Sen1p performs two genetically separable functions in transcription and processing of U5 snRNA in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* SEN1 gene codes for a nuclear-localized Superfamily I helicase. SEN1 is an ortholog of human SETX (Senataxin), which has been implicated in the neurological disorders ataxia-ocular apraxia Type 2 and Juvenile Amyotrophic Lateral Sclerosis. Pleiotropic phenotypes conferred by sen1 mutations suggest that Sen1p affects multiple steps in gene expression. Sen1p is embedded in a protein-protein interaction network involving direct binding to multiple partners. To test whether the interactions occur independently or in a dependent sequence, we examined interactions with the RNA polymerase II subunit Rpb1p, which is required for transcription, and Rnt1p, which is required for 3’ end maturation of many non-coding RNAs. Mutations were identified that impair one of the two interactions without impairing the other interaction. The effects of the mutants on the synthesis of U5 snRNA were analyzed. Two defects were observed, one in transcription termination and one in 3’ end maturation. Impairment of the Sen1p-Rpb1p interaction resulted in a termination defect. Impairment of the Sen1p-Rnt1p interaction resulted in a processing defect. The results suggest that the Sen1p-Rpb1p and Sen1p-Rnt1p interactions occur independently of each other and serve genetically separable purposes in targeting Sen1p to function in two temporally overlapping steps in gene expression.
Protein-protein interaction networks contribute the underlying basis for phenotypic pleiotropy. In *Saccharomyces cerevisiae*, global studies suggest that each protein interacts on average with five other proteins (Grigoriev 2003), leading to a complex network of interactions involving at least 16,000 individual protein-protein interactions that influence the functions of wild-type proteins and the phenotypes of mutants. The essential *Saccharomyces cerevisiae* SEN1 gene codes for a nuclear-localized nucleic acid helicase (DeMarini et al. 1992) that is embedded in a complex network of protein-protein interactions (Ursic et al. 2004). Furthermore, mutations in SEN1 confer pleiotropic phenotypes, including defects in transcription termination, RNA processing, and DNA repair (Rasmussen and Culbertson 1998; Steinmetz and Brow 1996; Steinmetz and Brow 1998; Steinmetz et al. 2001; Steinmetz et al. 2006; Ursic et al. 2004). The study of SEN1 therefore provides a useful paradigm to examine the impact of protein-protein interactions on mutant phenotypes and function.

Mutations in human SETX (Senataxin), the ortholog of yeast SEN1, cause two clinically distinct neurological diseases, ataxia-ocular apraxia 2 (AOA) and juvenile amyotrophic lateral sclerosis (ALS4) (Chen et al. 2004; Chen et al. 2006; Duquette et al. 2005; Moreira et al. 2004; Suraweera et al. 2007; Suraweera et al. 2009). The yeast and human proteins are strikingly similar in their organization. Some of the human mutations cause changes in the ATP-helicase domain, whereas others cause changes in the N-terminal region where protein-binding domains reside. Some of the clinical differences might be caused by mutations that differentially affect the function of Senataxin by disrupting different protein-protein interactions.
Sen1p interacts with the C-terminal domain of Rpb1p, the largest subunit of RNA polymerase II (RNAP II) (MYER and YOUNG 1998), Rad2p, a ssDNA endonuclease required for DNA repair (HABRAKEN et al. 1993; PRAKASH and PRAKASH 2000), Rnt1p, a dsRNA cleavage enzyme involved in 5' or 3' end processing (CHANFREAU et al. 1997; ELELA et al. 1996; LAMONTAGNE et al. 2000), and SmD3p (FROMONT-RACINE et al. 1997), a subunit of the heteroheptameric Sm complex that assembles snRNAs into ribonucleoprotein particles required for pre-mRNA splicing (KAMBAZ et al. 1999; ROY et al. 1995; ZHANG et al. 2001). Recently, it was shown that Sen1p interacts with Glc7p, a protein phosphatase subunit of the cleavage/polyadenylation factor, and Nab3p, a RNA-binding protein that interacts with other proteins involved in transcription termination of non-coding RNAs (CONRAD et al. 2000; NEDEA et al. 2008).

RNA processing, ribonucleoprotein assembly and transcription-coupled DNA repair occur concomitant with transcription (HANAWALT and SPIVAK 2008; KOMARNITSKY et al. 2000; MANIATIS and REED 2002; NEUGEBAUER 2002), suggesting a complex interplay between protein-protein interactions that potentially orchestrate co-transcriptional pathways. The interactions of Sen1p with proteins involved in transcription, processing and repair might occur independently of each other or they might occur in a dependent sequence of interactions.

To begin assessing the relationships between the different Sen1p protein-protein interactions, we analyzed the effects of sen1 mutations on the expression of SNR7, which codes for U5 snRNA. SNR7 serves as a diagnostic indicator of the relationship between Sen1p protein-protein interactions and Sen1p function because previous studies based on depletion assays suggested a role for SEN1 in U5 RNA 3’ end processing (URSIC et al.
Other reports indicated that Sen1p plays a role in transcription termination of non-coding RNAs (Rasmussen and Culbertson 1998; Steinmetz and Brow 1996; Steinmetz and Brow 1998; Steinmetz et al. 2001; Steinmetz et al. 2006). Furthermore, the Sen1p interacting partners Rpb1p and Rnt1p are required for U5 snRNA transcription and maturation, respectively (Myer and Young 1998).

