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Stimulation of the Mouse rRNA Gene Promoter by a Distal Spacer Promoter

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We show that the mouse ribosomal DNA (rDNA) spacer promoter acts in vivo to stimulate transcription from a downstream rRNA gene promoter. This augmentation of mammalian RNA polymerase I transcription is observed in transient-transfection experiments with three different rodent cell lines, under noncompetitive as well as competitive transcription conditions, over a wide range of template concentrations, whether or not the enhancer repeats alone stimulate or repress expression from the downstream gene promoter. Stimulation of gene promoter transcription by the spacer promoter requires the rDNA enhancer sequences to be present between the spacer promoter and gene promoter and to be oriented as in native rDNA. Stimulation also requires that the spacer promoter be oriented toward the enhancer and gene promoter. However, stimulation does not correlate with transcription from the spacer promoter because the level of stimulation is not altered by either insertion of a functional mouse RNA polymerase I transcriptional terminator between the spacer promoter and enhancer or replacement with a much more active heterologous polymerase I promoter. Further analysis with a series of mutated spacer promoters indicates that the stimulatory activity does not reside in the major promoter domains but requires the central region of the promoter that has been correlated with enhancer responsiveness in vivo.

Of the three classes of eukaryotic RNA polymerase studied to date, only RNA polymerase I is committed to transcribing a single type of DNA: the ribosomal DNA (rDNA) that ultimately gives rise to the mature 18S, 5.8S, and 28S rRNAs of the ribosome. Study of the organization of the tandem rRNA genes and of the RNA polymerase I transcription machinery has led to the identification of *cis*-acting elements and *trans*-acting factors involved in the regulated expression of these genes (40, 44, 46). The best-studied rDNA elements are those of *Xenopus laevis*, but the rDNA of other metazoans, including mice, rats, Chinese hamsters, and *Drosophila melanogaster*, appears quite conserved in organization, although greatly diverged in sequence. Fig. 1A illustrates the similarity in the organizations of the *X. laevis* and mouse rDNA repeats.

The conserved organization of rDNA begins with the promoter, the most important regions of which are an ~40-bp core element extending upstream from the initiation site and an upstream element from approximately residue −140 to approximately residue −120. Sequences upstream of the core element can be made dispensable in vitro but are critical in vivo. Just upstream of this complete gene promoter is a promoter-proximal transcriptional terminator element that is required for efficient initiation at the gene promoter in vivo, evidently by preventing polymerase read-in (2, 18) but possibly also by providing a position-dependent stimulation (28, 29). Immediately upstream of the promoter and terminator region in most animal and plant species examined are multiple copies of a repetitive sequence. In frogs and mice (Fig. 1A), these repeat elements have been called enhancers because they have been shown to stimulate transcription from an adjacent gene promoter (~5- to 20-fold in frogs [23, 33, 35, 38] and ~3- to 10-fold in mice [20, 37, 41]) in an orientation-independent but somewhat distance-dependent manner. The region of the gene promoter between −110 and −75 appears critical in allowing its stimulation by an adjacent enhancer in vivo (39). Enhancer elements can also depress transcription of a promoter that is on an unlinked DNA molecule (20, 23, 41; see also reference 12) or even distant on the same molecule (38), so they have been envisioned to function by binding an essential polymerase I transcription factor(s).

Finally, upstream of the frog, mouse, Chinese hamster, and rat enhancer repeats is a so-called spacer promoter, which has been the only other known RNA polymerase I promoter besides that for the pre-rRNA (21, 34, 52). (A very recent report suggests that an RNA polymerase I promoter also resides within an intron of the human ribosomal protein S14 gene [50].) A related rDNA organization also exists in *D. melanogaster*, but there the repetitive enhancer elements themselves are spacer promoters (14). The functional elements of spacer promoters appear to be organized similarly to those of the gene promoter (52); in addition, their sequences can be very similar to that of the gene promoter, as in the frog, or very different from that of the gene promoter, as in the mouse and Chinese hamster. Spacer promoters of various species also vary widely in their abilities to initiate polymerase I transcription. The isolated frog spacer promoter appears to be as active as the gene promoter (24, 32), the Chinese hamster and rat spacer promoters are somewhat less active (6, 52), while the mouse spacer promoter shows only a very low level of transcriptional initiation in vitro and below-detectable levels of transcription in vivo (generating $<10^{-6}$ as much RNA as the gene promoter upon transient transfection) (52). Since the presence of spacer promoters appears conserved in evolution but their transcripts generally do not accumulate in vivo (16, 32), the RNA polymerase I spacer promoter may provide an evolutionary advantage that does not involve generating a stable, abundant RNA.

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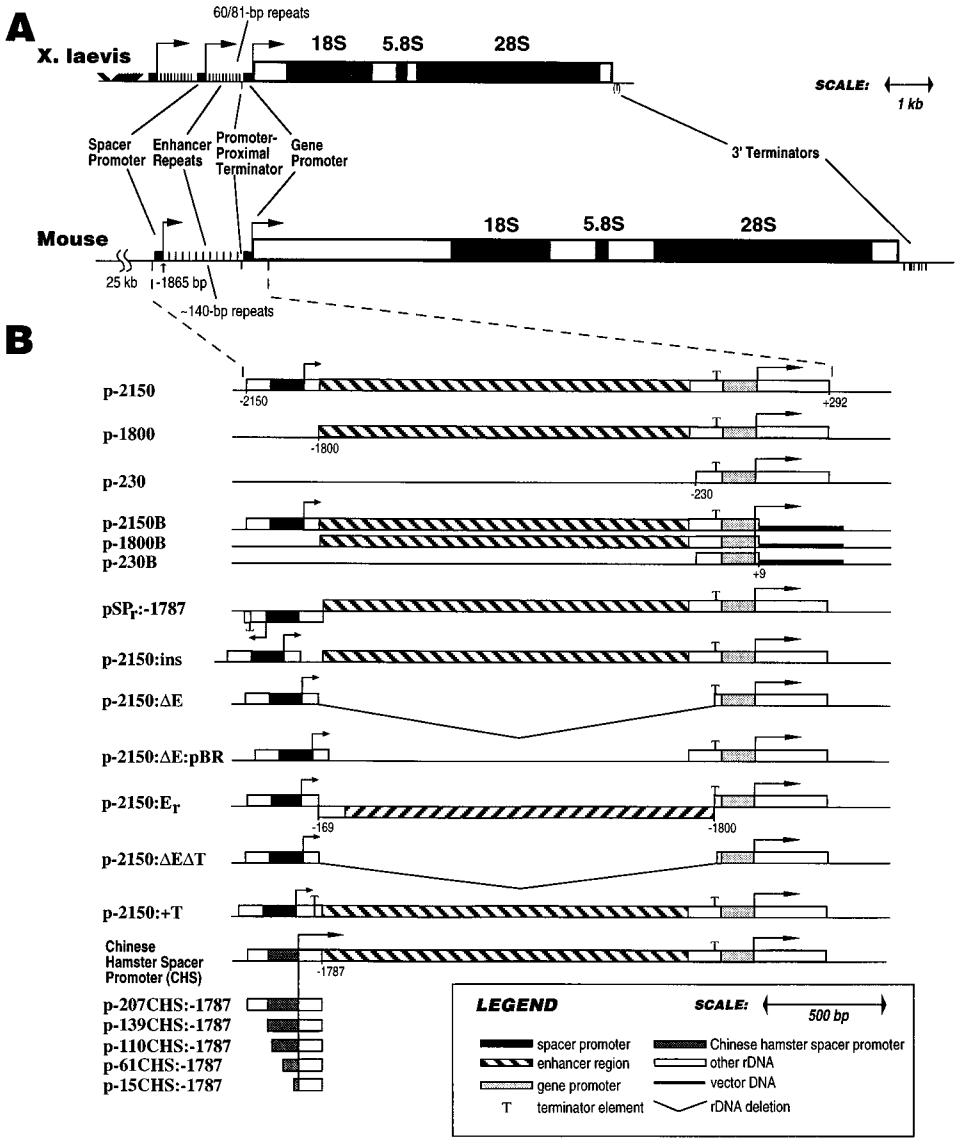

