INVOLVEMENT OF THE SPLICEOSOMAL U4 snRNA IN HETEROCHROMATIC GENE SILENCING AT FISSION YEAST CENTROMERES

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Running title: U4 snRNA is involved in centromeric silencing

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prp13-1 is one of the mutants isolated in a screen for defective pre-mRNA splicing at a nonpermissive temperature in fission yeast Schizosaccharomyces pombe. We cloned the prp13+ gene and found that it encodes U4 small nuclear RNA (snRNA) involved in the assembly of the spliceosome. The prp13-1 mutant produced elongated cells, a phenotype similar to cell division cycle mutants, and displays a high incidence of lagging chromosomes on anaphase spindles. The mutant is hypersensitive to the microtuble-destabilizing drug thiabendazole, supporting that prp13-1 has a defect in chromosomal segregation. We found that the prp13-1 mutation resulted in expression of the ura4+ gene inserted in the pericentromeric heterochromatin region and reduced recruitment of the heterochromatin protein Swi6p to that region, indicating defects in the formation of pericentromeric heterochromatin, which is essential for the segregation of chromosomes, in prp13-1. The formation of centromeric heterochromatin is induced by the RNA interference (RNAi) system in S. pombe. In prp13-1, the processing of centromeric noncoding RNAs to siRNAs, which direct the heterochromatin formation, was impaired and unprocessed noncoding RNAs were accumulated. These results suggest that U4 snRNA is required for the RNAi-directed heterochromatic gene silencing at the centromeres. In relation to the linkage between the spliceosomal U4 snRNA and the RNAi-directed formation of heterochromatin, we identified an mRNA-type intron in the centromeric noncoding RNAs. We propose a model in which the assembly of the spliceosome or a ‘sub-spliceosome complex’ on the intron-containing centromeric noncoding RNAs facilitates the RNAi-directed formation of heterochromatin at centromeres, through interaction with the RNA-directed RNA polymerase complex.

The recognition and removal of introns from pre-mRNAs are essential for gene expression in eukaryotic cells. Pre-mRNA splicing takes place in a large complex, the spliceosome, which assembles through ordered interactions of four small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U4/U6 and U5 snRNPs, and numerous non-snRNP proteins (for a review, see 1,2). During assembly of the spliceosome and catalysis of the splicing reaction, the U snRNPs undergo ordered dynamic changes in composition and structure. The U1 and U2 snRNPs initially bind to the pre-mRNA and generate the pre-spliceosome, or ‘complex A’. The pre-formed U4/U6.U5 tri-snRNP is then recruited to complex A to form a ‘pre-catalytic complex B’ containing all five snRNAs. Subsequently, a large conformational rearrangement occurs in the spliceosome in which U1 and U4 snRNPs are released, accompanied by base-pairing between U6 and U2, and generates ‘complex B’. After the first step of splicing, the spliceosome is converted into ‘complex C’. The second step generates the mature mRNA product and is followed by the release of the remaining snRNPs from the spliced-out intron (1,2). In this way, pre-mRNA splicing is performed via RNA-RNA and RNA-protein interactions and each snRNA plays a role in the recognition of pre-mRNA sequences or functions as a catalytic element like a ribozyme (3). U6 snRNA base paired with U4 snRNA has been believed to have a catalytic role.

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in pre-mRNA splicing. It forms two intermolecular RNA helices (helix I and helix II) with U4 snRNA, both of which are disrupted during the activation of the spliceosome resulting in subsequent release of U4 snRNA. U6 snRNA interacts with U2 snRNA and the 5’ end of the intron to form part of the catalytic center (3). Only U4 snRNA does not have a sequence for the recognition of pre-mRNA directly. U4 snRNA has been thought of as an RNA chaperone to keep U6 snRNA in a repressed state by masking its catalytic residues (4). Precise roles of U4 snRNA in the spliceosome, however, remain to be clarified.

We and others have isolated prp (pre-mRNA processing) mutants of the fission yeast Schizosaccharomyces pombe, which are defective in pre-mRNA splicing at a nonpermissive temperature (5-8). So far, 14 prp mutants (prp1-prp14) have been isolated, 8 of which produce elongated cells, like cell-division-cycle (cdc) mutants, suggesting a possible link between the pre-mRNA splicing and the cell cycle progression (7,8).

The centromere is the chromosomal region for the assembly of the kinetochore, which ensures equal segregation of chromosomes at cell division through its interaction with the microtubules of the mitotic spindle (9). In S. pombe, the centromeres are composed of innermost repeats and outer dh and dg repeats that flank the central kinetochore-binding domain (for a review, see 10). The outermost dh and dg repeats are packaged into heterochromatin that is important for the centromere’s functions.

It has been shown that factors related to RNA interference (RNAi) play essential roles in the formation of heterochromatin at the centromeres in fission yeast (for a review, 11). Noncoding RNAs transcribed from outer repeats are processed to siRNAs, which are responsible for recruiting the RNAi-induced transcriptional silencing (RITS) complex comprising Ago1, Chp1 and Tas3 to the pericentromeric outer regions (12). The generation of siRNA is dependent on dsRNA synthesis by the RNA-directed RNA polymerase complex (RDRC) and processing by the Dicer ribonuclease, which physically associate with RITS (13,14). The targeting of RITS by siRNAs then promotes the dimethylation of histone H3-lysine 9 (H3K9me2) by the Chr4/Rik1 histone methyltransferase complex, directing the localization of the chromodomain protein Swi6, a heterochromatin protein 1 (HP1) homologue, to the centromeric outer regions (12,15-17).

