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The *Xenopus* Ribosomal DNA 60- and 81-Base-Pair Repeats Are Position-Dependent Enhancers That Function at the Establishment of the Preinitiation Complex: Analysis In Vivo and in an Enhancer-Responsive In Vitro System

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Although it is generally believed that the 60- and 81-base-pair (60/81-bp) repeats of the *Xenopus laevis* ribosomal DNA (rDNA) spacer are position-independent transcriptional enhancers, this has not been shown directly. We have now developed a critical assay which proves that the 60/81-bp repeats do, in fact, stimulate transcription from promoters in *cis* and that they function in both orientations and when up to 1 kilobase pair from the initiation site. However, contrary to the widely accepted view, these elements are found to be highly position dependent, for they have no net effect when downstream of the initiation site within the transcribed region and they behave as transcriptional silencers of promoters in *cis* when moved >2 kilobase pairs upstream of the initiation site. The 60/81-bp elements therefore are position-dependent 5′ enhancers. We also found that this rDNA enhancer was polymerase I specific and that it was composed of duplicated, individually functional elements. Finally, we report an in vitro system that reproduces both *cis* enhancement and *trans* competition by the 60/81-bp repeats. Sequential-addition studies in this system demonstrated that the rDNA enhancer functions in *trans* at or before establishment of the stable transcription complex, not subsequently at each round of transcription.

DNA sequences that augment transcription independent of orientation and at various locations relative to a colinear promoter are termed enhancers. First defined in simian virus 40 (SV40) viral DNA, enhancers have since been identified for a large number of cellular and viral genes transcribed by RNA polymerase II (reviewed in references 18, 25, 40, and 50) and more recently also for a gene transcribed by RNA polymerase III (2). Eucaryotic polymerase II enhancers function 5′ and usually also 3′ of the initiation site and generally over many kilobase pairs (kb), although their effects can decrease significantly at increasing distance (2, 18, 25, 38, 40, 41, 43, 50, 60); analogous upstream activation sequences in yeast cells are located within ~1 kb upstream of the initiation site and do not appear to function 3′ of the initiation site (19, 56). Polymerase II enhancers are normally assessed by comparing the level of transcription obtained from two otherwise identical templates, one bearing and one lacking these elements, when introduced separately into cells (a *cis* stimulation assay). It has been reported that polymerase II enhancers do not markedly depress transcription of promoters on separate molecules (a *trans* competition assay [6, 49, 63, 64]); however, some polymerase II enhancers and promoters are now known to share common factors (14, 29, 35, 39). Sequences containing or related to polymerase II enhancer elements have also been shown to act as silencers of *cis*-located promoters. Although a great deal has been learned about sequences that direct RNA polymerase II enhancement and about factors that bind to these sequences (21, 69; reviewed in references 23, 48, 51, and 68), their mechanisms of action are not yet understood.

Spacer sequences that affect promoter function are not limited to genes transcribed by RNA polymerase II. Transcription of the *Xenopus laevis* rRNA gene is greatly affected by sequences in the ribosomal DNA (rDNA) spacer region (7, 36) upstream of the 150-base-pair (bp) promoter (66, 67). The organization of this spacer is diagrammed in Fig. 1A. A major effector role has been attributed to blocks of 60- and 81-bp (60/81-bp) repeats that make up about half of the intergenic spacer and clearly can compete with a promoter in *trans* (26; see Fig. 4A, lanes 7 and 8). The widely accepted view is that the 60/81-bp elements also stimulate transcription from an rDNA promoter in *cis* and that this stimulation is orientation- and position-independent enhancement (42, 44), occurring equally efficiently when the repeats are kilobase pairs distant from the promoter and when they are downstream of the promoter within the transcribed portion of the template (26, 27, 44, 47). These conclusions are based almost entirely on oocyte coinjection experiments using two competing, promoter-bearing templates (13, 26, 27, 45, 47); when one also carries the 60/81-bp repeats, the transcriptional balance is shifted toward that promoter and away from the one lacking the repeats (such an assay is illustrated in Fig. 4A, lanes 1 to 3 with two marked rRNA genes). As might have been anticipated and will be shown below, however, this is a complex assay, involving contributions from both *trans* competition and from whatever *cis* stimulation is occurring, and the relative contributions of these two effects cannot be readily discerned from the analysis as it is generally performed. Curiously, all published attempts to specifically demonstrate *cis* stimulation by the 60/81-bp elements in injected oocytes using the standard protocol for assessing polymerase II enhancement (introducing promoters bearing or lacking the 60/81-bp elements into separate cell populations) have reported no stimulation (13, 26, 46, 47). Separate experiments involving injected embryos have

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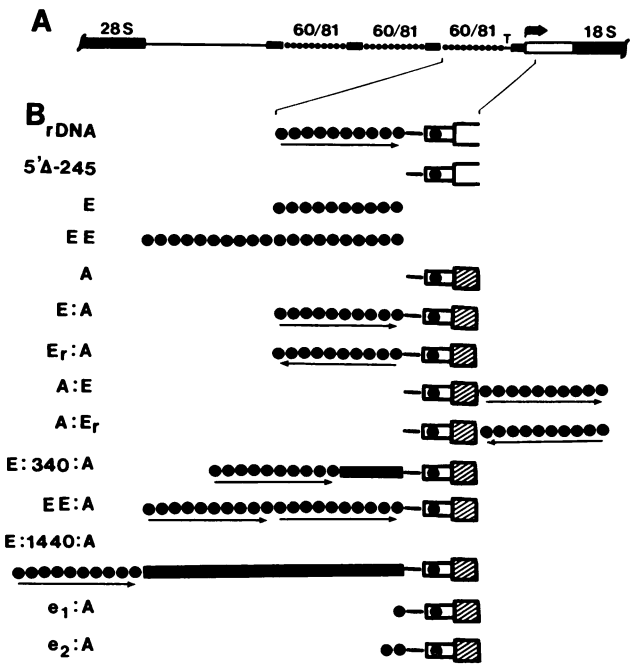

FIG. 1. rDNA organization and plasmids. (A) Organization of the *X. laevis* rDNA repeat. RNA reads through the 5′ transcribed spacer (▭) and into the 18S and 28S coding regions. The transcription initiation site (➡) is adjacent to the gene promoter (▬) and a terminator site (T). Each spacer also contains about two to seven blocks of ~7 to 10 repeating 60/81-bp elements (●; which we will show to be the rDNA enhancer) separated by spacer promoters. (B) Enlargement of the enhancer-promoter region. Plasmid 5′Δ-245 (54) contains the rDNA promoter and sequences up to residue +115. The E plasmid contains a block of 10 60/81-bp repeats, and the EE plasmid contains two such blocks. The A plasmid (and the B plasmid [67]) contains the *X. laevis* rDNA promoter (residues −245 to +13) joined to different procaryotic tester segments. The E:A and E_r:A plasmids contain the E segment cloned at its natural position in the normal and reverse orientations upstream of the A gene. A:E and A:E_r contain the E segment in either orientation ~170 bp downstream of the initiation site of the A gene. E:340:A and E:1440:A contain 340 and 1,440 bp, respectively, of pBR322 DNA (▬) separating the enhancer from the full promoter. EE:A contains two tandem E segments cloned in the normal position and orientation upstream of the A gene. e_1:A and e_2:A contain, respectively, a single 60-bp element and a 60- plus an 81-bp element cloned into the same site of the A gene. Analogous B-gene constructs are not shown.

clearly demonstrated a *cis* stimulatory effect of some rDNA spacer sequences (7), but these did not distinguish whether the stimulation was due to the 60/81-bp repeats, to the spacer promoters (also transcription effectors [12]), or to the other 1.5 kb of repetitive sequence elements contained in all of the *cis* stimulatory spacers examined. In any event, this embryo *cis* stimulation was completely orientation dependent (7), unlike the enhancement attributed to the 60/81-bp elements in oocytes. These considerations forced us to conclude that although the 60/81-bp elements clearly have transcription effects, their relationship to enhancers warrants further investigation.

