Characterization of the fission yeast ribosomal DNA binding factor: components share homology with Upstream Activating Factor and with SWI/SNF subunits

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ABSTRACT

A ribosomal DNA (rDNA) binding activity was previously characterized in fission yeast that recognized the upstream ribosomal RNA (rRNA) gene promoter in a sequence specific manner and which stimulated rRNA synthesis. It was found to share characteristics with *Saccharomyces cerevisiae*’s Upstream Activating Factor (UAF), an RNA polymerase I (pol I) specific transcription stimulatory factor. Putative fission yeast homologs of the *S.cerevisiae* UAF subunits, Rrn5p and Rrn10p, were identified. The *Schizosaccharomyces pombe* rDNA binding activity/transcriptional stimulatory activity was found to co-fractionate with both SpRrn5h and SpRrn10h. Analysis of polypeptides interacting with SpRrn10h uncovered a 27 kDa polypeptide (Spp27) homologous to a SWI/SNF component (now known to be homologous to Uaf30p). The contributions of the *S.pombe* and *S.cerevisiae* upstream rDNA promoter domains were assessed in cross-species transcriptional assays. Furthermore, comparative genomic analysis revealed putative Rrn5p, Rrn10p, Rrn9p and p27 homologs in multiple non-vertebrates. The *S.pombe* rDNA binding activity is proposed to be an RNA pol I specific SWI/SNF type factor.

INTRODUCTION

During active cellular growth, synthesis of the large ribosomal RNAs (rRNAs) accounts for nearly half of total RNA synthesis. This high level of rRNA synthesis is driven, in part, by the action of ribosomal DNA (rDNA) transcription factors that associate with the upstream region of rRNA gene promoters and/or intergenic regulatory sequences to mediate stimulation of this synthesis (1–3). In vertebrates, upstream binding factor (UBF), an HMG box protein, is such a factor that associates with rDNA promoters (4) and enhancers (5,6) in a sequence tolerant fashion (7) to increase levels of synthesis up to ~50-fold. UBF functions as a dimer (8); its activity is modified by phosphorylation (9,10), by acetylation (11,12) and by interaction with Retinoblastoma protein (13).

In baker’s yeast, an RNA polymerase I (pol I) transcriptional stimulatory factor, Upstream Activating Factor (UAF), was characterized both genetically and biochemically as being important for synthesis of the large rRNAs (14,15). It was found to stably associate with upstream rDNA promoter sequences in a sequence-specific manner and to consist of six subunits: Rrn5p, Rrn9p, Rrn10p, p30 (Uaf30p), and Histones H3 and H4 (14–16). Lesions in *RRN5*, *RRN9* or *RRN10* resulted in a severe decrease in growth rate and in synthesis of large rRNAs (15) and in an increase in switching of the catalytic enzyme for rRNA synthesis from RNA pol I to RNA polymerase II (pol II) (17).

It was unclear whether UBF was present in non-vertebrates or in lower eukaryotes and whether a UAF activity was present in higher eukaryotes. This study focuses on analysis of an rDNA binding factor in fission yeast that interacts specifically with the *Schizosaccharomyces pombe* upstream rDNA promoter and that increases levels of rRNA synthesis (18). Since it shares characteristics with *Saccharomyces cerevisiae* UAF, a search was conducted to identify potential *S.pombe* components of the rDNA binding factor based on homology to Rrn5p, Rrn9p and Rrn10p. Putative homologs were identified for Rrn5p and Rrn10p, and epitope-tagged versions were engineered for investigating molecular interactions and activities leading to species-specific activation of rRNA synthesis.
MATERIALS AND METHODS

Identification of putative SpRrn5 and SpRrn10 homologs

Database searches of the S. pombe genome (Wellcome Trust Sanger Institute) uncovered putative homologs of S. cerevisiae Rn5p and Rn10p: SpRrn5h (S. pombe Rn5p homolog, accession no. Q10360) and SpRrn10h (accession no. O14013, putative Rn10p homolog). Searches for putative homologs of the S. pombe SpRrn5h and SpRrn10h (and later S. pombe p27) were also conducted in other available databases [those at NCBI; Neurospora crassa database at the Whitehead Institute; databases at DOE Joint Genome Institute including white rot fungus (Phanerochaete chrysosporium); Aspergillus fumigatus at TIGR; Candida albicans at the Stanford Genome Technology Center website, http://www-genome.stanford.edu/group/candida; Cryptococcus neoformans at C.neoformans Genome Project, Stanford Genome Technology Center and The Institute for Genomic Research (TIGR); and the Genolevures database (19) (http://cbi.labri.u-bordeaux.fr/Genolevures/advanced_blast.php3) using BLASTP, position-specific iterated BLAST (PSI-BLAST; 20) and TBLASTN. Putative homologs of SpRrn5h were identified in N. crassa [686 residues, contig 1.115, Neurospora Sequencing Project. Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu; V. 3/12/01)], white rot fungus (504 residues, Scaffold 65, DOE Joint Genome Institute), A. fumigatus (preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org/); C.neoformans (399 residues, CN11020013 AA, database: cneo_orfs011005), K. marxianus (accession no. AL424621) and K. marxianus lactis (accession no. AL426913). Putative Rn10p homologs include: C. albicans Rn10h (Contig 6-1536), N. crassa (contig 2.273), A. fumigatus (contig 690), Saccharomyces bayanus (AL402119), K. lactis (AL428395), K. marxianus (AL422400) and Pichia farinosa (AL141722). Multiple protein alignments were conducted using ClustalW (21) [http://searchlauncher.bcm.tmc.edu (Baylor College of Medicine)] and displayed using Boxshade (http://www.ch.embnet.org/).