FIG. 1. rRNA gene templates. (A) Organization of the rRNA gene and spacer regions of *X. laevis* and the mouse. Regions conserved in organization and function, although not in sequence, are indicated. (B) rDNA transcription templates. The indicated rDNA regions (in boxes) are cloned between the *Eco*RI and *Pvu*II sites of pBR322 in each case, except for the three B constructs (p-230B, p-1800B, and p-2150B), which contain a prokaryotic marker segment in place of the region between +9 of the rDNA and the *Tth*111I site of pBR322. The construction of the plasmids is described in Materials and Methods.

Understanding the role of the spacer promoter in rRNA synthesis is far from complete. Studies have shown that the *X. laevis* spacer promoter can have a marked stimulatory effect on transcription of a downstream gene promoter relative to that of a coinjected competing promoter, and this effect appears to be dependent on sequences in the −120 region of the spacer promoter (9) and on the presence of intervening enhancer repeats (10). In contrast, no transcriptional stimulatory effect has been reported for the *X. laevis* spacer promoter by other investigators (42). Furthermore, the data showing stimulation by the *X. laevis* spacer promoter have come exclusively from *trans* competition assays (9, 10) in which the stimulation of a promoter in *cis* cannot be distinguished from repression of a promoter in *trans* (38). In fact, analyses conducted without added competitor templates did not show any stimulation of an *X. laevis* gene promoter by a spacer promoter in *cis* (9, 10). The only other reported studies are with *D. melanogaster*, in which the elements that have been termed enhancer repeats are duplications of the minimal gene promoter region and therefore may be considered spacer promoters. These elements, in the forward but not the reverse orientation, were found to stimulate transcription from a downstream gene promoter (13, 14).

Here we demonstrate that rodent rDNA spacer promoters function as true transcriptional *cis* stimulators of a downstream mammalian gene promoter in vivo. Mouse rDNA promoters bearing the spacer promoter and enhancer produce 3- to 10-fold more transcript than gene promoters bearing the enhancer but lacking the spacer promoter. This transcriptional stimulation by the spacer promoter even occurs under alternate conditions in which the mouse enhancer acts to repress transcription from a gene promoter in *cis*. In both these conditions, stimulation by the spacer promoter requires the presence of the enhancer repeats between the spacer and

gene promoters. Stimulation also requires that both the enhancer repeats and the gene promoter are in their natural orientations. However, the same extent of transcriptional stimulation is obtained when the mouse spacer promoter is replaced by the much more transcriptionally active Chinese hamster spacer promoter or when a functional transcriptional terminator is inserted between the spacer promoter and enhancer. Further supporting the conclusion that the mouse spacer promoter does not function by directing transcription through the enhancer region is the fact that the stimulation is abolished by deletion of the central region of spacer promoter, between the core and upstream domains, but not by deletion of the transcriptionally important upstream promoter domain.

MATERIALS AND METHODS

Mouse rDNA plasmid constructs. The mouse rDNA templates p-2150, p-1800, and p-230, which extend from the indicated rDNA position to position +292 (numbered relative to the transcription initiation site of the gene promoter) and which were cloned between the *Eco*RI and *Pvu*II sites of pBR322, have been described elsewhere (41), as have the 5′ deletions of the Chinese hamster spacer promoter (52) that were used to prepare constructs p-207CHS:-1787 through p-15CHS:-1787. All constructs were prepared by standard methods (1, 45). Mouse templates designated B, constructed to permit assay of their transcription in mouse cells (see Fig. 3B), are the same as their parental plasmids upstream of rDNA residue +9, but there they join to a 278-bp prokaryotic vector segment and then to the *Tth*111I site of pBR322; these B templates performed in a manner identical to that of their parallel rDNA templates in CHO cells (36). Template pSP_r:-1787 is identical to p-2150, except that the −2150-to-−1788 region (containing the spacer promoter sequences) was cloned in reverse and the sequences comprising the promoter-proximal transcriptional terminator (residues −168 to −147) were inserted immediately downstream of position −1788 in the same orientation as the spacer promoter to prevent transcriptional elongation from the spacer promoter from potentially interfering with the oppositely oriented rRNA gene promoter. The template p-2150:ins has a 72-bp stretch of nonspecific DNA inserted at position −1787 (the *Afl*II site) between the spacer promoter and the enhancer repeats. Template p-2150:ΔE was generated by removing the enhancer-containing *Sal*I fragment of p-2150 (positions −1800 to −169) and religating; this restores the *Sal*I site at position −168 (including sequences essential for the promoter-proximal terminator element [17]). Template p-2150:ΔE:pBR was constructed to replace the enhancer repeats (from positions −1800 to −231) with 1,477 bp of pBR322 DNA. In template p-2150:E_r, the *Sal*I fragment containing the enhancer repeats was recloned in a reverse orientation. Template p-2150:ΔEΔT is like p-2150:ΔE, except that the *Sal*I restriction site (residues −168 to −163) was resected with S1 nuclease (Bethesda Research Laboratories) to eliminate essential transcriptional terminator sequences. Template p-2150:+T contains the spacer promoter and transcriptional terminator sequences from p-2150:ΔE (through residue −147) inserted upstream of the enhancer repeats at position −1787, effectively inserting an efficient terminator element between the spacer promoter and the enhancer. The series of templates designated p-207CHS:-1787 through p-15CHS:-1787 were formed by replacing the mouse spacer promoter region (−2150 to −1788) with the Chinese hamster spacer promoter region (extending from the designated 5′ deletion site to position +70 relative to this spacer promoter +1 initiation site).

