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SUB2 helicase is a regulator of heterochromatic silencing in Saccharomyces cerevisiae.

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SUB2 HELICASE IS A REGULATOR OF HETEROCHROMATIC SILENCING IN *SACCHAROMYCES CEREVISIAE*

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by

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THESIS ACCEPTANCE

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SUB2 HELICASE IS A REGULATOR OF HETEROCHROMATIC SILENCING IN *SACCHAROMYCES CEREVISIAE*

Jennie C. Smith, MA

University of Nebraska, 2004

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Gene silencing is a normal part of eukaryotic development, and mis-regulation of silencing can affect cell-cycle regulation, differentiation, and genome stability. Our aim was to examine the role of SUB2 (a *Drosophila Hel1* homolog) in gene silencing, to better understand this complex regulatory process.

To determine whether the *SUB2* homolog functions like the *Drosophila HEL1* gene, we performed genetic and biochemical analyses of the *SUB2* gene and two *sub2* mutant alleles. We show Sub2p, a *Saccharomyces cerevisiae* RNA helicase, is capable of suppressing heterochromatic silencing at telomeres when overexpressed.

Silencing assays in a *S. cerevisiae* reporter strain where *URA3* is silenced by telomeric DNA, show a 27-fold increase in the number of Uracil positive (Ura +) colonies above background, when *SUB2* is overexpressed. Silencing assays using the *sub2-1* allele, which is mutated adjacent to two different ATP-binding motifs, showed a 14-fold increase above background. The *sub2-5* allele, which is mutated within a single nucleic acid binding motif, showed a 50-fold increase above vector alone. All alleles showed RNA and protein levels that correlated with their ability to overcome *URA3* silencing, as determined by Western and Northern blot assays. High copy *SUB2* constructs showed an average 3-fold increase in transcript levels over vector alone. The *sub2-1* and *sub2-5* mutant constructs showed 1.8-fold and 5-fold increases in transcript levels, respectively. In comparison, Sub2 protein levels were an average of 7-fold higher than background, Sub2-1

showed an average of 2.3-fold higher than background, and Sub2-5 showed an average of 10.3-fold higher than background.

One theory to explain these results invokes a SUB2 interaction within the TREX transcription and elongation complex to alter localization of proteins required for heterochromatin silencing. A second theory suggests SUB2 may act as a regulatory component of specific transcription-coupled chromatin remodeling complexes that recruit Set1 and Dot1 methyltransferases to the nuclear membrane. Future research will elucidate which theory may be more accurate.

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INTRODUCTION

Heterochromatic gene silencing

Gene silencing is a normal process in every cell and is required for cells to have different functions. Mis-regulation of silencing can affect cell-cycle regulation, differentiation, and genome stability, all of which can lead to developmental defects and cancer [1]. Heterochromatic or silenced regions are highly condensed and transcriptionally inactive, whereas euchromatin is less condensed and transcriptionally active. Heterochromatic gene silencing occurs when chromosome domains are packaged into specialized structures that limit access of DNA binding proteins, including transcription factors, to the regulatory regions as well as coding regions of genes [2]. For example, the telomeres and silent mating-type loci in *Saccharomyces cerevisiae*, which are normally in a silent state, consist of a repressive chromatin structure composed of nucleosomal core histones and nonhistone chromatin components, including the Sir (silent information regulators) silencing complex (Figure 1) [2]. Rap1 probably initiates assembly at telomeres when it binds to a 300 bp region of $C_{1\text{-}3}A$ repeats on single stranded DNA. Rap1 is thought to recruit Sir4, which recruits the

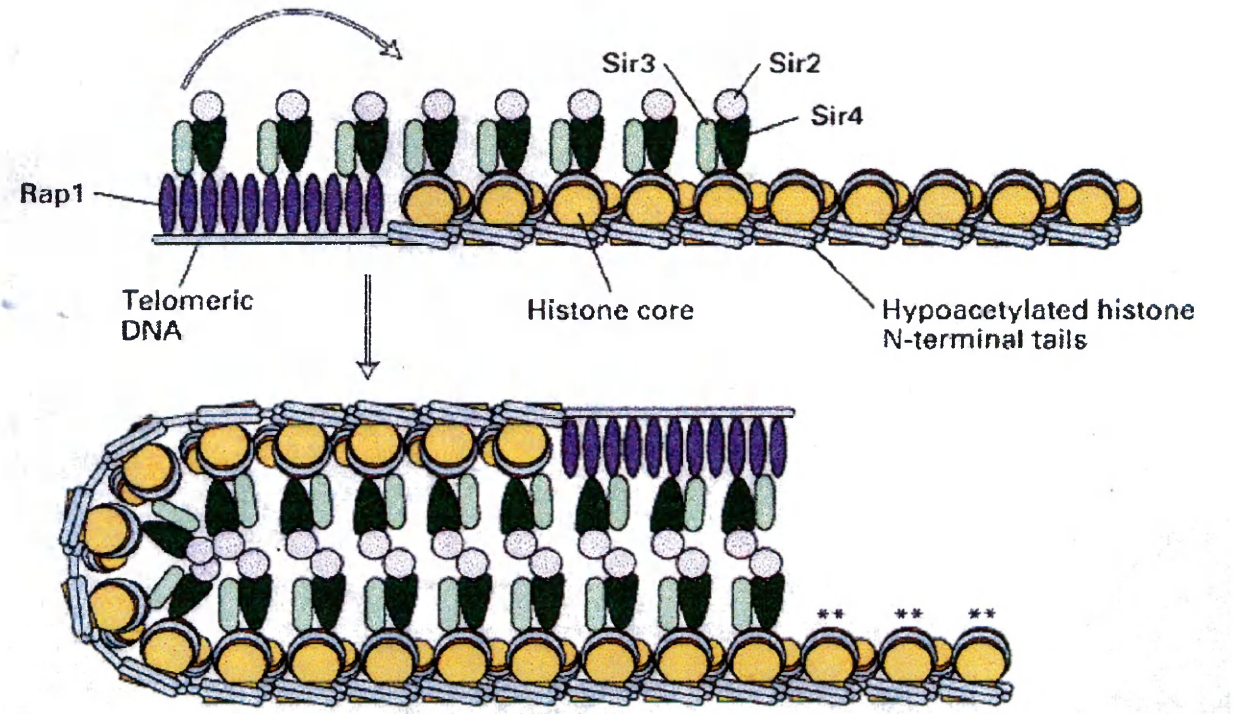
deacetylase Sir2, that enables Sir3 and Sir4 to bind hypoacetylated histone N-terminal tails [3]. The process of heterochromatin spreading based on interactions between the Sir proteins makes approximately 2-4 kb of DNA from the ends of chromosomes inaccessible until the Sir proteins are removed (Figure 1) [4]. The assembly of silencing proteins is thought to form a cap-like structure that protects telomeres and serves to maintain a heterochromatic state [4]. Recently, Kamakaka and others have questioned the traditional model of stably-bound non-histone components as necessary for silencing maintenance, asserting that these proteins are in a constant state of flux based on competition for binding between activators and repressors [5]. Supporting evidence for this model includes fluorescence recovery after photobleaching (FRAP) studies of a key component of condensed DNA, Heterochromatin protein 1 (HP1), that show a fusion protein of HP1-green fluorescence protein in ex-vivo resting murine T cells is almost equally mobile within heterochromatin as it is within euchromatin [6].

In addition, the subnuclear distribution of chromatin binding factors has been associated with gene silencing [7]. For example, the localization of telomeres to nuclear membranes puts them in a generally repressive transcriptional environment , thought to be based on a high local

concentration of repressor proteins [7]. Histone modifications including acetylation, methylation, and phosphorylation have also been associated with silencing activity, although the multiple mechanisms and pathways are not well understood [8].

Figure 1. Heterochromatic gene silencing at yeast telomeres. Adapted from M. Grunstein, 1997, *Curr Opin. Cell Biol.* 9:383[4].

Fig. 1.


Sir3
Sir2
Sir4
Rap1
Telomeric
DNA
Histone core
Hypoacetylated histone
N-terminal tails

The transcriptional activity of a gene may be affected by its position in a chromosome, a phenomenon called position effect variegation (PEV) in yeast and *Drosophila*. PEV occurs when euchromatic genes that are placed next to heterochromatic regions become transcriptionally inactivated, possibly due to chromosome condensation by expansion of heterochromatin into the euchromatic gene [9]. For example, when the *ADE2* gene in *S. cerevisiae* is expressed from its normal euchromatic location, yeast cells are white. If the *ADE2* gene is moved adjacent to a telomere, it is often silenced and the cells become red. This red color is due to a red-colored precursor in the adenine biosynthetic pathway that is not converted to adenine, and this precursor builds up within the cell. Silencing is stochastic at these locations, so a gene that was silenced in a cell may be stably silenced in subsequent daughter cells for multiple generations, yet can switch to active again. Additionally, heterochromatin does not always spread into adjacent genes, so some genes may not be silenced despite being adjacent to heterochromatin.

Our interest in how PEV works is based on research by Daniel Eberl that showed the overproduction or underproduction of Hel1 protein in fruit flies can suppress or enhance PEV [10]. Hel1, an essential, cell-cycle

regulated RNA helicase from *Drosophila melanogaster* [10], is homologous to the DEAD box family of RNA helicases [11]. We used *S. cerevisiae* as our model organism because yeast is easy to manipulate for biochemical and genetic studies. We chose to study the *S. cerevisiae* homolog of Hel1, Sub2, named for the Suppressor of Brr1-1 cold sensitive snRNP biogenesis mutant [12]. Eberl isolated Hel1 in a genetic screen of variegated flies to find suppressors and enhancers of PEV. Instead of normal red eyes, PEV flies have variegated eye color based on the transposition of the *white* gene (encoding red pigment) adjacent to heterochromatin. He found that deletion of a single copy of *Hel1* enhances PEV, deletion of both copies is lethal, and overexpression of *Hel1* overcomes PEV or counteracts silencing [10]. Adding an extra (third) wild type copy of Hel1 to the diploid flies can suppress PEV, restoring almost normal red eye color. This suggests that the Hel1 protein somehow promotes an open chromatin structure that allows transcription to occur.

SUB2 is 63 % identical at the amino acid level to *Drosophila* protein HEL [10], and 66 % identical to human protein UAP56/BAT1 [13]. Other homologs include *C. elegans* hel-1, which is 75 % identical to human UAP56 [14], and rat protein p47, which is 82 % identical to HEL in flies

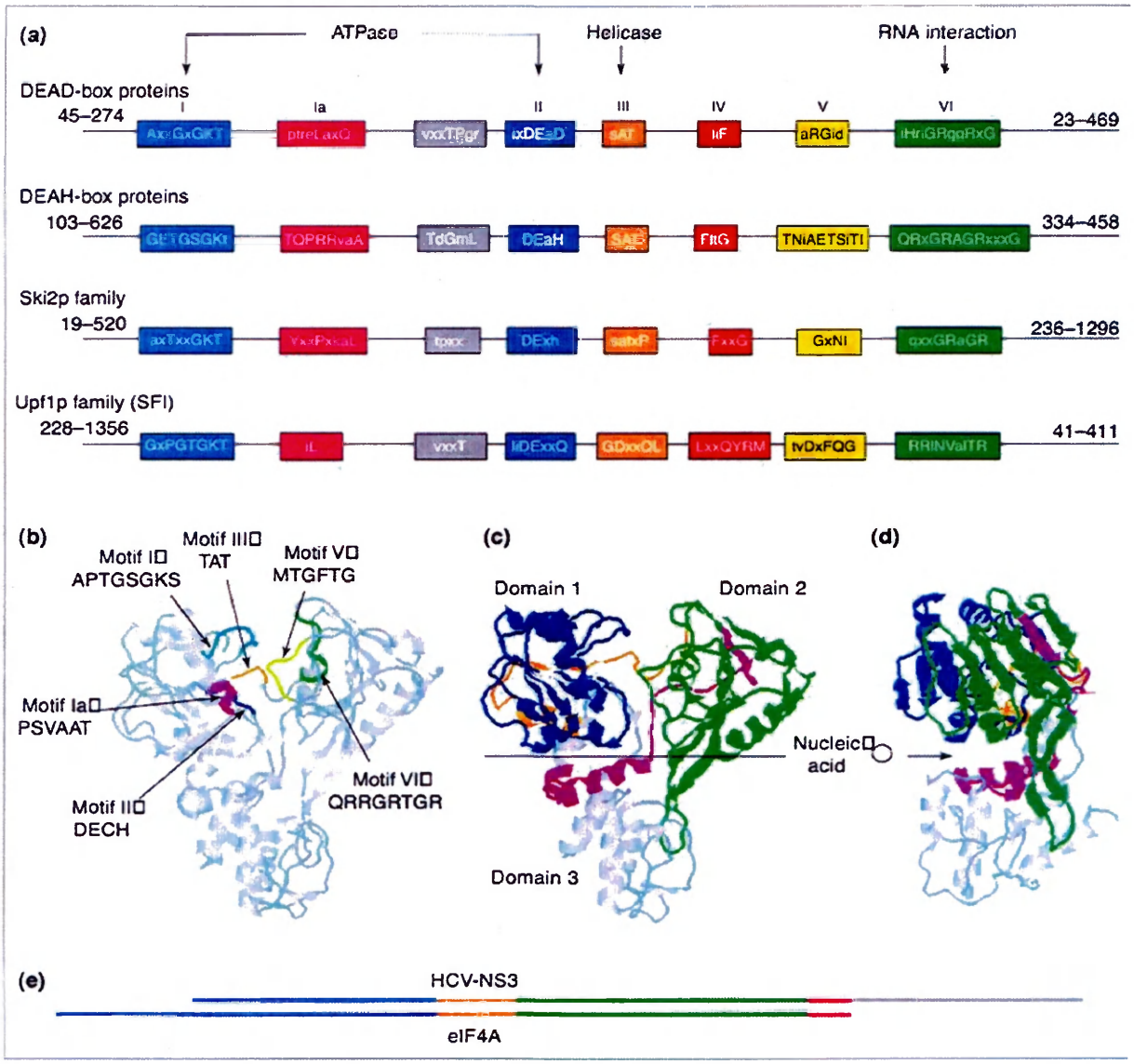
[10]. Based on the apparent homology of *Hel1* to the *S. cerevisiae SUB2* gene, we hypothesized that *SUB2* would be a true homolog of Hel1 and would be able to overcome heterochromatic silencing when overexpressed. A second question addresses whether this silencing suppression is associated with the helicase component of Sub2 or with some other associated function.

