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Activated levels of rRNA synthesis in fission yeast are driven by an intergenic rDNA region positioned over 2500 nucleotides upstream of the initiation site

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ABSTRACT

RNA polymerase I-catalyzed synthesis of the *Schizosaccharomyces pombe* ~37S pre-rRNAs was shown to be sensitive to regulatory sequences located several kilobases upstream of the initiation site for the rRNA gene. An *in vitro* transcription system for RNA polymerase I-catalyzed RNA synthesis was established that supports correct and activated transcription from templates bearing a full *S.pombe* rRNA gene promoter. A 780 bp region starting at –2560 significantly stimulates transcription of a *cis*-located rDNA promoter and competes with an rDNA promoter *in trans*, thus displaying some of the activities of rDNA transcriptional enhancers *in vitro*. Deletion of a 30 bp enhancer-homologous domain in this 780 bp far upstream region blocked its *cis*-stimulatory effect. The sequence of the *S.pombe* 3.5 kb intergenic spacer was determined and its organization differs from that of vertebrate, *Drosophila*, *Acanthamoeba* and plant intergenic rDNA spacers: it does not contain multiple, imperfect copies of the rRNA gene promoter nor repetitive elements of 140 bp, as are found in vertebrate rDNA enhancers.

INTRODUCTION

While *Schizosaccharomyces pombe* has emerged as a eukaryotic organism in which many aspects of RNA processing and transcriptional initiation are strikingly similar to these basic molecular processes in higher eukaryotic organisms (1–8), little was known about requirements and regulatory sequences that affected initiation of the ~37S rRNA genes. The *in vivo* initiation site was fine mapped and the promoter region sequenced (9), but molecular analysis of *cis*-acting regulatory sequences and factors required for rRNA synthesis and establishment of a transcription system for these studies had not been accomplished.

Synthesis of the ~37S–45S pre-rRNAs represents nearly 50% of total RNA synthesis for logarithmically growing eukaryotic cells (10,11). One mechanism for ensuring activation of the rRNA gene promoters is stimulation of initiation at the rRNA gene promoter by intergenic rDNA transcriptional enhancers, sequences that increase rRNA synthesis in an orientation-independent (12–18) and somewhat position-independent manner (13). The rRNA gene enhancers promote assembly of the transcription complex at the rDNA gene promoter (13,19,20), in part by association with the nucleolar factor upstream binding factor (UBF) that counteracts inhibition of transcriptional initiation by a repressor (21) and that stimulates initiation (14,21–24). Vertebrate rDNA enhancers can serve as repressors of initiation when located *in trans* to a promoter (13,25) and can compete with complex formation on an rDNA promoter *in trans* (26). However, the mechanism of enhancement is not at all clear.

The vertebrate rDNA enhancers are present in repeats of 130–140 bp units (13–16,25) located just upstream of the rDNA gene promoter. In contrast, the rDNA enhancer of the lower eukaryote, *Saccharomyces cerevisiae*, is present as a single ~320 bp unit downstream of the 3′-end of the 25S rRNA coding sequences. This region can stimulate initiation in an orientation- and position-independent fashion from 8- to 30-fold (12,27–30). Although the domains critical to the eukaryotic rDNA enhancer effect have been extensively analyzed both *in vivo* and *in vitro* (12,19,27–33), the means by which these rDNA enhancers function remains to be determined.

Nearly one-third of the ~11 kb repeat unit of the *S.pombe* rRNA gene comprises the intergenic spacer (IGS) (9,35), the region that separates each of the ~100 tandemly repeated copies (36) of the coding sequences for the pre-rRNA molecules. In our analysis of *cis*-acting regulators of rRNA synthesis in this intergenic rDNA, we found that spacer sequences contribute significantly to initiation events at the rRNA gene promoter. To further analyze these sequences and to enable cross-species comparison of the region of the rRNA gene that regulates rRNA synthesis, the IGS, we determined its nucleotide sequence.

MATERIALS AND METHODS

Yeast and bacterial strains; transformations

The bacterial strains used included XL1-Blue (*endA1*, *hsdR17* (r_{k-},m_{k+}), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *relA1*, *lac*, [F′, *proAB*, *lacI^qZΔM15*, Tn*10*, (tet^r)] and SURE™ (*mcrA*, Δ(*mcrBC-hsdRMS-mrr*)*171*, *endA1*, *supE44*, *thi-a*, λ^-, *gyrA96*, *relA1*, *lac*, *recB*, *recJ*, *sbcC*, *umuC*::Tn*5*(kan^r), *uvrC*, [F′, *proAB*, *lacI^qZΔM15*, Tn*10*(tet^r)] (Stratagene). The *S.pombe* strains used were wild-type 972 (h^-; kindly sent by Dr H.Levin) and MP6-10B (h^-, *ade1-51*, *ade6-M210*, *leu1-32*, *ura4-D18*; kindly provided by M.Moser and T.Davis). Bacterial cells were transformed by either standard $CaCl_2$–PEG procedures or by electroporation using a BioRad

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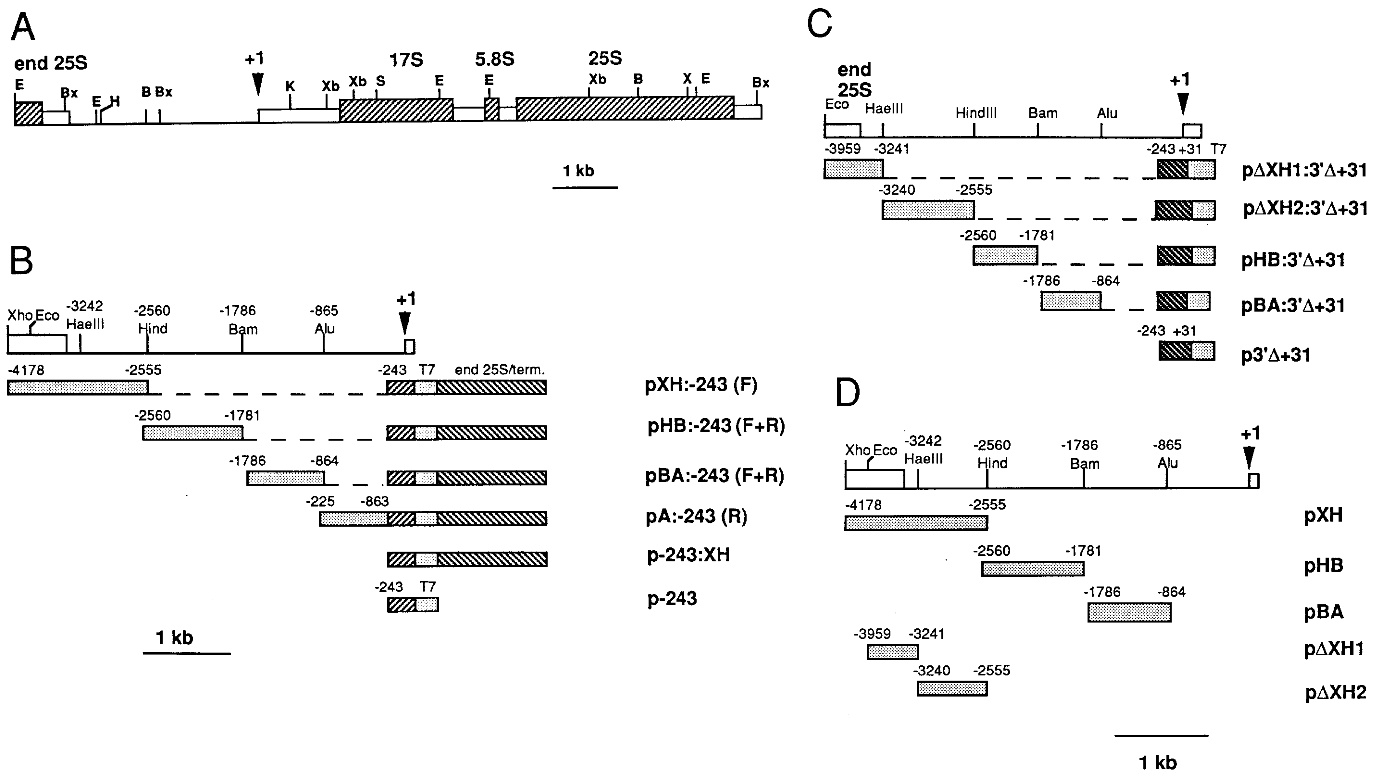

