4778–4785 *Nucleic Acids Research, 1997, Vol. 25, No. 23*

The yeast *SEN1* gene is required for the processing of diverse RNA classes

Doris Ursic*, Karen L. Himmel, Kyle A. Gurley, Frances Webb and Michael R. Culbertson

Laboratories of Molecular Biology and Genetics, 1525 Linden Drive, University of Wisconsin, Madison, WI 53706, USA

Received August 7, 1997; Revised and Accepted October 20, 1997

ABSTRACT

A single base change in the helicase superfamily 1 domain of the yeast *Saccharomyces cerevisiae SEN1* gene results in a heat-sensitive mutation that alters the cellular abundance of many RNA species. We compared the relative amounts of RNAs between cells that are wild-type and mutant after temperature-shift. In the mutant several RNAs were found to either decrease or increase in abundance. The affected RNAs include tRNAs, rRNAs and small nuclear and nucleolar RNAs. Many of the affected RNAs have been positively identified and include end-matured precursor tRNAs and the small nuclear and nucleolar RNAs U5 and snR40 and snR45. Several small nucleolar RNAs co-immunoprecipitate with Sen1 but differentially associate with the wild-type and mutant protein. Its inactivation also impairs precursor rRNA maturation, resulting in increased accumulation of 35S and 6S precursor rRNAs and reduced levels of 20S, 23S and 27S rRNA processing intermediates. Thus, Sen1 is required for the biosynthesis of various functionally distinct classes of nuclear RNAs. We propose that Sen1 is an RNA helicase acting on a wide range of RNA classes. Its effects on the targeted RNAs in turn enable ribonuclease activity.

INTRODUCTION

The yeast *Saccharomyces cerevisiae SEN1* gene was originally isolated using a screen for heat-sensitive mutations affecting tRNA splicing endonuclease activity. It was then discovered that the *sen1-1* mutation causes reduced *in vitro* endonuclease activity and *in vivo* accumulation of unspliced end-matured precursor tRNAs (1). We report here that the Sen1 protein (Sen1) has a role in RNA maturation much more global than previously thought.

SEN1 encodes an essential protein of 252.5 kDa that localizes to the nucleus in a granular distribution (2,3). It is a member of the superfamily I helicases, containing all seven conserved motifs typical of this group (4). A number of genes from this family of helicases have been identified from *Escherichia coli*, *S.cerevisiae*, human and mouse and their protein products have been purified and shown to contain both NTPase and helicase activities. Sen1 is homologous to *S.cerevisiae* Upf1 and Dna2, which are also members of this class of helicases (2,4–6). Upf1 is involved in nonsense-mediated mRNA decay (6,7). Biochemical analysis of Upf1 demonstrates that it contains RNA-dependent ATPase, 5′→3′ RNA helicase and RNA binding activities (8). Dna2, which is required for DNA replication, has DNA-dependent ATPase and 3′→5′ DNA helicase activities (5). The *sen1-1* mutation changes amino acid residue 1747G to 1747D in the helicase domain (2). The glycine residue is conserved in Upf1, Dna2 and a number of related putative helicases, and is therefore expected to disrupt ATPase and helicase activities.

Several observations indicate that Sen1 is not restricted in function to tRNA biosynthesis as originally conjectured. The *sen1-1* mutation confers a tight shutdown of growth after about two to three cell doublings at the restrictive temperature, whereas it takes at least six to eight cell generations to exhaust the stable tRNA pool (9). Furthermore, mutant cells mislocalize two nucleolar proteins, the fibrillarin homolog Nop1 and Ssb1 to the nucleoplasm (3,10,11). The nucleolus is the site of ribosomal biosynthesis, which requires the presence of nucleolar proteins including Ssb1 and Nop1, as well as small nucleolar RNAs (snoRNAs) (12,13). It was suggested that nucleolar localization is the result of binding interactions between various domains of nucleolar proteins and other nucleolar structures, possibly RNAs (14). Thus, the effects of *sen1-1* on the localization of some nucleolar proteins are consistent with a role for Sen1 in nucleolar RNA-mediated processes.

Other alleles of *sen1* confer additional phenotypes related to nuclear functions. *SEN1* was isolated as a *trans*-acting factor, *NRD2*, which modulates gene expression in yeast by altering the accumulation of a chimeric transcript. It has been proposed that Sen1 is playing a role in mRNA metabolism at a step subsequent to transcription initiation (15). Overproduction of a truncated Sen1 protein results in the accumulation of multinucleate cells, indicating a defect in nuclear migration (3). Likewise, another allele of *SEN1*, *cik3-1*, affects nuclear fusion and chromosome maintenance (16). Our research provides an explanation for the seemingly divergent phenotypes associated with *SEN1*.

Here we report that the *sen1-1* mutation either increases or decreases the relative abundance of many RNAs. The affected RNAs comprise representatives of several RNA classes, including snoRNAs, small nuclear RNAs (snRNAs), rRNAs and tRNAs and several previously unidentified RNAs. Moreover, Sen1-specific antibodies co-immunoprecipitate several snoRNAs, indicating that Sen1 is associated with them. Sen1 is one of only a few proteins known to interact with snoRNAs and is so far the only known one required for the biosynthesis of both small nuclear and nucleolar RNAs. Our results are consistent with Sen1 being an RNA helicase acting on many metabolically stable RNAs.

