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Srb7p is essential for the activation of a subset of genes

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Abstract The mediator complex in the RNA polymerase II holoenzyme is known to be involved in transcriptional activation. The role of the essential mediator component Srb7p has been difficult to investigate, since no conditional lethal allele has been available to date. While the expression of Srb7p under the control of a repressible promoter is not sufficient to reduce the level of Srb7p beneath the threshold for survival, we have been able to isolate a clone termed ts16 which confers a temperature sensitive phenotype. ts16 contains an insertion mutation that requires translational frameshifting for correct expression of Srb7p, leading to extremely low protein levels. Strains bearing the ts16 construct show mild defects in the transcription of constitutive genes like TDH1 but severely affect activated transcription, e.g. of the GAL1 gene. In contrast, CUP1, which is also independent of other holoenzyme components, is not affected by ts16.

Key words: Mediator; Holoenzyme; Transcription; Galactose induction; *Saccharomyces cerevisiae*

1. Introduction

Recruitment of the RNA polymerase II machinery is an important step in transcriptional activation [1,2]. Recent evidence suggests that the polymerase is brought to the promoter as a holoenzyme complex [3–5] containing a subset of the general transcription factors, the mediator subunit and the Swi/Snf complex [6]. The mediator subunit is sufficient to confer to an in vitro reconstituted transcription system the ability of responding to activator proteins [4]. It consists of 16 polypeptides including the Med proteins and a subset of the Srb (suppressor of RNA polymerase B) proteins [7]. The Med proteins have been identified biochemically because of their presence in the mediator [7,8] while the Srb proteins were discovered in a genetic screen with a truncated C-terminal domain (CTD) of the largest subunit of RNA polymerase II. Mutations in the Srb proteins were able to suppress the cold sensitive phenotype of yeast strains with such a CTD truncation [3,9]. Within the mediator, the Srb proteins 2, 4, 5 and 6 form a small subcomplex together with Med6p and Rox3p [10,11]. Of the Srb proteins, Srb proteins 4, 6 and 7 are essential. Studies with temperature sensitive (ts) alleles of SRB4 and SRB6 or deletion strains of SRB2 and SRB5 have shown that these proteins are needed for efficient transcription of the vast majority of RNA polymerase II genes [3,12,13]. One of the notable exceptions is the CUP1 gene, which is also independent of Kin28p, the kinase subunit of TFIIH [14,15]. In contrast to these Srb proteins, a ts mutant of Med6p was shown to selectively affect transcription of some activated yeast genes [16]. Even more specifically, mutations in Med9p or Med10p cause defects in the induction of only certain activated genes [17]. A conditional lethal mutant of Srb7p has not been available to date. Here we report that a ts allele of SRB7 negatively affects transcription of some but not all genes to a moderate degree, while impairing the activation of a subset of genes.

2. Materials and methods

2.1. Yeast strains and plasmids

Parental yeast strains were JD53 (matα *ura3–52 leu2–3,-112 his3Δ200 lys2–801 trp1Δ63*), the isogenic **a** strain JD52 [18] and strain NLY2 (matα *gal4- gal80- ade- ura3–52 leu2–1 his3Δ200 trp1–1 lys2Δ3*) [19]. Strains JD53Δsrb7 and NLY2Δsrb7 were generated by first transforming plasmid YCplac33 [20] containing SRB7 with its own promoter and terminator and then deleting the chromosomal copy of SRB7 by homologous recombination with a linearised construct consisting of the SRB7 promoter, the LYS2 gene and the SRB7 terminator. The resulting Lys+ strains are suitable for the plasmid shuffle method. The plasmid Pgal1-SRB7 contains 660 bp GAL1 promoter sequences and the coding sequence and terminator of SRB7 generated by polymerase chain reaction (PCR), with the second codon exchanged from ACA to TAC (see text) in vector YCplac111 [20]. Strain JD53::Pgal1SRB6 was made by replacing the chromosomal SRB6 promoter with the TRP1 gene and 660 bp GAL1 promoter sequences using a cassette consisting of SRB6 upstream sequences, the TRP1 gene, the GAL1 promoter and the SRB6 coding sequence with the second codon AGC replaced by TAC. The correct recombination was checked by growth phenotype and PCR with genomic DNA. Plasmid dihydrofolate reductase $(DHFR)^{ts}$SRB7 contains the CUP1 promoter and ubiquitin-$DHFR^{ts}$ sequences of pPW66R (*Bam*H1/*Bam*H1 and *Bam*H1/Asp718 fragments) [21] and the coding sequence and terminator of SRB7 in vector YCplac22 [20]. In the original ts16 construct, an *Escherichia coli* DNA sequence (see text) replaced the *Bam*H1-Asp718 fragment of pPW66R. In addition, there was a base insertion mutation in the third codon of SRB7. To eliminate the CUP1 promoter, a PCR generated fragment containing all sequences downstream of the ATG of ubiquitin was subcloned into YCplac111. This construct was used in most experiments. A construct starting with the *E. coli* DNA sequence (compare Fig. 2) was also generated and tested for ts and mating defect phenotypes. Plasmid Pcup1GAL4 contains 450 bp CUP1 promoter sequence and the coding sequence and terminator of GAL4 in vector pRS314 [22]. Yeast cells were grown in YPDA or dropout medium [23]. Galactose media contained 2% galactose instead of glucose. 5-Fluoro orotic acid (FOA) medium contained 0.5 g/l FOA [24].