In this paper, we show that the 60/81-bp repeats do, in fact, directly stimulate transcription of a promoter in *cis* as well as compete with a promoter in *trans* and that this *cis* stimulation is true enhancement, for it occurs similarly when the 60/81-bp elements are reversed or are moved several hundred base pairs upstream of the initiation site. However, we demonstrate that this *cis* stimulation is very position dependent. The 60/81-bp elements exert no net *cis* stimulatory effect when they are within the transcribed region, downstream of the initiation site; when they are ≥1.7 kb upstream of the initiation site, they actually substantially repress the promoter in *cis*. These findings demonstrate that the 60/81-bp elements are quite position dependent. We also show that this rDNA enhancer functions in a polymerase-specific fashion, at the transcriptional level, and that it is made up of repeated independently functional elements, for even a single 60-bp element exerts both *cis* stimulation and *trans* competition. Finally, we report the development of an in vitro system that reproduces both *cis* stimulation and *trans* competition of the rDNA enhancer; using this novel system, we demonstrate that the rDNA enhancer acts in *trans* at or before the establishment of the stable preinitiation complex, not subsequently at each round of transcription.

MATERIALS AND METHODS

Plasmid constructions: A, E, and derivatives. Plasmid constructions are shown in Fig. 1. The A plasmid is derived from plasmid 5′Δ-245/3′Δ+13 (54) and is identical to the B plasmid (67) except that a different segment of pBR322 (from the *Bam*HI site to the *Hae*III site at position 296) was inserted between the *Bam*HI and the *Eco*RV sites just downstream of the rDNA segment at residue +13. Since the A and B tester sequences are not present in the frog genome and since the 13-nucleotide segment of rRNA-coding sequence carried on these genes is too short to participate in trimolecular hybrid formation (32), accurate transcription of these genes in microinjected *X. laevis* oocytes can be readily assessed by S1 nuclease analysis using A- and B-gene-specific probes (Fig. 2B; 67). In all of the A- and B-gene constructs, the first termination site that the transcript encounters is at residue −200 (28), just upstream of the initiation site.

To construct the E plasmid, a 720-bp *Sma*I fragment containing a block of 10 60/81-bp repeats from pX1rl4 (5) was cloned into pBR322, using *Cla*I and *Sal*I linkers. EE contains a dimer of these repeats. To form E:A and E_r:A, the enhancer repeat, subcloned in both orientations and isolated as a *Cla*I-*Sal*I fragment, was joined to position −245 of the A gene (isolated as a *Sal*I-*Eco*RI fragment), using an *Eco*RI-*Cla*I-cut pBR322 vector. A:E was made by joining an *Eco*RI-*Sal*I fragment containing the enhancer repeats, the large *Eco*RI-*Pst*I fragment from the A plasmid, and the small *Pst*I-*Sal*I fragment from pBR322; A:E_r was constructed in the same manner except that the enhancer repeats were taken from a construct in which they had been cloned in the reverse orientation. To form e_2:A, the E plasmid was digested with *Pst*I, and a 140-bp fragment containing one 60- and one 81-bp element was isolated. For e_1:A, this 140-bp fragment was digested with *Hae*III (see sequence in reference 53). *Cla*I and *Sal*I linkers were added onto the ends of these fragments, and they were joined to the *Sal*I-*Eco*RI fragment of the A gene, using an *Eco*RI-*Cla*I pBR322 vector. To form A(pUC) and B(pUC), the *Sal*I-*Hin*dIII fragment of the A and B genes, respectively, was inserted into a *Sal*I-*Hin*dIII pUC18 vector. For E:A(pUC) and E:B(pUC), a *Cla*I fragment containing the E:A and E:B genes, respectively, was inserted into the *Acc*I site of pUC18. E:340:A and E:1440:A were made by inserting the 344-bp *Hin*fI fragment or the 1,444-bp *Taq*I fragment of pBR322 into the *Sal*I site of E:A(pUC) at residue −245 after S1 nuclease treatment of the overhanging ends. For EE:B, a *Cla*I fragment containing two blocks of 10 60/81-bp repeats each was cloned into the *Acc*I polylinker site of B(pUC).

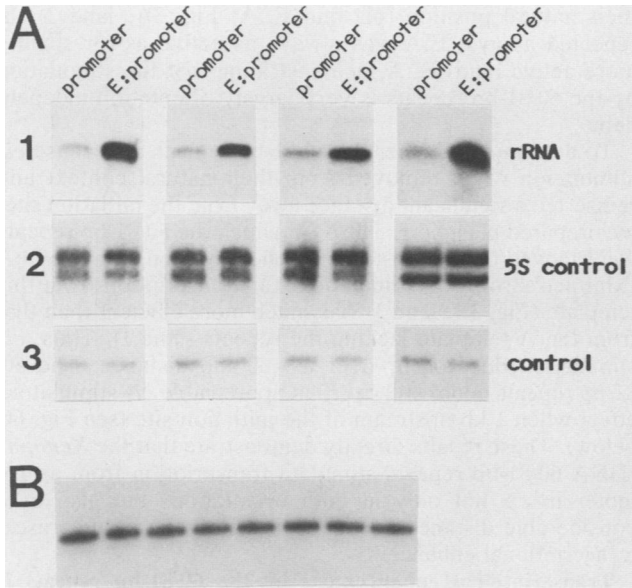

FIG. 2. Reproducible direct assessment of *cis* stimulation by the 60/81-bp repeats. (A) Plasmids containing the rDNA promoter or the promoter and upstream-joined 60/81-bp elements were coinjected into oocytes with a 5S maxigene plasmid. Transcription of the injected rDNA plasmid (rRNA), of the injected 5S maxigene (5S control), and of the cellular rDNA (control) was assessed by S1 analysis. The four panels represent separate injection experiments. (The 5S maxigene template generally yields a pair of closely spaced transcript bands.) (B) Lanes representing eight sets of oocytes injected with the 5S maxigene plasmid. Transcription was assessed by S1 analysis.

E:tk and E:5S plasmids. The 720-bp *Sal*I-*Bam*HI E fragment was inserted immediately upstream of the *Bam*HI-*Hin*dIII thymidine kinase (TK) gene region in the herpesvirus TK plasmid ptk5'Δ-119 (34). For SV40:tk, the enhancer region from pSV2-CAT (*Pvu*II-*Hin*dIII fragment) was inserted at position −119 of the TK plasmid (ptk5'Δ-119). The tubulin plasmid was previously described (30). For E:5S, two complete blocks of 60/81-bp repeats were cloned in tandem at the *Cla*I site immediately upstream of the *Hin*dIII 5S insert in maxi-5S, a derivative of the *Xenopus borealis* 5S gene plasmid that contains a duplication of the sequences +77 to +115 (4).

Transcription in microinjected oocytes and S1 nuclease analysis. Microinjection into *X. borealis* oocytes was performed as described previously (52, 66) unless otherwise noted. For each assay, ~30 oocytes were injected with ~30 nl each of plasmid DNA at a concentration of 25 or 50 μg/ml, as stated in the figure legends. Injected rDNA solutions contained 20 μg of α-amanitin per ml; 5S and TK injections did not. RNA was isolated from the pooled oocytes after ~6 h of incubation at 20°C. For sequential injections, oocytes were injected by using a very thin needle with ~5 nl of each plasmid DNA at a concentration of 125 μg/ml in 1× Gurdon buffer (88 mM NaCl, 15 mm Tris hydrochloride [pH 7.6], 1 mM KCl) plus 100 μg of α-amanitin per ml.

All experiments were repeated multiple times, with each injection performed in duplicate. Duplicate injections generally varied by <15%. Different frogs can show somewhat different quantitative but always identical qualitative responses. We find that this provides a reproducible measure of *cis* stimulation by enhancer sequences in the absence of plasmid *trans* competition effects. One can also use a coinjected polymerase III (5S) or polymerase II (TK) gene as a neutral internal control for the cis stimulation assays, but since these promoters can exhibit somewhat different transcriptional responses to varying template concentrations than the rDNA promoter (unpublished observations), they are not perfect internal controls.