*To whom correspondence should be addressed. Tel: +1 608 262 4381; Fax: +1 608 262 4570; Email: dursic@facstaff.wisc.edu

MATERIALS AND METHODS

Strains and constructions

Two isogenic strains containing the *SEN1* (strain 1971: MATα *leu2-3, -112 ura3-52 pep4-3*) and *sen1-1* (strain FWY1: MATα *leu2-3, -112 ura3-52 pep4-3 sen1-1*) alleles were used throughout. FWY1 was constructed by replacing the wild-type *SEN1* gene with a gene carrying the *sen1-1* mutation using a two-step gene replacement (17) as follows. The 4219 bp *Hin*dIII fragment, contained in plasmid YEp352sen1-1 (2) was cloned into vector YIp352 which resulted in plasmid pTR35. The clone contains the *sen1-1* ORF from position 3307 extending 447 bp 3′ to the translation termination site. The 3′ *Hin*dIII site is vector derived. pTR35 was linearized by endonuclease digestion with *Bsa*BI and used to transform the *SEN1* strain 1971 to a Ura^+ phenotype. Ura^- derivatives of transformants were selected in the presence of 5-fluoro-orotic acid (5-FOA) (18), and one transformant, called FWY1, was selected. Strain FWY1 has the same temperature-sensitive growth phenotype as strain tsx24.13c (1). We verified that correct gene replacement had occurred by performing Southern blot analysis. The restriction endonuclease patterns of FWY1 and 1971 are nearly identical. The exception is the serendipitously created *Cla*I site at position 5240 as the result of a G→A transition in the *sen1-1* mutant resulting in the amino acid residue change 1747G→1747D. We also confirmed that the mutation in FWY1 was within the *SEN1* gene by complementation and plasmid rescue as described previously (19,20).

Oligonucleotides used

Oli1(cggttttaattgtccta), Oli2(atgaaaactccacagtg), Oli3(aaaggccagcaatttcaagtta), 25S(gcaccgaaggtaccag), 18S(agctctagaattaccacgg), 5.8S(tttcgctgcgttcttcatc), 5S(aagttccaaaaaatatggcaagc), snR10(aatttgttctccagtccaagc), snR30(gccgttgtccgaagcgcc), snR31(gcactgaagcaacgccc), snR40(acttaatccttcataggacac), snR41(cgaaaaagggttgtcgacatgtag), snR45(cctcagatcgctccgagaaga), 5′-snR45(aaacttggaaggtcatt), 3′-snR45(aagaatcagaattttat), snR190(ggctcagatctgcatgtgttgtataacactggc), U3(gtggctcttttgaagagtcaaag), U2(tgtattgtaacaaattaaaagg), U5(aagttccaaaaaatatggcaagc).

The oligonucleotides were used to identify RNAs affected by Sen1. Sequences are orientated 5′→3′. They were purchased from Operon. Hybridization and washing conditions were described in (21). Oli1 and Oli2 are specific to ITS1 (5′ and 3′ to the A_2 cleavage site) and are complementary to nucleotides 189–205 and 219–235, respectively. Oli3 is specific to ITS2 (5′ of the C_2 cleavage site, complementary to nucleotides 46–68). The other oligomers are unique to the indicated mature RNAs.

RNA preparation and northern hybridization

Cells in exponential phase of growth were shifted from the permissive temperature of 24°C to the restrictive temperature of 37°C. Samples were taken at time points ranging from 0 to 7.5 h after the temperature shift. RNA was isolated in presence of a low ionic strength buffer by acid/hot phenol extraction as described in (7). RNA (10 µg) was fractionated on denaturing 1.2% agarose gel (high molecular weight RNA) or 9% polyacrylamide (29:1), 8 M urea gel (low molecular weight RNA) and transferred to GeneScreen Plus (DuPont). Low molecular weight RNA was transferred to membranes using a BIO RAD Trans Blot Cell.

Deoxyoligonucleotide probes used in northern blotting were radiolabeled using T4 polynucleotide kinase (Pharmacia) and [γ-^{32}P]ATP (Amersham) using standard protocols (22) and DNA fragments were radiolabeled with [α-^{32}P]CTP using an oligo-labeling kit (Pharmacia). The absolute amounts of radiolabeled probes that hybridized to specific bands were quantitated on a PhosphorImager (Molecular Dynamics). All values were normalized to the snRNA U2 (high molecular weight RNA) or 5.8S rRNA (low molecular weight RNA), which served as loading controls. Data presented are an average of five or more experiments. Typical standard deviations are ~10% of the averages.

Total small RNA was visualized by 3′-end-labeling for ~18 h at 4°C with [5′-^{32}P]pCp using T4 RNA ligase (Promega) following manufacturer's instructions in presence of RNAsin (Promega). The labeled RNA was purified using Sephadex G-25 (Sigma) spin columns, followed by acid phenol/chloroform extractions and ethanol precipitations in presence of (1 mg/ml) glycogen carrier (Boehringer Mannheim). RNA was fractionated in an 8% Long Ranger Gel (AT Biochem) and subjected to autoradiography and PhosphorImager analysis. T4 polynucleotide kinase labeled θX174 DNA, and digested with *Hin*fI, served as a size marker.

Antisera

Affinity purified rabbit polyclonal antibodies directed against a synthetic peptide (Peptide 1), designated Anti-1, were prepared as described in (3). Peptide 1 (Sen1 amino acids 1539–1551) maps to a non-conserved region within the helicase domain of Sen1. The yeast genome sequencing project uncovered a sequencing error, an additional A residue, at the 5′-end at position 388 in the *SEN1* DNA sequence (GenBank accession number U20939). Thus, *SEN1* codes for 2231 amino acids.

Immunoprecipitation and RNA isolation

Lysates from cells that were in exponential phase of growth were prepared using glass beads extraction in Lysis buffer (50 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 0.1% NP-40 and 500 mM NaCl). The following protease and RNAse inhibitors were added: aprotinin, pepstatin, chymostatin, leupeptin, antipain (final concentration of 1 µg/ml), 1 mM phenylmethyl-sulfonyl fluoride and 10 mM Vanadyl Ribonucleoside Complex (GIBCO BRL).

