mouse HR-peroxidase conjugated secondary antibody (BioRad). Signals were detected by enhanced chemiluminescence following the manufacturer’s protocol (Amersham).

Microscopy

Yeast strain W3031B was transformed with pGFP-N-Xtc1 or pGFP-C-Xtc1 and grown in selective 2% glucose media supplemented with 50 μg/ml adenine to mid-log phase. For microscopy, cells were fixed in 70% ethanol for 12 min, washed with phosphate-buffered saline and stained with 2.5 μg/ml 4,6-diamino phenyldinolide (DAPI). Cells were visualized using a Nikon Eclipse E1000 microscope.

RESULTS

Repression by Xtc1 in vivo

Previous studies suggested that Xtc1 might function as a regulator that modulates the activity of RNA polymerase II (37), but the mechanism by which Xtc1 acts in regulating gene expression is unclear. To better characterize the function of Xtc1 in transcription regulation, we tested the in vivo transcription function of a fusion of Xtc1 to the heterologous DNA binding domain of Gal4 (Gal4–Xtc1), using a chromosomally integrated lacZ reporter construct containing Gcn4 and Gal4 binding sites upstream of the yeast GAL1 core promoter (Fig. 1, reporter GG9). As shown in Figure 2A, Gal4–Xtc1 repressed transcription of the reporter gene to 40% of the activity obtained with the DNA binding domain alone. To eliminate the possibility that transcriptional repression is a result of steric hindrance in the context of GG9, where the repressor is bound downstream of the activator, we also tested the repression function of Gal4–Xtc1 using the reporter SS38-3, in which Gal4 binding sites are positioned upstream of Gcn4 binding sites (Fig. 1). A similar level of repression by Gal4–Xtc1 was observed in the context of SS38-3 (Fig. 2A), arguing against a steric hindrance mechanism of repression. Furthermore, repression by Gal4–Xtc1 is dependent on tethering Xtc1 to the promoter region by the Gal4 DNA binding domain, since no repression was observed in the absence of Gal4 binding sites in the reporter or with Xtc1 lacking the Gal4 DNA binding domain (Fig. 2A, reporter SS36; data not shown).

We next tested whether Xtc1 was also able to repress transcription in a different promoter context using a reporter plasmid containing the LexA operator upstream of the CYC1 promoter driving the expression of lacZ (Fig. 1, JK1621). Figure 2B shows that Lex–Xtc1 also repressed transcription of a reporter gene in the context of the CYC1 promoter.
In order to identify the regions of Xtc1 required for transcriptional repression, we sequentially deleted 50, 75 and 100 amino acids from the N-terminus and 50 and 100 amino acids from the C-terminus of Xtc1, and fused them to the Gal4 DNA binding domain. Figure 3A shows that deletion of 50 or 75 N-terminal amino acids, or 50 or 100 C-terminal amino acids did not compromise the ability of Xtc1 to repress transcription of the reporter gene. However, the deletion mutant that lacks the N-terminal 100 amino acids (ΔN100) no longer repressed transcription, but rather exhibited higher β-galactosidase activity than the DNA binding domain alone. Therefore, the repression domain of Xtc1 maps to the fragment between amino acids 75 and 100 (Fig. 3A). This fragment has a positive charge of +3 and a number of hydrophobic (Val+Leu) residues. The identified repression domain was sufficient to repress expression of the reporter gene (Fig. 3A, see construct RD 75–100), but to a slightly lesser extent than the full length protein.

The activation potential of the construct ΔN100 was also assayed using the reporter RY171 (Fig. 1), which is analogous to GG9, but lacks Gcn4 binding sites and it is, therefore, not activated. As shown in Figure 3B, the C-terminal domain of Xtc1 activated transcription of the reporter gene 3-fold, compared with the Gal4 DNA binding domain. Thus, in addition to the repression domain, Xtc1 bears a weak activation domain in the C-terminal part of the protein.

Expression of all Gal4 fusion proteins, except the construct containing the repression domain (RD 75–100), was confirmed by western blot (Fig. 4; data not shown). Since we were able to observe repression with the construct RD 75–100, we believe it is expressed in yeast but possibly to a lower level than the other fusions. Also, we were unable to demonstrate expression of the deletion mutant ΔN100 in strain GGY1ΔH::GG9, but were able to do so in strain GGY1ΔH::RY171, in which this construct also activated transcription. Therefore, we think that the construct ΔN100 is expressed in both yeast strains, but that it is either rapidly degraded or inefficiently extracted in our extract preparations from strain GGY1ΔH::GG9.

Xtc1 has been shown to co-purify with the RNA polymerase II holoenzyme complex (37) and could possibly functionally interact with other holoenzyme components in exerting its repression function. Therefore, we tested whether repression by Gal4–Xtc1 required the function of the holoenzyme components Srb10 and/or Srb11. The reporter SS38-3 was integrated in the URA3 locus of strains deleted for genes encoding either Srb10 or Srb11 and β-galactosidase activities were assayed. Table 1 shows that, in contrast to wild-type cells where Gal4–Xtc1 repressed transcription 2-fold compared with control levels, repression was abolished in the Δsrb10 and Δsrb11 strains. The expression of the Gal4–Xtc1 fusion was not affected in the deletion strains (data not shown).