DExD RNA helicases

A helicase is an enzyme that uses the energy from hydrolysis of nucleoside triphosphates, generally ATP, to unwind nucleic acid duplexes. Helicases have been categorized as DNA and RNA helicases, based on the nucleic acid substrate used by the helicase and the amino acid sequence conservation of the helicases themselves [15]. There are seven conserved motifs in RNA helicase families which are used to classify family members [16] (Figure 2a).

Figure 2. Conserved motifs common to RNA helicase family members, from de la Cruz *et al.* [16]. **a)** Conserved RNA helicase motifs: I (light blue), Ia (pink), II (dark blue), III (orange), IV (red), V (yellow), and VI (green). **b-d)** Hepatitis C Virus NS3 domain structure front view (**b**) depicts motifs I, Ia, II, III, V and VI. Front view (**c**) and side view (**d**) show domain 1 colored blue, domain 2 colored green, domain 3 colored purple or grey, and the hinged region colored orange. **e)** Schematic alignment of HCV-NS3 RNA helicase and yeast eIF4A core enzymatic regions colored as seen in views (**c**) and (**d**).

Fig. 2.


(a)
ATPase
Helicase
RNA interaction
DEAD-box proteins
45–274
I
Ia
II
III
IV
V
VI
23–469
DEAH-box proteins
103–626
334–458
Ski2p family
19–520
236–1296
Upf1p family (SFI)
228–1356
41–411
(b)
Motif Ia
PSVAAT
Motif Ib
APTGSGKS
Motif II
DECH
Motif III
TAT
Motif V
MTGFTG
Motif VI
QRRGRTGR
(c)
Domain 1
Domain 2
Domain 3
Nucleic acid
(d)
(e)
HCV-NS3
eIF4A

Though all seven motifs are not found in every helicase, the majority of helicases have four to six of these motifs, and mutagenesis studies have elucidated the functions of these different motifs for helicase activity [16, 17]. Motifs I and II are involved in binding and hydrolysis of nucleotides [18, 19], while motifs III and VI somehow link NTPase activity with conformational changes needed for unwinding nucleic acids [20]. For RNA helicases, motif VI is also associated with RNA interactions. Motifs Ia, IV and V are thought to be involved in nucleic acid substrate interactions [17].

The hepatitis C virus helicase NS3 (HCV NS3) has been crystallized [21] and a representative model of the core helicase region is shown in 3 different views (Figure 2b, c, d). As described in reference [16], the core helicase region is made up of 3 globular domains, shown in views c and d. Domain 1, in blue, is the N-terminal domain which contains the ATPase motifs I and II. Domain 2, in green, is the RNA-recognition domain that includes motif VI. Domain 3, in grey, is the C-terminal domain and lacks conserved motifs, but the magenta portion helps form the cleft that holds nucleic acids and binds ATP. The orange hinge region allows domain 2 to pivot relative to domains 1 and 3. In view b, motifs I and II, associated with ATPase function, are colored blue and line a cleft between the 3 domains.

Motif I forms a pocket to bind phosphates of NTP while motif II interacts with the β and γ phosphates and is involved in NTP hydrolysis [19]. Located near the cleft, motif Ia, colored purple, and motif V, colored yellow, are involved in nucleic acid substrate binding. Also within the cleft is motif VI, colored green, which contacts the phosphates of the ATP molecule bound by motifs I and II, to link helicase activity with ATP hydrolysis [20, 22] . Orange-colored motif III, associated with unwinding activity [23, 24], is in the flexible hinged region in the cleft that connects domains 1 and 2. Crystal structures show the cleft isn't wide enough to bind double-stranded DNA, but it is large enough to bind single stranded nucleic acids [20, 21]. So, the binding of ATP within cleft is thought to induce a conformational change once a nucleic acid is bound and the cleft is closed. Hydrolysis of ATP might open this cleft and translocate the helicase along the nucleic acid [16, 20]. The overall structure seems to be conserved among helicases as indicated by a schematic alignment of HCV NS3 with yeast translation initiation factor eIF4A (Figure 2e).

Sub2 is a member of the DExD box family of RNA helicases [25]. RNA often contains only short base-paired regions, so many DExD helicases, such as those involved in splicing, may act as "unwindases" or as

"RNA chaperones" that disrupt or rearrange RNA-DNA or RNA-protein interactions, rather than directly unwind nucleic acid duplexes [26] . Cellular roles for RNA helicases include transcription, ribosome biogenesis, pre-mRNA editing, nuclear transport, and chromatin remodeling [11, 27, 28] and reviewed in [17].

Sub2 cellular roles

Eberl showed that *Drosophila* Hel1 is capable of affecting PEV, is an essential protein that binds chromatin, and is cell-cycle regulated [10]. Since that publication, other researchers have shown *SUB2* is essential in yeast, as knockouts are lethal [13, 29], *SUB2* is probably cell-cycle regulated [30], and *SUB2* localizes to the nucleus [13, 31]. Furthermore, *SUB2* is capable of rescuing mitotic catastrophe in wee1/mik1 mutants of *S. pombe* [32], also indicating this protein may affect cell cycle regulation. Wee1 and mik1 are protein kinases that act as negative regulators at the G2 to M transition of the cell cycle via phosphorylation (inhibition) of CDC2.

Characterization of Sub2 in other labs has found multiple roles for this interesting protein, including transcription elongation, mRNA splicing and nuclear transport, genome stability, and silencing regulation.

Sub2 in transcription elongation

Sub2's involvement in transcription elongation is implied through interactions with RNA Polymerase II subunits and components of the THO complex. Genetic and functional interactions were reported between Sub2 and Rad3, an essential component of transcription initiation factor TFIIH within the RNA Pol II transcription machinery [33]. Radiation sensitive Rad3 is a 5' to 3' DNA helicase involved in nucleotide excision repair and transcription. Two *rad3* mutants were isolated in a loss of function suppression screen using UV-mutagenesis of a *sub2* null strain. When the mutants were placed in *sub2-201* mutant strains defective in mRNA export, mRNAs were released from transcription site foci [33]. Physical interactions between SUB2 and another subunit of Pol II, RPB3, are based on their coimmunoprecipitation in *hpr1* deletion mutant strain [13].

Sub2 also has physical and genetic associations with Hpr1 and Rlr1 (Tho2) of the THO complex, a complex involved in transcription elongation [34, 35]. The THO complex interacts genetically with subunits of the transcription Mediator complex [36, 37]. The Mediator complex, composed of Srb2, Srb5, Hrs1, Gal11, and Sin 4, plays a key role in promoter-dependent

transcription regulation [38]. Experimental evidence that Sub2 affects transcription elongation comes from West *et al.* who identified *SUB2* as a high-copy suppressor of *rlr1* mutants, which have defects associated with transcription elongation, arresting in G+C-rich, or very long transcripts [35]. Similarly, Jimeno *et al.* found that overexpression of *SUB2* suppresses the *hpr1*Δ transcription defect associated with the long G+C-rich *lacZ* sequence when fused to a strong P_{tet} promoter [39]. Investigation of gene expression in the *hpr1*Δ mutants indicated that "multicopy *SUB2* can fully substitute or bypass the need for Hpr1 and presumably the THO complex in the cell" possibly by an increased association of Sub2 rather than Hpr1 with nascent mRNAs [39]. Zenklusen *et al.* used chromatin immunoprecipitation (ChIP) assays to show that Hpr1 is required for efficient recruitment of Sub2 and Yra1, a nuclear export protein, to active genes during transcription elongation [40]. Additionally, Sub2 and Yra1 interact genetically and physically with the THO complex and genetically with Tex1, to form the highly conserved TREX complex, which stands for transcription/export [36]. Based on protein purification studies of the TREX complex, Sub2 is thought to link Yra1 with the THO complex [36]. Strasser *et al.* performed genetic studies using mutants of each THO component (Tho2, Hpr1, Mft1, Thp2) to

show that all four proteins have a role in efficient mRNA export of intronless genes [36]. Moreover, ChIP assays demonstrated that the TREX complex is recruited to DNA during transcription and travels with RNA Pol II the entire length of the reporter gene being transcribed [36]. Thus the interaction of Sub2 with components of the THO complex and TREX complex indicates Sub2 has a role in transcription coupled with mRNA processing and export.

Overexpression of Sub2 is also able to rescue the inviable double mutant *hpr1Δ cdc73Δ* grown at 30°C, and suppress the hyperrecombinant phenotype of *cdc73Δ* [13]. Transcription factor Cdc73 is complexed with Hpr1 and other components to form the Paf1 complex, which is involved in transcription initiation [34] and probably elongation as well [41]. The Paf1/RNA Pol II transcription complex, biochemically distinct from the Srb-mediator form of Pol II holoenzyme, is required for full expression of many cell wall biosynthetic genes and cell cycle-regulated genes [42]. Interactions between Sub2 and the Paf1 complex has not been shown, although the *SUB2* and *CDC73* genetic interaction suggests involvement with the Paf1 complex, providing another link for Sub2 playing a role in transcription based on associations with RNA Pol II [13]. Thus, via genetic versus

physical interactions with Hpr1, Rlr1 and Cdc73, Sub2 is associated with transcription regulation.

Sub2 in splicing

During transcription, pre-mRNA is processed by 5' capping, 3' cleavage and polyadenylation, and intron removal/splicing for some transcripts. Sub2 has an essential role in pre-spliceosome assembly and also appears to couple splicing with mRNA transport through its interactions with proteins from both splicing and export machines (Reviewed in [43]). Sub2 has been shown to be an ortholog of the essential human pre-mRNA splicing factor hUAP56, and is involved in multiple steps of spliceosome assembly [11, 27, 28]. Libri *et al.* have shown that genetic interactions of Sub2 with U1snRNP-associated proteins, Prp40 and Nam8, stabilize commitment complex 1 (CC1) formation in which U1snRNP interacts with the pre-mRNA in an ATP-independent manner [28]. Kistler and Guthrie reported that Sub2 also functions in collaboration with an inhibitory splicing protein, Mud2 (mammalian U2AF65), possibly by removing Mud2 to allow an ATP-independent interaction with the pre-mRNA substrate of commitment complex 2 (CC2). However, when Mud 2 is deleted, Sub2

function becomes dispensable and a Sub2-Mud2 independent pathway is followed [27]. In subsequent ATP-dependent steps of prespliceosome formation, Zhang and Green indicated Sub2 is also required for stable binding of U2snRNP to the pre-mRNA branchpoint [11]. Thus pre-mRNA splicing requires Sub2 for U1 binding to the pre-mRNA, MUD removal to allow pre-spliceosome assembly, and U2 binding to the branchpoint.

Sub2 in mRNA transport

Sub2 is involved in nuclear export of mRNAs from intronless genes as well as intron-containing genes via its role in splicing (reviewed in [44]). As part of the TREX complex, Sub2 interacts genetically and biochemically with Yra1, an essential nuclear export protein that directly interacts with conserved nuclear pore-associated protein Mex67/Mtr2 [45]. Yra1, a RNA annealing protein, associates with chromatin in a transcription dependent manner [46, 47]. Strasser and Hurt's studies indicate that Sub2 recruits Yra1 to the mRNA, then Sub2 is displaced by competitor Mex67/Mtr2 which binds to the same Yra1 domains as Sub2, resulting in export of the mRNA through the nuclear pore [45]. Similarly, Luo *et al.* demonstrated UAP56 (mammalian Sub2 homolog) links pre-mRNA splicing with mRNA

export by recruiting Aly (metazoan Yra1 homolog) to the spliced mRNA-protein complex [47]. Regarding intronless genes, Strasser *et al.* demonstrated Sub2 is required for efficient export of the heat shock SSA1 mRNA [36]. In addition, Kiesler *et al.* has shown that HEL (*C. tentans* Sub2 homolog) binds cotranscriptionally to the Balbiani ring pre-mRNP, independent of intron location, and accompanies it to the nuclear pore, where it appears to remain in the nucleus as the mRNP exits the pore [48]. Taken together, Sub2's essential role in splicing linked between transcription elongation and mRNA export provides for a direct influence of *SUB2* on gene expression.

Sub2 in DNA stability

Fan *et al.* implicated Sub2 in the maintenance of genome stability through a role in transcription-coupled repair, based on high-copy *SUB2*'s ability to suppress hyperrecombinants seen in *hpr1Δ* and *cdc73*Δ single and double mutants [13, 49]. According to Fan, Sub2 may be involved in regulating recombination between directly repeated DNA sequences and may substitute for either Hpr1 or Cdc73 during transcription elongation [13]. To study transcriptional defects in a *hpr1*Δ strain, Merker and Klein

conducted plasmid loss assays using 1.7 kb yeast chromosomal sequences inserted into a *CEN*-based plasmid with a bacterial *ori-amp* sequence behind a yeast promoter [49]. They found that inserts containing the *DED1* promoter apparently caused plasmid instability in *hpr1*Δ mutants unless transcription was terminated upstream of the *ori-amp* sequence. Surprisingly, when the plasmid construct was altered by adding a short unique DNA sequence to the *ded1* sequence , high copy *SUB2* was shown to stabilize plasmid loss rates while stimulating transcription of the *DED1* promoter in *hpr1*Δ cells above wild-type levels. As explained by Merker and Klein,

> "Hpr1p-deficient cells have difficulty with transcription elongation through a variety of DNA sequences and often result in a transcription block that is correlated with genetic instability between direct repeats...high-copy number *SUB2* does not suppress by returning transcription to a wild-type level. Rather, high-copy number *SUB2* appears to make the defective transcripts into productive transcripts that can be exported to the cytoplasm...[Sub2 may act] by aiding in the removal of a complex from the nascent RNA or the DNA template or the RNA-DNA hybrid region [49]."

