Repetitive 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 x 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 agreement 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 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...
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

**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.
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 codominance. 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 Ψ40 and Ψ52 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 Ψ40 and Ψ52, allowing their transcripts to be distinguished from one another and from endogenous X. borealis transcripts. The constructs whose names begin with “Ψ” use the Ψ40 minigene body, whereas constructs that begin with “Ψ5” use the Ψ52 minigene body. Constructs Ψ40, Ψ409, Ψ4060-10, Ψ4081-10, Ψ52, and Ψ52 were have been described previously (11, 37). Ψ5281-10 is identical to Ψ4081-10 (37) with the exception that the ten 81-bp repeats are attached to a Ψ52 minigene body rather than to a Ψ40 body. Ψbl1-52 is virtually identical to Ψbl1 described previously (35) with the exception that the X. borealis spacer sequences have been attached to the Ψ52 minigene body rather than the Ψ40 minigene body. Construct Ψ411 contains a 1.6-kb intergenic spacer fragment including region 0 and region 1 fused to a Ψ40 minigene body. Ψ411 was engineered by digesting Ψ409 with SalI and BamHI (near the 5’ end of the most upstream spacer promoter) isolating the 1.6-kb fragment, attaching a BamHI/Xhol adapter, and ligating the resulting SalI/Xhol fragment into the SalI site located at position –245 of Ψ40. The orientation of the inserted sequences relative to the promoter is reversed in Ψ411B. Construct Ψ411-01 had the 1600-bp spacer fragment of construct Ψ411 inserted into Ψ401 at the SalI site located just 5’ of the 60/81-bp enhancer block. Ψ410 was created from Ψ409 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 × 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 mg NaCl, 50 pg Tris-HCl (pH 7.5), 0.1 mg EDTA and 500 μg/ml 0-aminanit (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 5–000 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 5 μl 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 μl/oocyte and frozen for subsequent S1 analysis.

**S1 Nuclease Analysis—**DNA probes for S1 nuclease analysis were prepared by digesting the Ψ40 or Ψ52 minigenes with BamHI, which cuts at +46 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-32P]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 μl of 300 mm NaCl, 10 μl 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 μl of S1 nuclease digestion buffer (5% glycerol, 1 mm ZnSO4, 30 mm sodium acetate (pH 4.5), 50 mm NaCl, 130 units/ml S1 nuclease (Sigma)) was added. S1 digestion was for 30 min at 37 °C. Digestion reactions were stopped by removing 280 μl from the bottom of the tubes (to avoid the mineral oil at the top) to a fresh tube containing 30 μl of 7.5 m ammonium acetate, 5 μl of 0.5 m EDTA, and 3.3 μg of yeast tRNA. After mixing, 1 ml of cold (–20 °C) absolute ethanol was added to precipitate nucleic acids. Following centrifugation at 14,000 × g for 15 min, pellets were washed with 70% ethanol, dried, and resuspended in formamide-containing loading buffer supplemented with 10 μl 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 Ψ40 minigene body and the competing construct uses the Ψ52 minigene body. RNA isolated from the injected oocytes is then split into two equal aliquots, one of which is hybridized to a Ψ40-specific probe (odd numbered lanes) and the other is hybridized to the Ψ52-specific probe (even numbered lanes). When Ψ40 and Ψ52 minigenes are co-injected, both support comparable levels of transcription (compare lane 1 with 2), although Ψ40 signals are ~1.6-fold stronger because of higher specific activity of the Ψ40 probe (true in all of our experiments). To facilitate a comparison with other injected minigene pairs, the ratio of Ψ40 to Ψ52 signals in lanes 1 and 2 was defined as 1.0, and all subsequent injection signal ratios were normalized accordingly. Minigene Ψ401 has a wild-type block of 60/81-bp elements upstream of the Ψ40 minigene body. When Ψ401 is co-injected with Ψ52, Ψ401 is preferentially transcribed by >10-fold, such that transcription from Ψ52 is almost undetectable (compare lane 3 with 4). Note that the extremely weak signal from the Ψ52 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. Ψ4060-10 has 10 complete 60-bp enhancers cloned as a polym-
A. Enhancer activity of 60 and 81 bp repeats

