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The ribosomal spacer in *Xenopus laevis* is transcribed as part of the primary ribosomal RNA

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ABSTRACT

S1 mapping of *Xenopus laevis* ribosomal RNA transcripts, both in oocyte microinjection experiments and *in vivo*, shows that all but 212 bp of the so-called "non-transcribed" spacer (NTS) of the ribosomal DNA repeat is transcribed as part of the primary ribosomal transcript. The 40S pre-ribosomal RNA (pre-rRNA) is therefore a processing intermediate. The primary ribosomal transcript co-terminates with the previously described spacer transcripts [Moss (10)], at a site 213 bp upstream of the 40S pre-rRNA initiation site. This mode of transcription suggests a simple mechanism for the recently proposed phenomenon of "readthrough-enhancement", [Moss et al (9), Moss (10)], where readthrough transcription from an upstream gene may enhance transcription of a gene immediately downstream in the tandem ribosomal repeat.

INTRODUCTION

The gene coding for the 18S, 5.8S and 28S ribosomal RNAs (rRNAs) in eukaryotes are transcribed as part of a single oligocistronic transcript, (see refs 1, 2 for early reviews). For *Xenopus laevis* and other amphibia the "Miller" spread (3-5) elegantly supported earlier biochemical arguments that the tandemly repeated transcribed regions were separated from eath other by "non-transcribed" spacers (NTS). In agreement with these data the primary ribosomal transcript in *X. laevis* was identified as a 40S pre-rRNA molecule which terminated at the end of the 28S coding sequence (6,7). Although it was recently discovered that pre-rRNA transcription continued a little downstream of the 28S gene (8), this did not change the general view that a large untranscribed stretch of DNA separates consecutive transcription units. Even the earlier discovery of active RNA polymerase I promoters in the ribosomal DNA, (rDNA), NTS of *X. laevis*, *Drosophila melanogaster* and *D. virilis*, (see ref. 9 for review) did not affect this view.

It has been shown that the *X. laevis* ribosomal (rDNA) spacer sequences lying between the first spacer promoter or Bam Island I, (SpPr 1 in fig. 1) and the 40S promoter proximal termination site at -213 bp, (T in fig.

1) are transcribed (10). Thus if the 40S pre-rRNA transcript is terminated, this must occur either upstream of the first spacer promoter or at the promoter proximal -213 bp site. We have found that in oocyte microinjection experiments >40% of the transcripts initiated from the 40S pre-rRNA promoter on pXℓ108c (and derivative clones), run through the vector region of these circular (plasmid) constructs and back into the rDNA insert, (Mitchelson and Moss, in preparation). These transcripts are designated pre-rRNA in fig. 1. Hence transcription on each of these rDNA plasmids resembles that on the tandemly repeated rDNA of the *X. laevis* chromosome. It has also been shown that under the conditions used for microinjection, no detectable level of transcription is initiated at any site(s) other than the already identified polymerase I promoters (op. cit.). This situation was therefore used to study the effects of deleting putative termination sequences on the transcription of downstream NTS sequences. The results show that all but 212 bp of the ribosomal NTS in *X. laevis* is transcribed as part of the primary ribosomal transcript. Mapping of RNA from *X. laevis* tissue culture cells and oocytes shows that this mode of transcription also occurs *in vivo*.

MATERIALS AND METHODS

Enzymes

Restriction enzymes were obtained from Boehringer Mannheim, DNA T4 ligase from New England Biolabs, T4 DNA polymerase, T4 polynucleotide kinase and S1 nuclease from P-L Biochemicals. Enzymes were generally used as recommended by the suppliers.

