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A 140-base-pair repetitive sequence element in the mouse rRNA gene spacer enhances transcription by RNA polymerase I in a cell-free system

(*in vitro* transcription/enhancer/mouse rDNA)

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ABSTRACT **We show that the repetitive 140-base-pair (bp) elements present in the spacer of mouse rRNA genes function as enhancers for RNA polymerase I. Attachment of these elements to the rDNA promoter stimulates rRNA synthesis both *in vivo* and *in vitro*. The cis-activating effect of the spacer repeats is orientation-independent and increases with increasing numbers of the 140-bp elements. Competition experiments demonstrate that the spacer repeats bind one or more of the transcription factors interacting with the rDNA promoter. Both the 140-bp elements and the core promoter act cooperatively and thus are functionally linked. The 60/81-bp enhancer repeats from *Xenopus laevis* rDNA compete for a murine transcription factor(s) and stimulate transcription when fused to the mouse rDNA promoter. The results indicate that despite the marked species specificity of rDNA transcription initiation, common factors may interact with both the rDNA promoter and the enhancer.**

The intergenic spacers between individual rRNA transcription units have rapidly diverged between different species, varying in length from 2 kilobases (kb) in yeast to >30 kb in mammals. In spite of this marked sequence divergence, several regulatory elements that affect the readout of rRNA genes have been maintained. Functionally important sequence elements within the spacer include promoter and terminator elements, spacer promoters, promoter-proximal terminators, and origins of replication. Furthermore, repetitive blocks of interspersed 60- or 81-base-pair (bp) elements that behave in some respect like enhancer elements have been identified in the intergenic spacer of *Xenopus laevis* (1, 2). They confer a competitive transcriptional advantage on promoters located in cis but compete against promoters located in trans (3, 4). This cis stimulation of transcription is orientation- and position-independent and, therefore, these elements behave like transcriptional enhancers. To date, RNA polymerase I enhancers in rDNA spacers have been rigorously demonstrated only in yeast and in *X. laevis* and its relative *X. borealis*. In the mouse spacer there is a cluster of repetitive 140-bp sequence elements (5, 6) that lies between the spacer promoter (7) and the upstream terminator, T_0 (8, 9). Both their repetitive nature and their localization between the spacer promoter and the upstream terminator suggest that these elements may be functionally analogous to the frog 60/81-bp element. The results presented here demonstrate that this assumption is correct. We show that the 140-bp repeats stimulate in cis transcription from the rDNA promoter both in a cell-free system and after transfection into mouse cells. This activation appears to be mediated by transcription factor(s) that interact with both the promoter and the enhancer elements.

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METHODS

Plasmid Constructions. See Fig. 1. Plasmid pMrWT contains a 324-bp mouse rDNA promoter fragment (positions −169 to +155; ref. 10). pMrSP is identical to pMrWT except that the coding region extends to a *Pvu* II site at +292. The 5′-deletion plasmids pMrΔ-168, pMrΔ-144, pMrΔ-112, and pMrΔ-39 are derivatives of pMrWT. In the enhancer-containing clones pMrE-SP, pMrE-WT, etc., a 1762-bp *Sal* I fragment (−1930 to −169) containing 13 repeats of the 140-bp element was inserted upstream of the rDNA promoter. pMrE*-SP contains the 140-bp repeats in the opposite orientation. In pMrSP-E the enhancer is inserted into the single *Nar* I site of pUC9 located 168 bp downstream of the transcribed region. In pMrE-Δ168 the upstream terminator T_0 was destroyed by cleaving pMrWT with *Sal* I, and the *Hin*dII fragment containing the enhancer repeats was inserted by blunt-end ligation. The 60/81-bp repeats of the *X. laevis* rDNA enhancer were fused to the mouse promoter by ligating the 1115-bp *Bam*HI fragment from the *X. laevis* intergenic spacer region into the *Hin*dIII site of pMrSP (pX.I.-MrSP). For competition experiments the mouse and the frog enhancer fragments were cloned into pUC9. pMrE13 covers the murine 1762-bp *Sal* I fragment with 13 repeats of the 140-bp element. pMrE10 contains 10 and pMrE3 contains 3 repeats extending from position −1930 to −639 and −639 to −169, respectively. pX.I.60/81 contains the 1115-bp *Bam*HI fragment from the *X. laevis* spacer, including a block of ten 60/81-bp elements. pP160B is a pBluescript (Stratagene) derivative with a 140-bp *Pst* I fragment from the *Xenopus* rDNA spacer containing one 60/81-bp repeat (11). The constructs pMrCAT and pMrE-CAT contain a 1632-bp *Bam*HI–*Hin*dIII fragment from pSV2-cat (12) cloned into the *Sma* I site (position +155) of pMrWT or pMrE-WT. The plasmid DNA $pMrCAT_{SP}$ was constructed by inserting the 167-bp *Stu* I–*Pvu* II fragment from pSV2-cat (nucleotides 4855 to 19) into the *Sma* I site of pMrWT.

