Conservation and divergence of transcriptional coregulations between box C/D snoRNA and ribosomal protein genes in *Ascomycota*

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ABSTRACT

Coordinated assembly of the ribosome is essential for proper translational activity in eukaryotic cells. It is therefore critical to coordinate the expression of components of ribosomal programs with the cell's nutritional status. However, coordinating expression of these components is poorly understood. Here, by combining experimental and computational approaches, we systematically identified box C/D snoRNAs in four fission yeasts and found that the expression of box C/D snoRNA and ribosomal protein (RP) genes were orchestrated by a common Homol-D box, thereby ensuring a constant balance of these two genetic components. Interestingly, such transcriptional coregulations could be observed in most *Ascomycota* species and were mediated by different *cis*-regulatory elements. Via the reservation of *cis* elements, changes in spatial configuration, the substitution of *cis* elements, and gain or loss of *cis* elements, the regulatory networks of box C/D snoRNAs evolved to correspond with those of the RP genes, maintaining transcriptional coregulation between box C/D snoRNAs and RP genes. Our results indicate that coregulation via common *cis* elements is an important mechanism to coordinate expression of the RP and snoRNA genes, which ensures a constant balance of these two components.

Keywords: snoRNA; ribosomal protein; transcriptional coregulation; *cis* element; *Ascomycota*

INTRODUCTION

Ribosome biogenesis is one of the most complex processes in eukaryotic cells, requiring coordination of ribosomal proteins, rRNAs, and snoRNPs (Venema and Tollervey 1999; Lafontaine and Tollervey 2001; Fromont-Racine et al. 2003). Although the productions of different ribosomal components, as well as their assembly into active ribosomes, occur in different cellular compartments, normally growing cells do not contain pools of them. Therefore, there must be tightly coordinated controls to ensure the production of corresponding amounts of various ribosomal components in response to nutrient levels, environmental stress, and the presence of growth factors (Warner 1999).

The coordinating regulations of RP genes are well characterized, whereas those of other ribosomal components are still poorly understood, and how these different components ensure a constant balance is still unknown. For example, snoRNAs are important components of functional snoRNPs and represent a well-characterized group of noncoding RNAs (Balakin et al. 1996; Tollervey and Kiss 1997; Weinstein and Steitz 1999; Watkins and Bohnsack 2012), which mainly act as guides for the site-specific 2′-O-ribose methylation (box C/D snoRNAs) and pseudouridylation (box H/ACA snoRNAs) of rRNAs (Kiss-László et al. 1996; Bachellerie and Cavaillé 1997; Ganot et al. 1997) or are required for the nucleolytic processing steps of the pre-rRNA (Kass et al. 1990; Liang and Fournier 1995; Enright et al. 1996). In recent years, the Homol-D box (CAGTCACA) was demonstrated to not only mediate coordinated RP gene expression but also to control the transcription of *U3* box C/D snoRNA in *Schizosaccharomyces pombe* (Witt et al. 1993; Nabavi and Nazar 2008); RAP1 was found to mediate RP gene expression and control some snoRNA expressions in *Saccharomyces cerevisiae* (Qu et al. 1999; Lieb et al. 2001); and some snoRNA host genes were involved in ribosome biogenesis or function. These data suggest that the same transcriptional controls probably contribute to the coordinating

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expression of the RP gene and specific snoRNAs. However, whether such coordinating regulation exists in a genomic scalar and the detailed mechanisms are still unknown.

The transcription mechanisms of RP genes are complex in different fungi species (Tanay et al. 2005). FHL1, IFH1, and RAP1 were demonstrated to be functional in *S. cerevisiae* (Lieb et al. 2001; Martin et al. 2004; Wade et al. 2004); TBF1 and CBF1 mediate RP gene transcription in *Candida albicans* (Hogues et al. 2008); and the Homol-D box was proved to play a key role in regulating RP transcription in *S. pombe* (Witt et al. 1993). However, the transcriptional regulation of snoRNA genes is comparatively elusive. It has only been well-documented in *S. cerevisiae*, in which RAP1 and TBF1 are the primary transcription factors (Qu et al. 1999; Preti et al. 2010). snoRNAs exist in diverse eukaryotes and require different processing pathways that correspond to different methods of genome organization (Brown et al. 2003; Dieci et al. 2009). Given that the mechanisms of the coordinated regulation of RP genes are highly diverse and the genomic organizations of snoRNA genes are different in various species, it is also interesting and necessary to investigate how the synchronization of the RP and snoRNA expression is maintained in different species.

In this study, we systematically identified box C/D snoRNAs from four fission yeast genomes by combining experimental and computational approaches. By analyzing the motifs upstream of the box C/D snoRNA and RP genes in fission yeasts, we found that the Homol-D box mediates transcriptional coregulation of box C/D snoRNA and RP genes. Furthermore, transcriptional coregulation of box C/D snoRNA and RP genes is maintained in most *Ascomycota* species, among which both conserved and divergent features of transcriptional coregulation are observed.

RESULTS

Systematical identification of box C/D snoRNAs in fission yeast genomes by combining experimental and computational approaches

After screening of a *S. pombe* cDNA library generated from nuclear small RNA molecules, 44 cDNA sequences were assigned to the box C/D snoRNAs according to sequences and structural motifs (Supplemental Table S1). Among these, 16 cDNA sequences have been annotated by other studies (Bachellerie et al. 1995; Samarsky et al. 1996; Wood et al. 2002), and the remaining 28 snoRNAs were previously deposited to GenBank by us and are characterized here (Supplemental Table S2). To further confirm the stable accumulation of snoRNAs, Northern blot and reverse transcription were performed with *S. pombe* total cellular RNA and oligonucleotides specific for 23 random selected snoRNAs. As shown in Figure 1A and Supplemental Figure S1A, all the tested snoRNAs were positively detected in Northern blot. In reverse transcription analyses, a major cDNA product was obtained for each snoRNA (for examples, see Fig. 1B; more results can be found in Supplemental Fig. S1B). Since the primers were designed near the 3′ ends of the RNA species, the cDNA products in reverse transcription assays are a few nucleotides shorter than the RNA molecules detected by northern hybridization.

