MOLECULAR AND CELLULAR BIOLOGY, May 1997, p. 2866–2875 Vol. 17, No. 5
0270-7306/97/$04.00+0
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The Repeat Organizer, a Specialized Insulator Element within the Intergenic Spacer of the *Xenopus* rRNA Genes

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Received 20 December 1996/Returned for modification 7 February 1997/Accepted 26 February 1997

We have identified a novel activity for the region of the intergenic spacer of the *Xenopus laevis* rRNA genes that contains the 35- and 100-bp repeats. We devised a new assay for this region by constructing DNA plasmids containing a tandem repeat of rRNA reporter genes that were separated by the 35- and 100-bp repeat region and a rRNA gene enhancer. When the 35- and 100-bp repeat region is present in its normal position and orientation at the 3′ end of the rRNA reporter genes, the enhancer activates the adjacent downstream promoter but not the upstream rRNA promoter on the same plasmid. Because this element can restrict the range of an enhancer's activity in the context of tandem genes, we have named it the repeat organizer (RO). The ability to restrict enhancer action is a feature of insulator elements, but unlike previously described insulator elements the RO does not block enhancer action in a simple enhancer-blocking assay. Instead, the activity of the RO requires that it be in its normal position and orientation with respect to the other sequence elements of the rRNA genes. The enhancer-binding transcription factor xUBF also binds to the repetitive sequences of the RO in vitro, but these sequences do not activate transcription in vivo. We propose that the RO is a specialized insulator element that organizes the tandem array of rRNA genes into single-gene expression units by promoting activation of a promoter by its proximal enhancers.

The rRNA genes of yeasts and multicellular eukaryotes are organized in tandem arrays in which the genes are separated by intergenic spacers (25, 40). While the rRNA transcription units range from 8 kb in *Xenopus* and *Drosophila* spp. to 13 kb in mammals, the intergenic spacers range from 4 to 10 kb in *Xenopus* and *Drosophila* to 40 kb in mammals (22, 27). Thus, the rRNA loci constitute regions of chromosomes in which highly active transcription units occur every 12 to 53 kb. The intergenic spacer of rRNA genes in *Xenopus* and other metazoans is composed of repetitive sequence elements (reviewed in reference 40). Although the sequences of the intergenic spacers are not conserved among different eukaryotes, the presence and analogous arrangement of repetitive enhancer elements, promoter duplications, and terminator sequences in *Xenopus*, *Drosophila*, and mice suggest that there is a functional conservation of intergenic spacer elements across distantly related species (40). Despite intensive investigation of rRNA gene transcription and regulation, the function of a significant portion of the intergenic spacer region is not understood.

In *Xenopus laevis*, the majority of the intergenic spacer is composed of repeated sequence elements of 60 and 81 bp (60/81-bp repeats), which have the characteristics of transcriptional enhancers and activate transcription from the promoter of the 40S rRNA gene (reviewed in reference 38). These sequences constitute an array of binding sites for the transcription factor xUBF, which also binds to the RNA polymerase I (RNA pol I) promoters and is required for initiation (2, 12, 28, 36; reviewed in reference 34) (see Fig. 1). The remainder of the intergenic spacer is composed of four different repetitive sequence classes: 35-bp repeats, 100-bp repeats, *Taq* boxes, and spacer promoters (3, 23, 32, 43) (see Fig. 1). The individual repeated units within each class are virtually identical to one another in both sequence and length. The spacer promoters share high sequence identity with the 40S rRNA gene promoter (3, 32, 43) but are not regulated in concert with the gene promoter. In *X. laevis*, transcripts from endogenous spacer promoters are detected in tissue culture cells but not in oocytes, even though the gene promoters are highly active in both cell types (references 10 and 31 and references therein). Therefore, although the spacer promoters augment transcription under some circumstances (10, 11), their function is not clear (38). The transcriptional activities of the 35- and 100-bp repeats are also uncertain. Early experiments concluded that these elements did not greatly stimulate transcription from the rRNA gene promoter (11), but recently it has been reported that they function as enhancer sequences (33). The 35-bp repeats also coincide with a replication fork barrier in *X. laevis* tissue culture cells (45).

In this study, we characterized the *X. laevis* intergenic spacer region that contains the 35-bp repeats, 100-bp repeats, and *Taq* boxes, using two different transcription assays. First we tested the direct effect of this region on transcription from the 40S gene promoter. In contrast to experiments by other workers (33), this intergenic spacer region does not act as an enhancer sequence in microinjected *X. laevis* oocytes. We then tested the activity of this intergenic spacer region in plasmid constructs containing an enhancer and a simplified tandem arrangement of rRNA reporter genes. We found that this region restricts the enhancer to activation of a proximal promoter. Thus, this region possesses a characteristic of insulator elements and has the apparent ability to organize the tandem rRNA genes into single-gene expression units. Because of this, we suggest the name repeat organizer (RO) for this element. The transcription factor xUBF binds in vitro to the 35-bp repeat and 100-bp repeat sequences of the RO even though these elements do not stimulate transcription in vivo. Our results are consistent with a model in which the endogenous RO specifies polarity of enhancer action within the rRNA gene repeats.

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MATERIALS AND METHODS

Construction of plasmids. The *X. laevis* rRNA reporter genes used in all plasmids were the reporters pXlrψ40 and pXlrψ52 (20), which we refer to as 40 and 52, respectively. The reporters contain the full 40S rRNA gene promoter region to −245 and approximately 110 bases of the external transcribed spacer fused to the last 200 bases of the 28S region including 160 bases after the processed 3′ end of the transcript. These reporter genes contain 40- or 52-bp insertions so that their transcripts can be distinguished from each other and from the endogenous transcripts by S1 nuclease protection analysis. The enhancer module used in our studies was from the plasmid pXlr14F and contains 10 60/81-bp repeats (20). The wild-type RO element used in our constructs was the *Stu*I/*Bam*HI fragment from the *X. laevis* rRNA gene clone pXlr101A (1). This plasmid subclone is named p0,1. The block of six 35-bp repeats was a subcloned *Sac*II fragment from p0,1. The region containing the block of five 100-bp repeats with *Taq* boxes was generated by an *Afl*II digest of p0,1 which releases all of the 35-bp repeats. The clone pXlr164 was obtained from Garry Morgan (30). The 164RO was subcloned as a *Stu*I/*Bam*HI fragment.

Quantitative analysis of transcription in injected oocytes. Unless otherwise indicated, all quantitated data represent the results of at least three independent experiments. Portions of *X. laevis* ovary were removed surgically, digested with collagenase (Boehringer Mannheim Biochemical) to dissociate the ovary into single oocytes, and cultured in OR-2 buffer overnight (44). Each oocyte was injected with 20 nl of a mix containing 40 μg of each circular plasmid template/ml and 50 μg of α-amanitin/ml. At least 60 oocytes were injected for each template or coinjected pair of templates, and experiments were analyzed only when oocyte survival was >60%. Transcripts were allowed to accumulate for 6 to 20 h after injection, corresponding to steady-state levels of rRNA transcripts. The oocytes were then collected and total nucleic acid was purified as previously described (14). Transcripts from the injected templates were detected with probes specific for each template in an S1 nuclease protection assay (14). S1 protected bands were quantitated with a PhosphorImager (Molecular Dynamics). To control for probe specific activities, transcription ratios were normalized to the ratio from the coinjection of 40 with 52, which was set at a value of 1. Data for the tandem-reporter constructs were normalized by using the ratio of 40 to 52 from pXlr926.