Recently, Bayne et al. (18) reported that defects in specific splicing factors, such as Cwf10p and Prp10p, affect the generation of siRNAs from noncoding RNAs and consequently the centromeric heterochromatin integrity, although the molecular mechanism involved remains unknown. They revealed that Cid12p, a component of RDRC, interacts physically with those splicing factors. In this study, we revealed that a mutation in the spliceosomal U4 snRNA (the prp13-1 mutation) affects the formation of centromeric heterochromatin in S. pombe. We also found, for the first time, that the noncoding RNAs involved in the formation of heterochromatin have an intron typical of pre-mRNAs. A possible model for the involvement of splicing components, including U4 snRNP, in the formation of the centromeric heterochromatin is proposed.

**Experimental Procedures**

*S. pombe Strains and General Methods* - The S. pombe strains used in this study and their corresponding genotypes are listed in Table 1. Standard genetic procedures for S. pombe were as described (19,20). Transformation of S. pombe with a cosmid library was done as described (21).

*Cloning and Sequencing* - An S. pombe cosmid genomic library, which was constructed in pSS10 with the LEU2 marker (22), was introduced into prp13-1, and cosmids were recovered from transformants that grew at 36°C on MM plates. Isolated cosmids were reintroduced into prp13-1 to confirm their ability to complement the growth defects at the restrictive temperature. The insert of the cosmid was subcloned into pSP1, which is an S. pombe ars1 multicopy vector (23).

To identify the site of mutations in prp13-1, genomic DNA was prepared from cells, and the U4 snRNA and prp31+ genes were amplified by PCR. The amplified DNA fragments were directly sequenced with oligonucleotide primers, which are complementary to the coding sequences of the U4 snRNA or prp31+ gene.
Isolation of Total RNA- *S. pombe* strains were cultured in 10ml of YEALU medium to a mid-log phase. Cells were harvested, and disrupted by glass beads in TELS Buffer (10mM Tris-HCl pH7.5, 10mM EDTA, 100mM LiCl and 1%SDS). PCI (Phenol: Chloroform: Isonamyl Alcohol, 25: 24: 1) was added and the samples were vortexed for 20sec 4 times. After centrifugation, RNAs were precipitated by the addition of ethanol. Following treatment with DNase (Promega), RNA samples were treated with PCI and precipitated with ethanol. RT-PCR was performed using a PrimeScript RT-PCR kit (TAKARA). The primers used are listed in Table 2.

Silencing Assays- Cell suspensions (2×10³ cells/ml) were serially diluted five fold and spotted on N/S plates (nonselective YE medium supplemented with adenine, leucine and uracil), or 5-FOA plates (N/S plates containing 1mg/ml 5-fluoroorotic acid). The plates were then incubated at 26°C for *ts* mutants or 33°C for *cs* mutants for 3 to 4 days.

Chromatin immunoprecipitation- Immunoprecipitation of fixed chromatin with the Swi6 antibody (kindly provided by Dr. Jun-ichi Nakayama) was performed as described previously (15), except that protein A dynabeads (BERTAS) were used. Briefly, cells grown to mid-log phase were fixed with 3% formaldehyde for 30 min. The cells were then ruptured with a Multi beads shocker (Yasui, Co.), and the chromatin was shared using a Biorupter sonicator (BM Equipment, Co.). The samples were next incubated with the anti-Swi6p antibody overnight, then mixed with protein A dynabeads with rotation at 4°C for 1h. After being washed, the samples were decrosslinked at 65°C and treated with protease K.

Competitive PCR of the *ura4* gene to quantify Swi6p enrichment was done using the *ura4* DS/E Fw and Rv primers (Table 2). Real-time quantitative PCR was performed in the presence of SYBR Green on a Roche Diagnostics LightCycler with the primers listed in Table 2. *Ura4*-5 and *ura4*-6 primers can amplify the *ura4* gene inserted in the *otr1R* locus specifically. The *fbp1* gene was amplified as a control for the euchromatic region. Data were analyzed with Lightcycler Software ver. 3.5. The ChIP experiments were done independently three times.

Immunostaining- Cells grown to a mid-log phase in YE medium were fixed with 3% formaldehyde and 0.2% glutaraldehyde for 90min. After washing with PEM buffer (100mM PIPES pH6.9, 1mM EGTA and 1mM MgSO₄), the cells were treated with 0.5 mg/ml Zymolyase at 37°C for 8 to 10min, then incubated for 2min in PEMST (PEM containing 1M Sorbitol and 1% Triton X-100). After being washed with PEMS (PEM containing 1M Sorbitol), the cells were treated with the TAT1 monoclonal antibody against tubulin (kindly provided by Dr. Keith Gull) in PEMBAL (PEM containing 0.1M L-lysine, 1% BSA and 0.1% Na azide) at room temperature overnight, and then with anti-mouse IgG antibody conjugated with FITC (ICN Biomedicals). After counterstaining with DAPI, fluorescence images of the cells were obtained using an Olympus AX70 fluorescence microscope equipped with a Photometrics Quantix cooled CCD camera.

