

Xenopus Ribosomal RNA Gene Intergenic Spacer Elements Conferring Transcriptional Enhancement and Nucleolar Dominance-like Competition in Oocytes*

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Amy A. Caudy‡ and Craig S. Pikaard§

From the Department of Biology, Washington University, St. Louis, Missouri 63130

Repeated within the intergenic spacers that separate adjacent ribosomal RNA (rRNA) genes in *Xenopus laevis* are several distinct sequence elements. These include transcription terminators, “region 0” repeats, “region 1” repeats, duplicated spacer promoters, and 42-bp enhancer elements that are embedded within 60 or 81-bp repeats. All have been reported to stimulate RNA polymerase I transcription from an adjacent gene promoter. A greater number of 42-bp enhancers/gene have been suggested to explain the preferential transcription of *X. laevis* rRNA genes in *X. laevis* × *Xenopus borealis* hybrids, an epigenetic phenomenon known as nucleolar dominance. However, the possible contribution of regions 0/1 and/or spacer promoters to the preferential transcription of *X. laevis* (over *X. borealis*) rRNA genes has never been tested directly. In this study, we systematically tested the various intergenic spacer elements for their contributions to promoter strength and nucleolar dominance-like competition in oocytes. In disagreement with a previous report, region 0 and region 1 repeats do not have significant enhancer activity, nor do they play a discernible role in *X. laevis*–*X. borealis* rRNA gene competition. Minigenes containing *X. laevis* spacer sequences are only dominant over minigenes having complete *X. borealis* spacers if a spacer promoter is located upstream of the 42-bp enhancers; *X. laevis* enhancers alone are not sufficient. These results provide additional evidence that spacer promoters together with adjacent enhancers form a functional activating unit in *Xenopus* oocytes.

In *Xenopus* as in other eukaryotes, RNA polymerase I is dedicated to the transcription of ribosomal RNA genes, producing a 40 S primary transcript that is then processed into the 18 S, 5.8 S, and 28 S RNAs found within cytoplasmic ribosomes (1–5). There are hundreds (sometimes thousands) of rRNA genes in eukaryotic genomes. These rRNA genes are tandemly arrayed in head-to-tail clusters that are known as nucleolus

organizer regions because nucleoli, the sites of ribosome assembly, are formed at the loci where rRNA genes are actively transcribed (6–9).

Within the nucleolus organizer regions, adjacent rRNA genes are separated by an intergenic spacer that typically contains repetitive DNA sequences, some of which have defined roles in transcriptional regulation (10). Intergenic spacers of *Xenopus laevis* have been particularly well characterized (Fig. 1). In oocytes injected with plasmid minigenes, the 60- and 81-bp repeats located just upstream of the *X. laevis* rRNA gene promoter act as orientation- and distance-independent enhancers of transcription (11). These elements are very similar, 81-bp repeats being 60-bp enhancers with an additional 21-bp extension (12, 13). Within each 60/81-bp enhancer is a 42-bp sequence that is ~80% identical to an upstream domain of the gene promoter (nucleotides –114 to –72 relative to the transcription start site, +1). A synthetic oligonucleotide corresponding to this upstream promoter region is sufficient for strong orientation-independent enhancer function (14). Interestingly, a core promoter domain (–20 to +15) lacking similarity to *X. laevis* spacer repeats but similar to a 44-bp repeated spacer element in *Xenopus borealis* (matching promoter sequences –22 to +22) (15, 16) also displays enhancer activity in *X. laevis* oocytes (14). Collectively, these data support the hypothesis that the enhancers evolved from duplicated promoter domains that bind essential transcription factors. Injection into oocytes of a plasmid bearing only 60/81-bp enhancer repeats will inhibit transcription from a promoter on a second plasmid, consistent with the idea that enhancers and promoters bind one or more transcription factors in common (11). Indeed, the transcription factor UBF (upstream binding factor) was identified and purified from *Xenopus* based on its ability to bind both the 60/81-bp enhancers and the promoter (17, 18).

In *X. laevis* and *X. borealis* intergenic spacers, enhancer arrays are preceded by spacer promoters that share ~90% identity with the gene promoter (see Fig. 1) (19). Spacer promoters can program polymerase I transcription initiation, but their transcripts terminate upstream of the gene promoter at site T3 located at position –213 (20, 21). T3 is a “fail-safe” termination site in that it prevents spacer transcription from proceeding through the gene promoter. The function of spacer promoters in *Xenopus* is not entirely clear. The oocytes of most females display little or no spacer promoter activity with only rare individuals displaying significant numbers of spacer transcripts (22, 23). Nonetheless, several studies have presented evidence that the full enhancer function of 60/81-bp repeats is only realized in oocytes if at least one spacer promoter is located upstream (24, 25).

The 5' most portion of the intergenic spacer in *X. laevis* consists of 34- and 100-bp repeats known as region 0 and region 1, respectively (12, 13). A study using *X. laevis* minigenes

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§ To whom correspondence should be addressed: Dept. of Biology, Washington University, Campus Box 1137, 1 Brookings Dr., St. Louis, MO 63130. Tel.: 314-935-7569; Fax: 314-935-4432; E-mail: pikaard@biology.wustl.edu.