***In Vitro* Transcription Assays.** The cultivation of Ehrlich ascites cells, the preparation of extracts, the system for cell-free transcription, and the methods for RNA analysis have been described (13, 14). One to 100 ng of template DNA and various amounts of pUC9 or competitor DNA were incubated for 60 min at 30°C with 15 μl of a mixture of S-100 and nuclear extracts in a total volume of 25 μl containing 12 mM Hepes (pH 7.9), 0.1 mM EDTA, 0.5 mM dithioerythritol, 5 mM $MgCl_2$, 75 mM KCl, 0.66 mM each ATP, CTP, and UTP, 0.01 mM GTP, 1.5 μCi (55.5 kBq) of [α-^{32}P]GTP, 10 mM creatine phosphate, and 12% (vol/vol) glycerol. For some experiments the extract mixture was fractionated by chromatography on DEAE-Sepharose (15) and the fractions eluted at 280 mM KCl were used for transcription.

Abbreviations: UCE, upstream control element; UBF, upstream binding factor; CAT, chloramphenicol acetyltransferase.

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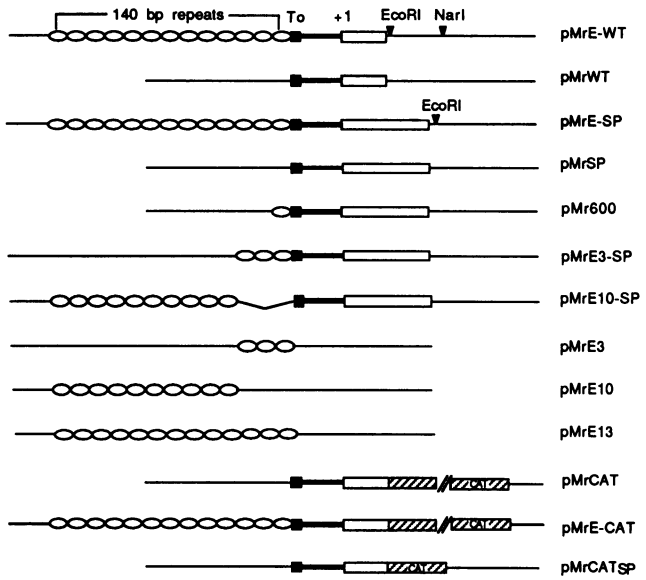

FIG. 1. Schematic of the recombinant plasmids used. Black bar, spacer sequences (positions −171 to +1) including the upstream terminator T_0 (black box); open bar, coding region; hatched bars, sequences derived from the bacterial chloramphenicol acetyltransferase (CAT) gene; ovals, 140-bp elements.

Transfection of Cells and Analysis of RNA. Subconfluent cultures of Ltk$^-$ cells (thymidine kinase-deficient mouse fibroblasts) were transfected with supercoiled plasmid DNA and RNA was isolated after 44–48 hr. Samples (50 μg) of total RNA were hybridized for 3 hr at 37°C to a 5′-labeled oligonucleotide (5×10^5 cpm) complementary to nucleotides 4936–4955 from pSV2-cat (12) and were analyzed by primer extension (7).