According to the antisense element conserved among organisms, a comparison with known snoRNAs from various organisms showed that of the 44 box C/D snoRNAs in

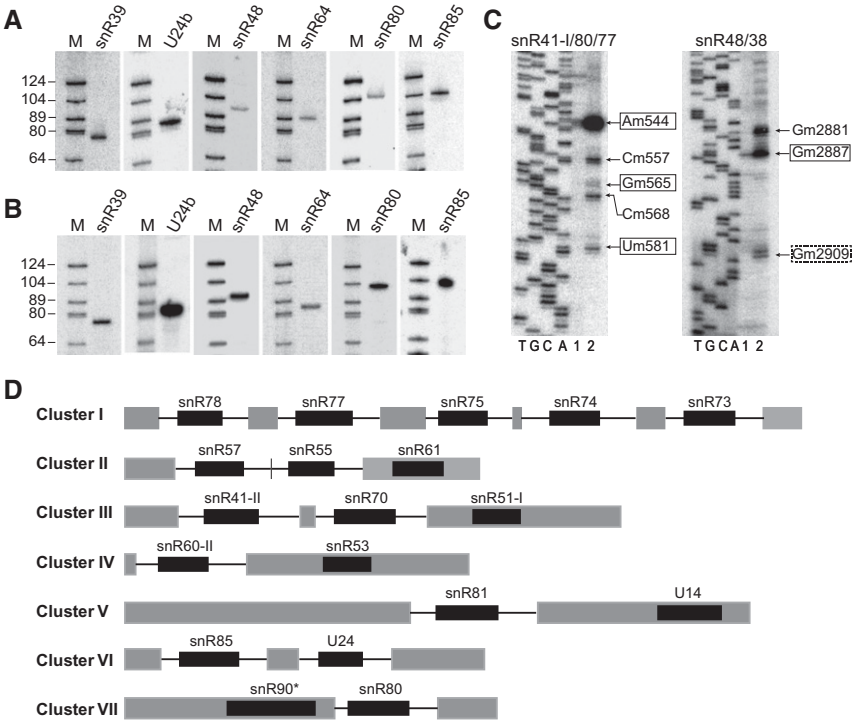

FIGURE 1. Characterization of *S. pombe* box C/D snoRNAs. Northern blot detection (*A*) and reverse transcription analyses (*B*) for selected box C/D snoRNAs: Lane M shows molecular weight markers. (*C*) Determination of selected rRNA methylation sites by primer extension; lanes T, G, C, and A show the rDNA sequence ladder; lane *1* is the control reaction at 1.5 mM dNTP; lane *2* is the primer extension at 4 μM dNTP. Arrows and solid boxed coordinates indicate potential methylation sites predicted by the novel snoRNAs. Arrows and dashed boxed coordinates indicate methylation sites modified by the previously known snoRNAs. Arrows without box refer to the modification sites with unknown molecules. (*D*) A schematic of snoRNA gene clusters (from transcription start site [TSS] to the last transcription terminating site [TTS] of each cluster). The exons (gray-filled rectangle), snoRNAs (black-filled rectangle), and introns (straight line) were drawn using the same scale. *snR90** is a box H/ACA snoRNA reported previously (Li et al. 2005). *snR57* and *snR55* are within two introns in juxtaposition without intercalating exon, and this special genomic organization has been reported previously (Mitrovich et al. 2010).

S. pombe, 40 have homologs in budding yeast *S. cerevisiae*, *Arabidopsis thaliana*, or humans, implying a conserved function of box C/D snoRNAs throughout the course of evolution (Supplemental Table S2). Although most of the *S. pombe* box C/D snoRNAs have functional homologs in *S. cerevisiae*, *A. thaliana*, and human genomes, the sequences of homologous snoRNAs generally have no significant similarity, except for the box C and box D motifs and their complementarity to target RNAs.

Based on the relationship between structure and function, using the D/D′ box plus 5 nt rules (Kiss-László et al. 1996), we predicted 32 rRNA methylation sites and 1 *U6* methylation site for these novel snoRNAs (Supplemental Table S2). Deoxyribonucleotide triphosphate (dNTP) concentration-dependent primer extension assays were employed to confirm the rRNA methylation sites. We identified a total of 36 methylation sites, of which 17 were newly methylated nucleotides guided by snoRNAs, seven were previously reported sites for snoRNAs *snR38*, *snR39*, *U24b*, *snR82*, *snR69*, and *snR56* (Supplemental Table S2), and 12 were novel sites but had no corresponding snoRNAs assigned (Fig. 1C; Supplemental Fig. S1C). Of the methylation sites identified, 26 are conserved in humans, plants, and budding yeast. Three sites (18S Gm565, 18S Am768, and 25S Am949, guided by the newly discovered *snR80*, *snR84*, and *snR83*, respectively) are not found in humans, plants, and budding yeast.