Figure 1. (**A**) Map of the *S.pombe* rRNA gene repeat unit based on Balzi *et al*. (9), Schaak *et al*. (35), data from this paper (accession no. Y09256) and B.Lapeyre (GenBank accession no. Z19578). The coding sequences for the~37S pre-rRNA are boxed, with the open boxed regions representing the 5′ external transcribed spacer, the two internal transcribed spacers and the 3′ external transcribed spacers, and the larger, diagonally striped boxed portions coding for the mature~17S, 5.8S and ~25S rRNAs. E, *Eco*RI; Bx, *Bst*XI; H, *Hin*dIII; B, *Bam*HI; K, *Kpn*I; Xb, *Xba*I; S, *Sal*I; X, *Xho*I. (**B**) Diagrams of *S.pombe* rDNA mini-genes containing the full promoter (from 5′Δ–243 to 3′Δ+31), downstream marker bacteriophage T7 sequences and an rDNA fragment containing the putative rDNA terminators (–4178 to –2555; 50) in the cloning vector YEp351 (41). The regions of the *S.pombe* rDNA IGS that were inserted directly upstream of the promoter are shown in the stippled boxes to the left of the promoter, with the boxes positioned below their normal location in the map of the rDNA IGS (top line). The *Alu*I site at –865 is not unique; an additional *Hae*III site at –4107 and *Eco*RI site at –2602 are not shown in (B–D) for simplicity. (**C**) Diagrams of a second series of *S.pombe* rDNA mini-genes containing the full promoter, from 5′–243 to 3′+31, downstream bacteriophage T7 sequences and regions of the *S.pombe* rDNA IGS inserted directly upstream of the promoter in the cloning vector pBS SK+. (**D**) Diagrams of plasmids containing different subcloned regions of the *S.pombe* rDNA IGS. These are all in pBS SK+ and the positions of the first and last nucleotide of the rDNA are listed relative to the initiation site (+1).

GenePulser (39). The transformation method used for introduction of plasmid DNAs into *S.pombe* was that of Allshire (49).

Construction of subclones bearing regions of the *S.pombe* rDNA IGS

Figure 1D illustrates five subcloned region of the *S.pombe* IGS. The parental plasmid, pSP329, bears the complete *S.pombe* rDNA repeat unit (36,37), kindly sent by Dr M.Yamagishi, and was used to make the following subclones of the *S.pombe* rDNA repeat unit: pXH contains a *Xho*I–*Hin*dIII fragment from –4178 to –2555; pHB contains a *Hin*dIII–*Bam*HI fragment from –2560 to –1781; pBA contains a *Bam*HI–*Alu*I partial fragment from –1786 to –864, inserted between *Bam*HI and *Sma*I sites of the vector. The cloning vector was pBlueScript SK+ (38), treated with the respective restriction enzymes, dephosphorylated and isolated (39). pΔXH1 was constructed by inserting an *Eco*RI–*Hae*III fragment from pXH, –3959 to –3241, between *Eco*RI and *Sma*I sites of pBS SK+; for pΔXH2, a *Hae*III–*Bam*HI fragment isolated from pXH, extending from –3240 to –2555, was inserted into pBS SK+ between an *Eco*RI site, which had been converted to a blunt-ended site, and a *Bam*HI site. Restriction enzymes and DNA modifying enzymes were from N.E. Biolabs, US Biochemical/Amersham or Promega.

Construction of *S.pombe* rDNA mini-genes

The original template containing a full *S.pombe* rDNA promoter and upstream intergenic sequences was constructed from an *Alu*I fragment extending from 5′Δ–863 to 3′Δ+89 inserted into pUC18 *Sma*I site. This template, p5′–863, supported accurate initiation. A p5′Δ–243/3′Δ+89 template was cleaved downstream of +89 at the *Eco*RI site and treated with BAL-31 nuclease; 3′ deleted promoter fragments were isolated following inactivation of the nuclease and cleavage upstream of position –243 at the vector *Bam*HI site. These were inserted into pBS SK+ treated with *Bam*HI, *Sma*I and phosphatase. The plasmid prR+31 contains the *S.pombe* rDNA promoter from 5′–243 to 3′+31 and served as the basis of all subsequent rDNA mini-genes reported in this study. This promoter contains only 31 nt of coding sequence to avoid the problem of trimolecular artifacts in S1 analysis (40). Bacteriophage T7 sequences isolated from the plasmid pSP-T7(+) (kindly sent by Dr J.Warner; Albert Einstein College of Medicine; 27) were used as a foreign DNA marker. pSP-T7(+) was cleaved by *Eco*RI, blunt-ended using Klenow fragment (39) and the T7 sequences were isolated following a second cleavage by *Hin*dIII. p5′–243/3′Δ+31 was cleaved by *Eco*RI blunt-ended, cleaved with *Hin*dIII, and the T7 sequences were inserted. A *Bam*HI–*Xba*I fragment carrying this mini-gene was isolated and reinserted into

pBS SK+ between *Bam*HI and *Xba*I sites to create p3′Δ+31. The *Bam*HI–*Hin*dIII fragment bearing the rDNA promoter (–243/+31) and T7 sequences was reinserted into the yeast/*E.coli* shuttle vector Yep351 (41) and named p5′–243. p5′–243 was cleaved with *Sal*I and *Hin*dIII, treated with Klenow fragment and a *Xho*I–*Hin*dIII fragment (–4178 to –2555) with both ends blunted was inserted; the resultant plasmid that contained the terminator sequences in the correct orientation was named p–243:XH.