RESULTS

rDNA Transcription Is Inhibited Both by the Murine 140-bp Repeats and by the Frog Enhancer in Trans. It has been shown that the RNA polymerase I transcription factor xUBF (11) or TFIS (16) interacts with both the promoter and the repetitive 60/81-bp enhancer of the *Xenopus* rRNA genes. If one accepts the view that despite marked sequence divergence between different organisms, the type and arrangement of regulatory elements within the spacer have been conserved, one would expect to find also in mammalian rDNA a polymerase I enhancer with similar properties located between the spacer promoter and the upstream terminator. Indeed, in this particular location there are 13 repeats of tandemly arranged 140-bp elements in the mouse spacer (5). To study whether the murine 140-bp elements are functionally analogous to the frog enhancer, they were used as competitors in a cell-free transcription assay. In the presence of the competitor DNA (pMrE13), transcription from the mouse rDNA promoter was progressively reduced (Fig. 2*A*, lane 2), indicating that the spacer fragment bound some factor(s) essential for promoter function. Surprisingly, the frog repeats (pX.I.60/81) exerted a similar inhibitory effect on murine rDNA transcription (lane 3) despite the lack of obvious sequence homology between the frog and the mouse repeats.

The competitive effect of the 140-bp elements was observed only before a stable transcription initiation complex had been assembled at the rDNA promoter. pMrWT was assayed in the presence of vector DNA or in the presence of pMrE13 competitor DNA. When test and competitor DNAs were added simultaneously (Fig. 2*B*, lanes 1–3) or when the competitor DNA was preincubated with extract before the test plasmid was added (lanes 4–6), concentration-dependent

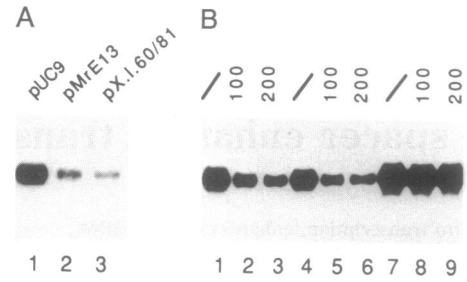

FIG. 2. Competition of rDNA transcription by the repetitive sequence elements present in the mouse and frog rDNA spacer. (*A*) pMrSP (100 ng) linearized with *Eco*RI was transcribed with extract proteins in the presence of 200 ng of pUC9 (lane 1), pMrE13 (lane 2), or pX.I.60/81 (lane 3). (*B*) pMrWT (100 ng) truncated with *Nar* I was transcribed in the absence (lanes 1, 4, and 7) or presence of 100 ng (lanes 2, 5, and 8) or 200 ng (lanes 3, 6, and 9) of pMrE13. The recombinant plasmids were added simultaneously to the reaction (lanes 1–3), or pMrE13 (lanes 4–6) or pMrWT (lanes 7–9) was preincubated with extract proteins for 10 min at 30°C before the second plasmid DNA was added and transcription was started by the addition of nucleoside triphosphates. The amount of total competitor DNA was adjusted to 200 ng with pUC9 DNA.

inhibition of transcription occurred. In contrast, when pMrWT was preincubated in the extract to form stable preinitiation complexes (17), it became resistant to the effects of subsequently added competitor DNA (lanes 7–9). This shows that the transcription factor(s) interacts less stably with the 140-bp repeats than with the promoter, and the competitive effect of the 140-bp repeats is lost after transcription initiation complexes have been formed.

The degree of competition depended on the number of repeats present in the competitor DNAs. The negative effect on transcription was cumulative, becoming weaker as the number of elements decreased from 13 to 3 (Fig. 3*A*). Plasmids containing 10 repeats of the 140-bp element (pMrE10, lanes 5–7) or only 3 repeats (pMrE3, lanes 8–10) competed less efficiently than pMrE13 (lanes 2–4). Quantification of the transcripts revealed that the inhibition was proportional to the number of repeats present. This correlation between the number of the repetitive elements and the degree of competition was observed not only with the mouse but also with the frog repeats (Fig. 3*B*). This finding suggests that (*i*) each repeated spacer element binds an essential