For all two-plasmid injection experiments, the molar ratios of the coinjected plasmids were determined by Southern blotting. Samples from each injection were digested with restriction enzyme(s) prior to electrophoresis to obtain fragments that transfer with similar efficiency. Blots were hybridized with a probe for the reporter portion of each construct. In cases where the actual ratio of injected templates was not 1:1, the transcription signals were normalized for this ratio. Variations in template concentration led to a linearly proportional change in transcription signal over the twofold range of template concentration differences in our experiments (data not shown). It was not necessary to correct for template concentrations for the tandem-reporter constructs as both reporters were contained on the same plasmid and were therefore in an equimolar ratio.

In vitro DNase I footprinting. xUBF was purified from *X. laevis* A6 tissue culture cell nuclear extracts by DNA-affinity chromatography as described previously (12), where it was designated TFIS. The footprinting protocol was essentially as previously described (12). Probes were made from linearized plasmid DNA that was treated with calf intestinal phosphatase (Boehringer Mannheim), 5′ end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England Biolabs), and then isolated from acrylamide gels. The 35-bp repeat probe is a subcloned *Sac*II fragment from p0,1. The 100-bp repeat probe was subcloned from a *Bgl*I partial digest of p0,1. The 60/81-bp repeat probe has been previously described (12). Footprinting reactions contained 130 mM KCl, 25 mM HEPES-KOH (pH 7.6), 12.5 mM $MgCl_2$, 1.0 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 8.3 ng of poly(dA-dT)/ml, and 3 to 5 fmol of probe DNA. The mixture was cooled on ice until the addition of xUBF, whereupon it was incubated for 10 min at room temperature. DNase I (8 to 33 ng) was then added to each reaction, and digestion was allowed to proceed for 30 s.

RESULTS

The RO element of the intergenic spacer has modest activation activity. The region of the intergenic spacer that we focused on extends from a unique *Stu*I site immediately downstream of the site of 40S transcript 3′ end formation to the *Bam*HI site that marks the first spacer promoter (Fig. 1). The subcloned 1.3-kb fragment contains six 35-bp repeats, five 100-bp repeats, and two 21-bp *Taq* boxes but does not include a complete spacer promoter. For clarity, we will refer to this DNA fragment as the RO (see below). In our characterization of the RO, we generated a number of plasmid constructs which were given names that indicate the identity and order of the sequence elements that they contained. Our constructs are based on two reporter genes, designated 40 and 52, which are derived from sequences of the *X. laevis* 40S rRNA gene promoter and gene body (20). These reporters contain 40- and 52-bp insertions so that their transcripts can be distinguished in an S1 nuclease protection assay. To assay for transcriptional activity, the experimental constructs were coinjected with a reference reporter gene into *X. laevis* oocyte nuclei, and the transcriptional activities of the two reporter genes were compared.

We first asked whether the repetitive sequences of the RO might directly stimulate transcription. When an enhancer module containing 10 60/81-bp repeats was placed upstream of the 40 promoter in the construct E40, transcription was increased almost 30-fold relative to the coinjected reference reporter, 52 (Fig. 2, construct 2, E40). In contrast, when the RO was placed upstream of the 40 promoter, transcription increased less than twofold (construct 3, RO40). When the orientation of the RO was reversed so that the 35-bp repeats were adjacent to the 40 promoter, transcription was stimulated fourfold (construct 4, OR40). This element had no effect on transcription when placed in its endogenous position downstream of the 40 reporter (construct 5, 40RO). We also tested each major repetitive region individually for effects on transcription. The block of six 35-bp repeats had a less than twofold stimulatory effect on transcription while the region with the block of five 100-bp repeats and the *Taq* boxes inhibited transcription somewhat (Fig. 2, constructs 6 [35rep40] and 7 [100rep*Taq*40], respectively). The level of stimulation from the RO or its repetitive sequences did not approach that observed for the enhancer module. We therefore conclude that the RO intergenic spacer region does not function as an enhancer.

The RO restricts enhancer activity in the context of tandem genes. Since the RO did not behave as a conventional enhancer, we devised a new assay for this intergenic spacer region. Because the rRNA transcription units are organized as direct repeats separated by the intergenic spacers, we reasoned that the effect of the 1.3-kb fragment on transcription might be detected by placing it in a tandem rRNA gene repeat. We therefore constructed a simplified tandem gene repeat containing two rRNA reporter genes separated by the RO and one enhancer module. For these constructs, the values for relative transcription are reported as the ratio of transcription from the downstream reporter to the upstream reporter.

In the plasmid pXlr926, the two rRNA reporter genes 40 and 52 are in a direct tandem repeat separated by one enhancer module and a 1.44-kb fragment of nonspecific DNA (Fig. 3A, construct 1). The enhancer module in this construct stimulated both promoters almost equally (Fig. 3B) (20). However, when the nonspecific DNA was replaced by the RO, the enhancer preferentially activated the downstream promoter 3.5-fold (construct 2, 40ROE52T). To extend the similarity of this construct to the endogenous rRNA gene repeats, we inserted a second RO at the 3′ end of the downstream reporter. Addition of the second element resulted in a further increase in transcription of the downstream reporter to 10-fold (construct 4, 40ROE52TRO). We interpret this as a further restriction of the enhancer's activity to the proximal promoter. Since both rRNA reporter genes had an RO placed at their 3′ ends in this tandem construct, and since the RO did not stimulate transcription at the 3′ end of the single-reporter constructs (Fig. 2B, construct 5, 40RO), it is unlikely that the element stimulated transcription directly. Because of its influence over the activity of an enhancer in the context of tandem rRNA gene promoters, we have named this intergenic spacer region the repeat organizer.