The U5 snRNA transcript matures through a branched pathway leading to the production of two functional end products, U5L (214 nucleotides) and U5S (180 nucleotides) (Chanfreau et al. 1997; Patterson and Guthrie 1987) (see Fig. 1B). During co-transcriptional maturation, Rnt1p cleaves at two locations in a stem/loop structure leading to accumulation of U5L-3’ RNA (240 nucleotides) and U5-3’a RNA (270 nucleotides). The exosome removes 3’ nucleotides from each of the cleavage products to form mature U5L and U5S RNA, respectively (Allmang et al. 1999). Despite this, a deletion of RNT1 only affects synthesis of U5L, indicating that a Rnt1-independent bypass pathway allows for U5S RNA synthesis in the absence of Rnt1p cleavage. Sen1p and Rnt1p are required for production of U5L RNA but not for U5S RNA (Ursic et al. 2004).

To distinguish whether the interactions with Rpb1p and Rnt1p have a dependent or independent relationship, sen1 mutations were identified that impair one interaction without impairing the other interaction. The phenotypes were assessed to determine their effects on SNR7 expression. Two defects were observed, one in transcription termination and one in 3’ processing. The genetic data supports a model in which the interactions occur independently of each other.
MATERIALS AND METHODS

Strains, genetic methods, and plasmids: Strains carrying sen1-1 were derived from FWY1 (MATa ura3-52 leu2-3, –112 pep4-3 trp1 sen1-1) (URSIC et al. 2004); sen1-2 from DDY86 (MATα ade2-101 his3-200 lys2-801 trp1-Δ1 ura3-52 leu2-Δ1::sen1-2) (DeMarini et al., 1992); sen1-K128E from JFY41 (MATα leu2Δ ura3Δ his3Δ1 trp1Δ sen1-K128E) (this study); sen1-R302W from DUY1513 (MATα leu2Δ ura3Δ his3Δ1 met15Δ sen1-R302W) (this study); rrp6Δ from BY4742 (MATα his3Δ1 leu2Δ lys2Δ ura3Δ rrp6Δ::KanMX4) (Open Biosystems, Inc.); rnt1Δ from JFY5 (MATα ade2-101 his3-200 leu2-Δ1::sen1-2 HIS3:pet56:rnt1) (URSIC et al. 2004); mtr4-1 from YSL402 (MATα ura3-52 lys2-801 pep4::HIS3 prb1-Δ1.6R mtr4-1) (LIANG et al. 1996). Isogenic sets of strains were created by two-step gene replacement (BOEKE et al. 1984). Standard yeast mating and dissection techniques were used to construct double mutants. Growth media were described previously (URSIC et al. 2004). Gene deletions were constructed using the PCR-based gene disruption method (WACH et al. 1994).

All strains were grown at 30°C. Strains carrying sen1-K128E and sen1-R302W grow at normal rates. Strains carrying sen1-2 in single copy are viable but grow at a reduced rate. Strains carrying sen1-1 are temperature sensitive for growth. 30°C is permissive for growth, but the changes in levels of accumulation of U5-related RNAs at 30°C resemble the changes observed at a non-permissive temperature of 37°C.

The plasmids pU5mt and pU5wt contain DNA starting 500 nucleotides upstream of SNR7 and ending 500 nucleotides downstream of the TOS2 open reading frame (ORF). pU5mt contains GAAA in the stem-loop recognized by Rnt1p in place of AGUC in pU5wt (see Fig. 4A). pJF89 contains the same DNA insert as in pU5wt except that the
TOS2 ORF was replaced with the *E. coli lacZ* ORF. Plasmids were introduced into strains by LiAc transformation (GIETZ and WOODS 2002).

**RNA methods:** Methods for RNA isolation and northern blotting were described previously (URSIC *et al.* 2004). RNAs (10 μg) were fractionated on 2% agarose or 6% acrylamide:8M urea (29:1) gels, transferred to GeneScreen Plus membranes (NEN Life Science Products), and cross-linked using a UV Stratalinker 2400 (Stratagene). Probes were labeled using T4 polynucleotide kinase (Pharmacia) in the presence of [γ-32P] ATP (Amersham). Riboprobes used in Fig. 6B were prepared using an *in vitro* transcription kit (Promega) in the presence of [α-32P]CTP and [α-32P]UTP (3,000 Ci/mmol) (Perkin-Elmer). Band intensities on the northern blots were quantitated using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences, Inc.). Oligonucleotide probes (Thermo Scientific) used for northern blotting to map the U5-3' 3' end were as follows: U5A (CGCCCTCCTTACTCATTG), U5D (TAATCCATCTTCGGTAATAAG), U5E (GCATTGCTGTCTGAGTTG), and TOS2 (TTACATGTACATTCTCG).

The rate of TOS2 mRNA decay was determined by measuring the temporal decline in mRNA by northern blotting following transcription inhibition using 10 μg/ml thiolutin (Pfizer, Inc., part number CP-4092) (GUAN *et al.* 2006). SCR1 RNA was monitored as a loading control. SigmaPlot was used to evaluate decay data using an exponential decay formula: y=a*exp(-b*x). Estimations of b, designated as B, and corresponding standard errors, were used to calculate standard error (t_{1/2}=log(2)/b). t_{1/2} ± s.e.(t_{1/2}) was calculated as [log(2)/(B+s.e.(B)), log(2)/(B-s.e.(B))].