All plasmid DNAs were purified by standard methods and isolated following banding in cesium density gradients during a 44 h, 42 000 r.p.m. centrifugation (39); concentrations were determined in a Perkin Elmer lambda/2 spectrometer.

The p–243 series (Fig. 1B) of containing intergenic rDNA spacer sequences upstream of the rDNA promoter was constructed as follows. The DNA fragment in pXH was cleaved with *Xho*I, blunt-ended and cleaved with *Bam*HI. For the reverse orientation, both sites were converted to blunt-ended sites. pHB was cleaved with *Hin*dIII, blunt-ended and cleaved with *Bam*HI. For the reverse orientation, both ends were blunted. These were inserted upstream of the promoter in p5′–243:XH cleaved with *Sma*I and *Bam*HI or, for the reverse orientations, with *Sma*I. The inserts in pBA and p5′-863/3′Δ–225 were isolated as *Bam*HI–*Eco*RI fragments and the ends were blunted. These were inserted upstream of the promoter in p5′–243:XH at a *Sma*I site. To construct deletions in pHB:–243, primers TGCTCTAGACTTTTTTTTTGGTGTG and T3 and TGCTCTAGATTTTTTTACACTTTTAACC and T7 were utilized to amplify pHB template DNA in PCR reactions (39). The amplified DNAs were isolated as *Bam*HI–*Xba*I and *Kpn*I–*Xba*I fragments respectively and inserted into *Bam*HI and *Kpn*I cleaved pBS SK+ vector DNA, creating pHBΔE. Primers TGCTCTA-GAGTTTAATTTATTTTTAAAAATAG and T3 and TGCTCTA-GAACTTTGGAAACAATTTTC and T7 were utilized to amplify pHB template DNA and isolated and cloned as above to form pHBΔP. Inserts containing a deletion in the enhancer-homologous (HBΔE) or promoter-homologous (HBΔP) regions of the HB intergenic sequences were then isolated as *Hin*cII–*Bam*HI fragments and inserted into *Sma*I and *Kpn*I sites upstream of the promoter of p–243:XH. pHBΔE:–243 contains a deletion from –2094 to –2064, while pHBΔP:–243 contains a deletion from –1900 to –1879.

A second series (pXH:3′Δ+31, pΔXH1:3′Δ+31, pΔXH2:3′Δ+31, pHB:3′Δ+31, pHBΔE:3′Δ+31, pHBΔP:3′Δ+31 and pBA:3′Δ+31) was constructed from the parental template, p3′Δ+31. To create these, pXH:–243, pHB:–243 and pBA_F:–243 were cleaved with *Kpn*I and *Xba*I and the fragments bearing the upstream intergenic sequences, promoter and T7 marker DNA were purified and inserted between the *Kpn*I and *Xba*I sites of the vector pBS SK+. The intergenic sequences were isolated from pΔXH1 as an *Eco*RI–*Bam*-HI fragment and inserted between the *Eco*RI and *Bam*HI sites of p3′Δ+31. To create pΔXH2:+31, the intergenic sequences in pΔXH2 were isolated as a *Xho*I–*Bam*HI fragment and inserted between the *Xho*I and *Bam*HI sites of p3′Δ+31. The templates pHBΔE:3′Δ+31 and pHBΔP:3′Δ+31 were made by ligating *Kpn*I–*Bam*HI fragments with the HBΔE and HBΔP inserts upstream of the promoter in p3′Δ+31.

Preparation of S-100 transcription extract from *S.pombe*

An overnight culture of wild-type *S.pombe* cells (*h*–972; kindly provided by Dr H.Levin) was grown at 30°C and 250 r.p.m. overnight in rich yeast media, YPD or YE (39,42), until the OD_{600} was ~2.0. Once determined to be in mid-logarithmic growth, the *S.pombe* cells were cooled, collected and the S-100 extract prepared using a modified procedure of Schultz *et al.* (43). Frozen *S.pombe* cells (39,43) were later extracted under liquid nitrogen in a Waring commercial stainless steel blender (39) or by extensive manual grinding in a porcelain mortar and pestle (43). The extract was thawed in the presence of 13 ml 1× extraction buffer (containing protease inhibitors) per 10 g starting cells and the supernatant (S-100) collected following a 2 h 100 000 *g*, 4°C ultracentrifugation in a Beckman Ti50 rotor. The supernatant was diluted 1:3 with storage buffer (60% glycerol, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM DTT). Alternatively, it was dialyzed against 50 vol. 20 mM HEPES–KOH, pH 7.9, 50 mM KCl, 5 mM EGTA, 0.05 mM EDTA, 2.5 mM DTT, 20% glycerol (43). Aliquots of the *S.pombe* S-100 transcription extract were stored at –75°C.

Assay of correctly initiated rDNA mini-gene transcripts catalyzed by RNA polymerase I *in vitro*

For *in vitro* analysis of rRNA synthesis, 10 µl extract (5–10 mg/ml) and 0.025–5.0 µg/ml template DNA were used in 20 mM HEPES–KOH, pH 7.9, 90 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, pH 7.9, 0.05 mM EDTA, pH 7.9, 10% glycerol, 1.3 mM DTT, 100–500 µM each rNTP (Pharmacia) and 10 µg/ml α-amanitin. Reactions were stopped after 45 min at 26°C by adding 180 µl Stop Mix and the RNA was isolated as described (44). For Figure 6D, the reactions also contained 3% PEG-10,000, with template DNA at 40 ng/ml and competitor p5′Δ–850 at 80 ng/ml.

For S1 analysis, the radioactive probes were obtained by labeling the 5′-end of the template strand of p5′Δ–243 or p3′Δ+31 at a *Xba*I site at +340 with [γ-^{32}P]ATP (4500 Ci/mmol; ICN) and T4 polynucleotide kinase (39). Following cleavage with *Bam*HI and denaturation, the single-stranded end-labeled fragment was resolved on a 4% native polyacrylamide gel (39). Alternatively, the probe was treated with exonuclease III (100 U) for 15 min at 37°C to render it single-stranded, followed by extraction with buffered phenol and ether. Hybridization reactions were in 20 µl solution [80% ultrapure formamide (US Biochemical), 40 mM PIPES, pH 6.4, 2 mM EDTA, pH 7.9, 0.4 M NaCl, 0.01 pmol probe] at 44°C overnight. The reactions were subjected to S1 analysis as described (45) and resolved by electrophoresis on 4% acrylamide–9 M urea gels. Gels were dried and exposed to Kodak XAR-5 film with L-plus intensifier screens at –70°C. Quantitation was performed using a GS-250 Molecular Imager™ (BioRad). The size markers were 5′-^{32}P-labeled pBR322 *Hpa*II fragments. All experiments were done multiple times using different batches of cesium-purified plasmid DNAs and of transcription extract.