Transient transfections of rodent cell lines. Transient transfections of CHO and mouse L TK^- cells involved DEAE dextran-dimethyl sulfoxide shock as described by Lopata et al. (26), with the pretransfection cell density modified to ensure logarithmic growth during the experiment (41). Cell cultures were transfected with the same amount of supercoiled template (2 to 0.065 pmol/ml, supplemented with supercoiled pUC18 plasmid DNA to give a final total DNA concentration of 3 μg/ml) by using transfection medium (Dulbecco modified Eagle medium [Gibco BRL] plus 10 mg of DEAE dextran [Sigma] per ml) as indicated in the figure legends. All plasmids used for transient transfections were purified either by cesium chloride density gradient centrifugation or by anion exchange chromatography (Diagen, Inc.); no transcriptional differences between the two preparation methods were detected (36). Transfected cells were harvested after 10 to 48 h, as indicated in the figure legends, and whole-cell RNA was prepared with guanidinium isothiocyanate (by a method described in reference 7 and modified in reference 17). All transfections were performed in duplicate with highly reproducible results, and all experiments were repeated at least twice and generally more times. In some experiments, an *X. laevis* 5S RNA gene (5) was cotransfected to further verify the reproducibility of the results.

Analysis of mouse rDNA transcripts from transient-transfection experiments. Aqueous hybridization and S1 nuclease protection assays, performed as described by Henderson and Sollner-Webb (17), were used to assess the expression of the transfected templates. The DNA probes used to assess the mouse rRNA transcripts were 5′ end labeled at position +155 or +134 and diverged from the sequence of the transfecting DNA upstream of position −168. (Such divergence probes not only detect correctly initiated RNA but also distinguish RNA that reads into the promoter region from undigested probe.) Specifically, the +155 probe (used in the experiment depicted in Fig. 2A) was prepared by 5′ end labeling plasmid p5′Sal-Pvu at residue +155 (*Sma*I) and then by subjecting it to exonuclease III treatment (30), and the +134 probe was the 522-bp *Ava*I-*Nhe*I fragment of plasmid pSPG-1 (containing mouse rDNA from −168 to +155 cloned into pGEM-1) that was 5′ end labeled and strand separated on 5 to 6% nondenaturing polyacrylamide gels (45). The probe for the B clones was the 5′-end-labeled and strand-separated 556-bp *Ava*I-*Pvu*II fragment of plasmid p-168/+9pUC (which contains residues −168 to +9 of mouse rDNA and 257 bp of the pBR322 reporter sequence cloned into pUC18); this probe specifically protects the first 270 nucleotides of the transcript of the B clones. After hybridization with 5 μg of transfected-cell RNA and S1 nuclease treatment, samples were extracted, run on 4% polyacrylamide–7 M urea sequencing gels, and analyzed by autoradiography (17). Most of the autoradiographs, which were exposed within the linear range of the film, were quantitated by densitometric scanning with an LKB Ultrascan XL.

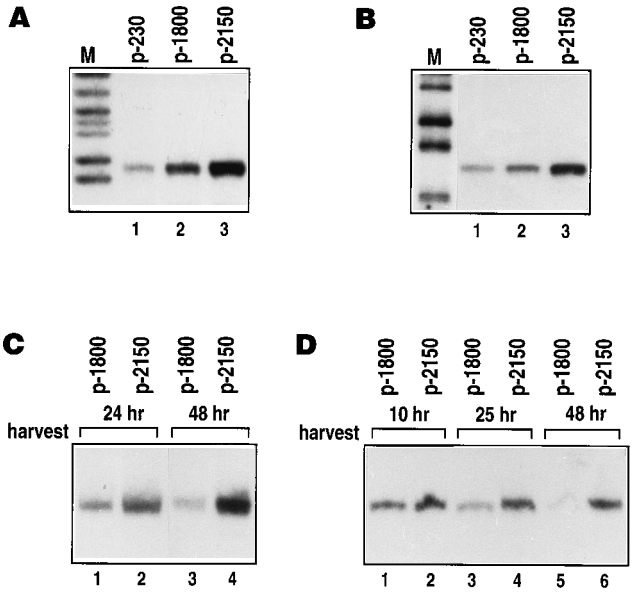

FIG. 2. The mouse rDNA spacer promoter stimulates gene promoter transcription. (A and B) CHO-1 cells (~3×10^5 per ml) were transiently transfected with the indicated plasmids at (A) 1 pmol/ml and (B) 0.065 pmol/ml. Five micrograms of whole-cell RNA prepared at 24 h posttransfection was assayed by S1 nuclease protection analysis for transcription from the gene promoter. (The probe used for panel B was hotter than that used for panel A.) In this particular experiment, relative to that for p-230, p-1800 and p-2150 exhibited 3.5- and 7.5-fold (A) and 2.5- and 6-fold (B) as much transcription, respectively. (C) CHO-1 cells were transfected as described in the legend for panel B, and whole-cell RNA was prepared for analysis at 24 h and 48 h posttransfection. Relative to that for lane 1, lanes 2 to 4 exhibited 3-, 0.4-, and 4-fold as much transcription, respectively. (D) CHO-1 cells were transfected as described in the legend to panel A, and whole-cell RNA was prepared for analysis at 10, 24, and 48 h posttransfection. Relative to that for lane 1, lanes 2 to 6 exhibited 1.7-, 0.5-, 2-, 0.15-, and 1.7-fold as much transcription, respectively. M, end-labeled *Hpa*II-cut pBR322 size markers.