2.2. Primer extension

Yeast strain JD53Δsrb7 was transformed with plasmids SRB7wtYCplac111 or ts16, streaked on FOA medium to eliminate the URA3 marked SRB7wt plasmid and cells were grown in YPDA medium. RNA was prepared as described [25] and primers (TTGCAGCTACCACATTGGCATTGGCACTCA for CUP1 and

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Abbreviations: CTD, C-terminal domain; DHFR, dihydrofolate reductase; FOA, 5-fluoro orotic acid; ORF, open reading frame; Srb, suppressor of RNA polymerase B

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AAACGAAATAAATCTCTTTGTAAAACGGTTCA for U6) were labelled with polynucleotide kinase [26]. Primer extension reactions were carried out following the protocol for aqueous hybridisation [27]. Samples were further treated according to [26] and resolved on a 6% polyacrylamide 7 M urea gel.

2.3. Northern blots

RNA preparation and Northern blot were carried out as described [25,26]. For the probes, gel purified PCR fragments of the ACT1, TDH1, GAL1 and MFα1 coding sequences were labelled with Klenow fragment and random hexanucleotide primers. For CUP1, the promoter and coding sequence was used to generate a longer probe.

3. Results

3.1. Expression of Srb7p from a glucose repressed promoter still supports cell viability

In order to investigate the function of an essential protein like Srb7p, it is necessary to create a conditional lethal allele. One way to achieve this is to place the coding sequence of the gene in question under the control of a repressible promoter, e.g. the GAL1 promoter. The resulting yeast strain should be viable under inducing conditions (galactose) but non-viable under repressing conditions (glucose). To achieve faster degradation of the protein after the switch from galactose to glucose medium, we changed the codon following the ATG to TAC, coding for tyrosine instead of threonine. According to the N-end rule, this should increase the turnover of the protein [28]. Surprisingly, the Pgal1-SRB7 construct supports cell viability also under repressing conditions, even at elevated temperature (Fig. 1A). However, a Western blot using αSrb7p antibody confirmed that Srb7p expression in this strain was regulated in a carbon source dependent manner (Fig. 1B). In contrast to the results with Srb7p, placing SRB6 under the control of the GAL1 promoter in a similar strategy led to a conditional lethal strain as predicted (data not shown). For both the SRB6 and the SRB7 deletion, the entire open reading frame (ORF) had been deleted and the respective expression vectors contained the ORFs under the control of heterologous promoters and terminators. Therefore, we can exclude homologous recombination as a way for the promoterless SRB7 or the ts16 clone to support viability. In case that the unexpected result for Srb7 was a peculiarity of the GAL1 promoter, we also tested the MET3 promoter which is repressed in the presence of methionine. But like Pgal1-SRB7, Pmet3-SRB7 also supports cell viability under inducing as well as repressing