This provides further support that Sub2 has a role in transcription in addition to roles in mRNA splicing and export.

Sub2 in silencing regulation

Based on its sequence homology with Hel1, we believe Sub2 has a role in heterochromatic silencing regulation in *S. cerevisiae*, as it does in *Drosophila*. Our initial question was how an RNA helicase is involved in regulation of heterochromatin structure. Sub2's potential activity with heterochromatin implied an RNA helicase binding double stranded DNA. Since we started this project, many labs have elucidated roles for Sub2 in *S. cerevisiae* and for Sub2 homologues. The function of Sub2 in silencing regulation may be tied to Sub2's ability to bind and recruit transcription complexes, rather than directly binding DNA and remodeling chromatin. But the question remains whether yeast Sub2 is a functional homolog of drosophila Hel1, and what role Sub2 has in silencing regulation in *S. cerevisiae*. We have cloned *SUB2* and two *sub2* mutants with mutations adjacent or within conserved RNA helicase motifs I, Ia, and IV, to analyze the effect of these mutations on Sub2 function.

We want to examine whether *SUB2* overexpression can overcome PEV at telomeres in yeast. If so, is this a global transcription effect or is it specific to telomeres? Toward this end, we used yeast genetic silencing assays developed by Gottschling [50], to determine the effect of *SUB2* on

telomeric silencing. We assayed for survival on media lacking uracil (-Ura) by an *S. cerevisiae* reporter strain where *URA3* is silenced by telomeric DNA, using overexpressed *SUB2,* and *sub2* mutants *sub2-1* and *sub2-5*. We examined whether RNA and protein levels from our constructs correlated with the percentage of Ura positive colonies in our assays. We also looked at RNA levels for *RPB2*, a constitutively Pol II-transcribed gene whose product is a core component of RNAP II, to see whether effects on transcription by overexpression of *SUB2* is limited to heterochromatin or if the effect is global. Concurrent with our research, Fan *et al.* showed that low copy number *SUB2* had low effect on silencing at telomeres in *S. cerevisiae* [13], while West and Milgrom found that overexpression of *SUB2* increases rDNA silencing [35].

MATERIALS AND METHODS

Strains and media

Bacterial Strains

E. coli HB101: *supE*44 *hsdS*20($r^-_B m^-_B$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1.

E. coli DH5α: *supE*44 Δ*lac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1.

Yeast Strains

S. cerevisiae UCC 3505: *MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 ppr1::HIS3 adh4::URA3-TEL-VIIL DIA5-1* kindly provided by Daniel E. Gottschling [50].

S. cerevisiae S288C: *MATα SUC2 mal mel gal2 CUP1 flo1 flo8-1.*

Media

YPD – per liter: 10g Yeast extract, 20g Peptone, 20g Dextrose, plus 18g agar for plates. Synthetic Complete dropout media – per liter: .28 mM Asp, .28 mM Ile, .22 mM Phe, .35 mM Ser, .31 mM Thr, .20 mM Tyr, .32

mM Val, 20g Glucose , 1.7g Yeast Nitrogen Base without amino acids and Ammonium Sulfate, 1g Ammonium Sulfate, plus the following amino acids, minus those being "dropped out": 1.0 mM Ade, 1.65 mM Ura, 2.38 mM His, 2.82 mM Leu, 2.02 mM Lys, 1.81 mM Trp, 1.76 mM Arg, or 2.48 mM Met, plus 17.5g agar for plates.

Plasmids

Shuttle vectors used for cloning in *E. coli* and for transforming *S. cerevisiae* include plasmid *pRS 314* (*CEN*, TRP1) [51] and plasmid *pRS 424* (2μ origin, TRP1) [52]. The expression vector *pUC19* (pMB1 ori; encoding lac*Z'*) was used for subcloning in *E. coli*.

Sub2 cloning

A 2075 bp fragment that included 388 bp upstream of AUG and 347 bp downstream of the termination codon was subcloned from a plasmid containing YCGYDL084w (EUROSCARF). This fragment was assumed to contain adequate upstream sequence for the promoter and was inserted into NotI digested plasmids *pRS 314* and *pRS 424* to generate clones of wild type *SUB2*. The insertion of *SUB2* into *pRS 314* and *pRS 424* was verified by

sequencing and restriction digestion with NotI to produce a 2075 bp insert and 4.7 Kb or 5.6 Kb vector, respectively.

Isolation and preparation of *sub2* mutants

The cold sensitive/temperature sensitive (cs/ts) *sub2-1* ($D_{22}G$, $E_{83}G$, $L_{142}M$, $I_{146}T$) and cold sensitive (cs) *sub2-5* ($Q_{308}R$) mutants were kind gifts from Christine Guthrie [27]. Plasmids containing the *sub2* mutants were subcloned from their original plasmids and cloned into both *pRS 314* and *pRS 424* plasmids. Specifically, *pCG839* (*sub2-1* in *pRS 315*) and *pCG 838* (*sub2-5* in *pRS 315*) were isolated from donated yeast strains using plasmid rescue. Approximately 50 µl of cells were scraped from streaked plates of donated strains. The cells were washed in 500 µl water and collected by centrifugation for 30 seconds. Cell pellets were gently resuspended with the pipet tip in 200 µl of yeast lysis buffer (10 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 2% (v/v) Triton X-100, 1% (w/v) SDS). Approximately 200 µl of 425-600 µm glass beads (Sigma, Catalog #G8772) and 200 µl of buffer-saturated phenol:chloroform were added, then cells were vortexed vigorously, 6 times for 30 seconds each, and placed on ice for 30 seconds between each vortexing. Following centrifugation for 1 minute,

the top/aqueous phase was removed to a new tube and nucleic acids were precipitated by the addition of 20 µl of 3M sodium acetate (pH 6.0) and 500 µl of 100% ethanol. Samples were incubated for 30 minutes at -20°C, then centrifuged for five minutes to pellet the DNA. Pellets were washed with 100 µl of 70% ethanol, spun again, the supernatant was removed, and the pellets were dried at room temperature. The phenol:chloroform extraction and ethanol precipitation steps were repeated and pellets were redissolved in 20 µl of TE. One µl of plasmid DNA was used to transform chemically competent DH5α *E. coli*. Transformants were selected on LB plus 50 µg/ml ampicillin (LB+amp) plates, and plasmids were recovered from transformants using the QIAGEN QIAprep Miniprep Kit.

To prepare *pRS 314 sub2-1, pRS 314 sub2-5, pRS 424sub2-1* and *pRS 424 sub2-5,* restriction enzymes ApaI and XbaI were used to excise the 2120 bp fragments containing *sub2-1* or *sub2-5* from *pCG839* and *pCG838*, respectively. This fragment contains 409 bp of DNA upstream of +1 and 372 bp of DNA downstream of the stop codon in the ORF. Restriction digests were fractionated on 1% agarose TBE gels and the *sub2-1* and *sub2-5* fragments were excised from the gel and extracted using the QIAGEN QIAquick gel extraction kit. Digestion of *sub2* mutant fragments with AccI

produced a 1Kb fragment, confirming the extracted DNA contained *SUB2*. The purified mutant DNA fragments were ligated into ApaI-SmaI-cleaved *pRS 314* and *pRS 424* using T4 DNA ligase (400 units per reaction) in 1X T4 DNA Ligase Buffer (50mM Tris-HCl pH 7.5, 10MM $MgCl_2$, 10mM dithiothreitol, 1mM ATP, 25 μg/ml bovine serum albumin) at 16°C overnight to connect the ApaI ends. The XbaI C-terminal ends of *sub2* mutant fragments were filled in to blunt ends, using 1/2 unit Klenow fragment and 500 μmol dNTPs for 15 minutes at room temperature. The reaction was stopped by the addition of 10 mmol EDTA, then phenol:chloroform extracted and ethanol precipitated to concentrate the DNA in 8 μl of water. T4 DNA ligase (400 units per reaction) was added to the concentrated initial ligation reaction and was incubated at 16°C overnight to ligate the blunted ends. Ligation products were transformed into either HB101, or DH5α *E. coli,* and selected on LB+amp plates. Plasmid DNA from transformants was purified with the QIAGEN QIAprep miniprep kit and subjected to restriction digest analysis to verify the insertion of *sub2-1 and sub2-5.* Plasmids *pRS 314 sub2-1*, *pRS 314 sub2-5*, *pRS 424 sub2-1* and *pRS 424 sub2-5* were digested with restriction enzymes

ApaI and SacI then loaded onto a 1% agarose TBE gel to produce a 6 Kb band (vector backbone) and a 2 Kb band (entire *sub2-1* or *sub2-5* insert).

Silencing Assays

S. cerevisiae host strain UCC3505 has *URA3* adjacent to an artificial telomere on chromosome VII-L, which silences *URA3*, allowing us to monitor the suppression of telomeric silencing by assaying for *URA3* positive colonies. We transformed this reporter strain with the *pRS 314* and *pRS 424* vectors alone, and with the same vectors containing *SUB2* or a *sub2* mutant. For each silencing assay fresh transformations of the *SUB2*-construct, and the corresponding vector alone, were performed using the quick and easy lithium acetate method of Geitz [53]. For each transformation, a 25µl cell pellet from fresh yeast cells grown in YPD liquid or on solid media (Yeast extract-peptone-dextrose medium) was suspended in 1 ml 0.1M Li Ac (lithium acetate), incubated for 5 minutes at 30°C, then pelleted in a microcentrifuge. Pellets were resuspended in a transformation mixture of 240 µl 50% (w/v) PEG (polyethylene glycol), 36 µl 1.0M LiAc, 50 µl 2.0 mg/ml salmon sperm DNA, 5 µl (100 ng-5 µg) plasmid DNA, and 20 µl water, then heat shocked for 20 minutes at 42°C. The cells were

pelleted and the supernatant removed, then the pellets were resuspended in 1 ml of water. After 100 µl of the transformation mix was plated onto selective media, the remainder was pelleted and resuspended in 100 µl of water, then also plated. Transformations were platcd onto SC-Trp (synthetic complete minus tryptophan) to select for the plasmid, and incubated at 30°C for 5 days.

From each transformation, four large colonies from vector alone and 4 from *SUB2*-containing plasmid transformations were selected, and each colony was resuspended in 1 ml H_20 (dilution 1). Six 10-fold serial dilutions were generated from each colony in water and 100 µl of each dilution was plated on selective agar. Dilution 0 consisted of the remaining 900 µl of dilution 1, spun down and resuspended in 100 µl of water. One hundred µl of dilutions 0-3 were plated onto SC –Ura -Trp to determine activity of *URA3*, and 100 µl of dilutions 4-6 were plated on SC -Trp, to determine total viable cells in each colony. After 4-5 days of incubation at 30°C, the total of colonies that grew on SC –Ura –Trp, and SC –Trp, were counted. The total colony numbers for all four repetitions were averaged for vector alone and each *SUB2* or *sub2* mutant construct. The average total number of cells capable of growth on -Ura plates was divided by the average number of total

viable cells (capable of growth on -Trp plates) for both the vector alone constructs, and for *SUB2* constructs. This gives the percentage of the total viable cells that express *URA3*. We then divided the percent of cells expressing *URA3* from the *SUB2* or mutant *sub2* construct by the percent of viable cells expressing *URA3* from the vector alone, to give us the fold increase in *URA3* positive cells above background. Each experiment was repeated a minimum of 4 times. The fold increases from each experiment were averaged to enable us to determine a standard deviation. A fold effect of 1 indicates no effect, while a positive number indicates silencing was suppressed (*URA3* transcription increased) and a negative number indicates silencing was enhanced. We arbitrarily set 10-fold as our benchmark for meaningful activity.

Vector alone $\quad \frac{\text{Avg -Ura growth}}{\text{Avg -Trp total growth}} = X$

Constructs $\quad \frac{\text{Avg -Ura growth}}{\text{Avg -Trp total growth}} = Y$

Fold Increase $= \frac{Y}{X}$ *SUB2* construct (sample) / vector alone (control)

Northern Blots

RNA extraction

Total RNA was extracted from yeast cells using the hot phenol method of Leeds [54]. Cells were grown to an OD_{600} of .4-.6 in 10 mls SC-Trp (YPD for UCC 3505 only), harvested, washed in sterile DEPC-treated dH_20, then pelleted, and pellets were frozen at –80°C until ready to process. Cell pellets were resuspended in 500 µl of Complete Buffer A [90% Buffer A (50mM NaOAc,10mM EDTA), 1% SDS, with 1% DEPC immediately prior to use], mixed with 600 µl of Buffer A saturated phenol, and incubated at 65°C with mixing every 30 seconds for 5-6 minutes. Following centrifugation, the phenol layer was removed, another 600 µl aliquot of Buffer A saturated phenol was added, and the mixing and incubation cycles were repeated. The aqueous layer was collected after centrifugation and mixed with 600 µl of 1:1 phenol, buffered with TE:chloroform. After centrifugation, the resulting aqueous layer was removed to a clean tube and ethanol precipitated twice by addition of 50 µl of 3 M NaOAc (pH 5.2) and 1 ml of 100% ethanol, incubated for 15 minutes on ice. RNA was pelleted by centrifugation and the pellet was air-dried. Pellets were resuspended in 400 µl of DEPC-treated dH_20 and ethanol precipitated again. The

precipitated RNA was washed in 1 ml of 70% ethanol, vortexed, centrifuged, and ethanol removed. RNA pellets were air-dried then dissolved in 50 µl of DEPC-treated dH_2O, heating for 10 minutes at 65°C to assist solubilization. Absorbence at A_{260} and A_{280} was determined using 5 µl aliquots of each RNA sample in 495 µl of dH_2O. RNA was diluted to a 1µg/µl concentration in TE. Two volumes of ethanol were added to each sample and samples were stored at –80°C.