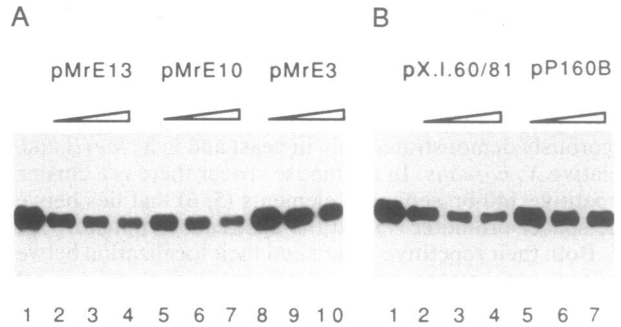

FIG. 3. Trans competition by different numbers of the murine and frog repeats. (*A*) pMrWT (25 fmol, 50 μg) linearized with *Nar* I was transcribed in the presence of either 115 fmol (200 ng) of pUC9 (lane 1) or 25 fmol (lanes 2, 5, and 8), 50 fmol (lanes 3, 6, and 9), or 75 fmol (lanes 4, 7, and 10) of the competitor DNAs indicated above the lanes. The amount of total competitor DNA was adjusted to 200 ng with pUC9 DNA. (*B*) *In vitro* transcription was carried out as described in *A* except that 25 fmol (lanes 2 and 5), 50 fmol (lanes 3 and 6), or 75 fmol (lanes 4 and 7) of the competitor DNA pX.I.60/81 (lanes 2–4) or pP160B (lanes 5–7), containing ten or one 60/81-bp repeat, respectively, was added to the reaction mixtures.

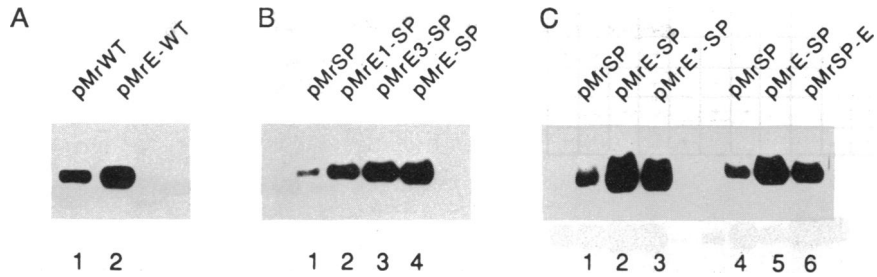

FIG. 4. Stimulation of rDNA transcription in cis by the 140-bp elements. (*A*) Recombinant plasmid (5 fmol) pMrWT (lane 1) and pMrE-WT (lane 2) truncated with *Nar* I were transcribed in standard transcription mixtures containing 150 ng of total DNA. (*B*) Fifteen microliters of the DEAE fraction eluted at 280 mM KCl was used to transcribe 0.5 fmol of pMrSP (lane 1), pMrE1-SP (pMr600, ref. 17; lane 2), pMrE3-SP (lane 3), or pMrE-SP (lane 4). The total amount of DNA in the reactions was brought up to 150 ng by addition of pUC9 vector DNA. (*C*) Recombinant plasmids (5 fmol) pMrSP (lanes 1 and 4), pMrE-SP (lanes 2 and 5), pMrE*-SP (lane 3), and pMrSP-E (lane 6) linearized with *Eco*RI were transcribed with the DEAE fraction in the presence of 150 ng of pUC9 DNA. Truncation of pMrSP-E with *Eco*RI places the 140-bp repeats ≈2.5 kb upstream of the promoter.

transcription factor(s) and (*ii*) binding of this factor(s) to the individual repeats is not cooperative.