Construction recombinant plasmids

pXℓΔNS9c, pXℓΔNS10c and pXℓΔNS11c were all constructed by restricting pXℓ108c with BamH1 totally, (for pXℓΔNS9c), or partially, (for pXℓΔNS10c and pXℓΔNS11c), followed by religation and transformation into E. coli HB101. pXℓΔNS13f was constructed by totally restricting pXℓ108f with HindIII and BamH1. pXℓ108f is a vector deletion mutant of pXℓ108c lacking the pBR322 HindIII-PvuII fragment and contains only the two BamH1 sites in the rDNA insert (11). The two HindIII-BamH1 fragments of pXℓ108f were separated on low melting agarose. The smaller fragment was totally digested with Hinf1, the HindIII-Hinf1, Hinf1-BamH1 and larger Hinf1-Hinf1 fragments isolated, ligated into the larger HindIII-BamH1 fragment from pXℓ108f and transformed into E. coli HB101. As the HindIII-Hinf1 and Hinf1-BamH1 fragments could not self ligate due to the different sequences of the Hinf1 sites, only the Hinf1-Hinf1 fragment was included in the recombinant plasmid. Transformants were

analysed by restriction enzyme analysis. The resultant recombinants are shown in figure 1.

Probes

Probes specific for 40S pre-rRNA transcripts and spacer promoter initiated transcripts have been described previously (10,12). In brief, the 40S pre-rRNA specific probe is the PstI-TaqI fragment crossing the 40S initiation site. This probe was also used for detecting the 3' terminus of spacer transcripts by labelling the Pst1 end as described previously (10), Fig. 1. The probe specific for spacer promoter initiated transcripts is the BamH1-AvaII fragment from the first spacer promoter (12), (see also Fig. 1). This fragment was cloned and recovered with the smaller EcoR1-BamH1 fragment from pBR322 as an extension. A second spacer transcript probe was constructed by cloning the AvaII-AvaII fragment from the first spacer promoter linked to the larger BamH1-PvuII fragment of pBR322. Fragment and vector were filled out with T4 DNA polymerase before ligation. In this way, at the PvuII-AvaII junction the AvaII site was restored, whereas at the BamH1-AvaII junction the AvaII site was destroyed. By choosing the appropriate orientation of the AvaII fragment, it was then possible to recover a DdeI to AvaII fragment containing the original AvaII-AvaII rDNA fragment but extended by vector sequences. When 5' end labelled, this fragment could distinguish transcripts reading through the spacer promoter I from those reading through more downstream spacer promoters, see fig. 4. DNA 5' and 3' end labelling with ^{32}P have previously been described (10,11,12).

Microinjection and transcript detection

Equimolar amounts of plasmid were microinjected in *X. borealis* oocytes in the presence of α-amanitin (BCL) and transcripts were detected by S1-nuclease mapping as described previously (10,11,12). Endogenous tissue culture and oocyte transcripts were also detected by S1 mapping as previously described (10,11,12).

RNAase sensitivity of transcripts

After hybridisation to the plasmid extended BamH1-AvaII probe, the samples were incubated under the standard S1 digestion conditions for 2 hours at 45°C with 12.5U of S1 nuclease in the presence or absence of 50 µg of RNase (Sigma).

RESULTS AND DISCUSSION

Deletion mutants of pXℓ108c and a derivative, pXℓΔNS11c, fig. 1, were created and assayed for their capacity to terminate 40S transcription