All hybridization probes were 5' end labeled and single stranded. The probe for transcription of the family of B genes was the *Eco*RI-*Ava*I fragment (residues +89 to −60 relative to the initiation site) of the B plasmid (67), except for B(pUC), where the probe was a *Hin*dIII-*Ava*I fragment (residues +60 to −60 relative to the initiation site); the probe for transcription of the family of A genes was the *Hin*dIII-*Ava*I fragment (residues +97 to −60 relative to the initiation site) of the A plasmid. For transcripts of plasmid 5'Δ-245, an rDNA fragment extending from residue +55 to −125 was used (52). The probe for the maxi-5S transcripts was the *Bam*HI-*Eco*RI fragment of the 5S maxigene plasmid, and the probe for the TK transcripts was the *Bgl*II-*Bam*HI fragment of ptk5'Δ-119. The probe for the *X. borealis* endogenous pre-rRNA was a *Taq*I-*Bgl*I fragment of pXbr101a. Two oocyte equivalents of RNA was hybridized to 0.01 pmol of probe (in some experiments, 0.1 or 0.5 oocyte equivalent of RNA was used to assess TK transcription or *X. borealis* rRNA transcription, respectively), and the reactions were subjected to S1 analysis as described previously (52). The intensity of the autoradiographic bands was determined by densitometry of various exposures of the gels within the linear range. A few lanes from Fig. 3A, 4A, and 7 were shown in a recent review (37).

In vitro assay of the *X. laevis* rDNA enhancer action. The in vitro *Xenopus* rDNA transcription system (65) was modified as follows: the homogenate of the manually isolated *X. borealis* oocyte nuclei (at 2 nuclei per μl) was diluted 1:5 in the 1× J buffer (3). In a 16-μl reaction mixture, the final concentration of each DNA was 2.5 μg/ml, the α-amanitin concentration was 0.3 mg/ml, ribonucleoside triphosphates (rNTPs) were 100 μM, and 12 μl of the diluted homogenate was present, making the final salt concentration 3/4× J buffer. In the sequential-additional studies, the promoter-bearing plasmid was preincubated in the reaction mixture in the absence of rNTPs before addition of the second DNA; final DNA concentrations were the same as in the simultaneous addition experiments. We found that the level of enhancement reported in this paper was not increased when the reactions were conducted at altered NH_4Cl, KCl, spermidine, pH, or polyethylene glycol concentrations. The magnitude of the in vitro *trans* competition effect appeared to vary with the batch of extract between 1.5- and 8-fold, but this variation could have been at least partly due to the rather sharp DNA optimum. The transcriptional activity, but not routinely the enhancer activity, of the oocyte nuclear extract is preserved upon freezing at −80°C when diluted 1:5 in 1× J buffer containing 6% polyethylene glycol 20000.

RESULTS

***cis* stimulation by the 60/81-bp repeats.** To date, the effect of the *Xenopus* rDNA 60/81-bp repeats has been studied only by measuring the ratio of transcription of two coinjected plasmid templates, an assay that contains contributions both from the *trans* competition effect of the repeats and from whatever *cis* stimulatory effect is occurring (see Fig. 3 and 4 below). With the aim of specifically assessing the *cis* stimulatory role of the 60/81-bp repeats independently of their *trans* competitive effect, we constructed the marked rRNA

gene family A, in which a segment of procaryotic reporter DNA was cloned just downstream of an rDNA promoter region (residues −245 to +13, forming the A gene), and then a block of 10 60/81-bp repeated elements was inserted at its natural position just upstream of the promoter (forming the E:A gene) (Fig. 1). When these or analogous templates were injected singly into mature *X. borealis* oocytes (Fig. 2A, row 1), a markedly higher level of transcription was supported by the promoter bearing the 60/81-bp repeats than by the promoter alone. Thus, a *cis* stimulatory effect of the 60/81-bp repeats can be clearly observed under such noncompetitive transcription conditions. This result argues strongly that the 60/81-bp elements in plasmid E:A directly stimulate transcription.

We have reproducibly observed *cis* stimulation in over 50 separate experiments, both with the A-gene family and with several other analogous pairs of templates (see examples in Fig. 2A, 3, 6, and 7). In all experiments, each template is assayed in duplicate or triplicate sets of injections, and these yield virtually identical results. Furthermore, in some experiments we have coinjected a 5S RNA gene, whose presence does not affect the rDNA transcription (data not shown), for use as an internal control. The same levels of control 5S transcription (±15%) are observed in the oocytes coinjected with the rDNA plasmids lacking and bearing the 60/81-bp repeats (Fig. 2A, row 2), further confirming the dependability of this *cis* stimulation assay. The reproducibility of our injections is further emphasized by the fact that we obtained very comparable transcriptional signals when eight sets of oocytes were injected with the control plasmid (Fig. 2B). As a final control, transcription of the endogenous rDNA of the injected oocytes is also very consistent in the various injections (the control bands in Fig. 2A and 3A). From these data, we conclude that *cis* stimulation is a true reproducible effect of the 60/81-bp elements.

***cis* stimulation is observed in oocytes from mature frogs.** In contrast to our data showing *cis* stimulation, others have uniformly reported failing to detect it in similar assays involving singly injected oocytes (e.g., references 13 [Fig. 7], 26 [Fig. 5], and 46 [Fig. 4]). This discrepancy can almost assuredly be attributed to the condition of the recipient oocytes. In all of our *cis* stimulation studies, we use oocytes from older *X. borealis* frogs, which we have found give the greatest *cis* stimulatory effect; oocytes from younger *X. borealis* or from *X. laevis* frogs (as have been used in the past) generally exhibit only minimal net *cis* stimulation. In fact, we have now followed two batches of *X. borealis* frogs as they have grown older in the laboratory. The oocytes from both batches initially showed no significant *cis* stimulation, but when the frogs reached approximately 5 years in age (3 years ago and very recently, respectively), their oocytes became active for *cis* stimulation. A third batch of frogs was examined only when older, and these animals showed *cis* stimulation; several other batches that were examined only when relatively young all showed no net *cis* stimulation. Presumably, as the animals mature, the ratio of available factors changes in a manner that allows the *cis* stimulatory effect of the 60/81-bp repeats to be observed.

The 60/81-bp elements cause *cis* stimulation in both orientations and over considerable distances. The orientation and position dependence of *cis* stimulation was directly assayed by singly injecting various 60/81-bp element-bearing templates (diagrammed in Fig. 1) into *X. borealis* oocytes. *cis* stimulation occurred to a comparable extent whether the 60/81-bp repeats were present in their normal orientation (plasmid E:A; Fig. 3A, lane 2) or in the reverse orientation at their natural position (plasmid E_r:A; Fig. 3A, lane 3). In repeated assays, E:A was always as active as (or slightly more active than) E_r:A, demonstrating that the stimulation by the 60/81-bp repeats is very largely orientation independent.

To determine whether the 60/81-bp repeats also cause *cis* stimulation when removed from their natural context and reinserted a significant distance away from the initiation site, we prepared plasmid E:340:A, in which the 60/81-bp repeats were moved 600 bp upstream of the initiation site. E:340:A exhibited strong *cis* stimulation, for transcription from this template (Fig. 3B, lane 3) was much more efficient than that from the A template lacking the repeats (lane 1). Thus, *cis* stimulation does occur over a >600-bp distance. The 60/81-bp repeats could still exert an appreciable *cis* stimulatory effect when 1 kb upstream of the initiation site (see Fig. 6C below). These results directly demonstrate that the *Xenopus* rDNA 60/81-bp repeats stimulate transcription from a promoter in *cis* not only in both orientations but also over considerable distances. Hence, they are true polymerase I transcriptional enhancers.

Transcriptional enhancement by the 60/81-bp repeats is markedly position dependent. Although the 60/81-bp repeats exert a *cis* stimulatory effect over many hundred base pairs, this effect diminishes sharply with distance. Even when moved only 600 bp upstream of the initiation site (plasmid E:340:A), they exerted less enhancement than when located at their natural position. This decrease ranged from ~30% (Fig. 3B, lane 3 versus lane 2) to 50% (data not shown), depending on the batch of oocytes. To further examine the distance dependence of *cis* enhancement, we constructed plasmid A:E, in which the 60/81-bp repeats were placed within the transcribed region, 170 bp downstream of the initiation site. Surprisingly, at this position the repeats exerted no detectable net *cis* stimulatory effect (Fig. 3A, lane 4). In the closed circular A:E template, the 60/81-bp repeats are 4.6 kb upstream, as well as 170 bp downstream, of the initiation site. Thus, the *cis* enhancement effect is lost when the 60/81-bp repeats are moved 4.6 kb upstream or just downstream of the initiation site in their normal orientation.

In an analogous plasmid, A:E_r (Fig. 1), in which the 60/81-bp repeat block is positioned in the reverse orientation 170 bp downstream of the initiation site, it has a small but reproducible stimulatory effect (Fig. 3A, lane 5). Notably, the end of the 60/81-bp repeat block that is nearest the promoter is the same in A:E_r and in the natural rDNA but is the opposite in A:E. Since the two ends may not be strictly equivalent, there is no a priori reason to expect identical levels of transcription from templates A:E and A:E_r.