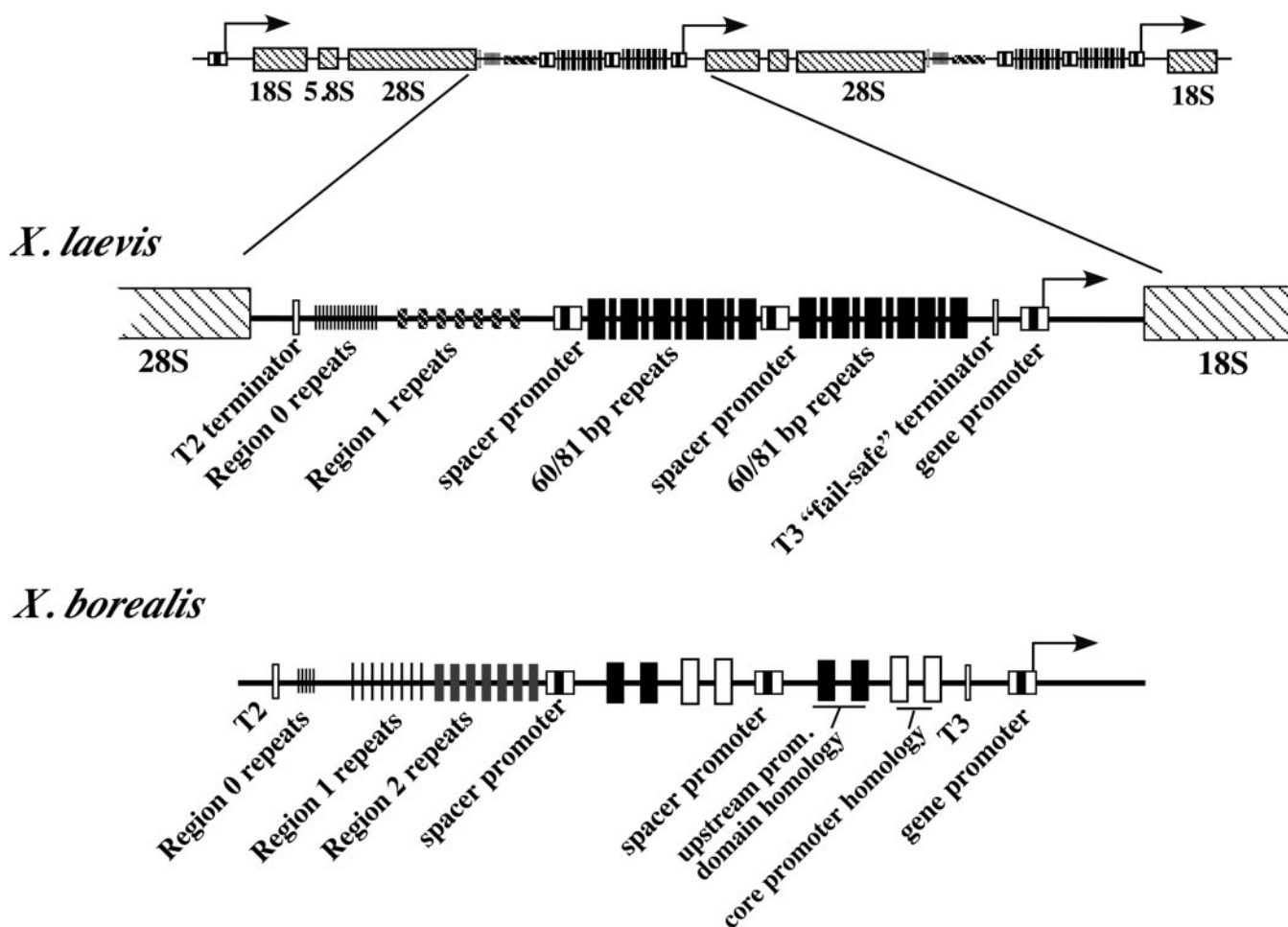


FIG. 1. Organization of *Xenopus* ribosomal RNA genes and intergenic spacers. The rRNA genes are arranged head-to-tail in tandem arrays with coding sequences separated by intergenic spacers. Representative intergenic spacers of *X. laevis* and *X. borealis* are shown with the various classes of repetitive elements labeled. Arrows denote the sites of transcription initiation from the gene promoters. Duplications of the gene promoter known as spacer promoters occur multiple times within the intergenic spacers. In the spacers of both species are elements that share similarity with a 42-bp upstream promoter domain. White rectangles in the *X. borealis* spacer represent 44-bp elements that share similarity with the promoter region surrounding the transcription start site. Within an individual, intergenic spacers of different rRNA genes can vary substantially in size because of differences in the number of spacer promoters and the repetitive elements located between them. At the 5' end of the intergenic spacers, region 0 repeats of *X. laevis* and *X. borealis* share an identical core sequence. Region 1 repeats of *X. laevis* share a similarity with region 2 repeats of *X. borealis*.

microinjected into *X. borealis* oocytes led to the conclusion that regions 0/1 are strong enhancers of transcription, perhaps even stronger than 60/81-bp repeats (26). These data combined with prior studies of 60/81-bp repeats and spacer promoters have collectively suggested that essentially all of the intergenic spacer serves to stimulate transcription from the downstream gene promoter.

In many interspecific hybrids, the ribosomal RNA genes of only one parent are transcribed. This phenomenon is known as nucleolar dominance, because only transcribed (dominant) rRNA genes induce the formation of a nucleolus (27, 28). Nucleolar dominance was first observed in plants (29) but also occurs in *Xenopus* (30, 31) and *Drosophila* (32, 33). When *X. laevis* and *X. borealis* are crossed to form a hybrid by *in vitro* fertilization, only the *X. laevis* rRNA genes are active in the embryos and young tadpoles (34). Reeder and Roan (35) showed that nucleolar dominance could be mimicked using *X. laevis* and *X. borealis* minigenes injected into oocytes. An *X. laevis* minigene with a complete intergenic spacer suppressed transcription from an analogous *X. borealis* minigene when both were co-injected into *X. borealis* oocytes. The *X. laevis* and *X. borealis* promoters were shown to be indistinguishable in their activity; it was the intergenic spacer of *X. laevis* that conferred

dominance. In one experiment, a construct bearing only a block of *X. laevis* 60/81-bp repeats upstream of the promoter suppressed transcription from a co-injected construct bearing a complete *X. borealis* spacer, leading the authors to conclude that these enhancer repeats whose 42-bp core sequence is more numerous in *X. laevis* than in *X. borealis* spacers were responsible for the phenomenon (35). However, in other experiments, only the constructs bearing a full *X. laevis* spacer showed dominance. One explanation favored by the authors (35, 36) was that the full spacer simply had more of the 60/81-bp repeats. However, a role for spacer promoters and/or regions 0 and 1 has never been ruled out. The report that regions 0 and 1 possess strong enhancer activity (26) in oocytes has underscored the need to re-investigate the sequences responsible for nucleolar dominance-like rRNA gene competition.

In this report, we show that region 0 and region 1 repeats do not display significant enhancer activity and thus cannot withstand competition from 60/81-bp enhancer repeats in *X. laevis* oocytes. Likewise, region 0 and region 1 repeats play no detectable role in the preferential transcription of *X. laevis* spacer-containing minigenes competing with *X. borealis* minigenes in *X. borealis* oocytes. A full *X. laevis* spacer construct having two spacer promoters and two blocks of 60/81-bp elements or an

internally deleted construct bearing only one spacer promoter and one block of 60/81-bp repeats is able to completely suppress transcription from a construct bearing a full *X. borealis* spacer. In contrast, a construct bearing only one block of *X. laevis* 60/81-bp repeats upstream of the promoter shows only co-dominance. Collectively, these data suggest that a spacer promoter in addition to 60/81-bp enhancers is needed for nucleolar dominance-like minigene competition in oocytes. The results of this assay are consistent with those of the Moss laboratory that showed that a spacer promoter is needed for full enhancer function (24, 25).