RESULTS

The mouse rRNA spacer promoter stimulates transcription from a downstream gene promoter. We earlier showed that the ~1.5-kb block of ~140-bp repeats extending upstream from the mouse rRNA gene promoter enhanced transcription from that promoter when rDNA constructs were transiently transfected into rodent cells, much as the similarly positioned enhancer repeats in *X. laevis* rDNA did (41). We also demonstrated the presence of an RNA polymerase I spacer promoter immediately upstream of these enhancer repeats in mouse and Chinese hamster rDNA (52). To examine whether the mouse spacer promoter has an effect on transcription of the downstream gene promoter, we transiently transfected CHO cells

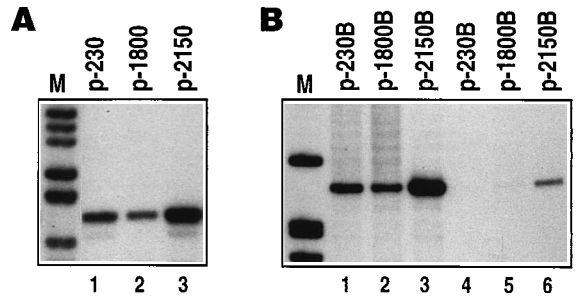

FIG. 3. The spacer promoter relieves the enhancer-mediated *cis* repression of the gene promoter. (A) CHO-2 cells (which have somewhat different growth and morphological characteristics than CHO-1 cells) were transiently transfected with the indicated templates (at 0.065 pmol/ml), and the RNA was analyzed as described in the legend for Fig. 2B. In this particular experiment, relative to that p-230, p-1800 and p-2150 exhibited 0.2- and 8-fold as much transcription, respectively. (B) Mouse fibroblastoid L TK$^-$ cells were transfected with the indicated marked templates as described in the legend to Fig. 2A and B (lanes 1 to 3, 1 pmol/ml; lanes 4 to 6, 0.065 pmol/ml). Relative to that for p-230 in lane 1, p-1800 and p-2150 showed 0.6- and 6-fold as much transcription in lanes 2 and 3, respectively; relative to that for p-230 in lane 4, p-1800 and p-2150 showed 2 and 50 times as much transcription in lanes 5 and 6, respectively. M, end-labeled *Hpa*II-cut pBR322 size markers.

with the same molar amounts of plasmids that contain the first 292 nucleotides of the mouse rRNA transcript driven by (i) the gene promoter region (promoter plus promoter-proximal terminator extending upstream to residue −230 [construct p-230]), (ii) the gene promoter and enhancer repeat regions (extending upstream to residue −1800 [construct p-1800]), and (iii) the gene promoter, enhancer repeat, and spacer promoter regions (extending upstream to residue −2150 [construct p-2150]) (diagrammed in Fig. 1B). Their expression was assessed by S1 nuclease protection by using equal amounts of transfected cell RNA and an end-labeled, single-stranded probe unique to the 5′ end of the transcript of the transfected template (17).

In these transfected cells, the enhancer- and spacer promoter-bearing p-2150 construct consistently supported 5- to 10-fold more transcription of the downstream gene promoter than did the enhancerless gene promoter construct p-230 and 3- to 7-fold more transcription than did the enhancer-bearing construct p-1800 (Fig. 2A, lanes 1 to 3). Since these results were obtained by transfecting single kinds of rDNA plasmids, they do not depend on a competition between two different kinds of transfecting rDNA plasmids. It should be noted that although single gel lanes are shown throughout this article, every transfection was performed in duplicate with highly reproducible results (with a transcript signal of almost invariably plus or minus ~25%, as judged by densitometric scanning), and each experiment was repeated several times. Repeat experiments were also qualitatively very reproducible, although quantitatively the extents of stimulation differed somewhat for experiments performed on different days (see above), depending on the precise condition of the recipient cells and possibly other factors. Additionally, in some of our experiments, the rDNA plasmid was cotransfected with a plasmid encoding *Xenopus* 5S RNA, and S1 analysis of the resultant 5S transcript showed a constant signal (plus or minus ~25%) in the various samples, in agreement with the reproducibility of the duplicate transfections. Finally, additional studies have shown that these p-230, p-1800, and p-2150 transcripts all have the same short half-life (~2.5 min [25]), so the observed differences in transcript abundance from these templates indeed reflect differences in the levels of transcription and not differences in RNA half-life.

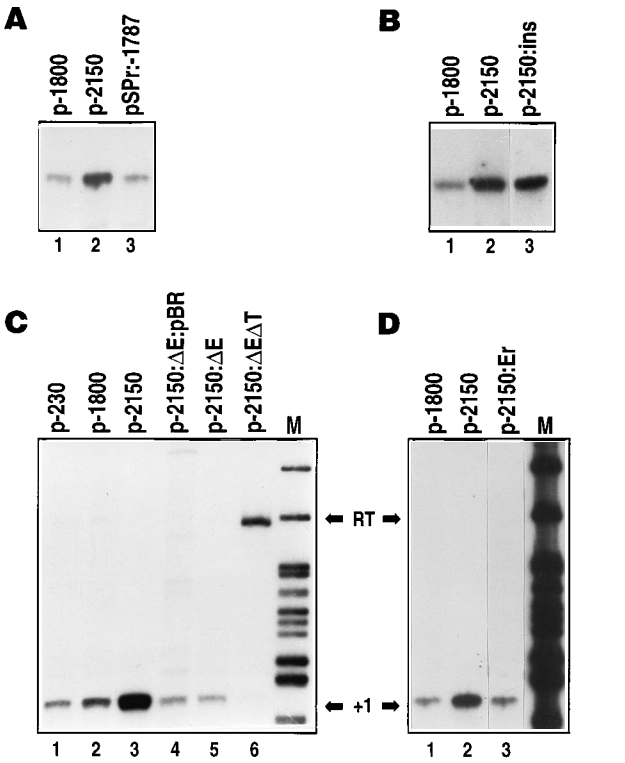

FIG. 4. The spacer promoter is an orientation-dependent stimulator of the downstream gene promoter and requires the intervening enhancer repeats in their native orientation. CHO-1 cells were transiently transfected with the indicated templates (described in the legend to Fig. 1B) at 0.065 pmol/ml, and the transcripts were analyzed as described in the legend to Fig. 2B. The arrow labeled RT in panels C and D indicates the signal from any transcription that reads through the promoter-proximal terminator region and into the promoter region from upstream; this length fragment results because the probe sequence diverges from the template sequences upstream of position −168. The four panels represent separate experiments. M, end-labeled *Hpa*II-cut pBR322 size markers. Relative to that for lane 1 in each panel, lanes 2 and 3 showed 6 and 1.3 times as much transcription (A); lanes 2 and 3 showed 5- and 6-fold as much transcription (B); lanes 2 to 6 showed 2.5-, 10-, 0.9-, 0.7-, and 0-fold as much +1 transcription (C); and lanes 2 and 3 showed 5- and 1.4-fold as much transcription (D), respectively.

Since the level of transcription of an rRNA gene promoter transiently transfected into cultured cells is known to depend on the amount of transfecting plasmid and on the time of the assay posttransfection (26), we examined whether the level of transcriptional stimulation by the spacer promoter region was also dependent on these variables. We observed the same hierarchy of transcriptional level over a 15-fold range in the amounts of transfected p-2150, p-1800, and p-230 templates (compare Fig. 2A [1 pmol/ml] and Fig. 2B [0.065 pmol/ml]) and over a considerable range in the densities of the recipient cells (36). However, variations in the posttransfection times at which the RNA was analyzed did appreciably affect the levels of transcriptional stimulation afforded by the spacer promoter region. As illustrated by the experiments (Fig. 2C and D) performed with low and high amounts of transfecting plasmid DNA, respectively, the levels of transcriptional stimulation by the spacer promoter increased at longer posttransfection times. In general, the signal from p-1800 and p-230 slowly decreased with posttransfection time, whereas the signal from p-2150 remained constant or increased with time (Fig. 2C and D) (36).