To assess the effect of the 60/81-bp repeats when positioned still further away from the initiation site, we constructed plasmid E:1440:A, in which the 60/81-bp repeats were moved 1,440 bp upstream of their natural position (i.e., 1,700 bp upstream of the initiation site; Fig. 1). With this template, not only was *cis* enhancement no longer apparent, but transcription was and is reproducibly depressed about two- to fourfold relative to that of the parental A template lacking the 60/81-bp repeats (Fig. 3C). It appears that when the 60/81-bp repeats are ≥1.7 kb from the initiation site, their *cis* stimulatory effect is so diminished that the net effect of the repeats is a competition with the promoter in *cis*, akin to their competition with promoters in *trans* (see Discussion). Presumably, if moved very far away from a promoter, the repeats would repress transcription as strongly as they do with promoters in *trans*.

We conclude that although the 60/81-bp repeats enhance

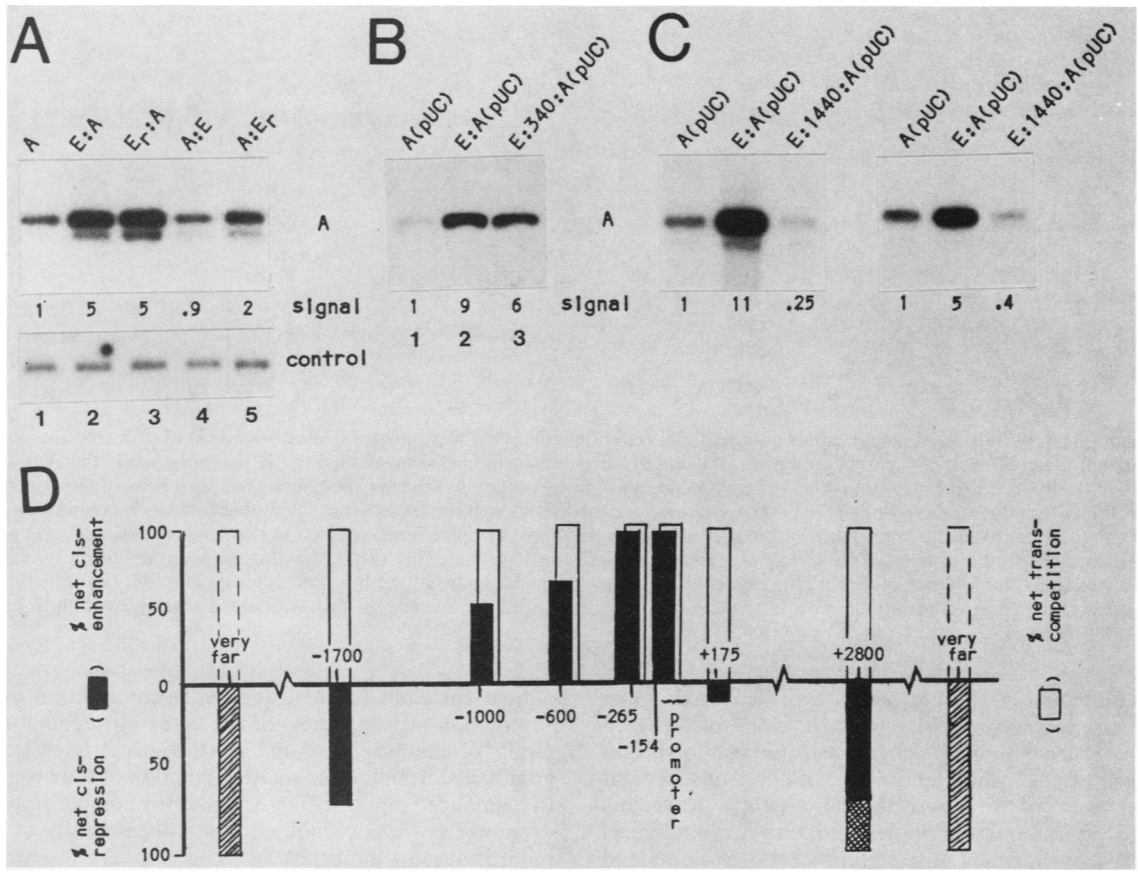

FIG. 3. *cis* stimulation by the rDNA 5' enhancer. (A to C) The indicated rDNA constructs, cloned in pBR322 (A) or in pUC (B and C), were injected into *X. borealis* oocytes at a DNA concentration of 9 (A) and 18 (B and C) pmol/ml (equivalent to ~25 μg/ml), and transcription was assessed by using the A-gene probe. The magnitude of the *cis* stimulation (signal; shown in numbers below the lanes) varied with the batch of frogs but was very consistent within a set of sibling frogs. (The faint lower band is due to a small fraction of the polymerase molecules reading around the plasmid and through the *Bam*HI-*Hae*III segment of the vector that was duplicated to form the A-gene tester sequence [see Materials and Methods]; it lacks the first 13 nucleotides of rRNA sequence that are present in the A-gene transcript.) The control band is as described for Fig. 2. (C) Results of injections into two different batches of *Xenopus* frogs. (D) ■, Percentage of *cis* stimulation or *cis* repression caused by the 60/81-bp repeats at various locations (indicated in base pairs) relative to that exerted when in the normal location at residue −265. Data were derived from Fig. 3A to C, Fig. 6C, and other experiments. Since the +2800 datum point is the same as the −1700 datum point, its inherent activity may be overestimated (▩). □, Approximate level of *trans* competition exerted by the repeats at each location (Fig. 4 and 5 and data not shown); ▨, □, extrapolations from our data showing the expected *cis* and *trans* effects of the repeats located extremely far from the *cis* promoter.

promoters in *cis* over several hundred base pairs, their action is strongly position dependent, and they actually become net negative transcriptional effectors when moved distant from the promoter (solid and hatched bars in Fig. 3D). The ability of rDNA enhancer sequences to depress transcription from distant promoters in *cis* is reminiscent of the enhancerlike silencers of transcription by RNA polymerase II.

Given the data presented thus far, both in this paper and in all previous articles on the action of the 60/81-bp repeats, it could have been argued that their effect is not one of *cis* stimulation of rDNA transcription at all but rather one of selective transcript stabilization. (The transcripts of all of these plasmids should extend through the 60/81-bp region and up to the natural T3 terminator [28] at position −200.) To address this question, we measured the stabilities of the transcripts of various members of the A-gene family. Batches of oocytes were injected with plasmid A, E:A, or E:1440:A; after a 4-h transcription period, actinomycin D was injected into their nuclei and incubation was continued for additional short periods. A long exposure of the gel assessing RNA abundance in the actinomycin D-treated oocytes looked virtually identical to a short exposure of the gel showing the RNAs from oocytes incubated without actinomycin D (as in Fig. 3C; data not shown), indicating that the transcripts of the A, E:A, and E:1440:A templates all had virtually the same stabilities under these conditions. This finding indicated that the observed effects of the 60/81-bp repeats, both the *cis* enhancement of nearby promoters and the *cis* depression of distant ones, were due to alterations in transcriptional efficiency and not to effects of differential RNA half-life.