MATERIALS AND METHODS

Minigene Constructs—The minigenes $\Psi 40$ and $\Psi 52$ served as the foundations for all constructs tested. These minigenes described previously (11) have complete *X. laevis* promoters and sequences extending 5' to -245, thus including the T3 terminator site. Shortly downstream of the transcription start site, sequences from the 3' end of the gene are attached including the 3'-terminal 28 S coding sequences and flanking intergenic spacer sequences. Separating the promoter region and 28 S sequences are linkers whose size is slightly different in $\Psi 40$ and $\Psi 52$, allowing their transcripts to be distinguished from one another and from endogenous rRNA transcripts. The constructs whose number begins with "4" use the $\Psi 40$ minigene body, whereas constructs that begin with "5" use the $\Psi 52$ minigene body. Constructs $\Psi 401$, $\Psi 409$, $\Psi 4060-10$, $\Psi 4081-10$, $\Psi 52$, and $\Psi 521$ have been described previously (11, 37). $\Psi 5281-10$ is identical to $\Psi 4081-10$ (37) with the exception that the ten 81-bp repeats are attached to a $\Psi 52$ minigene body rather than to a $\Psi 40$ body. $\Psi 411-52$ is virtually identical to $\Psi 411$ described previously (35) with the exception that the *X. borealis* spacer sequences have been attached to the $\Psi 52$ minigene body rather than the $\Psi 40$ minigene body. Construct $\Psi 411$ contains a 1.6-kb intergenic spacer fragment including region 0 and region 1 fused to a $\Psi 40$ minigene body. $\Psi 411$ was engineered by digesting $\Psi 409$ with *SalI* and *BamHI* (near the 5' end of the most upstream spacer promoter) isolating the 1.6-kb fragment, attaching a *BamHI-XhoI* adapter, and ligating the resulting *SalI-XhoI* fragment into the *SalI* site located at position -245 of $\Psi 40$. The orientation of the inserted sequences relative to the promoter is reversed in $\Psi 411B$. Construct $\Psi 411-01$ had the 1600-bp spacer fragment of construct $\Psi 411$ inserted into $\Psi 401$ at the *SalI* site located just 5' of the 60/81-bp enhancer block. $\Psi 410$ was created from $\Psi 409$ by partial *BamHI* digestion to cut at the homologous *BamHI* site within the two spacer promoters followed by re-ligation, thus deleting one spacer promoter and one 60/81-bp enhancer block.

Oocyte Injection—*X. laevis* and *X. borealis* females were obtained from Nasco International. Thirty oocytes (stages V and VI) were subjected to centrifugation for 5 min at $30 \times g$ to cause the nucleus to become localized at the top of the oocyte. Injection mixes consisted of 500 pg of test construct, an equimolar concentration of its competitor minigene, 50 mM NaCl, 5 mM Tris-HCl (pH 7.5), 0.1 mM EDTA and 500 μ g/ml α -amanitin (added to inhibit transcription by RNA polymerases II and III, Sigma). With the aid of a dissecting microscope, minigenes were co-injected directly into each nucleus in a total volume of 40 nl. Injection needles were formed from 5- μ l glass capillary tubes drawn to a fine point using a pipette puller (David Kopf Instruments). After incubation overnight at room temperature, oocytes were pooled and homogenized in 50 mM Tris-HCl (pH 7.6), 1 mg/ml proteinase K, and 1% (w/v) sodium dodecyl sulfate. Following protease digestion for 1 h at 37 °C, the homogenate was extracted sequentially with 1 volume of phenol, 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v), and 1 volume of chloroform:isoamyl alcohol (24:1 v/v). Sodium acetate was added to the aqueous phase to a final concentration of 0.3 M, and nucleic acids were precipitated by the addition of 2.5 volumes of absolute ethanol. Pellets were collected by centrifugation, washed once with 70% ethanol, and air-dried. Pellets were resuspended in sterile water using 10 μ l/oocyte and frozen for subsequent S1 analysis.

S1 Nuclease Analysis—DNA probes for S1 nuclease analysis were prepared by digesting the $\Psi 40$ or $\Psi 52$ minigenes with *BamHI*, which cuts at +40 or +52 relative to the transcription start site. Following dephosphorylation with calf intestine alkaline phosphatase, 5' ends were labeled using T4 polynucleotide kinase and [γ - 32 P]ATP using standard protocols (38). The plasmids were then digested with *SalI*, which cuts at -245. Labeled antisense single-stranded probe fragments were purified from strand-separating gels according to standard protocols (38) and co-precipitated with RNA (3–5 oocyte equivalents). RNA/probe pellets were resuspended in 30 μ l of 300 mM NaCl, 10 mM

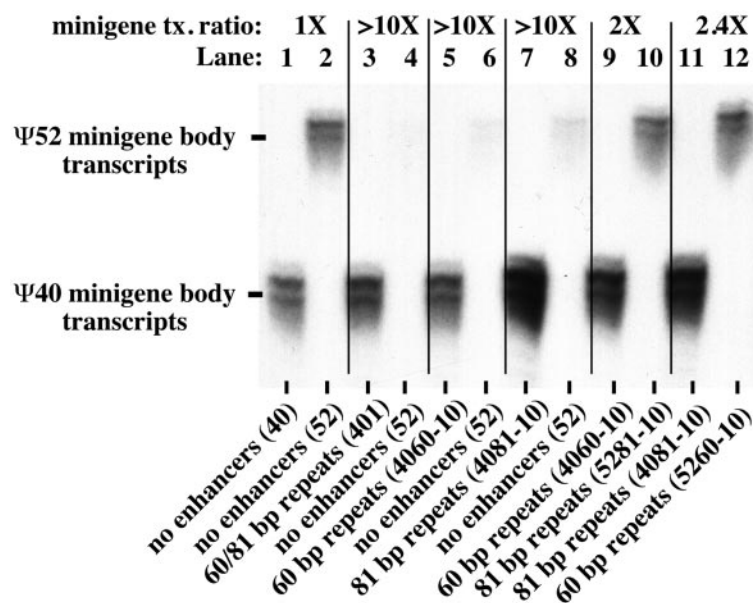
Tris-HCl (pH 7.5), and 1 mM EDTA and overlaid with mineral oil. After brief denaturation at 95 °C, hybridization was at 65 °C overnight. Reactions were placed on ice, and 270 μ l of S1 nuclease digestion buffer (5% glycerol, 1 mM ZnSO₄, 30 mM sodium acetate (pH 4.5), 50 mM NaCl, and 130 units/ml S1 nuclease (Sigma)) was added. S1 digestion was for 30 min at 37 °C. Digestion reactions were stopped by removing 280 μ l from the bottom of the tubes (to avoid the mineral oil at the top) to a fresh tube containing 30 μ l of 7.5 M ammonium acetate, 5 μ l of 0.5 M EDTA, and 3.3 μ g of yeast tRNA. After mixing, 1 ml of cold (-20 °C) absolute ethanol was added to precipitate nucleic acids. Following centrifugation at 14,000 $\times g$ for 15 min, pellets were washed with 70% ethanol, dried, and resuspended in formamide-containing loading buffer supplemented with 10 mM NaOH to degrade any RNA. S1 digestion products were subjected to electrophoresis on a 6% denaturing urea-polyacrylamide gel. Following electrophoresis, gels were transferred onto filter paper and dried using a vacuum gel dryer. Radioactive S1 digestion products were visualized following exposure of dried gels to x-ray film (Eastman Kodak Co.). Autoradiogram band intensities were estimated using a Umax 1100 scanner and ImageJ software (version 1.27, Wayne Rasband, National Institute of Mental Health, Bethesda, MD). X-ray film was not pre-flashed prior to autoradiography.