Construction of pREP-Sp-MH-Rrn5h, pREP-Sp-FLAG-Rrn10h and pREP-Sp-MH-Rrn10h

The forward and reverse primers for PCR amplification of SpRrn5h were 5′-CCCATATGTTCTTCATCTCTTCAAAACGGG and 5′-GAGATCTCTATTTTAAAGGGAGTGAC, and the template was an S. pombe pACT cDNA library (22; purchased from American Type Culture Collection). For cloning in general, PCR products were first inserted into pCR2.1-Topo™ (Invitrogen) before sub cloning into the target vector. The SpRrn5h PCR product was isolated as an NdeI/BglII fragment and ligated into pREP42MH N (23) cleaved with NdeI and BamHI. Coding sequences of the putative S. pombe Rn10 homolog were amplified from a S. pombe pACT cDNA library (since the coding sequences are interrupted by an intron) using the primers SpR10f9 GCCATATGGCATCAAAATCCACC- AACTCG and SpR10re2, CGGATCCTCATCTTTTC- AATTC. The PCR product was isolated as an NcoI-partial BamHI fragment and cloned into pREP-HA-FLAG cleaved with NcoI and BamHI. To construct pREP-HA-FLAG, coding sequences for the HA and the FLAG epitope tags were amplified from pGEM:HA-FLAG (kindly provided by Drs Alexander Hoffman and R. Roeder) with 5′-CCGCGTCGACTTCATGTCGACGATGCTC and 5′-GGAATTCCA- TATTGGAATCCC. This fragment was cleaved with NdeI and SalI, purified, and ligated to NdeI/SalI cleaved pREP, creating pREP-HA-FLAG. pREP-Sp-MH-Rrn10h was made by inserting a PCR fragment (primers SpR10f9 GCCATATGTTCTTCATCTCTTCAAAACGGG and SpR10re9 CGGATCCTCATCTTTTC- AATTC) into NdeI/BamHI cleaved pREP42MH N.

Preparation and fractionation of extracts from fission yeast

Whole cell extracts (WCEs) were prepared from ~5 l of MP6-10b pREP-Sp-FLAG-Rrn5h and pREP-Sp-MH-Rrn5h (‘Sp-MH-Rrn5h/Sp-FLAG-Rrn5h’ cells grown in selective Edinburgh minimal medium (24), as well as MP6-10b pREP-Sp-FLAG-p27 and pREP-Sp-MH-Rrn5h (‘Sp-FLAG-p27/Sp-MH-Rrn5h’) or pREP-Sp-MH-Rrn10h (‘Sp-FLAG-p27/Sp-MH-Rrn10h’). Control WCEs were prepared from 2.5 l of cells expressing just Sp-MH-Rrn5h, Sp-FLAG-Rrn10h or Sp-MH-Rrn5h. The extract proteins (94.1 mg, Sp-MH-Rrn5h/Sp-FLAG-Rrn10h; 89.1 mg, Sp-FLAG-p27/Sp-MH-Rrn5h; and 78 mg, Sp-FLAG-p27/Sp-MH-Rrn10h) were fractionated by ammonium sulfate precipitation, the pellet recovered and dialyzed, and proteins were then subjected to anion exchange chromatography using three 5 ml HiTrapQT™ columns (Amersham Pharmacia Biotech) as described (18). The columns were developed using a KCl step gradient [0.1 M, 0.35 M, 0.7 M KCl–column buffer (CB: 25 mM HEPES pH 7.9, 0.2 mM EDTA, 5 mM MgCl2, 20% glycerol, 1 mM PMSF and 1 mM DTT)]. All buffers contain PMSF and DTT, added just prior to use. The RNA pol I transcription activity eluted with 0.35 M KCl–CB. Peak fractions were pooled (27 mg total for ‘SpRrn5/SpRrn10h’; 31 mg for ‘SpRrn5h’; and 24 mg for ‘SpRrn5h’), converted to 1× wash buffer (WB: 25 mM HEPES pH 7.9, 0.15 M KCl and 20% glycerol) and rotated with 1 ml anti-FLAG™ M2 affinity matrix (Kodak/IBI) or 1 ml Ni-NTA agarose matrix (Ni-nitrilotriacetic acid; Qiagen; 1× WB, 0.1% NP-40) for 2 h at 4°C, followed by transfer to a column. For the M2 affinity matrix: following five, 1 ml washes with 1× WB, polyepides were eluted with 1× WB–125 μg/ml FLAG peptide (five ×1 ml). For the nickel affinity matrix, the column was washed five times with 1 ml WB, 0.1% NP-40, 10 mM imidazole, and myc-his-tagged proteins were eluted with 1× WB, 0.1% NP-40, 0.3 M imidazole. Approximately 30 mg of the control extracts (MP6-10b expressing just Sp-MH-Rrn5h; Sp-FLAG-Rrn10h or Sp-MH-Rrn10h) were fractionated as above and in Figure 2D [Sp-FLAG-Rrn10h (Fig. 2C)] or in Figure 7G [Sp-MH-Rrn5h (Fig. 7C) or Sp-MH-Rrn10h (Fig. 7F)] but using one 5 ml HiTrapQ matrix. Concentration and buffer exchange was performed by ultrafiltration using Centricon 10 units.

For western analysis, fractions were resolved on a 16 or 12% SDS–polyacrylamide gel and transferred to nitrocellulose membrane. Concentrations of fractions (in μg/ml) of Figure 2A and B (Sp-MH-Rrn5h/Sp-FLAG-Rrn10h) were Hi Trap Q (Q)—Q-0.1, 1.8 μg/μl; Q-0.35, 2.0; Q-0.7, 0.8; Ni-NTA FT, 1.3; Ni-NTA El, 0.1; Figure 2C (Sp-FLAG-Rrn10h): Q-0.1, 1.9 μg/μl; Q-0.35, 1.8; Q-0.7, 0.6; Ni-NTA FT, 1.3;
Ni-NTA El, <0.1; Figure 7A and B (Sp-FLAG-p27/Sp-MH-Rrn5h); Q-0.1, 2.2 µg/µl; Q-0.35, 2.1; Q-0.7, 0.9; M2 aff.-FT, 1.5; M2 aff.-El, <0.1; Figure 7C (Sp-MH-Rrn5h); Q-0.1, 2.2 µg/µl; Q-0.35, 2.3; Q-0.7, 0.7; M2 aff.-FT, 1.6; M2 aff.-El, <0.1; Figure 7D and E (Sp-FLAG-p27/Sp-MH-Rrn10h), Q-0.1, 2.0 µg/µl; Q-0.35, 1.9; Q-0.7, 0.7; M2 aff.-FT, 1.5; M2 aff.-El, <0.1; Figure 7F (Sp-MH-Rrn10h): Q-0.1, 2.1 µg/µl; Q-0.35, 1.8; Q-0.7, 0.6; M2 aff.-FT, 1.6; M2 aff.-El, <0.1. Either anti-FLAG, M2 monoclonal antibody (Kodak/IBI; for Sp-FLAG-Rrn10h and Sp-FLAG-p27) or anti-myc monoclonal antibody [9E10 (BabCo); Sp-FLAG-Rrn10h and Sp-FLAG-p27] was used at 1:1500 dilution for immunodetection as described (25), with the secondary peroxidase linked anti-mouse antibody at 1:5000 dilution. Antibody/polypeptide complexes were detected with Enhanced Chemiluminescent reagents (Amersham Pharmacia Biotech). Polypeptide size markers were Kaleidoscope and broad range standards (Bio-Rad).