The 140-bp Repeats Enhance rDNA Transcription in Cis. If the spacer sequences function as transcription enhancers, they should exert a positive effect on rDNA transcription when linked to the gene promoter. To study cis effects of the repeated elements on rDNA promoter activity, we inserted 13 copies of the 140-bp element at their natural position upstream of the promoter (pMrE-WT and pMrE-SP) and assayed *in vitro* the transcriptional activity of these constructs in parallel to control plasmids without spacer fragments (pMrWT and pMrSP). The templates having the 140-bp repeats attached to the mouse promoter were transcribed 2–3 times better than the constructs containing only the promoter fragment (Fig. 4*A*). This stimulation of transcription was reproducibly observed at optimized DNA concentrations in several different extracts. A higher degree of enhancement was achieved after fractionating the extracts by chromatography on DEAE-Sepharose. As demonstrated before, the fractions eluted at 280 mM KCl contain high levels of RNA polymerase I and the auxiliary transcription initiation factors (15). With these fractions, usually a 4- to 12-fold stimulation of transcription was observed with templates containing the 140-bp repeats.

Next, we examined whether the extent of cis stimulation also correlates with the number of repeats, or whether one repeat is sufficient to exert the full stimulatory effect. At low template concentrations the stimulating effect became stronger as the number of repeats fused to the promoter increased from 1 to 13 (Fig. 4*B*). Apparently, the more factor molecules that are bound to the repeats the more transcripts are initiated.

By definition, transcription enhancers act independently of orientation and over far distances. According to this definition the 140-bp repeats are true polymerase I enhancer elements. They stimulate transcription in both orientations although the degree of enhancement is reproducibly slightly lower in the reverse (pMrE*-SP) as compared to the correct (pMrE-SP) orientation (Fig. 4*C*). Furthermore, augmentation of transcription is still observed when the repeats are moved 2.5 kb upstream of the initiation site (pMrSP-E). However, the degree of enhancement is higher and less variable if the 140-bp elements are placed in the correct position next to the transcription start site.

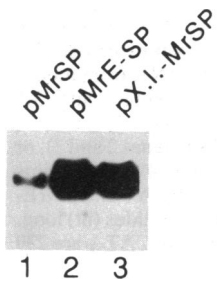

FIG. 5. The *Xenopus* 60/81-bp repeats enhance mouse rDNA transcription. The indicated templates (5 fmol) were transcribed in the cell-free system containing 15 μl of the DEAE fraction and 150 ng of pUC9 DNA.

The *Xenopus* 60/81-bp Elements Stimulate Mouse rDNA Transcription. As shown above, mouse rDNA transcription is sensitive to competition by both the 140-bp elements of the mouse spacer and the 60/81-bp repeats of the *Xenopus* spacer, suggesting that the same factor may interact with the frog and mouse enhancer. We therefore studied whether the frog 60/81-bp repeats would enhance mouse rDNA transcription when attached to the mouse promoter in cis. For this, the block of ten 60/81-bp repeats was inserted upstream of the mouse promoter (pX.I.-MrSP) and the template activity of this construct was assayed in parallel to pMrE-SP, the plasmid containing the mouse 140-bp repeats. Interestingly, the heterologous enhancer also stimulated mouse rDNA transcription (Fig. 5). When fractionated extracts were used the activity of the template containing the frog enhancer was usually significantly higher than that of the parental clone lacking the repeats. However, it generally showed a somewhat lower level of enhancement than the homologous construct containing the mouse repeats, suggesting that the factor(s) responsible for enhancement has differing affinities for the 140- and 60/81-bp elements.

The 140-bp Repeats Interact Cooperatively with the Promoter. That the 140-bp repeats reduce transcription initiation from the rDNA promoter when present on a separate plasmid, and stimulate transcription when present on the same template in cis, implies a functional cooperativity between the promoter and the enhancer. To elucidate the molecular mechanism of this complex interaction, the ability of different rDNA constructs to sequester transcription factors was measured by simultaneously incubating two templates that contained either the promoter (pMrSP) or the promoter plus the enhancer (pMrE-SP) with various competitor DNAs (Fig. 6). The transcripts derived from the test and the competitor rDNAs can be distinguished from each other by the different lengths of the run-off RNAs (158 and 297 nucleotides for pMrWT and pMrSP, respectively). The competitors used were pMrE13 containing the block of 140-bp repeats and pMrWT plus or minus the enhancer linked to the promoter. Addition of a 2-fold molar amount of pMrE13 to the template lacking the enhancer (pMrSP) reduced transcription about 3-fold (Fig. 6, lane 5). Mixing the two rDNA templates pMrSP and pMrWT resulted in equal transcription of both DNAs (lane 6), which was further reduced by the presence of pMrE13 in trans (lane 7). However, when the enhancer-containing fragment was linked to the promoter (pMrE-WT), transcription of the reference plasmid pMrSP was almost completely suppressed whereas the other template was pref-