In the tandem-reporter constructs above, the relative orientation of the promoters and RO corresponded to that of the endogenous rRNA gene repeats. We tested the importance of

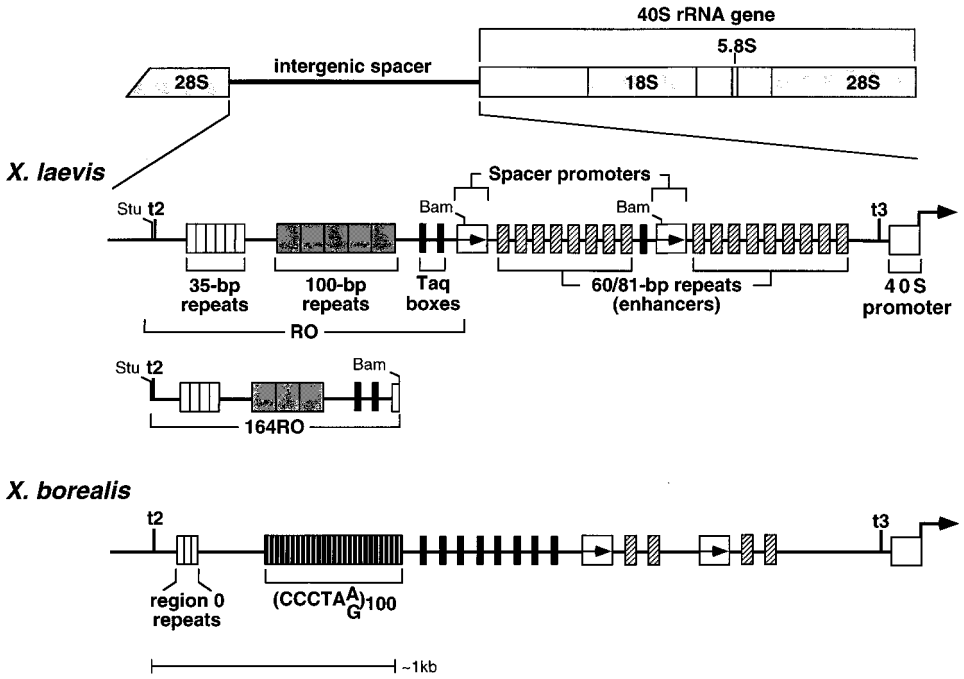

FIG. 1. Organization of the intergenic spacers of the *Xenopus* rRNA genes. This diagram shows blow-ups of typical intergenic spacers that separate the 40S rRNA genes of *X. laevis* and *X. borealis*. The 35-bp repeats of *X. laevis* are represented by white boxes, while the 100-bp repeats are represented by dark grey boxes. (These sequences are also known as the region 0 repeats and region 1 repeats, respectively [32].) The *Taq* boxes are a 21-bp element identified by their similarity to a repeated sequence in the *X. borealis* spacer and are indicated by black boxes for both species (21, 22). The *X. laevis* *Stu*I/*Bam*HI fragment, which encompasses the 35-bp repeats, 100-bp repeats, and accompanying *Taq* boxes region, is designated RO. The 164RO is an element that contains fewer 35-bp repeats and 100-bp repeats than typical intergenic spacers. The 60/81-bp repeats contain a 42-bp sequence, represented by striped boxes, that shares homology with the 40S promoter. These repeats bind xUBF and constitute the rRNA gene enhancers (12, 20, 36). The spacer promoters and the 40S rRNA gene promoter are indicated by white boxes with arrows that indicate the direction of the transcription units. The site of 40S transcript 3′ end formation immediately precedes the *Stu*I site. The t3 element is a fail-safe terminator of RNA pol I transcription, while the t2 element is a nonfunctional sequence variant (21). The intergenic spacer of *X. borealis* contains three 13-bp region 0 repeats that are identical to sequences within the 35-bp repeats of *X. laevis*. The $(CCCTAA/G)_{100}$ region, indicated by the box with vertical stripes, is similar to telomeric sequences and has no homologous sequences to the *X. laevis* spacer (23). The *Taq* boxes of *X. borealis* are embedded in repeated sequences of 100 bp that share homology with the 100-bp repeats of *X. laevis* (33). The spacer promoters and 42-bp sequences are as described above. The t2 and t3 sites in *X. borealis* are both functional terminators (23). The number of spacer promoter-enhancer block units can vary between intergenic spacers, resulting in length heterogeneity for this region. The drawing is approximately to scale.

the tandem organization of the RO and the promoters for the enhancer restriction activity. When the element was placed in a reverse orientation relative to the tandem promoters, the transcription ratio for the downstream to the upstream reporters decreased, indicating that the enhancer restriction activity of the RO had been diminished (Fig. 3, construct 3, 40ORE52T). Similarly, when the orientation of the upstream 40 reporter was reversed so that the promoters were in a divergent configuration, the enhancer restriction activity was reduced (construct 5, 40ROE52D). Further, the level of transcription from the upstream reporter, 40, is consistently increased relative to the other constructs. This may be a result of the modest activation we observed when the RO was placed in reverse orientation adjacent to a promoter (see Fig. 2, construct 4, OR40). From these results we conclude that the presence of the RO between the enhancer and the upstream reporter is not sufficient for enhancer restriction activity. Rather, this activity requires a tandem organization in which the RO and at least the upstream reporter are in the same orientation, suggesting a defined interaction among the elements.

The enhancer restriction activity of the RO is reminiscent of insulator elements, which are proposed to delineate the active range of regulatory elements within the genome (16). A diagnostic feature of insulator elements for RNA pol II genes is the ability to block an enhancer from activating a promoter when interposed between the two (16, 19). We therefore tested the RO for enhancer-blocking activity by positioning it between the rRNA enhancer module and a promoter. To reduce the possibility of steric interference between the various elements in the construct, we flanked the RO with nonspecific DNA. Although the nonspecific DNA used in this construct reduced transcription from the reporter gene (Fig. 4, construct 2, 1k40), insertion of the RO increased this value about twofold (construct 3, dROd40). This was the same level of stimulation seen when the RO was positioned adjacent to a promoter (Fig. 2B, construct 3, RO40). In contrast, placement of an enhancer module upstream of the nonspecific DNA activated transcription sevenfold (Fig. 4, construct 4, E1k40). This was a lower level of stimulation than was seen for the construct E40 (Fig. 2, construct 2) and is presumably due to the nonspecific DNA, which reduces transcription (see above). We then inserted the RO into the nonspecific DNA between the enhancer module and promoter (Fig. 4, construct 5, EdROd40). In this context, the RO did not block enhancer activation of the promoter. Addition of a second RO at the 3′ end of the reporter gene still showed no blocking activity (construct 6, EdROd40RO). In fact, transcription from this construct was unexpectedly stimulated compared to that of EdROd40. This is the only case in which we saw high levels of transcriptional stimulation by the RO fragment; we have observed no more than a twofold stimulation in other single-reporter constructs in which an RO is placed at the 3′ end of an enhancer-reporter pair (13). Since