For each set of immunoprecipitation experiments equal amounts of lysate prepared from the equivalent of 5 OD_{600} U of cells (~10^8 cells) were analyzed. The lysate was allowed to bind to antibodies or pre-immune serum that had previously been immobilized on Protein A agarose (GIBRO BRL). For immuno-competition, first, competitor Peptide 1 (50 µg) was pre-bound for 30 min to Anti-1 antibodies (10 µg) followed by the incubation conditions indicated above. Under these conditions, the antibodies are present in excess and Sen1 is limiting. Protein A agarose had been equilibrated with TN buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.5). The incubations proceeded for 2 h at 8–11°C, followed by three washes with 1 ml TTNE (150 mM NaCl, 50 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 0.5% Tween 20).

RNA products that co-precipitated with the final pellet were extracted either directly or subsequent to deproteination using 0.2 mg/ml proteinase K (Boehringer Mannheim) in PK buffer (0.1 M Tris–HCl pH 7.4, 12.5 mM EDTA, 150 mM NaCl, 1% SDS). The RNA products were isolated using phenol/chloroform extraction, followed by ethanol precipitation in presence of (1 mg/ml) glycogen carrier. In parallel experiments total RNA

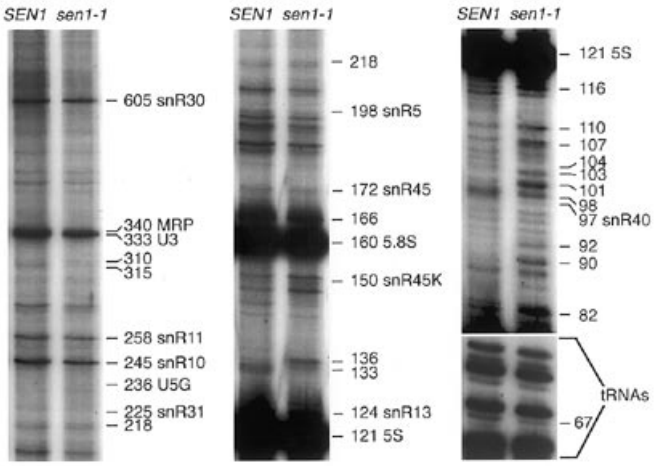

Figure 1. The *sen1-1* mutation affects the levels of small RNAs. Total RNA from *SEN1* and *sen1-1*-containing cells after a 5 h shift from 24 to 37°C. The RNA was extracted using a low ionic strength buffer, and labeled with [5′-^{32}P]pCp prior to electrophoresis in an 8% gel. Equal amounts of ^{32}P-labeled material were applied to the gel. The bands that change in intensity between the wild-type and mutant are marked with their nucleotide sizes and names, where known. Some characteristic RNAs that do not change are also marked for reference. Refer to Table 1 for a detailed listing of the marked RNA molecules that are affected by the *sen1-1* mutation. Autoradiographs of gel subsections were obtained with exposure times appropriate to the respective signal levels. Sections corresponding to RNAs with sizes down to 121 nt were exposed for 2 days, from 121 to 80 nt for 1 day, and the tRNAs for 1 h.

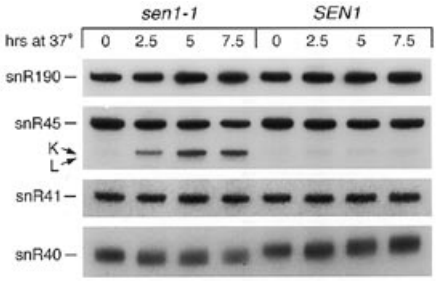

Figure 2. Sen1 is required for the normal accumulation of several snoRNAs. (**A**) Northern blots of small RNAs from *SEN1* and *sen1-1* cells that were shifted to the restrictive temperature of 37°C for the indicated times. The RNAs were fractionated in a 9% polyacrylamide gel. Probes specific to the snR40, snR41, snR45 and snR190 snoRNAs were applied to the blots. The arrow points to snR45K and snR45L that are 5′-end truncations of the mature snR45 snoRNA. They exhibit a temperature-dependent accumulation in *sen1-1* cells. The intensity of snR45H, also detectable by the snR45-specific probe, was too weak to reproduce.

was extracted directly using the above procedure but in absence of protease inhibitors and antibody affinity purification. The RNA was fractionated and transferred to membranes as described for low molecular weight RNA.

RESULTS

The cellular abundance of many RNAs is altered by Sen1 inactivation

The presence of a helicase domain and effects on tRNA intron removal point to a role for Sen1 in RNA biogenesis. We therefore tested whether Sen1 acts globally on all RNAs or targets selectively a subset by determining the levels of small metabolically stable RNAs in wild-type *SEN1* and mutant *sen1-1* cells.

Two isogenic strains carrying *SEN1* or *sen1-1* were constructed by gene replacement. Thus, we can attribute all observed RNA changes to a single mutation at the *SEN1* locus. After temperature shift from 24 to 37°C the number of viable cells in the mutant increases for ~5 h followed by a gradual drop in cell viability (3).

We compared the steady-state levels of small RNAs from wild-type and mutant cells after a 5 h temperature shift. Total RNA was extracted using a standard low ionic strength buffer and the RNAs were 3′-end-labeled prior to fractionation by high resolution gel electrophoresis. Although the labeling efficiencies of different RNAs are likely to vary, the labeling efficiency for any one RNA is expected to be identical between strains, making comparisons of relative abundance possible (23).