Genomic analysis of box C/D snoRNA genes identified in *S. pombe* revealed that most are intron-encoded (Supplemental Table S2). Notably, of the 40 intronic snoRNAs in *S. pombe*, only seven are located in the introns within the open reading frames (ORFs) of protein-coding genes, and the remaining are all found in the introns of either untranslated regions (UTRs) of protein-coding genes or noncoding RNA genes. Seven polycistronic RNA genes composed of 18 box C/D snoRNA genes and one box H/ACA gene were found, one of which was previously reported (Li et al. 2005). We termed these polycistronic RNA genes cluster I, II, III, IV, V, VI, and VII, respectively (Supplemental Fig. S2). All tandemly arrayed snoRNA genes within the same cluster are oriented on the same strand and arranged in a head-to-tail fashion (Fig. 1D), and each cluster was transcribed as a polycistronic transcript with a single transcriptional start site (TSS) (Supplemental Fig. S3A) and poly(A) tail (Supplemental Fig. S3B, C). These polycistronic transcripts have no protein-coding potential and resemble the U snoRNA host gene (UHG) structures discovered in mammals (Tycowski et al. 1996).

As several genomes of other fission yeasts have recently been sequenced (Rhind et al. 2011), we used a homology search to predict box C/D snoRNAs in these fission yeast genomes. Homologous genes of all 44 *S. pombe* box C/D snoRNAs were found in *Schizosaccharomyces octosporus*, *Schizosaccharomyces japonicus*, and *Schizosaccharomyces cryophilus*, except *snR80* and *snR83*, which were not identified in *S. japonicus* (Supplemental Data set S1), indicating that most box C/D snoRNAs are well conserved in fission yeasts.

Homol-D box mediates the transcriptional coregulation of box C/D snoRNA and RP genes in fission yeasts

A previous study showed that the Homol-D box mediates coordinated RP gene expression in *S. pombe* (Witt et al. 1993). Recently, the Homol-D box was demonstrated to also control the transcription of *U3*, a box C/D snoRNA (Nabavi and Nazar 2008). These results raised the possibility that the same motif, the Homol-D box, may synchronously control the transcription of box C/D snoRNA and RP genes in *S. pombe* (Dieci et al. 2009). Since it is not enough to prove this hypothesis based on some specific genes, we first built a position weight matrix from previously identified sites (Tanay et al. 2005) to identify the Homol-D box and examined whether this motif was enriched upstream of box C/D snoRNA and RP genes on a genome scalar.

In *S. pombe*, there are four snoRNAs located within the introns of four RP genes. Homol-D box is upstream of three of these RP genes. Since these intronic snoRNAs are likely cotranscribed with their host RP genes, we excluded these snoRNAs and their host RP genes from Homol-D box enrichment analysis for more solid conclusion. Compared to the randomized upstream sequences, the Homol-D box was indeed significantly overrepresented upstream of both box C/D snoRNA and RP genes. As shown in Figure 2A, ~50% of the box C/D snoRNA and RP genes were associated with the Homol-D box, compared to only 11.6% protein genes in *S. pombe* genome (Fig. 2C). These results indicated that the Homol-D box can coordinate the transcription of box C/D snoRNA and RP genes in *S. pombe*. We performed the same motif enrichment analysis on the box C/D snoRNA and RP genes in three other fission yeast genomes and found that the Homol-D box was also simultaneously overrepresented in upstream regions of box C/D snoRNA and RP genes (Fig. 2A,C), suggesting that the coregulated transcriptional control of box C/D snoRNA and RP genes mediated by the Homol-D box is a conserved mechanism in fission yeast genomes.

It had been demonstrated that the Homol-D box mediates the transcription initiation of RP genes and is within 100 bp upstream of the ATG start codon (Witt et al. 1993). Indeed, the location of the identified Homol-D box of the RP genes in fission yeast genomes peaked in 100 bp regions. Interestingly, the Homol-D box associated with box C/D snoRNA genes in fission yeast genomes were also position-biased (Fig. 2B) and peaked in 200-bp regions, suggesting that the TSSs of box C/D snoRNA genes are within 200 bp upstream of matured snoRNAs in fission yeasts. This observation was supported by the fact that the TSSs of the identified snoRNA clusters were mostly (six of seven) within 200 bp upstream of the first snoRNA members (Supplemental Fig. S2).

To test whether the Homol-D box was functional, we selected cluster I, which represents other kinds of genomic organization (intronic and clustered snoRNA) compared to the independent transcript *U3* snoRNA, for experimental examination. We replaced regions that include the TATA-box