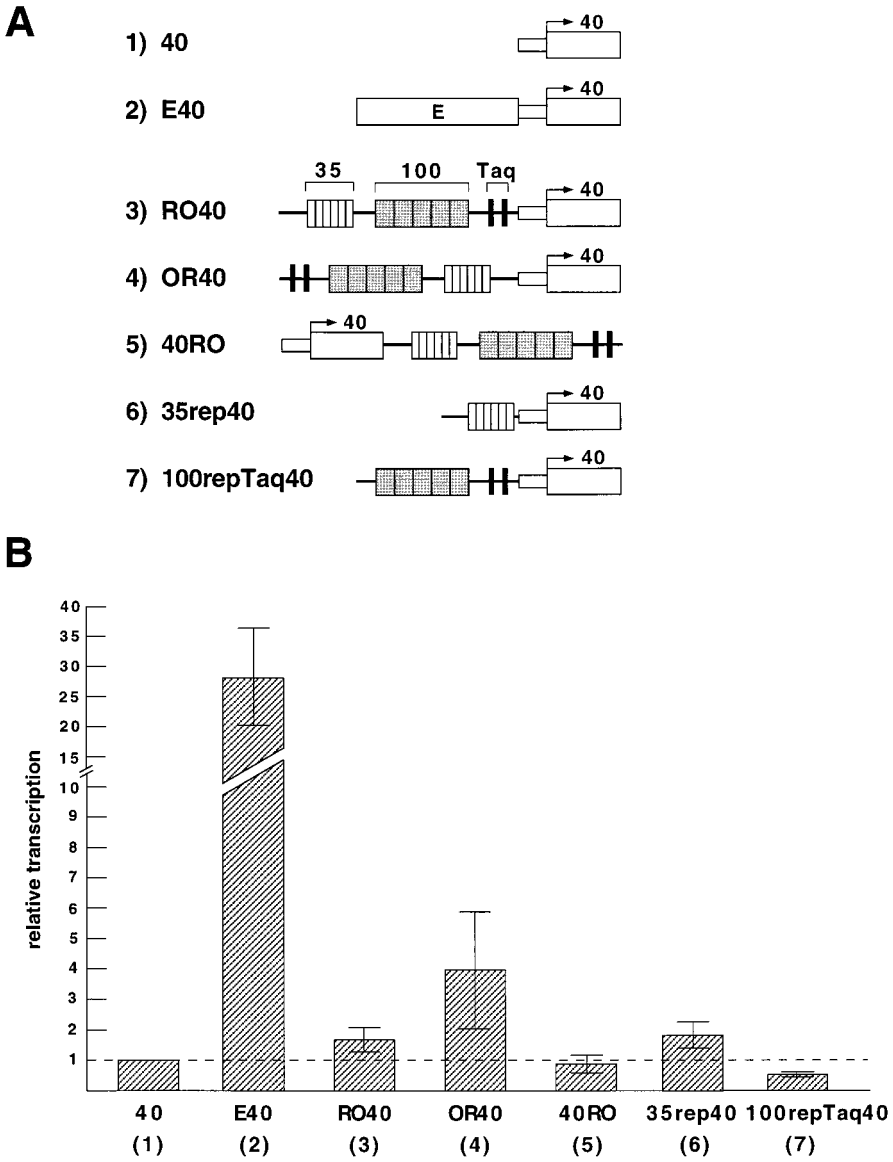

FIG. 2. The RO does not significantly affect transcription from an adjacent promoter. (A) Diagram of experimental constructs. Plasmids constructs were made in which an enhancer module or the repetitive sequences of the RO were cloned adjacent to the promoter of the 40 reporter gene. The enhancer module contains 10 60/81-bp repeats and is represented by the open box labeled E. The RO was subcloned as a *Stu*I/*Bam*HI fragment and includes the t2 site, a block of six 35-bp repeats (small white boxes), a block of five 100-bp repeats (shaded boxes), and two *Taq* boxes (black boxes) but does not contain a complete spacer promoter. Nonrepetitive sequences of the RO *Stu*I/*Bam*HI fragment are represented by a black line. The 35-bp repeats in 35rep40 are in the same orientation as for RO40. For cloning details see Materials and Methods. (B) Histogram of the values for relative transcription for the constructs in panel A. Approximately 1 ng of each experimental plasmid in panel A was coinjected with an equal molar ratio of the 52 rRNA reference reporter gene into *X. laevis* oocyte nuclei. In all cases circular plasmids were injected. Transcripts from both reporters were assayed separately by S1 nuclease protection. Data were quantitated and normalized for probe specific activity and template concentration as described in Materials and Methods. The values for each construct were calculated from the S1 assay data as the ratio of transcription from the experimental construct to the reference construct. The means and standard errors of at least three independent experiments are reported for this and all subsequent figures. The large standard error is due to variation between the batches of oocytes. (Note the change in scale on the graph for the value of relative transcription.)

the RO did not block the enhancer from activating the promoter when it was positioned between them, it did not behave as previously described insulator elements. Therefore, the RO requires the correct context to restrict enhancer activity, suggesting that its insulator-like activity is specialized for the rRNA gene tandem array.

Deletion analysis of the RO. To assess the participation of the different repeated sequence classes of the RO in the enhancer restriction activity, we tested several deletion mutants of the wild-type element in tandem-reporter constructs. In this experiment, the value for relative transcription for 40ROE52TRO is nearly 14-fold (Fig. 5, construct 2). This value differs from that reported in Fig. 3 because here we have included only those transcription values for 40ROE52TRO that came from the same oocyte batches into which the deletion constructs were injected. The deletion element containing the 35-bp repeats alone (Fig. 5, construct 3, tan/35rep) retained 75% of the enhancer restriction activity of the complete wild-type element. The deletion element lacking the 35-bp repeats (construct 4, tan/100rep*Taq*) and that lacking the *Taq*