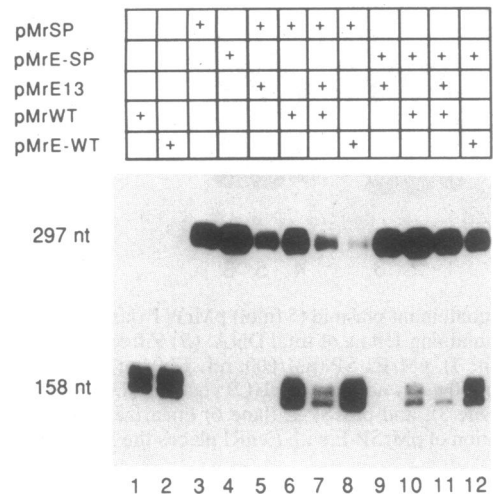

FIG. 6. Cooperative interaction between the 140-bp repeats and the rDNA promoter. Test plasmids (20 fmol) pMrSP (lanes 5–8) and pMrE-SP (lanes 9–12) were transcribed with extract proteins in the presence of 40 fmol of pMrE13 (lanes 5, 7, 9, and 11), pMrWT (lanes 6, 7, 10, and 11), or pMrE-WT (lanes 8 and 12). Lanes 1–4 show the transcriptional activity of 20 fmol of pMrSP and pMrE-SP in the absence of competitor DNAs and of 40 fmol of pMrWT and pMrE-WT. The total amount of DNA was brought up to 175 ng by addition of linearized pUC9. The length of the run-off transcripts derived from the template DNA pMrSP and pMrE-SP is 297 nucleotides (nt), and from pMrWT and pMrE-WT, 158 nt.

erentially transcribed (lane 8), indicating that a cooperative interaction between promoter and enhancer accounts for the transcriptional advantage of the plasmids bearing the 140-bp repeats. When the reciprocal experiment was performed—i.e., the enhancer was fused to the test plasmid (pMrE-SP, lanes 9–12)—the 140-bp repeats alone or together with a separate promoter DNA did not significantly inhibit transcription. Reduction of transcription of pMrE-SP accompanied by a concomitant increase in 158-nucleotide transcripts generated from the competitor template was observed only if both templates had the enhancer fragment attached to the promoter (lane 12). This result suggests that the enhancer either facilitates the assembly of the initiation complex or stabilizes this complex.

Upstream Promoter Elements Are Not Required for Enhancement. Sequences located upstream of the core promoter, including a terminator element (T_0, between nucleotides −171 and −154) and the upstream control element (UCE, between nucleotides −144 and −112), positively affect transcription initiation (8, 9, 15, 18, 19). To get insight into the mechanism of enhancer function we tested whether the stimulatory effect of the 140-bp repeats is exerted via these distal sequence elements. pMrEΔ-168 is similar to pMrE-WT except that the upstream terminator T_0 was inactivated by deleting 3 nucleotides from the *Sal*-box terminator sequence (8). This construct showed the same degree of transcription stimulation as pMrE-WT (Fig. 7, lanes 1–3). Thus transcriptional enhancement does not require a functional upstream terminator.

Next, the block of repeats was fused to a series of 5′-deletion mutants to find out whether the enhancement is mediated by the UCE, the core, or both promoter elements. The transcriptional activity of the enhancerless deletion mutants was progressively reduced when nucleotides beyond position −144 were removed (Fig. 7, lanes 4, 6, 8, and 10). When the analogous constructs containing the enhancer repeats were assayed in parallel with their parental clones, each of the individual mutants was stimulated by the 140-bp repeats (lanes 5, 7, 9, and 11). Even pMr-Δ39, which showed only about 20% of the template activity of the wild-type