The *trans* competition assay detects a complex mixture of *cis* stimulatory and *trans* inhibitory effects. Our results from the direct *cis* stimulation assay (Fig. 3) showing that enhancement by the 60/81-bp repeats is very position dependent refute prior assertions (26, 27, 42, 44) that the 60/81-bp repeats augment transcription equally when located far upstream of the initiation site or when downstream of the initiation site within the transcribed region. These earlier conclusions derived from results obtained with *trans* competition assays, in which the relative amount of transcription

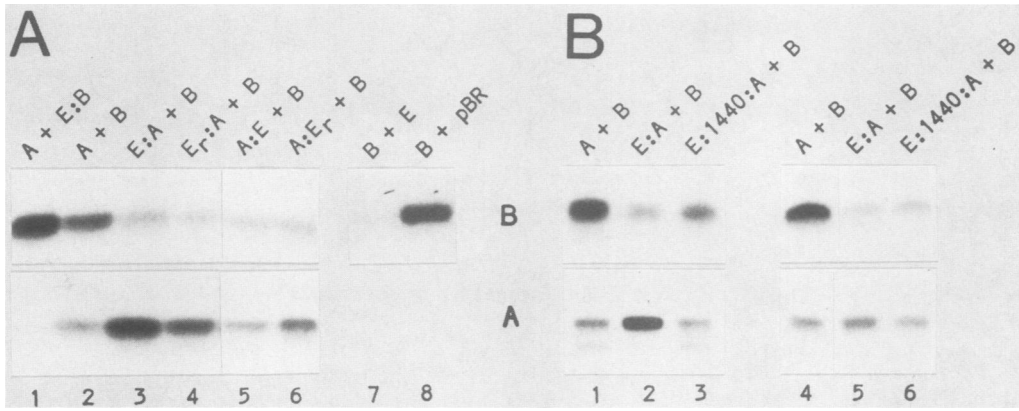

FIG. 4. Action of the rDNA enhancer under competition conditions. Oocytes were microinjected with ~30 nl of a solution containing a 50-μg/ml concentration of each of the indicated pairs of plasmids, and transcription was assessed by S1 mapping, using the A probe (which hybridizes specifically to transcripts of the A-gene family) and the B probe (which hybridizes specifically to transcripts of the B-gene family). In all panels, the autoradiogram assessing B-gene expression was exposed about three times longer than that for A-gene expression to enable detection of the faint bands in the latter lanes of the gels. (A) In lanes 1 to 6, samples were injected in the same experiment and assayed on the same gel. In lanes 7 and 8, transcription was assessed only with the B probe. This result showing *trans* competition by a subcloned 60/81-bp block extends the findings of a previous report (26) demonstrating a similar effect but of smaller magnitude. (B) Injections into *X. borealis* oocytes that exhibited *cis* stimulation in the direct assay (lanes 1 to 3) and into *X. laevis* oocytes that showed only minimal *cis* stimulation (lanes 4 to 6).

from two coinjected rDNA promoters was measured; when one of the coinjected plasmids carried a block of 60/81-bp repeats, the transcriptional balance was observed to shift in the direction of the promoter in *cis* with the repeats and away from the one in *trans* to the repeats. It is this qualitative shift in the transcript ratio that has been taken as diagnostic of enhancement (e.g., reference 45). We obtained results from the *trans* competition experiments basically similar to those of the previous workers (Fig. 4), but our interpretations are quite different, based on a quantitative analysis of the *trans* competition data and on the results of the direct *cis* stimulation assay.

To examine the behavior of templates carrying the 60/81-bp repeats at various positions in the *trans* competition assay, various members of the A-gene family were coinjected with the B or E:B gene (analogous templates in which a different segment of procaryotic tester DNA than of the A gene was cloned downstream from the initiation site). When the A and B genes (both lacking 60/81-bp repeats) were coinjected into oocyte nuclei, they were transcribed at the same level (Fig. 4A, lane 2). (In this figure, the autoradiograms assessing transcription of the B genes were exposed approximately threefold longer to favor detection of the inhibited B signal in the latter lanes of the gels.) As anticipated from the earlier data (26, 47), with the pairs A plus E:B (lane 1) and E:A plus B (lane 3), in which the enhancer-bearing plasmids exhibited strong *cis* stimulation in the direct assay of Fig. 3, the transcriptional balance was shifted substantially in favor of the gene bearing the 60/81-bp repeats. The striking result, however, is that the same qualitative effect was observed with the pairs E:1440:A plus B (Fig. 4B, lanes 3 and 6) and A:E plus B (Fig. 4A, lane 5), in which the 60/81-bp repeat-bearing plasmids actually showed large negative *cis* effects and no net *cis* stimulation, respectively, in the direct assay (Fig. 3A and C). Thus, all of the enhancer-bearing templates appear to score positive in the *trans* competition assay even though they exhibited large differences in the direct *cis* stimulation assay. We are forced to conclude that the *trans* competition assay as used standardly is not diagnostic of absolute *cis* effects that are shown in the direct single-injection assay (Fig. 2 and 3). This is almost certainly because the *trans* competition assay is a summation of two effects of the 60/81-bp repeats: (i) stimulation of the promoter in *cis* (the effect of which varies greatly according to the relative position of the promoter and the enhancer repeats; Fig. 3D) and (ii) competition with the promoter in *trans* (which occurs independently of the plasmid location of the repeats).

Closer examination of our data (Fig. 4 and multiple duplicate experiments; data not shown) reveals that transcription from the B gene was reduced to a similar extent when competed with almost any of the 60/81-bearing plasmids or with a subcloned promoterless block of 60/81-bp repeats (the E plasmid; Fig. 4A, lanes 7 and 8). When these injections were into oocytes of mature frogs, which showed strong *cis* stimulation (see above), the coinjected A-gene derivatives were transcribed to an extent that largely reflected their activities in the *cis* stimulation assay; i.e., E:A and E_r:A were transcribed the most efficiently, E:340:A was transcribed to an intermediate extent, and A:E and E:1440:A were transcribed no better or even slightly worse than the A gene lacking 60/81-bp repeats (Fig. 4A and B, lanes 1 and 2; also data not shown). However, when the *trans* competition instead involved injections into oocytes of younger *X. laevis* frogs, there was only a minimal increase in signal from E:A relative to that from the A gene (Fig. 4B, lanes 4 and 5), consistent with the minimal level of *cis* stimulation seen in these oocytes in the direct assay. Thus, when performed and examined quantitatively, the *trans* competition assay can provide information about *cis* effects. However, since there is a large and constant contribution from the *trans* competition effect of the 60/81-bp repeats, the ratio of the signals from two coinjected templates (transcriptional balance) is not a very sensitive measure of *cis* effects. Furthermore, since the absolute level of transcription from each template is influenced by the activity of the coinjected template through promoter competition effects (67; Fig. 3B, lanes 2 and 3 and lanes 5 and 6), the absolute signals in this assay are not a true measure of what occurs in the absence of plasmid competition.

Promoter specificity of the *X. laevis* rDNA enhancer. In light of the recent findings that polymerase II enhancers are

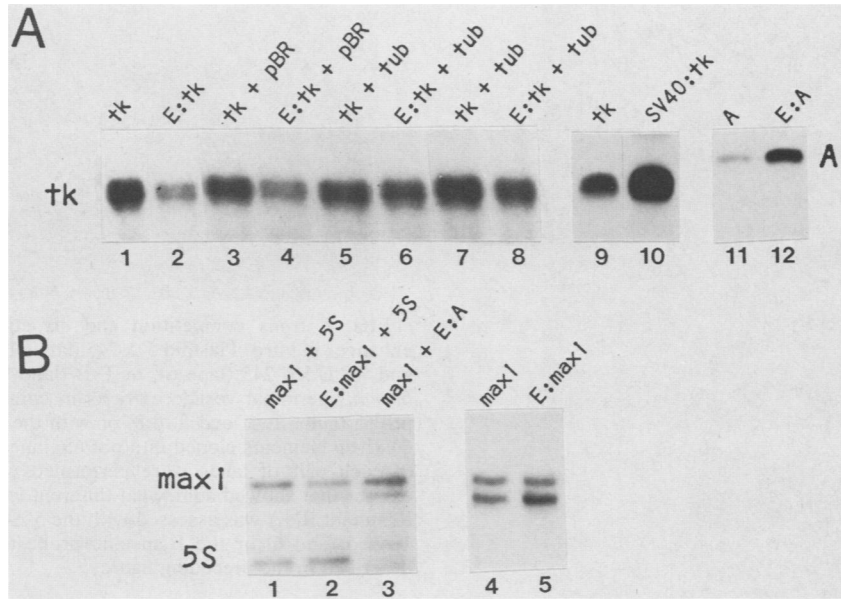

FIG. 5. Effect of the rDNA 5' enhancer on transcription by RNA polymerases II and III. (A) TK plasmids. The indicated plasmids were microinjected into *Xenopus* oocytes at 10 μg of each DNA per ml (lanes 1 to 6) or at 10 μg of TK plasmid plus 1 μg of β-tubulin plasmid per ml (lanes 7 and 8). Lanes 9 and 10, Injections of the TK plasmid and the SV40 enhancer-bearing TK plasmid (SV40:tk); lanes 11 and 12, injections of the A and E:A plasmids. RNA was detected by S1 mapping. (B) 5S plasmids. The indicated plasmids were microinjected into *Xenopus* oocytes at 50 μg/ml. In lanes 1 to 3, 0.4 μCi of [α-^{32}P]CTP (410 Ci/mmol) was also injected, and RNA from one oocyte equivalent was directly electrophoresed and visualized by autoradiography. In lanes 4 and 5, the transcripts were unlabeled and detected by S1 mapping.