Two-hybrid analysis

For cloning into the parental vectors pGAD and pGBDU (26), SpRrn5h sequences were amplified using 5'-GGATT-CATGTCTTCTTCAATAAAACGGATATAATG and 5'-TGAAGATCTTTTTTAAGGGAGTGACAAC. The PCR fragment was isolated as an EcoRI/BglII fragment and inserted into pGAD-C1, creating pGAD-SpRrn5h, while SpRrn10h cDNA was amplified using CGAATTCACTGTAATACTCCA-CCAACTCGGC and CTGCTGACCTCATCTTTCAATAATC. It was inserted into EcoRI/Sall cut pGAD-C1 and pGAD-C1, creating pGAD-SpRrn10h and pGAD-SpRrn10h. Plasmids were transformed into PJ69-4A (MATa trpl-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-AD2 LYS2::GAL1-HIS3 met2::GAL7-lacZ (26), and activation of the three reporters, ADE2, HIS3 and lacZ, was assessed. PJ69-4A pGAD-SpRrn10h was transformed with an S.pombe Matchmaker™ cDNA library (in pGAD; Clontech), to yield 5.5 million Ura+ transformants. Transformants that independently activated all three reporters (i.e., ADE+ and activated beta galactosidase) were further assessed. (M.Tripp and L.Pape, manuscript in preparation). One of interest contained coding sequences for a predicted 27 kDa polypeptide (Sanger Centre, SPCC285.17).

Construction of pREP-Sp-FLAG-p27 and full-length pGBD-Spp27

The primers used to amplify full-length S.pombe p27 (Spp27) sequences from a S.pombe pACT cDNA library (to amplify the three introns) were 5'-CCCATGGCCGAGGAGTACGAAACAGAC and 5'-CCGATGCTCTCAAGCCCTGGATTCCGGCG. The resultant NotI/BamHI fragment was cloned into pREP-HA-FLAG cut with the same enzymes. The primers 5'-CGGATCATGGAGAGTACGAAACAGAC and 5'-CGATGCTCTCAAGCCCTGGATTCCGGCGTAG were used to amplify Spp27 cDNA for insertion into BamHI and BglII cleaved pGBD-C1 as a BamHI/BglII fragment. The resultant plasmid was pGBD-Spp27.

Alignment of S.pombe p27/S.cerevisiae Uaf30p homologs

Putative S.pombe p27 (AL031545) homologs include: white rot fungus (P. chrysosporium), DOE JGI, Scaffold 27; N.crassa p27 homolog, contig 1.821, NCU02204.1, Neurospora Sequencing Project, Whitehead Institute/MIT Center for Genome Research (www-genome.wi.mit.edu; V. 3/12/01); Drosophila melanogaster p27 homolog, AAF47684.1; Arabidopsis thaliana AAD43149.1 (386 amino acid); AAL24145 (385 amino acid) BABA1706 (452 amino acid), and ~13 additional ones; Bombyx mori (AU006413.1); S.cerevisiae Uaf30p (YOR295W; 16), Caenorhabditis elegans p27 homolog (AA50726.1); Orzya sativa (AAK63939.1); Apis mellifera (bee; BI516000); Hordeum vulgare (BF621939); Chlamydomonas reinhardtii (B1528153); Chlamydia muridarum (NP_297118). Additional putative p27 homologs include cotton, tomato, corn, moss, soybean, C.albicans, A.funigatus, Debaryomyces hansenii, etc.

Electrophoretic mobility shift analysis with SpRrn5h/SpRrn10h fraction

A 32P-5'-end-labeled fragment (0.01 pmol) bearing the S.pombe rDNA promoter (~243 to +31; labeled at the BamHI and EcoRI sites in p37A+31 and electrophoretically purified) was incubated with 7.5 µl of immunopurified Sp-FLAG-Rrn10h/Sp-MH-Rrn5h and associated polypeptides (~60 ng/µl) for 30 min at 25°C. Competitive binding reactions contained purified, unlabeled fragments bearing S.pombe rDNA reporter sequences (~150, ~150 to +89; ~84 to +89; ~57 to +89 isolated from p57Δ5-150, p57Δ84, and p57Δ57, or a 190 bp pBS SK* HpalI fragment) at a 10- or 30-fold molar excess to the labeled rDNA promoter fragment. Electrophoresis was as described (18) except that the gel was 4% acrylamide-0.068% bisacrylamide-1× gel shift buffer-5% glycerol.

Construction of S.cerevisiae and S.pombe rDNA templates

The S.pombe wild-type template p37A+31, containing a full promoter from ~243 to +31, was previously described (27,28). An analogous full-length S.cerevisiae rDNA template was constructed by PCR amplification of sequences between ~243 and +44 with ScF-243 5'-CCGAGATCTCTT-CCGCAGTAAAAAATAG-3' and ScR+44 5'-CCGGAAATCCGGTTTCTTCTTCTC-3'. Template DNA was YEP88 (kindly sent by Dr J. Warner; 29). The fragment was cleaved with BamHI and EcoRI, gel isolated, and ligated between the BamHI and EcoRI sites of pBS SK* to yield Sc-243/+44. PCR amplifications were cycled at: 94°C, 1 min; 55°C, 2 min; 72°C, 2 min for 27 cycles. A BamHI-PstI fragment containing sequences from ~243 to ~56 of the S.cerevisiae rDNA promoter was amplified with ScF-243, and ScR-56, 5'-AGAAGTGCAGTCCTCCATTACAATGAAATCG-3' and S.cerevisiae rDNA template was amplified with ScF-243- and ScR-56, 5'-AGAAGTGCAGTCCTCCATTACAATGAAATCG-3'. Schizosaccharomyces pombe rDNA sequences from ~49 to +31 were amplified with SpF-49 5'-AGAAGTGCAGTCCTCCATTACAATGAAATCG-3' and SpR+31 as a template. The PCR product was cleaved with PstI, isolated and ligated into PstI cleaved pSc-243-56. The orientation of the S.pombe rDNA was assessed by dyeoxy sequence analysis. The primers ScR44 (see above) and ScF-50, 5'-AGAAGTGCAGTCCTCCATTACAATGAAATCG-3' were used to amplify the S.cerevisiae core rDNA promoter (~50 to +44); the fragment was cleaved with PstI and EcoRI and inserted into pBS SK* linearized with these same enzymes, to create ‘Sc core’.
Characterization and molecular tagging of SpRrn5h and SpRrn10h