The relative levels of U5-related RNAs detected by northern blotting were determined as follows. U5S RNA was used as an internal standard in each gel lane since
U5S levels are not affected by mutations in <i>SEN1</i> or <i>RNT1</i> (Chanfreau et al. 1997; Chanfreau et al. 1998; Ursic et al. 2004). U5-related RNAs were named as described previously (Chanfreau et al. 1997). The relative levels of U5L-3’ RNA, U5-3’b RNA, and the sum of the RNAs ranging in size from 271-960 nucleotides (designated RNA271-960) were measured by calculating the band intensities of the RNAs divided by the band intensity corresponding to U5S RNA in the same lane. Fold changes were calculated by dividing the ratios in mutant strains by the ratio in the corresponding wild-type strain.

**Protein and immunological methods:** β-galactosidase activity was measured as described previously (Stahl et al. 1995). Immunoprecipitation (IP) and western blotting were described previously (Ursic et al. 2004). Primary antibodies that recognize epitope-tagged proteins were as follows: mouse monoclonal anti-HA antibody (clone HA-7, Sigma) recognizes Rpb1p-HA; mouse monoclonal anti-cMyc antibody (clone 9E10, Sigma) recognizes cMyc-Sen1p, cMyc-sen1-K128Ep, and cMyc-sen1-R302W; and rabbit anti-TAP antibody (Thermo Scientific) recognizes Rnt1p-TAP, Sen1p-TAP, sen1-K128Ep-TAP, and sen1-R302Wp-TAP. Membranes for western blotting were probed with anti-mouse or anti-rabbit peroxidase-conjugated antibodies (Thermo Fisher Scientific). Protein bands were visualized by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fisher Scientific) and quantified using a Typhoon 9200 Variable Mode Imager (Amersham Biosciences, Inc.). For IP experiments, 10 μg/ml RNase A (Sigma) was added to pre-IP lysates. The relative amount of protein that co-purified with an immunoprecipitated protein was determined by comparing the ratio of the band intensity of the immunoprecipitated protein to the band intensity of the co-purifying protein detected by western blotting of IP lysates. The effects of <i>sen1</i> mutations
on the extent of co-purification were determined by calculating the ratio of band intensities of the immunoprecipitated and co-immunoprecipitated proteins in the mutant divided by the ratio of band intensities in wild-type.

RESULTS

**Sen1p affects two steps in the expression of SNR7:** Two alleles of *SEN1* that affect the expression of genes for non-coding RNAs were described previously (Fig. 1A) (DEMARINI *et al.* 1992). The *sen1-1* mutation (G1747D) is located in a conserved motif in the ATP-helicase region of *SEN1*. Based on its location, *sen1-1* most likely impairs helicase activity. The *sen1-2* mutation is a partial deletion producing a stable, truncated protein lacking the first 975 amino acids of Sen1p. The deletion removes binding domains required for interaction with the RNase III cleavage enzyme Rnt1p and the largest RNA polymerase II subunit, Rpb1p (URSIC *et al.* 2004). Depletion of the *sen1-2* protein causes a time-dependent accumulation of U5L-3’ RNA, the product of Rnt1p cleavage, at the expense of mature U5L RNA (URSIC *et al.* 2004). This establishes that elevated accumulation of U5L-3’ RNA is a diagnostic indicator of a Sen1-mediated processing defect.

Using a U5-specific probe, we examined the steady-state levels of U5-related RNAs by northern blotting of RNA from strains carrying the *sen1-1, sen1-2,* and/or *rnt1Δ* mutations (Fig. 1B). When the relative levels of U5L-3’ RNA were analyzed (Fig. 1C), the *sen1-1* mutation caused a $2.9 \pm 0.2$ fold increase in accumulation, whereas the *sen1-2* mutation caused a $2.5 \pm 0.2$ fold increase. The strains carrying *rnt1Δ* caused a significant reduction in the level of U5L-3’ RNA since this RNA is the product of Rnt1p cleavage.
A small amount of U5L-3’ RNA can still be detected in rnt1Δ strains, presumably due to inefficient degradation of a longer precursor by the exosome which may stall at the stem-loop recognized by Rnt1p. These results are consistent with data showing that sen1-2 causes a time-dependent increase in the accumulation of U5L-3’ at the expense of U5L RNA but with no effect of U5S accumulation (Ursic et al. 2004).

The longest detectable U5-related RNA, U5-3’b, is approximately 960 nucleotides in length (Fig. 1B, see below). This RNA accumulated to a 2.8 ± 0.8 fold higher level in a sen1-1 strain (Fig. 1D). However, excess accumulation of U5-3’b RNA was not observed in a sen1-2 strain. When double mutants were analyzed, U5-3’b RNA accumulated in excess in both sen1-1 rnt1Δ and sen1-2 rnt1Δ strains (8.9 ± 1.3 fold and 9.3 ± 1.1 fold, respectively). These results suggest that Rnt1p cleavage limits the accumulation of U5-3’b RNA and partially or completely masks the effects of sen1-1 and sen1-2 on accumulation. Overall, the results show that sen1-1 and sen1-2 affect the accumulation of two RNAs, U5L-3’, and U5-3’b. Furthermore, both the N-terminal and helicase regions of Sen1p are required for efficient expression of SNR7.