highly conserved in evolution (functioning between species as divergent as yeast and mammals [24, 61]) and that a polymerase II enhancer can augment transcription from a polymerase III promoter (2), it was important to determine whether the *Xenopus* rDNA enhancer would function with a polymerase II or III promoter. To examine its effects on a polymerase II promoter, the herpesvirus TK gene (the 5'Δ-119 template of reference 34) or a derivative of this gene in which the block of 60/81-bp rDNA enhancer elements had been joined immediately upstream (E:tk) was microinjected into *Xenopus* oocytes. No matter whether the plasmids were injected alone (Fig. 5A, lanes 1 and 2) or were coinjected with pBR322 (lanes 3 and 4), with a competitor polymerase II-transcribed gene (the chicken β-tubulin gene; lanes 5 to 8), or with an rDNA promoter (the B gene; data not shown), the 60/81-bp rDNA enhancer failed to augment transcription from the *cis*-located polymerase II promoter. (In fact, it reproducibly decreased transcription somewhat, possibly by inhibiting transit of a polymerase II transcription component along the DNA to the promoter, akin to an effect described in procaryotes [22]). Furthermore, the E plasmid in *trans* had no adverse effect on the level of transcription of the TK gene (data not shown). Nonetheless, control injections showed enhancement of the polymerase I promoter by the polymerase I enhancer and of the TK promoter by a polymerase II enhancer (Fig. 5A, lanes 9 to 12). The ability of polymerase II enhancers to function in injected *Xenopus* oocytes has also been well documented previously (1, 8, 16, 55). Thus, the rDNA enhancer does not function properly in conjunction with a polymerase II promoter.

The rDNA enhancer also failed to stimulate transcription when cloned upstream of a polymerase III promoter (Fig. 5B). In this experiment, a 5S maxigene (4) either lacking or bearing the 60/81-bp rDNA repeats (maxi or E:maxi, respectively) was singly injected (lanes 4 and 5) or coinjected with a control 5S gene (lanes 1 and 2) or with pBR322 (data not shown). In all cases, the rDNA enhancer did not stimulate transcription from the *cis*-located polymerase III promoter. Yet under analogous *Xenopus* oocyte injection conditions, expression of a polymerase III-transcribed gene is stimulated by its own enhancer (2). Furthermore, when located in *trans*, the rDNA enhancer did not reduce transcription from a coinjected maxi-5S gene (Fig. 2A and Fig. 5B, lane 3). Thus, the activities of the rDNA enhancer, both *cis* stimulation and *trans* competition, are specific for the RNA polymerase I promoter and are not compatible with promoters for polymerase II or polymerase III.

Enhancement is exerted by a single 60-bp repeat. All of the initial studies on the *X. laevis* 60/81-bp repeats used intact ~700-bp blocks consisting of ~10 repeating units (26, 36, 47). To assess whether *cis* enhancement is dependent on the highly repetitive nature of this structure or whether a single enhancer element is also functional, we constructed plasmids in which the A gene was located adjacent to one 60-bp element (e_1:A) or to one 60- and one 81-bp element (e_2:A) (Fig. 1). When the various A-gene constructs were injected into oocytes individually (Fig. 6B), the *cis*-located 60-bp monomer element reproducibly had about a twofold positive effect on transcription, the 60/81-bp dimer element had a greater effect, and the decamer element had a still greater *cis* enhancement effect. Evidently, each of the 60- or 81-bp elements can cause *cis* enhancement, and the effects of the elements are cumulative. *trans* competition analysis (Fig. 6A) confirmed that the single 60-bp element (lane 2), the 60/81-bp element (lane 3), and the intact decamer element (lane 4) all also competed with the promoter in trans.

To determine whether *cis* stimulation would be further increased as more 60/81-bp elements were added, we formed plasmid EE:B, in which 20 60/81-bp elements (two E blocks) were positioned upstream of the B gene (Fig. 1). Figure 6C shows typical *cis* stimulation assays in which plasmid B, E:B, or EE:B was singly injected into oocytes. EE:B was

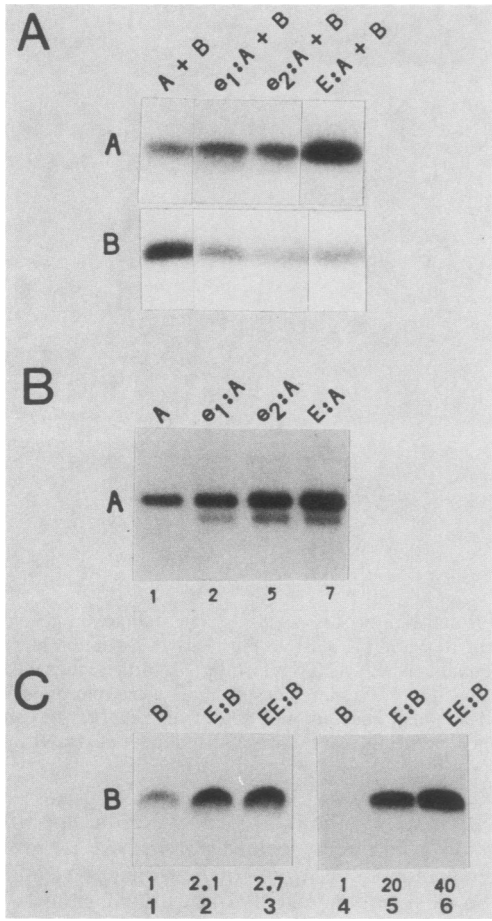

FIG. 6. Enhancement by various numbers of 60/81-bp repeats. (A) The indicated pairs of plasmids were injected with 50 μg of each DNA per ml, and transcription was assessed by S1 mapping. All of the samples were injected in a single experiment and assayed on a single gel, appropriate lanes of which have been aligned. The lanes using the B probe were exposed about three times longer than the lanes using the A probe to favor detection of the faint bands in the latter lanes. (B) The indicated plasmids were injected singly into *X. borealis* oocytes at 25 μg/ml, and transcription was assayed by S1 mapping. The faint lower band is explained in the legend to Fig. 3. (C) The indicated pUC-based plasmids were injected singly into *X. borealis* oocytes at a total DNA concentration of 50 μg/ml (18 pmol of rDNA plasmid per ml, with the remainder pUC9 DNA), and transcription was assayed by S1 analysis. Lanes: 1 to 3, transcription in oocytes from one frog; 4 to 6, results from oocytes from a different frog that exhibited a very large degree of *cis* enhancement. Numbers below the lanes indicate the magnitude of *cis* stimulation.

found to be the most actively transcribed member of our B-gene family. Depending on the batch of oocytes, EE:B was between 1.3 and 2 times as active as E:B (Fig. 6C, lanes 2 versus 3 and 5 versus 6, respectively), which in turn was between 3- and 20-fold as active as the B gene. This is precisely the extent of *cis* stimulation that would be expected from doubling the number of the 60/81-bp elements, given that their efficiency decreases at increasing distance from the initiation site. Thus, the extent of *cis* enhancement increases with increasing numbers of 60/81-bp repeats, at least up to ~20 elements. As one might anticipate, *trans* competition also increases with additional 60/81-bp repeats, for transcription from the B plasmid was depressed to a greater extent when coinjected with the EE plasmid than when coinjected with the E plasmid (data not shown).

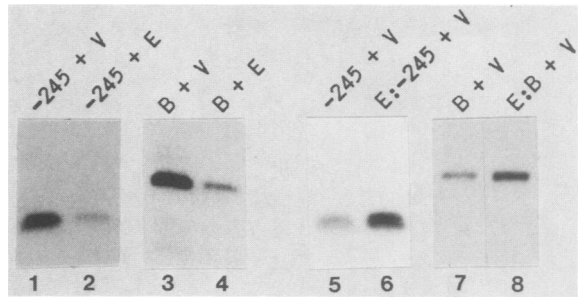

FIG. 7. *trans* competition and *cis* stimulation by the rDNA enhancer in vitro. Plasmid 5′Δ-245 (lanes 1, 2, and 5), B (lanes 3, 4, and 7), E:5′Δ-245 (lane 6), or E:B (lane 8) was transcribed in *X. borealis* germinal vesicle extracts in combination with the vector pSP65 (lanes 1, 3, and 5 to 8) or with the E plasmid containing 10 60/81-bp elements cloned into pSP65 (lanes 2 and 4). The reactions of each pair of lanes were performed with different batches of extract that showed somewhat different levels of enhancer effects. Resultant RNA was assessed with the 5′Δ-245-specific probe (lanes 1, 2, 5, and 6) or the B-specific probe (lanes 3, 4, 7, and 8) as described in the preceding figures.