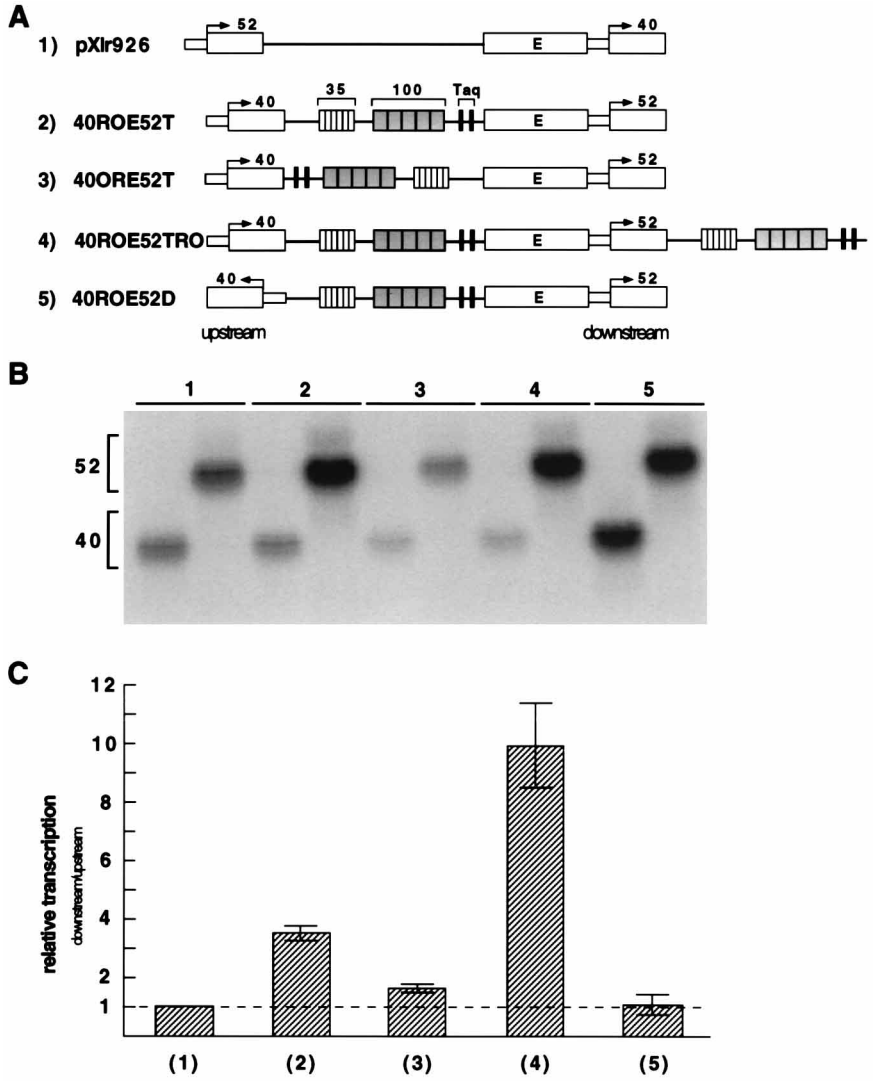

FIG. 3. The RO restricts enhancer activity in the context of a tandem gene repeat. (A) Diagram of experimental constructs. The RO and enhancer module were cloned in constructs containing both the 40 and 52 reporter genes in a head-to-tail tandem organization. Elements used are the same as those shown in Fig. 2. In the construct names, T indicates tandem promoters and D represents divergent promoters. Note that in the case of pXlr926, the upstream reporter is 52 and the downstream reporter is 40. The reporters in this construct are separated by 1.44 kb of nonspecific DNA (20). (B) Representative S1 assay. Approximately 1 ng of each construct was injected. Transcripts were analyzed as described in Materials and Methods. Lanes: 1, pXlr926; 2, 40ROE52T; 3, 40ORE52T; 4, 40ROE52TRO; 5, 40ROE52D. Note that for 40ORE52T only half as much DNA was injected. (C) Histogram of the values for relative transcription for the constructs in panel A. The values per construct are calculated from the S1 assay data as the ratio of transcription from the downstream reporter to the upstream reporter. Data were analyzed as described in the legend for Fig. 2 except that transcription values for 40 and 52 reporters were normalized to the values from pXlr926. Only two experiments are reported for 40ORE52T.

boxes region (construct 5, tan/35rep100rep) were 50% as efficient as the complete element in restricting enhancer action. Therefore, all of the deletions had some ability to restrict the scope of enhancer activity.

In addition to these deletions of whole repetitive regions from the RO, we tested a naturally occurring element that contains fewer 35-bp repeats and 100-bp repeats than the element we have characterized. This element, the 164RO, was derived from a rRNA gene-intergenic spacer repeat cloned from a female *X. laevis* whose oocytes, unlike those of most females, showed consistent transcription from the spacer promoters (30). This unusual transcriptional activity was also observed from the cloned DNA when it was injected into the oocytes of other *X. laevis* females; however, analysis of the spacer promoters indicated they were essentially wild type (31). Sequence analysis of the 164RO indicated that it has four 35-bp repeats and three 100-bp repeats, while the RO used in our studies contains six 35-bp repeats and five 100-bp repeats (data not shown). Both clones have two *Taq* box sequences. The number of 35-bp repeats from intergenic spacer clones from various laboratories ranges from three to nine, while the number of 100-bp repeats varies from five to eight (reference 32 and data not shown). Notably, those clones reported to have only three 35-bp repeats have more than five 100-bp repeats. Thus the 164RO does not contain the typical number of repeated sequence units. Although there are some single-base differences between the repeated sequences of the 164RO and those of the RO sequence reported in the database, these differences appear to be no greater than the base heterogeneity between repeats of the same class within the RO or between the RO regions of different intergenic spacer clones.

In a tandem-reporter construct, the 164RO did not have the

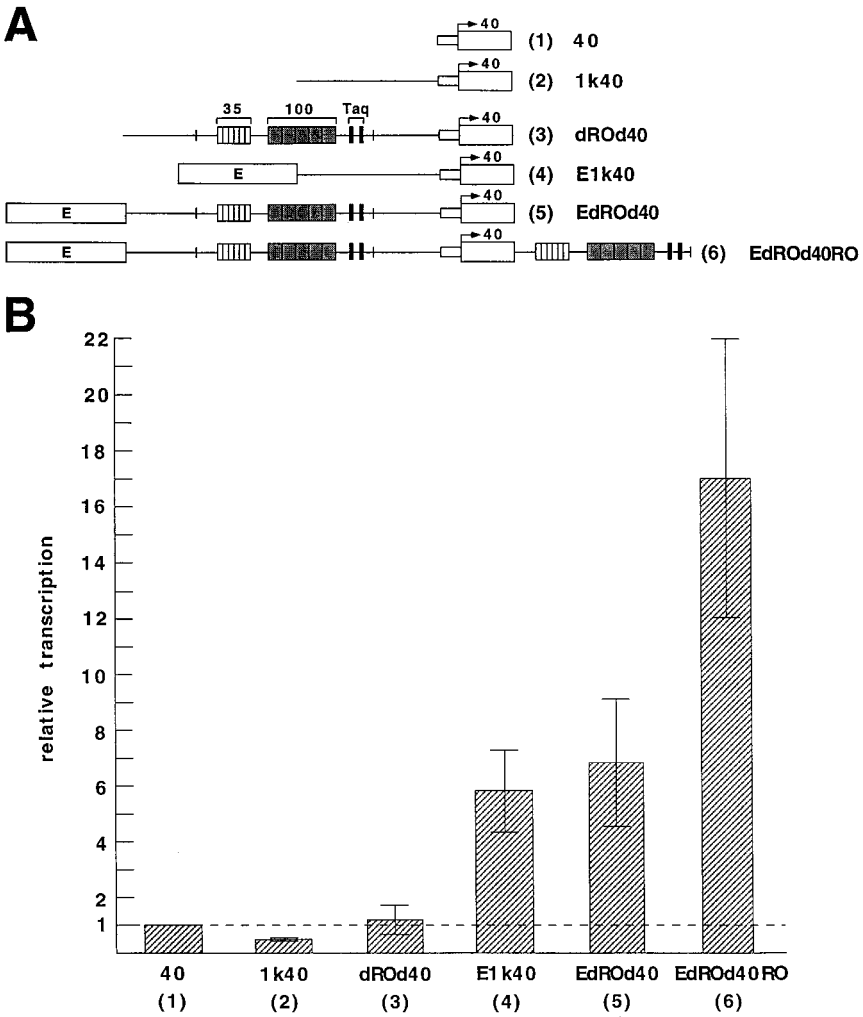

FIG. 4. The RO does not block enhancer activity in the absence of tandem promoters. (A) Diagram of experimental constructs. Constructs were made from DNA elements as described for Fig. 2. A 1-kb eukaryotic cDNA, represented by a black line, was used as nonspecific DNA in 1k40. The RO was inserted into the nonspecific DNA to make dROd40 where d represents 500 bp of spacing. Short vertical lines demarcate the ends of the nonrepetitive sequences of the RO *Stu*I/*Bam*HI fragment. (B) Histogram of the values for relative transcription for the constructs in panel A. Data were analyzed as described in the legend for Fig. 2. Note that the 1-kb fragment inhibits the promoter somewhat. Only two trials are reported for EdROd40RO.

full activity of the RO but was comparable to other deletions (Fig. 5, construct 6, tan/164RO). This result suggests that the presence of each of the three repetitive sequence classes of the RO is not sufficient to restrict enhancer activity and that the number of repetitive sequence units is important for the activity of the RO. However, the 35-bp repeats seem to be the most important element for the activity of the RO since they were sufficient for 75% of the full element's activity and deletion of these elements was the most deleterious of the deletions tested.