RESULTS

The basic design for all experiments was to co-inject equimolar amounts of two plasmids whose transcripts can be differentiated by S1 nuclease protection. Under such a competitive situation, intergenic spacer sequences with stimulatory activity provide an advantage to an adjacent promoter (11, 20, 24, 39, 40). In some experiments, enhancers cause stimulation of the adjacent promoter (*cis*-effect) but have little effect on the transcription of the competing minigene in *trans*. In other batches of oocytes, enhancer effects are displayed primarily by reducing transcription from the competing plasmid (*trans*-effect only) rather than stimulating the adjacent promoter. Most commonly, a combination of *cis*- and *trans*-effects is observed. Regardless of whether a given batch of oocytes displays primarily *cis*- or *trans*-effects, the ratio of test construct transcripts to competitor transcripts is highly reproducible (37). Thus, the competition assay allows for reliable comparisons of co-injected minigenes.

Just upstream of the gene promoter and T3 terminator in the *X. laevis* rRNA gene intergenic spacer is a cluster of ten, mostly alternating 60- or 81-bp repeated elements, each of which includes a 42-bp core sequence shared by the promoter. The equivalence of the 60/81-bp elements as enhancers is demonstrated in Fig. 2. In Fig. 2 and in all of the figures shown, each pair of lanes corresponds to a single co-injection in which one construct is built using the $\Psi 40$ minigene body and the competing construct uses the $\Psi 52$ minigene body. RNA isolated from the injected oocytes is then split into two equal aliquots, one of which is hybridized to a $\Psi 40$ -specific probe (*odd numbered lanes*) and the other is hybridized to the $\Psi 52$ -specific probe (*even numbered lanes*). When $\Psi 40$ and $\Psi 52$ minigenes are co-injected, both support comparable levels of transcription (compare *lane 1* with 2), although $\Psi 40$ signals are ~1.6-fold stronger because of higher specific activity of the $\Psi 40$ probe (true in all of our experiments). To facilitate a comparison with other injected minigene pairs, the ratio of $\Psi 40$ to $\Psi 52$ signals in *lanes 1* and 2 was defined as 1.0, and all subsequent injection signal ratios were normalized accordingly. Minigene $\Psi 401$ has a wild-type block of 60/81-bp elements upstream of the $\Psi 40$ minigene body. When $\Psi 401$ is co-injected with $\Psi 52$, $\Psi 401$ is preferentially transcribed by >10-fold, such that transcription from $\Psi 52$ is almost undetectable (compare *lane 3* with 4). Note that the extremely weak signal from the $\Psi 52$ plasmid in *lane 4* (also in *lanes 6* and *8* and in similar lanes in other figures) precluded the calculation of a precise transcription ratio. $\Psi 4060-10$ has 10 complete 60-bp enhancers cloned as a polymerized array with a 65-bp periodicity upstream of the $\Psi 40$ minigene body (37). When co-injected with $\Psi 52$, $\Psi 4060-10$ is

A. Enhancer activity of 60 and 81 bp repeats



B. Minigenes tested

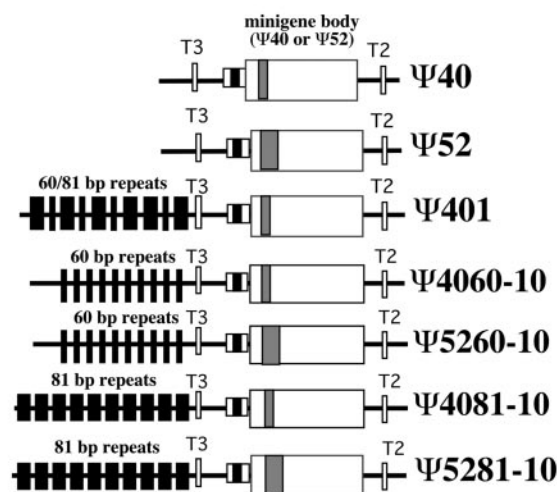


FIG. 2. Assaying relative enhancer activity by minigene competition. *X. laevis* oocytes were co-injected with pairs of minigenes, one engineered using the Ψ40 minigene body and the other based on the Ψ52 minigene. These two minigenes differ only in the size of the linker sequence (denoted by a gray rectangle) inserted just downstream from the transcription start site, allowing their transcripts to be distinguished from one another. Each pair of lanes shows the S1 nuclease protection products from the competing minigenes. Lanes 1 and 2 display transcription signals following co-injection of the two minimal minigenes, Ψ40 and Ψ52. Lanes 3 and 4 show the results of competition between Ψ401, which has a block of ten 60/81-bp enhancer repeats, and Ψ52. Lanes 5 and 6 represent the competition between Ψ4060-10, which has 10 of the 60-bp repeats, and Ψ52. In lanes 7 and 8, Ψ4081-10, which has 10 of the 81-bp enhancer repeats, is competed with Ψ52. Lanes 9 and 10 show the signals resulting from direct competition between minigenes with ten 60-bp enhancers (Ψ4060-10) and ten 81-bp enhancers (Ψ5281-10). In lanes 11 and 12, minigenes with ten 60-bp enhancers (Ψ4060-10) and ten 81-bp enhancers (Ψ5281-10) are again competed against one another, but the enhancer repeats are swapped onto the other minigene body relative to the constructs tested in lanes 9 and 10. Transcription ratios in this and subsequent figures represent the Ψ40/Ψ52 minigene body signals estimated from scanned x-ray films. Ratios were normalized to reflect differences in probe specific activity.

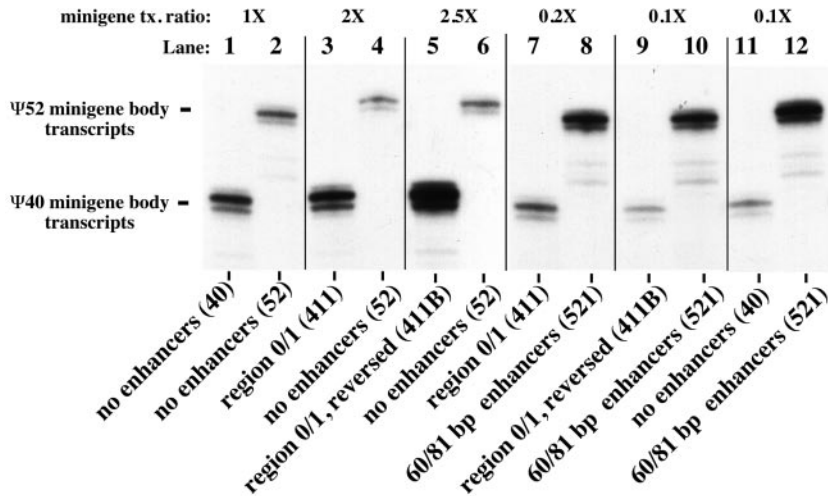
preferentially transcribed by >10-fold (compare lane 5 with 6), mirroring the results obtained using Ψ401 (lanes 3 and 4). A similar result is observed following the co-injection of Ψ4081-10 and Ψ52 (lanes 7 and 8, transcription ratio is >10). Ψ4081-10 contains ten slightly truncated 81-bp elements polymerized as 76-mers (37). Collectively, the results of lanes 1–8 suggest that cloned 60- or 81-bp repeats with different periodicities still retain the enhancer function of a wild-type 60/81-bp enhancer block, in agreement with prior results (37). The addition of ten 60- or 81-bp enhancers to Ψ52 (constructs Ψ5260-10 and Ψ5281-10) counteracts the competitive advantage of Ψ4060-10 or Ψ4081-10, making Ψ52 minigene body transcripts readily detectable (compare lanes 9 with 10 and 11 with 12). However, an unexplained 2-fold bias in favor of the Ψ40 minigene body (in addition to the 1.6-fold higher specific activity of the Ψ40 probe) is apparent in lanes 9 and 11 regardless of whether 60- or 81-bp repeats are located in *cis*. The latter bias was not apparent in other repetitions of this experiment (data not shown).