The data of Fig. 6B and C provide direct evidence that the *cis* enhancement effect of the 60/81-bp elements is cumulative, becoming stronger as the number of elements increases from 1 to 2 to 10 to 20. Furthermore, our *trans* competition data (Fig. 6A and data not shown) extend previous *trans* competition results showing increasing effects in the number of 60/81-bp elements increases through 10 (13, 42, 45) and that further increases in numbers make the gene *trans* compete either far less well (13), with unchanged efficiency (47), or considerably better (7, 47); however, the plasmids in these studies also carried additional transcriptional potentiators.

In vitro assay of the *X. laevis* rDNA enhancer. Although a great deal can be learned about the *X. laevis* rDNA enhancer from injection experiments, analysis of the mechanism of action of rDNA enhancement would be greatly facilitated by the availability of an in vitro system in which transcription is responsive to the 60/81-bp elements. We therefore have endeavored to reproduce both the *trans* competition and *cis* stimulation effects in vitro. We have now found that rDNA transcription in a *Xenopus* oocyte nuclear extract (65) is sensitive to *trans* competition by the 60/81-bp repeats, for considerably less transcript was directed by an rDNA promoter when the reactions were supplemented with the E plasmid (carrying the 60/81-bp repeats) than when supplemented with an equal amount of vector DNA (Fig. 7). This effect was seen with a plasmid carrying the natural rRNA 5′ coding region (lanes 1 and 2) and with the B gene (lanes 3 and 4).

cis stimulation of an rDNA promoter by an enhancer located on the same plasmid molecule could also be observed in this nuclear extract by using either the natural rRNA initiation region (Fig. 7, lanes 5 and 6) or the B gene (lanes 7 and 8). Duplicate reactions in the extracts positive for *cis* stimulation confirmed the existence of this effect, even though as yet it has not been observed in every extract.

The rDNA enhancer acts at the establishment of the preinitiation complex. To begin to understand the mechanism of action of the rDNA enhancer, it is important to determine at what stage it exerts its effect. Although ultimately the enhancer must serve to alter the frequency at which polymerases productively initiate on rRNA genes, increasing the frequency when the repeats are juxtaposed to the promoter

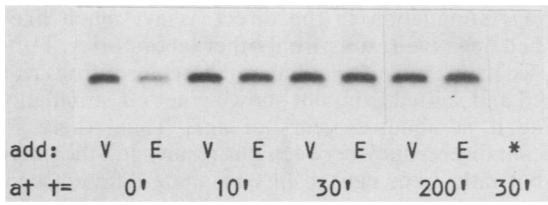

FIG. 8. Demonstration that the *trans* enhancer acts before the onset of transcription. The B plasmid was added at $t = 0$, and the vector (V) or enhancer (E) plasmid was added at the indicated times. rNTPs were also added at the indicated times, and incubations were continued for 8 h except for the reaction of the last lane, which was terminated at $t = 30$, before the addition of rNTPs. Transcription was assayed by S1 mapping, using the plasmid vector (pSP65) of this E plasmid [E(pSP65)].

in *cis* and decreasing the frequency when in *trans*, this effect could arise from at least two different mechanisms of action. On the one hand, the enhancer could act by directly binding polymerase molecules, thereby increasing their local concentration. On the other hand, the enhancer could act on polymerase indirectly by increasing the local concentration of a factor that facilitates establishment of the rDNA transcription complex and which only then interacts with the polymerase. In the former scenario, the presence of the 60/81-bp elements should be important primarily at each round of transcription; in the latter, they should act before the onset of transcription. By performing sequential-addition studies in the in vitro enhancement system, we have now been able to directly assess this question (Fig. 8). Confirming the ability of the in vitro system to reproduce *trans* competition by the 60/81-bp repeats, the B gene was transcribed less efficiently when added to the reaction mixture in the presence of E plasmid than when added in the presence of an equal amount of vector DNA (lanes 1 and 2). In contrast, when the B gene was preincubated in the cell extract for a short period to allow establishment of a stable preinitiation complex (9; data not shown) before addition of the vector or the E plasmid, it became immune to the effects of subsequently added 60/81-bp repeats; the same amount of B-gene transcription was then obtained in both the absence and the presence of the 60/81-bp repeats (lanes 3 to 8). Transcription did not take place during the preincubation period (lane 9) and began only upon addition of rNTPs; therefore, all of the detected RNA reflects transcription that occurred when both plasmids were present. Furthermore, since RNA synthesis was occurring throughout the transcription period (data not shown), an established transcription complex remained intact and resistant to enhancer *trans* competition throughout the 8-h incubation period (Fig. 8). These data showing that the rDNA promoter becomes unresponsive to the 60/81-bp repeats once it has established a stable transcription complex indicate that the rDNA enhancer acts in *trans* (and presumably also in *cis*) by affecting the stable binding of important transcription components; it makes the alternative model that the enhancer acts primarily at each round of transcription very unlikely.

We have also performed analogous studies in intact oocytes which support the in vitro results. These sequential-injection experiments are technically demanding, first because the success rate for both injections must be shown to be very high (e.g., by coinjecting noncompeting 5S and TK templates). More important, however, we have found that sequential injections are not meaningful when performed by the standard protocol of introducing ~20 to 30 nl of solution into the nucleus; this is because DNA injected within ~45 min of such a first injection is virtually inactive, even if only buffer was introduced by the first injection (data not shown). (An earlier sequential-injection study which reported that a gene bearing 40 60/81-bp repeats does not prevent transcription from a previously introduced gene bearing 10 60/81-bp repeats used 20-nl injection volumes [47] may have similarly suffered from such artifactual inhibition of the second template.) However, by injecting only ~5 nl per oocyte with fine needles, preinjection of buffer does not affect transcription of subsequently injected rDNA promoters. With this modified injection protocol, transcription of a preinjected plasmid bearing the rDNA promoter but no 60/81-bp repeats was seen to be resistant to an enhancer plasmid that was injected >60 min later, although it was fully inhibited by an enhancer plasmid injected within 10 min (data not shown). We therefore conclude that both in vitro and in vivo, the 60/81-bp rDNA enhancer acts by affecting the establishment of the transcription complex and does not act directly at each round of transcription.

DISCUSSION

The *X. laevis* rDNA 60/81-bp repeats are position-dependent polymerase I enhancers. To directly examine the action of the *X. laevis* rDNA 60/81-bp elements, we have developed a selective oocyte injection assay that exhibits *cis* stimulation by these repeats in the absence of a competitor plasmid (Fig. 2 and 3A). We have also developed an in vitro transcription system that is responsive to both the *trans* competitive and this *cis* stimulatory effect of the 60/81-bp repeats (Fig. 7). *cis* stimulation was thereby shown to be a distinct effect of the rDNA 60/81-bp repeats, not dependent on their ability to exert plasmid *trans* competition.

By assaying the ability of the 60/81-bp repeats to augment transcription at various orientations and positions relative to the promoter, *cis* stimulation was seen to occur with virtually equal efficiencies whether the repeats were in their normal or reverse orientation (Fig. 3A). *cis* stimulation was also quite efficient when the repeats were moved over 600 bp upstream of the initiation site (Fig. 3B) and still occurred when they were over 1 kb upstream of the start site (Fig. 6C). The 60/81-bp repeats thus exert true enhancement, acting to stimulate promoters in *cis* in a relatively orientation independent manner and over considerable distances.

Using the direct *cis* stimulation assay, we have observed that the 60/81-bp repeats are quite position dependent (see, for example, reference 42). The action of the repeats is already diminished when they are moved 600 bp upstream of the initiation site (Fig. 3B). Furthermore, they exhibit no net *cis* stimulatory effect when located near the beginning of the transcribed region, 170 bp downstream (or 4.6 kb upstream) of the initiation site (Fig. 3A). Even more surprising, when the 60/81-bp repeats are moved 1.7 kb upstream of the initiation site, they become transcriptional silencers, depressing transcription from the distant promoter in *cis* (Fig. 3C). It seems likely that the net action of the 60/81-bp repeats in E:1440:A is to repress the distant promoter in *cis* as a result of their minimal *cis* stimulation at this position and their competition with that promoter, much as they compete with promoters in *trans*. As will be detailed below, a strong position dependence is also seen with a number of polymerase II enhancer-promoter pairs.