Alignment of a putative fission yeast Rrn5p homolog with S.cerevisiae Rrn5p is shown in Figure 1A. The putative S.pombe Rrn5p homolog (SpRrn5h, 556 residues) has a calculated molecular weight of 64.0 kDa and a 147 amino acidic C-terminus not present in S.cerevisiae Rrn5p. The S.cerevisiae Rrn5p and putative S.pombe homolog share 28% identical and 41% similar residues over 176 amino acids (E-value, 6 × 10⁻⁵ in a PSI-BLAST search of fungal sequences with S.cerevisiae Rrn5p as query). In addition, SpRrn5h was shown to localize to the nucleolus (31). SpRrn5h contains a domain of particular interest: a SANT domain, located between residues 71 and 112 (E-value, 1 × 10⁻⁴, RPS-BLAST). The SANT domain was originally identified from SWI3, ADA2, N-CoR and TFIIIB and it overlaps a myb domain (32).

When fungal protein coding sequences are queried with S.cerevisiae Rrn10p, a putative S.pombe homolog (SpRrn10h) is aligned, yielding an E-value of 1.4 [PSI-BLAST (20)]. Although this value is less than threshold, the putative SpRrn10h is the top match besides Rrn10p itself (see Fig. 1B). The putative SpRrn10h shares 29% identical and 49% similar residues with S.cerevisiae Rrn10p over 55 residues. Furthermore, when fungal sequences are searched with SpRrn10h as the query, the S.cerevisiae Rrn10p is the top match after SpRrn10h (E-value, 0.61, PSI-BLAST).

Sp-FLAG-Rrn10h cofractionates with Sp-MH-Rrn5h

A fission yeast strain, MP6-10B pREP-Sp-MH-Rrn5h pREP-Sp-FLAG-Rrn10h, was engineered to express N-terminal myc-epitope and poly-histidine (MH)-tagged SpRrn5h fusion protein and FLAG-tagged SpRrn10h. Fractionation of WCE from these cells was conducted to determine whether the putative SpRrn5h and SpRrn10h copurified with each other, with transcriptional stimulatory activity, and/or with rDNA binding activity (Fig. 2D). Western analysis of WCE proteins revealed an anti-myc antibody immunoreactive polypeptide with an apparent molecular weight of ~75 kDa (Fig. 2A, lane 1), corresponding to Sp-MH-Rrn5h (its calculated molecular weight is 68.3 kDa), while a 15.1 kDa polypeptide, corresponding to Sp-FLAG-Rrn10h (calculated molecular weight, 14.5 kDa), was detected with M2 (anti-FLAG) monoclonal antibodies (Fig. 2B, lane 1). Neither of these immunoreactive polypeptides was detected in WCE prepared from control S.pombe cells (data not shown).

WCE proteins were subjected to ammonium sulfate precipitation, followed by fractionation on the anion exchange matrix, HiTrapQ™, and the nickel affinity matrix as described (18). Polypeptides that eluted from the HiTrapQ matrices with 0.35 M KCl column buffer contained all factors required for specific rRNA synthesis (18,25), as well as the putative SpRrn5h and SpRrn10h (as seen in the immunoblot of Fig. 2A and B, lanes 3). The peak fractions of the 0.35 M KCl HiTrapQ eluate were pooled, applied to a nickel affinity matrix and, following extensive washing of the matrix, polypeptides were eluted with 0.3 M imidazole buffer. In a parallel fractionation, extracts were prepared and fractionated as above, but the affinity purification in this alternate fractionation was an M2 (anti-FLAG) affinity matrix for purification of Sp-FLAG-Rrn10h and associated polypeptides.

RESULTS

Synthesis of wild-type levels of large rRNAs in fission yeast is dependent on the presence of the upstream rDNA promoter domain (28) and on a sequence-specific rDNA binding activity that stimulates rDNA transcriptional initiation (18). Since several properties of the fission yeast rDNA binding activity are reminiscent of S.cerevisiae UAF (14,15), S.pombe polypeptides uncovered as potential homologs of S.cerevisiae UAF subunits Rrn5p and Rrn10p were molecularly analyzed.
As seen in Figure 2A, Sp-MH-Rrn5h ('SpRrn5h') was detected in fractions eluted from nickel affinity matrix with 0.3 M imidazole, as expected (lane 6, 'Ni-NTA El'; compare to lane 5, 'Ni-NTA FT'). A parallel immunoblot of proteins in the nickel affinity purified fractions was challenged with anti-FLAG (M2) antibody and revealed Sp-FLAG-Rrn10h largely in the bound fraction ('Ni-NTA El', Fig. 2B, lane 6) with a portion in the flow-through fraction ('Ni-NTA FT', lane 5). Further evidence for interaction came from co-immunoprecipitation analysis of Sp-MH-Rrn5h with Sp-FLAG-Rrn10h (data not shown). To assess the extent of binding of the putative Sp-FLAG-Rrn10h to the Ni-NTA matrix in the absence of Sp-MH-Rrn5h, WCE was prepared from S.pombe expressing just Sp-FLAG-Rrn10h and fractionated as in Figure 2D. As seen in Figure 2C, lane 5, SpRrn10h does not bind the Ni-NTA matrix in the absence of Sp-MH-Rrn5h but is present in the flow-through fraction (compare lane 5 with 6).