The distance between the 3’ end of U5L-3’ RNA and the beginning of the downstream TOS2 ORF is 466 nucleotides. The estimated length of U5-3’b RNA suggested it might extend into the downstream TOS2 gene and could be a read-through transcript. To test this possibility, the 3’ end of U5-3’b RNA was approximated by northern blotting of RNA from a sen1-1 rnt1Δ strain using probes complementary to sequences spanning the region from the SNR7 gene to the end of the TOS2 ORF (Fig. 1E). Probe U5A, which is complementary to sequences near the 3’ end of mature U5L RNA, hybridized to U5-3’b RNA, whereas the TOS2 probe detected TOS2 mRNA but
not U5-3’b RNA. When the two probes were mixed, both RNAs were detected, verifying that RNAs of different sizes were detected with U5-3’b RNA being the smaller of the two. The approximate 3’ end of U5-3’b RNA was located by observing that probe U5D (3’ end at nucleotide 950) hybridized to U5-3’b and TOS2 RNA, whereas probe U5E (5’ end at nucleotide 970) hybridized only to TOS2 mRNA.

The 3’ end of U5-3’b RNA is therefore likely to be located at a position between 940 and 980 nucleotides from the beginning of transcription of SNR7. Since the 3’ end is located between 260-300 nucleotides downstream of the beginning of the TOS2 ORF, U5-3’b RNA is most likely a read-through transcript that accumulates in sen1-1 rnt1Δ strains. Overall, these results suggest that sen1-1 and sen1-2 affect accumulation of two RNAs: an intermediate in RNA processing and a read-through transcript presumed to result from impaired termination of transcription.

**Impact of Sen1p-Rnt1p and Sen1p-Rpb1p interactions on Sen1p function:** Mutations in SEN1 that reduce the efficacy of specific protein-protein interactions might provide insights on the role of the interactions in Sen1p function. To pursue the genetic approach, the boundaries of the binding domains for interaction with Rnt1p and Rpb1p were approximated using polypeptide fragments of Sen1p in two-hybrid studies (Fig. 1A). Direct physical interactions of Sen1p with Rnt1p and Rpb1p were previously demonstrated using two-hybrid and co-IP analyses (URSIC et al. 2004). Phylogenetic comparisons of sequences within the domains were used to identify conserved sites potentially important for each interaction. By screening a collection of candidate mutations for their effects in two-hybrid tests, it was found that sen1-K128E and sen1-R302W impaired the Sen1p-Rnt1p and Sen1p-Rpb1p interactions, respectively.
The alleles were examined by co-IP of proteins in native complexes (Fig. 2). To examine *sen1-K128E*, protein extracts from strains expressing epitope-tagged cMyc-Sen1p or cMyc-sen1-K128Ep with Rnt1p-TAP were analyzed (Fig. 2A, left panel). Proteins were bound to anti-TAP antibodies, eluted from beads, and analyzed by western blotting using anti-TAP and anti-cMyc antibodies. The results indicate that wild-type Sen1p co-purified with wild-type Rnt1p. However, the *K128E* mutation abolished the ability of the proteins to co-purify. To test whether the loss of co-purification was specific for the interaction with Rnt1p, protein extracts from strains expressing Sen1-TAP or sen1-K128E-TAP and Rpb1p-HA were analyzed (Fig. 2A, right panel). Western blotting of the IP lysates shows that the *K128E* mutation had no discernable effect on the extent of co-purification of Sen1p and Rpb1p.

Similar experiments were performed to analyze the effects of *sen1-R302W*. Protein extracts from strains expressing Sen1p-TAP or sen1-R302W-TAP with Rpb1p-HA were examined by co-IP of proteins bound to anti-HA antibodies (Fig. 2B, left panel). Western blotting of the IP lysates indicated that wild-type Sen1p co-purified with Rpb1p, but sen1-R302Wp failed to co-purify. However, when protein extracts from strains expressing cMyc-Sen1p or cMyc-sen1-R302Wp with Rnt1p-TAP were analyzed (Fig. 2B, right panel), the R302W mutation had no discernable effect on co-purification.

Although the results described above do not necessarily indicate that protein-protein binding is completely abolished, it was reasoned that the interactions might be sufficiently impaired that the mutations would have unique phenotypic effects on Sen1p function. To assess the functional consequences, strains expressing chromosomally integrated *sen1-K128E* and *sen1-R302W* alleles were analyzed by northern blotting with
a U5-specific probe (Fig. 3A). The \textit{K128E} mutation caused a 1.9 ± 0.4 fold increase in the relative accumulation of U5L-3’ RNA, the product of Rnt1p cleavage (Fig. 3B). The increase was abolished in a \textit{sen1-K128E rnt1Δ} double mutant. In a \textit{sen1-K128E RNT1} strain, there was no significant effect on the relative accumulation of U5-3’b RNA, the read-through product (Fig. 3C). In the \textit{sen1-K128E rnt1Δ} double mutant, a 2.1 ± 0.2 fold increase was observed. This was similar to that observed for a \textit{SEN1 rnt1Δ} strain, indicating that the increase is attributable to \textit{rnt1Δ} and not to \textit{sen1-K128E}.