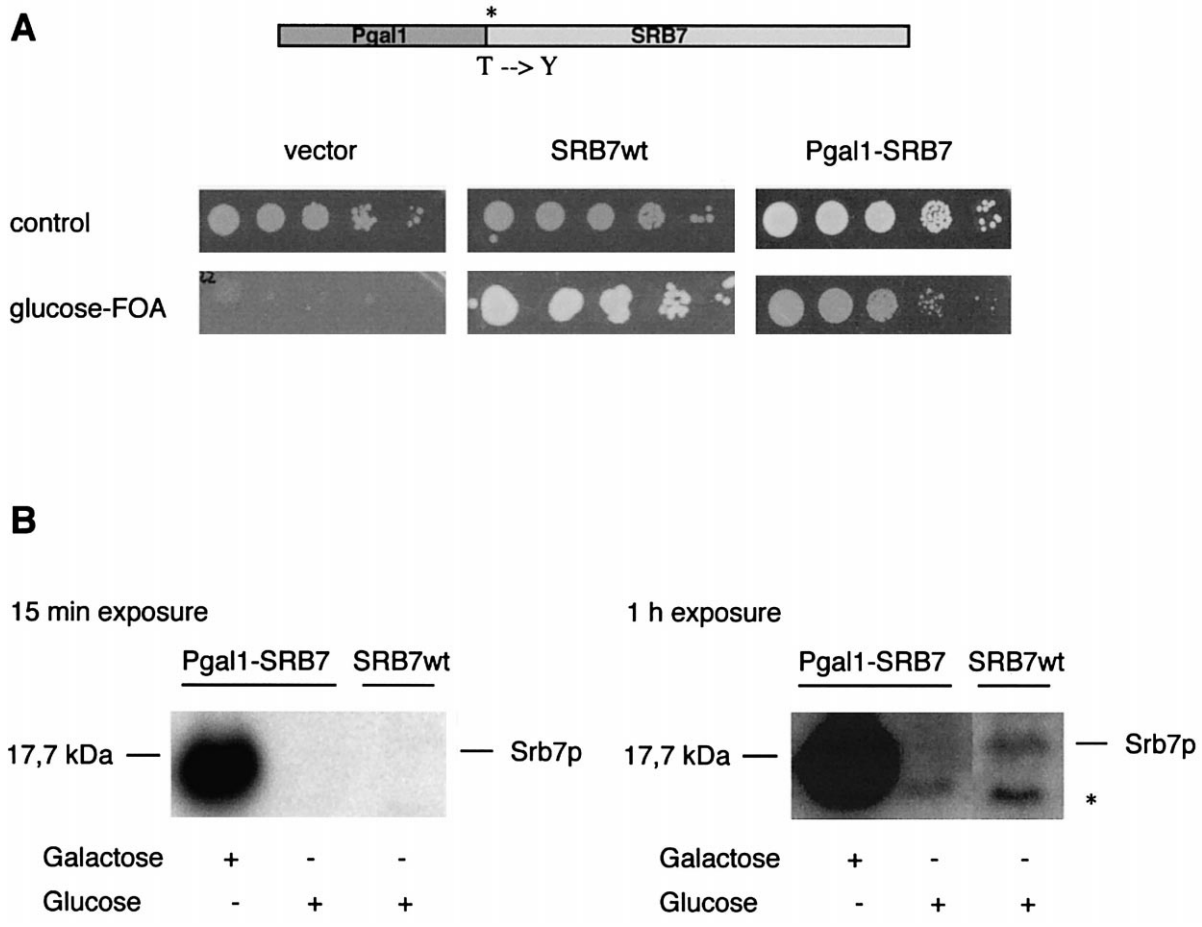

Fig. 1. Expression of Srb proteins from a repressible promoter. A: Strain JD53Δsrb7 was transformed with empty vector (left), SRB7wt on a LEU2-marked plasmid (middle) or a plasmid carrying the SRB7 coding sequence under the control of the GAL1 promoter (right). The construct is depicted on top; T→Y is a mutation of the second amino acid to achieve faster degradation, see text for details. The Pgal1-SRB7 strain can still grow under repressive conditions (glucose FOA medium). The control plates do not contain FOA and thus allow retention of the URA3 marked SRB7wt plasmid. The strain with empty vector does not contain any other source of SRB7 and cannot grow on FOA medium (left). B: Western blot showing the expression of Srb7p in strain JD53Δsrb7 from the Pgal1-SRB7 plasmid in galactose medium (left) and its repression in glucose medium (middle). For comparison, the expression of Srb7p from its own promoter (SRB7wt) is shown (right). Two different exposures of the same blot are shown. The asterisk marks a non-specific band recognised by the αSrb7p antibody.