In genomic rDNA, blocks of 60/81-bp repeats alternate with rRNA gene regions in long tandem arrays. In principle, these enhancer repeats could stimulate both the upstream

and downstream promoters. However, their directional and spatial specificity (Fig. 3) argues that in vivo, their primary target in the tandem rDNA array is the downstream promoter, not the upstream one.

Resolution with previous data. This marked position dependence of the 60/81-bp repeats is in striking contrast to the common view of their action (42, 44, 46). Prior studies of the 60/81-bp elements have concluded that these elements are position independent, functioning equally efficiently at their natural location, when removed from the promoter by >4 kb of vector sequences or when positioned a short distance downstream of the initiation site within the transcribed region (26, 27, 42, 44). These conclusions were based on *trans* competition experiments in which oocytes were coinjected with two templates, one bearing and one lacking the 60/81-bp repeats, and the transcriptional balance was shifted toward the promoter in *cis* with the repeats and away from the one in *trans* (13, 26, 27, 46, 47).

We have repeated such *trans* competition studies using our templates (Fig. 4) and find basically the same results. Namely, all of the enhancer bearing plasmids, even the ones that show negative or no net *cis* stimulatory effects in the direct assay, exhibit a qualitative shift in transcriptional balance in the *trans* competition assay. However, the *trans* competition assay reflects a complex mixture of (i) the competition of the enhancer with the promoter in *trans* (which occurs in all oocytes examined and regardless of position of the enhancer), (ii) the effect of the enhancer on the promoter in *cis* (which is very dependent both on the position of the repeats and on the recipient oocytes), and (iii) competition between the two coinjected promoters (67). When the *trans* competition assay is performed and evaluated quantitatively, using oocytes that show direct *cis* enhancement (Fig. 4), the absolute level of transcription of each template is not unlike that resulting from the individual *cis* stimulation and *trans* competition effects of the 60/81-bp repeats. Oocytes that exhibit minimal or no *cis* stimulation in the direct assay also appear to show mainly competition with the promoter in *trans* to the enhancer and only minimal stimulation of the promoter in *cis* (Fig. 4B, lanes 4 and 5), further underscoring the complex nature of the *trans* competition assay. We conclude that the direct *cis* stimulation assay is far more suited to detecting quantitative variations in *cis* stimulation than is the classical *trans* competition assay, which is insensitive in detecting even the large degree of position-dependent rDNA enhancement.

In the prior *trans* competition experiments, the actual quantitative effect of the 60/81-bp repeats appeared to vary. Sometimes the repeats depressed only the promoter in *trans* and did not appear to affect the one in *cis*, occasionally they stimulated the promoter in *cis* while showing no effect on the promoter in *trans*, and sometimes they showed both effects of various magnitudes (13, 26, 27, 45, 47). These differences could have been due to variability in the *cis* stimulatory capacity of the different batches of oocytes or to other causes.

It also needs to be addressed why all previously published attempts to demonstrate *cis* stimulation by the 60/81-bp elements by using singly injected (rather than coinjected) oocytes have proven negative, with no more transcript obtained from enhancer-bearing than from enhancerless templates (13, 26, 46). Almost assuredly, this is largely due to differences in the oocytes used, for we have found that oocytes from *X. borealis* over ~5 years of age exhibit *cis* stimulation (as shown in Fig. 3), whereas oocytes from all young *X. borealis* or *X. laevis* frogs showed no or only very minor *cis* stimulation in the direct assay, much like the published negative results from other laboratories. Furthermore, we have tracked two different batches of frogs as they matured and shifted from not showing net *cis* stimulation to showing it at about 5 years of age. Thus, there is no significant discrepancy between our results and the previous data, but rather our new results mandate different conclusions.

Relationship to other studies on enhancer action. In many ways, the rDNA 5′ enhancer appears quite similar to classical polymerase II enhancers (18, 25, 40, 50, 57). Like polymerase II enhancers, the rDNA enhancer augments transcription from a promoter located in *cis* in the absence of added competitor templates (Fig. 2 and 3A), exerts *cis* stimulation when in the reverse orientation (Fig. 3A), and still functions in *cis* >1 kb upstream of the initiation site (Fig. 3B and 6C). However, the position dependence we have demonstrated for the rDNA enhancer also has its analog in the position-dependent quantitative differences that are frequently observed for the action of metazoan polymerase II enhancers (10, 38, 41, 60). For instance, activity of the SV40 enhancer has been reported to decrease by 60% when separated from a test promoter by only 275 bp (60) and not to work when inserted 3′ of another test promoter (38). The position dependence of upstream activation sequences in yeast polymerase II transcription systems is also well documented (reviewed in references 17 and 57). It remains to be determined whether there is any functional significance to the similarity between our observation that distant 60/81-bp enhancer repeats act as polymerase I transcriptional silencers and the observations of others that polymerase II silencer sequences frequently contain polymerase II enhancer elements. Also like many polymerase II enhancers (15, 20, 21, 69), the polymerase I enhancer is composed of multiple, independently functioning elements whose *cis* stimulatory effects are cumulative (Fig. 6B and C; see reference 42 for analysis in the *trans* competition assay). Finally, both the *Xenopus* 60/81-bp repeats and polymerase II enhancers appear to be active at or before the establishment of a stable transcription complex (Fig. 8; 33, 59, 62).

On the other hand, polymerase II enhancers appear to differ from the *Xenopus* polymerase I enhancer in some features, for the latter competes with a promoter in *trans* (26; Fig. 4A), whereas the SV40, polyomavirus, adenovirus type 2, and mouse immunoglobulin heavy-chain polymerase II enhancers have been reported not to compete in *trans* (6, 49, 63, 64; but see also references 14, 29, 35, and 39). Furthermore, the observation (Fig. 5) that the rDNA enhancer neither stimulates transcription from a polymerase II or III promoter in *cis* nor depresses transcription from a polymerase II or III promoter in *trans* demonstrates that the enhancers for polymerase I and II promoters are not functionally interchangeable. This conclusion is further strengthened by our previous complementary observation that a polymerase II enhancer did not stimulate transcription from a polymerase I promoter located in *cis* (31). This lack of functional exchange between polymerase I and II enhancers suggests that the polymerase I enhancer does not function by providing acidic regions that stimulate a component of the transcription apparatus, as occurs with many polymerase II enhancers (43, 51).

The action of the rDNA enhancer. From sequential-addition studies (Fig. 8) performed in the newly developed in vitro system that is responsive to the rDNA enhancer (Fig. 7) as well as in vivo, the rDNA enhancer is inferred to function before the onset of transcription. This inference

strongly suggests that the rDNA enhancer acts at the establishment of the stable transcription complex. An alternative possible scenario, that the 60/81-bp repeats function by directly providing a constant supply of polymerase I molecules to the rDNA promoter at each round of subsequent transcription (36), is greatly disfavored.

Most likely, the rDNA enhancer augments the formation of the stable transcription complex by binding rDNA transcription factors and attracting them to the promoter region, as was suggested earlier on the basis of the high degree of sequence similarity between the 60/81-bp repeats and the central region of the rDNA promoter (44). In vitro sequential-addition experiments have now shown that prebinding of the 60/81-bp repeats in the cell extract further augments the *trans* competitive effect of the repeats (37), demonstrating that a common factor or factors can bind stably both to the rDNA enhancer and to the rDNA promoter. Very recent experiments have indicated that the rDNA-binding component UBF is one such common binding factor (C. Pikaard and R. Reeder, personal communication). However, our current exonuclease III footprinting competition studies have suggested that another factor (possibly the frog analog of the mouse essential polymerase I transcription factor D [11, 58]) can bind both to the enhancer complex and to the essential rDNA promoter domains at residue ~−140 and at residue ~−30. The rDNA enhancer repeats may serve as high-density initial binding sites to draw this transcription factor to the vicinity of the rDNA promoter, which then favors binding of the factor to the promoter itself. We are hopeful that the newly developed enhancer-responsive in vitro system will prove useful in further analyzing how the rDNA enhancer acts.

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