RESULTS

Cloning of the *prp13* Gene. To clone the *prp13* gene, we transformed the *prp13*-1 mutant with the *S. pombe* genomic library constructed in the cosmid pSS10 (22). We isolated six cosmids that rescue the temperature-sensitive growth of *prp13*-1 (Fig.1A). Four clones including cosmid #3 showed the same restriction enzyme-cutting patterns, suggesting that genomic DNA fragments contained in these cosmids are identical (data not shown). Cosmids #7 and #19 showed restriction enzyme-cutting patterns different from that of cosmid #3. Several DNA fragments from cosmid #7 were subcloned into pSP1, the *S. pombe* *ars1* multicopy vector. We found that a 1.6-kb SpeI fragment complemented the temperature-sensitive phenotype of *prp13*-1 at 36°C (Fig.1B). After subcloning, a 3.6- kb SacI fragment [398x169]BamHI fragment of cosmid [307x157]also complemented the temperature-sensitive phenotype of *prp13*-1 at 36°C. After subcloning, a 3.6-kb SacI fragment was found to complement the *prp13* mutation (data not shown). The fragment contained the *prp31* gene that encodes a component of
that the U4 snRNA gene is the authentic gene and the prp13-1 gene in the U4/U6.U5 tri-snRNP (24).

To determine the site of mutation in prp13-1, we amplified the gene for U4 snRNA and the prp31+ gene from the prp13-1 genomic DNA, and sequenced them directly. We identified one nucleotide change in the U4 snRNA gene in prp13-1; G at position +35 in the 5’-stem loop structure was replaced with A (Fig. 2). There was no nucleotide change in the prp31+ gene in prp13-1. These results indicate that the U4 snRNA gene is the authentic prp13+ gene and the prp31+ gene is a multi-copy suppressor for prp13-1.

The 5’ stem-loop structure in the Y-shaped U4/U6 domain is essential for the late stage of spliceosome assembly (25,26), and is a binding site for the 15.5 kD protein (Snu13p in yeasts) in the U4/U6.U5 tri-snRNPs (27). To elucidate effects of the prp13-1 mutation, we examined if the binding of Snu13p to U4 snRNA is impaired in prp13-1. To that end, we expressed Snu13p tagged with HA in prp13-1 and the wild-type strain HM123. The immunoprecipitation assay showed that U4 snRNA bound to HA-Snu13p significantly decreased in prp13-1, suggesting that the mutation in the 5’-stem-loop structure reduced the ability of U4 snRNA to bind Snu13p (Supplemental Fig. 1).

The U4 snRNA Mutation Causes a High Incidence of Lagging Chromosomes. It has been shown that some S. pombe splicing mutants including prp13-1 produce elongated cells at a nonpermissive temperature, a typical phenotype of cell division cycle mutants, cdc (7,8,28). To examine a cause of the cdc-like phenotype of prp13-1, we stained cells with DAPI to see a manner of the nuclear division in prp13-1. As a result, we found that prp13-1 yields lagging chromosomes on anaphase spindles at both permissive and nonpermissive temperatures (Fig. 3A and Supplemental Fig. 2).

It has been shown that fission yeast mutants defective in kinetochore-microtubule attachment essential for chromosomal segregation are generally hypersensitive to the microtubule-destabilizing drug thiabendazole (TBZ). We examined the sensitivity to TBZ of prp13-1 and other prp mutants at a permissive temperature. Δ ago1 and Δ dcr1, the wild-type genes of which are required for the accurate segregation of chromosomes, were used as TBZ-sensitive controls. Serial dilutions of mutant cells were spotted onto YEALU plates containing TBZ (10µg/µl) and incubated at 26°C for ts or 33°C for cs. As a result, prp13-1 was found to be hypersensitive to TBZ (Fig. 3B). prp2-2, prp8-1, prp10-1, and prp14-2 were also sensitive to the TBZ treatment like prp13-1.

The U4 snRNA Mutation Results in Defective Centromeric Silencing. To clarify the causes of the abnormal chromosomal segregation and hypersensitivity to TBZ in prp13-1, we examined if the mutation in U4 snRNA affects the formation of centromeric heterochromatin. In S. pombe, the centromeric region consists of a central core, where the kinetochore is assembled, surrounding inner (imr) and outer (otr) repeats (Fig. 4A) (10,11). The outer repeat domain, the otr and some of the imr repeats, is packaged as heterochromatin that silence transcriptional activity. We constructed strains that have the prp13-1 mutation and the ura4- marker gene inserted in the imr and otr regions. In those strains, the authentic ura4+ gene was partially deleted (ura4 DS/E). Serial dilutions of the cells were spotted onto plates with or without 5-fluoroorotic acid (5-FOA). In wild-type cells, as the outer repeat domains are silenced by the formation of heterochromatin (11), the ura4- transgene was not expressed, and there was no sensitivity to 5-FOA. In contrast, the prp13 cells with the ura4+ transgene in the outer repeat domain (imrR::ura4+ and otrR::ura4+) could not grow on the FOA plates, suggesting that the ura4- transgene was expressed. Expression of the ura4- genes inserted in the pericentromeric outer repeat domain was also confirmed by RT-PCR (Fig. 4C).