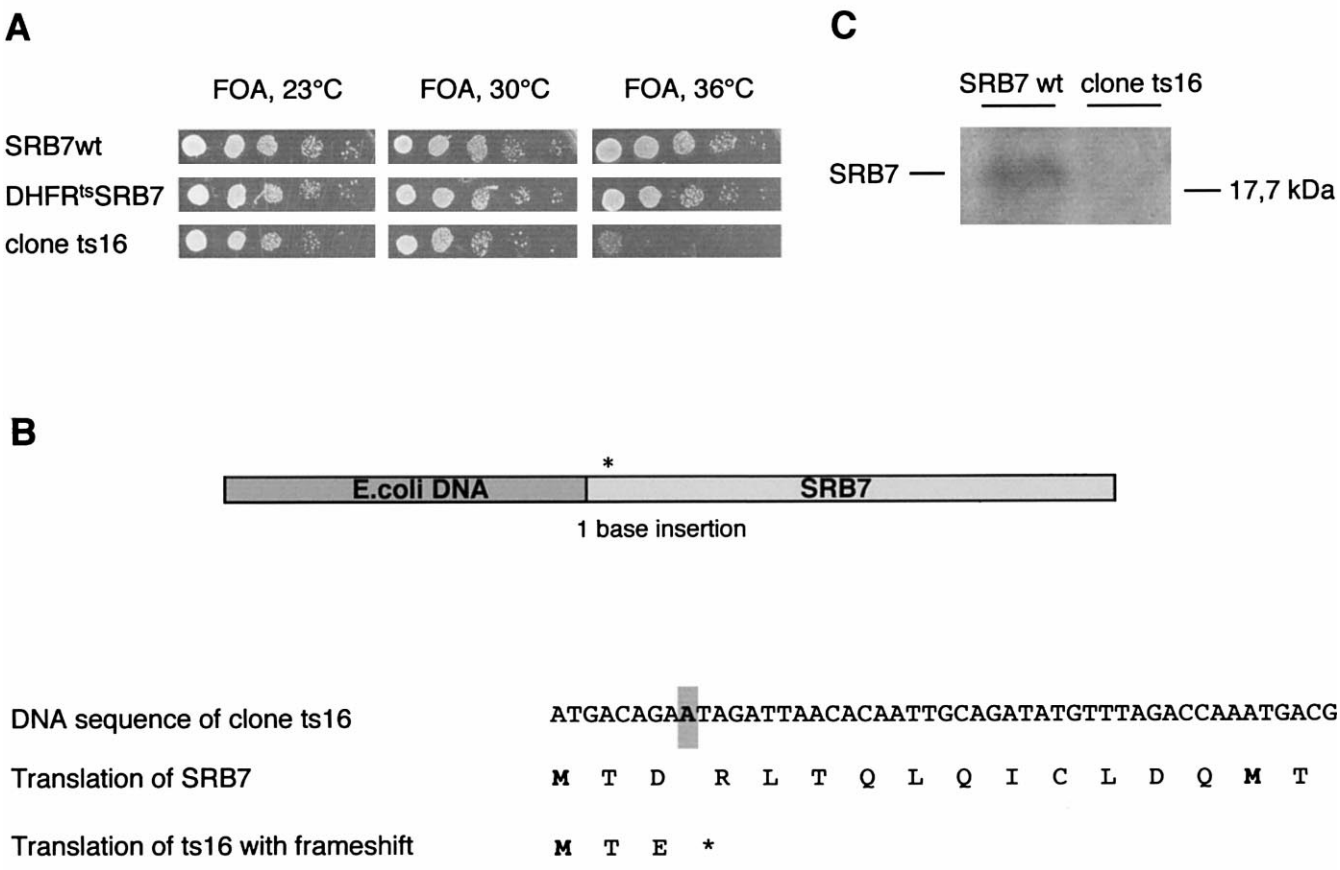

Fig. 2. Clone ts16 is a ts allele of SRB7. A: Growth phenotype of different SRB7 constructs. Strain JD53Δsrb7 was transformed with plasmids carrying the SRB7wt sequence (top), the heat inducible degron based construct DHFR^{ts}SRB7 (middle) or clone ts16 (bottom). Growth was tested on FOA containing media at different temperatures. While the DHFR^{ts}SRB7 construct unexpectedly supports growth even at 36°C, the clone ts16 confers a ts phenotype. B: Clone ts16 contains DNA sequences from the *E. coli* genome instead of a promoter and a base insertion mutation leading to a frameshift just after the ATG. Top: Schematic representation of clone ts16. Bottom: Effect of the base insertion. The inserted A is marked in grey. The amino acid sequence of both native Srb7p and the predicted translation product of clone ts16 is given. The asterisk marks a stop codon generated by the frameshift. C: Western blot with αSrb7p antibody. Left: Strain JD53Δsrb7 expressing wild-type Srb7p. Right: Strain JD53Δsrb7 expressing Srb7p from clone ts16.

conditions even though the amount of Srb7 protein was reduced in medium containing methionine (data not shown). These results indicate that very low levels of Srb7p expression are sufficient for cell viability.