The relative amounts of 25 RNAs were detectable as changing between wild-type and mutant (Table 1 and Fig. 1). The *sen1-1* affected RNAs were initially identified with their estimated size and band pattern (24–26). They fall into at least three classes, including tRNA, snoRNA and snRNA. Among the 12 RNAs that showed reduced accumulation, six were tentatively identified as snoRNAs. Six out of 13 RNAs that either over-accumulated or were not detectable in the wild-type could not be identified. However, two of these RNAs were tentatively identified as RNA species homologous to the snRNA U5 and the snoRNA snR45 (see below), and five as end-matured intron-containing tRNAs previously shown to accumulate in the *sen1-1* mutant (1). Because the experimental conditions used do not resolve RNA species of similar size and because inefficiently labeled or non-abundant RNAs may not be detectable, it is likely that additional RNAs accumulate abnormally in *sen1-1* mutant cells.

We verified the identity and levels of two snoRNAs that were changed in the mutant and, for comparison, two RNAs that appeared to be unaltered, using northern blotting (Fig. 2). RNAs from either wild-type or mutant cells were analyzed following the temperature-shift for various periods of time ranging from 0 to 7.5 h. Probes specific to the snoRNAs snR40, snR45, snR41 and snR190 were used (24). Quantitative analysis of the RNA levels confirmed both the RNA identification and the observed changes in the amounts of RNA between wild-type and mutant as summarized in Table 1. Whereas the snR40 and snR45 under-accumulated ~2-fold in the mutant relative to the wild-type, snR41 and snR190 RNA altered little in abundance, providing evidence that Sen1 does not globally affect all RNAs.

Furthermore, after shift to 37°C, snR40 RNA extracted from the mutant consistently had a slightly higher rate of mobility, indicating that *SEN1* affects both the size and levels of this transcript. Also, three additional RNAs with estimated sizes of 800–850, 150 and 146 nt (designated snR45H, -K and -L, respectively) hybridized to a probe unique to the mature snR45 (172 nt) in *sen1-1* cells within 2.5 h at 37°C. Because snR45H was barely detectable (data not shown), reliable quantitative measurements were not possible. Very low levels of the snR45K and snR45L species were present in the wild-type, which increased 2-fold over the course of the analysis. Like in the wild-type, the mutant accumulated low levels of snR45K and snR45L at 24°C. However, by 7.5 h at 37°C it accumulated up to 9.5-fold. Because snR45K and snR45L migrated as a doublet, this value represents the two species. For each time point the sum of the mature snR45 and snR45K and snR45L levels were similar for both wild-type and mutant. Thus, while the mature snR45 species decreased, truncated RNAs increased by corresponding amounts.

Table 1. RNA species whose relative intensities changed between wild-type *SEN1* and mutant *sen1-1* cells

Size	Name	Method of identification	RNA change	Salt extraction
315	–	–	+	–
310	–	–	–	+
258	snR11	size	–	–
236	U5G	northern	+	–
225	snR31	size	–	–
218	–	–	+	+
198	snR5	size	– –	–
172	snR45	northern	–	+
166	–	–	– –	+
150	snR45K	northern	+	+
136	pre-tRNA IleUAG	size	+ +	–
133	–	–	– –	+
124	snR13	size	–	+
116	pre-tRNA LeuCAA	size	+ +	+
110	–	–	+ +	–
107	–	–	+ +	–
104	–	–	– –	+
103	pre-tRNA SerCGA	size	+ +	+
101	pre-tRNA SerGCU	size	+ +	–
98	–	–	–	+
97	snR40	northern	–	–
92	pre-tRNA TyrGUA	size	+ +	–
90	–	–	+ +	–
82	–	–	+ +	–
67	–	–	–	+

The table lists the RNA species shown in Figure 1 whose relative labeling intensities changed between wild-type and mutant after shift to the restrictive temperature.
Size: inferred size (in nucleotides) based on molecular weight standards and gel electrophoretic fractionation pattern of yeast snoRNAs (24). Size errors of a few percent are possible, especially on the larger unknown RNA species.
Name: Name identified snRNA, snoRNA or pre-tRNA species (24–26). Many fragments could not be identified with existing named RNAs, either because these RNAs are as yet unidentified or because they are a consequence of the mutation and hence *sen1-1*-specific.
Method of identification: method used for band identification, tentatively by size and band patterns using gel electrophoresis, followed by northern blotting using RNA-unique probes (Figs 2 and 5B).
RNA change: relative band-intensity changes from wild-type to mutant, using a low ionic strength extraction buffer (hot phenol extraction). – –, strong decrease; –, decrease; + +, strong increase; +, increase.
Salt extraction: effects of 500 mM NaCl in the RNA lysate buffer on RNA extraction and 3′-end-labeling as indicated by the band intensity (Fig. 3). + means that band differences are stronger compared to hot phenol extraction; – indicates no difference.

Next, we ascertained if snR45K and snR45L were 5′- or 3′-end deletions, using hybridization probes that are specific to the terminal 17 nt of the mature snR45. Our results showed that whereas the 5′-end probe only recognized the mature snR45, the 3′-end probe detected both the mature and the truncated snR45 snoRNAs (data not shown). Therefore, the 150 and 146 nt RNAs are 5′-end-truncations of the mature snR45 snoRNA. Furthermore, the presence of these transcripts in the wild-type indicates that they are normal processing products that exhibit a temperature-dependent accumulation in the *sen1-1* mutant.