The spacer promoter also relieves an enhancer-mediated *cis* repression of RNA polymerase I transcription. Three different

rodent cell lines that support accurate transcription of the mouse rDNA gene promoter were transfected with the p-230, p-1800, and p-2150 constructs. While CHO-1 (the cell line used previously and for most of the experiments in this report) supported stimulation of the gene promoter by both the enhancer and the spacer promoter (Fig. 2A and B, lanes 1 to 3; see also reference 41), CHO-2 (a separately passaged CHO line possessing slightly different morphological and growth characteristics) showed a marked diminution in transcription from the transfected gene promoter when the enhancer repeats were present in *cis* (p-230 versus p-1800) (Fig. 3A, lanes 1 and 2). Nonetheless, cell line CHO-2 exhibited a strong transcriptional stimulation of the gene promoter when both the spacer promoter and enhancer repeats were present (p-2150) (Fig. 3A, lane 3). In CHO-2, p-2150 supported approximately 4-fold more transcription than 5′Δ-230 and approximately 10-fold more transcription than p-1800.

Similar results were obtained with the mouse fibroblastoid cell line L TK$^-$. In order to detect transcription from these mouse promoters amid the endogenous cellular mouse rRNA, we transfected alternate mouse template constructs which had the same rDNA upstream sequences but contained a prokaryotic reporter sequence following residue +9 (constructs p-230B, p-1800B, and p-2150B; see Materials and Methods). The presence of the enhancer repeats did not substantially alter the transcriptional level from the gene promoter in this cell line (it slightly depressed +1 transcription at high template concentrations and slightly augmented transcription at low template concentrations). However, in all conditions, the additional presence of the spacer promoter greatly augmented transcription from the gene promoter, relative both to the construct lacking the spacer promoter, p-1800B, and to the one lacking the spacer promoter and the enhancer repeats, p-230B (Fig. 3B). Thus, in three different mammalian cell lines, we consistently observe stimulation of gene promoter transcription by the upstream spacer promoter region.

The spacer promoter augmentation of transcription is orientation dependent, somewhat position independent, and enhancer dependent. To better understand the nature of the spacer promoter's stimulatory effect on gene promoter transcription, we constructed plasmids containing rearrangements, insertions, deletions, and substitutions of either the spacer promoter or the rDNA connecting the spacer promoter to the gene promoter (Fig. 1B). Construct pSP$_r$:-1787, which contains the same spacer promoter region (−2150 to −1788) upstream of the enhancer region as that in p-2150 but which is cloned in the reverse orientation (Fig. 1B), exhibited much less gene promoter transcription than either p-2150 (Fig. 4A, lanes 1 to 3) or a control construct in which the spacer promoter was inserted in the plasmid in the forward direction 72 bp upstream from its native site (p-2150:ins) (Fig. 4B, lanes 1 to 3). In fact, pSP$_r$:-1787 transcribed approximately at the level of p-1800, which lacked the spacer promoter altogether. Thus, the stimulatory effect of the mouse spacer promoter region is dependent on its orientation.

We next tested whether the rodent spacer promoter could stimulate gene promoter transcription in the absence of intervening enhancer repeats. In construct p-2150:ΔE (Fig. 1B), the sequence from −1800 to −169 was deleted from p-2150, moving the spacer promoter closer to the gene promoter. Gene promoter transcription from p-2150:ΔE was reproducibly much lower than that from p-2150, approximately at the level of that from p-230 (Fig. 4C). To examine whether p-2150:ΔE failed to show transcriptional stimulation because the spacer promoter region must be separated from the gene promoter by a considerable distance (as in p-2150), we constructed p-2150:ΔE:pBR (Fig. 1B), in which the enhancer repeats of p-2150 were replaced with a comparably sized segment from pBR322 (a region that previous experiments showed had no adverse effect on rDNA promoter transcription) (8). This construct also did not exhibit stimulation of the gene promoter by the spacer promoter (Fig. 4C). Thus, the transcriptional stimulation of the gene promoter by the upstream spacer promoter is dependent on the presence of an intervening enhancer repeat region.

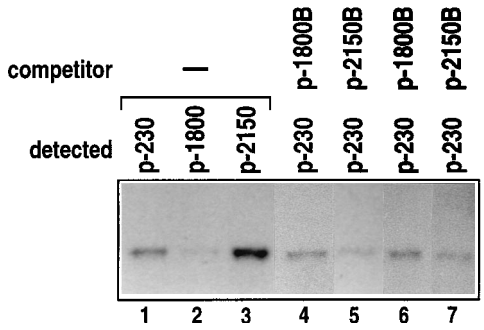

FIG. 5. The spacer promoter does not abrogate an enhancer's repression of a gene promoter in *trans*. CHO-2 cells were transfected with the indicated constructs (lanes 1 to 3, 0.065 pmol/ml) or were cotransfected with equimolar amounts of the indicated constructs (lanes 4 to 7, 0.065 pmol/ml of each). Equal amounts of whole-cell RNA were probed separately for each transcript; the signals from p-230, p-1800, and p-2150 are shown. Corroborating analysis of the B gene transcripts showed the same low levels of transcription from p-1800B and the same high levels of transcription from p-2150B in the cotransfections as in the single transfections of p-1800 and p-2150. Lanes 6 and 7 represent separate transfections duplicating lanes 4 and 5.

Although one might have anticipated that the spacer promoter could stimulate gene promoter transcription with the enhancer repeats present in the reverse orientation, this did not turn out to be the case. Construct p-2150:E$_r$, which is like p-2150 except that the −1800-to-−169 enhancer region was flipped (Fig. 1B), exhibited much less gene promoter transcription than p-2150 (Fig. 4D). In fact, it transcribed at the level of p-1800 (Fig. 4D). Thus, the spacer promoter stimulation of the gene promoter requires that the enhancer repeat block be in the wild-type orientation. This result was unexpected because transcriptional stimulation of the gene promoter by the enhancer repeats alone occurs in both mouse and frog rDNA whether the repeats are present in the forward or reverse orientation (20, 24, 37, 38).