3.2. Identification of a ts allele of SRB7

As an alternative method to create a conditional lethal allele of SRB7, we used the heat inducible degron system [21]. But the DHFR^{ts}SRB7 construct supported cell viability also at high temperatures and did not confer a conditional lethal phenotype. However, one clone tested in this set of experiments showed the expected ts phenotype (Fig. 2A). This clone, termed ts16, turned out to be a cloning artefact with two alterations compared to the DHFR^{ts}SRB7: first, it contained *E. coli* DNA sequences (640 bp comprising upstream sequences and the first 191 codons of the araB gene) instead of the DHFR sequence. Second, there was a one base insertion in the third codon of the SRB7 sequence. This insertion mutation leads to a frameshift and, as a result, to a stop codon at position 4 of SRB7 (Fig. 2B). Clone ts16 does not contain a bona fide yeast promoter. Several control constructs were tested to determine the nature of the effect elicited by this cloning artefact. Different observations were made: (i) the SRB7 coding sequence lacking any kind of promoter is sufficient to support cell viability; (ii) the *E. coli* DNA fused to the SRB7 coding sequence without the base insertion supports cell viability also at 36°C; (iii) the *E. coli* DNA alone or the SRB7 coding sequence with the frameshift mutation alone cannot support viability even at 30°C. Therefore, both the *E. coli* DNA and the frameshift mutation are needed to give the conditional lethal phenotype. Since the frameshift creates a stop codon just after the ATG of SRB7, it is a plausible assumption that translation in clone ts16 may start only at the second ATG in the SRB7 sequence, corresponding to codon 15. However, a construct with the first 14 codons deleted but under the control of the natural SRB7 promoter does not support cell viability, indicating that the N-terminus is an essential part of the Srb7 protein. It therefore seems that, despite the frameshift mutation, the N-terminus of Srb7p is correctly expressed from clone ts16, probably via a rare translational frameshifting event (see Section 4). In a Western blot, Srb7p is not detectable in a strain bearing the ts16 construct (Fig. 2C). Although the exact nature of its ts phenotype is not understood, clone ts16 fulfils the requirements for a conditional lethal allele and can thus be used to study the function of Srb7p.

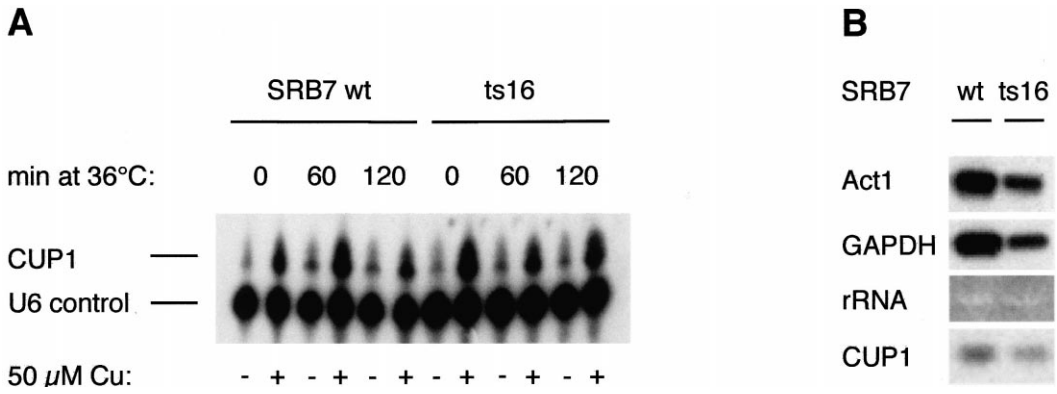

Fig. 3. Transcription in strain JD53Δsrb7 bearing an SRB7wt plasmid or clone ts16. A: Primer extension analysis using a CUP1 specific primer. As a control for the reaction and loading efficiency a primer for the snRNA U6 was used in the same reaction (bottom). CUP1 transcription was induced by adding $CuSO_4$ to a final concentration of 50 μM 30 min prior to harvesting where indicated. Some cultures were incubated at the restrictive temperature for ts16 (36°C) for the indicated times. B: Northern blot with ACT1 and TDH1 probes. The rRNA and a blot with a CUP1 probe are given as controls.