Many snoRNAs, precursor tRNAs (pre-tRNA) and other RNAs that are associated with stable complexes are not easily extractable in low NaCl concentrations (27,28). We therefore analyzed the accumulation of small RNAs using a buffer system that included 500 mM NaCl. When extracts from wild-type and mutant cells were compared, many of the differences in the accumulation observed using low salt extraction were accentuated when high salt extraction was used (Fig. 3; Table 1). The differential extraction probably is the result of the disruption of RNA–protein complexes under conditions of high salt buffers and indicates that Sen1 affects these structures.

Co-precipitation of snoRNAs using Sen1-specific antibodies

Sen1 contains a sequence motif known to affect RNA binding (4,29). To test whether Sen1 physically associates with these molecules, we analyzed the ability of Sen1-specific antibodies to immunoprecipitate RNAs from total yeast extracts of wild-type *S.cerevisiae* cells. The Sen1-co-precipitated RNAs were examined by northern blotting using probes specific for individual RNAs. The large number of yeast snoRNAs (upward of 100) makes a systematic analysis of all species a costly undertaking. Three snoRNAs, U3, snR30 and snR10 were chosen for a more detailed analysis for the

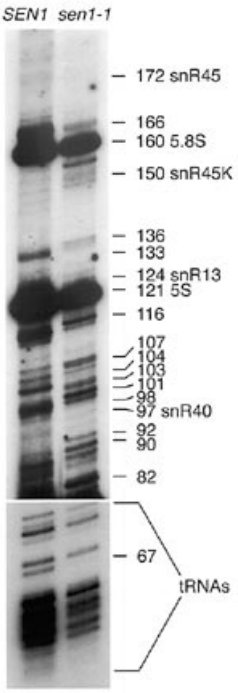

Figure 3. Extracted amounts of RNAs associated with *SEN1* and *sen1-1* are sensitive to salt levels. RNA was extracted from cells in presence of lysis buffer that contains 500 mM NaCl, instead of the standard low ionic strength buffer as used for the data in Figure 1. The RNAs were labeled with [5′-^{32}P]pCp prior to electrophoresis in an 8% gel. Only the gel subsection that contained the most pronounced salt effects on RNA intensities between the wild-type and the mutant is shown. RNA band legends are as in Figure 1. The tRNAs were exposed ~25-fold less than RNAs ranging in size from 130 to 80 nt.

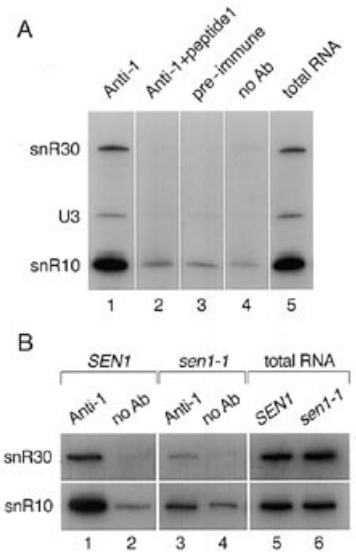

Figure 4. Northern analysis of snoRNAs that immunoprecipitate with Sen1-specific antibodies. (**A**) Total cell extracts from equal amounts of wild-type *SEN1*-containing cells, based on OD600, were analyzed. Cell lysates were immunoprecipitated by the Anti-1 antibodies (lanes 1 and 2). The same reactions were carried out in presence of competitor-Peptide 1 (lane 2), with pre-immune serum (lane 3) and in absence of antibodies (lane 4). Material in lanes 2, 3 and 4 represents non-specific binding of RNA to agarose beads. RNA from total cellular RNA (1% of total extract) is shown in lane 5. The blot was hybridized simultaneously with all three snoRNA-specific probes. The snR10-specific probe recognizes two RNAs of similar size. (**B**) Comparisons of snR30 and snR10 snoRNA accumulation and co-immunoprecipitation using Anti-1 antibodies between wild-type *SEN1* and mutant *sen1-1* cells. The experimental conditions were the same as described for (A). The material in lanes 2 and 4 represents non-specific binding of RNA to agarose beads in absence of antibodies. An amount equivalent to 1% of the *SEN1* and *sen1-1* cell extracts used for precipitation reactions is shown in the lanes marked 'total RNA' (lanes 5 and 6). The same blots were sequentially hybridized with the snoRNA-specific probes.

following reasons. First, these snoRNAs are essential or important for rRNA biogenesis (30). Second, these snoRNAs fall into two different structural classes. U3 contains C/D box elements and snR10 and snR30 contain H/ACA box elements (24,31,32). Third, deletion of snR10 and depletion of Sen1 result in similar pre-rRNA processing defects (see below).

All three RNAs co-precipitated with Sen1 (Fig. 4A, lane 1). This association is indicative of specific interactions. Only a small fraction of the RNAs did bind non-specifically to agarose beads in immunoprecipitations using Anti-1 antibodies and in the presence of competitor-Peptide 1 (lane 2), with pre-immune serum (lane 3) or in absence of antibodies (lane 4). An amount of RNA equivalent to ~1% of the cell extracts used for immunoprecipitation is presented in lane 5. PhosphorImager data allowed us to conclude that from ~0.5 to 3% of total U3, snR10 and snR30 snoRNAs co-fractionated with Sen1.

We also analyzed the ability of the mutant protein product of the *sen1-1* allele to co-immunoprecipitate snR10 and snR30 snoRNAs using lysates from both wild-type and mutant cells that were shifted for 5 h to 37°C. The amounts of snR10 and snR30 that co-fractionated with the mutant protein in presence of Anti-1 sera were decreased ~10-fold compared to the wild-type protein (Fig. 4, lanes 1 and 3). Their levels were slightly higher (1.5–3-fold) than the observed non-specific RNA binding to agarose beads (Fig. 4B, lanes 2 and 4). The decreased efficiency of co-purification of these snoRNAs using the mutant lysate can not be reconciled by reduced accumulation of the snoRNAs. As shown in Figure 4B (lanes 5 and 6), snR10 was only marginally reduced in the mutant compared to wild-type, whereas similar levels of snR30 were present in both strains. Thus, the low amounts of snoRNA co-precipitation with the mutant protein may be the result of either impaired RNA–protein interaction or reduced cellular levels of the *sen1-1* protein product. However, because Sen1 is essential for cell viability and the *sen1-1* containing cells are still viable, it is likely that the mutation significantly alters the RNA binding properties.