preferentially transcribed by \(>10\)-fold (compare lane 5 with 6), mirroring the results obtained using \(\Psi401\) (lanes 3 and 4). A similar result is observed following the co-injection of \(\Psi4081\) and \(\Psi52\) (lanes 7 and 8, transcription ratio is \(>10\)). \(\Psi4081\) and \(\Psi52\) 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 \(\Psi52\) (constructs \(\Psi5260\) and \(\Psi5281\)) counteracts the competitive advantage of \(\Psi4060\) or \(\Psi4081\), making \(\Psi52\) minigene body transcripts readily detectable (compare lanes 9 with 10 and 11 with 12). However, an unexplained 2-fold bias in favor of the \(\Psi40\) minigene body (in addition to the 1.6-fold higher specific activity of the \(\Psi40\) 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 \(\Psi411\) or \(\Psi411B\) over a co-injected \(\Psi52\) minigene, but in some hatches of oocytes, there was a discernible stimulatory effect. Fig. 3 shows one of the latter experiments. In this experiment, \(\Psi40\) and \(\Psi52\) programmed comparable levels of transcription when in competition with one another (compare lane 1 with 2, again the specific activity of the \(\Psi40\) probe was \(\sim 1.6\)-fold higher). Constructs \(\Psi411\) and \(\Psi411B\) have the 5’ end of the intergenic spacer including T2, region 0 repeats, and region 1 repeats cloned in both orientations upstream of the \(\Psi40\) minigene. Both \(\Psi411\) and \(\Psi411B\) out-performed a co-injected \(\Psi52\) minigene \(\sim 2–2.5\)-fold (compare lanes 3 with 4 and 5 with 6). However, the \(\Psi411\) and \(\Psi411B\) constructs were out-competed \(\sim 6:1\) or \(10:1\), respectively, by \(\Psi52\), a \(\Psi52\) minigene with a block of ten 60/81-bp enhancers (compare lanes 7 with 8 and 9 with 10). In fact, \(\Psi411\) and \(\Psi411B\) fared no better in competition with \(\Psi52\) than did \(\Psi40\) (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 \(\Psi411\) 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). \(\Psi52\), which lacks enhancers, was co-injected with eight different test constructs built on a \(\Psi40\) minigene body. Following co-injection of \(\Psi40\) and \(\Psi52\), transcripts from both minigenes were readily detected (lanes 1 and 2). The addition of a block of 60/81-bp repeats to the \(\Psi40\) minigene (construct \(\Psi401\)) results in a strong trans-competition effect (7-fold), such that \(\Psi52\) transcripts are only barely detectable (lanes 3 and 4). In contrast, construct \(\Psi411\) containing regions 0 and 1 upstream of the \(\Psi40\) gene promoter has no competitive advantage over \(\Psi52\) in this experiment (lanes 5 and 6). Constructs \(\Psi411-01\) and \(\Psi411B-01\) 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, \(\Psi411-01\) has all of the

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classes of repeats found in the wild-type spacer with the sole exception of a spacer promoter(s). The \( \Psi 411 \) constructs 0.1X and 0.1X out-compete the enhancer-less \( \Psi 40 \) minigene 7:1 (lanes 7–10), 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 \( \Psi 411 \) constructs. To test this possibility, we added a block of ten 60-bp enhancers (same used in construct \( \Psi 4060 \)–10) to \( \Psi 411 \), such that the enhancers were upstream of T2 and regions 0 and 1. This construct, \( \Psi 411 \) was still able to out-compete a \( \Psi 52 \) minigene 6:1 (lanes 11 and 12) similar to \( \Psi 411 \) (lanes 3 and 4), suggesting that 60/81-bp enhancers remain functional when moved an additional 1.6-kb upstream of
the promoter. Finally, we tested two constructs, \( \Psi 410 \) and \( \Psi 409 \), 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). \( \Psi 410 \) and \( \Psi 409 \) suppressed all transcription from the competing \( \Psi 52 \) minigene (see lanes 14 and 16, >10-fold competition effect). This result indicates that promoter strength is higher in \( \Psi 410 \) and \( \Psi 409 \) than in constructs that have only 60/81-bp repeats (with or without region 0 and region 1 repeats). \( \Psi 410 \) appears to be as active as \( \Psi 409 \), suggesting that full promoter strength requires only one set of 60/81-bp repeats and one spacer promoter. The increased strength of \( \Psi 410 \) relative to \( \Psi 411-01 \) 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. laevis* 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* \( \Psi 52 \) minigene body. When co-injected with \( \Psi 40 \), 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 \( \Psi 409 \), the analogous construct with a full *X. laevis* spacer, the \( \Psi 409 \) 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 \( \Psi 409 \) of one spacer promoter and one block of enhancers to form \( \Psi 410 \) 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 \( \Psi 410 \) represented by minigene \( \Psi 411-01 \) 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 \( \Psi 401 \) is of no consequence, such that \( \Psi 401 \) and pbl1 remain co-dominant (lanes 9 and 10). Likewise, \( \Psi 4060-10 \), 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, \( \Psi 411 \) 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 \( \Psi 40 \). 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 (\( \Psi 409 \) or the almost identical \( \Psi 209 \)) 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 \( \Psi 401 \) could suppress transcription from a co-injected construct with a complete *X. borealis* spacer, whereas in two other experiments, \( \Psi 401 \) 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 pH1-52). 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 x X. borealis hybrids. The co-transfection of rRNA minigenes into plant cells at a copy number of \(\sim 3000\) molecules/cell has revealed 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 x 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.

Acknowledgments—We thank Judy Roan and Ron Reeder (Fred Hutchinson Cancer Research Center, Seattle, WA) for providing numerous minigene constructs for this study. We thank Tom Moss (Laval University, Quebec) for sharing ideas concerning possible spacer promoter functions.

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\(^1\) C. S. Pikaard and R. H. Reeder, unpublished data.
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