For the *trans*-competition assay, prior to addition of the rDNA template, S-100 extract was preincubated with 40 fmol plasmid DNAs, pXH, pHB, pΔXH1 or pΔXH2, and 100 ng pBS SK+ for 30 min at 26°C. 8.5 fmol plasmid p–243 was subsequently added, as well as rNTPs to initiate transcription. The reaction was terminated after 45 min.

Nucleotide sequence determination of the *S.pombe* rDNA IGS

The entire sequence of the ~3.5 kb IGS was determined on both strands by standard dideoxy sequencing reactions (39) using Sequenase Version 2.0 (US Biochemical), 7-deaza-dGTP to clarify the compression regions and [α-^{35}S]dATP (>1000 Ci/mmol, >37 TBq/mmol; Amersham Corp.). Primers were T3 and T7 and primers designed from known IGS sequence (synthesized by Bio-synthesis Inc., Lewisville, TX). Programs utilized to assess the presence of

repeated elements in the IGS and homologies included the Genetics Computer Group Dotplot, Bestfit, Gap (47,48) and Findpatterns programs, on line at the NYU Medical Center Research Computing Resources.

The oligonucleotides synthesized for sequencing are listed below, with the position of the first nucleotide given. For the pXH subclone, the primers included pr-XH3 (5′-TTGGTGGGAAAGTACTC-3′; –3061), pr-XH4 (GTGGTAGGGTAGGTCG, –2845) and, for the template strand, pr-XH1 (5′-AAATTTGAAAAGGGGGA-3′; –2714) and pr-XH2 (5′-AAAACTTTGTTGAATA-3′; –2942). For the pHB subclone, the sense strand was sequenced using pr-HB3 (5′-AGTTAAAAGATCGTT-3′; –2406) and pr-HB4 (5′-ACAACAGGTTGGGTT-3′; –2081) and the template strand was using pr-HB1 (5′-CACCATTCCATCATGAT-3′; –1978) and pr-HB2 (5′-CTATATCTATATCTC-3′; –2260). For the pBA subclone, the primers included for the sense strand pr-15B1 (5′-AGGTCAGGTATCTGC-3′; –1421) and pr-15B2 (5′-ACAGGCAAATGGGTC-3′; –1180) and for the template strand pr-15E1 (5′-TTCTCATACAACTACC-3′; –1125). For the p5′–863 plasmid, the M13 universal primer was used to sequence from the sense strand and for the template strand pr-850 (5′-TGACTCTACCGACCC-3′; –445). The series of subclones of the IGS used included those shown in Figure 1D as well as p–863/–225, p–513/+89, p–342/+89, p–863/–448, p–863/–594, p–1786/–1496, p–1786/–954, p–1540/–864, p–1711/–864 and pSP329.

RESULTS

Analysis of control sequences for rRNA synthesis in fission yeast required construction of rDNA mini-gene templates and establishment of an *in vitro* transcription system for *S.pombe* rRNA synthesis that supports accurate and activated initiation of cloned *S.pombe* rDNA mini-genes. This system could then be exploited to assess the presence of transcriptional regulatory elements present in the *S.pombe* rDNA IGS. A parental rDNA mini-gene was engineered to contain a full rDNA promoter, extending from 5′–243 to 3′+31, bacteriophage T7 DNA sequences as a foreign marker (27) and rDNA sequences containing the putative terminator(s) for RNA polymerase I transcription (50). The inclusion of rDNA terminators ensures that initiation would not be inhibited by RNA polymerases reading around the template and dislodging bound promoter complexes (51,52). The resultant template, p–243:XH, was tested initially for its ability to support correct initiation *in vivo*. When introduced into *S.pombe* cells, this rDNA mini-gene template supports accurate initiation, with rRNA synthesis levels reduced at higher cell densities (see Fig. 2, compare last lane, OD_{600} 3.9, with other lanes).

A fission yeast S-100 extracts supports accurate and activated transcriptional initiation

Given the down-regulation of rRNA synthesis at higher cell densities, the *S.pombe* S-100 transcription extracts were prepared from logarithmically growing cells cooled and collected before the cells reached an OD_{600} of 3.0. The rDNA mini-gene, p5′–243:XH, as well as p5′–243, supported accurate initiation *in vitro* as well as *in vivo* and the transcription supported by the template p5′–243:XH, containing a single copy of the intergenic sequences with the terminator sites located downstream of the initiation site, was slightly increased relative to p5′–243, which lacks this region (compare lane 7, p–243, with lane 1, p′-243:XH, in Fig. 3A and lane 5 with lane 1 in Fig. 3B). Transcription

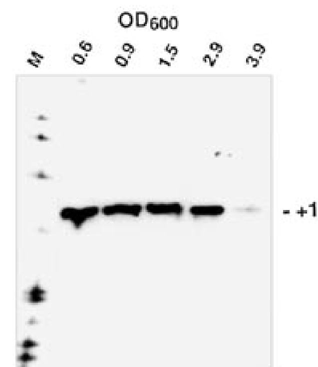

Figure 2. Correct RNA polymerase I transcriptional initiation supported by the *S.pombe* rDNA mini-gene *in vivo*. The *S.pombe* rDNA mini-gene, p–243:XH, was introduced into *S.pombe* MP6-10B cells (42,49) and the levels of correctly initiated mini-gene transcripts synthesized in stably transformed cells were assessed at different cell densities. Cells were collected at OD_{600} of 0.6, 0.9, 1.5, 1.9 and 3.9 and S1 analysis performed using 5 µg total RNA and a specific, single-stranded probe labeled at +340 of the template strand. The S1 protected fragment representing correctly initiated RNAs is labeled +1. The marker lane (M) contains ^{32}P-5′-end-labeled pBR322 *Hpa*II fragments.

supported by a plasmid bearing the full rDNA promoter was insensitive to concentrations of α-amanitin of 10–100 µg/ml and initiation was fine mapped to the same site utilized by endogenous *S.pombe* rDNA genes (data not shown; 9)

In vitro analysis of *cis*-acting regulatory sequences in the *S.pombe* IGS of the rRNA gene

The templates constructed to assess the presence of stimulatory or inhibitory transcriptional regulatory sequences in the *S.pombe* rDNA IGS are illustrated in Figure 1B and C and were designed to position an IGS region of ~700–1000 bp, comparable with the length of the known vertebrate enhancers, the *Xenopus* 60/81 bp repeats and the mouse 140 bp repeats, upstream of the *S.pombe* rDNA promoter (13,14,16,25). Regions of the IGS have been inserted in the wild-type orientation (F, forward) or the reverse orientation (R, reverse). Transcription levels were measured using stringent *in vitro* transcription conditions that are sensitive to the activities of eukaryotic rDNA enhancer sequences (13,15,19).