The formation of heterochromatin at the centromeric outer repeat domain in fission yeast is directed by the RNA interference (RNAi) machinery (11). Thus, we examined if the dg noncoding transcripts transcribed from the otr regions, which are processed into siRNAs that target the RITS complex to the centromere, is accumulated in prp13-1 and other prp mutants. Total RNAs isolated from the prp strains and the Δ dcr1 strain were subjected to a RT-PCR assay using primers for the dg transcript. Reverse transcription was done using the oligo dT. As shown in Fig. 4D, the dg noncoding transcripts were accumulated in prp13-1, like Δ dcr1, suggesting the processing of the centromeric transcripts into siRNA by Dicer to be impaired. Accumulation of the dg transcripts was also observed in prp3-3, prp4-2, prp8-1, prp10-1, prp12-1 and prp14-2.
In fission yeast, histone H3 in the heterochromatin is methylated at lysine 9 (H3K9me2) and this modification becomes a target of the chromodomain protein Swi6 to form silent heterochromatin (29,30). To examine if the heterochromatin’s structure is affected by the prp13-1 mutation, we carried out a chromatin immunoprecipitation (ChIP) assay using the anti-Swi6 antibody. As shown in Fig. 5, we found that the prp13-1 mutation results in a decreased level of Swi6p in the pericentromeric outer domain, which is consistent with the finding that the processing of noncoding RNAs into siRNAs is severely inhibited in this mutant. Introduction of the wild-type U4 snRNA gene into the prp13-1 mutant recovered reproducibly the Swi6p level to that in the wild-type cells (Fig. 5, the lane denoted as prp13-1+pSP1U4), demonstrating that the mutation in the U4 snRNA gene is responsible for the reduction of Swi6p at the pericentromeres. **Defects in Centromeric Silencing in prp13-1 are not a Secondary Effect of Impaired pre-mRNA Splicing.** prp13-1 was isolated by the screening of a ts’ bank with the northern blot analysis to detect splicing defects in tbp1+ pre-mRNA and U6 pre-snRNA at the nonpermissive temperature (7). In contrast to typical splicing mutant prp2-2, prp13-1 shows weak splicing defects at a nonpermissive temperature (Fig. 6A).

As the ago1+, hrr1+ and sir2+ genes essential for the RNAi system contain introns, it is possible that the defect in heterochromatic silencing in prp13-1 is caused by the splicing defects in intron-containing RNAi-related genes. To exclude that possibility, we examined the splicing efficiency of ago1”, hrr1” and sir2” in prp13-1 by RT-PCR and found that transcripts from these genes are spliced at 26°C and even at 37°C with almost the same efficiency as in the wild-type cells (Fig. 6B). This result suggests that the defect in centromeric silencing in prp13-1 did not result from impaired splicing of these RNAi components.

We also confirmed that expression of the wild-type U4 snRNA in prp13-1 could complement the defective gene silencing at centromeres (Fig. 6C). Transformants with the plasmid containing the wild-type U4 snRNA gene grew on the plates with 5-FOA (Fig. 6C, rows 5 and 6), like wild-type cells, whereas the swi6 otr1R::ura4 and prp13-1 otr1R::ura4 strains did not (Fig. 6C, rows 2 and 4), indicating that the expression of the wild-type U4 snRNA rescues the defective centromeric silencing. In addition, introduction of the prp31” gene, a multiplicity suppressor for prp13-1, complemented the defective heterochromatin silencing at the pericentromeric outer repeat domain in prp13-1 (Fig. 6C, rows 7 and 8).

### The Centromeric dg Noncoding RNA has an mRNA Type Intron.

The results presented above suggest that spliceosomal U4 snRNA is involved in the processing of centromeric noncoding RNAs into siRNAs and formation of centromeric heterochromatin. In the course of our analysis of the relationship between U4 snRNA and centromeric gene silencing, Bayne et al. (18) also reported that specific splicing factors such as Cwf10p and Prp10p play a role in the generation of siRNA in RNAi-directed centromere silencing. The molecular basis for that phenomenon is, however, unknown.

In relation to the crosstalk between the spliceosomal components and RNAi-directed heterochromatic silencing, we speculated that the centromeric noncoding RNAs have signals to allow the spliceosomal components to assemble on them. We thus searched for sequences matching the consensus sequences of the splice and branch sites in the pericentromeric noncoding RNAs. Interestingly, we found intron-like sequences that contain sequences suitable for the 5’ and 3’ splice sites and the branch site in the centromeric dg (Fig. 7A) and dh (Supplemental Fig. 3) noncoding RNAs.

To examine if these intron-like sequences are actually spliced, we performed RT-PCR using primers to amplify the dg and dh genomic regions containing the intron-like sequences. As a result, we detected smaller bands corresponding to the spliced form of the dg centromeric noncoding RNAs, in addition to the band derived from the primary transcripts (Fig. 4D). We then sequenced DNAs purified from the shorter band and confirmed that the intron-like sequence is actually spliced (Fig. 7B). We could not detect the splicing of the intron-like sequence in the dh noncoding centromeric transcript (Supplemental Fig. 3).

### DISCUSSION

In this study, we revealed that a mutation in the spliceosomal U4 snRNA causes a defect in the formation of heterochromatin at the centromere, suggesting a linkage between...
the splicing machinery and the RNAi-directed formation of centromeric heterochromatin. As the present study was being conducted, Bayne et al. also reported that defects in specific splicing factors affect the generation of centromeric siRNAs and integrity of centromeric heterochromatin (18). They showed that csp4 and csp5 (centromere: suppressor of position effect), which alleviated silencing of marker genes inserted in the otr of centromere 1, are alleles of the splicing factors Cwf10p and Prp39p, respectively. Interestingly, Cwf10p is a homologue of the *Saccharomyces cerevisiae* U5 small nuclear ribonucleoprotein Snu114p that is required for the unwinding of U4/U6 snRNA (31). Prp39p in *S. cerevisiae* is known to be a factor associated with U1 snRNA and involved in the early step of the splicing reaction, that is, commitment to the splicing of pre-mRNA (32).