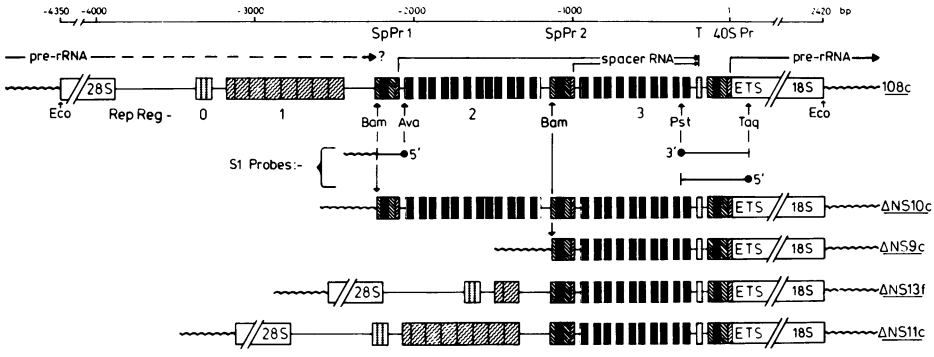
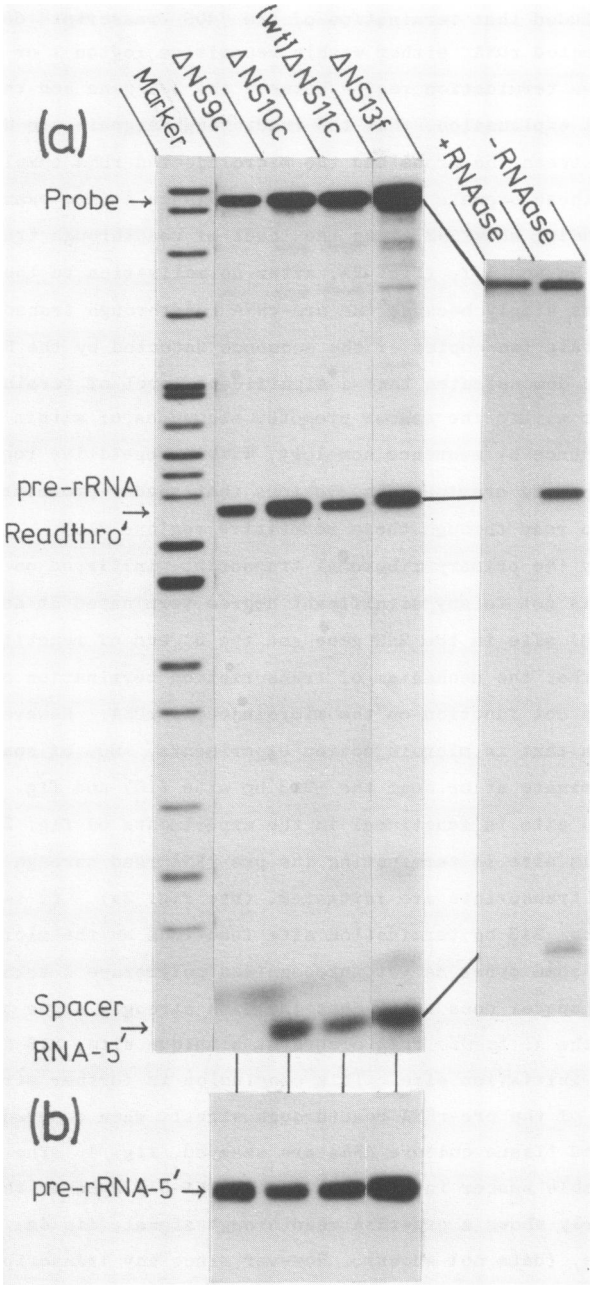


Figure 1. Structure of the *X. laevis* rDNA spacer of pXℓ108c and derived spacer mutants. Shading indicates common sequences, a wavy line, the plasmid vector and SpPr, 40S Pr and T respectively the spacer and 40S pre-rRNA promoters and the -213 bp terminator. 28S and 18S indicate the gene regions, ETS the external transcribed spacer and Rep Reg the repetitive sequence regions, which are numbered. Eco, Bam, Ava, Pst and Taq refer to the restriction enzymes, EcoR1, BamH1, AvaII, PstI and TaqI. The position of the terminal ^{32}P label in the S1 probes is indicated by a solid circle.