The \textit{R302W} mutation had no effect on the relative accumulation of the U5L-3’ RNA processing intermediate in \textit{RNT1} or \textit{rnt1Δ} strains (Fig. 3B). When the accumulation of the U5-3’b read-through RNA was examined, \textit{sen1-R302W} caused a 2.2 ± 0.4 fold increase in a \textit{RNT1} strain (Fig. 3C). A similar increase of 2.2 ± 0.6 fold was observed in a \textit{SEN1 rnt1Δ} strain (Fig. 3C). A 4.7 ± 1.3 fold increase was observed in a \textit{sen1-R302W rnt1Δ} double mutant (Fig. 3C). The synthetic phenotype of the double mutant suggests that the R302W amino acid substitution specifically affects accumulation of the read-through RNA. Overall, the results suggest that \textit{sen1-R302W} causes a defect in transcription termination without affecting 3’ end processing.

**Functional relationship between Sen1p and the exosome:** Since the product rather than the substrate of Rnt1p cleavage accumulates in strains carrying \textit{sen1-2} and \textit{sen1-K128E}, the Sen1p-Rnt1p interaction is not functionally related to cleavage itself. We reasoned that the interaction could leave Sen1p, a 5’ to 3’ helicase, bound to the 3’ end of the RNA cleavage product. From that location, Sen1p could unwind the stem/loop (Fig. 4A) and separate the cleaved RNAs. This might allow more efficient access for the exosome, which processes the 3’ end of the Rnt1p cleavage product in conjunction with
the Trf4p/Air2p/Mtr4p polyadenylation complex (TRAMP) (LACAVA et al. 2005; LIANG et al. 1996; MILLIGAN et al. 2005). To test potential relationships of Sen1p with the exosome and TRAMP, we analyzed the phenotypes of sen1-K128E combined with rrp6Δ, a viable deletion of RRP6, which codes for an exosomal subunit required in U5 snRNA processing (ALLMANG et al. 1999), and mtr4-1, an allele of MTR4, which codes for a 3’ to 5’ helicase subunit of TRAMP (LIANG et al. 1996).

Single mutant strains carrying sen1-K128E or rrp6Δ/rrp6Δ compared with a sen1-K128E rrp6Δ double mutant by assaying effects on the accumulation of two sets of RNAs: the processing intermediate U5L-3’ RNA and the sum of RNAs in the 271-960 nucleotide size range, which includes the U5-3’b read-through RNA. For the latter, we settled on the broader range of larger RNAs so as to include intermediates in exosomal degradation of the read-through transcript.

A synthetic phenotype was observed for U5L-3’ RNA accumulation in the K128E rrp6Δ double mutant (Fig. 4B). In the single mutants, the sen1-K128E mutation caused a 2.0 ± 0.3 fold increase in the relative accumulation of U5L-3’ RNA, whereas rrp6Δ caused a 2.3 ± 0.4 fold increase. However, a 4.6 ± 1.6 fold was observed in the double mutant, which is significantly higher than in either single mutant. When RNAs in the 271-960 nucleotide size range were examined, the sen1-K128E and rrp6Δ single mutants had no significant effect on relative accumulation. In the double mutant, no synthetic increase was observed. If anything, accumulation was marginally reduced, indicating that the synthetic interaction is restricted to effects on the U5L-3’ processing intermediate.

Similar experiments were performed to compare sen1-K128E and mtr4-1 single mutants with a sen1-K128E mtr4-1 double mutant (Fig. 4C). Consistent with previous
results, U5L-3’ RNA increased 1.9 ± 0.4 fold in the sen1-K128E single mutant. No significant increase was observed in the mtr4-1 single mutant. In the double mutant, U5L-3’ RNA accumulation increased 2.1 ± 0.3 fold, which was statistically the same as that observed in the sen1-K128E single mutant. No significant effects on the accumulation of RNAs in the 271-960 nucleotide size range were observed in either the single or the double mutants.

Overall the results reveal a synthetic interaction between sen1-K128E and rrp6Δ that is related to RNA processing and unrelated to transcription termination, suggesting that the Sen1p-Rnt1p protein-protein interaction may serve a role in promoting TRAMP-independent exosomal processing of the Rnt1p cleavage product. If this model is correct, then sen1-K128E should have no effect on the expression of U5 RNA when RNT1 is deleted or when U5 RNA is altered by nucleotide substitutions that prevent Rnt1p recognition of the stem/loop in which Rnt1p cleaves. To test this, an allele of SNR7 was created in which four nucleotides, AGUC, at the top of the Rnt1p recognition loop, were changed to GAAA (U5mt, Fig. 4A). It was shown previously that this alteration prevents Rnt1p cleavage (Chanfreau et al. 2000). The wild-type and mutant versions of SNR7 were expressed from plasmids in a strain carrying the wild-type SNR7 gene. Due to overexpression, the mutant snr7 RNAs outnumber the wild-type RNAs in strains producing U5mt from the plasmid.

The effects of sen1-K128E on the accumulation of the read-through transcript and on RNAs in the 271-960 nucleotide size range were examined in strains carrying rnt1Δ or expressing cleavage-defective U5 RNA (U5mt) (Fig. 5). The results indicate that sen1-K128E has no significant effect on either set of RNAs in rnt1Δ or U5mt-expressing
strains. The results suggest that the effects of sen1-K128E on SNR7 expression are limited to exosomal processing of the immediate product of Rnt1p cleavage.