The transcription factor xUBF binds the 35-bp repeat and 100-bp repeat sequences. In preliminary in vitro DNase I footprinting experiments, we had tested a nuclear extract from *X. laevis* tissue culture cells for activities that bound to the 35-bp repeat and 100-bp repeat sequences. We noted that the extract produced extensive protections and enhanced cleavages on the 35-bp repeat and 100-bp repeat probes that were analogous to those observed for the 60/81-bp enhancer probe in control experiments (data not shown). Because the 60/81-bp repeats are bound by the transcription factor xUBF, we repeated the footprinting experiments with DNA-affinity-purified xUBF (12, 36). Indeed, xUBF bound to the 35-bp repeat and 100-bp repeat sequences in vitro with similar extended protections and enhanced DNase I cleavages similar to those seen for the enhancer element 60/81 (Fig. 6). Titrations of xUBF indicated that its binding affinities for the 35-bp repeat, 100-bp repeat, and 60/81-bp repeat sequences differ by less than twofold (Fig. 6). Although there is strong sequence conservation between the enhancer repeats and an xUBF binding site in the *X. laevis* rRNA promoter, there is no similarity between sequences in the enhancer and the 35- and 100-bp repeats aside from a high G+C content. However, it has been reported that the 100-bp repeats bear strong sequence homology to the upstream core element (−167 to −105) of the 40S gene promoter (33). Nevertheless, xUBF binds to a variety of sequences with no obvious sequence similarity such as the *X. laevis* and human rRNA gene promoters (36). In agreement with our results, the binding of xUBF to the 35- and 100-bp repeats has recently been detected by electrophoretic mobility shift assay (33).

The titration of xUBF with the 35-bp repeat probe displayed a sharp transition from undetectable binding to extensive protection of the 35-bp repeats and adjacent sequences from the

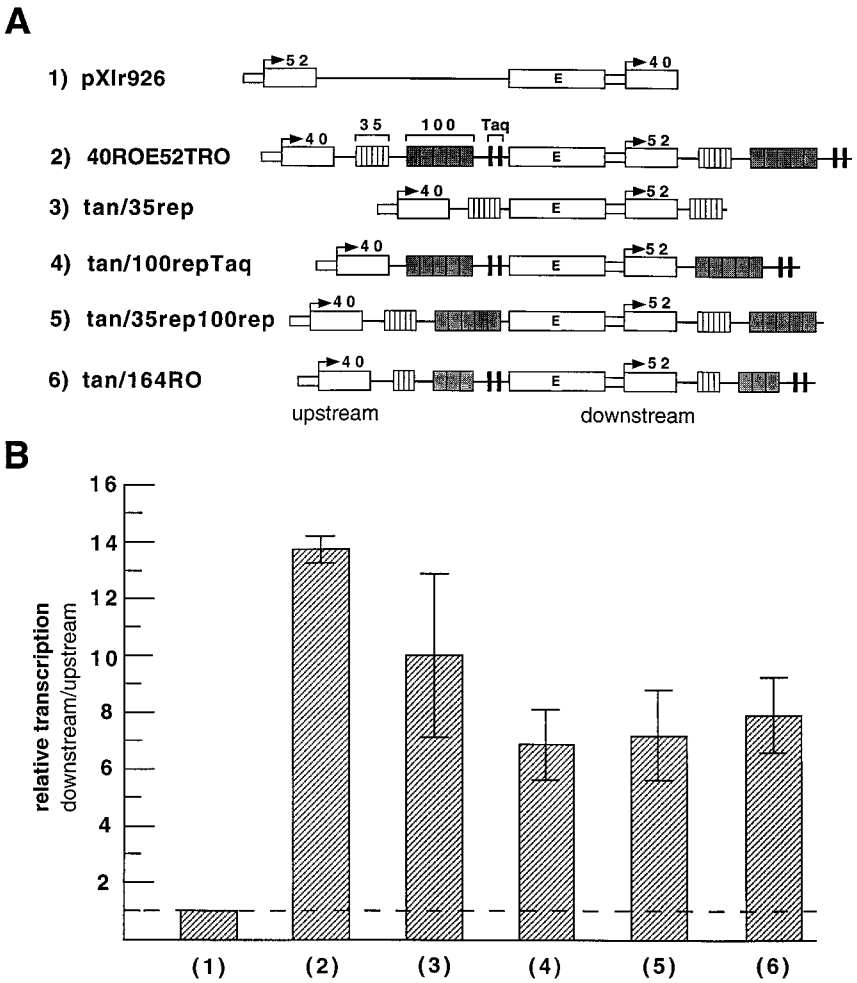

FIG. 5. Deletion analysis of the RO. (A) Diagram of experimental constructs containing deletions of the RO in the tandem-reporter construct. In constructs 3 to 5, the major repetitive regions of the RO were subcloned and used to flank an enhancer-promoter pair. The orientation of the 35-bp repeats in tan/35rep is the same as in 40ROE52TRO. The 164RO contains the RO region subcloned from pXlr164, a cloned mutant rDNA gene-intergenic spacer unit associated with a high frequency of spacer transcription. It contains four 35-bp repeats, three 100-bp repeats, and a complete *Taq* box region. Other DNA elements are as described in the legend for Fig. 2. (B) Histogram of the values for relative transcription as described in the legend for Fig. 3. Three batches of oocytes were injected with each of the constructs shown in panel A. Relative transcription is reported as the ratio of transcription from the downstream reporter to the upstream reporter, as in Fig. 3.

spacer, corresponding to a twofold increase in protein concentration (Fig. 6, 35rep, lanes 3 and 4). As the 35-bp repeat probe contains an array of binding sites, we attribute this effect to cooperative binding of xUBF to the multiple repetitive sequences. Such cooperativity has been observed for the binding of xUBF to multiples of the 60/81-bp repeats (reference 37 and data not shown). The titrations of xUBF with the 100-bp repeat and 60/81-bp repeat probes, each of which contains fewer repeat units, showed a linear increase in the intensity of the enhanced cleavages with increased protein concentrations. Thus, a transcription factor that binds to the promoter of the 40S rRNA gene and the enhancer elements to activate transcription also binds to the RO, an element that acts to restrict enhancer activity in the context of tandem genes.