Formaldehyde gel and blotting

For each sample, 10 µg of RNA were ethanol precipitated and brought up in 10 µl of DEPC-treated H_2O, mixed with 20 µl of 1.5X loading buffer [75% formamide, 7.5% formaldehyde, 10X running buffer (50 mM NaOAc, 0.2M MOPS pH 7.0, 10 mM EDTA), 75µg/ml EtBr], incubated at 65°C for 5 minutes, and cooled on ice. Two µl of loading dye (bromophenol blue in 50% glycerol, 1X running buffer) were added to each sample. RNA samples were run on a formaldehyde gel (0.9% agarose, 5% formaldehyde) at 65 volts for 4.5 hours. The buffer was stirred after 2 hours. The gel was washed twice in ~150 mls of DEPC-treated H_2O for 15 min, soaked for 15

minutes in ~150 mls of 10X SSC (3M NaCl, citric acid), photographed under 312 nm UV light (FOTODYNE FOTO/Prep® UV Transilluminator) then blotted onto a 0.45 micron nylon transfer membrane (Osmonics Laboratory Products #N00HYB0010) overnight in 10X SSC. The transfer method was sponges assembled like a sandwich to form a wick. Blotting was assembled from bottom to top in this order: sponges dampened with H_20 were arranged to form a block larger than the gel. Then two pieces of Whatman paper were soaked in 10XSSC and laid on top. Nylon membrane presoaked in dH_20 and 10XSSC, was placed on top of the filter paper, then the gel (wells facing up), and 2 more pieces of Whatman blot paper soaked in 10XSSC. The assembly was wrapped loosely with plastic wrap and a glass plate and weight were placed on top to allow the transfer of liquid into the sponges. Blotting was left overnight. The damp membrane was UV crosslinked with a Stratolinker (Stratagene, automatic setting) and stored in plastic wrap at -20°C until hybridization.

Radioactive hybridization

The membrane was soaked in 15 mls of pre-hybridization buffer (5X SSC, 50% formamide, 100µg/ml SS DNA, 5X Denhardt's, 0.2% SDS) for 1

hour at 42°C. A DNA fragment from +33 to +1293 of *SUB2* coding sequence was used for a probe. Radioactive probes were prepared using Ambion's DECA primeII™ DNA labeling kit according to manufacture's instructions. Free radionucleotides were removed from labeled DNA using QIAGEN QIAquick spin PCR purification columns. The 50 µl radiolabeled probes were boiled for 5 minutes, cooled on ice, mixed with 500 µl of hybridization buffer, and added to the blot which was in 12 mls of prehybridization buffer. Blots were hybridized overnight at 42°C in a rotisserie hybridization oven (ProBlot 6 from Labnet). Membranes were subsequently washed in Wash 1(2X SSC, 0.1% SDS) twice for 30 minutes at 55°C and 2 times for 15 minutes in Wash 2 (.5X SSC, 0.1% SDS) then wrapped in Saran wrap. Kodak X-OMAT AR film or the Cyclone Storage Phosphor System with OptiQuant image analysis software (Packard Instrument Company) was used to analyze the Northern blots. Membranes were stripped and reprobed using ^{32}P labeled *18S rDNA* followed by stripping and reprobing with ^{32}P labeled *RPB2*. The 1.6 Kb DNA fragment from + 1012 to +2664 of *RPB2* coding sequence was produced by PCR as described below. To reprobe the membranes, membranes were incubated 1hr at 65°C in Stripping solution (5mM Tris-HCl- pH 8, 2mM EDTA-pH 8,

.1X Denhardt's solution), checked for no residual CPM using hand-held Geiger-Muller monitor, then reprobed with the new probe.

Quantitation of Northern Blots

Quantitation of transcripts was accomplished by exposing the Northern blot to the Cyclone™ storage phosphor screen, followed by data acquisition using the Cyclone™ scanner with OptiQuant™ Software (Packard Instrument Company). Regions of interest (ROIs) were established for each sample, and the total gross intensity in Digital Light Units (DLU) was determined. For the 18S rRNA blot, the total gross intensity of each 18S rRNA band was divided by the total gross intensity of the 18S rRNA band for the *pRS424* vector alone sample. The resulting values of Blot A and Blot B served as conversion factors for normalizing RNA loading. The total gross intensity of each *SUB2* or *sub2* construct was divided by the corresponding conversion factor to provide the estimated total transcript intensity, normalized for loading. This data is presented graphically as normalized digital light units.

Western Blots

Whole cell lysates of UCC 3505 containing the indicated plasmids, were prepared using a glass beads method [55]. Cells taken from saturated overnight cultures were added to fresh media to an OD_{600} equal to 0.3 per ml. When cultures reached an OD_{600} of 0.6 to 0.8 per ml, cells were harvested by centrifugation, washed with 1 ml of H_20 and centrifuged again. Cell pellets were resuspended in 100 µl of ice cold Sample Buffer (0.06M Tris-HCl pH 6.8, 10% v/v glycerol, 2% w/v SDS or sodium dodecyl-sulfate, 5% v/v 2-mercaptoethanol, 0.0025% w/v bromophenol blue) containing 0.5mM phenylmethylsulfonyl fluoride (PMSF) and 0.5mM Benzamidine protease inhibitors. Samples were vortexed with ~100 µl of 425-600 µm glass beads (Sigma, Catalog no. G8772) for 45 seconds then cooled 30 seconds on ice. The vortex step was repeated 5 more times. The cell lysates were centrifuged for 5 minutes, boiled at 95°C for 5 minutes, and spun again briefly. Protein samples were loaded onto duplicate 10% SDS polyacrylamide protein gels. The gel for Western blotting had 10 µl of each sample loaded and 20 µl of each sample was loaded on the second gel for Coomassie Brilliant Blue staining. The molecular markers used were low range biotinylated SDS-PAGE standards (BIO-RAD) for Westerns and low

range SDS-PAGE standards (BIO-RAD) for coomassie gels. Electrophoresis was performed at 100 volts constant voltage. Gels for Western blotting were transferred to Hybond-P PVDF transfer membrane (Amersham Pharmacia Biotech) for one hour at 0.5 amps in blotting buffer (25 mM tris, 192mM glycine, 20% Methanol). Membranes were blocked overnight in 5% blocking buffer (1X Phosphate Buffered Saline (PBS), 0.05% Tween-20, 4% dry milk). Membranes were subsequently rinsed twice in wash buffer (1X PBS, .05% Tween-20), followed by a 15-minute wash then three, 5-minute washes in approximately 20 mls of wash buffer. The membranes were then incubated in 1/6000 dilution of rabbit polyclonal anti-Sub2 antibody in blocking buffer for 1-2 hours with gentle rocking at room temperature. Membranes were rinsed twice in wash buffer, followed by one 15-minute, then three 5-minute washes. Membranes were incubated in blocking buffer with anti-rabbit-HRP (BIO-RAD, 1:6000 dilution) and anti-biotin-HRP (New England Biolabs, 1:6000 dilution) secondary antibodies for one hour. Following one 15-minute wash and three 5-minute washes, the blot was developed using the chemiluminescent ECL+plus Western Blotting Detection System (Amersham Biosciences) according to manufacture's directions. The gel run in parallel for coomassie staining was

soaked overnight in Fairbanks stain (10% glacial acetic acid, 25% Isopropanol, .625% Coomassie Brilliant Blue R250 in 70% methanol) with gentle rocking, then de-stained in 10% acetic acid.

Non-specific binding of secondary antibodies

We measured background cross reactivity of secondary antibodies to determine a possible source for the presence of a 46 kD band detected on our Western blots (Figure 3). Assays were performed by incubating one blot in the primary antibody, anti-Sub2 (rabbit) for 1 hour, performing washes as described previously, and incubating in anti-biotin (HRP) one hour, followed by development on Kodak X-OMAT AR film (Figure 4A). A second blot was incubated 2 hours in the secondary antibody: anti-biotin (HRP), a third blot was incubated 2 hours in the secondary antibody anti-rabbit (HRP), and a fourth blot was incubated 2 hours in both secondary antibodies: anti-biotin (HRP) and anti-rabbit (HRP), followed by development on Kodak X-OMAT AR film (Figure 4B-D). The lower band had slight non-specific binding of secondary antibodies with or without Anti-Sub2. Although more evident in initial Western assays, as seen in Figure 3, the 46 kD bands were lighter in subsequent blots.

Figure 3. Initial Western blots show non-specific background band at 46kD. Lanes 1, 8, & 9, N/A; lane 2, Biotinylated Marker; lane 3, UCC3505; lane 4, pRS424; lane 5, SUB2; lane 6, sub2-1; lane 7, sub2-5; lane 10, bacterial Sub2p. The bacterial Sub2p has a His_6tag, contributing to the slower migration compared with yeast Sub2 proteins of 51kD, lanes 3-7. Below the Sub2 protein bands is a 46 kD band which appears in our Western blots and was attributed to non-specific binding of the secondary antibodies rather than the anti-Sub2 antibody.

Fig. 3.

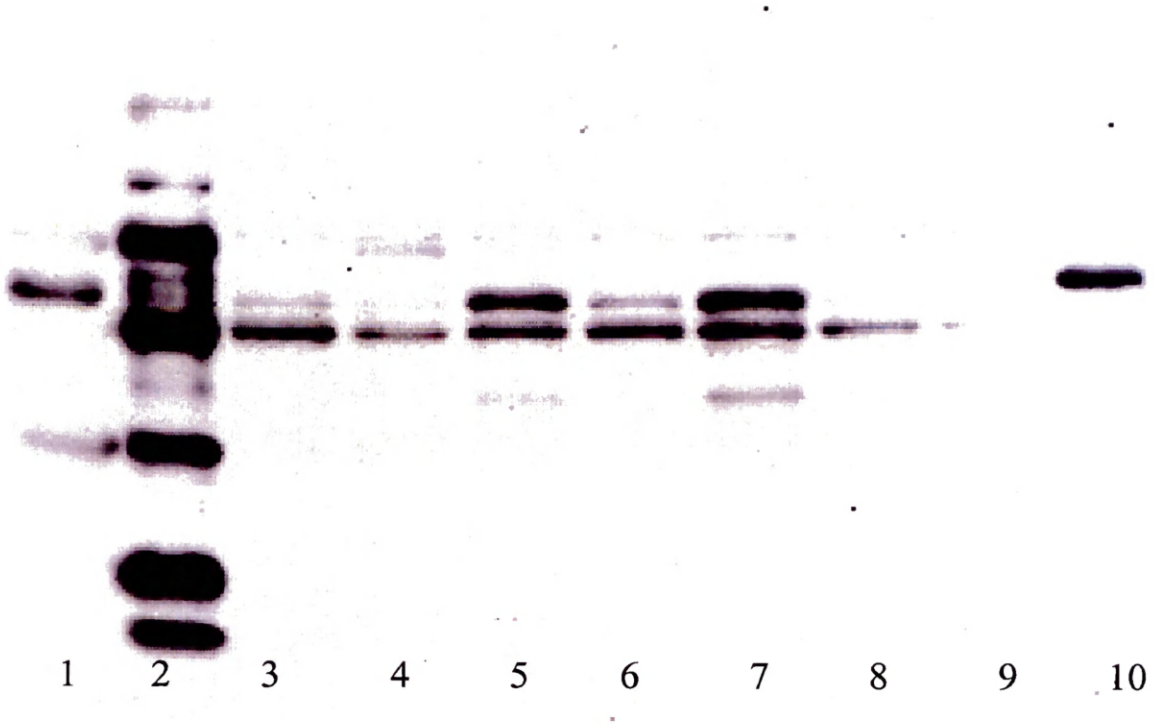
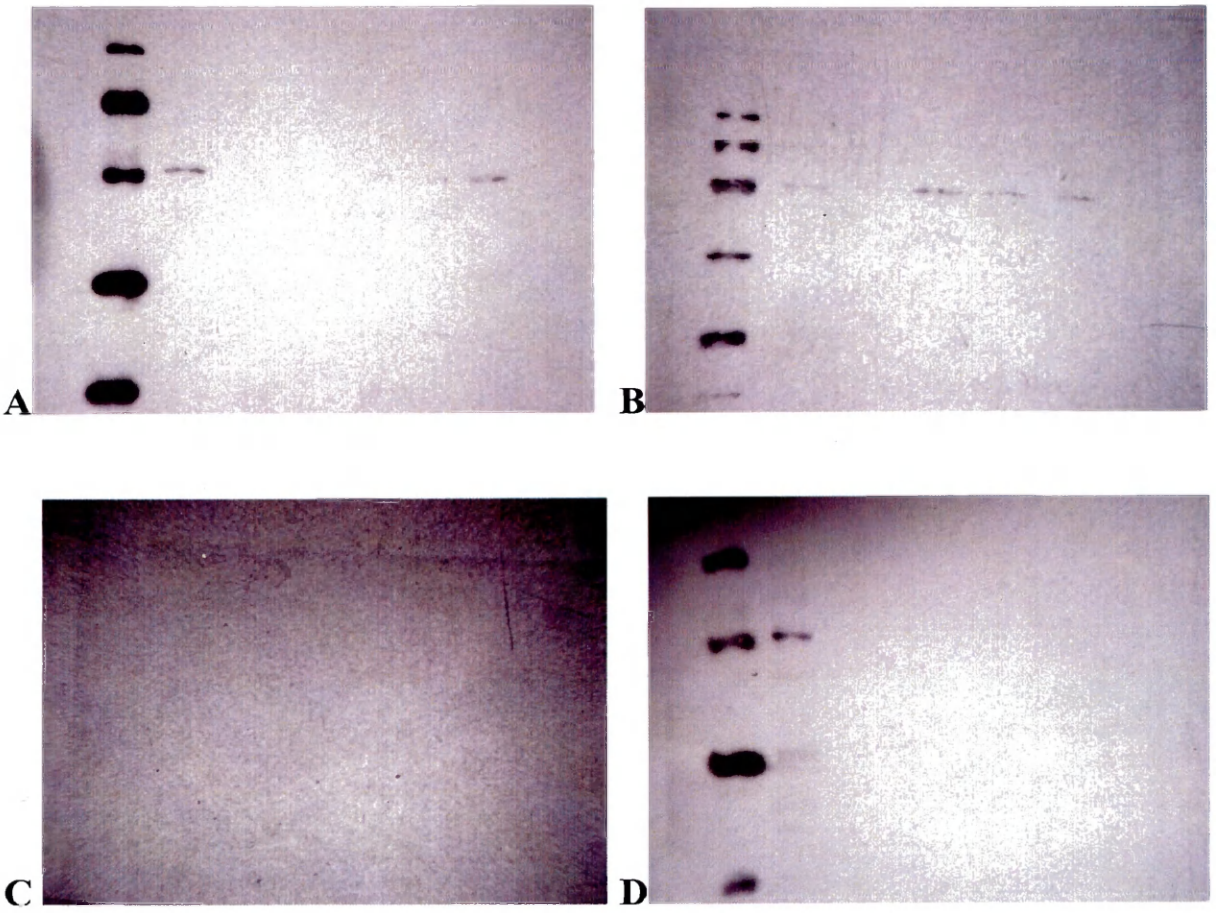


Figure 4. Slight non-specific binding of secondary antibodies is responsible for 46 kD band on Western blots of Sub2 constructs. Western blotting was performed on SDS polyacrylamide gels loaded with protein samples: biotin standard, pRS 424, SUB2, sub2-1, sub2-5, control 1, control 2, and bacterial Sub2p. Blots were incubated in: **A)** Anti-Sub2 and Anti-biotin (HRP), **B)** Anti-biotin, **C**) Anti-Rabbit, and **D)** Anti-biotin (HRP) & Anti-Rabbit (HRP).