We are confident that the low levels of transcription from pSP$_r$:-1787, p-2150:E$_r$, and p-2150:ΔE, relative to that from p-2150, are due to the reversal and/or removal of the spacer promoter or enhancer regions and not to several other potentially artifactual causes. Specifically, construction of these plasmids did not damage the promoter-proximal terminator, which begins at residue −168 and is necessary for efficient transcription on closed circular mouse rDNA templates because it prevents promoter occlusion (17, 18). The functioning of this terminator can be assessed by a lack of transcription that reads completely around the plasmid and into the gene promoter region from upstream (17); efficient transcriptional termination for these constructs is shown by the absence of such a read-through transcription band (Fig. 4C, lanes 4 and 5, and Fig. 4D, lane 3 [positions labeled RT]). In contrast, construct p-2150:ΔEΔT (Fig. 1B), which bears a small deletion of the essential promoter-proximal terminator sequences of p-2150:ΔE, generated copious amounts of read-through transcription and very little +1 transcription (Fig. 4C, lane 6), as was expected (17).

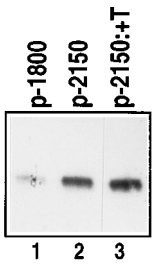

FIG. 6. Transcription from the spacer promoter into the enhancer repeat region is not required for the stimulation of gene promoter transcription. The indicated templates were transfected into CHO-1 cells (at 0.065 pmol/ml), and their transcripts were analyzed as described in the legend to Fig. 2B. Relative to that for lane 1, lanes 2 and 3 showed 4.5- and 5.5-fold as much transcription, respectively.

Furthermore, a particular relative position or junction sequence between the spacer promoter and the enhancer regions is not required, because the construct p-2150:ins, which contains a 72-bp segment of foreign DNA separating the spacer promoter and the enhancer regions, directed gene promoter transcription at the same level as the parental p-2150 does (Fig. 4B, lanes 2 and 3). These results argue strongly that transcriptional stimulation of the gene promoter by the spacer promoter region requires the presence of intervening enhancer repeats and that the enhancer and spacer promoter regions must both be in the wild-type orientation but that this phenomenon permits some flexibility in the relative positions of and sequences between the spacer promoter and the enhancer regions.

The spacer promoter does not stimulate a gene promoter located in *trans*. The observation that the forward-oriented spacer promoter (Fig. 4A) stimulates transcription from a downstream gene promoter in conjunction with intervening enhancer repeats, even if these repeats repress transcription by themselves (Fig. 3A and B), could support a variant of the "read-through enhancement" model that has been proposed for such stimulation (9–11, 33). In this model, the spacer promoter functions by driving polymerase I molecules through the enhancer repeats, thereby releasing a transcription factor(s) that was sequestered on these repeats and making it available to bind to gene promoters. Prior data have confirmed that polymerase I transcription factors can become sequestered on enhancer repeats (38, 41) and that transcription through factor-bound polymerase I promoters releases these factors and makes them available for binding to other promoters (2, 17). If such were the case for the action of the mouse spacer promoter with the enhancer, transcription from a gene promoter construct might be less impaired by cotransfection with a construct bearing both the spacer promoter and enhancer repeats than by cotransfection with a construct lacking the spacer promoter but bearing the enhancer repeats. We thus tested this hypothesis in the cell line that would appear most limiting for transcription factors, CHO-2. However, the results showed that template p-230 transcribed less efficiently, not more efficiently, when cotransfected with spacer promoter- and enhancer-bearing construct p-2150B (Fig. 5, lanes 5 and 7) than when cotransfected with the construct bearing the enhancer repeats but lacking the spacer promoter, p-1800B (lanes 4 and 6). Thus, the spacer promoter does not appear to stimulate activity of the gene promoter by initiating transcription through the enhancer repeats and releasing sequestered transcription factors free into the solution. Additional evidence against other forms of a read-through enhancement model is presented in the following section.

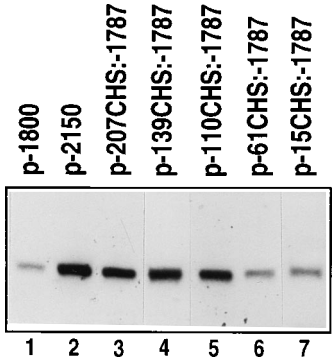

FIG. 7. The Chinese hamster spacer promoter and 5′ deletions of it replace the mouse spacer promoter in stimulating a downstream gene promoter. The indicated chimeric templates bearing intact and deleted versions of the Chinese hamster spacer promoter in place of the mouse spacer promoter region (diagrammed in Fig. 1B) or control mouse templates were transfected into CHO-1 cells (at 0.065 pmol/ml), and the transcripts were analyzed as described in the legend to Fig. 2B.

Transcription from the spacer promoter region into the enhancer repeats is not necessary for the gene promoter stimulatory effect. Although the data in Fig. 5 suggest that the spacer promoter does not cause stimulation by releasing free into solution the enhancer-bound transcription factors, the spacer promoter could act by transcribing through the enhancer and pushing bound factors along the DNA toward the downstream gene promoter. To test this model, an active RNA polymerase I transcriptional terminator was positioned between the spacer promoter and the repetitive enhancer elements. Specifically, the spacer promoter region of p-2150 was replaced by the region containing the spacer promoter and the active promoter-proximal terminator of p-2150:ΔE, forming p-2150:+T (Fig. 1B). Strikingly, p-2150:+T directs substantially more gene promoter transcription than does p-1800 and directs at least as much as p-2150 (Fig. 6). Thus, the spacer promoter stimulates downstream gene promoter transcription just as efficiently whether or not an active transcriptional terminator is located between the spacer promoter and the enhancer regions. Note that the effectiveness of the inserted terminator in this spacer promoter-terminator segment was shown earlier by the lack of read-through transcription from p-2150:ΔE compared with the large amount of read-through transcription from construct p-2150:ΔEΔT, which had critical residues of the terminator signal deleted (Fig. 4C, lanes 5 and 6). The terminator's effectiveness was further substantiated by our observation that transcription reading into the enhancer region can be readily observed from p-2150 but not from p-2150:+T (36). Thus, the spacer promoter's transcriptional stimulation of the gene promoter is not dependent on the spacer promoter directing transcribing polymerases into or through the enhancer repeat region. Read-through enhancement models of the transcriptional stimulatory effect of the rDNA spacer promoter are not compatible with this experimental result.