To exclude the possibility that the defect in heterochromatic silencing is a secondary effect of the impaired splicing in the *prp* mutants, we showed that splicing of the transcripts for major factors related to the RNAi pathway, whose genes have introns, is not blocked significantly at the permissive temperature of 26°C, the temperature at which chromatin silencing was disrupted (Figs. 4 and 6). To demonstrate that defective splicing in the *prp* mutants is not a cause of the impaired RNAi-directed gene silencing, Bayne et al. replaced the endogenous intron-containing *ago1* and *hrr1* genes with corresponding intron-less cDNAs and showed that the *prp10-1* mutation still alleviated heterochromatic silencing in the recombinant strains as in wild-type cells (18). It is thus likely that splicing factors including U4 snRNP play roles directly in the RNAi-directed formation of heterochromatin in *S. pombe*, although we cannot exclude the minor possibility that splicing of unknown essential genes involved in the RNAi system is specifically impaired even at the permissive temperature, resulting in the defective gene silencing.

We revealed that centromeric noncoding RNAs contain introns typical of pre-mRNAs (Fig. 7). This is the first report that centromeric noncoding RNAs have introns. The presence of the mRNA-type intron results in the assembly of the splicing factors on nascent centromeric noncoding RNAs. Interestingly, it has been shown that spliceosome subunits are co-purified with affinity-selected Cid12p, a component of RDRC involved in dsRNA synthesis, in cells lacking Rdp1 (13). Cid12-FLAG was also co-immunoprecipitated with specific splicing factors, such as Cwf10p, in wild-type cells (18), indicating the physical interaction between the splicing factors and the RNAi component Cid12p. It is noteworthy that not all the spliceosomal factors were co-purified with the tagged Cid12p (18).

Figure 8 shows a hypothetical model for the involvement of the splicing factors and snRNPs in the formation of pericentromeric heterochromatin. During transcription of the centromeric noncoding RNAs by RNA polymerase II, a group of splicing factors and snRNPs recognize the splice sites and the branch site in the intron of the nascent transcripts from the centromeric region to form a spliceosome or a ‘sub-spliceosome complex’ consisting of specific factors that affect processing of centromeric noncoding RNAs to siRNAs. The complex assembled on the noncoding RNAs then facilitates recruitment of RDRC through interaction with Cid12p. The assembled spliceosome or the sub-spliceosome complex might function as a platform to facilitate the processing of centromeric noncoding RNAs by RDRC, which synthesizes double-stranded RNAs used for the production of siRNA (18).

High-throughput sequencing of Argonaute-associated siRNAs revealed that siRNAs are generated from the intron of the *dg* centromeric noncoding RNA, as well as its exons (33). This means that splicing of the noncoding RNAs itself is not necessary for production of the centromeric siRNAs. Recognition of the intron sequences and assembly of the pre-spliceosome seem to be important for the recruitment of RDRC. Further analysis of the roles of the intron sequence in the RNAi pathway for heterochromatic gene silencing is now underway.

We and Bayne et al. (18) showed that some specific *prp* mutants are defective in heterochromatic gene silencing, thereby suggesting that subsets of splicing factors and snRNPs are involved in the silencing process. We cannot exclude the possibility that exhibition of the defective chromatin silencing phenotype might be dependent on the site of mutation in each *prp* mutant, as we found allele-specific sensitivity to TBZ in the *prp2* mutants, the wild-type gene of which encodes U2AF59 (34) (Fig. 3). *prp2-1* was moderately sensitive to TBZ,
whereas prp2-2 was highly sensitive to the drug. Both alleles cause severe splicing defects at a nonpermissive temperature (34,35). However, we could not detect the accumulation of noncoding RNAs in the prp2-1 or prp2-2 mutant, suggesting that sensitivity to TBZ in prp2-2 is not caused by the defect in the RNAi-directed formation of heterochromatin (Supplemental Fig. 4). The TBZ sensitivity in prp2-2 might be due to the abnormal nuclear structure in this mutant (36). Thus, it is unlikely that Prp2p is involved in the RNAi-directed centromeric gene silencing.

We revealed that the prp13 mutation, the mutation in the U4 snRNA gene, caused weak splicing defects at the permissive temperature, although it caused severe defects in heterochromatic gene silencing at the same temperature. We demonstrated that the prp13-1 mutation in the 5' stem-loop structure resulted in decreased binding of U4 snRNA to Snu13p even at the permissive temperature (Supplemental Fig. 1). Unstable binding of Snu13p to U4 snRNA might affect the structure of U4/U6 snRNP and the sub-spliceosome complex assembled on the centromeric noncoding RNAs, leading to the decreased recruitment of RDRC and impairment of siRNA amplification. Analyses of the RDRC recruitment to the centromeric noncoding RNAs in the presence of the prp13 mutation, or in the absence of the intron, are now underway.