These data suggest that Xtc1 functionally interacts with the kinase–cyclin pair Srb10/11 to repress gene transcription in vivo.

Subcellular localization of Xtc1

As mentioned previously, Xtc1 was implicated not only in regulating RNA polymerase II dependent transcription (37), but also to function in the maintenance, recombination and repair of the mitochondrial genome (38,39). These two processes occur in separated cellular compartments, the nucleus and the mitochondria, which prompted us to determine the subcellular localization of Xtc1.
To that end, we fused GFP to the C- or N-terminus of Xtc1 and looked at its subcellular localization by fluorescence microscopy.

Wild-type yeast cells were transformed with plasmids containing either N- or C-terminal fusions of Xtc1 to GFP and grown in selective media at 30°C to mid-log phase. Nuclear and mitochondrial DNA were visualized by DAPI staining.

As shown in Figure 5A, when GFP was attached to the C-terminus of Xtc1, GFP–Xtc1 localized exclusively in the mitochondria; however, the N-terminal GFP fusion to Xtc1 could be seen in both nuclear and mitochondrial compartments (Fig. 5B). The C-terminal GFP–Xtc1 fusion expressed from plasmid pGFP-C-Xtc1 is functional for complementation of the petite phenotype of an XTC1 deletion strain. Interestingly, the N-terminal fusion could not complement the petite phenotype of the XTC1 deletion strain, even though it localized to the mitochondria, suggesting that the N-terminal fusion of GFP obstructs an interaction required for full mitochondrial function. Similarly, the fact that the N- but not the C-terminal fusion localizes to the nucleus suggests that fusing GFP to the N-terminus of Xtc1 precludes an interaction important for nuclear localization. At present, we have been unable to define a specific nuclear phenotype for the XTC1 deletion strain given the pleiotropic effect of the petite phenotype and thus cannot test for nuclear function complementation.

The fact that we could localize Xtc1 to both the nucleus and the mitochondria is in agreement with the two proposed functions for this protein.

### DISCUSSION

The yeast protein Xtc1 was identified as a protein that binds directly and specifically to the activation domains of the acidic activators E2F-1, Gal4 and VP16, and co-purifies with the RNA polymerase II holoenzyme complex. Binding to acidic activators positively correlated with their ability to activate transcription and Emili et al. suggested that Xtc1 may act as a factor that modulates the response of RNA polymerase II to transcriptional activators (37). Various activator binding proteins and RNA polymerase II holoenzyme components are able to influence reporter gene expression when tethered to the promoter region by a heterologous DNA binding domain (4–14,42,43). Therefore, to further address the transcription function of Xtc1, we used a Gal4–Xtc1 fusion and assayed its ability to regulate expression of a synthetic lacZ-based reporter containing Gcn4 and Gal4 binding sites upstream of a GAL1 core promoter. Our results show that Gal4–Xtc1 inhibits reporter gene expression in vivo. Repression by Gal4–Xtc1 was observed regardless of whether Gal4 binding sites were upstream or downstream of Gcn4 binding sites. These data argue against a steric hindrance mechanism of repression, in which DNA-bound Gal4–Xtc1 would simply sterically impede
activation by Gcn4. In addition, Xtc1 was also able to repress transcription in the CYC1 promoter context.

By sequentially deleting 50, 75 and 100 amino acids from the N-terminus, or 50 and 100 amino acids from the C-terminus, we have shown that, as is the case for other repressors such as Eve, Krüppel or Mig1 (44–46), Xtc1 contains a small, 25 amino acid long repression domain, which is located between amino acids 75 and 100. This domain bears a positive charge of +3, and positively charged fragments have been found to repress transcription when fused to the Gal4 DNA binding domain (47). However, in contrast to the repression domain of Xtc1, the peptides identified by Saha et al. had, in principle, a higher degree of basicity, and it was shown that the degree of basicity positively correlates with the strength of repression (47). In that respect, the small positive charge of the Xtc1 repression domain could account for the relatively weak repression of 2–2.5-fold, observed with Gal4–Xtc1. The 25 amino acid long repression domain was sufficient to inhibit transcription when fused to the Gal4 DNA binding domain, although to a lesser extent than the full length protein.

Interestingly, the construct which lacked the N-terminal 100 amino acids of Xtc1 showed higher β-galactosidase activities than the DNA binding domain alone, both in the context of the Gcn4-activated reporter and with the reporter which lacks Gcn4 binding sites. These data demonstrate that Xtc1 bears an activation domain in its C-terminal portion. The activation potential of this domain is weak, activating transcription 3-fold, in contrast to strong activators, such as VP16, which activates transcription 1000-fold in the same reporter context (48). Other transcriptional regulators, such as some hormone receptors or p53, also contain separate activation and repression domains (49–52), and many coactivators have been implicated to function in both activation and repression of transcription (36,53–56). It has recently been shown that the yeast co-repressor complex Tup1/Ssn6 can switch to a coactivator function, in response to mitochondrial dysfunction (57). The Ssn6 component was critical for both the interaction with Rtg3 and the co-activator function, and different domains of the Ssn6 protein were required for the function of the complex in activation versus repression of transcription (57). Therefore, it is possible that Xtc1 can positively regulate transcription at certain genes, or in a different physiological context, and that this function is exerted through its C-terminal domain.