after microinjection into *X. borealis* oocytes. All transcripts reading through the putative region of 40S pre-rRNA termination and into the first spacer promoter were detected using a cloned BamH1 to AvaII probe, derived from the first spacer promoter, which had been extended at the BamH1 site with a pBR322 derived sequence, fig. 1. This probe could not however distinguish transcripts which read through the first spacer promoter from those reading through subsequent downstream spacer promoters. To avoid this problem the derivative pXℓΔNS11c, which has a unique spacer promoter and in all transcription assays is indistinguishable from the wild type, pXℓ108c, (ref. 11 and De Winter and Moss, in preparation), was used as "wild type" control. Fig. 2b (pre-rRNA-5') shows that the levels of 40S transcription initiating on and hence reading around (see introduction), each mutant and the control plasmid are very similar. As expected,initiation from the unique spacer promoters of pXℓΔNS10c, 11c and 13f can be detected. (Spacer RNA-5' in fig. 2a), but since the spacer promoter of pXℓΔNS9c lacks sequences upstream of the BamH1 site, no transcripts initiate at this promoter (11). Transcripts reading through to the unique spacer promoter of pXℓΔNS11c can also be easily detected, (pre-rRNA readthro' in fig. 2a). However, neither deletion of most of repetitive region 1, as in pXℓΔNS13f, nor deletion of the complete EcoR1 to BamH1 region, containing all sequences upstream of the

first spacer promoter and including part of the 28S coding region, as in pXℓΔNS9c, causes any increase in the level of this readthrough transcription, (+/-30% after normalisation to the 40S pre-rRNA signals in fig. 2b). Hence it must be concluded that termination of the "40S transcript" does not occur on the microinjected rDNA, either within repetitive region 1 or within the complete putative termination region between the 28S gene and the first BamH1 site. A trivial explanation, that the readthrough signals are due to hybridisation between the probe and the microinjected rDNA templates, can be excluded since these signals are sensitive to RNAase A (see example in fig. 2a). pXℓΔNS10c shows >2 times the level of readthrough transcription seen on ΔNS11c, 9c and 13f, (fig. 2a, after normalisation to the pre-rRNA 5' signal). This is simply because the pre-rRNA readthrough transcripts on ΔNS10c each contain two copies of the sequence detected by the Bam-Ava probe. This observation demonstrates that a significant level of termination does not occur either within the spacer promoter sequences or within repetitive region 2, (and hence by sequence homology, within repetitive region 3, fig. 1), and supports previous observations that spacer promoter derived transcripts also read through these repetitive regions (10).

Clearly the primary ribosomal transcript, initiated on the micro-injected rDNA, is not to any significant degree terminated at any point(s) between the EcoR1 site in the 28S gene and the 3' end of repetitive region 3. It is possible that the mechanism of transcription termination occurring *in vivo* simply does not function on the microinjected rDNA. However we have previously shown that in microinjection experiments, >90% of spacer transcripts terminate at or near the -213 bp site (10) and fig. 3 shows that this termination site is functional in the experiments of fig. 2. In fact on pXℓΔNS9c, this site is terminating the pre-rRNA read through transcripts, since no spacer transcripts are initiated, (see fig. 2a). It is extremely unlikely that the -213 bp termination site functions on the microinjected template, while some other as yet unrecognised polymerase I termination site within the rDNA spacer does not. Thus the data strongly argue that termination on the *X. laevis* rDNA occurs at a unique site, 213 bp upstream of the pre-rRNA initiation site. This conclusion is further strengthened by the observation of the pre-rRNA readthrough signal, when endogenous *X. laevis* oocyte and tissue culture RNAs are assayed, fig. 4. The oocyte RNA shows no detectable spacer initiation (Spacer RNA-5') even at the highest RNA input, but clearly shows a pre-rRNA readthrough signal, fig 4a, which is RNAase sensitive, (data not shown). However since any transcripts initiated