Precursor-rRNA accumulation is altered in the *sen1-1* mutant

Because inactivation of Sen1 function affects the levels of several snoRNAs known to affect rRNA biogenesis, we determined whether rRNA processing is altered in the *sen1-1* mutant. The rDNA repeat encodes the divergently transcribed 5S rRNA and 35S precursor rRNA. The 35S pre-rRNA contains sequences for the mature 18S, 5.8S and 25S rRNAs (5′→3′), two external transcribed spacers, 5′ ETS and 3′ ETS, and two internal transcribed spacers, ITS1 and ITS2. The synthesis of mature rRNA molecules requires snoRNPs and ribonucleases to remove the transcribed spacers (12,13,33).

The accumulation of precursor and mature rRNAs was compared between cells that are wild-type and mutant for *SEN1*, by analyzing the RNAs extracted from cells that were shifted to 37°C for various periods of time, by northern blotting. One

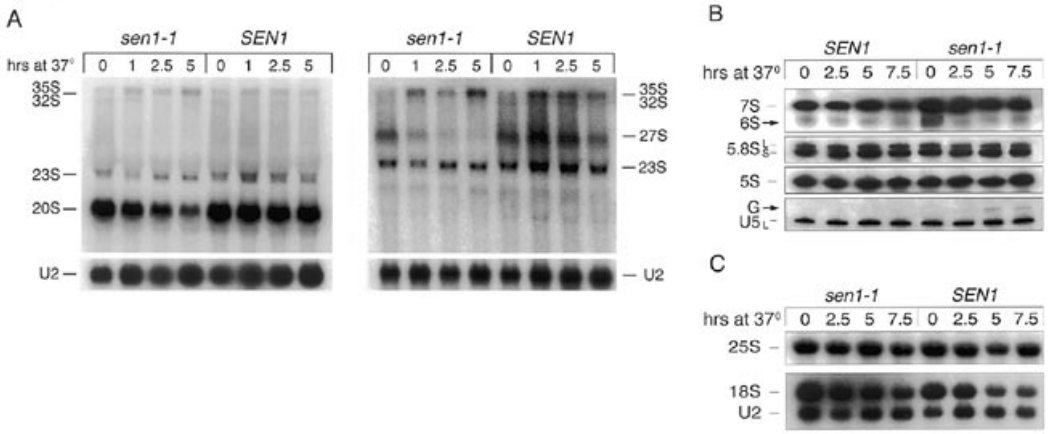

Figure 5. Effects of the *sen1-1* mutation on the levels of precursor and mature rRNAs and the snRNAs U5 and U2. Northern blot analysis of total RNA from *SEN1* and *sen1-1* cells that were shifted from 24 to 37°C for the indicated times. Hybridization probes specific for 5S, 18S, 5.8S, 25S, ITS1 (Oli1 and Oli2), ITS2 (Oli3), U2 and U5 were used. The migration positions of the RNA molecules are indicated. (**A**) Northern blot of pre-rRNA hybridized with Oli1 (left) and Oli2 (right). These probes are specific for the 5′ and 3′ regions of ITS1. Note the increase in the band intensities of the 35S–32S pre-rRNA and the decrease of the 20S, 23S and 27S pre-rRNAs in the *sen1-1* mutant after temperature-shift. (**B**) Northern blot of low molecular weight RNAs. Oli3 is specific to ITS2 and detects 7S and 6S pre-rRNAs. The arrows point to the abnormal RNA processing products (6S pre-rRNA and U5G snRNA) in the *sen1-1* mutant. (**C**) Northern blot of mature 18S and 25S rRNAs. Also shown is the U2 snRNA.

hybridization probe unique to each mature rRNA and three probes specific to pre-rRNA processing intermediates were chosen. Two of the latter (Oli1 and Oli2) detect pre-rRNAs 5′ and 3′ of a cleavage site in ITS1, defined as A_2. Processing at this site separates the 32S pre-rRNA into the 20S and a 27S intermediate that become the mature rRNAs for the small and large ribosomal subunits, respectively. The third probe (Oli3), detects processing intermediates 5′ of the cleavage site located in ITS2, named C_2, which cleaves a 27S fragment to form 5.8S and 25S rRNA.

We found that the *sen1-1* mutation affected the accumulation of 35S, 27S, 23S, 20S and 6S pre-rRNAs, but not the amounts of mature rRNAs (Fig. 5A–C). Compared to wild-type cells, ~2-fold higher levels of the 35S pre-rRNA accumulated in mutant at the non-permissive temperature. Moreover, the 20S, 23S pre-rRNAs and a 27S pre-rRNA species under-accumulated from 2- to 4-fold within 1 h at 37°C (Fig. 5A).

We also observed 2-fold higher levels of 6S pre-rRNA in *sen1-1* cells grown at the permissive temperature (Fig. 5B, marked by an arrow). The elevated accumulation of the 6S pre-rRNA was only observed at 24 and not at 15, 30 (data not shown) or 37°C. 7S pre-rRNA is processed to mature 5.8S rRNA via a 6S intermediate by Rrp4, a 3′→5′ exoribonuclease (34). Our data show that the *sen1-1* mutation impedes maturation and therefore presumably the processive ribonuclease activity of Rrp4 at a temperature that is permissive for growth.