Recently, it was reported that the first cleavage reaction by the spliceosome generates the mature 3' end of telomerase RNA in S. pombe (37). The results of the present study provide another line of evidence that the spliceosome is potentially multifunctional and has evolved to play essential roles not only in pre-mRNA splicing, but also in other biological processes, such as the maturation of telomere RNA and heterochromatic gene silencing, during evolution.

REFERENCES


FOOTNOTES

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The abbreviations used are: prp, pre-mRNA processing; snRNP, small nuclear ribonucleoprotein particle; RITS, RNAi-induced transcriptional silencing; RDRC, RNA-directed RNA polymerase complex; TBZ, thiabendazole; 5-FOA, 5-fluoroorotic acid; ChIP, chromatin immunoprecipitation; siRNA, short interfering RNA

FIGURE LEGENDS

Fig. 1. Cloning of the prp13+ gene
(A) Complementation of temperature-sensitive phenotype of prp13-1. Cosmids #3, #7 and #19 complemented the temperature-sensitive phenotype of prp13-1 at 36°C. (B) Three DNA fragments were isolated from cosmid #7 after digestion with BamHI and subcloned into the pSPI vector (pSPI-4.2kb, 4.8kb and 6.2kb). The 8.4kb fragment is a self-ligated clone of the BamHI-digested
Enrichment at the quantitative PCR analyses were carried out with the samples analyzed in (A) to assess the Swi6p wild-type U4 snRNA gene (lanes in performed with the indicated strains. Relative enrichment was calculated as the ratio of enrichment values for three independent immunoprecipitations are shown. The reduction in the recruitment of Swi6p to the cells containing the inserted rescue the cosmids #7 (pSS10-8.4kb). All subclones were introduced into prp13-1 and tested for complementation. Only the 8.4kb self-ligated fragment complemented the prp13-1 mutation. Subcloning of the restriction fragments from pSS10-8.4kb revealed that the 1.6kb fragment could rescue the prp13-1 mutation. The 1.6kb fragment contains the entire coding region for U4 snRNA.

Fig. 2. Schematic representation of the U4/U6 structure and the mutation site in prp13-1. (A) The structure of the U4/U6 snRNA. U4 snRNA base pairs with U6 snRNA and has a 5’ stem-loop (5’-SL) structure (27). Snu13p and Prp31p bind directly with the 5’-SL region of U4 snRNA (38). (B) The mutation in U4 snRNA results in defective gene silencing at the centromere. (A) The structure of the U4/U6 snRNA. U4 snRNA base pairs with U6 snRNA and has a 5’ stem-loop (5’-SL) structure (27). Snu13p and Prp31p bind directly with the 5’-SL region of U4 snRNA (38). (B) In prp13-1, G35 in the 5’-SL region of U4 snRNA is changed to A.

Fig. 3. prp13-1 yields lagging chromosomes and is sensitive to TBZ treatment. (A) Mitotic chromosomal segregation is impaired in prp13-1, resulting in a high incidence of lagging chromosomes during late anaphase. The prp13-1 cells were double stained with DAPI that binds DNA and the TAT1 antibody that binds tubulin. In the merged images (Merge), green and red denote DNA and tubulin, respectively. Two independent prp13-1 cells cultured at the permissive temperature are shown. The arrowheads indicate the lagging chromosomes. (B) Sensitivity to TBZ of the prp mutants. Serially diluted cells were spotted on YEALU plates without TBZ (N/S), or with 10µg/ml of TBZ (+TBZ). The plates were incubated at 26°C. Total RNA was isolated from the indicated strains and subjected to RT-PCR using primers corresponding to the dg noncoding RNA or act1 mRNA. No bands were detected in any samples without the reverse-transcription reaction (-RT). Act1 mRNA was amplified by RT-PCR as an internal control. Upper and lower arrows denote bands derived from the authentic dg noncoding RNA and its spliced form, respectively.

Fig. 4. The mutation in U4 snRNA results in defective gene silencing at the centromere. (A) Schematic representation of the structure of the fission yeast centromere 1. The vertical lines in the imr1L and imr1R regions indicate clusters of (or single) tRNA genes that have been proposed to function as boundary elements (39). The central core domain is comprised of cut and the inner part of the imr elements. The outer repeat domain encompasses the dg/dh elements and a small part of the imr elements. The ura4+ marker gene was inserted in the imr1R or otr1R region. (B) Serially diluted cells containing the inserted ura4+ gene were spotted on YEALU plates (N/S), or YEALU plates containing 1mg/ml of 5-FOA, and incubated at 26°C. prp13-1 with the ura4+ gene inserted in the imr1R or otr1R region was sensitive to 5-FOA, indicating that the ura4+ marker gene was expressed in these strains. The swi6 mutant containing the ura4+ transgene, the wild-type of which encodes a chromodomain protein, was used as a control for defects in the formation of centromeric heterochromatin. (C) Expression of the ura4+ mRNAs from the genes inserted in the pericentromere region was confirmed by RT-PCR. Total RNA was isolated from indicated strains and subjected to a RT-PCR assay. Reverse transcription was done with an oligo dT primer. Ura4DS/E indicates bands amplified from the authentic gene with the 269bp deletion. (D) Accumulation of the centromeric dg noncoding RNA in the prp mutants. The prp mutants were cultured at the permissive temperature of 26°C. Total RNA was isolated from the indicated strains and subjected to RT-PCR using primers corresponding to the dg noncoding RNA or act1 mRNA. No bands were detected in any samples without the reverse-transcription reaction (-RT). Act1 mRNA was amplified by RT-PCR as an internal control. Upper and lower arrows denote bands derived from the authentic dg noncoding RNA and its spliced form, respectively.