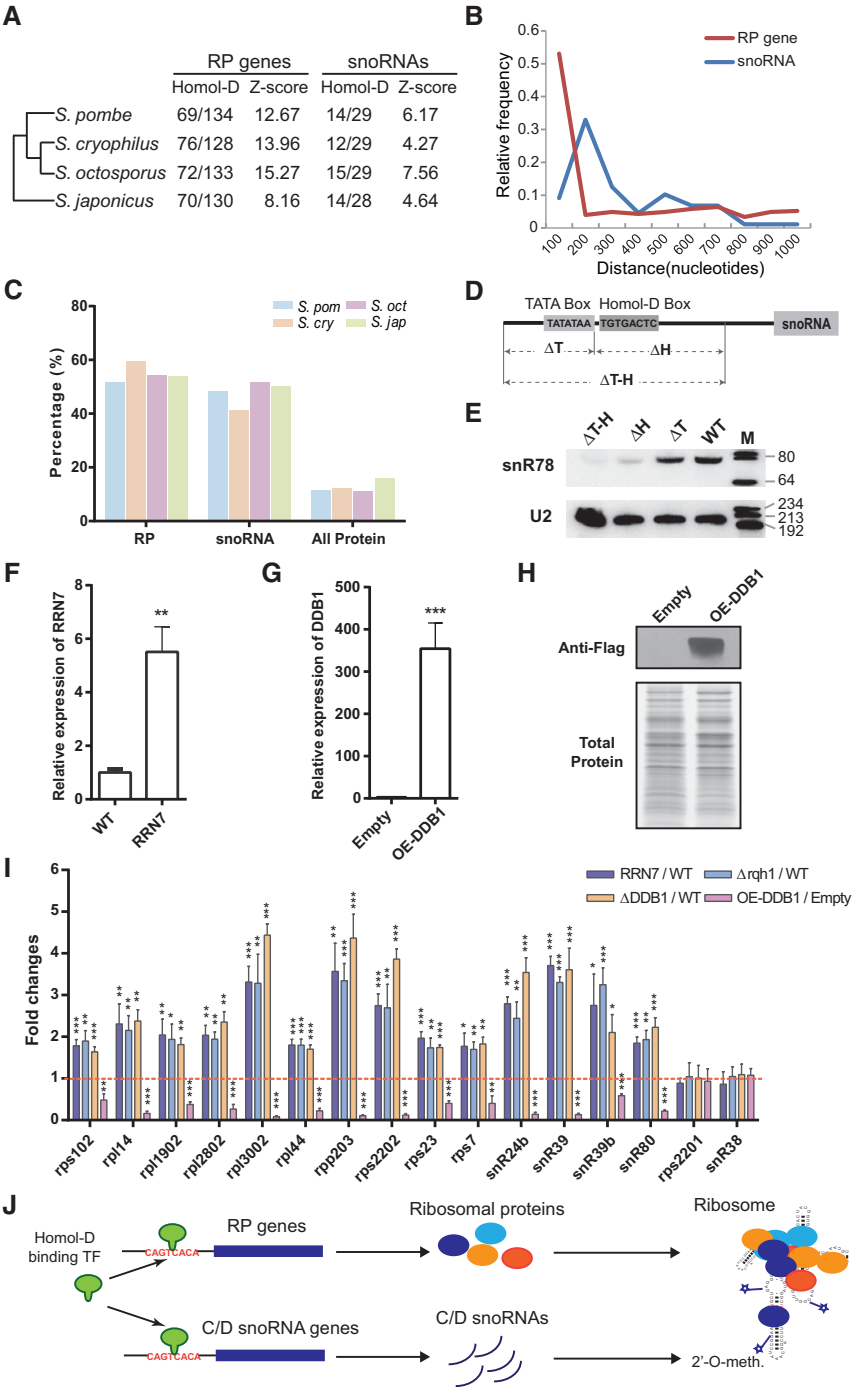

FIGURE 2. Homol-D *cis*-regulatory elements mediate the transcriptional coregulation of box C/D snoRNA and RP genes in fission yeasts. (*A*) Homol-D box was overrepresented upstream of box C/D snoRNA and RP genes in *S. pombe*, *S. cryophilus*, *S. octosporus*, and *S. japonicus*. *Z*-scores were calculated as described in Materials and Methods. (*B*) Distribution of distances from the Homol-D sites to the box C/D snoRNA and RP genes. (*C*) The percentages of RP genes, snoRNA genes, and genome-wide protein genes with Homol-D box in promoter regions in four fission yeasts. (*D*) Schematic diagram of deletions in the cluster I promoter region. (*E*) Northern blot: (WT) wild type *S. pombe*; (*ΔT*) TATATAA-box deleted strain; (*ΔH*) Homol D-box deleted strain; (*ΔT-H*) TATATAA-Homol D-box deleted strain; (M) molecular weight markers. *U2* was used as a control. (*F*) RRN7 gene relative expressions detected by real-time PCR. (WT) wild type; (*RRN7*) RRN7 up-regulated strain. (*G*) DDB1 gene relative expressions detected by real-time PCR; (*Empty*) the strain stably expressing HA tag vector; (*OE-DDB1*) DDB1 up-regulated strain stably expressing DDB1 protein-HA tag vector. (*H*) Western blot: (*Empty*) the strain stably expressing HA tag vector; (*OE-DDB1*) DDB1 up-regulated strain stably expressing DDB1 protein-HA tag vector. Anti-FLAG indicates FLAG-tagged DDB1 protein. The *lower* gel shows Coomassie blue-stained total proteins from samples. (*I*) Fold changes of random selected RP and snoRNA genes expression detected by real-time PCR when Homol-D binding proteins were changed. (*RRN7*/WT) RRN7 up-regulated strain compared to wild type; (*Δrqh1*/WT) rqh1 knockout strain compared to wild type; (*ΔDDB1*/WT) DDB1 knockout strain compared to wild type; (*OE-DDB1*/*Empty*) DDB1 overexpressed strain compared to empty vector strain. Error bars indicate SEM; two-sided student's *t*-test; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. (*J*) A model for Homol-D box mediating the transcriptional coregulation of box C/D snoRNA and RP genes.

only (*ΔT*), the Homol-D box only (*ΔH*), or the TATA-box-Homol-D box (*ΔT-H*) with a Kan^R selectable marker module (Fig. 2D). The results showed that the expression of snoRNA (*snR78*) in cluster I was nearly not affected by the deletion of the TATA-box, compared to the significantly lower level of *snR78* in *ΔH* and *ΔT-H* strains (Fig. 2E), indicating that the down-regulation of snoRNA expression in *ΔH* and *ΔT-H* strains is due to the deletion of the Homol-D box, which is critical for cluster I transcription. This result, along with previous studies, that Homol-D box could control the expressions of RP genes (Witt et al. 1993) and *U3* snoRNA (Nabavi and Nazar 2008), showed that Homol-D box could be an important functional *cis* element for both box C/D snoRNA and RP genes.