Fig. 4.



Quantitation of Western Blots

The KODAK Image Station IS440CF with ID Image Analysis Software was used to capture and analyze images of chemiluminescent Western blots and coomassie-stained gels. ROIs were defined for each protein sample. Analysis of the ROI data determined the net intensity of each sample as the gross intensity minus the background intensity level (automatically determined by the software as the median pixel intensity in the frame of each ROI). For comparison, relative intensity levels were calculated by dividing the net intensity of each *SUB2* construct with the value for the vector alone. To normalize the data by controlling for protein loading effects, the relative intensity of each Western blot sample was divided by a conversion factor, calculated from the coomassie-stained gel run in parallel with each Western gel. The conversion factors were calculated by first dividing the net intensity of bands at approx. 57 kD and 37 kD for each sample with the net intensity of the 2 corresponding bands (same molecular weight) for the 424 vector alone sample. Then the relative net intensity units were averaged together to provide conversion values for normalization of protein loading. Sub2 protein levels (measured in

normalized relative intensity units) in high copy *SUB2* and *sub2* mutants were averaged from 5 separate gels, and standard deviations calculated.

Polymerase Chain Reaction (PCR)

PCR was used to amplify fragments from *RPB2* for use as DNA probes for our Northern blot analysis. For each PCR reaction, we combined 1 µl (estimated 100ng) of RNAse A-treated yeast genomic DNA isolated from strain S288C with 45 µl PCR cocktail (5 µl 10X thermo pol buffer, 10 mm dNTP, 10 mg/ml BSA), 2 units of deep vent polymerase, and 50 µM of the following primers:

RPB2 forward: ATTCGAGGATGAAAGTGCACC

RPB2 reverse: TGGAATAATAACTTCGCGGC

The samples were run for 1 cycle at 94°C for 5 minutes, 30 cycles at 94°C for 1 min, 40°C for 1 min, and 68°C for 1.5 minutes, 1 cycle at 72°C for 5 minutes, then held at 4°C. Samples were analyzed on a 1% agarose TAE gel and a 1650 bp band of *RPB2* was excised. The *RPB2* fragment was isolated using the Qiaquick gel extraction kit (QIAGEN) and eluted into 50 µl of

Buffer EB (10 mM Tris-HCl, pH 8.5). Five µl of the eluted *RPB2* fragment was used to prepare the P^{32}- labeled *RPB2* probe.

RESULTS

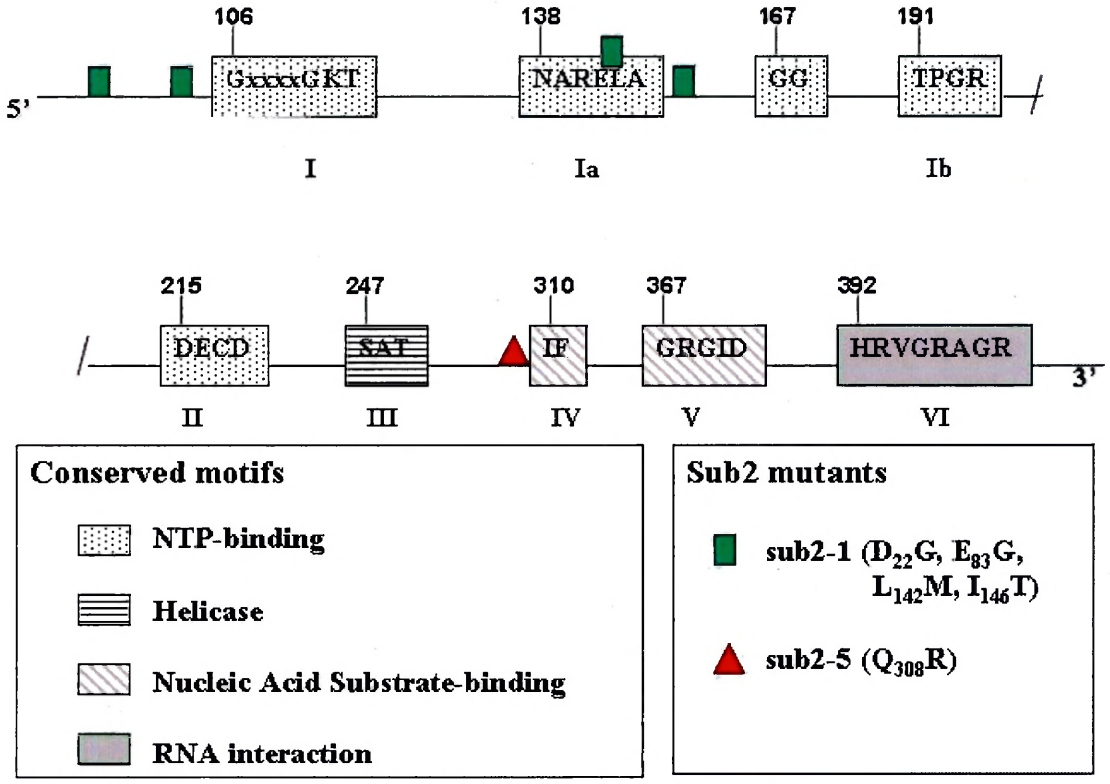
Overexpression of *SUB2* can increase expression of silent *URA3* reporter.

To determine whether overexpression of *SUB2* can overcome heterochromatic silencing as does the *Drosophila* homolog *Hel1,* we conducted silencing assays that places the *URA3* reporter gene adjacent to telomeric heterochromatin (UCC3505) [50]. The wild-type haploid copy of *SUB2* was not removed from the reporter strain because *SUB2* deletions are lethal. This reporter strain has a background ratio of *URA+* colonies of about 1 in 10^5 cells. UCC 3505 was transformed with *SUB2* or one of two *sub2* mutant constructs in *pRS 314*, a low copy vector utilizing a *CEN* sequence for replication. The reporter strain was also transformed with *SUB2* or one of two *sub2* mutant constructs in *pRS 424*, a high copy vector with a 2 micron origin of replication [51]. Thus, we assayed both low numbers and high numbers of extra copies of *SUB2* and *sub2* mutants, to measure the percentage of *URA+* colonies above background. Mutations in mutants *sub2-1* and *sub2-5* helicase motifs are illustrated in figure 5. *Sub2-1* has 4 conservative mutations: $D_{22}G$, $E_{83}G$, $L_{142}M$, and $I_{146}T$ located within

motif I and Ia, the NTP binding motifs, resulting in growth impairment at 16°C and 37°C. Accumulation of uncleaved pre-mRNA occurred when extracts grown at 25°C were preincubated at 37°C, but not when preincubated on ice, as described by Guthrie [27]. *Sub2-5* contains a conservative amino acid change $Q_{308}R$, adjacent to the nucleic acid binding motif IV, resulting in a defective growth phenotype at 16°C, yet splicing appeared unaffected.

Figure 5. *Sub2* mutants contain mutations near conserved RNA helicase motifs. The locations of mutations within the helicase motifs of *SUB2* are illustrated by a green rectangle (*sub2-1*) and red triangle (*sub2-5*). Adapted from Tanner et al., 2001[17]

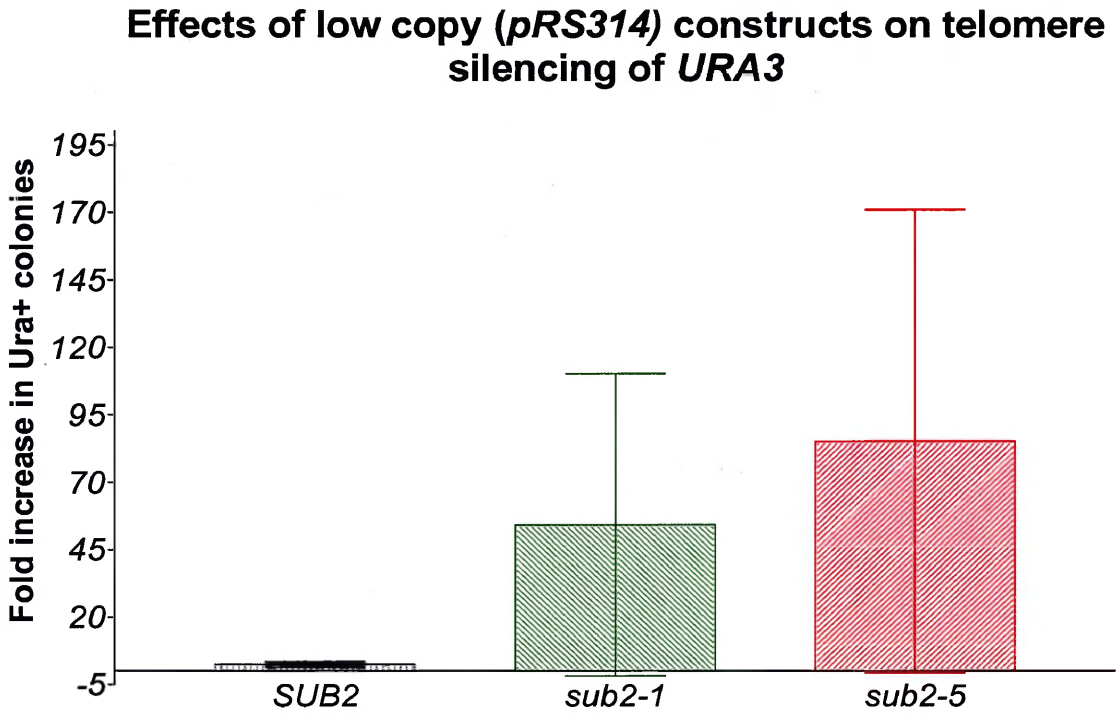
Fig. 5.


106
138
167
191
5'
GxxxxGKT
NARELA
GG
TPGR
I
Ia
Ib
215
247
310
367
392
DECD
SAT
IF
GRGID
HRVGRAGR
3'
II
III
IV
V
VI
Conserved motifs
NTP-binding
Helicase
Nucleic Acid Substrate-binding
RNA interaction
Sub2 mutants
sub2-1 ($D_{22}G$, $E_{83}G$, $L_{142}M$, $I_{146}T$)
sub2-5 ($Q_{308}R$)

Assays of overexpression with *pRS 314 SUB2* constructs showed a 2.5 fold increase above background Ura+ colonies, but was not statistically significant (Figure 6). We were unable to draw conclusions about the *sub2-1 and sub2- 5* mutants in *pRS 314* due to high variability with the 314 series assays. Interpretation of the few assays that were quantitated suggests there does not appear to be a statistical difference in *URA3* levels above background (Figure 6).

Figure 6. Low copy overexpression of *SUB2* does not affect silencing. The average of 3 or more silencing assays is presented with the standard error of the mean (SEM) indicated by error bars. Values were determined as described in Methods. Low copy *SUB2* (black vertical stripe) shows a 2.5 fold increase in Ura+ colonies. Mutant *sub2-1*(green diagonal stripe) shows an average 54-fold increase in Ura+ colonies. Mutant *sub2-5* (red diagonal stripe) shows an average 85-fold increase in Ura+ colonies. However, the standard deviations (not illustrated) are so high that values for the mutants are not statistically significant.

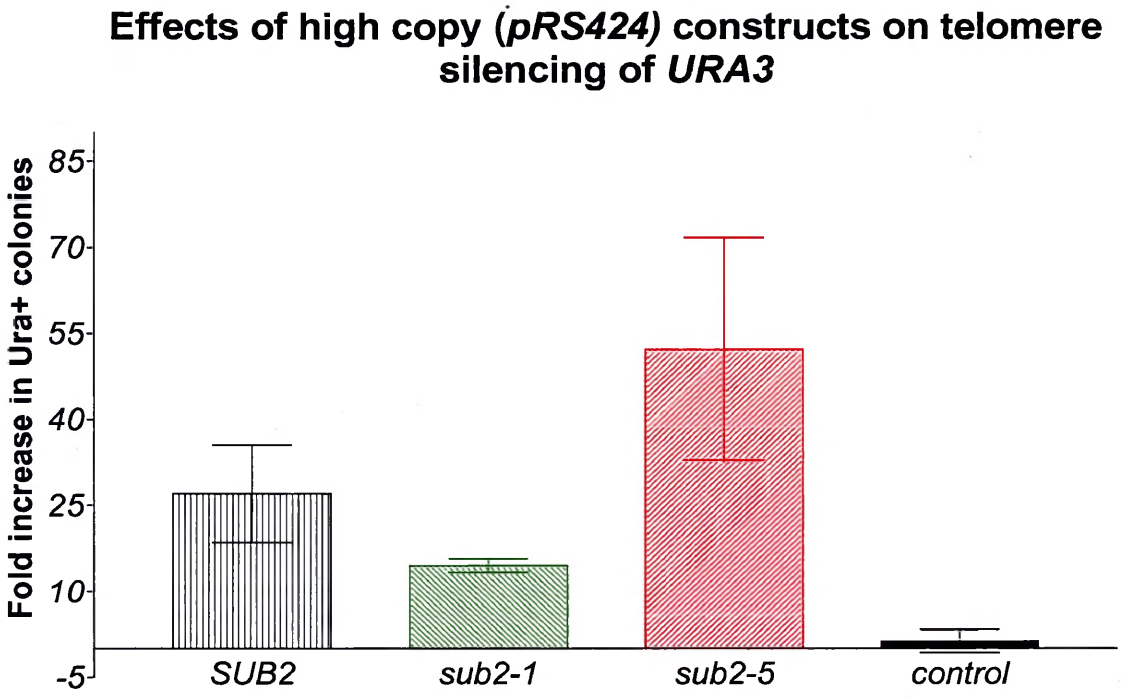
Fig. 6.