Marker
ΔNS9c
ΔNS10c
(wt)ΔNS11c
ΔNS13f
+RNAase
-RNAase
(a)
Probe →
pre-rRNA
Readthro'
Spacer
RNA-5'
(b)
pre-rRNA-5'→

at the spacer promoters of the endogenous rDNA, read through the more downstream spacer promoters, in the case of the tissue culture RNA the BamH1-AvaII probe detects the sum of tissue pre-rRNA readthrough and spacer initiated transcripts. Thus a further probe, the AvaII to AvaII fragment from spacer promoter I of pXℓ108c, see fig. 1 and fig. 4c, was cloned in pBR322, such as to reconstruct only the downstream AvaII site (Materials and Methods). Using this probe, the transcripts reading into the spacer promoter I, (the true pre-rRNA readthrough), form hybrids 24 bp longer than those which read through more downstream spacer promoters, such as SpPr2 of pXℓ108c in fig. 1. Fig. 4b then shows that a true pre-rRNA readthrough signal can be detected (Readthro' 1). Thus it can be concluded that a normal mode of *in vivo* transcription is for the pre-rRNA transcript to read through the downstream rDNA spacer.

DNA sequence comparisons have shown that sequences surrounding the -213 bp termination site of the *X. laevis* rDNA, (T in fig. 1), are also found in exactly analogous positions in the rDNAs of *X. borealis* and *X. clivii* (9). The putative termination sequence consists of two distinct boxes, having a consensus sequence :

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  AAA
G   TTTnnCAaAgTG(13-14 bp) GACtTGCnCG
  TTT
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After scanning the spacer sequence of pXℓ108c, (of which all but ~40bp are known, see ref. 9), a single weak homology to the upstream "termination" box was found to occur within each RNA polymerase promoter sequence at -27 bp relative to the initiation base. Labhart and Reeder (13) noted that an homology to the downstream "termination" box is found close to an RNA processing site, 251 bp downstream of the 28S gene coding sequence. We have noted a further weak homology to this box, "GACcTG", occurring at the 5' boundaries of repetitive regions 2 and 3. The significance of these homologies, if any, cannot be judged at present. However the lack of an homology to both the -213 bp "termination" boxes at any one site supports

Figure 2. S1 mapping of the rDNA spacer transcripts from the microinjected control ("wt"), and mutant plasmids. a, the pre-rRNA readthrough transcript, (pre-rRNA Readthro') and the 5' terminii of the spacer promoter initiated transcripts, (Spacer RNA-5'). b, the 5' terminus of the 40S pre-rRNA transcript, (pre-rRNA-5'). + and - RNAase refers respectively to the mapping of pXℓΔNS13f derived transcripts in the presence or absence of RNAase. In (a) the RNAs were assayed with the 5' end-labelled, plasmid extended, BamH1-AvaII probe and in (b) the same RNAs were assayed with the 5' end-labelled PstI-TaqI probe, see fig. 1. The "Marker" track contains pBR322 restricted with HpaII.