A fragment containing both region 0 and region 1 has been reported to possess strong enhancer activity in *X. borealis* oocytes (26). Using *X. laevis* oocytes, we conducted similar tests of regions 0 and 1 in competition with enhancer-less and enhancer-bearing minigenes. In most experiments, we observed no competitive advantage of Ψ411 or Ψ411B over a co-injected Ψ52 minigene, but in some batches of oocytes, there was a discernible stimulatory effect. Fig. 3 shows one of the latter experiments. In this experiment, Ψ40 and Ψ52 programmed comparable levels of transcription when in competition with one another (compare lane 1 with 2, again the specific activity of the Ψ40 probe was ~1.6-fold higher). Constructs Ψ411 and Ψ411B have the 5' end of the intergenic spacer including T2,

region 0 repeats, and region 1 repeats cloned in both orientations upstream of the Ψ40 minigene. Both Ψ411 and Ψ411B out-performed a co-injected Ψ52 minigene ~2–2.5:1 (compare lanes 3 with 4 and 5 with 6). However, the Ψ411 and Ψ411B constructs were out-competed ~6:1 or 10:1, respectively, by Ψ521, a Ψ52 minigene with a block of ten 60/81-bp enhancers (compare lanes 7 with 8 and 9 with 10). In fact, Ψ411 and Ψ411B fared no better in competition with Ψ521 than did Ψ40 (compare lanes 7–10 with 11 and 12). We conclude that region 0 and region 1 repeats have only weak enhancer activity in *X. laevis*. This conclusion was further supported by testing a variety of deletion derivatives of Ψ411 in which the relative contributions of region 0 repeats and region 1 repeats could be evaluated (data not shown). No cryptic enhancer activity was uncovered among the latter deletion constructs.

The possibility that regions 0 and 1 might increase the enhancer strength of 60/81-bp repeats in *X. laevis* oocytes was examined using a variety of constructs having natural and engineered arrangements of intergenic spacer elements (Fig. 4). Ψ52, which lacks enhancers, was co-injected with eight different test constructs built on a Ψ40 minigene body. Following co-injection of Ψ40 and Ψ52, transcripts from both minigenes were readily detected (lanes 1 and 2). The addition of a block of 60/81-bp repeats to the Ψ40 minigene (construct Ψ401) results in a strong *trans*-competition effect (7-fold), such that Ψ52 transcripts are only barely detectable (lanes 3 and 4). In contrast, construct Ψ411 containing regions 0 and 1 upstream of the Ψ40 gene promoter has no competitive advantage over Ψ52 in this experiment (lanes 5 and 6). Constructs Ψ411-01 and Ψ411-01B have regions 0 and 1 in natural and reversed orientation, respectively, inserted upstream of a block of 60/81-bp repeats (see lanes 7–10). Thus, Ψ411-01 has all of the

A. Region 0/1 assays



B. Minigenes tested

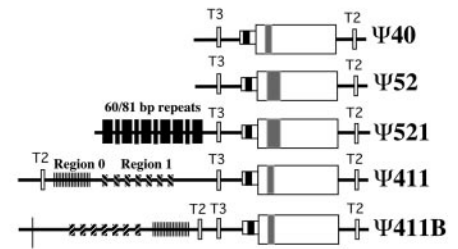
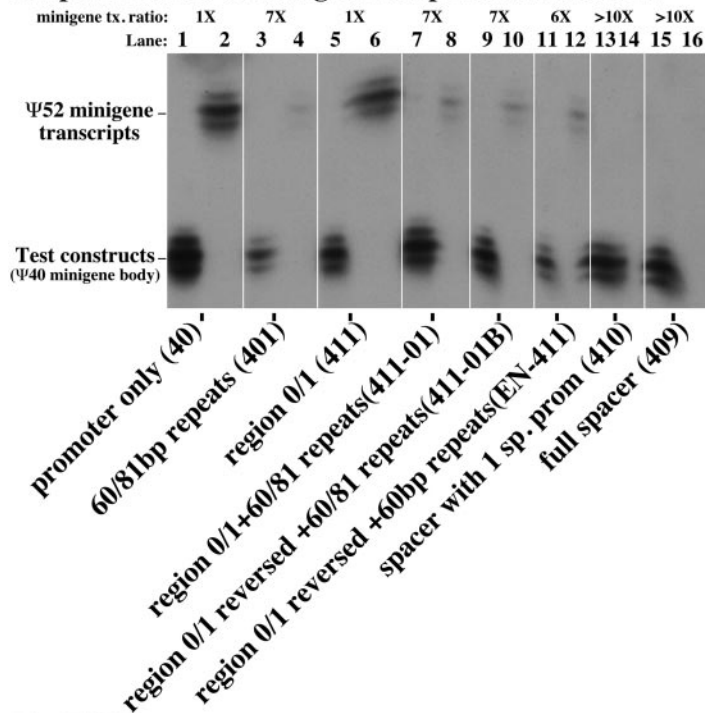


FIG. 3. Region 0 and region 1 repeats stimulate transcription only weakly and do not withstand competition from 60/81-bp enhancer repeats in *X. laevis* oocytes. Lanes 1 and 2 display the S1 nuclease protection products of transcripts resulting from co-injection of the two basic minimal minigenes, Ψ40 and Ψ52. Lanes 3 and 4 are the products resulting from co-injection of Ψ411, which has region 0 plus region 1, and Ψ52. Lanes 5 and 6 show the products of Ψ411B (region 0 and region 1 in reversed orientation relative to Ψ411) in competition with Ψ52. Lanes 7–10 show the results of competitions in which Ψ411 and Ψ411B were co-injected with a minigene bearing a block of ten 60/81-bp repeats (Ψ521). Products resulting from co-injection of Ψ40, which lacks enhancers, and Ψ521 are shown in lanes 11 and 12.