**SNR7 transcriptional read-through increases expression of downstream TOS2:** Mutations in SEN1 typically decrease the expression of downstream genes due to read-through. For example, sen1-mediated read-through of the snoRNA gene SNR13 reduces expression of the downstream gene, TRS31 (Steinmetz and Brow 1996; Steinmetz and Brow 1998; Steinmetz et al. 2001; Steinmetz et al. 2006). Since the SNR7 read-through transcripts end in the ORF of downstream TOS2 (Fig. 1E), we examined the effect of read-through on TOS2 expression.

In a SEN1 RNT1 strain, TOS2 mRNA was below the level of detection even when four times the standard 10μg of RNA were loaded on the gel (Fig. 6A). However, in a sen1-1 rnt1Δ strain, TOS2 mRNA was readily detected, suggesting that the accumulation of U5-3’b RNA that occurs in sen1-1 rnt1Δ strains might cause increased expression of TOS2. To test this hypothesis, TOS2 expression levels were compared by northern blotting with a high specific activity riboprobe in a wild-type strain, a strain carrying a multi-copy CUP1-TOS2 plasmid, and a strain carrying sen1-1 rnt1Δ (Materials and Methods, Fig. 6B). TOS2 was detected at a low level in the wild-type strain. TOS2 mRNA expressed from the CUP1-TOS2 plasmid was detected at levels comparable to the level observed in a sen1-1 rnt1Δ strain lacking the plasmid.

Over-expression of TOS2 from a multi-copy plasmid disrupts cytokinesis, leading to aberrant cell morphology including multiple elongated buds (Gandhi et al. 2006). When the cell morphology of a wild-type strain was compared to that of a strain expressing CUP1-TOS2 from a multi-copy plasmid and a strain carrying sen1-1 rnt1Δ,
the elongated, multi-bud morphology was observed for the latter two strains compared to wild-type (Fig. 6C). Collectively, these results suggest that read-through causes increased expression of TOS2.

TOS2 mRNA levels might be elevated in sen1-1 rnt1Δ strains as the result of an increased rate of TOS2 transcription or a decreased rate of mRNA decay. To distinguish between these models, the half-life of TOS2 mRNA was determined. Transcription was inhibited with 10 μg/ml thiolutin. Northern blotting with a TOS2-specific end-labeled probe was used to monitor the disappearance of the pre-existing mRNA at time intervals following inhibition (Fig. 6D). The half-life of TOS2 mRNA in a wild-type strain is 51 ± 9 minutes, which is statistically indistinguishable from the 59 ± 4 minutes (p= 0.13) observed in the sen1-1 rnt1Δ strain. This indicates that read-through has no effect on the stability of TOS2 mRNA.

One way that TOS2 expression might be elevated is through positive auto-regulation mediated by Tos2p itself. To test this model, pJF89, which expresses DNA including the SNR7-TOS2 region but with lacZ replacing the TOS2 ORF (Materials and Methods), was transformed into strains carrying SEN1 and sen1-1. β-galactosidase assays revealed that the sen1-1 strain had a 4.3 ± 2.3 fold increase in activity compared to wild-type. Northern blotting revealed a 7.4 ± 1.8 fold increase in accumulation of the lacZ transcript in a sen1-1 strain compared to wild-type. These results indicate that increased expression is not dependent on the TOS2 ORF, and must therefore depend on upstream sequences.
DISCUSSION

The goal of this study was to assess whether a genetic approach could be used to distinguish between alternative models for the functional relationships between protein-protein interactions. According to one model, interactions could occur in a dependent sequence of protein-protein binding reactions (Fig. 7A). One way this could be achieved is if two binding domains overlap such that occupation of the domain for one protein precludes occupation by a second protein. Alternatively, protein-protein interactions might occur independently of each other and there might be no obligate order of binding (Fig. 7B). For a protein that interacts with multiple partners, coexistence of dependent and independent sets of binding reactions is possible.

To determine whether the Sen1p-Rnt1p and Sen1p-Rpb1p interactions depend on each other, we asked how they affect the function of Sen1p in the expression of SNR7 coding for U5 snRNA. Two previously described alleles, sen1-1, containing a point mutation in a conserved helicase motif, and sen1-2, which is missing DNA coding for the first 975 amino acids (URSIC et al. 2004), each cause two defects in SNR7 expression, one in transcription termination and the other in 3’ end processing. Two new alleles described in this study, sen1-K128E and sen1-R302W, specifically impair the interactions with Rnt1p and Rpb1p, respectively. The mutants served as primary tools to ask how the termination and processing defects might be related. As summarized in Fig. 7C, sen1-K128E caused elevated accumulation of the U5L-3’ RNA without detectable accumulation of the read-through RNA, whereas sen1-R302W caused accumulation of the read-through RNA without excess accumulation of U5L-3’ RNA. The evidence
supports a model for independent binding and independent function in transcription and processing since the phenotypic effects of mutations are genetically separable (Fig. 7B).

It is likely that the defect in SNR7 expression caused by \textit{sen1-K128E} is entirely related to impaired Sen1p-Rnt1p binding because \textit{sen1-K128E} had no phenotypic effects on RNA accumulation when the \textit{RNT1} gene was deleted or when \textit{SNR7} RNA was altered to a form that is immune to Rnt1p cleavage. Based on this and the synthetic effect of a \textit{sen1-K128E rrp6Δ} exosome-defective double mutant on U5L-3’ RNA accumulation, we suggest that the primary purpose of the Sen1p-Rnt1p interaction is to assist the exosome in the processive shortening of U5L-3’ RNA to mature U5L by the exosome. The binding of Sen1p to Rnt1p might place Sen1p in physical proximity to the 3’ end of the exosomal substrate. The interaction could play a similar role in the processing of other non-coding RNAs that depend on Sen1p, Rnt1p, and the exosome.