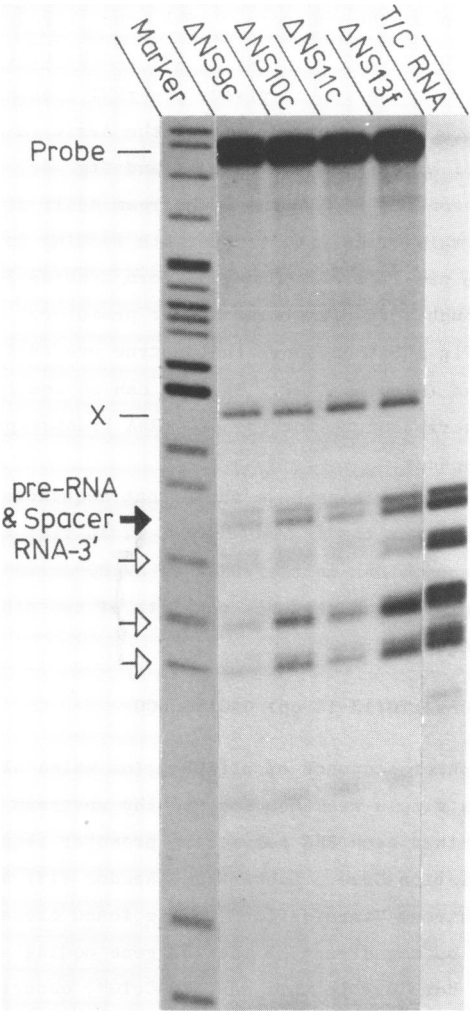

Figure 3. The termination of spacer and pre-rRNA readthrough transcripts at the -213 bp site on the microinjected rDNA. The solid arrow indicates transcription termination and the open arrows, 3' terminii probably derived from internal cutting of the RNA-DNA hybrids, see ref (10). "X" refers to a probe-derived artifact band found to be independent of input RNA. The RNA preparations used were the same as those mapped in Fig. 2. T/C RNA is 4 µg of RNA from an *X. laevis* tissue culture line (10). All were S1 mapped with the 3' end-labelled PstI-TaqI probe shown in fig. 1. Marker as in fig. 2.

the conclusion that the *X. laevis* rDNA repeat contains only one termination site.

Transcription of the ribosomal "NTS" as part of the primary ribosomal transcript is probably not peculiar to *X. laevis*. Recent data strongly

suggest that this is also the case for *D. melanogaster* (14), although in this case termination does not occur at a unique site. Thus it may be a common feature of the ribosomal genes of eukaryotes that some or most of the "NTS" constitutes a second external transcribed spacer (ETS). It would then be better to rename this part of the "NTS" the 3'-ETS and to distinguish it from the present ETS, ie the leader sequence of the 40S pre-rRNA, by referring to this as the 5'-ETS. In mouse the available data suggest that the 45S pre-rRNA is terminated proximal to the 28S coding sequence giving rise to a short 3' ETS (15,16). Hence the large rDNA spacers found in mammals may function differently to those in other eukaryotes.

CONCLUSION

The mode of ribosomal transcription described here, necessitates some reappraisal of data previously obtained on the *X. laevis* rDNA. Firstly the interspersion of the "Christmas-tree" pattern of transcriptionally active DNA with "smooth", untranscribed DNA, as seen on electron micrographs of oocyte rDNA (3-5), must be due to rapid processing of the pre-rRNA just downstream of the 28S gene coding region. Why polymerase molecules are only rarely seen in the 3'-ETS on electron micrographs (17) is puzzling. Possibly 3'-ETS transcription complexes are not well crosslinked because they are associated with too little reactive protein. Early electron microscope studies of microinjected rDNA (18,19) showed that on transcriptionally active plasmids, the chromatin structure of vector sequences was "beaded", ie had obvious nucleosome structure, while the 3'-ETS (NTS) did not show such structures and was therefore typical of the endogenous 3'-ETS. It now seems extremely likely that both vector and 3'-ETS sequences were equally well transcribed in these experiments. Hence the difference in chromatin structure probably did not reflect a difference in transcriptional activity, but in DNA function, base sequence or both of these. We and others have proposed models for the function of the *X. laevis* rDNA spacer, (see refs. 9 and 20 for reviews). These models require little or no modification to be compatible with the mode of transcription described here. We have suggested that polymerase molecules completing transcription of one rDNA gene unit, can be captured and recycled by a subsequent unit and this process has been called "readthrough-enhancement", (refs. 9,10). Since it is now clear that on the *X. laevis* rDNA, polymerase molecules transcribe each gene repeat to within 212 bp of the downstream pre-rRNA promoter, it is perhaps feasible for this promoter to recapture the polymerase. Readthrough-enhancement could then