To further test whether RP and snoRNA genes could be coregulated when Homol-D box binding proteins were changed, we up-regulated or knocked out three known Homol-D binding proteins and then detected the primary transcript expression of Homol-D box bearing box C/D snoRNA and RP genes. RRN7, DDB1, and RECQL, homologous to RRN7/DDB1/rqh1 in *S. pombe*, were proved to be the binding proteins of Homol-D box in *S. pombe* and human, respectively (Rojas et al. 2011; Contreras-Levicoy et al. 2012). We up-regulated the essential gene RRN7's expression by deleting a 26-bp sequence within its 3′ UTR, overexpressed DDB1 protein by using a protein expression shuttle vector, and knocked out DDB1 and rqh1 genes by deleting a section of their exons (Supplemental Fig. S4). Then, we used real-time RT-PCR to investigate the primary transcript level of box C/D snoRNA and RP genes. We randomly selected 10 RP genes and four box C/D snoRNA genes for testing, compared with the negative controls rps2201 and *snR38*, upstream of which no Homol-D box were found. When expression of Homol-D box binding genes changed (Fig. 2F–H), all randomly selected RP and snoRNA genes showed significant and accordant changes in primary transcript expression levels, whereas two negative-control genes showed no significant changes (Fig. 2I). Furthermore, our results suggested that RRN7 induces the RP and snoRNA genes, whereas DDB1 and rqh1 repress these genes. These results indicated that the transcription factors bind to both Homol-D elements and control the transcription of both box C/D snoRNA and RP gene, implying this could be the regulatory mechanism that is employed to achieve the transcriptional coregulation.

Taken together, we propose a model in which the Homol-D box coordinates the transcription of box C/D snoRNA and RP genes in fission yeasts (Fig. 2J). Through regulation by the same *cis*-regulatory element, the fission yeast cells ensure a constant balance of box C/D snoRNA and RP expression levels.

Evolution of transcriptional coregulation between box C/D snoRNA and RP genes in *Ascomycota*

As the genome sequences and box C/D snoRNA genes have been systematically annotated in other nine *Ascomycota* species (*S. cerevisiae*, *Aspergillus fumigatus*, *Neurospora crassa*, *C. albicans*, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Kluyveromyces waltii*, *Yarrowia lipolytica*, and *Zygosaccharomyces rouxii*), we expanded our Homol-D box enrichment analysis to box C/D snoRNA and RP genes in these species. Although Homol-D box enrichment upstream of RP or snoRNA genes could be observed in five species other than fission yeasts (Fig. 3), Homol-D box enrichment was not simultaneously present upstream of both box C/D snoRNAs and RP, indicating that the coregulated transcription control of box C/D snoRNA and RP genes mediated by Homol-D box is unique to the fission yeasts.

Previous studies have shown that different species may use different transcription factors to coordinate the expression of RP genes (Witt et al. 1993; Lieb et al. 2001; Martin et al. 2004; Wade et al. 2004; Tanay et al. 2005; Hogues et al. 2008). It is possible that the coregulated transcription controls may involve different transcription factors in different species of *Ascomycota*. To test this hypothesis, we systematically identified the overrepresented motifs upstream of box C/D snoRNA and RP genes in 13 *Ascomycota* species and compared these motifs to the JASPAR Fungi Core TFBS database (Supplemental Table S3). Consistent with previous studies, we successfully identified the Homol-D box and TBF1 binding motif as the master *cis* elements of RP genes in *S. pombe* and *C. albicans* (Hogues et al. 2008), respectively. In contrast, there were no overrepresented motifs predicted in the shuffled sequences upstream of box C/D snoRNA and RP genes. These results showed that our identification approach could indeed discover bona fide overrepresented motifs. We observed coenrichment of the same motif upstream of box C/D snoRNA and RP genes in eight species (Fig. 4). Such motifs could be employed to synchronize expression of the box C/D snoRNA and RP genes.

Four interesting evolutionary patterns of transcriptional coregulation between species were observed. First, the same *cis*-regulatory elements have been used in different species

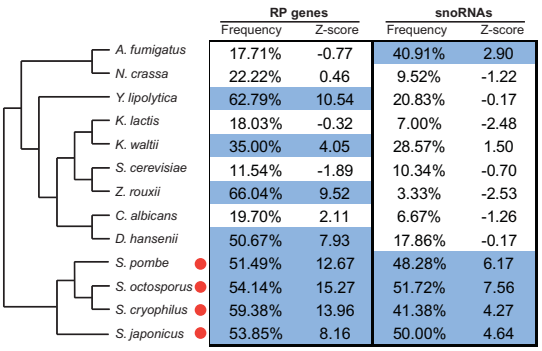

FIGURE 3. Homol-D sites discovered upstream of box C/D snoRNA and RP genes in 13 *Ascomycota* species. Significant Homol-D enrichment is colored; *Z*-score > 2.33. Transcriptional coregulation of box C/D snoRNA and RP genes mediated by Homol-D box was found in *S. pombe*, *S. octosporus*, *S. cryophilus*, and *S. japonicus* (indicated by red-filled circle).