DISCUSSION

We have identified a novel activity for a region of repeated DNA sequences in the intergenic spacer of the *X. laevis* rRNA genes. We have shown that the region encompassing the 35-bp repeats, 100-bp repeats, and *Taq* boxes, which we call the repeat organizer, does not directly activate transcription. Rather, in plasmid constructs containing two tandem promoters separated by intergenic spacer elements in their usual position and orientation, the RO prevents the 60/81-bp repeats from activating the upstream promoter. These results suggest that, in vivo, the RO might ensure that an enhancer will activate the gene promoter within the same intergenic spacer. Although we have assayed the RO for its ability to restrict enhancer-promoter interactions, the endogenous RO may have a more positive action that guides the enhancer to activate the proximal gene promoter. The consequence of either interpretation is that the RO effectively separates the tandem array of rRNA genes into single-gene expression units.

The RO bears functional similarity to insulator elements by virtue of its ability to insulate a promoter from the influence of an enhancer in a position-dependent manner. Insulator elements such as the *Drosophila* scs, scs′, and the array of su(HW) binding sites from the *gypsy* retrotransposon and the insulator in the chicken β-globin locus insulate a reporter gene from both positive and negative chromosomal position effects as well as "block" the activity of outlying enhancers (7, 18, 19, 42).

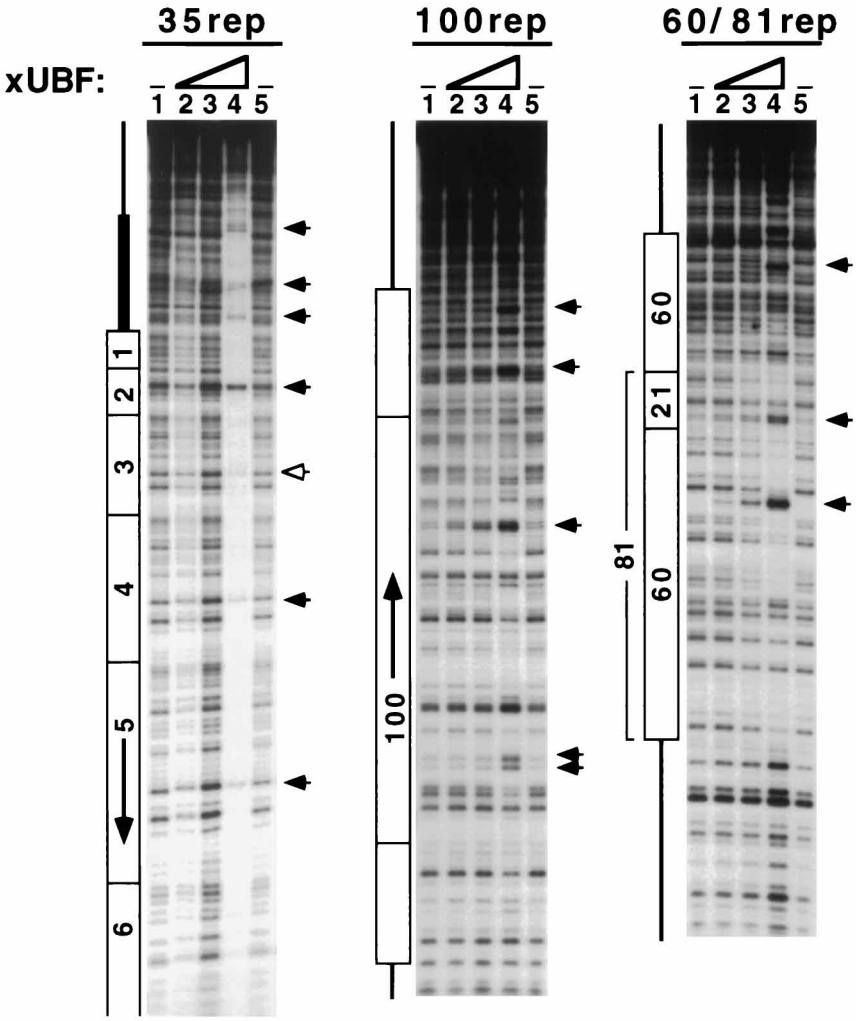

FIG. 6. xUBF binds to the sequences of the RO in vitro. DNase I footprinting experiments were conducted using 5′ end-labeled probes and xUBF purified by DNA affinity-chromatography. The 35-bp probe (35rep) contains the block of six 35-bp repeats and 100 bp of nonrepetitive intergenic spacer sequence (bold line). The 100-bp repeat probe (100rep) contains one complete 100-bp repeat flanked by 15 bp and 80 bp of partial 100-bp repeats. We mark the beginning of each 100-bp repeat as the sequence (C/G)TCCCCCC. The 60/81-bp repeat probe (60/81rep) contains two 60-bp repeats separated by a 21-bp repeat. Increasing amounts of xUBF were incubated with 2 to 4 fmol of probe for 10 min at room temperature prior to digestion with DNase I as described in Materials and Methods. Lanes 1 and 5 for each probe are DNA without protein. For the 35rep, lanes 2 to 4 contain 5, 10, or 20 μl of xUBF, respectively. For the 100rep and 60/81rep, lanes 2 to 4 contain 1, 3, or 10 μl of xUBF, respectively. Major enhanced cleavages are indicated with solid arrowheads. Less obvious but reproducible enhancements on the 35rep probe are indicated with a hollow arrowhead. Relevant sequences as determined by Maxam-Gilbert sequencing are diagrammed next to the autoradiogram; thin black lines represent vector sequences. The arrows indicate the orientations of the probe sequences relative to the diagram in Fig. 1.

The versatility of these elements is demonstrated by their effective insulator activity in the context of heterologous RNA pol II enhancers and promoters and by the orientation independence of the *Drosophila* elements (6, 19). Remarkably, the scs and scs′ elements are able to block activation of the rRNA gene promoter by the rRNA enhancer module in *X. laevis* oocytes (15). In contrast, the insulator activity of the RO appears to be specialized since it is dependent on the organization of surrounding elements. For example, the RO does not behave as an insulator element in the construct EdROd40RO (Fig. 4, construct 6). Although RO elements flank both sides of the single reporter, their presence is not sufficient to exclude the enhancer from interacting with the promoter. However, when RO elements flank an enhancer-promoter pair in a tandem-reporter construct, the enhancer is prevented from activating the outlying promoter on the same plasmid (Fig. 3, construct 4, 40ROE52TRO).