Sen1p is a 5’ to 3’ helicase (Kim \textit{et al.} 1999), whereas Mtr4p, a component of TRAMP that assists in exosomal degradation, is a 3’ to 5’ helicase (Lacaiva \textit{et al.} 2005; Liang \textit{et al.} 1996; Milligan \textit{et al.} 2005). No synthetic interaction was observed in \textit{sen1-K128E mtr4-1} double mutants. We propose that Mtr4p helicase activity is sufficient to aid the exosome in degrading most RNA structures. However, following Rnt1p cleavage, the RNA products may remain based-paired in the stem/loop region of the Rnt1p binding/cleavage domain. Because of this, the 5’ to 3’ helicase activity of Sen1p may promote unwinding of the stem to force separation of the cleaved RNAs, allowing for efficient 3’ access to the exosome. Sen1p could assist the exosome in other regions of the RNA by providing a 5’ to 3’ unwinding activity, but it is not likely that this would depend on the Sen1p-Rnt1p protein-protein interaction.
The Sen1p-Rnt1p interaction may play a role in other RNA biosynthetic pathways besides U5 snRNA. We observed that both sen1 and rnt1 mutations cause changes in the accumulation of RNAs detected with probes complementary to the snoRNAs SNR40 and SNR47. Furthermore, sen1 rnt1 double mutants exhibited novel patterns of accumulation for SNR40- and SNR47-related RNAs (unpublished observations), suggesting a potential role for the Sen1p-Rnt1p interaction in these pathways. However, other snoRNAs such as SNR13 and SNR10 were only affected by sen1 mutations, and the effects are most likely restricted to defects in transcription termination (RASMUSSEN and CULBERTSON 1998; STEINMETZ and BROW 1996; STEINMETZ and BROW 1998; STEINMETZ et al. 2001; STEINMETZ et al. 2006; URSIC et al. 2004).

It seemed possible that all functional roles for Sen1p during the transcription cycle might depend on the binding of Sen1p to the RNAP II subunit Rpb1p. Our genetic evidence argues against this model. Strains carrying sen1-R302W produce a protein that fails to bind to Rpb1p, but there is no observable defect in the processing of U5L-3’ RNA. Thus, despite the fact that transcription and processing are temporally coupled, the genetic data indicate that coupling is not enforced by a dependent sequence of protein-protein interactions. This does not necessarily mean that dependent sequences do not exist. Although it has not yet been tested, the interactions of Sen1p with Rpb1p and Rad2p (URSIC et al. 2004) could form a dependent sequence in which transcription-coupled DNA repair might require binding to Rpb1p as a prerequisite to Sen1p-Rad2p binding. The protein-protein interaction network in which Sen1p is embedded could be comprised of sets of proteins that form both dependent and independent interactional and functional relationships. The broader possibilities remain to be tested.
The binding of Sen1p to Rpb1p is required for termination of transcription of the SNR7 gene, since sen1-R302W causes accumulation of a read-through transcript that extends into the downstream TOS2 gene. Similar effects of sen1 mutants on transcriptional termination have been reported for many other non-coding RNA genes (RASMUSSEN and CULBERTSON 1998; STEINMETZ and BROW 1996; STEINMETZ and BROW 1998; STEINMETZ et al. 2001). What distinguishes the read-through effect for SNR7 is that the extended transcript causes increased rather than decreased expression of TOS2.

Typically, the expectation is that promoter occlusion would decrease expression of the downstream gene. We ruled out the possibility that increased expression is mediated at the level of mRNA stability or by Tos2p-mediated auto-regulation, because there was no effect on the TOS2 mRNA half-life and because increased expression was observed when the TOS2 ORF was replaced with the lacZ ORF. Since increased expression must involve sequences upstream of the TOS2 ORF, an elevated rate of TOS2 transcription is likely. One way this could occur is if the read-through transcript displaces a transcriptional repressor. Such displacement could override the potential effects of promoter occlusion leading to a net increase in transcription.

ACKNOWLEDGMENTS

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LITERATURE CITED


NEDEA, E., D. NALBANT, D. XIA, N. T. THEOHARIS, B. SUTER et al., 2008 The Glc7 phosphatase subunit of the cleavage and polyadenylation factor is essential for transcription termination on snoRNA genes. Mol Cell 29: 577-587.