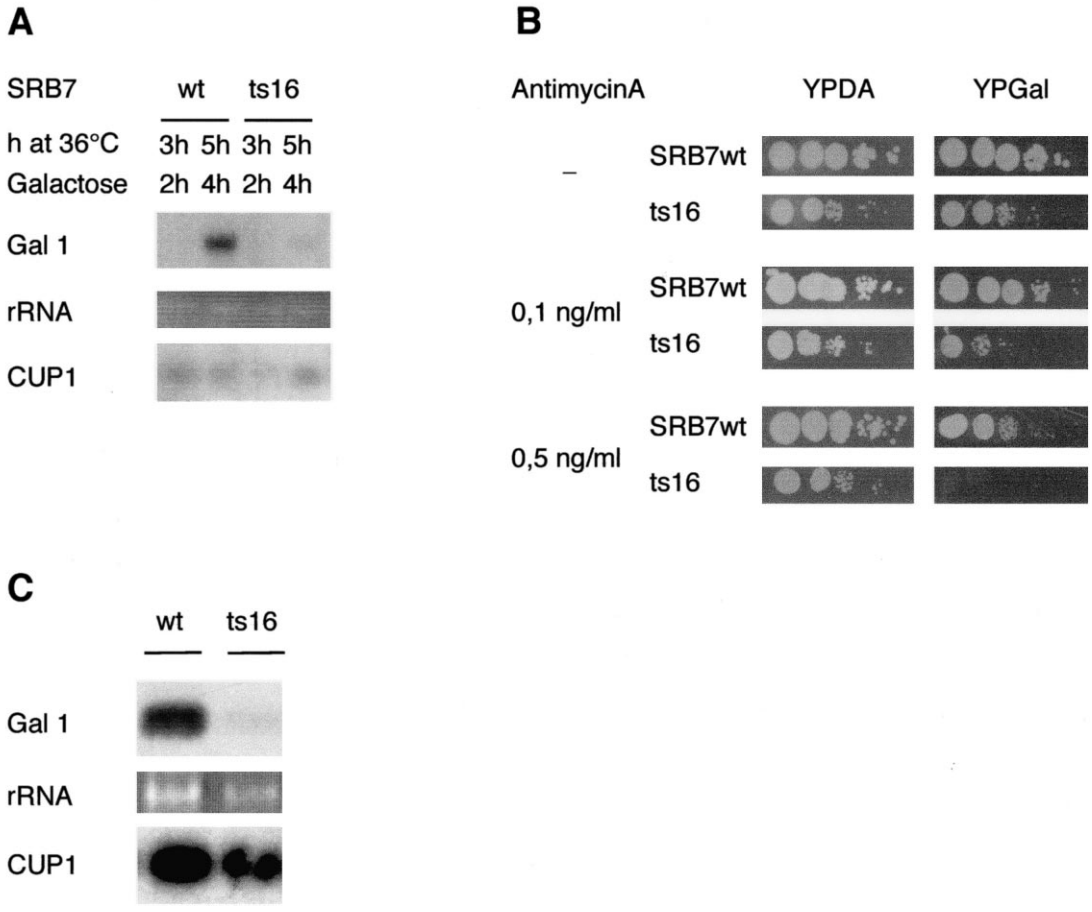

Fig. 4. Effect of ts16 on GAL1 transcription. A: Northern blot with a GAL1 probe with strain JD53Δsrb7 bearing an SRB7wt plasmid (left) or clone ts16 (right). Cultures were induced by growth in 2% galactose medium for the indicated times. The incubation temperature was switched to 36°C 1 h prior to galactose induction. However, the marked decrease in GAL1 transcription caused by ts16 is also seen at 30°C (compare C). The rRNA and a CUP1 blot are given as controls. B: Growth phenotype on galactose medium. 10-fold serial dilutions of strain JD53Δsrb7 bearing an SRB7wt plasmid or clone ts16 were spotted onto glucose or galactose medium containing the indicated amounts of the respiration inhibitor antimycin A. C: Northern blot with a GAL1 probe with strain NLY2Δsrb7 bearing an SRB7wt plasmid or clone ts16 and a plasmid with the coding sequence of Gal4p under the control of the CUP1 promoter to ensure equal expression of Gal4p. Cells were grown at 30°C and induced in Trp^- galactose medium (selecting for the GAL4 plasmid) for 4 h. The rRNA and a blot with a CUP1 probe are given as controls.

3.3. Clone ts16 has no effect on transcription of CUP1 and only minor effects on ACT1 and TDH1

To investigate the role of Srb7p in transcription, the effects of clone ts16 were analysed on the level of RNA. First, we looked at the CUP1 message. CUP1 was previously shown to be independent of both Srb4p [14] and Kin28p [15]. Fig. 3A shows a primer extension experiment with a strain bearing the clone ts16 and a CUP1 specific primer. As a control, a primer for the snRNA U6 was used. U6 is transcribed by RNA polymerase III and is therefore independent of the polymerase II specific Srb7p. CUP1 is transcribed at equal levels in SRB7wt and ts16 strains under induced and non-induced conditions, even after incubation at the restrictive temperature for the ts16 clone. Thus CUP1 transcription is independent not only of Srb4p and Kin28p, but also of Srb7p, and can be used as a control for further experiments. In Fig. 3B, the ACT1 and TDH1 messages were analysed by Northern blot. Both are expressed below wild-type level in the ts16 strain even at the permissive temperature. Incubation at the restrictive temperature yields comparable results. In the example shown, both ACT1 and TDH1 expression were normalised to the CUP1 expression that served as a control and quantitated to be two-fold reduced compared to the strain bearing the SRB7wt plasmid. In other experiments, the effect was even less pronounced. Clone ts16 therefore has a detectable but minor effect on the expression of constitutive genes like ACT1 and TDH1.