Comparison of transcription levels reveals that two regions of the IGS sequences can significantly stimulate rRNA synthesis *in vitro*. The intergenic rDNA sequences present in both pXH:–243 and in pHB_F:–243 support dramatically increased transcriptional levels from the *S.pombe* rDNA promoter (compare lanes 4 and 6 with lane 1 in Fig. 3A and lanes 2 and 3 with 1 in Fig. 3B). It should be noted that the intergenic rDNA sequences in the template pXH:–243 (from –4178 to –2555) are present in two copies, one upstream of the promoter and the other 330 bp downstream of the initiation site; these serve to stimulate transcriptional levels >3-fold relative to the control template, p–243:XH. To assess whether the sequences present in pHB_F:–243 could stimulate transcription in an orientation-independent manner, the template pHB_R:–243 was constructed, with IGS sequences positioned in the reverse orientation upstream of the rDNA mini-gene (see Fig. 1B). Although more effective in the forward orientation, this region serves to stimulate initiation *in vitro* in the reverse orientation

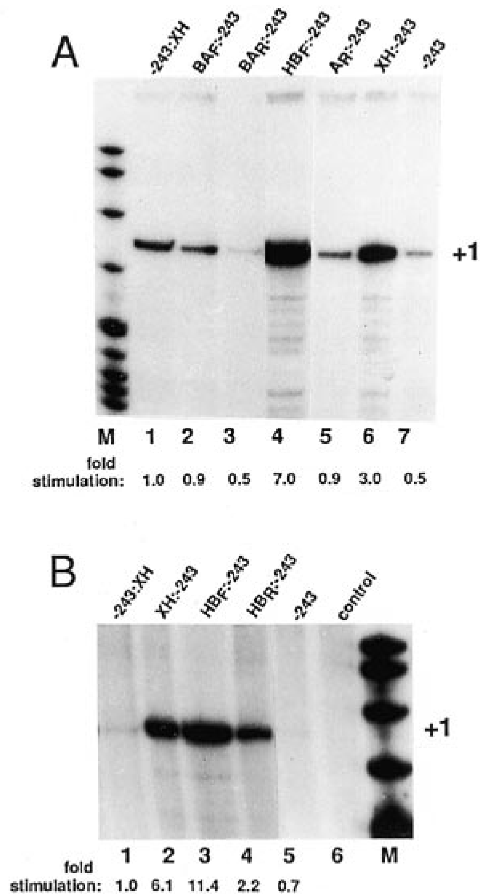

Figure 3. Activation of the *S.pombe* rDNA promoter by intergenic rDNA sequences *in vitro*. (**A**) The *S.pombe* rDNA templates (Fig. 1B) were transcribed *in vitro* at low template concentration (1.3 fmol in a 40 µl reaction) with ~50 µg *S.pombe* transcription extract and a final concentration of 2.5 µg/ml non-specific pBS SK+ DNA. Transcription supported by each template, p5′–243:XH, pBA_F:–243, pBA_R:–243, pHB_F:–243, pA_R:–243; pXH:–243 and p5′–243, was assessed by S1 analysis of the synthesized RNAs. The 340 nt S1 protected fragment representing correctly initiated RNAs is marked +1. The markers are *Hpa*II fragments of pBR322. These stimulatory transcriptional effects have been observed in multiple extracts and with different preparations of purified plasmid DNAs. Relative transcription levels supported by each template are listed relative to the parental p5′–243:XH template, set as 1.0. (**B**) The templates p5′–243:XH, pXH:–243, pHB_F:–243, pHB_R:–243 and p5′–243 were assessed for their ability to support initiation as in (A). Lane 6 is a control transcription reaction conducted in the absence of template DNA.

~2-fold relative to the control (compare lanes 3 and 4 with lane 1 in Fig. 3A).

The sequences between –1786 and –243, when assessed under the most stringent conditions, do not stimulate initiation of the gene promoter (see Fig. 3A, lanes 2, 3 and 5, pBA_F:–243, pBA_R:–243 and pA_R:–243). Thus, it is the upstream half of the *S.pombe* IGS that contains sequences that serve to dramatically stimulate initiation of a *cis*-located rDNA promoter.

A second series of templates was constructed to determine whether dissection of the IGS rDNA present in pXH:–243 would reveal which regions stimulated initiation *in vitro*, independent of a second, downstream copy of this same intergenic rDNA region (see Fig. 1C). Although this series lacked a terminator downstream of the promoter, promoter occlusion did not apparently occur on these templates (data not shown). As seen in Figure 4A, the sequences from –2560 to –1781 also significantly stimulated transcription in this rDNA mini-gene (pHB:3′Δ+31). The level of stimulation was nearly 6-fold for pHB:3′Δ+31 relative to the transcription level supported by the template bearing just the full rDNA promoter (compare lanes 3 and 2, Fig. 4A).

The two templates engineered to dissect the 5′- or the 3′-half of the IGS present in pXH:–243 and assess transcriptional effects of each half on a *cis*-located rDNA promoter were pΔXH1:3′Δ+31 and pΔXH2:3′Δ+31 (see Fig. 1C). Neither of these templates supported a significantly higher level of transcriptional initiation than the wild-type p3′Δ+31 template (see Fig. 4). In fact, transcription supported by the pΔXH2:3′Δ+31 template was 3-fold lower than that supported by the wild-type template (compare lane 6 with lane 2, Fig. 4A). Thus, dissection of the rDNA IGS present in pXH:–243 failed to reveal any region of the ~–3900/–2555 rDNA that contained regulatory sequences that significantly stimulated transcriptional initiation of the rDNA gene promoter. The region of the rDNA IGS containing the sequences that direct termination of RNA polymerase I (50) and which contain the *S.pombe* Reb1p binding sites (A.Zhao and L.Pape, unpublished data) were present in the pΔXH2:3′Δ+31 template. The inability of the intergenic sequences in either pΔXH1:3′Δ+31 or pΔXH2:3′Δ+31 to significantly stimulate initiation may reflect a requirement for interactions dependent on multiple *cis*-acting domains that were dissected or for the presence of an additional, second copy of this rDNA IGS region, downstream of the rDNA mini-gene coding sequences, as in pXH:–243. The intergenic sequences present in the pBA:3′Δ+31 template served to increase initiation 3.6-fold (compare lane 4 with lane 2, Fig. 4A).

The ability of these intergenic sequences to stimulate initiation from a *cis*-located rDNA promoter was further analyzed under competitive transcription conditions, where the ability of the test template to compete for required transcription factors was assessed. As seen in Figure 4B, lanes 1–5, neither pΔXH1:3′Δ+31 nor pΔXH2:3′Δ+31 supported higher levels of initiation than p3′Δ+31. In fact, transcription levels supported by pΔXH2:3′Δ+31 were even more repressed than was apparent under non-competitive conditions (compare Fig. 4A and B). The relative transcriptional level of pΔXH2:3′Δ+31 was 0.03, compared with 1.0 for the reference p3′Δ+31 template, while pΔXH1:3′Δ+31 supported nearly the same level as p3′Δ+31 (compare lanes 2 and 3 with lane 1, Fig. 4B). The intergenic rDNA sequences in the pHB:3′Δ+31 template also stimulated initiation significantly in this assay (compare lane 4 with lane 1).