Effects of low copy (pRS314) constructs on telomere silencing of URA3
Fold increase in Ura+ colonies
195
170
145
120
95
70
45
20
-5
SUB2
sub2-1
sub2-5

Interestingly, silencing assays of *SUB2* in *pRS 424* showed an average 27-fold increase in Ura+ colonies, above background (Figure 7). Expression levels from high copy *sub2-1* constructs showed a 14-fold increase in Ura+ colonies, above background (Figure 7) and a 13-fold increase when incubated at the permissible temperature of 24°C (data not shown). High copy *sub2-5* constructs showed a 52-fold increase in Ura+ colonies, above background (Figure 7). As a control to quantitate background growth levels, expression levels of the vector construct alone were quantitated against each other, which gave a 1.3 fold (*pRS 424*) increase in Ura+ colonies, but was not significantly different than background (Figure 7). These results confirm that *SUB2* affects silencing when it is overexpressed at high copy number in yeast but not at low copy number, and that mutations in *SUB2* can alter the level of the silencing effects.

Figure 7. Overexpression of *SUB2* relieves silencing and *sub2* mutants alter this effect. The average of 4 or more silencing assays is presented with the SEM indicated by error bars. Values were determined as described in Methods. High copy *SUB2* (black vertical stripe) shows a 27-fold increase in Ura+ colonies. Mutant *sub2-1* (green diagonal stripe) shows a 14-fold increase in Ura+ colonies. Mutant *sub2-5* (red diagonal stripe) shows a 52-fold increase in Ura+ colonies. The control compares *pRS 424* vector (black solid) with itself and shows 1.3 fold, or no significant difference, in Ura+ colonies.

Fig. 7.


Effects of high copy (pRS424) constructs on telomere silencing of URA3
Fold increase in Ura+ colonies
85
70
55
40
25
10
-5
SUB2
sub2-1
sub2-5
control

mRNA transcript levels correlate with silencing results.

We performed 2 separate Northern blot assays to analyze *SUB2* transcript levels in high copy *SUB2* and *sub2* constructs to see if they correlated with the results from our silencing assays. A P^{32} labeled DNA fragment from the *SUB2* coding region was used to probe Northern blots as described in Methods (Figure 8a). To control for differences in loading, we used a P^{32} labeled probe of *18S rDNA* which is transcribed uniformly throughout the cell cycle by RNA Polymerase I (Figure 8a). The gross digital light units (DLU) were arbitrarily set to 1.0 for the *pRS424* vector alone samples. Although the values from both blots follow a similar trend, differences between the two blots may be attributed to variation in RNA preparation and isolation, RNA transfer, washing conditions, and other human error. The gross DLUs for blots A and B were divided by their corresponding conversion values to give the average normalized DLU counts for each construct from the two blots together (Figure 8b). Quantitation of the *18S rRNA*-probed blots provided the conversion values used to normalize the data obtained from the *SUB2* -probed blots (Figure 8c). However, it is possible that changes in *SUB2* copy numbers do affect rDNA transcription, based on our lab's acquisition of 5 separate rDNA

clones from a two-hybrid screen for Sub2. The *RPB2* probe was used to assess general RNA Polymerase II transcription effects because *RPB2,* a component of the RNA Pol II core complex, is also transcribed by RNA Pol II. If overexpression of *SUB2* causes a global effect on RNA Pol II transcription, then we should see an effect on *RPB2* transcript levels. Sub2 may affect *RPB2* transcription because the *RPB2* levels from Northern blot A did not appear equal nor was the same trend seen as in our silencing assays (Figure 8d).

Figure 8. Transcript levels reflect *SUB2* construct silencing activity. **a)** Intensity of RNA levels depicted in color ranging from blue (low) to green/yellow (medium) to red (high). For Northern blot A, the top row shows the blot probed against P^{32}-labeled *SUB2* DNA. The middle row shows the same blot probed with P^{32}-labeled *18S ribosomal DNA*. The bottom row shows the same blot probed with *RPB2*. For Northern blot B, the top row shows the blot probed with P^{32}-labeled *SUB2*. The bottom row shows the same blot probed with *18S ribosomal DNA*. **b)** Northern Blot quantitation of UCC 3505 containing high copy *SUB2* constructs was performed as described in Methods. Quantitation of *SUB2* transcripts for 3505, 424, SUB2, sub2-1 and sub2-5 on Northern blots A and B is averaged together and presented as normalized DLUs. **c)** Conversion values for normalization come from Northern blots A and B that compare *18S rRNA* transcript levels for 3505, 424, SUB2, sub2-1 and sub2-5, presented in DLUs. **e)** Northern blot A shows *RPB2* transcript levels for 3505, 424, SUB2, sub2-1 and sub2-5, presented in normalized DLUs.

Fig. 8a.

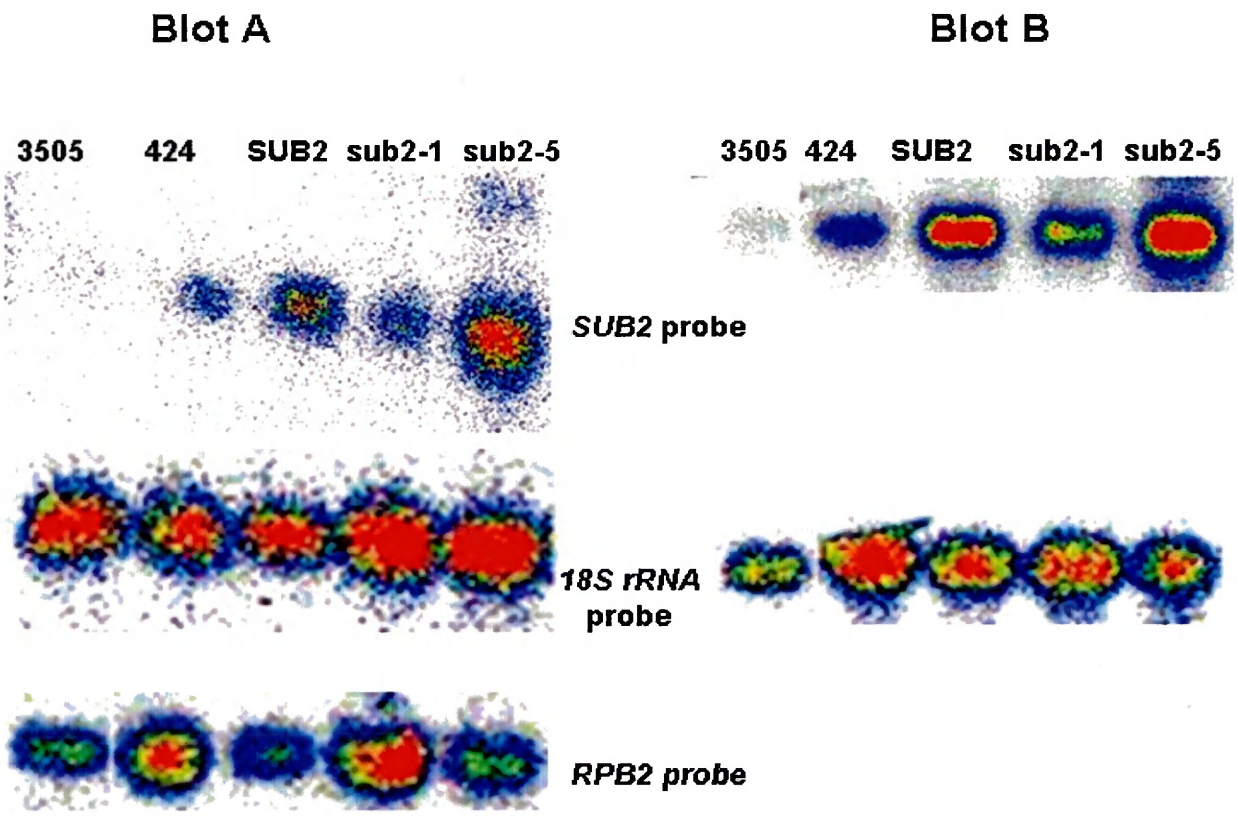

Blot A
Blot B
3505
424
SUB2
sub2-1
sub2-5
3505
424
SUB2
sub2-1
sub2-5
SUB2 probe
18S rRNA probe
RPB2 probe

Fig 8b.

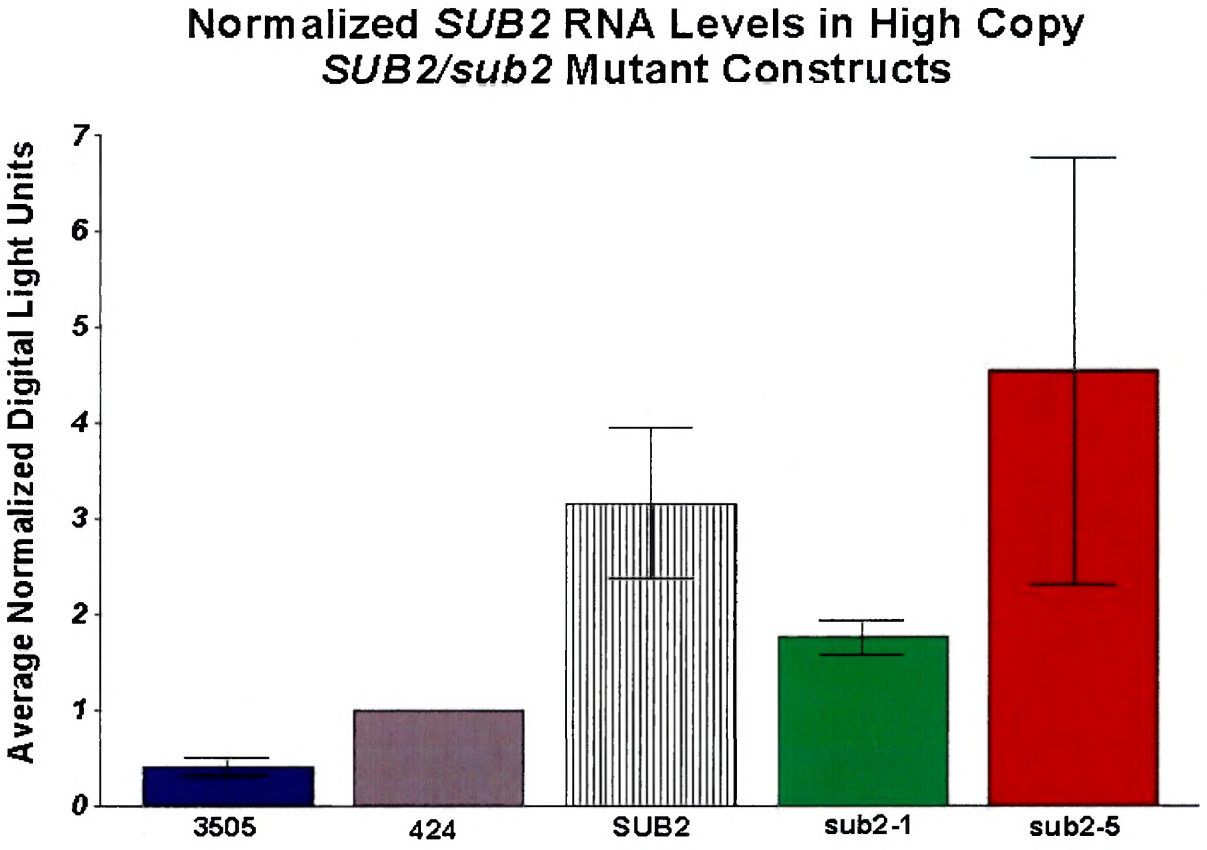

Normalized SUB2 RNA Levels in High Copy
SUB2/sub2 Mutant Constructs
Average Normalized Digital Light Units
0
1
2
3
4
5
6
7
3505
424
SUB2
sub2-1
sub2-5

Fig. 8c.