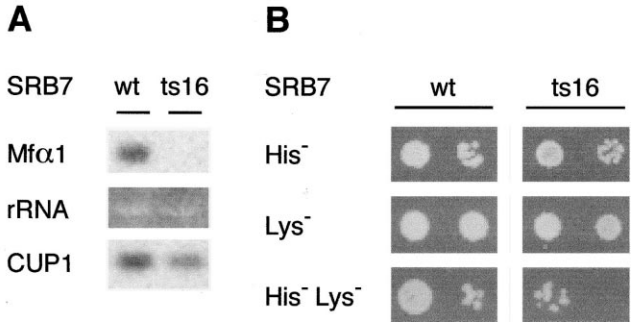

Fig. 5. Effect of ts16 on mating factor expression. A: Northern blot with an MFα1 probe with strain JD53Δsrb7 bearing an SRB7wt plasmid (left) or clone ts16 (right). The rRNA and a blot with a CUP1 probe are given as controls. B: Mating efficiency defect caused by ts16. Mixed cultures of JD53Δsrb7 bearing an SRB7wt plasmid or clone ts16 (both Lys+) and JD52::His3 (His+) were grown overnight and 10-fold serial dilutions were spotted onto plates lacking histidine or lysine to control for equal concentrations of cells of both mating types and onto plates lacking both histidine and lysine selecting for diploid cells (bottom). Mating in the strain with clone ts16 is less efficient.

3.4. Activation of GAL1 and MFα1 is impaired in strains bearing the ts16 mutant

As an example for an inducible gene, we looked at the GAL1 mRNA. As seen in Fig. 4A, GAL1 expression is severely compromised in the strain bearing the ts16 construct. This effect is observed at both 30 and 36°C. The defect in GAL gene expression also leads to a growth defect on galactose medium for the ts16 strain. Transcription defects at the

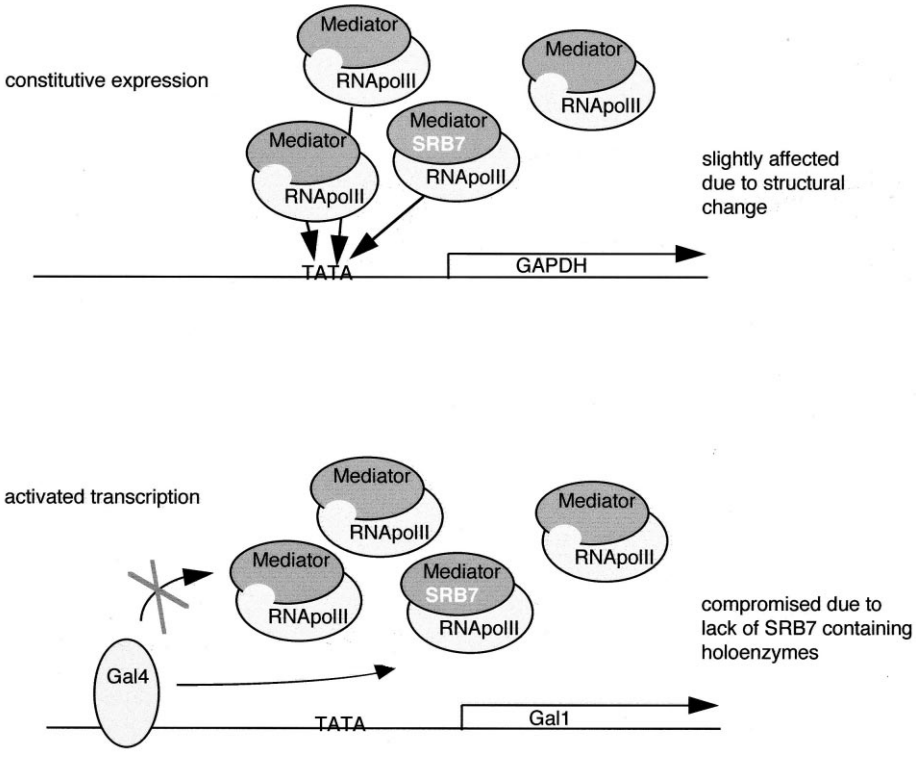

Fig. 6. Model for the effects of clone ts16. In a strain with ts16 as the only source for Srb7p, Srb7p is expressed at extremely low levels. Thus the majority of RNA polymerase II holoenzymes does not contain Srb7p. In the case of constitutive expression, the lack of Srb7p leads to only minor effects. Srb7p is not essential for this process, but structural changes in the holoenzymes lacking Srb7p may lead to minor defects (top). In contrast, Srb7p is required for activated transcription, e.g. at the GAL1 promoter. If only a small fraction of holoenzymes contains Srb7p, transcription from these promoters is severely compromised (bottom).