Fig. 5. The prp13-1 mutation abolished heterochromatin modifications at the otr1R::ura4+ locus. (A) ChIP analyses of Swi6p at otr1R::ura4+ relative to a euchromatic control locus (ura4 DS/E) were performed with the indicated strains. Relative enrichment was calculated as the ratio of otr1R::ura4+ to ura4 DS/E in the immunoprecipitate, IP(+), relative to whole cell extract, WCE. The average enrichment values for three independent immunoprecipitations are shown. The reduction in the recruitment of Swi6p to the otr1R::ura4+ locus in prp13-1 was recovered by transformation of the wild-type U4 snRNA gene (lanes in prp13+pSP1U4 ). A representative gel is shown. (B) Real-time quantitative PCR analyses were carried out with the samples analyzed in (A) to assess the Swi6p enrichment at the otr1R::ura4+ locus relative to that of the fib1 euchromatic control locus. Error bars represent standard deviations.

Fig. 6. Impaired pre-mRNA splicing is not a cause of defective centromeric silencing. (A) prp13-1
shows weak or no defects in pre-mRNA splicing. Total RNA was isolated from strains cultured at 37°C for the periods indicated and analyzed by RT-PCR using primers for \textit{tbp1} or \textit{cdc2}. White and black arrowheads indicate bands for pre-mRNAs and mature mRNAs, respectively. The \textit{cdc2} gene has two introns. (B) No severe defects were observed in the splicing of \textit{hrr1}+, \textit{ago1}+ and \textit{sir2}+ pre-mRNAs, the products of which are essential for the RNAi pathway. Total RNA extracted from the wild-type 972 cells or \textit{prp13-1} cells cultured at 26°C or 37°C for 2 h was subjected to a RT-PCR assay using primers for the indicated genes. Ago1 pre-mRNA is spliced with a low efficiency in both the wild-type and \textit{prp13-1} cells. (C) Introduction of the genes for U4 snRNA, Prp31p and Snu13p recovered the centromeric gene silencing in \textit{prp13-1}. Each strain or transformant was spotted on YEALU plates with 5-FOA (+FOA) or without 5-FOA (N/S), and incubated at 26°C for 5 days. Two independent clones (1 and 2) were spotted for each transformant.

**Fig. 7.** The centromeric \textit{dg} noncoding RNA contains an mRNA-type intron. (A) Nucleotide sequence of the region containing the mRNA-type intron in the centromeric \textit{dg} element. A part of the genomic region, where the \textit{dg} noncoding RNA is transcribed from, is shown. The intron region is written in red lower-case. The sequences that matched the consensus sequence of the 5’ and 3’ splice sites in \textit{S. pombe} are boxed. The putative branch site is underlined. Arrows indicate primers used for the RT-PCR analysis. (B) The intron-like region in the \textit{dg} noncoding RNA is precisely removed in the cDNA corresponding to the lower band observed in Fig. 4D. Sequence data determined using the ABI 310 sequencer are shown. A vertical arrow indicates the position of the intron-like sequence in the \textit{dg} noncoding RNA.

**Fig. 8.** A hypothetical model for the involvement of the spliceosomal components in the RNAi-directed formation of heterochromatin. A specific set of splicing factors, including the U4/U6 snRNP, or ordinal spliceosomal components are assembled on the mRNA-type intron in the nascent noncoding RNAs transcribed from the pericentromeric \textit{otr} region. Using the assembled sub-spliceosome complex or spliceosome as a platform, RDRC is recruited to the noncoding RNAs through interaction between Cid12p in RDRC and splicing factors, such as Cwf10p. RDRC then convert the centromeric noncoding RNAs into the double-stranded RNAs that are processed into siRNAs by Dicer.
Table 1. List of *S. pombe* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
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<tbody>
<tr>
<td>972</td>
<td><em>h</em></td>
</tr>
<tr>
<td>UR470</td>
<td><em>h</em> <em>leu1</em>-32 <em>ura4</em>-D18</td>
</tr>
<tr>
<td>UR520</td>
<td><em>h</em> <em>prp13</em>-1 <em>leu1</em>-32</td>
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<tr>
<td>MC1</td>
<td><em>h</em> <em>prp13</em>-1 <em>leu1</em>-32 <em>ura4</em>-D18</td>
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<tr>
<td>SP473</td>
<td><em>h</em> <em>leu1</em>-32 <em>ura4</em>-D18 <em>Ddc1::KanR</em></td>
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<td>SP587</td>
<td><em>h</em> <em>leu1</em>-32 <em>ura4</em>-D18 <em>Dago1::KanR</em></td>
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<td>SU34-5D</td>
<td><em>h</em> <em>prp1</em>-1</td>
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<td>UR104</td>
<td><em>h</em> <em>prp1</em>-4</td>
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<td>SU35-3A</td>
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<td>SU50-5B</td>
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<td>SU13-11B</td>
<td><em>h</em> <em>prp3</em>-3</td>
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<td>SU42-6C</td>
<td><em>h</em> <em>prp4</em>-2</td>
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<td>SU38-12</td>
<td><em>h</em> <em>prp5</em>-1</td>
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<td>SU31-8B</td>
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<td>UR390</td>
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<tr>
<td>FY498</td>
<td><em>h</em> <em>imr1R</em>(dg-glu)<em>NcoI::ura4</em> <em>oril</em> <em>leu1</em>-32 <em>ura4</em>-D/E <em>ade6</em>-210</td>
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<td>FY648</td>
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<td>FY711</td>
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Table 2. List of primers used in this study