Trans-competitive effect of intergenic rDNA spacer sequences on initiation

The IGS regions that had the largest stimulatory effect when located *in cis* to the *S.pombe* rDNA promoter were subjected to a *trans*-competition assay. In this assay, plasmid DNAs containing subcloned regions of the rDNA IGS were assessed for their ability to competitively inhibit transcription supported by an rDNA mini-gene when located *in trans* to the rDNA promoter. Control reactions contained non-specific vector DNA (pBS SK+) as competitor. As seen in Figure 5, incubation of both pXH (containing rDNA from –4178 to –2555) and pHB (–2560 to –1781) with transcription components resulted in a decrease in transcription from a subsequently added rDNA promoter by

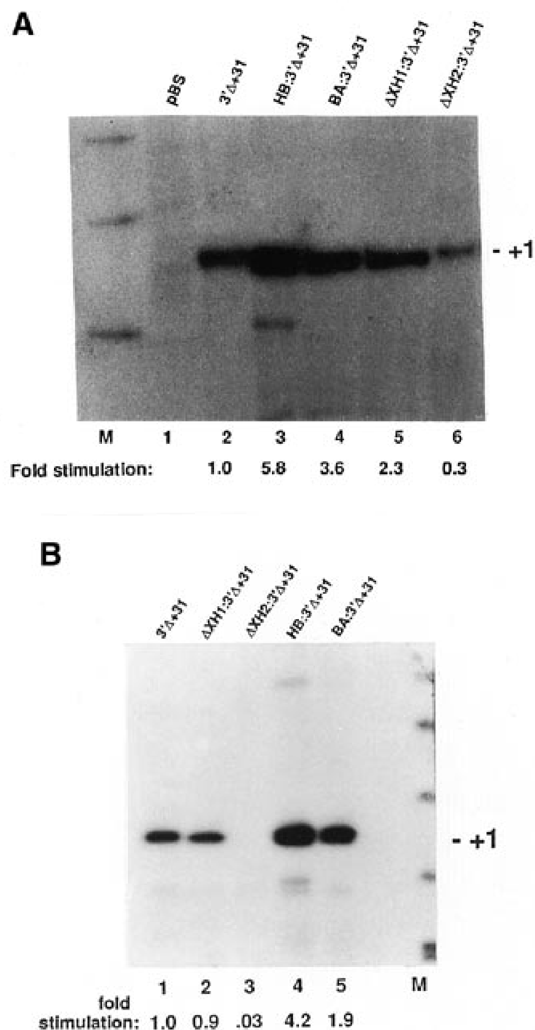

Figure 4. The *S.pombe* rDNA IGS sequences between –2560 and –1781 stimulate transcription of an independent rDNA mini-gene. (**A**) A second series of *S.pombe* rDNA templates containing regions of the IGS inserted upstream of a full rDNA promoter, 5′–243 to 3′+31, was designed to test activation independent of the downstream *S.pombe* intergenic sequences from –4178 to –2555 (see Fig. 1C). Correct initiation of rRNA synthesis supported by these templates was assessed in the *S.pombe* S-100 extract under conditions sensitive to the activity of upstream stimulatory sequences. 2.1 fmol each template, non-specific pBS SK+ at 2.5 µg/ml and ~50 µg S-100 protein were present. The marker lane (M) contains ^{32}P-end-labeled pBR322 *Hpa*II fragments and lane 1 is a control reaction with no template DNA. Lanes 2–5 show the S1 protected fragment (340 nt) representing correctly initiated RNAs produced from: lane 2, the template containing the promoter alone (p3′Δ+31); lane 3, pHB:3′Δ+31 (with sequences from –2560 to –1781 inserted upstream of the promoter); lane 4, pBA:3′Δ+31 (with sequences from –1786 to –864); lane 5, pΔXH1:3′Δ+31 (with sequences from –3959 to –3241); lane 6, pΔXH2:3′Δ+31 (with sequences from –3240 to –2555 upstream of the promoter). The transcription levels were quantitated using a BioRad Molecular Imager and the fold stimulation relative to the template bearing the promoter alone (set as 1.0) is noted below each lane. (**B**) In this competitive assay, transcription components were allowed to preincubate for 15 min in the presence of a competitor rDNA plasmid, p–863/+89 (50 ng/ml), prior to addition of the *S.pombe* rDNA mini-gene template (0.85 fmol). Following a second 15 min incubation period, ribonucleoside triphosphates were added to initiate transcription, as well as the rNTP regenerating reagents creatine phosphate and creatine kinase.

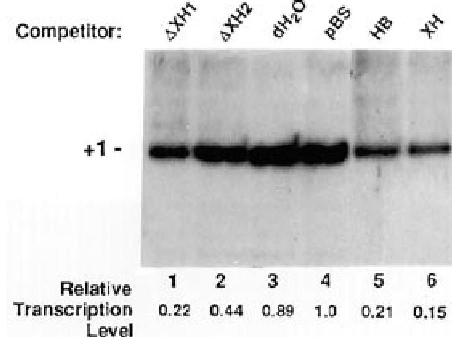

Figure 5. The *S.pombe* rDNA far upstream intergenic stimulatory region inhibits initiation supported by the rDNA promoter when located *in trans*. The plasmid DNAs pΔXH1 (lane 1), pΔXH2 (lane 2), pHB (lane 5), pXH (lane 6) or the controls containing the cloning vector DNA, pBS SK+ (lane 4) or no DNA (lane 3) were allowed to preincubate with transcription components prior to addition of the rDNA template, p3′Δ+31, and to initiation of transcription. Transcription supported by the rDNA template, p3′Δ+31, in each reaction was assessed relative to the control containing pBS SK+. The relative transcription levels are listed on the bottom line relative to the pBS control, set as 1.0.

≥5-fold (compare lanes 5 and 6 with lane 4, Fig. 5). The two plasmids derived from pXH, pΔXH1 (–3959 to –3241) and pΔXH2 (–3240 to –2555), showed a lesser competitive effect than the parental pXH (compare lanes 1 and 2 with lane 4). The *cis*-stimulatory and *trans*-competitive activities of the far upstream intergenic rDNA were not due to 'spacer promoters'. All of the plasmids bearing regions of the *S.pombe* rDNA IGS (Fig. 1D) have been assayed for rDNA spacer promoters (53–58), but virtually no transcription initiated from any putative spacer promoters was detected *in vitro* (data not shown).

Schizosaccharomyces pombe rDNA IGS elements homologous to known rDNA enhancers and to the *S.pombe* rDNA promoter

The nucleotide sequence of the entire *S.pombe* IGS was determined to enable analysis of the regions containing regulatory sequences and a search for the presence of repeats longer than ~100 bp, for regions homologous to rDNA promoter domains or for elements homologous to known rDNA enhancers. The DNA sequence was determined from both strands of the IGS (see Fig. 6A; accession no. Y09256).