The enhancer-responsive domain of the spacer promoter is required for spacer promoter-mediated gene promoter stimulation. We next wished to examine whether it indeed was the spacer promoter sequences of the −2150-to-−1800 region that were responsible for the observed transcriptional stimulatory effect and whether the level of this stimulation was correlated with the initiation efficiency of the spacer promoter. Compared with the mouse spacer promoter, which is an extremely weak

promoter under all conditions examined, the Chinese hamster spacer promoter is a much stronger promoter, both in vivo and in vitro and in both mouse and CHO systems (52). To examine whether the transcriptional strength of the spacer promoter correlates with its ability to stimulate gene promoter transcription, the mouse spacer promoter region (the segment from −2150 to −1788 of p-2150, which corresponds to positions −285 to +77 relative to the spacer initiation site) was replaced by the Chinese hamster rDNA spacer promoter (positions −207 to +70 relative to the spacer initiation site) (diagrammed in Fig. 1B). With this p-207CHS:-1787 construct, gene promoter transcription is stimulated to the same extent as with p-2150 (Fig. 7), even though the hamster spacer promoter directs at least 100-fold more transcription than does the mouse spacer promoter under all conditions examined. Thus, the level of stimulation does not correlate with the transcription level of the spacer promoter.

To map the sequences of the spacer promoter region that confer transcriptional stimulation on the downstream gene promoter, the complete Chinese hamster spacer promoter region was then substituted with a series of deletion mutants (Fig. 1B). Prior studies of rDNA promoters have shown that the upstream domain (approximately positions −140 to −120) and core domains (approximately positions −40 to +1) of the promoter are the most critical for directing transcriptional initiation while the sequence between approximately −110 and −73 (the enhancement domain) is critical for allowing the promoter to be stimulated by an adjacent rDNA enhancer in vivo (15, 19, 39, 49, 52, 53). Promoter-unrelated upstream sequences proved unnecessary for stimulation, since p-139CHS:-1787, bearing a 5′Δ-139 deletion of the hamster spacer promoter, the largest deletion of the hamster spacer promoter region to retain full promoter activity (52), conferred the same level of gene promoter stimulation as p-2150 and p-207CHS:-1787 (Fig. 7). While the quantitative level of stimulation relative to p-1800 varied between 4-fold and 12-fold in different experiments involving transfection into CHO-1 or CHO-2 cells, in each experiment, the levels of stimulation by p-2150, p-207CHS:-1787, and p-139CHS:-1787 were very similar (approximately ±25%). Notably, p-110CHS:-1787, which lacks the upstream domain of the hamster spacer promoter, also exhibited this same level of gene promoter stimulation. In contrast, p-61CHS:-1787, which lacks the region of the spacer promoter corresponding to the in vivo enhancement domain (39), failed to cause appreciable transcriptional stimulation (Fig. 7). The gene promoter of this construct transcribes at approximately the same level as those of p-15CHS:-1787 (bearing a 5′Δ-15 deletion of the hamster spacer promoter and exhibiting no promoter activity) and p-1800 (which lacks all spacer promoter sequences). Thus, the transcriptional stimulation by the spacer promoter region is indeed directed by sequences within the functional spacer promoter but requires the region corresponding to the enhancement-responsive domain and not the regions most critical for promoter activity.

DISCUSSION

We have shown that the mouse rDNA spacer promoter, itself a very weak initiator of RNA polymerase I transcription located 2 kb upstream of the gene promoter in natural mouse rDNA (52), markedly stimulates transcription from the downstream gene promoter in vivo (Fig. 2A and B). This spacer promoter stimulation of gene promoter transcription is dependent on the presence of intervening rDNA enhancer repeats in their natural orientations (Fig. 4C and D) and surpasses the stimulation conferred by the enhancer repeats alone. The spacer promoter-enhancer combination appears to be a more faithful stimulator of gene promoter transcription than the enhancer repeats alone, since under certain conditions the enhancer repeats actually repress their adjacent gene promoter while the additional presence of the spacer promoter not only derepresses the gene promoter but further stimulates its transcription (Fig. 3A). Although the stimulation of the gene promoter requires the spacer promoter to be in its natural orientation (Fig. 4A), it does not depend on transcription from the spacer promoter reading into the intervening enhancer repeats. Several lines of evidence lead to this conclusion. First, insertion of a functional RNA polymerase I transcriptional terminator between the spacer promoter and the enhancer does not impair the stimulatory effect (Fig. 6). Second, the level of stimulation does not correlate with the initiation capacity of the spacer promoter (Fig. 2 and 7). And finally, the upstream domain of the spacer promoter, which is needed for efficient initiation, is not critical for this stimulation; instead, a central segment of the spacer promoter, in a region that is not essential for promoter function, is critical for the stimulation (Fig. 7).

Our basic observations of stimulation of an rDNA gene promoter by a spacer promoter located upstream of the rDNA enhancer (Fig. 2A and B and 4C) are reminiscent of findings for *X. laevis* rDNA (9–11, 33), with the major exception that the frog spacer promoter is as strong an initiator of RNA polymerase I transcription as the frog gene promoter (24, 32) while the mouse spacer promoter initiates RNA polymerase I transcription orders of magnitude less well than the mouse gene promoter (36, 52). In addition, the spacer promoter effect in the frog was observed only when large amounts of two different kinds of input genes are introduced in competition with each other (9, 10), under conditions in which stimulation of a promoter in *cis* is not distinguished from a different phenomenon, repression of a promoter in *trans* (38, 39). However, the rodent spacer promoter stimulation in our studies also occurs under "normal" transcription conditions in which a single kind of template is introduced and transcribed (Fig. 2). Nonetheless, the fundamental observation of an rDNA spacer promoter affecting the transcription (Fig. 2) or relative transcription (Fig. 5) (9, 10) of a downstream rDNA gene promoter, dependent on intervening enhancer repeats, appears to be so similar for rodents and amphibians that our findings with the rodent spacer promoter will very likely apply to the many other metazoan species whose rDNA is organized as spacer promoter→repetitive enhancer→gene promoter. In addition to demonstrating the similarity of the spacer promoter effects in rodents (this study) and frogs (9–11, 33), most of our experiments (Fig. 2C and D and 3 to 7) address new issues and provide novel information about how the stimulation by the rDNA spacer promoter does and does not function.