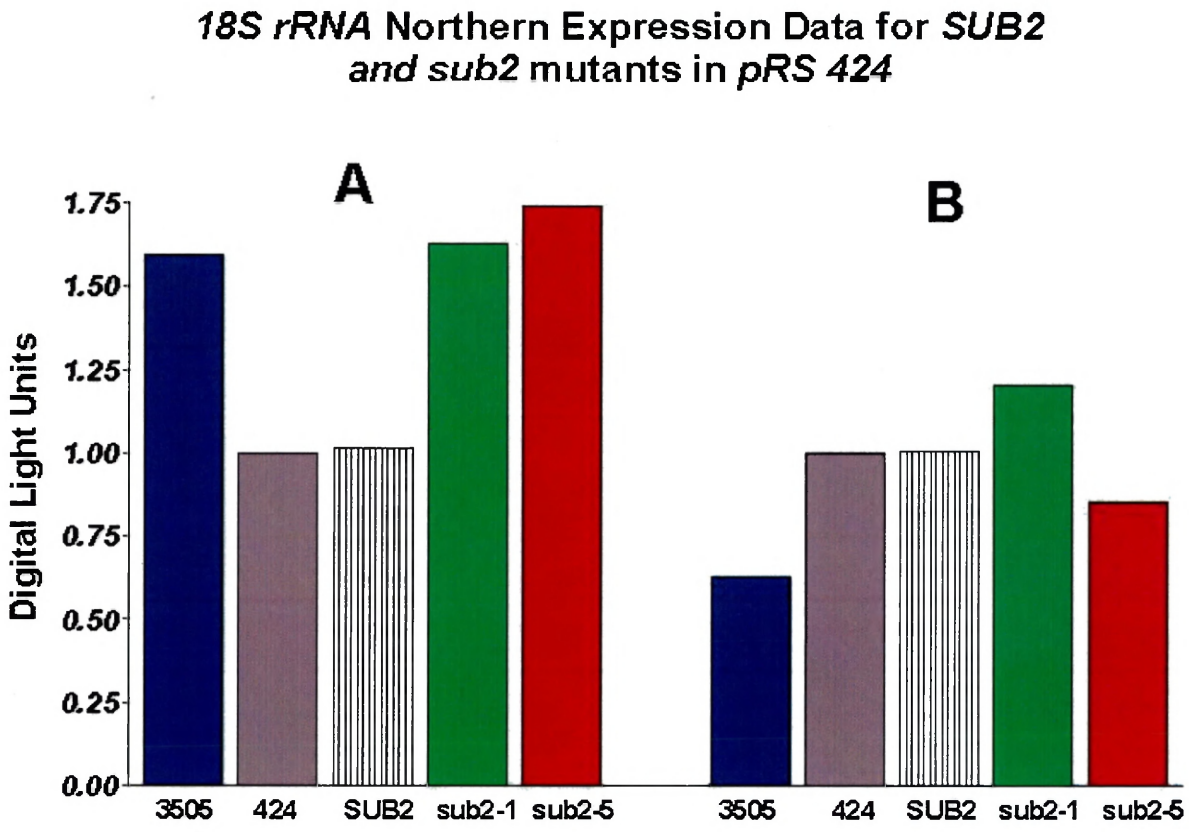
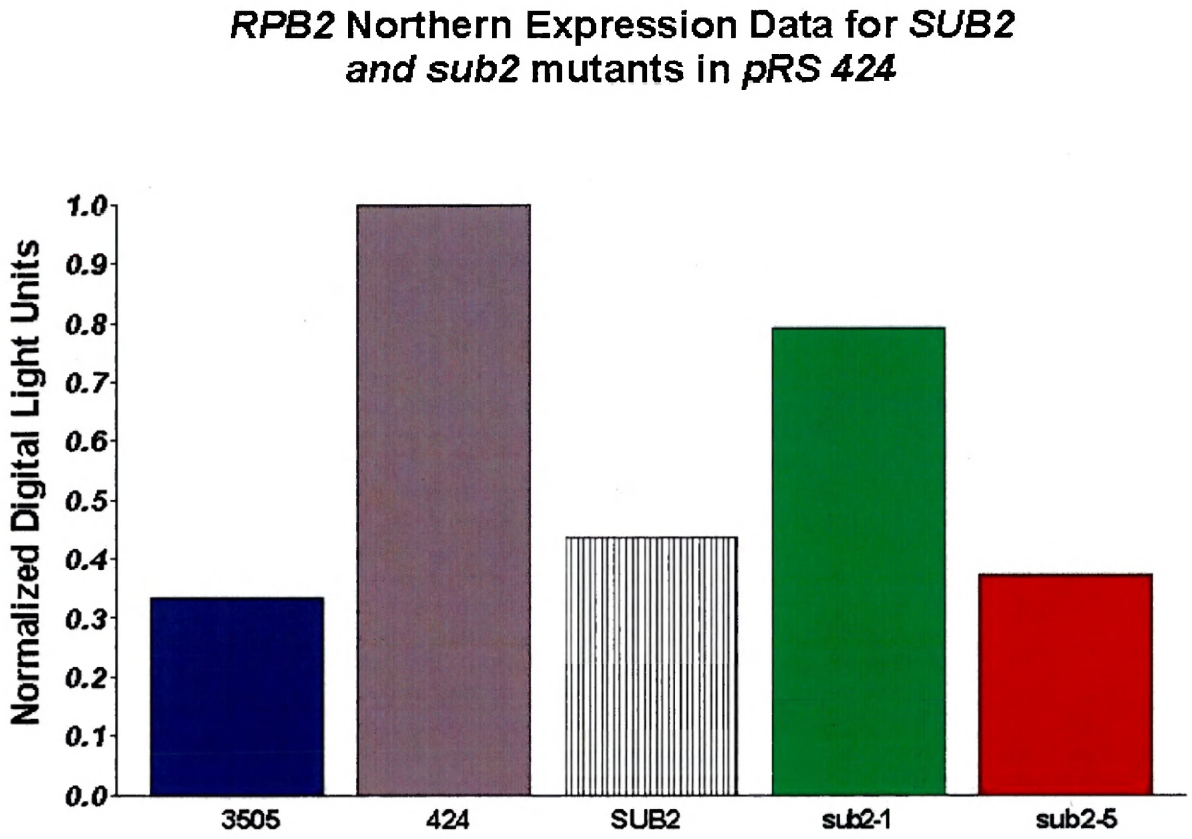

18S rRNA Northern Expression Data for SUB2 and sub2 mutants in pRS 424
A
B
Digital Light Units
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
3505
424
SUB2
sub2-1
sub2-5
3505
424
SUB2
sub2-1
sub2-5

Fig. 8d.


RPB2 Northern Expression Data for *SUB2* *and sub2* mutants in *pRS 424*
Normalized Digital Light Units
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
3505
424
SUB2
sub2-1
sub2-5

Sub2 protein levels correlate with silencing results.

Western blot assays were performed to determine if Sub2 protein levels also correlate with the results of our silencing assays. Protein levels were quantified in light intensity units and normalized for loading effects by quantitation of 2 bands on a coomassie-stained gel that was run in parallel with the same samples. The average of five Western assays is presented along with two representative blots (Figure 9 a,b) and a representative coomassie-stained gel used for normalization (Figure 10). The normalized values are the net intensity units calculated from the Western assays, divided by the corresponding conversion values determined from the coomassie gels to equalize for loading effects. UCC 3505, the reporter strain alone, indicates endogenous Sub2 protein levels. Normalized protein levels indicate UCC3505 has 2.6-fold higher Sub2p levels than *pRS424* alone. The Sub2 protein levels in *pRS 424* alone strains was set arbitrarily at 1.0 as the background level for comparison with *SUB* and mutant *sub2* constructs. Overexpressed *SUB2* shows an average 6.98-fold increase in protein level above background. The *sub2-1* mutant shows a 2.33-fold increase in protein above background. Interestingly, the *sub2-5* mutant had an average 10.26 fold higher protein level than background. These results indicate Sub2

protein levels follow the same general trend seen in the silencing assays and the Northern blot analysis.

Figure 9. Protein levels reflect silencing activity in *SUB2* constructs.

Western Blots were performed as described in Methods.

a) The average of five Western assays shows normalized intensity values for Sub2 protein levels. Protein levels were normalized for loading effects by quantitation of 2 bands on coomassie-stained gels run in parallel. The UCC3505 cell strain alone, which reflects endogenous Sub2 levels , shows normalized values at 2.65 compared with levels in *pRS424* vector alone arbitrarily set to a value of 1. In the *SUB2* constructs, a 6.98-fold increase in protein level above vector alone is seen. The *sub2-1* allele shows a 2.3fold increase above background and the *sub2-5* allele shows a 10.26-fold increase above background.

b) Representative Western blots A and B were used to determine average protein levels of high copy *SUB2* and mutant *sub2* constructs. Lanes for each blot are: UCC3505, *pRS424*, *SUB2*, *sub2-1*, and *sub2-5*. Western blot A also includes a lane with *sub2-1* grown at 24°C.

Fig. 9a.

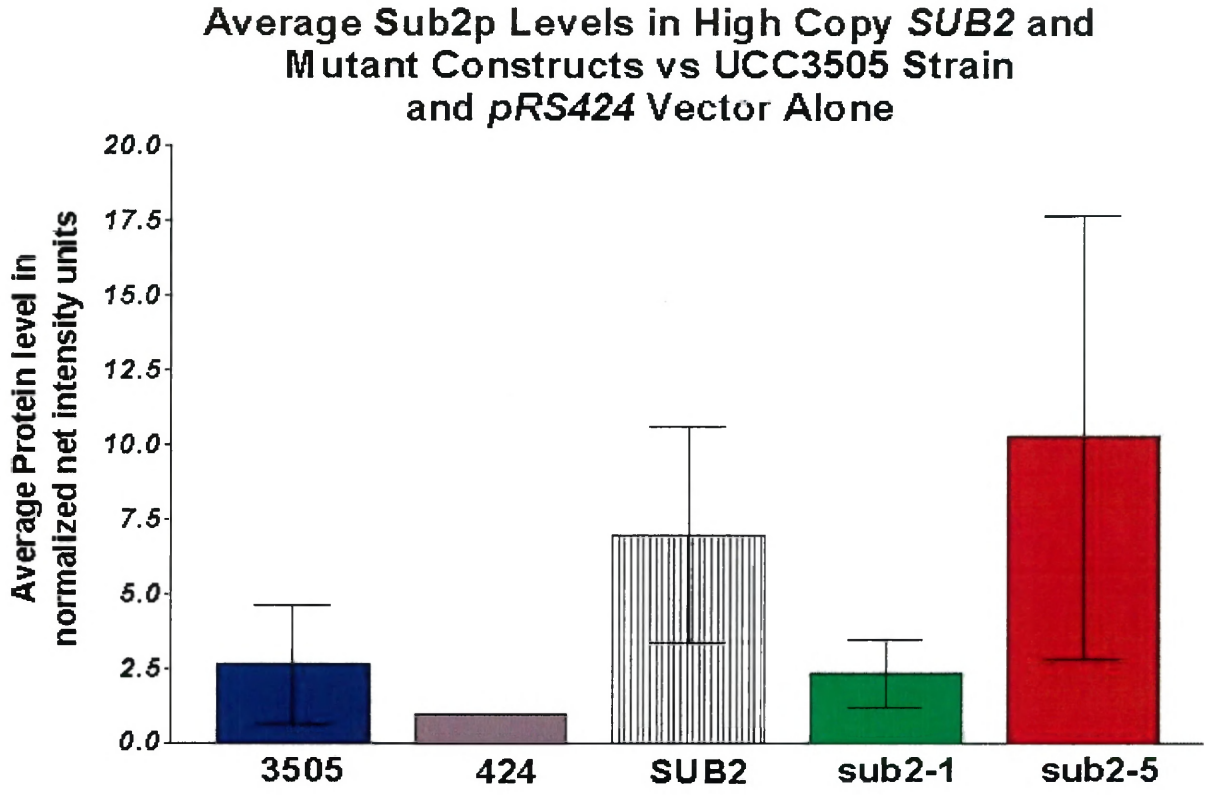
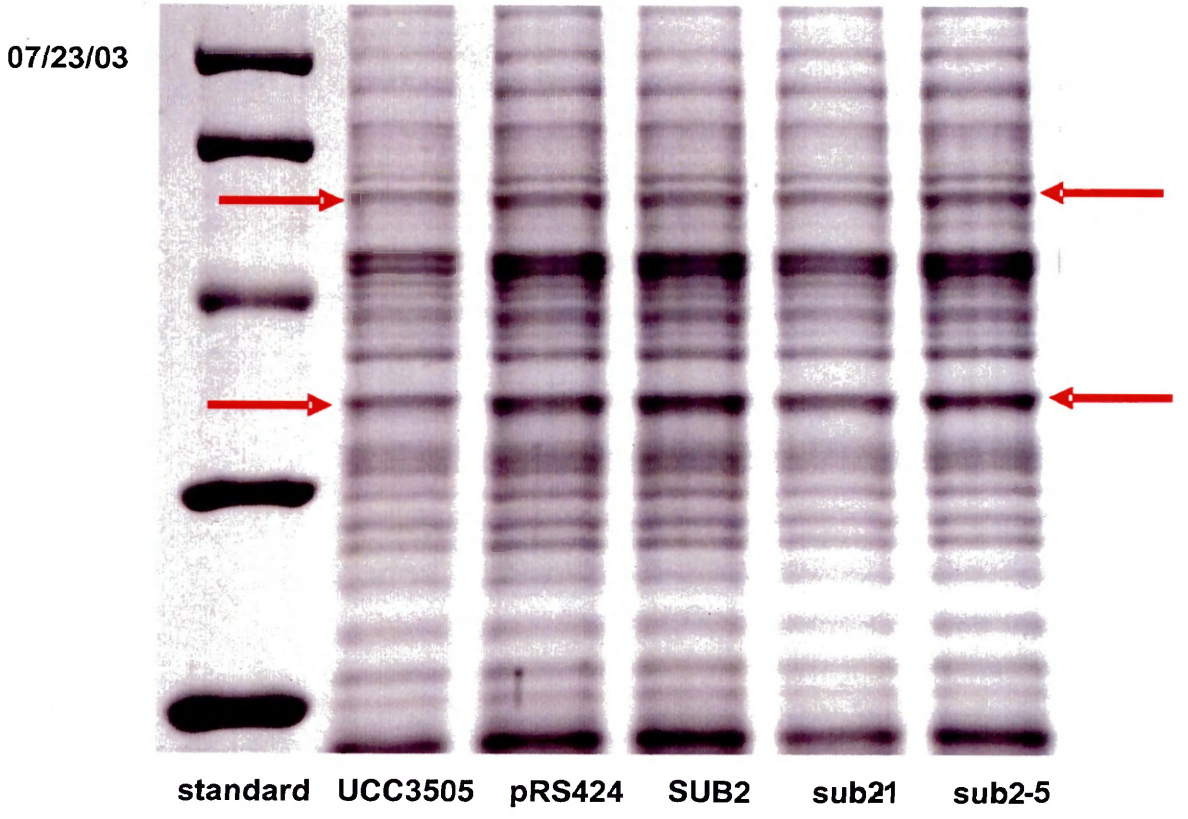

Average Sub2p Levels in High Copy SUB2 and Mutant Constructs vs UCC3505 Strain and pRS424 Vector Alone
Average Protein level in normalized net intensity units
20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
3505
424
SUB2
sub2-1
sub2-5

Fig. 9b.