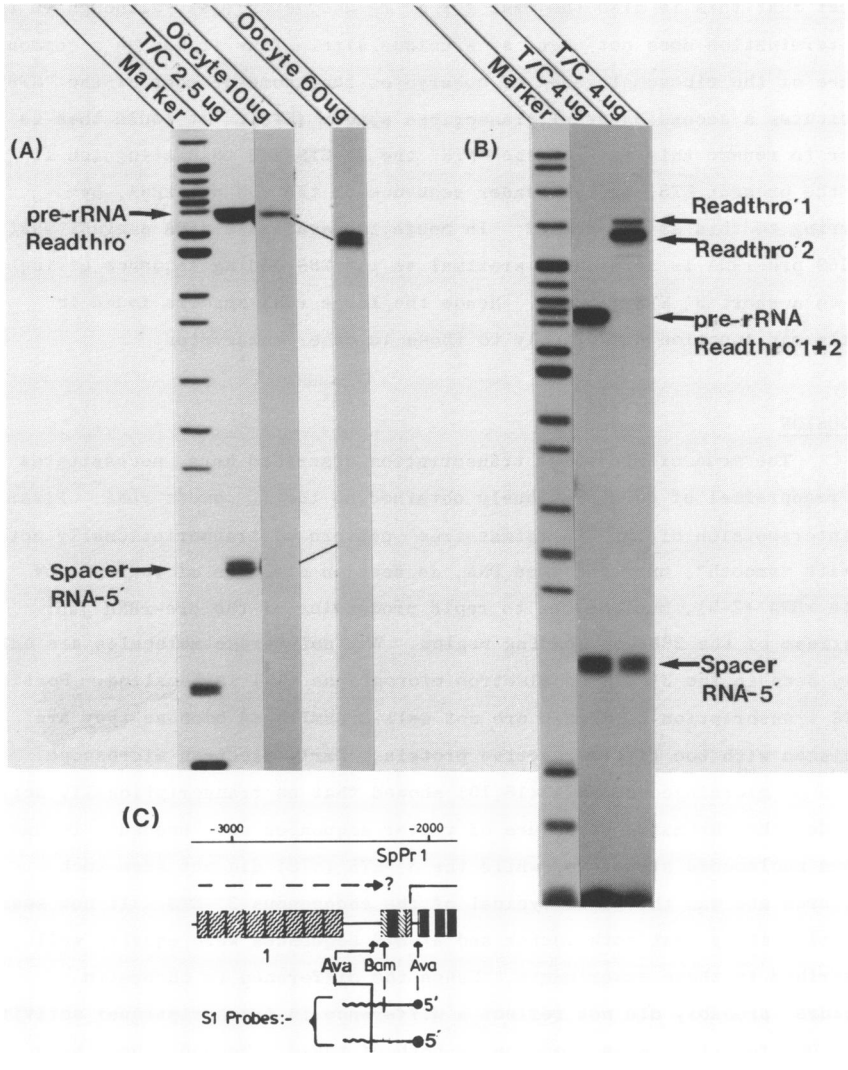

Figure 4. Pre-rRNA readthrough on the endogenous rDNA of *X. laevis* oocytes and tissue culture cells. (A) 2.5 ugm of tissue culture RNA (T/C) and 10 or 60 ugm of oocyte RNA were S1 mapped, as in figure 2, with the 5' end-labelled, plasmid extended, BamH1-AvaII probe, see figure 1 and (C) below. (B) 4 ug of tissue culture RNA was S1 mapped with the same 5' end labelled, plasmid extended BamH1-AvaII probe or a plasmid extended AvaII-AvaII probe also derived from spacer promoter I of pXℓ108c, see fig. 1 and (C) below. (C) The extent of the cloned probes used in (A) and (B). Nomenclature is as in figs. 1 and 2 except that in (B) Readthro' 1 and 2 refer respectively to pre-rRNA transcripts crossing spacer promoter 1 and those crossing subsequent spacer promoters. pre-rRNA Readthro' 1+2 is the sum of Readthro' 1 and Readthro' 2 when T/C RNA is mapped with the plasmid extended BamH1-AvaII probe.

occur because, after terminating transcription at the -213 bp site, the polymerase slides along the DNA to the nearby promoter. If however the polymerase is released from the DNA at termination, there may still be a high probability of it being recaptured by the proximal promoter. By causing premature termination of the pre-rRNA we have recently shown that readthrough-enhancement occurs on microinjected rDNA templates, (Mitchelson and Moss, in preparation). In *D. melanogaster* at least some polymerase molecules may transcribe more than one ribosomal cistron without terminating (14). If this is a major pathway, it is yet another mechanism by which ribosomal expression can be enhanced.

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