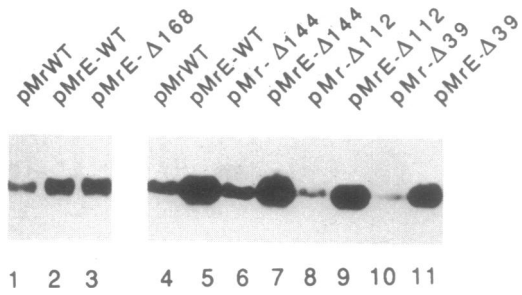

FIG. 7. Enhancement is mediated via the core promoter. Individual templates (5 fmol) linearized with *Nar* I were complemented with 150 ng of pUC9 DNA and were transcribed in the presence of a mixture of S-100 and nuclear extract proteins (lanes 1–3) or the DEAE fraction (lanes 4–11).

control, was efficiently stimulated by the enhancer. Therefore, the stimulating effect of the 140-bp repeats involves neither the function of the upstream terminator nor the presence of the UCE, but these elements appear to exert their activating effect via the core promoter.

Enhancement of rDNA Transcription Is Also Observed in Whole Cells. We tested the ability of the 140-bp repeats to enhance rDNA transcription in whole cells. For this, mouse Ltk$^-$ cells were transfected with different plasmid DNAs ($pMrCAT_{SP}$, pMrCAT, and pMrE-CAT) carrying the rDNA promoter and a foreign reporter gene fragment but differing by the absence or presence of the 140-bp repeats. The different length of the CAT gene fragments present in the constructs allowed a distinction of transcripts derived from the individual templates. The transiently expressed CAT-specific RNA was quantified by primer extension (Fig. 8). By cotransfection of two constructs lacking the enhancing elements (pMrCAT and $pMrCAT_{SP}$), the transcriptional activity of both promoters was roughly balanced (lane 3). However, when $pMrCAT_{SP}$ was cotransfected with pMrE-CAT, the plasmid containing the 140-bp repeats, the enhancer-bearing construct was preferentially transcribed (lane 4), indicating that the stimulatory effect of the 140-bp repeats observed *in vitro* is also functioning *in vivo*.

DISCUSSION

The results show that an enhancer for polymerase I transcription is present in the intergenic spacer of a mammalian rDNA transcription unit and that it is analogous in function to the repetitive 60/81-bp elements in the spacer of *X. laevis* rDNA (1, 11, 20, 21). Like the frog repeats the murine 140-bp elements exert a negative effect on the rRNA gene promoter when added to the transcription reaction in trans because they compete for one or more auxiliary factors required for transcription initiation. When fused to the murine rDNA promoter, plasmid DNAs containing these 140-bp elements in cis show significantly increased transcriptional activity.

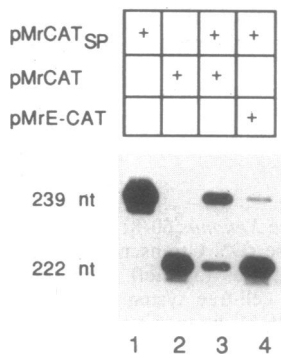

FIG. 8. The 140-bp repeats enhance rDNA transcription in whole cells. Primer extension was performed with RNA from mouse Ltk$^-$ cells transfected with 3.5 pmol of $pMrCAT_{SP}$ (lanes 1, 3, and 4), pMrCAT (lanes 2 and 3), or pMrE-CAT (lane 4). Transcripts derived from pMrCAT and pMrE-CAT are 222 nucleotides (nt) long, and those from $pMrCAT_{SP}$ are 239 nt long.

When two similar rDNA constructs that differ in the absence or presence of the block of 140-bp elements are simultaneously transfected into mouse cells, a preferential transcription of the enhancer-containing plasmid is observed, indicating that the 140-bp repeats confer a strong transcriptional advantage on the rDNA promoter in cis.