FIGURE LEGENDS

**Figure 1.** Sen1p mutations and interactions. (A) Schematic representation of Sen1p showing the locations of the interaction domains for Rbp1p and Rnt1p (NEDEA et al. 2008; URSIC et al. 2004). Sen1 alleles that affect interactions (*sen1-K128E, sen1-R302W, sen1-2*) or function (*sen1-1*) are shown. Numbers refer to amino acids. (B) Alternative pathways for U5 snRNA synthesis. RNAs are named as previously described (CHANFREAU et al. 1997; ÉLELA et al. 1996; LAMONTAGNE et al. 2000). The length of the primary transcript is unknown. The figure shows a representative northern blot in which
RNAs were fractionated by PAGE and detected using a complementary probe spanning nucleotides 1-320 of the SNR7 gene, which codes for U5 RNA (Materials and Methods). (C) Effects of sen1-1 and sen1-2 on the relative accumulation of U5L-3′ RNA, an intermediate in 3′ end processing. (D) The effects of sen1-1 and sen1-2 on the relative accumulation of U5-3′b RNA, a read-through transcript. Fold changes are indicated with standard error in parentheses. p values are indicated below the fold changes. Methods of quantitation are described in the Materials and Methods. (E) To delimit the location of the 3′ end of U5-3′b RNA, PAGE-fractionated RNAs from a sen1-1 rnt1Δ strain were analyzed by northern blotting with probes complementary to segments as shown in the figure.

Figure 2. Effects of amino acid substitutions on the interactions of Sen1p with Rnt1p and Rpb1p. (A) sen1-K128E impairs the interaction of Sen1p with Rnt1p (left) but does not affect the interaction with Rpb1p (right). Proteins were immunoprecipitated from cell lysates in the presence of 10 μg/ml RNase A using anti-TAP antibodies. The pre- and post-IP lysates were assayed by western blotting using anti-cMyc and anti-TAP antibodies (Materials and Methods). (B) sen1-R302W impairs the interaction of Sen1p with Rpb1p (left) but does not affect the interaction with Rnt1p (right). Experiments were performed as described in (A) except that the pre- and post-IP lysates were assayed by western blotting using anti-HA and anti-TAP antibodies.

Figure 3. Effects of sen1-K128E and sen1-R302W on U5 snRNA synthesis. (A) The figure shows a representative northern blot in which RNAs from RNT1 and rnt1Δ strains carrying alleles of SEN1 were analyzed as described in the Fig. 1 legend. The effects of
*sen1-K128E* and *sen1-R302W* on the relative accumulation of the processing intermediate U5L-3’ RNA (B) and the read-through RNA U5-3’b (C), were quantitated as described in the Materials and Methods. Fold changes are indicated with standard error in parentheses. *p* values are indicated below the fold changes.

**Figure 4.** Interactions between Sen1p with the exosomal nuclease Rrp6p and the TRAMP helicase Mtr4p. (A) Structure of the 3’ extended region of U5 RNA. Nucleotide substitution of AGUC in wild-type *SNR7* with GAAA at the top of the Rnt1p recognition stem/loop impairs the ability of Rnt1p to cleave the RNA (Chanfreau et al. 1997; Elela et al. 1996; Lamontagne et al. 2000). (B) The representative northern blots and bar charts show the effects of *sen1-K128E* and *rrp6Δ* alone and in combination on the relative accumulation of U5L-3’ RNA and the sum of RNAs in the 271-960 nucleotides size range (RNA271-960). (C) The northern blots and bar charts show the effects of *sen1-K128E* and *mtr4-1* alone and in combination on RNA accumulation as described in (B). Relative accumulation was quantitated as described in the Materials and Methods. Fold changes are indicated with standard error in parentheses. *p* values are indicated below the fold changes. The brackets and numbers below the bar charts indicate the *p* values for single versus double mutants.

**Figure 5.** Effects of *sen1-K128E* on *SNR7* expression in Rnt1p cleavage-defective strains. (A) RNA from strains carrying *SEN1* or *sen1-K128E* and either *rnt1Δ* or a plasmid expressing U5mt were analyzed by northern blotting using the probe described in Fig. 1B. The figure shows a representative northern blot (see Fig. 1 legend and Materials and Methods). (B) The bar chart compares the effects on the accumulation of U5-3’b
RNA and the sum of RNAs in the 271-960 size range (RNA271-960). Relative accumulation was quantitated as described in the Materials and Methods. Fold changes are indicated with standard error in parentheses. p values are indicated below the fold changes.

**Figure 6.** Effect of *SNR7* transcriptional read-through on expression of a downstream gene. (A) Increased expression of *TOS2* mRNA in a *sen1-1 rnt1Δ* strain. RNA levels were examined by northern blotting using an end-labeled probe that anneals to the *TOS2* 3’ end. (B) *TOS2* RNA levels compared with over-expression of *CUP1-TOS2* from a multi-copy plasmid (designated Tos2p OE). RNA levels were examined by northern blotting using a riboprobe (Materials and Methods). (C) Cellular morphology of a strain that over-expresses *CUP1-TOS2* from a multi-copy plasmid and a strain that carries *sen1-1 rnt1Δ*. (D) Half-life of *TOS2* mRNA in *SEN1* and *sen1-1 rnt1Δ* strains.

**Figure 7.** Protein-protein interactions and their relationships to function. (A,B) The figure summarizes models for the relationship between the binding of Rpb1p and Rnt1p to Sen1p and the corresponding functional roles for Sen1p in transcription and processing. Sen1p, which binds to the C-terminal domain of Rpb1p (*URSIC et al. 2004*), is depicted as a helicase domain comprised of stacked α-helices and an N-terminal segment containing multiple protein binding domains. (C) Relationship between protein-protein interactions and function based on the phenotypes of *sen1* mutants.
**A**

**sen1-K128E (LYS → GLU)**

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**loss of interaction with Rnt1p**

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**B**

**sen1-R302W (ARG → TRP)**

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**loss of interaction with Rpb1p**

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