A
UCC3505
pRS424
SUB2
sub2-1
sub2-1@24°C
sub2-5
B
UCC3505
pRS424
SUB2
sub2-1
sub2-5

Figure 10. Coomassie gels were used to normalize protein loading of gels for Western assays. Average protein intensity levels were determined by the averaging the relative net intensities of 2 bands per lane, as indicated by red arrows, from each coomassie gel. A representative gel shows lanes contain protein samples: low molecular weight standards, UCC3505, pRS424, SUB2, sub2-1 and sub2-5.

Fig. 10.



DISCUSSION

SUB2 and *sub2* mutants affect telomeric silencing when overexpressed.

Previous studies have implicated roles for *S. cerevisiae SUB2* in transcription elongation, mRNA splicing and nuclear transport, and DNA stability. The goal of our studies is to examine the role of *SUB2* in gene silencing to better understand this complex regulatory process. We present data that indicates *SUB2* partially overcomes silencing at yeast telomeres, when overexpressed. Silencing assays using a *S. cerevisiae* reporter strain where *URA3* is silenced by telomeric DNA, show a 27-fold induction of the reporter gene expression when *SUB2* is overexpressed. This suggests *SUB2* can affect heterochromatin structure, as is seen with *HEL1* in *Drosophila* [10].

We assayed two *sub2* mutants to determine if conserved helicase motifs were important for the silencing activity associated with high copy *SUB2*. Silencing assays using overexpressed *sub2-1,* which has four mutations around the ATP binding motif, showed 14-fold more Ura+ colonies than the vector alone. Thus, mutations adjacent to the ATP-binding

motifs I and Ia appear to reduce *URA3* expression relative to overexpressed wildtype Sub2, indicating a lesser effect on heterochromatin activation. It is possible the mutations near ATP binding have a negative effect on ATPase function, suggesting that the ATP binding region may be important in silencing suppression. Alternatively, the effect on silencing may result from an overall stability effect on protein folding. Silencing assays of single mutations created by site-directed mutagenesis could help determine the mutation(s) in the *sub2-1* mutant responsible for possibly affecting ATPase activity.

In contrast, silencing assays using the high copy *sub2-5* mutant, containing a single mutation adjacent to the nucleic acid binding motif IV, showed 52-fold higher Ura+ colonies than the vector alone. In this case, the mutation adjacent to the nucleic acid substrate-binding motif IV appears to improve expression of *URA3* relative to wildtype, indicating a stronger effect on heterochromatin activation. Based on the location of this mutation, efficient substrate binding may be important for Sub2's effect on silencing suppression. Perhaps this mutation strengthens RNA substrate binding by Sub2 to alter RNA-protein interactions important for transcription. Alternatively, the mutation may prevent binding of a specific nucleic acid

substrate, freeing Sub2 to bind with a different substrate such as other proteins or RNA. Without evidence to support these hypotheses, we are only able to speculate. Other experiments that could provide insight as to whether these mutations affect substrate binding, include additional yeast two-hybrid assays to pull up other protein interactors, and ChIP assays to see if Sub2 associates with particular gene regions.

The ability to suppress telomeric silencing is roughly correlated with RNA and protein levels.

The high copy *SUB2* and *sub2* mutant alleles had RNA and protein levels that roughly correlated with their ability to overcome *URA3* silencing, as determined by Northern and Western blot assays. Average transcript levels from two Northern blots gave estimates of *SUB2* transcript levels, showing high copy *SUB2* constructs have approximately 3-fold higher *SUB2* RNA levels than cells containing the *pRS424* vector alone. Furthermore, high copy mutant *sub2-1* had only a 1.8-fold higher transcript level than vector alone, while *sub2-5* had approximately a 5-fold higher RNA level than vector alone. The transcript levels correlate with activity levels seen in our silencing assays, indicating that increased SUB2 RNA levels influence

increased transcription through silenced regions. The variation among RNA levels may be attributed to differing rates of transcription or degradation of mutant *sub2-1* and *sub2-5* transcripts, compared with wild type. Precedence for an effect due to differences in mRNA decay rates comes from studies with the yeast decapping enzyme, DCP1, that indicate degradation rates from decapping without poly (A) tail-shortening may be based on varied interactions of DCP1 with individual transcripts [56]. Besides mRNA degradation in the cytoplasm, there is also a nuclear mRNA degradation (DRN) system [57], which may involve surveillance proteins that target splicing mutants [58], cleavage and/or polyadenylation mutants [59-61], hyper- or hypo- adenylation mutants [62, 63], or accumulated transcripts resulting from inefficient export [40].

Quantitation of five Western blots was used to determine an average Sub2 protein level for our high copy constructs, and protein levels showed a similar profile to the RNA levels reflected in the Northern assays. When normalized for loading differences, Sub2 protein was almost 7-fold higher than in the *pRS424* vector alone strains. Sub2 protein levels from the mutant *sub2-1* constructs were 2.3-fold higher than *pRS424* vector, while *sub2-5* constructs had 10.3-fold higher levels than *pRS424* vector. This suggests

that an increase in Sub2 protein levels directly affects the ability of Sub2 to suppress telomeric silencing, but does not prove it.

Comparison of Sub2 protein levels from *sub2-5* constructs to wildtype constructs shows improved efficiency in silencing suppression by the *sub2-5* mutant, possibly due to altered nucleic acid substrate binding by the Sub2-5 protein. Alternatively, increased total Sub2 protein levels might titer away a second protein necessary for telomeric silencing, such as Sir2. Increased levels of Sub2 protein could directly increase the number of functional TREX complexes for transcription elongation, improving overall transcription efficiency, if Sub2 is a limiting factor. Support for this hypothesis comes from the stoichiometric association of Sub2 with the THO complex to form the TREX complex [36], and the ability of THO-Sub2 to associate with RNA or dsDNA *in vitro* [39]. The greater availability of Sub2 may result in protein-protein competition, interference with, or altered sequestering of, proteins that regulate transcription. We suggest the excess Sub2 provides an over abundance of protein for essential roles in mRNA splicing and transport, to allow for a role in enhanced recruitment of chromatin modifiers.

Although *sub2-1* shows a loss of function in splicing at 37°C, but not at the permissible temperature of 25°C [27], our high copy *sub2-1* silencing assays showed a similar lower chromatin remodeling function at both 30°C and 24°C (data not shown). Although cold-sensitive for growth, the *sub2-5* extracts showed no defects in splicing activity or spliceosome assembly [27], yet our high copy *sub2-5* silencing assays indicate a higher level of silencing suppression than wild type at 30°C. This suggests the mechanism of Sub2 activity in silencing suppression is distinct from that of mRNA splicing. Despite the evidence for *SUB2*'s effect on heterochromatic silencing at telomeres, the underlying mechanism(s) involved have yet to be determined.

Site-directed mutagenesis to alter key amino acids within other DExD box motifs or target the unique 5' and 3' ends of the *SUB2* gene mutagenesis could help narrow down those motifs involved in Sub2's silencing ability. The N- and C-terminal ends may be substrate-specific, so mutating them could show an effect on silencing if interactions between Sub2 and other proteins or RNA are disrupted.

Partial toxicity due to *SUB2* overexpression, as reported by other labs [30], may account for some of our difficulties in quantitation of our assays that were excluded due to slow growth of colonies or high background.

Yeast cells from UCC 3505 transformed by our *SUB2/sub2* mutant constructs were slow growers, so our lab has begun to reaccomplish the silencing assays using another yeast reporter strain, YEP100-10, that places the *URA3* gene adjacent to the left telomere of chromosome XI. This strain does not have artificial telomeres and appears to be healthier when transformed with the high copy *SUB2/sub2* mutant constructs. Preliminary results show that *sub2-5* has higher ability to overcome heterochromatic silencing at a native telomere than wild type *SUB2*.

Does *SUB2* affect silencing at other heterochromatic locations in yeast?

In response to this question, we also performed silencing assays using *S. cerevisiae* strain UCC 3515, which places the *URA3* reporter gene adjacent to silent mating type locus HMLα. Unfortunately, we experienced technical difficulties due to contamination problems, as well as high levels of background growth with pinpoint colonies that were difficult to quantitate. This effect may be overcome by assaying on 5FOA rather than – Ura media. As initial assays with UCC 3515 did not indicate a noticeable effect on silencing, we postponed further assays and focused instead on the

UCC3505 series. Fan [13] and West [35] both show no effect of *SUB2* on yeast mating type loci silencing, which our preliminary data supports. Comparison of wild-type yeast strains with *sub2*Δ strains plus *SUB2* in a CEN plasmid showed an increase in *SUB2* copy number had no affect at HMRa [13, 35].

In contrast to our studies with telomeres, overexpression of *SUB2* has been associated with increased silencing of *URA3* adjacent to ribosomal DNA [35]. The rDNA array is localized to the nucleolus, a subnuclear structure in which ribosome assembly occurs. The inclusion of hUAP (Sub2 human homolog) within the nucleolus [64] supports the inference that Sub2 may localize to silenced DNA within the nucleolus. Consequently, overexpressed *SUB2* appears to have opposite effects on chromatin activation at telomeres and rDNA in yeast.

Examples of other proteins that have opposite effects on telomere and rDNA silencing include Sir2. Immunofluorescence studies showed Sir2 co-localizes with Sir3, Sir 4, and Rap 1, and this complex is found in foci near the nuclear membrane, forming a telomeric repression complex [65]. Studies using *in situ* cross-linking with immunoprecipitation have localized Sir2 to the nucleolus, cross-linked to the rDNA locus on chromosome XII,

though what targets it there has not been determined [65]. Systemic deletions of *SIR2* are viable, but *SIR2* disruptions result in telomeric delocalization of Rap1, Sir3 and Sir4, supporting Sir2 as an integral component of telomeric chromatin [65]. As others have proposed, perhaps the grouping of telomeres near the nuclear periphery provides a "reservoir of silencing factors" that can be targeted to other loci as needed [66-68]. For example, when yeast cells become old, Sir3 and Sir4 are redistributed from telomeres to the nucleolus by Uth4 and Ygl023, to extend life span [69] .

SUB2 may influence localization of silencing machinery components or may act as a regulatory component of specific transcription-coupled chromatin remodeling complexes.

A second model for how *SUB2* might influence telomeric silencing is tied to Sub2's function within the TREX complex. Although this scenario is unproven, a similar model comes from studies of Sus1, a nuclear pore-associated protein component of mRNA export machinery, that is also required for SAGA-dependent gene transcription [70]. Models for Sus1 function in gene expression suggest that the SAGA histone acetylase complex binds active genes while tethered to nuclear pores, via Sus1

interactions with the SAGA complex and mRNA export proteins Sac3 and Thp1. Sub2 interacts with other export proteins as part of the TREX complex which couples transcription elongation with mRNA transport to the nuclear pore. During mRNA nuclear export, Sub2 is displaced from Yra1 by the nuclear pore-associated protein Mex67/Mtr2 [46, 47], which leaves Sub2 in close proximity to telomeres localized at the nuclear membrane. An increased presence of Sub2 near telomeres could recruit histone remodelers to telomeres, or compete with other proteins that influence localization of the Sir-containing silencing complex by redirecting the Sir proteins to the nucleolus so that telomeric silencing is decreased.

Alternatively, Sub2's physical and genetic interactions with Hpr1 and genetic interactions with Cdc73 suggest Sub2 might also associate with the Paf1 complex as well as the THO complex to influence transcription elongation resulting from chromatin remodeling. The Paf1 complex regulates RNA Polymerase II transcription of a subset of genes involved in recombination and cell wall integrity [34], and recruits the Set1-containing COMPASS and Dot1 methyltransferases to an elongating transcript [41]. Set1 of COMPASS has been shown to methylate histone 3 at lysine 4 (H3 K4), and Dot1 is responsible for histone 3 at lysine 79 (H3 K79) methylation

[41, 71]. Methylation on the tail domain of H3 K4 H3 K79 is associated with silencing of expressed genes near yeast telomeres, presumably mediated by the recruitment of chromatin remodeling proteins [41]. However, Santos-Rosa *et al.* demonstrated that transcriptional activation of Set1-regulated genes was actually determined by changes in methylation status [71]. Set1-mediated methylation of H3 K4 can also result in di- or tri-methylation. Di-methylation of H3 K4 is associated with both silenced and active genes, but tri-methylation only appears on active genes. This may help explain how the recruitment of methyltransferases to chromatin can have both positive and negative effects on transcription activation. Although Sub2 has not been shown in direct association with the PAF1 complex, an increase in Sub2 near telomeres and Sub2 interaction with transcription machinery components, could alter Paf1 recruitment of histone remodelers. We propose that when Sub2 is overexpressed, it may out-compete repressive proteins, influence the redistribution of silencing factors, or recruit other activators, to upregulate transcription at heterochromatic promoters, thus suppressing silencing. Other than an increase in Sub2 protein near nuclear pores or the possible association of Sub2 with Sir proteins, the mechanism of Sub2 specificity for heterochromatin remains elusive. Future directions

should include biochemically determining if there is a physical interaction between Sub2 and the Paf1 complex or Sir proteins. Using ChIP assays with Sub2, we should also look for altered distribution of Sir proteins.

In conclusion, our quantifiable silencing assays show that high-copy *SUB2* suppresses heterochromatic silencing, as seen in *Drosophila*, and that mutations near the conserved helicase motifs involved in ATP hydrolysis and nucleic acid substrate binding can alter the silencing effect. Northern and Western blot analysis correlates upregulation of transcription through the silenced *URA3* gene with increased levels of *SUB2* mRNA transcripts and protein levels.

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