Interestingly, this stimulation of initiation by the enhancer repeats is also reproduced in a cell-free transcription system. In this system the 140-bp repeats stimulate transcription from an adjacent rDNA promoter. The degree of stimulation depends on the extract preparation and on the relative amount of template and nonspecific competitor DNA present in the assay. In fractionated extracts, enhancement is considerably higher (up to 12-fold) as compared to unfractionated extracts, which may be due to removal of inhibitory proteins and/or increasing the concentration of essential factors. Since enhancement is not observed at high template concentrations, we suggest that some limiting factor is required for transcription activation. Differential transcriptional activity is observed only at low amounts of template DNA. This dependence of enhancement on the DNA concentration is in accord with competition experiments demonstrating that a common factor(s) binds to both the rDNA promoter and the enhancer. However, when a promoter-containing plasmid is preincubated with extract before addition of the 140-bp repeats, it remains resistant to trans competition, indicating that (*i*) the common factor binds more stably to the promoter than to the enhancer and (*ii*) the enhancer acts before or during the establishment of stable transcription initiation complexes. We suggest that the 140-bp repeats augment the formation of preinitiation complexes by attracting rDNA transcription factors and interacting with the promoter via proteins bound to the DNA. The data shown in Fig. 6 support this hypothesis by demonstrating a cooperative interaction between the promoter and the enhancer repeats. In this scenario, the DNA between the promoter and the enhancer is thought to loop out, a model that has been implicated in the mechanism of transcriptional stimulation of RNA polymerase II genes by distant enhancer elements. Experimental support for the looping model of enhancer function has been provided by Müller *et al.* (22), who demonstrated that an enhancer from simian virus 40 or human cytomegalovirus can stimulate transcription even when noncovalently linked to the β-globin promoter via a protein bridge. Even the stimulation of the frog promoter by the 60/81-bp elements can be observed in trans when both elements are brought in close proximity by concatenating two plasmids containing the promoter and the enhancer, respectively (23).

Although the present study did not identify the target sequences and the factors that are responsible for trans competition and cis stimulation, we postulate that the murine upstream binding factor (mUBF) is involved in this process. This polymerase I transcription factor has been purified from human and *Xenopus* cells (termed hUBF and xUBF). hUBF and xUBF differ in size but recognize functionally analogous regions within the *Xenopus* enhancer and the rDNA promoter from both species, despite extensive sequence divergence of their binding sites (11, 24). Interestingly, hUBF and xUBF are not functionally interchangeable in terms of transcription activation. This result indicates that the two factors share a similar DNA-binding domain, but this similarity does not extend to the regions of the protein required for transcriptional activation. On the other hand, we have found that the frog and mouse enhancers can functionally complement each other. The 60/81-bp repeats both compete for an essential mouse factor(s) and also stimulate transcription when fused to the mouse promoter. Although the degree of stimulation by the frog elements is generally more variable and less pronounced in crude extracts than stimulation by the homologous mouse repeats, it has been reproducibly observed in a number of experiments. This activation by the heterologous enhancer indicates that binding of UBF to the murine or frog elements is involved in transcriptional activation. Whether or not UBF on its own is sufficient for enhancement is not known. However, recent preliminary results suggest that other transcription factors in addition to UBF are required for enhancement. First, we did not succeed in overcoming the enhancer-dependent trans competition by addition of partially purified UBF. Second, when extracts were fractionated on a DEAE column the degree of enhancement varied depending on the template used. Constructs containing either the frog or the mouse enhancer elements were differently activated by individual column fractions (unpublished results). Therefore, we conclude that other factors, which have different affinities for the frog and mouse sequences, need to associate with UBF to form a productive complex, which in turn mediates enhancer function.

The availability of an *in vitro* system in which transcription is responsive to the 140-bp elements may greatly help to dissect the mechanism of enhancer function. It will also facilitate studies on the functional interrelation of the different regulatory elements present in the intergenic spacer (i.e., spacer promoter, enhancer, upstream terminator, and upstream and core promoter elements). As a first step in this direction we have inserted the block of enhancer repeats into rDNA constructs in which part of the upstream terminator T_0 or the UCE was deleted. Transcription of these constructs was increased by the 140-bp repeats similarly as the control templates, indicating that neither of the two elements is required for enhancement. Therefore, enhancer-mediated augmentation of transcription probably occurs via the core rather than upstream regulatory elements.

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