Affinity purified SpRrn5h/SpRrn10h fraction harbors sequence-specific rDNA binding and transcriptional stimulatory activities

Subsequently, the affinity purified Sp-MH-Rrn5h/Sp-FLAG-Rrn10h fraction ('SpR5/SpR10') was tested for its ability to confer stimulation of transcription supported by a full-length S.pombe rRNA gene promoter in in vitro reactions. These reactions were conducted under stringent conditions that reflect requirements for the upstream rDNA promoter region and for stimulatory transcription factors in directing efficient initiation. Correctly initiated rRNAs are represented by 76 bp S1-resistant products. Virtually no accurately initiated rDNA transcripts are detected when reactions contain either the RNA pol I fraction (RNA pol I/S.pombe Rrn3p; see Fig. 3A, lane 2) or RNA pol I and the rDNA transcription initiation factor ['init. factor', lane 3 (28)]. However, when the affinity purified SpRrn5h/SpRrn10h fraction was also present in the reactions, the level of rDNA transcriptional initiation was significantly stimulated (see lane 4). The SpRrn5h/SpRrn10h fraction does not support correct initiation on its own (data not shown). Furthermore, Sp-MH-Rrn5h as well as the rDNA transcriptional stimulatory activity were found to immunopurify with Sp-FLAG-Rrn10h using an anti-FLAG affinity matrix (data not shown).

If SpRrn10h and SpRrn5h are components of the S.pombe sequence-specific rDNA binding activity, this activity should copurify with these tagged polypeptides. The presence of an rDNA binding activity was assessed by electrophoretic mobility shift analysis. When an end-labeled fragment bearing S.cerevisiae rDNA sequences from ±243 to +31 was incubated in the presence of the affinity purified SpRrn5h/SpRrn10h fraction, the electrophoretic mobility of the labeled rDNA promoter fragment was retarded (see 'complex', compare Fig. 3B, lane 2, with lane 5). In order to assess the specificity of this association, binding reactions were conducted in the presence of 10- or 30-fold molar excess of unlabeled competitor fragments bearing rDNA sequences from -150 to +89 (lanes 3 and 4) and -84 to +89 (lanes 5 and 6).
An illustration of the importance of this rDNA promoter region is seen in transcriptional analysis of rDNA promoters mutated in this region. The rDNA promoter of the template LS-68/-58 is full-length (from −243 to +89) but bears non-specific bases between −68 and −58. Comparison of its promoter strength with that of a wild-type template (3′ΔA+31) or a neutral mutant rDNA template, LS-106/-97, reveals that it is severely impaired in its ability to support rRNA synthesis (compare Fig. 4A, lane 2 with lanes 1 and 4). In contrast, LS-35/-28, bearing non-specific bases between −35 and −28 (28), is only somewhat impaired (compare lane 3 with its wild-type control, lane 4).

Analysis of cross-species function of *S.cerevisiae* and *S.pombe* upstream rDNA promoter regions

Since the respective *S.cerevisiae* or *S.pombe* upstream rDNA promoter domains mediate the effects of *S.cerevisiae* UAF (15) or the *S.pombe* rDNA binding activity (18), the cross-species activities of these domains were tested in *in vitro* *S.cerevisiae* and *S.pombe* transcription assays on templates bearing hybrid *S.pombe/S.cerevisiae* rDNA promoters. The hybrid templates bore the *S.pombe* upstream rDNA promoter fused to an *S.cerevisiae* core rDNA promoter (Sp/Sc) or the *S.cerevisiae* upstream promoter fused to the *S.pombe* core promoter (Sc/Sp), while the control rDNA templates consisted of full-length *S.pombe* (Sp) or *S.cerevisiae* rDNA promoters (Sc) or the ‘Sc core’ and ‘Sp core’ rDNA templates that lacked the upstream promoter domains entirely (see Fig. 4D and E).

When the promoter strength of this series was assessed in *S.pombe in vitro* transcription reactions (see Fig. 4B), no correctly initiated RNAs were detected supported by the *S.cerevisiae* core (Sc core; lanes 3 and 4) or full-length rDNA promoter (Sc; lanes 1 and 2). In contrast, the Sp/Sc template does direct accurate rRNA synthesis (see Fig. 4B, lanes 5 and 6), and its start site was fine-mapped to +1 (data not shown). This demonstrates that the *S.pombe* upstream rDNA promoter can mediate correct initiation of the *S.cerevisiae* core rDNA promoter with *S.pombe* RNA pol I transcription factors. In contrast, a *cis*-located *S.cerevisiae* upstream rDNA promoter (Sc/Sp) functions to repress basal level transcription of the *S.pombe* core rDNA promoter (Sp core) (see Fig. 4B, lanes 7 and 8, compare to Sp, lane 10, and Fig. 4C, compare lane 4 with lanes 1 and 2). The molecular basis underlying this repressive effect is unknown.

When transcription reactions were conducted with *S.cerevisiae* WCE components, the *S.cerevisiae* full-length rDNA promoter (‘Sc’), as expected, supported efficient transcriptional initiation (see Fig. 5, lanes 3 and 4), while levels supported by the *S.cerevisiae* core rDNA promoter (from −49 to +44) were reduced 6-fold or more (compare lane 2 with lane 4, and lane 1 with lane 3, Figure 5). A *cis*-located *S.pombe* upstream rDNA promoter mediated a modest increase in transcription supported by the *S.cerevisiae* rDNA core promoter [compare lanes 1 and 2 (Sc core) to lanes 5 and 6 (Sp/Sc)]. In the Sc/Sp template, the *cis*-located *S.cerevisiae* upstream rDNA promoter domain increases transcription supported by the *S.pombe* rDNA promoter 50% compared to the wild-type Sp template (compare lane 9 with lane 11) and several-fold more compared to Sp core (lanes 12 and 13). As seen previously, transcription of the *S.pombe* rDNA promoter initiates at an altered site [see Fig. 5, lanes 8 to 13 (25)] in