Accumulation of a new RNA with sequence identity to the small nuclear RNA U5

We investigated the steady-state levels of two snRNAs, U2 and U5, by northern blotting. Figure 5A and C shows that the sizes and levels of the U2 and the two U5 RNAs of 180 nt (S) and of 214 nt (L) (25) were comparable in *SEN1* and *sen1-1* cells (U5S is not shown). However, within 2.5 h after temperature-shift, a probe unique to U5 hybridized to an additional RNA in the *sen1-1* cells. This RNA, named U5G, is larger than U5L by ~22 nt (Fig. 5B, marked by an arrow). This RNA corresponds to a species detected by 3′-end-labeling of total RNA that was noticeable in the mutant cells after temperature-shift (Fig. 1 and Table 1).

DISCUSSION

The results of our investigation revealed that Sen1 is implicated in the biosynthesis of a diverse set of RNA classes, including snRNA, snoRNA, rRNA and tRNA. Therefore, Sen1 appears to target some not yet determined aspect of their structure and not their function.

Sen1 is required for the production of several snoRNAs

Only recently have we become aware that eukaryotic cells contain a complex population of snoRNAs numbering in excess of 100 (24,31,32,35–39). Most of the snoRNAs have been classified into two functionally and structurally distinct groups. One group acts as guide RNAs in site-specific ribose methylation and synthesis of pseudouridine in rRNA. snoRNAs required for ribose methylation are in the C/D box family, whereas snoRNAs necessary for pseudouridine formation are in the H/ACA box family. The other group of snoRNAs is necessary for the correct structure and cleavage of the pre-rRNA. It is likely that other not yet determined functions will be also attributed to the snoRNAs. Notwithstanding their ubiquity, so far only a few proteins besides Sen1 are known to interact with them (10,11,24,40–44).

The relative amounts of 25 small RNAs were detected as being altered in the *sen1-1* mutant when compared to the wild-type. About half of them are uncharacterized. Six of the changed RNAs were tentatively identified as snoRNAs. Among those, snR40 and snR45 were further analyzed using RNA-specific probes and verified to under-accumulate in the mutant. Furthermore, compared to wild-type, in the mutant the snR40 transcript was slightly shorter and several snR45-like RNAs (extended as well as 5′-end-truncated) accumulated. *SEN1* was also shown to be required for the accumulation of three other snoRNAs, snR11, snR13 and snR31 (45, our unpublished data). The Sen1-affected snoRNAs, or the ones that are not affected, do not share any known structural or functional similarities (24,28,32,37,46).

Sen1 associated *in vitro* with at least three snoRNAs (U3, snR10 and snR30), in agreement with the presence of a putative RNA binding domain in Sen1 and its nuclear localization (3,29). The Sen1 interactions with the snoRNAs appear to be strong, since they did not change even in presence of up to 500 mM NaCl. A fraction of each of the snoRNAs co-purified with Sen1. This is consistent with it being a rare cellular protein (3), and suggests a transient interaction with the RNAs.

To date, only one other protein, namely Nop1, is known also to affect cellular levels of snoRNAs (11). Depletion of Nop1 changes the accumulation of two snoRNAs, snR190 and U14, which are synthesized from the same polycistronic transcript (38). They are not the snoRNAs that we have verified to be affected by the *sen1-1* mutation. Hence, Nop1 and Sen1 appear to affect different aspects of snoRNA processing. Nevertheless, the *sen1-1* mutation causes Nop1 to mislocalize (3). Abnormal amounts of Nop1 are detected in the nucleoplasm, rather than its normal target, the nucleolus. Since nucleolar RNAs play a role in localizing proteins to this organelle (14), defects in snoRNA levels and pre-rRNA processing (see below) in *sen1-1* cells can explain the abnormal distribution of the nucleolar proteins.

Defects in precursor rRNA accumulation

The *sen1-1* mutation caused abnormal pre-rRNA maturation, because the mutant cells contain an excess of 35S primary rRNA transcript and reduced levels of rRNA processing intermediates. These rRNA processing intermediates lead to the synthesis of the mature 18S rRNA (23S and 20S) and the 25S and 5.8S rRNAs (a 27S species). The observed phenotypes can be reconciled by the effects of the mutant on snoRNA processing as follows.

The accumulation of 35S pre-rRNA shows that the initial processing at the A_0 and A_1 cleavage sites is slowed in the *sen1-1* mutant. In addition, the under-accumulation of the 20S, 23S and the 27S pre-RNA intermediates indicates abnormal nucleolytic processing within ITS1 (12). This phenotype can not be fully reconciled with the current model of the pre-rRNA processing pathway. Cleavage in ITS1 at site A_3 results in reduced steady-state levels of 20S and the 27S pre-rRNA species, which are generated by cleavage at the A_2 site in ITS1. However, cleavage at the A_3 site also leads to increased levels of 23S pre-rRNA, which is incompatible with the observed under-accumulation of the 23S pre-rRNA in the *sen1-1* mutant. We speculate that this RNA became unstable, possibly resulting from the depletion of one or more of the snoRNAs that are affected by the *sen1-1* mutation. Although snR40 and snR5, snR11 and snR31 were shown to serve as guides for ribose methylation and pseudouridine synthesis, respectively, in rRNA, the functions of these modified nucleotides is unknown (24,31,32,35,46). A possible hypothesis is that the hydrophobic properties of the methyl groups and the ability of pseudouridines to form versatile hydrogen bonds may mediate rRNA folding and RNA–protein or RNA–RNA interactions (31,50).