One of the hallmarks of vertebrate rDNA enhancers is the presence of longer, repetitive regions, as found in the *Xenopus* 60/81 bp repeats and the mouse 140 bp rDNA enhancer elements (13–17,25). However, extensive searching for such regions in the *S.pombe* IGS failed to uncover any. A comparison of the entire *S.pombe* rDNA IGS with the active region of the *S.cerevisiae* rDNA enhancer revealed the most significant homology to be located at –2092, in the region of the IGS that serves to significantly stimulate initiation of a downstream rDNA promoter (see Fig. 6B). A search of elements present in both this rDNA intergenic region and the *X.laevis* 60/81 bp repeats, also reveals a homology in this same region.

To test whether this enhancer-homologous region had a role in the stimulatory effect of this *S.pombe* far upstream intergenic region, templates were made bearing a deletion in this intergenic region from –2094 to –2064 (pHBΔE:–243 and pHBΔE:3′Δ+31). Transcription supported by pHBΔE:–243 (Fig. 3D) and pHBΔE:+31 (data not shown) was compared with that supported by the

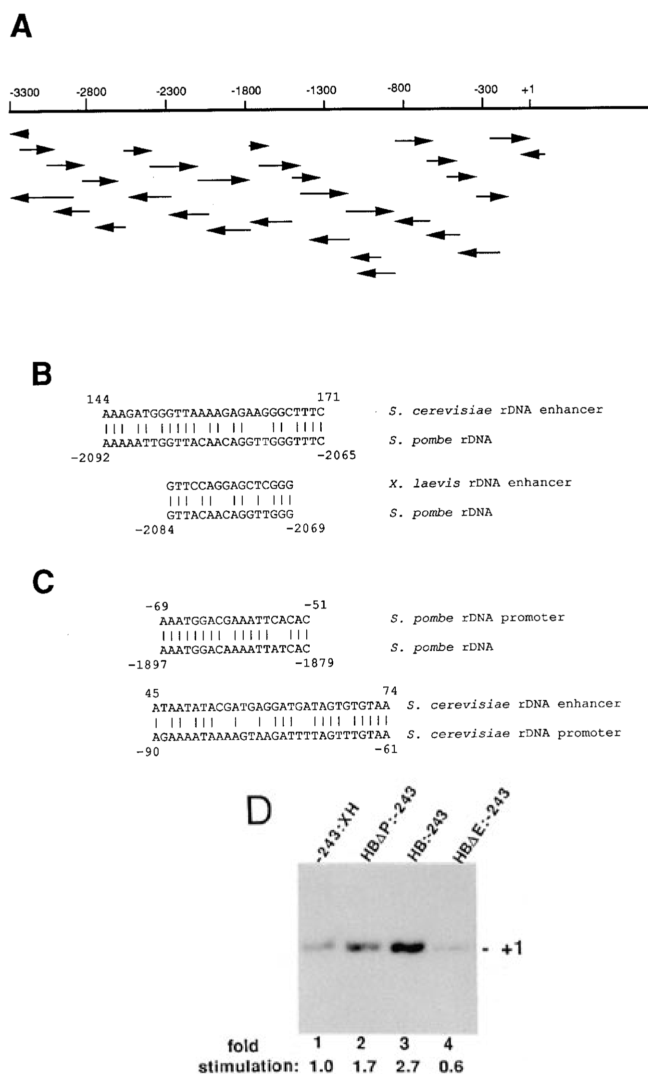

Figure 6. Comparative analysis of the *S.pombe* rDNA IGS. (**A**) Sequencing strategy. The nucleotide sequence of the entire ~3450 bp rDNA IGS that separates the tandemly repeated rRNA coding sequences was determined on both strands (EMBL/GenBank accession no. Y09256). The 33 bp sequence just 3′ of the end of the mature ~25S rRNA coding sequences is not included but has been published (50). Thus, the first nucleotide of this sequence is –3423. The sequencing strategy is depicted in diagrammatic form and the primers and templates utilized are listed in Materials and Methods. (**B** and **C**) Sequences homologous to the *S.cerevisiae* and the *X.laevis* rDNA enhancers are present in the region of the *S.pombe* rDNA IGS displaying some activities of rDNA enhancers. (B) The most homologous sequences in the entire *S.pombe* IGS to the active region of the *S.cerevisiae* rDNA enhancer (19,80; numbering as in 19) is between –2092 and –2065 in the *S.pombe* rDNA far upstream stimulatory region (71% homology). Sequences between –2084 and –2069 of the *S.pombe* rDNA far upstream stimulatory region were found to have the highest homology with the *X.laevis* rDNA enhancer. (C) The *S.pombe* intergenic rDNA spacer region displaying some activities of rDNA enhancers contains a 19 bp segment that is 84% identical to a critical, central rDNA promoter domain. An interesting homology was noted between a *S.cerevisiae* 5′ enhancer domain (29,30) and the *S.cerevisiae* central rDNA promoter region. (**D**) Transcription supported by the templates p–243:XH (lane 1), pHBΔP:–243 (lane 2; the rDNA sequences between –1900 and –1879, bearing the promoter homology, are deleted in this template), pHB:–243 (lane 3) and pHBΔE:243 (lane 4; the rDNA sequences between –2094 and –2064 bearing enhancer-homologous sequences are deleted) was assessed using stringent *in vitro* transcription conditions (see Materials and Methods). The S1 protected fragment representing correctly initiated mini-gene rRNAs is marked +1. The analogous deletions were introduced into the second series of templates, creating pHBΔE:3′Δ+31 and pHBΔP:3′Δ+31, and resulted in analogous effects (data not shown).

parental template pHB:–243 and pHB:+31. This deletion reduced transcriptional stimulation in both of these templates (compare lanes 3 and 4 in Fig. 6D for pHBΔE:–243; data not shown for pHBΔE:3′Δ+31).

Enhancement is mediated via critical promoter domains (13,59–61) and a core 50 bp region of the *Xenopus* rDNA enhancer is homologous to the central rDNA promoter. Comparison of the *S.pombe* rDNA promoter with the far upstream IGS stimulatory region revealed a 19 bp stretch that is 84% identical to a critical domain in the central rDNA promoter (see Fig. 6C). This intergenic region does not support rRNA synthesis via a spacer promoter (data not shown). Templates bearing deletions in this promoter-homologous region (pHBΔP:–243 and pHBΔP:3′Δ+31) showed a somewhat reduced level of transcriptional stimulation (compare lanes 2 and 3 with lane 1 in Fig. 6D). It is also of interest that 20 out of 30 bp of the *S.cerevisiae* rDNA central promoter domain are shared with its enhancer and are located in a region that contributes to *in vivo* stimulation of rRNA synthesis (29,30).

DISCUSSION

Nearly one third of the ~11 kb rRNA gene repeat unit of fission yeast is composed of an IGS and its upstream half was shown to house *cis*-acting sequences that modulate levels of transcriptional initiation of the *S.pombe* rRNA gene promoter. We have established an *S.pombe in vitro* transcription system that supports both accurate and activated transcriptional initiation from *S.pombe* rDNA mini-gene templates (see Figs 3–5) to investigate these interactions and our analyses were conducted using stringent *in vitro* transcription conditions that reflect the contributions of eukaryotic rDNA enhancer sequences (13,15,19).