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**Figure 2.** Fractionation of WCE expressing Sp-FLAG-Rn10h and Sp-MH-Rn5h. WCEs prepared from fission yeast expressing both Sp-MH-Rn5h and Sp-FLAG-Rn10h were fractionated via ammonium sulfate precipitation followed by chromatographic separation on HiTrapQ columns and affinity purification using a nickel chelating matrix or anti-FLAG (M2) monoclonal antibody matrix. Polypeptides were separated on a 12% (A) or 16% (B) acrylamide gel, transferred to nitrocellulose membrane, and western analysis was performed with anti-FLAG (A) or anti-FLAG (B) monoclonal antibodies. Lane 1, −120 μg S-100 protein (WCE); lanes 2−4, 15 μl of 0.1, 0.35 and 0.7 M KCl-1× CB eluates from HiTrap Q matrix; lanes 5 and 6, 15 μl of flowthrough (Ni-NTA FT) or 0.3 M imidazole wash of the nickel affinity matrix (Ni-NTA El). The immunoreactive bands corresponding to the ~15.1 kDa immunoreactive band detected with M2 monoclonal antibody and corresponding to Sp-FLAG-Rn10h is marked ‘SpRn10h’. (C) Fractionation of WCE prepared from MP6-10B pREP-Sp-FLAG-Rn10h. 120 μg of WCE protein and 15 μl of fractions were analyzed as in (B). The −15.1 kDa immunoreactive band detected with M2 monoclonal antibody and corresponding to Sp-FLAG-Rn10h is marked ‘SpRn10h’. (D) Diagram of fractionation scheme.

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6). Both competitors served to largely abolish the retarded complex. In contrast, 10-fold molar excess of a fragment bearing rDNA from −57 to +89 (lane 7) or non-specific vector fragment (pBS; lanes 9) does not affect formation of the complex (while 30-fold molar excess of −57 to +89 does; lane 8). Thus, the 5′ border of the *S.pombe* rDNA promoter that is critical for directing association of the SpRn5h/SpRn10h fraction with rDNA lies between −84 and −57. This correlates with the rDNA promoter region, between −80 and −56, shown to be stably bound by the *S.pombe* rDNA binding activity (18), and with the 5′ border required for mediating stimulated levels of rRNA synthesis that was also found to lie between −84 and −57 (28).
S. cerevisiae in vitro transcription reactions. A minor transcript supported by Sc/Sp (lane 9) may represent initiation at the correct +1 site or may be due to non-specific initiation.

A 27 kDa S. pombe polypeptide interacts in vivo with SpRrn10h

To investigate interactions of the putative SpRrn10h and SpRrn5h as well as to search for specifically interacting polypeptides, their coding sequences were cloned in frame with the GAL4 DNA binding domain or activation domain in the vectors pGBDU and pGAD (26). In a large-scale two-hybrid screen, polypeptides that specifically associated with SpRrn10h were searched for in a library of S. pombe GAL4AD-cDNAs (Clontech). One of the GAL4AD-cDNAs that encoded a fusion protein capable of activating three independent reporters together with GAL4BD-SpRrn10h, indicating interaction, was found to encode a ~27 kDa polypeptide. This S. pombe 27 kDa polypeptide was homologous to S. cerevisiae YOR295W [now reported as a subunit of UAF, Uaf30p (16)] and YMR233W (16) and shared some homology with the mammalian ~60 kDa SWI/SNF subunit (33). As seen in Figure 6, the activation of the ADE2 and the HIS3 reporters in PJ69-4A bearing both pGBDU-SpRrn10h (SpRrn10h) and pGAD-Spp27 (Spp27) allowed growth on medium lacking adenine or histidine (the lacZ reporter was also activated; data not shown). In contrast, no activation was observed in strains expressing GAL4AD-Spp27 (Spp27) and GAL4BD-SpRrn5h (SpRrn5h); GAL4BD-Spp27 and S. cerevisiae GAL4AD-ScRrn9p (ScRrn9p); Spp27 and vector; GAL4DB-SpRrn10h (SpRrn10h) and ScRrn9p; or SpRrn10h and SpRrn5h. The reason for the negative two-hybrid result between SpRrn5h and SpRrn10h is unknown, since they co-fractionate with each other (perhaps mediated by another associated factor) and with the S. pombe rDNA binding activity. Furthermore, the documented interaction of S. cerevisiae Rrn9p with Rrn10p (34,35) appears to be species-specific in nature and is not seen between S. cerevisiae Rrn9p and S. pombe SpRrn10h.

Sp-FLAG-p27 cofractionates with Sp-MH-Rrn5h and with Sp-MH-Rrn10h

To test whether Spp27 co-fractionated with SpRrn5h—as expected if Spp27 were a component of the rDNA binding complex—pREP-Sp-FLAG-p27 encoding N-terminal FLAG-tagged Spp27 was introduced into S. pombe MP6-10B together with pREP-Sp-MH-Rrn5h. WCE was prepared and fractionated as described above, except that the affinity purification step used anti-FLAG affinity matrix (see Fig. 7G). Duplicate aliquots of the fractions were resolved on 16 and 12% SDS–polyacrylamide gels, transferred to nitrocellulose membrane and challenged with anti-FLAG (Fig. 7A) or anti-myc (Fig. 7B) monoclonal antibodies. Both Sp-MH-Rrn5h and Sp-FLAG-p27 co-eluted from the HiTrap Q matrix (Fig. 7A and B, lane 3) together with the RNA pol I transcriptional activity.
Transcription reactions were conducted with Sp-FLAG-p27 and Sp-MH-Rrn10h in E. coli, consistent with their interaction with Sp-FLAG-p27 (Fig. 7A, lane 6), as above. Sp-MH-Rrn10h co-fractionates with Sp-FLAG-p27, suggesting that SpRrn5h, SpRrn10h and S. pombe p27 (Sp27) associate in a UAF-like complex.

WCEs were also prepared and fractionated from S. pombe expressing Sp-FLAG-p27 and Sp-MH-Rrn10h (see Fig. 7D and E, lane 1), as above. Sp-MH-Rrn10h co-elutes from the HiTrapt Q matrix with Sp-FLAG-p27 and is largely present in the bound fraction of the M2 affinity matrix (see Fig. 7D and E, lanes 3 and 6), consistent with their in vivo interaction (Fig. 6). In contrast, when WCE prepared from S. pombe cells expressing just Sp-MH-Rrn10h was fractionated as in Figure 7G, Sp-MH-Rrn10h was found in the flowthrough fraction of the M2 affinity matrix (Fig. 7F, lane 5).