The activity of the RO could be most simply modeled by proposing an interaction between the RO and the enhancer that would give asymmetry and polarity to enhancer activity. We have suggested a similar model for the action of the scs and scs′ insulators from *Drosophila* (15). In the context of a single-reporter construct, the RO would be expected to have little effect on transcription since the enhancer has only one possible promoter target on the small circular plasmid. However, in a tandem-reporter construct, the enhancer has two possible promoter targets. In the absence of the RO, transcription from these promoters is roughly equivalent (see Fig. 3, construct 1, pXlr926) indicating that the enhancer is equally as likely to interact with one promoter as the other. The values of relative transcription change from a value of 1 in the constructs containing the RO, indicating that this element biases the enhancer's ability to interact with these promoters. A model in which the RO directly interacts with the enhancer necessitates a

higher-order structural organization of the intergenic spacer. However, our analysis does not identify the targets of such interactions.

The question of how enhancers find and activate the correct promoter applies to both RNA pol I and II genes. For an RNA pol II gene, the inability of an enhancer to locate the correct promoter would result in either the activation of inappropriate genes or the failure to activate appropriate ones. While random enhancer-promoter interactions within the tandem rRNA gene array would not have the consequence of activating the wrong gene, it seems likely that the gene cluster has a structural organization that would promote efficient and specific interactions between enhancers and promoters. Since the RO can be thought of as an element that gives direction to the enhancer but is not itself strongly activating, it would contribute to the organization of enhancer-promoter interactions. This might be important for regulating the level of rRNA gene transcription at the level of gene activation, particularly in somatic cells where only a fraction of the rRNA genes appear to be transcriptionally active (8, 26). In *X. laevis* and mouse tissue culture cells, the rRNA genes exist in two distinct states of chromatin structure, and the transcriptionally active genes are associated with only one of these states (8, 26). Although it is not understood how the subset of transcriptionally active genes is established, the enhancer restriction activity of the RO might contribute to the maintenance of the distinct transcriptional states by ensuring that enhancers activate only proximal promoters.

Despite the ability of the transcription factor xUBF to bind to the 35-bp repeat and 100-bp repeat sequences in vitro, these sequence elements do not activate transcription of an adjacent promoter in vivo (see Fig. 2 and 6). In contrast, the 60/81-bp repeats are bound by xUBF in vitro and dramatically enhance transcription in vivo. Pikaard has previously reported that the binding of xUBF to a sequence in vitro is not necessarily an indication that the sequence will activate transcription in vivo (35). Because the RO and 60/81-bp repeats differ by activity and sequence, additional factors may act in conjunction with xUBF to mediate the distinct activities of enhancer restriction and direct transcriptional activation. Alternatively, the conformation of xUBF bound to the RO may be different from its conformation when it is bound to the promoter or enhancer. When xUBF is bound to enhancer sequences, the resultant complex is able to bind to *X. laevis* Rib1, the complex of TATA-box-binding protein (TBP) and pol I TBP-associated factors (TAFs) which is required for initiation at the rRNA gene promoter (28, 29). This suggests that the role of the rRNA enhancers is to recruit this limiting transcription factor (29). Although xUBF and human UBF are equally competent for enhancer-activated transcription and both interact with Rib1 when complexed with enhancer DNA, xUBF is essential for activity of the *Xenopus* rRNA gene promoter. Taken together, these results suggest that either the exposed surface of UBF bound to a specific sequence or the sequence context modulates the activity of the complex. Because the RO does not strongly stimulate transcription in vivo, the xUBF-RO complex may not interact with Rib1, or it interacts in a manner that precludes direct activation of an adjacent promoter.

Our observation that the 35- and 100-bp repeats do not activate transcription in vivo directly contradicts the report of Mougey and coworkers which suggests that these same sequences are as effective as the 60/81-bp repeats in activating transcription from the 40S gene promoter (33). The most obvious difference in these two sets of experiments is the use of two different species of *Xenopus* oocytes for the transcriptional analysis. Mougey and coworkers used *Xenopus borealis*, while we used *X. laevis*. Despite sequence homologies to the 35- and 100-bp repeats as well as the *Taq* boxes, the RO analogous region of the *X. borealis* intergenic spacer differs significantly in sequence and organization from the RO of *X. laevis* (23, 33) (Fig. 1). It is possible that the RO's activity is species specific and that the disparate effects of this *X. laevis* element in *X. laevis* and *X. borealis* can be explained by this species specificity.

It has generally been assumed that the RNA pol I transcriptional machinery and regulation of transcription are equivalent between these two species, and they have been used interchangeably to study these processes. A notable exception to this view is the phenomenon of nucleolar dominance, which describes the differential expression of rRNA genes in interspecies hybrids. In a hybrid between *X. laevis* and *X. borealis*, transcription of the *X. laevis* rRNA genes is dominant in the embryo regardless of which species supplies the egg (reference 39 and references therein). This effect can be mimicked in the *X. borealis* oocyte and embryo by coinjection of *X. laevis* and *X. borealis* genes (41). In this case, the dominance of the injected *X. laevis* genes has been attributed to the greater number of repeated enhancer sequences within this species' intergenic spacer relative to that of *X. borealis*, but other sequences, such as the promoter, may also be involved in nucleolar dominance (41). A second example where conflicting results correlate with the use of these two *Xenopus* species has been the analysis of spacer promoter transcription. Although the endogenous spacer promoters are transcriptionally silent in the oocyte (38), the activity of plasmid-borne spacer promoters in the context of *X. laevis* rRNA gene repeats microinjected into oocytes is less clear. Electron microscopic analyses have failed to detect gradients of nascent transcripts within the intergenic spacer (reference 31 and references therein), which implies that the spacer promoters are inactive, yet biochemical analyses have detected abundant spacer promoter-initiated transcripts (9, 11, 31). The fact that the electron microscopic analyses were conducted with *X. laevis* whereas the biochemical analyses were conducted with *X. borealis* raises the possibility that these disparate observations are actually species dependent. Based on this evidence and the experiments reported here, we suggest that the issue of species-specific effects on rRNA gene transcription merits more serious consideration.

Our experiments address the role of the RO in transcription, but this element may have multiple functions. For example, a replication fork barrier (RFB) has been described in the rRNA gene repeats of *Saccharomyces cerevisiae* (4, 5). Because it ensures that replication forks proceed only in the direction of gene transcription, this RFB would prevent head-on collisions between the DNA replication machinery and the transcribing RNA polymerases, although the detriment of such collisions is unclear (24). Like the RO, this RFB is positioned at the 5′ end of the intergenic spacer. Similar RFBs have been reported in the rRNA gene repeats of the pea *Pisum sativum* (17) and *X. laevis* (45). In fact, the RFB of *X. laevis* maps within the 35-bp repeats of the RO. Taken together, these results suggest that the RO is a multifunctional element that may be a universal feature of eukaryotic rRNA gene repeats.

ACKNOWLEDGMENTS

We thank Lynn Zechiedrich, Susan Uptain, Jocelyn Krebs, Susanne Ullmann, and Andrew Dillon for critical commentary on the manuscript. We thank Hon-Ling Yuen for construction of plasmid p0,1.

This work was supported in part by a grant from the National Science Foundation and by grant GM49944 from the National Institute of General Medical Sciences.

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