A. Spacer effects on minigene competition in *X. laevis*

B. Minigenes tested

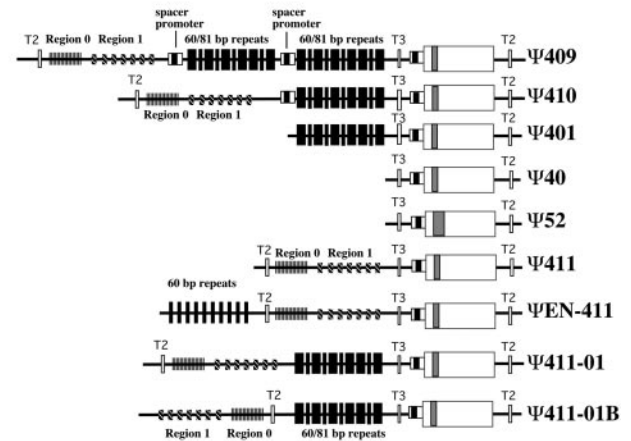


FIG. 4. At least one spacer promoter is needed in addition to 60/81-bp enhancer repeats for maximal enhancer strength in *X. laevis* oocytes. Lanes 1 and 2 display transcription signals following co-injection of the two minimal minigenes, Ψ40 and Ψ52. Lanes 3 and 4 show the results of competition between Ψ401 (ten 60/81-bp enhancer repeats) and Ψ52. Lanes 5 and 6 show the results of competing Ψ411 (region 0 + region 1) with Ψ52. In lanes 7–10, constructs with region 0 + region 1 repeats in each orientation adjacent to a block of 60/81-bp repeats (Ψ411-01, Ψ411-01B), were competed with Ψ52. A construct with 60-bp enhancer repeats located upstream of region 0 + region 1 repeats (ΨEN-411) was competed with Ψ52 in lanes 11 and 12. Lanes 13–16 show the results of competing Ψ52 with minigenes that have all of the various classes of spacer repeats upstream of the promoter but differ in the number of spacer promoters and 60/81-bp enhancer blocks (Ψ409 and Ψ410).

classes of repeats found in the wild-type spacer with the sole exception of a spacer promoter(s). The Ψ411-01 constructs out-compete the enhancer-less Ψ52 minigene 7:1 (lanes 7–10) similar to Ψ401 (lanes 3 and 4), suggesting that regions 0 and 1 contribute nothing discernible to the enhancer strength of a single 60/81-bp enhancer block. One possibility is that the region 0/1 repeats are too far removed from the promoter to be

effective in the Ψ411-01 constructs. To test this possibility, we added a block of ten 60-bp enhancers (same used in construct Ψ4060-10) to Ψ411, such that the enhancers were upstream of T2 and regions 0 and 1. This construct, ΨEN-411 was still able to out-compete a Ψ52 minigene 6:1 (lanes 11 and 12) similar to Ψ401 (lanes 3 and 4), suggesting that 60/81-bp enhancers remain functional when moved an additional 1.6-kb upstream of

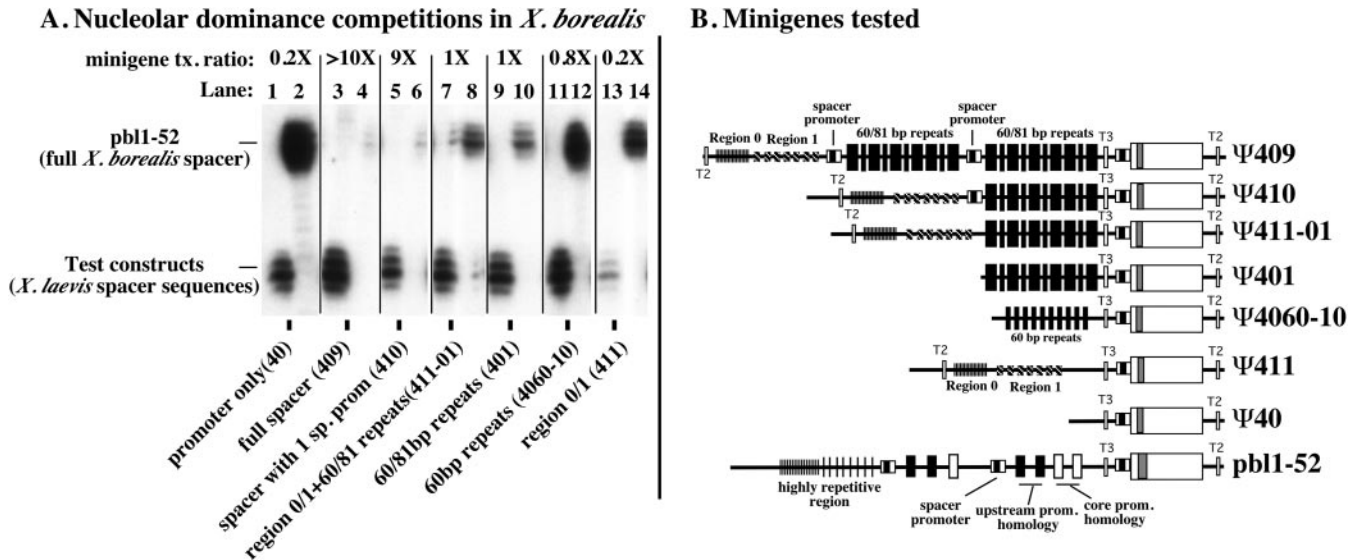


FIG. 5. At least one spacer promoter is needed in addition to 60/81-bp enhancer repeats to suppress transcription from a minigene with a full *X. borealis* spacer. *X. borealis* oocytes were injected with pbl1-52, a minigene that has a complete *X. borealis* intergenic spacer attached to a Ψ52 minigene body, in competition with a series of minigenes bearing various portions of an *X. laevis* intergenic spacer. Ψ40 was the competitor for pbl1-52 in lanes 1 and 2. The full *X. laevis* intergenic spacer construct Ψ409 was the competitor for pbl1-52 in lanes 3 and 4. The internally deleted derivative of Ψ409 having only one spacer promoter and block of 60/81-bp enhancers (Ψ410) was the competitor of pbl1-52 in lanes 5 and 6. The construct with regions 0 and 1 plus a block of 60/81-bp enhancer repeats but lacking a spacer promoter (Ψ411-01) was the competitor in lanes 7 and 8. In lanes 9 and 10, the minigene competing with pbl1-52 was Ψ401, which has a single block of 60/81-bp repeats. The minigene with ten 60-bp repeats was tested as a competitor in lanes 11 and 12. In lanes 13 and 14, a construct bearing only regions 0 and 1 upstream of the gene promoter was competed with pbl1-52.

the promoter. Finally, we tested two constructs, Ψ410 and Ψ409, that had regions 0 and region 1, spacer promoters, and 60/81-bp repeats, all in their normal orientation relative to one another (lanes 13–16). Ψ410 and Ψ409 suppressed all transcription from the competing Ψ52 minigene (see lanes 14 and 16, >10-fold competition effect). This result indicates that promoter strength is higher in Ψ410 and Ψ409 than in constructs that have only 60/81-bp repeats (with or without region 0 and region 1 repeats). Ψ410 appears to be as active as Ψ409, suggesting that full promoter strength requires only one set of 60/81-bp repeats and one spacer promoter. The increased strength of Ψ410 relative to Ψ411-01 leads us to conclude that at least one spacer promoter is essential for full enhancer function, in agreement with the conclusions of DeWinter and Moss (24).

