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

Zhao Liu, Annie Zhao, Ling Chen* and Louise Pape*

Department of Chemistry, New York University, New York, NY 10003, USA

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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 rDNA 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 the basic molecular processes in higher eukaryotic organisms (1–8), little was known about requirements and regulatory sequences that affect 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 function 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 rDNA gene promoter. To further analyze these sequences and to enable cross-species comparison of the region of the rRNA gene that regulates RNA synthesis, the IGS, we determined its nucleotide sequence.

MATERIALS AND METHODS

Yeast and bacterial strains; transformations

The bacterial strains used included XL1-Blue (endA1, hisD17 (rK–,mK+), supE44, thi-1, λ−, recA1, gyrA96, relA1, lac, [F′, proAB, lacZΔM15, Tn10, (tet)] and SURE™ (mcrA, Δ[mcrBC-hsdRMS-mrr]171, endA1, supF44, thi-1, λ−, λ, gyrA96, relA1, lac, recB, recJ, sbcC, umuC/TnSn kan3), wortC, [F′, proAB, lacF′ZΔM15, Tn10(ter)] (Stratagene). The S.pombe strains used were wild-type 972 (λ−; kindly sent by Dr H.Levin) and MP6-10B (λ−, ade1-51, ade6-M210, leu1-32, ura4-D18; kindly provided by M.Moser and T.Davis).

Bacterial cells were transformed by either standard CaCl2–PEG procedures or by electroporation using a BioRad

* To whom correspondence should be addressed. Tel: +1 212 998 8444; Fax: +1 212 260 7905; Email: papel01@mcrcr.med.nyu.edu

*Present address: Department of Microbiology, Ohio State University, Columbus, OH 43210, USA
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 spacer, and the larger, diagonally striped boxed portions coding for the mature −175S, 5S and −25S rRNAs. E, EcoRI; B, BamHI; H, HindIII; K, KpnI; Xh, Xhol; K, KspI; X, XhoI. (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 in the 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).

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

Figure 1A 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 *XhoI*-HindIII fragment from −4178 to −2555; pHB contains a HindIII–BamHI fragment from −2560 to −1781; pBA contains a BamHI–AluI partial fragment from −1786 to −864, inserted between BamHI and Smal 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 EcoRI–HaeIII fragment from pXH, −3959 to −3241, between EcoRI and Smal sites of pBS SK+. pΔXH2, a HaeIII–BamHI fragment isolated from pXH, extending from −3240 to −2555, was inserted into pBS SK+ between an EcoRI site, which had been converted to a blunt-ended site, and a BamHI 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 Smal site. This template, p5′−863, supported accurate initiation. A p5′Δ−243/3′Δ+89 template was cleaved downstream of +89 at the EcoRI 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 BamHI site. These were inserted into pBS SK+ treated with BamHI, Smal and phosphatase. The plasmid prR+31 contains the *S. pombe* rDNA 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+.

GenePulser (39). The transformation method used for introduction of plasmid DNAs into *S. pombe* was that of Allshire (49).
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 OD₆₀₀ 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, 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 p5Δ–850 at 80 ng/ml.

For S1 analysis, the radioactively labeled samples were obtained by labeling the 5′-end of the template strand of p5Δ–243 or p3Δ–31 at a XbaI site at +340 with [γ-³²³P]ATP (4500 Ci/mmol; ICN) and T4 polynucleotide kinase (39). Following cleavage with BamHI 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′-³²³P-labeled pBR322 HpaII 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, pXH1 or pXH2, 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 dyeoxy sequencing reactions (39) using Sequenase Version 2.0 (US Biochemical). 7-deaza-dGTP to clarify the compression regions and [γ-³²³P]ATP (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′-TTGTTGGGAAAG-TACTC-3′; –3061), pr-XH4 (GTTGAAGGTAGTCG, –2845) and, for the template strand, pr-XH1 (5′-AAATTTGAAAAGGG-GGA-3′; –2714) and pr-XH2 (5′-AAACACCTTGGTTGAATA-3′; –2942). For the pHB subclone, the sense strand was sequenced using pr-HB3 (5′-AGTAAAAGATCGTT-3′; –2406) and pr-HB4 (5′-ACACACGGTTGGTT-3′; –2081) and the template strand was using pr-HB1 (5′-CACCATTCATCATGAT-3′; –1978) and pr-HB2 (5′-CTATAATCATAATCTC-3′; –2260). For the pBA subclone, the primers included for the sense strand pr-15B1 (5′-AGGTCAAGTATCTCG-3′; –1421) and pr-15B2 (5′-ACAG-GCAAATGGTGC-3′; –1180) and for the template strand pr-15E1 (5′-TTCTCACTAACTACAC-3′; –1125). For the p5′–863 plasmid, the MAL primer was used to sequence from the sense strand and for the template strand pr-850 (5′-TGACTCTAC- CGACCC-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/–489, 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 RNA 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 (α) 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, OD600 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 OD600 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, p5′–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 OD600 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 [5P]–5′-end-labeled pBR322 HpaII fragments supported by a plasmid bearing the full rDNA promoter was insensitive to concentrations of α-aminatin 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 pHBe–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 pHBe–243 could stimulate transcription in an orientation-independent manner, the template pHBe–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.
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, pBAp:–243, pBAp:–243 and pAr:–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:3Δ+31). The level of stimulation was nearly 6-fold for pHB:3Δ+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 pAXH1:3Δ+31 and pAXH2:3Δ+31 (see Fig. 1C). Neither of these templates supported a significantly higher level of transcriptional initiation than the wild-type p3Δ+31 template (see Fig. 4). In fact, transcription supported by the pAXH2:3Δ+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 pAXH2:3Δ+31 template. The inability of the intergenic sequences in either pAXH1:3Δ+31 or pAXH2:3Δ+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 pBA3Δ+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 pAXH1:3Δ+31 nor pAXH2:3Δ+31 supported higher levels of initiation than p3Δ+31. In fact, transcription levels supported by pAXH2:3Δ+31 were even more repressed than was apparent under non-competitive conditions (compare Fig. 4A and B). The relative transcriptional level of pAXH2:3Δ+31 was 0.03, compared with 1.0 for the reference p3Δ+31 template, while pAXH1:3Δ+31 supported nearly the same level as p3Δ+31 (compare lanes 2 and 3 with lane 1, Fig. 4B). The intergenic rDNA sequences in the pHB:3Δ+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...
≥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 locate at –2092, in the region of the IGS that serves to

Figure 4. The S. pombe rDNA 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 RNA synthesis supported by these templates was assessed in the S. pombe S100 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 S100 protein were present. The marker lane (M) contains 32P end labeled pBR322 HpaII 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, pHB;3′Δ+31 (with sequences from –1786 to –864); lane 5, pXH1;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 containing 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), pXH2 (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.
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. Y09236). The 33 bp sequence just downstream (Figs 3A and 4) but this is apparently dependent on the presence of a second copy of the rDNA IGS promoter (data not shown). Templates bearing deletions in this homologous region abolished the stimulatory effect (Fig. 6D). The intergenic rDNA sequences present in the pXH−243 template stimulate transcriptional initiation from a 5′ promoter (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).

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 pHBp−243 and pHBp−3Δ+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 EcoRI-Hind 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 pXH2.3+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 intrinsic rDNA spacer is not interrupted by a SS 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-fractions 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 conferring 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 RNA gene promoter include, in addition to the promoter itself, rDNA enhancer elements, the promoter-proximal terminator for RNA polymerase II 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 —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 conferring 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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