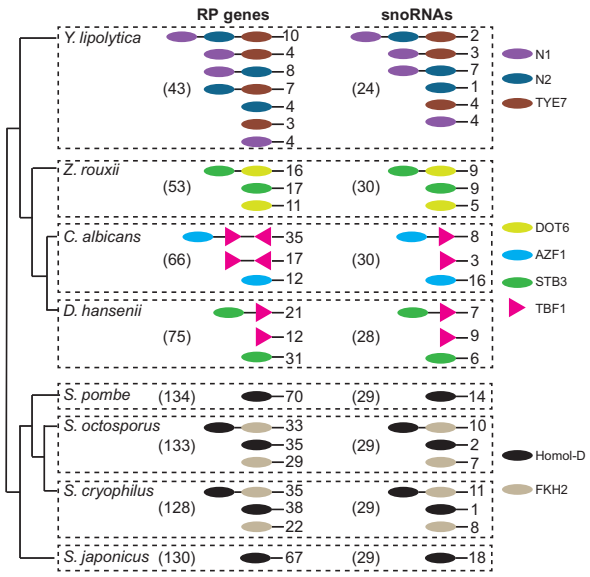

FIGURE 4. Evolution of *cis*-regulatory elements mediating the transcriptional coregulations of box C/D snoRNA and RP genes in eight *Ascomycota* species. A schematic phylogenetic tree (branches are not drawn to scale) representing the known phylogeny (Kurtzman and Robnett 2003) of the eight analyzed species is shown, together with the schematic *cis*-regulatory elements mediating the transcriptional coregulation of box C/D snoRNA and RP genes in each species. The total number of box C/D snoRNA or RP genes is given in parenthesis, and the number after each schema represents the number of box C/D snoRNA or RP genes that contain the corresponding motif. One putative binding transcription factor was listed for each motif. Two novel motifs found in *Y. lipolytica* were named N1 and N2, respectively. Multiple motifs were predicted in *C. albicans*, and only two motifs were shown. Full annotation of all motifs can be found in Supplemental Table S3.

to provide coregulated transcription control of box C/D snoRNA and RP genes. For example, Homol-D box have been used in four fission yeast species, and TBF1 binding elements have been used in *C. albicans* and *D. hansenii*. Second, even when the same elements are used, the spatial configuration of these elements was changed in box C/D snoRNA and RP genes in different species. For example, the TBF1 binding sites upstream of RP genes in *C. albicans* usually contained two palindromic TBF1 elements, whereas the TBF1 binding sites upstream of box C/D snoRNA genes contained only one TBF1 element. Such changes could also be observed between RP genes in *C. albicans* and in *D. hansenii*. Third, *cis* element substitution could arise in closely related species. For example, the TBF1 and AZF1 binding motifs in *C. albicans* changed to TBF1 and STB3 binding motifs in *D. hansenii*. Finally, *cis* elements may be lost or acquired during evolution. For example, the FKH2 binding motifs may have been acquired in *S. octosporus* and *S. cryophilus*, or may have been lost in *S. pombe* and *S. japonicus*. These patterns suggested that the regulatory networks of box C/D snoRNA and RP genes have evolved simultaneously, which maintain the transcriptional coregulations of these two components of ribosomal programs.

DISCUSSION

Coordinating the expression of ribosomal components is essential to ribosome biogenesis and is critical for cellular viability. However, our current knowledge about such coordinated expression in fungi is limited to the transcriptional networks that coordinate the RP genes (Witt et al. 1993; Lieb et al. 2001; Martin et al. 2004; Wade et al. 2004; Tanay et al. 2005; Hogues et al. 2008). The transcriptional networks controlling the regulation of other components of the ribosomal expression program are much less explored, and the coregulation of these different components in a genomic scalar is unexplored. By combining experimental and computational approaches, we have systematically identified the box C/D snoRNAs in four fission yeast genomes, including *S. pombe*, *S. octosporus*, *S. japonicus*, and *S. cryophilus*. We found that the expression of most box C/D snoRNA genes is orchestrated by Homol-D box. Most importantly, these fission yeast box C/D snoRNA genes were coregulated with RP genes by sharing the same *cis*-regulatory elements. Such a mechanism has the potential to ensure tight transcriptional coregulation of box C/D snoRNAs with RP genes. Box C/D snoRNAs and ribosome proteins are functionally coupled for ribosome biogenesis, and our results indicated that these two components of the ribosome biogenesis program are also coupled in their expression.

Although our analysis focused on box C/D snoRNAs, box H/ACA snoRNAs are likely to use the same mechanism to coregulate their expression with the expression of RP genes. The information content of motifs in box H/ACA snoRNAs is quite low and does not provide a sufficient basis for accurate prediction of box H/ACA snoRNA genes in *Ascomycota* genomes. Currently, box H/ACA snoRNAs have been systematically identified in only a few fungi species (Li et al. 2005; Schattner et al. 2006). Interestingly, a similar overrepresented motif discovery analysis performed on *S. pombe* box H/ACA snoRNAs indicated that the Homol-D box could also coordinate the expression of these snoRNAs (50% box H/ACA snoRNA genes bearing Homol-D box and the *Z*-score is 5.96), implying that coregulated transcriptional control between box H/ACA snoRNA and RP genes also exists. It will be interesting to expand such analysis to other box H/ACA snoRNAs when these gene annotations are available in other fungi species.

The transcriptional coregulation of box C/D snoRNAs and RP genes and the corresponding regulatory networks are tightly conserved within fission yeasts. The Homol-D box was found enriched upstream of box C/D snoRNA and RP genes in four fission yeasts but had diverged more in the regions upstream of box C/D snoRNAs than that of RP genes in nine other *Ascomycota* species (Fig. 3). Homol-D box has been also found in *D. melanogaster* (Ma et al. 2009), indicating that such *cis* elements may belong to an ancient regulation mechanism. It is likely that the divergence between species might be due to the loss of Homol-D box upstream of RP

and snoRNA genes. Furthermore, Homol-D box was diverged faster in upstream regions of C/D snoRNA genes than that of RP genes, indicated by the fact that Homol-D motif was enriched upstream of box C/D snoRNA genes in fewer species (Fig. 3). Such phenomenon may be due to the different selection pressures between RP and snoRNA genes.