We tested the involvement of spacer elements in nucleolar dominance-like minigene competition in *X. borealis* oocytes (Fig. 5). As was shown by Reeder and Roan (35), the *X. laevis* and *X. borealis* promoters have identical activity in *X. borealis* oocytes. Thus, the spacer effects can be monitored by competing minigenes bearing *X. borealis* or *X. laevis* intergenic spacer sequences attached to an *X. laevis* promoter. Minigene pbl1-52 (a minor modification of the pbl1 construct used by Reeder and Roan (35)) has a complete *X. borealis* intergenic spacer attached to a *X. laevis* Ψ52 minigene body. When co-injected with Ψ40, the pbl1-52 minigene is dominant by a ratio of 5:1, showing that the full *X. borealis* spacer also includes enhancer activity (compare lane 1 with 2). However, when pbl1-52 is co-injected with Ψ409, the analogous construct with a full *X. laevis* spacer, the Ψ409 minigene is strongly dominant (>10 times), such that transcription from pbl1-52 is barely detectable (compare lane 3 with 4). These results match those of Reeder and Roan (35) using almost identical constructs. Deletion from Ψ409 of one spacer promoter and one block of enhancers to form Ψ410 does not significantly diminish its competitive advantage relative to pbl1-52 (transcription ratio of 9:1, compare lanes 5 and 6 with 3 and 4). However, the removal of the remaining spacer promoter in Ψ410 represented by mini-

gene Ψ411-01 results in a significant loss in the competitive advantage of the *X. laevis* spacer compared with pbl1-52 (lanes 7 and 8), such that the two minigenes are co-dominant (expressed 1:1). Further removal of region 0 and region 1 repeats represented by Ψ401 is of no consequence, such that Ψ401 and pbl1 remain co-dominant (lanes 9 and 10). Likewise, Ψ4060-10, a construct bearing polymerized 60-bp enhancer repeats is also co-dominant with pbl1-52 (lanes 11 and 12, transcription ratio 0.8). In contrast, Ψ411 bearing only region 0 and region 1 repeats upstream of the promoter is out-competed 1:5 by pbl1-52 (compare lane 13 with 14) and fares as poorly as the promoter-only construct Ψ40. We conclude that region 0 and region 1 have no detectable enhancer activity in the nucleolar dominance-like competition assay. We also conclude that a single block of *X. laevis* 60/81-bp enhancers confers on an adjacent promoter approximately the same advantage conferred by the complete *X. borealis* spacer. Full suppression of a *X. borealis* spacer-bearing construct, analogous to nucleolar dominance in hybrid frogs, requires that at least one spacer promoter be located upstream of a 60/81-bp enhancer block.

DISCUSSION

In 1984, Reeder and Roan (35) showed that a *X. laevis* promoter is out-competed 10:1 by a minigene bearing a complete *X. borealis* spacer (pbl1). However, a minigene having a full *X. laevis* spacer (Ψ409 or the almost identical Ψ209) will completely suppress transcription from an analogous minigene (pbl1) bearing a complete *X. borealis* spacer regardless of whether the *borealis* spacer is attached to a *borealis* promoter (construct Xbr6) or a *laevis* promoter (construct pbl1) promoter (35). Our results are in agreement with these prior findings. In one of three experiments, Reeder and Roan (35) showed that Ψ401 could suppress transcription from a co-injected construct with a complete *X. borealis* spacer, whereas in two other experiments, Ψ401 was only co-dominant with the construct bearing a complete *X. borealis* spacer. Our results are in agreement with the latter two experiments but not the first. Given that 60/81-bp repeats clearly contributed to the competitive

strength of the *X. laevis* spacer sequences and that the full spacer construct $\Psi 409$ had twice as many of these elements as $\Psi 401$, it was reasonable to deduce that the 60/81-bp elements alone were likely to explain the dominance of the full *X. laevis* spacer (35). However, the formal possibility has remained that other spacer sequences in $\Psi 409$, in particular the two spacer promoters or the region 0 and region 1 repeats, might have played a role. By testing additional constructs, our data suggest that region 0 and region 1 repeats play no apparent role in this phenomenon, but that *X. laevis* 60/81-bp elements and at least one spacer promoter are required for the complete suppression of competing genes bearing full *X. borealis* spacers.

Consideration of the data in Figs. 4 and 5 suggests that the various spacer elements confer the same improvements to promoter strength regardless of whether the competition assay is conducted in *X. laevis* or *X. borealis* oocytes and regardless of whether the competitor is an enhancer-less promoter or a promoter with full *X. borealis* spacer sequences. In both assays, full enhancer effect is only observed if at least one spacer promoter is located upstream of a block of 60/81-bp repeats. The addition of a second block of 60/81-bp repeats and a second spacer promoter provides no additional benefit. The latter conclusion that a spacer promoter is needed to observe full 60/81-bp repeat enhancer function supports the findings of DeWinter and Moss (24, 25). These authors showed that a spacer promoter alone does not stimulate transcription from an adjacent gene promoter, but the insertion of one, three, or ten 60- or 81-bp elements between the gene promoter and spacer promoter results in a degree of enhancement proportional to the number of 60/81-bp repeats. They proposed that a spacer promoter and an adjacent block of 60/81-bp elements act together as a functional unit. Our data are consistent with this model. Nonetheless, the mechanism by which spacer promoters and 60/81-bp enhancers might work together are still not clear. The possibilities include spacer transcription clearing away nucleosomes or other chromatin proteins to allow transcription factor recruitment by the enhancers or displacement of transcription factors bound to enhancers attributed to spacer transcription (20). However, spacer promoters are not active in the oocytes of most individuals; thus, the mechanisms by which they synergize with enhancer elements in oocytes remain elusive.

A role for region 0 and/or region 1 repeats in transcriptional enhancement is made controversial by our results, which do not fully support those of Mougey *et al.* (26). These authors found that region 0 repeats could act as enhancers whose strength was proportional to the repeat copy number. They found the same to be true for region 1 repeats and for the wild-type combination of both region 0 plus region 1 repeats. However, in our hands, region 0 plus region 1 typically imparted little or no advantage to an adjacent promoter. A series of constructs we made that contained varying numbers of region 0 or region 1 repeats also lacked apparent activity (data not shown). In only rare cases (as in Fig. 3) did regions 0/1 show enhancer function, and even in these experiments, the region 0/1 repeats did not confer on an adjacent promoter the ability to withstand competition from a promoter bearing 60/81-bp enhancers. One possible explanation could be that Mougey *et al.* (26) used *X. borealis* oocytes exclusively, whereas most of our studies were done using *X. laevis* oocytes. However, the fact that in our hands regions 0 and 1 also failed to reveal enhancer function in *X. borealis* oocytes (Fig. 5) argues against this possibility. At present, we are unable to identify a probable cause for our different results.