The Spp27/SpRrn5h fraction was assayed to determine whether it contained an activity leading to stimulated levels of rDNA transcriptional initiation. Addition of the affinity purified Spp27/SpRrn5h fraction to a reaction containing basal level pol I transcription initiation components and a full-length rDNA promoter resulted in a significant increase in rRNA synthesis (compare lane 5 with lane 3, Fig. 3A), while it supported no initiation itself (data not shown). This Spp27/SpRrn5h fraction also harbored sequence specific rDNA binding activity (data not shown).

Identification of p27, SpRrn5h and SpRrn10h homologs in other eukaryotes

Sequence alignment of the S. pombe Spp27 with other eukaryotic homologs is seen in Figure 8A (and Supplementary Material, Fig. 9A). Extensive database searches reveal that non-vertebrates have a clearly identifiable homolog of Spp27 as well as a homolog of the SWI/SNF 60 kDa subunit. The latter polypeptide, however, shares less sequence homology with Spp27 than the putative Spp27 homologs. A clearly identifiable homolog of Spp27 was not found in the proteomes of vertebrates, although the 60 kDa SWI/SNF subunit homolog was.

The genomes of N. crassa, P. chrysosporium (white rot fungus), A. fumigatus, C. neoformans, K. marxianus and K. lactis encode putative Rrn5p homologs that align with S. pombe SpRrn5h and S. cerevisiae Rrn5p as seen in Figure 8B. (Full-length alignments are shown in Supplementary Material, (18). Peak fractions eluted with 0.35 M KCl–1× CB were applied to an anti-FLAG, M2 monoclonal antibody affinity matrix. Following extensive washing, bound polypeptides were eluted with FLAG peptide. As expected, Sp-FLAG-p27 is specifically bound to the M2 affinity matrix and dissociates upon addition of FLAG peptide (Fig. 7A, lane 6). The reason for the discrepancy between its apparent molecular weight of ~42 kDa and the calculated value of 30.1 kDa is unknown, as is the presumptive modification underlying the slower migrating immunoreactive band of ~44 kDa. Sp-MH-Rrn5h (~75 kDa apparent molecular weight) is detected in the fraction bound to the M2 affinity matrix, presumably via interactions with Sp-FLAG-p27 (‘SpRrn5h’, Fig. 7B, lane 6, ‘M2 aff.-El’; for Sp-FLAG-p27, see Fig. 7A, lane 6 (‘Spp27’)), although a portion is also present in the flowthrough fraction (‘M2 aff.-FT’). Figure 7B, lane 5). In addition, fractionation of an extract prepared from fission yeast expressing just the epitope-tagged Sp-MH-Rrn5h revealed that it does not specifically bind to the M2 affinity matrix in the absence of Sp-FLAG-p27 (Fig. 7C). Thus, Sp-MH-Rrn5h co-fractionates with Sp-FLAG-p27, suggesting that SpRrn5h, SpRrn10h and S. pombe p27 (Sp27) associate in a UAF-like complex.
The N. crassa, white rot fungus and A. fumigatus Rrn5p homologs, like SpRrn5h, also harbor a clearly identifiable SWI3-like SANT domain and all share conserved residues in this domain. While no definitive Rrn5p homologs have been found in eukaryotes other than in fungi, this may be due to a divergence in primary sequence that masks their recognition.

Similarly, putative Rrn10p homologs identified recently in several fungal databases are shown in Figure 8C. A search of C. albicans genomic sequences homologous to Rrn10p revealed a putative homolog (CaRrn10h). When fungal sequences are queried with the putative CaRrn10h, S. cerevisiae Rrn10p aligns with an E-value of $1 \times 10^{-6}$ (PSI-BLAST; 32% identities and 50% similarities over 98 residues), and SpRrn10h with an E-value of $2.2 \times 10^{-2}$ (29% identical and 53% similar residues over 57 amino acids). A putative N. crassa Rrn10 homolog (NcRrn10h) was also identified based on homology with CaRrn10h that is 50% identical and 63% similar over 52 residues, from 51 to 102, of CaRrn10h. Alignment of other putative yeast Rrn10p homologs from the hemiascomycetous class of yeast (S. bayanus, K. marxianus, K. lactis and P. farinosa), identified in the Genolevures database (19) with S. cerevisiae Rrn10p as query, is also shown in Figure 8C. (See Supplementary Material, Fig. 9C, for full-length alignments.)

DISCUSSION

Stimulation of rRNA synthesis in eukaryotes is mediated by cis-acting sequences in the upstream rDNA promoter as well as by the stimulatory factors, UBF in vertebrates and UAF (15) in S. cerevisiae (1–3 and references therein). These factors are vastly different in subunit structure: UBF is a homodimer composed of UBF1 or UBF2 molecules, while UAF is composed of six subunits: Rrn5p, Rrn9p, Rrn10p (15), histones H3 and H4 (14) and Uaf30p (16). UBF is abundant (up to 100 000 molecules per cell), while UAF is present at ~200 copies per cell (15). Both UAF and the S. pombe rDNA binding activity recognize their species’ upstream rDNA promoter in a sequence-specific manner (15,18), in contrast to UBF, which associates with rDNA in a sequence tolerant fashion (7,36). Both serve important roles in stimulating levels of rRNA synthesis.
The presence of a UAF-like activity and UAF subunit homologs that co-purify with this activity in *S. pombe* demonstrate that UAF is not restricted to *S. cerevisiae*. As reported here, epitope-tagged versions of putative fission yeast SpRrn5h, SpRrn10h and Spp27 were found to co-fractionate with each other and with the *S. pombe* rDNA binding/rDNA transcriptional stimulatory activity. A fundamental question raised when UAF was first characterized (15) was whether other eukaryotes harbor UAF, and if they do, whether they regulate levels of rRNA synthesis via both UBF and CoR, and a putative Rrn9p homolog (32). Homology to Swi3p, however, extends beyond this domain (see Supplementary Material, Fig. 10). One function of a SANT domain was recently reported: a SANT domain in SMRT and N-CoR was shown to be important for interaction with and activation of histone deacetylase (38).