The regulatory networks of ribosomal proteins are remarkably divergent in fungi (Tanay et al. 2005). For example, RAP1, FHL1, IHF1, and SFP1 have been found to regulate RP genes in *S. cerevisiae* (Lieb et al. 2001; Fingerman et al. 2003; Martin et al. 2004; Marion et al. 2004; Wade et al. 2004), TBF1 was found to be associated with RP gene promoters in *C. albicans* (Hogues et al. 2008), and the Homol-D box has been found to be involved in the transcriptional control of most ribosome proteins in *S. pombe* (Witt et al. 1993). Interestingly, the regulatory networks of box C/D snoRNAs have evolved to maintain the transcriptional coregulation between box C/D snoRNAs and RP genes. In *Ascomycota*, such coregulatory networks are divergent due to changes in the spatial configuration of binding sites, the substitution of *cis* elements and the gain or loss of *cis* elements, illustrating the extreme adaptability and flexibility of transcriptional regulatory networks. In some species, although overrepresented motifs are detected upstream of snoRNA or RP genes, the transcriptional coregulations are not identified. For example, TBF1 motifs are significantly overrepresented upstream of RP genes in *A. fumigatus* and *N. crassa* and are also significantly overrepresented upstream of snoRNA genes in *K. lactis* and *K. waltii*. RAP1 motifs are overrepresented upstream of RP genes in *S. cerevisiae*, *K. lactis*, and *K. waltii*. However, there are not any significant motifs for both RP and snoRNA genes in these species. The coordinated effects of snoRNA and RP genes in these species may be mediated by the collaboration of several transcription factors, causing them to be overlooked by our prediction procedure.

MATERIALS AND METHODS

Identification of box C/D snoRNA genes

Box C/D snoRNA cDNA library was constructed as described in Supplemental Methods. To identify candidate snoRNAs from three fission yeasts, we used BLAST to search for similarities to *S. pombe* snoRNAs in *S. octosporus*, *S. japonicus*, and *S. cryophilus* genomes (Rhind et al. 2011) with a weak cutoff (E-value ≤ 1). Blast hits were then extended 100 bp on both side, and box C (RUGAUGA, one mismatch allowed) and D (CUGA) motifs and conserved guide sequences (requiring at least 10 bp guide sequences conserved) were searched for. Sequences bearing box C and D motifs and conserved guide sequences were considered to be candidate snoRNAs.

Northern hybridization and reverse transcription analysis

Total RNA of wild-type and mutant-type strains was extracted using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). For Northern blot, 15 μg total RNA was analyzed by electrophoresis on 10% polyacrylamide/8 M urea gels, electrotransfered onto nylon membranes (Hybond-N+; Amersham), and followed by UV irradiation for 3 min on each side. Hybridization with 5′-labeled probes was performed as previously described (Qu et al. 1995). After overnight incubation at 42°C and washing, the membrane was exposed to a phosphor screen and analyzed by a Typhoon 8600 variable mode imager or by a STORM 820.

DNA contamination was removed with extensive DNaseI (TaKaRa) before reverse transcription analysis. Reverse transcription was carried out in a 20-μL reaction mixture containing 12 μg total RNA and 8 ng 5′-end-labeled primer in the presence of 250 μM dNTPs. After denaturation for 5 min at 65°C and cooling to 42°C, 200 units of M-MLV reverse transcriptase (Promega) were added and the extension carried out for 1 h at 42°C. Then the cDNAs were separated on 8% polyacrylamide, 8 M urea gels, and analyzed by a Typhoon 8600 variable mode imager.

Prediction and detection of methylated nucleotide sites in rRNA

Box C/D snoRNA target prediction was performed using the in-house snoRNA mining platform (snoRMP) (Chen et al. 2003; Huang et al. 2007), which is based on the SnoScan (Lowe and Eddy 1999) and SnoGPS (Schattner et al. 2006) algorithms. Target sequences were at least 10 nucleotides complementary (Watson-Crick and G:U base pairs) to an rRNA or snRNA sequence.

Ribose-methylated nucleotides of *S. pombe* rRNAs were detected by reverse transcription at low dNTP concentrations as follows: two reverse transcription reactions containing 5 μg total cellular RNA and 0.1 pmol oligodeoxynucleotides labeled at the 5′ end with [γ-^{32}P]ATP, carried out in the presence of either 4 μM or 1.5 mM dNTPs. For mapping the ribose methylation position precisely, an rDNA sequence ladder was prepared and used as a molecular weight marker. The rDNA fragments of *S. pombe* 18S and 25S rRNA were amplified by PCR with the primer pairs Sp18F/Sp18R and Sp25F/Sp25R, respectively, and then cloned into the pMD18-T vector. The plasmid's DNA insert was directly sequenced with the same primer used for rRNA methylation mapping and run in parallel with the reverse transcription reaction as a molecular weight marker.

Prediction of Homol-D sites upstream of snoRNA and RP genes

For *A. fumigatus*, *N. crassa*, and *S. pombe*, genome annotations were obtained from Ensembl (Kersey et al. 2012). For *Y. lipolytica*, *K. lactis*, *Z. rouxii*, and *D. hansenii*, genome annotations were from Génolevures (Sherman et al. 2009). For *S. octosporus*, *S. cryophilus*, and *S. japonicus*, annotations were from *Schizosaccharomyces* group database (Rhind et al. 2011). For *K. waltii*, *S. cerevisiae*, and *C. albicans*, annotations were obtained from Yeast Gene Order Browser (Byrne and Wolfe 2005), Saccharomyces genome database (Cherry et al. 2012), and Candida genome database (Inglis et al. 2012), respectively. A 1-kb flank sequence upstream of the RP genes were used to search for Homol-D sites. Box C/D snoRNAs in *S. cerevisiae*, *A. fumigatus*, *N. crassa*, *C. albicans*, *D. hansenii*, *K. lactis*, *K. waltii*, *Y. lipolytica,* and *Z. rouxii* were collected from previous studies—*S. cerevisiae*

from SGD (Cherry et al. 2012), *A. fumigatus* from Jöchl et al. (2008); *N. crassa* from Liu et al. (2009); and others from Mitrovich et al. (2010). A 1-kb flank sequence upstream of the mono-independent transcription snoRNAs, of the first snoRNA member (for snoRNA clusters), or of the host protein-coding genes (for snoRNAs coded in protein-coding gene introns) were chosen for Homol-D box searching. RP gene hosting snoRNAs and their host genes are excluded from analysis. Homol-D sites were predicted with a binding matrix based on sequences from a previous study (Tanay et al. 2005) using the following formula:

$$\text{score}(i) = \sum_b f_{b,i} \log_2 \frac{f_{b,i}}{p_b},$$

where i is the position within the site; p_b is the relative frequency of base b in the genome; and $f_{b,i}$ is the observed relative frequency of base b at that position (from the matrix). Scores were normalized to a 100-point scale. The scores of training sites were above 85, whereas the scores of shuffled training sites were below 75. Sites with scores greater than 85 and that matched the invariant Homol-D core consensus (AGTCAC) were considered to be predictions.