The *X. borealis* spacer has repeated sequences that are similar to the 42-bp upstream promoter domain present in each *X. laevis* 60- or 81-bp repeat. Unlike *X. laevis*, which has ~10 such

repeats between the gene promoter and the nearest spacer promoter, *X. borealis* has only two of these elements in the analogous location (15, 16). In the same region, *X. borealis* has additional types of repeats, none of which has been tested directly for enhancer activity. One of these, a 44-bp element present four times in the *X. borealis* spacer sequence of Bach *et al.* (15) but only twice in the sequence of Labhart and Reeder (16), is highly homologous to sequences of the core promoter surrounding the transcription site. A sequence matching this same portion of the *X. laevis* promoter has been shown to possess enhancer activity when polymerized and cloned upstream of a promoter (14), suggesting that the 44-bp elements in *X. borealis* are likely to be enhancers as well. Hence, one is likely to underestimate the true enhancer content of *X. borealis* spacers if one counts only the number of spacer elements homologous to 60/81-bp repeats and the upstream promoter domain. Nevertheless, *X. laevis* genes still appear to have a 10:4 or 10:6 (depending on which available *X. borealis* sequence best represents the situation in nature) numerical advantage in enhancer content compared with *X. borealis* genes in the region just upstream of the promoter (Fig. 5, see diagrams of $\Psi 409$ and pbl1-52). Aside from this difference, *X. laevis* and *X. borealis* spacers are similar, both having spacer promoters and region 0/region 1-like repeats that share substantial sequence similarity between the two species. Taken together, these observations and available experimental results suggest that differences in the number of enhancer elements and/or differences in spacer promoter activity could explain the competitive superiority of *X. laevis* over *X. borealis* intergenic spacers in the oocyte injection assay.

One question not resolved by these studies is whether the enhancer competition effects observed in injected oocytes can be taken as definitive evidence for the nucleolar dominance mechanism(s) at work among chromosomally encoded genes in *X. laevis* \times *X. borealis* hybrids. The co-transfection of rRNA minigenes into plant cells at a copy number of ~3000 molecules/cell has failed to reveal any competition effects analogous to those observed in *Xenopus* oocytes regardless of whether the minigenes have minimal promoters or complete intergenic spacers (41). Likewise, intergenic spacer sequences confer no transcriptional advantage on *Xenopus* rRNA minigenes transfected by electroporation into cultured *Xenopus* somatic cells derived from kidney.¹ One possibility is that *Xenopus* intergenic spacer repeats only display their enhancer function in oocytes and early embryos. Another possibility is that it is simply the high copy numbers of minigenes injected into oocytes that explain the differences in spacer effects in oocytes versus somatic cells. An observation favoring the former explanation is that in *X. laevis* \times *X. borealis* hybrids, the silencing of *X. borealis* rRNA genes is essentially complete in embryos and tadpoles but becomes leaky in the organs of adults (34). The fact that spacer repeats display strong enhancer activity in oocytes and early embryos (representative of early development) but not in cultured kidney cells (perhaps representative of adult tissues) is consistent with hypothesis (35) that enhancer activity is correlated with nucleolar dominance at least in *Xenopus*.

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REFERENCES

1. Paule, M. R., and White, R. J. (2000) *Nucleic Acids Res.* **28**, 1283–1298
2. Moss, T., and Stefanovsky, V. Y. (1995) *Prog. Nucleic Acid Res. Mol. Biol.* **50**, 25–66

¹ C. S. Pikaard and R. H. Reeder, unpublished data.

3. Hannan, K. M., Hannan, R. D., and Rothblum, L. I. (1998) *Front. Biosci.* **3**, 376–398
4. Grummt, I. (1999) *Prog. Nucleic Acid Res. Mol. Biol.* **62**, 109–154
5. Reeder, R. H. (1999) *Prog. Nucleic Acid Res. Mol. Biol.* **62**, 293–327
6. McClintock, B. (1934) *Z. Zellforsch. Mikrosk. Anat.* **21**, 294–328
7. Wallace, H., and Birnstiel, M. L. (1966) *Biochim. Biophys. Acta* **114**, 296–310
8. Scheer, U., and Weisenberger, D. (1994) *Curr. Opin. Cell Biol.* **6**, 354–359
9. Shaw, P. J., and Jordan, E. G. (1995) *Annu. Rev. Cell Dev. Biol.* **11**, 93–121
10. Reeder, R. H. (1989) *Curr. Opin. Cell Biol.* **1**, 466–474
11. Labhart, P., and Reeder, R. H. (1984) *Cell* **37**, 285–289
12. Boseley, P., Moss, T., Machler, M., Portmann, R., and Birnstiel, M. (1979) *Cell* **17**, 19–31
13. Moss, T., Boseley, P. G., and Birnstiel, M. L. (1980) *Nucleic Acids Res.* **8**, 467–485
14. Pikaard, C. S. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 464–468
15. Bach, R., Allet, B., and Crippa, M. (1981) *Nucleic Acids Res.* **9**, 5311–5330
16. Labhart, P., and Reeder, R. H. (1987) *Nucleic Acids Res.* **15**, 3623–3624
17. Dunaway, M. (1989) *Genes Dev.* **3**, 1768–1778
18. Pikaard, C. S., McStay, B., Schultz, M. C., Bell, S. P., and Reeder, R. H. (1989) *Genes Dev.* **3**, 1779–1788
19. Moss, T., and Birnstiel, M. L. (1979) *Nucleic Acids Res.* **6**, 3733–3743
20. Moss, T. (1983) *Nature* **302**, 223–228
21. Labhart, P., and Reeder, R. H. (1986) *Cell* **45**, 431–443
22. Morgan, G. T., Reeder, R. H., and Bakken, A. H. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 6490–6494
23. Morgan, G. T., Roan, J. G., Bakken, A. H., and Reeder, R. H. (1984) *Nucleic Acids Res.* **12**, 6043–6052
24. DeWinter, R., and Moss, T. (1986) *Cell* **44**, 313–318
25. DeWinter, R., and Moss, T. (1987) *J. Mol. Biol.* **196**, 813–827
26. Mougey, E. B., Pape, L. K., and Sollner-Webb, B. (1996) *J. Biol. Chem.* **271**, 27138–27145
27. Pikaard, C. S. (2000) *Plant Mol. Biol.* **43**, 163–177
28. Pikaard, C. S. (2000) *Trends Genet.* **16**, 495–500
29. Navashin, M. (1934) *Cytologia* **5**, 169–203
30. Blackler, A. W., and Geckling, C. A. (1972) *Dev. Biol.* **27**, 385–394
31. Cassidy, D. M., and Blackler, A. W. (1974) *Dev. Biol.* **41**, 84–96
32. Durica, D. S., and Krider, H. M. (1977) *Dev. Biol.* **59**, 62–74
33. Durica, D. S., and Krider, H. M. (1978) *Genetics* **89**, 37–64
34. Honjo, T., and Reeder, R. H. (1973) *J. Mol. Biol.* **80**, 217–228
35. Reeder, R. H., and Roan, J. G. (1984) *Cell* **38**, 39–44
36. Reeder, R. H. (1985) *J. Cell Biol.* **101**, 2013–2016
37. Pikaard, C. S., and Reeder, R. H. (1988) *Mol. Cell. Biol.* **8**, 4282–4288
38. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
39. Busby, S. J., and Reeder, R. H. (1983) *Cell* **34**, 989–996
40. Reeder, R. H., Roan, J. G., and Dunaway, M. (1983) *Cell* **35**, 449–456
41. Frieman, M., Chen, Z. J., Saez-Vasquez, J., Shen, L. A., and Pikaard, C. S. (1999) *Genetics* **152**, 451–460