A second component of the *S. pombe* rDNA binding activity also bears homology to a SWI/SNF subunit: the SpRrn10h interacting factor Spp27 to SWI/SNF subunit *SWI3, Ad3*, N-CoR, and a TFIIIB component (32). Homology to Swi3p, however, extends beyond this domain (see Supplementary Material, Fig. 10). One function of a SANT domain was recently reported: a SANT domain in SMRT and N-CoR was shown to be important for interaction with and activation of histone deacetylase (38).

While a homolog of the fourth RNA pol I specific subunit of UAF, Rrn9p, has yet to be characterized in *S. pombe*, putative Rrn9p homologs have been identified in multiple yeasts (see Supplementary Material, Fig. 11A and B). An intriguing observation obtained in a search of the *S. pombe* proteome with the putative *Kluyveromyces thermotolerans* Rrn9p homolog, *Kl*Rrn9h, as query was that *S. pombe* Snf2p was the top match...
The polypeptides with the highest degree of homology to Spp27 are clearly distinct from the mammalian 60 kDa SWI/SNF subunit class. While Spp27 shares some homology with this SWI/SNF subunit, non-vertebrates including S.pombe, S.cerevisiae Uaf30p (16), white rot fungus, N.crassa, D.melanogaster, C.elegans and A.thaliana, have Spp27 homologous coding sequences (see Fig. 8A). A search of eukaryotic coding sequences with these Spp27 homologs reveals that they align, in general, to yield E-values significantly higher with each other than with the SWI/SNF 60 kDa subunit homologs. With the exception of the

Figure 8. (A) Multiple alignment of S.pombe p27 (Sp.p27; 117–190 of 233 residues total) with putative homologs: white rot fungus (wrf.p27h; 264–337 of 363 total), N.crassa (Nc.p27h 186–259 of 265 total), A.thaliana (At.p27h; 251–324 of 386; note—there are ~16 putative A.thaliana p27 homologs), O.sativa (Os.p27h; 67–141 of 144 total), H.vulgaris (Hv.p27h), C.mauritani (Cm.p27h; 9–83 of 86 total), S.cerevisiae Uaf30P (Sc.UAF30, 120–192 of 228 total), D.melanogaster (Dm.p27h; 168–241 of 244 total), bee (Bm.p27h), B.mori (Bm.p27h), C.elegans (Ce.p27h; 264–337 of 415 total) and C.reinhardtii (Cr.p27h).

(B) Multiple alignment of S.pombe SpRrn5h (residues 68–160) and S.cerevisiae ScRrn5p (56–139) with putative Rrn5p homologs: K.marxianus KmRrn5h, K.lactis KlRrn5h, white rot fungus wrfRrn5h (66–165), C.neoformans CnRrn5h (52–147), A.fumigatus AfRrn5h (108–196) and N.crassa NcRrn5h (182–275).

SpRrn5h, NcRrn5h, wrfRrn5h and AfRrn5h have clearly identifiable SANT domains: SpRrn5h, between residues 71 and 112 (E-value 1.3 × 10^{-4} RPS-BLAST (20)); N.crassa Rn5h, between 147 and 185 (E-value 1.3 × 10^{-2}); white rot fungus Rn5h, between 72 and 107 (E-value 6.4 × 10^{-2}) and A.fumigatus from 114 to 158 (E-value 2.1 × 10^{-3}). All have conserved residues in this domain.

(C) Alignment of putative Rrn10p homologs: S.cerevisiae ScRrn10p (63–110 of 145 total residues), S.bayanus SbRrn10h (63–110 of 145 total), K.marxianus KmRrn10h, K.lactis KlRrn10h (49–96 of 125), P.farinosa PfRrn10h (93–143 of 183 total), C.albicans CaRrn10h (residues 102–155 of 195 total), A.fumigatus AfRrn10h, N.crassa NcRrn10h (residues 89–157, with 7 residues removed '//' (of 261 total)), and S.pombe SpRrn10h (residues 39–87 of 97 total).
Anopheles gambiae genome, the Spp27 homologs are found in eukaryotic genomes that do not have a clearly identifiable UBF.

The upstream rDNA promoter domain is recognized in a species-specific manner: the S.cerevisiae upstream rDNA promoter domain does not mediate any increase in rRNA synthesis when 5’ to either an S.cerevisiae or S.pombe rDNA core promoter domain in S.pombe in vitro transcription reactions. The possibility that different spacing between the upstream promoter and the core domains in the S.cerevisiae and S.pombe hybrid rDNA templates would increase their transcriptional efficiency, as was seen in the case of a Xenopus rDNA template in a heterologous murine transcription system (42), remains to be tested. While the S.pombe upstream rDNA promoter domain mediates a modest increase in rRNA synthesis in S.cerevisiae transcription reactions, it is critical for activating rRNA synthesis directed from the S.cerevisiae core rDNA promoter in in vitro S.pombe transcription reactions. Despite the homology observed between SpRrn5p and S.cerevisiae Rrn5p, and between SpRrn10p and S.cerevisiae Rrn10p, the S.pombe factors do not complement an S.cerevisiae Rrn5 mutant strain, respectively (M.Tripp, K. van den Heuvel and L.Pape, data not shown). For SpRrn10p, this may be due to its inability to interact with S.cerevisiae Rrn9p (Fig. 6), in contrast to the interaction between S.cerevisiae Rrn10p and Rrn9p (34).

Recent studies point to nucleolar histone modification and chromatin remodeling activity playing an important role in regulation of eukaryotic rRNA synthesis. Histone modifications and DNA methylation were shown to play a crucial role in activation of rRNA genes in hybrid plants (43,44). A histone acetyltransferase activity was found to co-purify with Xenopus RNA pol I (45), and mammalian rDNA transcription was shown to increase in vivo and in vitro when histone deacetylase was inhibited (12). Furthermore, a nucleolar, chromatin remodeling activity that associates with the RNA pol I termination factor, TTF-I, was characterized in mammals (46), which aids in repressing rRNA synthesis by targeting histone deacetylase (47). The RSC nucleosome remodeling complex has been found to target RNA polymerase III transcribed promoters as well as its pol II targeted promoters (48). The hypothesis that fungal UAF complexes are RNA pol I specific chromatin remodeling factors, suggested based on the homology several subunits share with those of the SWI/SNF complex, can now be molecularly tested.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at NAR Online.

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