Assessment of overrepresentation of the Homol-D box

To assess the overrepresentation of Homol-D sites upstream of box C/D snoRNA and RP genes, the upstream sequences were shuffled through nine runs. The shuffled sequences were subjected to Homol-D site prediction. The significance of overrepresentation was calculated using the following:

$$Z\text{-score} = \frac{F_{\text{real}} - \text{Mean}_{\text{ran}}}{\text{Std}_{\text{ran}}},$$

where F_{real} is the frequency of genes bearing Homol-D motifs; Mean_{ran} is the mean frequency of genes bearing Homol-D box in shuffled sequences; and Std_{ran} is the standard deviations of nine-run shuffled sequences.

This final Z-score can be interpreted as the number of standard deviations above the mean raw score for the shuffled upstream regions of the box C/D snoRNA or RP genes. The Z-score cutoff was set to 2.33, corresponding to a P-value of 0.01.

Genomic deletions

The different nucleotide sequences deletions were performed as below. A DNA fragment containing the entire flanking sequences of the deletion region was amplified using PCR with specific primers. After purification, it was cloned into the pMD18-T simple vector (TaKaRa), which does not contain a restriction site. Using inverse PCR, the flanking sequences were amplified without the region to be deleted. After digestion by KpnI/SalI, the amplified fragment was linked with a 1.4-kb selectable marker module from pFA6-kanmx4 (Wach et al. 1994) at the corresponding restriction sites. The resulting plasmid contains two homologous flanking segments and a selectable marker between them and was linearized by PCR with specific primers to obtain a close to a 2-kb fragment. Then, the wild-type yeast cells were transformed by a lithium acetate procedure. Transformants were screened on selective YPD medium with G418 (200 μg/mL), and colonies that exhibited specific recombination were examined by PCR.

Gene overexpression

The coding sequences of DDB1 were amplified with specific primers, digested by SacI/ApaI, and cloned into the corresponding restriction sites of plasmid pAUR224 DNA (protein expression shuttle vector for *S. pombe*, TaKaRa). The HA tag sequences, amplified from a plasmid ptub-HA_N-terminal_with_features, which is a gift from Dr. Hugo D. Lujan (Prucca et al. 2008), were digested by ApaI/SmaI and then cloned into the corresponding restriction sites of the aforementioned plasmid. The tag sequences are located at the 3′ end of DDB1 coding sequences, ahead of TAA of DDB1. The empty plasmid only contains HA tag sequences without DDB1 coding sequences. The resulting plasmids were then transformed into the wild-type yeast cells by a lithium acetate procedure. Transformants were screened on selective YPD medium with Aureobasidin A (AbA) (200 ng/mL), and colonies that contained overexpression plasmid were examined by PCR.

Western blot and total protein analysis

Three milliliters yeast culture with an A_{600} of about 0.6–1.0 was centrifuged and the pellet resuspended in RIPA buffer (50 mM Tris-HCl, pH 8.0/150 mM sodium chloride/1% NP-40/0.5% sodium deoxycholate/0.1% sodium dodecyl sulfate/2 mM EDTA) containing 1× protease inhibitor cocktail (Roche). After rigorous votex for 3 min, total protein of empty and DDB1 overexpression strains was extracted. The yeast suspension was heated for 10 min to 99°C followed by centrifugation at 13,000 rpm for 1 min. Equivalent total protein extracts were loaded onto a SDS-PAGE gel. For Coomassie Blue staining, the gel was stained for 4 h, destained overnight with gentle agitation, and analyzed by Odyssey (LI-CDR). For Western blot, the proteins were transferred to a nitrocellulose transfer membrane (Whatman) after electrophoresis. Anti-FLAG antibody (H9658, Sigma) was used at 1:5000 dilution in this study. Immunoreactivities were determined using the ECL method (CST).

Real-time RT-PCR

Total RNA was reverse-transcribed to cDNA using the Primescript RT reagent kit with gDNA Eraser (Takara). Real-time PCR was performed using SYBR Premix ExTaq II (Takara) according to the manufacturer's instructions. The relative expressions of genes were normalized to ACTIN and were calculated using the comparative $2^{\Delta\Delta Ct}$ method.

Overrepresented motifs prediction

For each species, 1-kb flank sequences upstream of box C/D snoRNA and RP genes were pooled. RP gene hosting snoRNAs and their host genes are also excluded from this analysis. Overrepresented motifs in pooled sequences were discovered by RSAT peak-motifs pipeline (Thomas-Chollier et al. 2012a,b), including position-analysis, oligoanalysis and dyad-analysis. Criteria for overrepresented motifs prediction were as follows: Sig score > 10, motif is presented in >30% RP and >30% box C/D snoRNA genes, and motif is presented in >50% RP or >50% box C/D snoRNA genes. Detected motifs were compared against the JASPAR core Fungi database (Portales-Casamar et al. 2010) for known TF binding motifs.

Primers and oligonucleotides

The sequences of the primers and oligonucleotides used in this study are listed in Supplemental Table S4.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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