A 780 base intergenic region starting at –2560 was shown to significantly stimulate transcription of a *cis*-located rDNA promoter compared with a control plasmid bearing the full rDNA promoter. These sequences functioned optimally in the forward orientation (Figs 3A and 4), with pHB_F:–243 and pHB_F:3′Δ+31 supporting significantly increased transcription levels. Deletion of a 30 bp enhancer-homologous region abolished the stimulatory effect (Fig. 6D). The intergenic rDNA sequences present in the pXH:–243 template stimulate transcriptional initiation *in vitro*, but this is apparently dependent on the presence of a second copy downstream (Figs 3A and 4).

In a separate assay that also reflects the presence of transcriptional regulatory sequences, the plasmids pXH and pHB (Fig. 1C) were shown to exert a significant *trans*-competitive effect on the rDNA mini-gene (Fig. 5) analogous to the action of vertebrate rDNA enhancers and an rDNA enhancer in the *A.castellanii* rDNA IGS (13–18,25,63). These same plasmid DNAs (pXH and pHB) did not contain any apparent spacer promoters (53–58): they did not support production of stable transcripts from an rDNA IGS promoter *in vitro* (data not shown).

The sequence of the rDNA IGS region of *S.pombe* differs from that of higher eukaryotes in that it lacks repetitive elements analogous to the ~140 bp intergenic rDNA enhancer repeats (13–17,25). However, it can be functionally replaced by the *Xenopus* rDNA enhancer (data not shown), suggesting a conserved mode of activated polymerase I transcription. The presence of repetitive elements is not a prerequisite for an rDNA enhancer: the *S.cerevisiae* enhancer is present in a 320 bp *Eco*RI–*Hpa*I fragment as a single copy ~2 kb upstream of the start site for ~37S rRNA synthesis (12,19,28–33). If the two divergent yeasts, *S.cerevisiae*

and *S.pombe*, had rDNA transcriptional enhancers analogous in position and function, the sequences just downstream of the putative *S.pombe* rDNA terminators would be expected to confer transcriptional stimulatory activity. The template pΔXH2:3′Δ+31, which contains this region upstream of the *S.pombe* rDNA promoter, directs significantly less transcription than the rDNA promoter alone (see Fig. 4), arguing against the presence of a transcriptional enhancer in this intergenic region.

The genomic organization and structure of the *S.pombe* rDNA repeat unit also differs from that of *S.cerevisiae*: the *S.pombe* rDNA repeat unit contains a larger IGS sequence [3.45 kb (9,35; this paper) versus 2.5 kb for *S.cerevisiae* (64)]; its intergenic rDNA spacer is not interrupted by a 5S rRNA gene (35,64,65) and its 5′ external transcribed spacer region is larger [1355 bp in *S.pombe* (9) versus 695 bp in *S.cerevisiae* (66,67)]. In addition, it contains multiple sites for apparent termination of transcription, located between 260 and 450 nt downstream of the mature 3′-end of the 25S rRNA coding sequences (50), analogous to multiple termination elements in the mouse rDNA IGS (68).

The activity of vertebrate rDNA enhancers appears to be due, in part, to their association with the nucleolar stimulatory factor UBF (14,21–23,69), which functions to counter repression of the promoter (24), induces structural changes in the rDNA enhancer sequences (69,70) and interacts with the Rb protein (71). Vertebrate UBF interacts with rDNA enhancers and promoters in a somewhat sequence-specific manner (72) and its interaction with the essential RNA polymerase I initiation factor may contribute to its effects on rRNA synthesis (46,73,74). In fact, both UBF and the *Acanthamoeba* enhancer binding factor (EBF) serve to stabilize interactions of the essential initiation factor with the rDNA promoter (23,46,58,75,76). Alternatively, a multi-subunit complex identified genetically as important for RNA polymerase I-catalyzed rRNA synthesis may represent a universal, rDNA-specific upstream activating factor (77). We have evidence that *S.pombe* contains an activity (L.Chen, A.Zhao and L.Pape, unpublished data) that co-fractionates with multiple polypeptides and which specifically associates with the *S.pombe* rDNA intergenic stimulatory region and with rDNA promoter sequences. An rDNA enhancer binding protein, the *S.cerevisiae* REB1 factor (78), directs RNA polymerase I termination *in vitro* via sequence-specific DNA binding (79), but this site is not essential for transcriptional enhancement (19,29–31). It is sequences downstream of this site that are apparently critical in confering enhancement (19,29,30,80). Comparison of this active region of the *S.cerevisiae* rDNA enhancer with the entire *S.pombe* rDNA IGS pinpointed 28 bp that are 71% identical and that lie within the *S.pombe* rDNA intergenic region that supports activated rRNA synthesis *in vitro* (see Fig. 6B). The interactions mediating enhancement via this *S.cerevisiae* domain are unknown, but this homology may point to cross-species conservation of activating factors for rRNA synthesis.

cis-acting sequences in eukaryotic rRNA genes that modulate the levels of transcriptional initiation at the rRNA gene promoter include, in addition to the promoter itself, rDNA enhancer elements, the promoter-proximal terminator for RNA polymerase I and spacer promoters (reviewed in 10,11,62,65). The rDNA enhancer apparently augments initiation by increasing the number of activated promoters (13,19,20), akin to the action of enhancers for RNA polymerase II transcribed genes (81,82), but the molecular basis for this stimulation is uncertain. For RNA polymerase II transcribed genes, a complex interplay of activators and co-activators with different basal level general transcription initiation factors serves to increase promoter activation (83–85).

Additional regions of the *S.pombe* IGS can affect initiation, albeit to a lesser extent than the ~–2600 region. An ~0.8 kb region starting at –1786 augments initiation of the rDNA promoter under less stringent conditions (data not shown and Fig. 4). In the widely studied *Xenopus* rDNA IGS, divergent classes of repeats upstream of the 60/81 bp repeats can also enhance transcription of the *Xenopus* rDNA gene promoter (86), while the spacer of the *Drosophila* rRNA gene is composed of repetitive elements homologous to the promoter that stimulate initiation (57,87). The implications are that nearly the entire rDNA IGS plays a role in modulation of the levels of rRNA synthesis.

These studies were conducted using *in vitro* transcription conditions sensitive to the action of vertebrate RNA polymerase I-specific enhancers that reflect the *in vivo* activity of these regulatory sequences (13,15,19). While the molecular interactions confering enhancement of transcriptional initiation remain to be fathomed, several independent lines of evidence point to interactions in addition to those with UBF that are required to mediate RNA polymerase I transcriptional enhancement (15,17,26,61,76). The establishment of an *in vitro* system for analysis of activated rRNA synthesis in fission yeast, the determination of the primary sequence of its rDNA IGS and identification of regions regulating transcriptional initiation lay the foundation for future studies on the interactions obligatory to transcriptional enhancement of eukaryotic rRNA genes.

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