Our findings that the rDNA spacer promoter must be oriented toward the enhancer region (Fig. 4A) and that it requires the enhancer to cause stimulation (Fig. 4C) could appear to support a model of spacer promoter function in which RNA polymerase I molecules, which are directed by the spacer promoter to transcribe through the enhancer, release rDNA transcription factors from the enhancer and make them available to the gene promoter, thereby stimulating gene promoter transcription. The factors postulated to be delivered to the gene promoter in such "read-through enhancement" models (11) could be RNA polymerase I molecules themselves (33) or other rDNA transcription factors (31), such as UBF, which can bind both the enhancer and the promoter (41). Reports of experiments have been published to argue both against (24, 27) and for (31) the feasibility of a polymerase delivery model, but

on reconsideration, although the data leading to these arguments appear strong, they could be subject to alternate interpretations. (For instance, if the UV-treated nuclei reported on by Labhart and Reeder [24] allowed the sliding of polymerases once the transcripts were released, read-through enhancement could still be occurring. The *cis* stimulation assay reported to show no effect of psoralen treatment by Lucchini and Reeder [27] also showed no effect of the enhancer repeats in another study by this laboratory [23]; therefore, it remains to be determined whether psoralen also has no effect under conditions in which the enhancer is demonstrated to be active. In addition, if the truncated RNAs reported on by Mitchelson and Moss [31] had reduced half-lives, their conclusions favoring read-through enhancement would need to be reconsidered.) Similarly, the model supposing a polymerase-assisted liberation of other transcription factors from the enhancer, although consistent with the liberation of transcription factors when RNA polymerase I transcribes into an active promoter (18), has not been directly tested when the polymerase transcribes into an rDNA enhancer. Thus, models of spacer promoter stimulation involving its directing transcription into the enhancer repeats have remained attractive but have not been validated.

Such read-through enhancement models are now directly addressed, and refuted, by experiments reported on in this article. First, insertion of a polymerase I terminator between the spacer promoter and the enhancer repeat does not diminish the level of stimulation imparted by the spacer promoter (Fig. 6), even though this terminator appears completely efficient (Fig. 4C) (17). Second, the level of stimulation caused by the spacer promoter does not correlate with the initiation capacity of the spacer promoter, since the mouse and Chinese hamster spacer promoters stimulate gene promoter transcription to the same extents (Fig. 7) and yet the former spacer promoter initiates transcription less than 1% as efficiently as the latter (36, 52). Third, constructs bearing a series of deletion mutations of the spacer promoter region have shown that its upstream domain, which is important in achieving transcriptional initiation (52), especially in vivo (19), is not relevant to its stimulation of the gene promoter (Fig. 7). Instead, the central portion of the spacer promoter, which has not shown as strong effects on transcriptional initiation capacity (50, 51), is critical for its stimulation of the gene promoter (Fig. 7). Fourth, in p-1800, the active transcription from the gene promoter on this circular template continues through the enhancer repeats (and stops just upstream of the gene promoter) (17); yet the weak spacer promoter in p-2150 stimulates gene promoter transcription beyond that seen in p-1800, in which such enhancer read-through already occurs. We thus conclude that stimulation of the gene promoter by the spacer promoter does not depend on the spacer promoter directing transcription into the enhancer region, as is envisioned by all forms of read-through enhancement models.

A corollary of read-through enhancement models has been that the spacer promoter acts somehow to amplify or increase the effect exerted by the enhancer repeats alone (10) and that the level of this stimulation is proportional to the promoter strength of the spacer promoter (9). Our data show that this hypothesis is also not generally true. First, while the enhancer repeats exhibit the same stimulatory effects on an adjacent gene promoter regardless of whether they are oriented in the forward or reverse direction (36, 52), stimulation of the gene promoter by the spacer promoter requires that the enhancer repeats be in their natural orientation (Fig. 4D). (Control experiments confirm that the lack of stimulation by the spacer promoter when the enhancer repeats are in the reverse orientation cannot be attributed to spacing alterations [Fig. 4B] or impairment of the promoter-proximal terminator [Fig. 4C and D].) The second line of evidence that the spacer promoter does not merely serve to increase the magnitude of the enhancer's effect is that in certain cell lines (Fig. 3A) and under conditions of high template concentration (Fig. 3B) in which essential components may be more limiting, the enhancer repeats themselves actually repress transcription of an adjacent gene promoter and yet the additional presence of the spacer promoter reactivates and further stimulates gene promoter transcription (Fig. 3). The complete spacer promoter-enhancer block thus appears to stimulate gene promoter transcription more reliably than do the enhancer repeats lacking the spacer promoter. Note that the *Drosophila* rDNA enhancer, which is a series of duplicated spacer promoters, also appears to stimulate only in the natural orientation (13, 14), consistent with its stimulatory effect being more akin to that of the mouse spacer promoter-enhancer segment than to the mouse enhancer element alone.

If the spacer promoter does not function by a type of read-through enhancement mechanism, then how might it act? An important clue seems to be that the 5′Δ-110 deletion of the spacer promoter region is fully active in stimulating gene promoter transcription but that the 5′Δ-61 deletion of the spacer promoter exhibits virtually no stimulatory activity (Fig. 7). While this segment identified between the 5Δ-110 and 5Δ-61 regions does not correlate with domains found to be critical in directing transcriptional initiation either by this spacer promoter or by rDNA promoters in general (48, 52), it does correspond to the rDNA promoter region found critical for enhancer-promoter interaction (39). In that study, which analyzed linker-scanning mutations of the *X. laevis* rDNA promoter by the *cis* enhancement assay, almost all mutations mapping within the −126-to-−73 region (located between the upstream [−140 to −128] and core [−36 to +1] promoter domains) directed robust levels of transcription by themselves (50) but failed to exhibit any stimulation when the enhancer was present in *cis* (39). In fact, transcription from several of these mutant promoters was significantly depressed by enhancer repeats in *cis*! The importance of a region similarly positioned between the upstream and core promoter domains in the spacer promoter (where it acts with the enhancer to stimulate the gene promoter [Fig. 7]) and in the gene promoter (where it allows stimulation by the enhancer [39]) suggests (i) that these two possibly related phenomena involve similar aspects of the promoter and (ii) that these represent interactions different from those of the upstream and core promoter domains that largely direct transcriptional initiation. Since the UBF transcription factor footprints the promoter most strongly in this central region (approximately positions −75 to −115 [4, 41]), but also footprints on the enhancers (41), which in some cases are duplications of the central region of the promoter (positions −72 to −119 in *X. laevis* [47]), as well as on other DNA sequences, UBF may well be involved. Furthermore, the propensity of DNA-bound UBF to autooligomerize (43) and to constrain the bound DNA in a particular configuration (3) suggests that the spacer promoter, acting with the enhancer, might serve to hold the promoter in a manner or at a site that favors active transcription. This effect could be a positive promoter stimulation. However, an alternative model is suggested by our recent in vitro studies of rDNA enhancer action (37) in conjunction with earlier studies of promoter action (22). These show that the promoter is subject to an ATP-dependent inhibition that the enhancer serves to alleviate. The indication that the stimulatory effect of the spacer promoter becomes more pronounced at later posttransfection times (Fig. 2C and D) would be consistent with augmentation of the longevity of the active transcription complex by the spacer promoter, possibly

through such an antiinhibition mechanism. We thus speculate that the spacer promoter acts in a novel manner in conjunction with the enhancer repeats to counteract a natural inhibition of transcription from the rDNA promoter that occurs in vivo and that the spacer promoter thereby augments the level of gene promoter transcription.

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