We propose that Sen1 is mediating rRNA processing through snoRNAs. This is corroborated by the fact that Sen1 physically associates with snR10, snR30 and U3, RNAs required for pre-rRNA cleavages at the A_0, A_1 and A_2 sites. Furthermore, the abnormal accumulation of several snoRNAs in the *sen1-1* mutant, which are likely contributing to the normal pre-rRNA processing pathway, provides additional support for a direct role for Sen1 in the manufacture of snoRNAs.

Roles for Sen1 in snRNA, mRNA and tRNA processing

Sen1 is also involved in the proper maturation of a small nuclear RNA, as shown by the *in vivo* accumulation of U5G in the mutant, a 3′-end extended form of the snRNA U5L (this report and our unpublished data). Together with four other snRNAs, U5 constitutes a key component of the spliceosome required for mRNA intron removal (25). To date, besides the *SEN1* gene, two other genes are known to affect U5 snRNA biogenesis. Mutations in the *S.pombe SNM1* and *S.cerevisiae NPL3* (also named *NOP3*, *MTS1*, *NAB1*, *MTR13*) genes result in multiple RNA processing defects, including the under-accumulation of the snRNA U5 (48,49). However, Sen1 is the first known *trans*-acting factor in 3′-end formation of the U5 snRNA.

This effect on snRNA might implicate Sen1 in mRNA splicing. Furthermore, another *sen1* allele increased the expression of a reporter gene containing a fortuitously generated sequence, when inserted into an intron (15). It is not known whether expression of the chimeric gene is intron dependent. However, the U5G RNA amounted to only ~3–5% of the mature U5 snRNA level. This suggests that Sen1 does not directly affect mRNA processing. In support of this, the *sen1-1* mutant does not inhibit splicing of the *ACT1* and *CYH2* pre-mRNAs, showing that Sen1 is not required for pre-mRNA intron removal (our unpublished data).

Consistent with previous findings (1), five end-matured intron-containing pre-tRNAs accumulate in *sen1-1* cells. Sen1 is not likely to affect tRNA intron removal directly because Sen1 is not a component of the catalytic tRNA splicing endonuclease (51) and the two proteins do not co-localize. The endonuclease localizes near the nuclear membrane whereas Sen1 distributes throughout the nucleus (3). We speculate that Sen1 affects the tRNA intron removal through some not yet identified small RNA molecules.

The intron-containing tRNAs that accumulate in *sen1-1* cells are known to be transient intermediates, not normally detectable in wild-type cells. Moreover, most of the snoRNAs and several snRNAs are synthesized as larger transcripts that are post-transcriptionally processed (40,48,52–58). Thus, it is likely that some of the unidentified RNAs that are detectable in the mutant are pre-snoRNAs and pre-snRNAs attributable to faulty processing.

Evidence that Sen1 may associate with nucleases

Together with Sen1, Upf1 and Dna2 are also members of the helicase superfamily 1. They exhibit ATPase and helicase activities (5,8). Mutations in either of the highly conserved ATPase or helicase motifs of Upf1 result in loss of both of these activities, and affect Upf1–RNA complex formation (29). The *sen1-1* mutation is thus likely to disrupt these functions. Consistent with the phenotypes of the *sen1-1* mutation, depletion of the RNA helicases RlhB and Suv3 result in accumulation of RNA processing intermediates, thus linking the helicase activity directly with RNA processing (59,60). Several recent reports have shown that helicases associate with nucleases. The RlhB and Suv3 helicases are stably associated with an endo- and/or exonuclease.

The profile of Sen1 phenotypes is similar to profiles observed for the *S.cerevisiae* exoribonucleases Xrn1 and Rat1. Xrn1 (also named Kem1, Dst2, Sep1, Rar5, Ski1 and Stpß) and Rat1 (also named Hke1, Xrn2, Tap1) share strong sequence homology. They have been implicated in various seemingly unrelated processes, including nuclear fusion, meiotic arrest, plasmid replication, chromosome stability, microtubule function and exoribonuclease activity important for mRNA degradation as well as pre-rRNA processing

(61–63). Both Xrn1 and *snR10* genetically interact with Rok1, a putative RNA helicase, which is necessary for pre-rRNA processing (64,65). Another putative RNA helicase, Ski2, also interacts with Xrn1 and is part of a system to protect cells from double stranded RNA viruses (66). The *sen1-1* mutant cells also have reduced *in vitro* tRNA endonuclease activity (1). Furthermore, the processing of 7S pre-rRNA to 5.8S rRNA is hindered, a step carried out by the exonuclease Rrp4. Thus, the observed alterations in the levels of many RNA species and the detection of RNA fragments not previously observed are consistent with a model in which Sen1 functions as a helicase enabling RNA cleavage.

As we can assign a helicase activity to Sen1, and helicase and ribonuclease activities have been extensively linked, the most likely defect introduced by the *sen1-1* mutation is widespread inaccurate RNA processing. This model explains the association of the defects of the mutant with many cellular activities connected with faulty RNA processing. The precise molecular actions carried out by Sen1 remain unknown.

ACKNOWLEDGEMENTS

This research was supported by the Medical School and the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and by the US Public Health Service grant RO1 GM-40310. K.A.G. is a Hilldale Fellow. This is paper no. 3478 of the University of Wisconsin, Laboratory of Genetics. We thank L.Olds for preparing the illustrations. We also thank D.A.Brow for discussions on RNA and A.G.Balakin, D.A.Brow and T.P.Rasmussen for supplying strains, deoxyoligonucleotides and snoRNA sequence information. We are particularly grateful to M.J.Fournier, A.G.Balakin and J.Ni for providing information prior to publication, the advice we received in identifying snoRNA molecules and for fruitful discussions.

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