For the immunoprecipitation analysis

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<tr>
<th>RNA</th>
<th>Primer 1</th>
<th>Primer 2</th>
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<tbody>
<tr>
<td>U4 snRNA</td>
<td>5'-CTTTGTGCACGGGTATTACG-3'</td>
<td>5'-TAACGAGAGTGGAGCGGTC-3'</td>
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<tr>
<td>U3 snRNA</td>
<td>5'-CACGGATGATGATAGGAGGC-3'</td>
<td>5'-GTCAGAAACACCAGCTGCC-3'</td>
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For the centromeric silencing assays

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<th>RNA</th>
<th>Primer 1</th>
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<tbody>
<tr>
<td>ura4+</td>
<td>5'-GCTATTCAGCTAGAGCTG-3'</td>
<td>5'-TTTCTCTATATCTTTG-3'</td>
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<tr>
<td>dg noncoding RNA</td>
<td>5'-CCCATCCGCAGTTGGGAG-3'</td>
<td>5'-TACCATGCTTTTAGTGCGG-3'</td>
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<tr>
<td>dh noncoding RNA</td>
<td>5'-ACCATAATGATATGGCTATG-3'</td>
<td>5'-GTCAATGTTCTGCTGTGC-3'</td>
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<tr>
<td>act1+</td>
<td>5'-CAAACGTTGAGAGATGAC-3'</td>
<td>5'-GAAGCCTACACGTTAAC-3'</td>
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For the analysis of the splicing defects

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<th>Primer 1</th>
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<td>Tbp1-5-2</td>
<td>5'-CTATTGCAGCTACATGCAC-3'</td>
<td>5'-GTCATCCTCGGATTTGCC-3'</td>
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<td>Ex3-s</td>
<td>5'-ATGAATCTGAGGGAGTTCC-3'</td>
<td>5'-TACTTCAGGAGCACGATACC-3'</td>
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<td>Cdc2-1</td>
<td>5'-CTACTCAGGAGCTGCATACC-3'</td>
<td>5'-GGGAATATACTCGGTTGAC-3'</td>
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<td>Cdc2-2</td>
<td>5'-CTTTCAAGAGCTGCATACC-3'</td>
<td>5'-CTCTCCTACCACTTCAGC-3'</td>
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<td>Hrr1-1</td>
<td>5'-GGAATATACCTCGGAGTGC-3'</td>
<td>5'-GGTTGCTGCTGGCTATCTATG-3'</td>
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<td>Hrr1-2</td>
<td>5'-CTAACATGCTGCTGTGC-3'</td>
<td>5'-TGGATAAGCAAAACACCACCACCACCAC-3'</td>
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For the ChIP analysis

Competitive PCR

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<tr>
<td>ura4 DS/E Fw</td>
<td>5'-GAGGGATGAAAAATCCCAT-3'</td>
<td>5'-TTCGACAACAGGATACGACC-3'</td>
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Quantitative PCR

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<tr>
<td>ura4-5</td>
<td>5'-ACTTGTTCTACACAGAG-3'</td>
<td>5'-GGATGTTAAGGAGACGAGC-3'</td>
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<tr>
<td>ura4-6</td>
<td>5'-GGATGTTAAGGAGACGAGC-3'</td>
<td>5'-GGTTGCTGCTGCTATATCATG-3'</td>
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<tr>
<td>fbpl Fw</td>
<td>5'-TTCGACAACAGGATACGACC-3'</td>
<td>5'-TGGATAAGCAAAACACCACCACCAC-3'</td>
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</table>
Fig. 2

(A) U6 snRNA

(B) U4 snRNA

5'-stem loop

Prp31p

Snu13
Fig. 4 (A) outer repeat domain central core domain outer repeat domain

otr1L invr1L ent1 invr1R otr1R
dh dg dg dh

(B) N/S + 5-FOA

 WT invr1F:ura4 psp13
 swi8 otr1R:ura4
 prp13 Δura4
 invr1F:ura4 otr1F:ura4

(C) + RT - RT

 + prp13

 - prp13

(D) Maker WT Δatr1 prp1 prp2 prp3 prp4 en3 prp3-1 prp3-2 prp2-1 prp2-2 prp1-1 prp1-2 prp1-3 prp1-4

 - RT

act1
Fig. 5

(A)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>swi6</th>
<th>prp13</th>
<th>prp13+pSP1U4</th>
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<tr>
<td></td>
<td>WCE</td>
<td>IP(-)</td>
<td>IP(+)</td>
<td>WCE</td>
</tr>
<tr>
<td>imr::ura4*</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ura4 DS/E</td>
<td></td>
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<td>Relative enrichment:</td>
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<td>1.8</td>
<td>1.6</td>
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</table>

(B)

enrichment relative to fip1

WT swi6 prp13 prp13+pSP1U4
Fig. 7

(A) SPNCRNA.232

(B)
Involvement of the spliceosomal U4 snRNA in heterochromatic gene silencing at fission yeast centromeres

Madoka Chinen, Misato Morita, Kazuhiro Fukumura and Tokio Tani

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