permissive temperature are commonly found for the ts alleles of transcription factors [29–31], especially if they display a slow growth phenotype as does the ts16 strain. However, while the slow growth phenotype of a ts16 strain is more pronounced on galactose than on glucose medium, the growth defect only becomes really apparent in the presence of the respiration inhibitor antimycin A (Fig. 4B). The effect of ts16 on GAL1 expression could be a direct or indirect effect. Since GAL1 transcription is activated by Gal4p, ts16 might simply reduce Gal4p levels and thus prevent GAL1 activation. To rule out this possibility, we used a GAL4 deletion strain with a construct containing the GAL4 coding sequence under the control of the CUP1 promoter. In this strain, Gal4p should be expressed at equal levels in the wt and ts16 strain, because the CUP1 promoter is not influenced by ts16 (Fig. 3A). The experiment depicted in Fig. 4C demonstrates that under these conditions, GAL1 expression is still impaired in strain ts16, arguing that the effect is direct. To test if clone ts16 has a similar effect on other activated genes, we looked at the mRNA levels of the mating factor MFα1. Similarly as for GAL1, MFα1 expression was reduced to background levels in a ts16 strain (Fig. 5A). Again, this transcriptional defect corresponds to a phenotype, since mating efficiency in strain ts16 is reduced (Fig. 5B).

4. Discussion

In experiments with SRB7 constructs under the control of repressible promoters or with the heat inducible degron system we have been able to demonstrate that low levels of Srb7p are still sufficient for cell survival. When using repressible promoters, another explanation might also account for the lack of an effect: if Srb7p were itself necessary for repression, a kind of feedback loop would prevent the protein level from dropping beneath the threshold necessary to keep up the repression at the promoter used in the SRB7 construct. The question of repression, however, was not addressed in this study. We used the conditional lethal SRB7 allele ts16 to study the role of Srb7p in constitutive and activated transcription. The ts16 construct lacks a bona fide yeast promoter and contains *E. coli* DNA sequences instead. In addition, it contains a frameshift mutation that creates a stop codon just after the ATG. Since the N-terminus turned out to be essential for Srb7p function it must, at least in part, be expressed correctly from clone ts16. The event of such a translational frameshifting leading to a 100-fold reduction in protein levels is a known phenomenon in yeast [32]. We propose that frameshifting occurs at a low frequency and that the extremely low level of Srb7p in ts16 cells is responsible for the ts phenotype. However, the *E. coli* DNA sequences are also needed in the ts16 clone to allow cell survival. They may exert an effect at the level of transcription or RNA stability, but most likely they influence the frameshifting event. Since the araB coding sequence in the *E. coli* DNA is in frame with the ATG of SRB7, a possible explanation is that ts16 cells express a fusion protein, with the N-terminal extension facilitating the translational frameshifting that would otherwise have to occur extremely close to the translational start site. In this case, the N-terminal extension might also contribute to the effects elicited by clone ts16. A direct demonstration of the putative fusion protein has not been possible, due to too many cross-reacting bands produced by the anti-Srb7p antibody in the relevant area of the Western blot (data not shown).

The analysis of transcription in strains bearing the ts16 clone defined three kinds of genes. First, transcription of the CUP1 gene was not affected by ts16. Since CUP1 is also independent of Srb4p and Kin28p [14,15], this result confirms its exceptional status. All other genes tested were affected by ts16, but to very different degrees. While the expressions of the constitutive genes ACT1 and TDH1 was only mildly reduced, expression of the activated genes GAL1 and MFα1 was almost completely impaired. We propose that the minor effects on e.g. TDH1 transcription are not due to a direct role of Srb7p in this process but rather to an indirect effect such as a structural change in holoenzymes lacking the Srb7 protein (Fig. 6). The dramatic effect on GAL1 and MFα1 transcription, however, argues that Srb7p is essential for activated transcription from these promoters. This behaviour of the ts16 strain resembles that of a Med6p ts mutant, which also selectively affects activated transcription [16]. In a more recent study, it was proposed that signals of activator proteins to different mediator subunits might be transmitted to Med6p as a central regulatory subunit [17]. Since Med6p is part of the small mediator subcomplex while Srb7p, like potential activator targets such as Med9p or Med10p, is localised in the large subcomplex [11], one might speculate that Srb7p acts between the two. It will be very interesting to more closely define the relationship between Med6p and Srb7p.

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