Since it has been shown that Xtc1 co-purifies with the RNA polymerase II holoenzyme complex, it seemed plausible to assume that it functionally interacts with other coactivators to repress gene expression. Among coactivators, components involved in repression, the best characterized is the Srb10/11 kinase–cyclin pair that negatively regulates transcription by phosphorylating the C-terminal domain of the largest subunit of RNA polymerase II, thereby preventing stable preinitiation complex formation (34). Srb10/11 function is also required for repression by the artificial repressor protein Lex–Tup1 (30,58). We tested whether Gal4–Xtc1 functionally interacted with the kinase–cyclin pair Srb10/11 in repressing gene expression, by assaying its repression function in strains deleted for SRB10 or SRB11 and found that repression by Gal4–Xtc1 was abolished in the ∆srb10 and ∆srb11 mutants, showing that Gal4–Xtc1 requires Srb10 and Srb11 to efficiently repress transcription in vivo. The fact that Xtc1 was able to repress transcription in different promoter contexts also suggests that it may act through the general machinery to inhibit gene expression. Since Srb10 does not globally affect transcription and represses only a subset of genes (35), there must be a mechanism to differentiate between Srb10-dependent and -independent promoters. It is possible that different RNA polymerase II holoenzyme complexes are recruited at Srb10-dependent versus Srb10-independent promoters, and that the presence or absence of Xtc1 discriminates between different types of holoenzyme. Xtc1 could help recruit the RNA polymerase II holoenzyme at Srb10-dependent genes by interacting with a promoter specific factor. It is also possible that the presence of Xtc1 stimulates the kinase activity of

![Figure 5](image-url)
Srb10, which is presumably inactive at genes that are not negatively regulated by this kinase (34). If Gal4–Xtc1 recruited a repression-specific type of holoenzyme with an active Srb10 kinase to the promoter, this would result in the observed repression of transcription. Similarly, Srb10 also represses transcription of a reporter gene when fused to a heterologous DNA binding domain (34,36).

In addition to regulation of RNA polymerase II-dependent transcription, it was also proposed that Xtc1 functions in homologous recombination, maintenance and repair of mitochondrial DNA in yeast cells (38,39). Since these two processes occur in different cellular organelles, namely the nucleus and the mitochondria, we determined the subcellular localization of Xtc1 using a fusion to the GFP (40). By using fusions of GFP to either the C- or N-terminal part of the protein, we found an interesting localization pattern: when GFP was fused to the C-terminus of the Xtc1 protein, the fusion localized to the mitochondria and was excluded from the nucleus. However, the N-terminal GFP–Xtc1 fusion localized to both organelles. There are a number of possible explanations for this localization pattern. Perhaps the C-terminus of Xtc1 has to be free for it to be localized to the nucleus, possibly by interacting with another protein required for its transport into the nucleus. Alternatively, two different forms of Xtc1 could exist in the cell: the full length protein that localizes to the mitochondria and the protein truncated at the C-terminus, which localizes to the nucleus. The latter would not be seen when GFP is attached to the C-terminal part of Xtc1, because any putative processing/cleavage events at the C-terminus would also result in release of the GFP moiety.

It is interesting to note that there is precedence for both yeast proteins, such as the helicase Pif1 (59,60) or the DNA repair protein Ntg1 (61), and mammalian proteins (for example the human DNA glycosylases hOGG1 and hNTH1) (62), localizing to the nucleus and the mitochondria and having either the same or a totally different function in each organelle.

The fact that we found that Xtc1 could be localized to both the nucleus and the mitochondria is consistent with the two proposed functions for this protein, one in the regulation of RNA polymerase II-dependent transcription and the other in mtDNA physiology. Although we cannot rule out the possibility that the nuclear and mitochondrial functions of Xtc1 are related, the dual localization in both organelles suggests that the two functions could be functionally and physically separated.

Future efforts will focus on finding Xtc1 target genes using whole genome transcription profiling of mutant strains and on identifying its protein interaction partners in the cell, both of which should allow us to better characterize the in vivo function of Xtc1.

ACKNOWLEDGEMENTS

We are grateful to Dr C. J. Ingles for his support in the initial stages of this work. We also thank Drs M. Ptashne, K. Struhl, S. Saha and J. Hegemann for strains and plasmids. This work was supported by a grant to M.S. from the Croatian Ministry of Science and Technology. A.T., L.S. and M.A. are recipients of a graduate scholarship from the Croatian Ministry of Science and Technology.

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