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**The origin of the rRNA precursor from *Xenopus borealis*, analysed *in vivo* and *in vitro***

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**ABSTRACT**

We have determined the origin of the major transcript of *Xenopus borealis* rDNA by the use of an SI nuclease protection assay. The DNA surrounding the origin of this transcript was sequenced, and the region upstream of the origin was shown to have strong sequence homology with that region from *X.laevis* rDNA. We have also demonstrated faithful transcription from this origin using cloned *X.borealis* rDNA in an extract derived from *X.laevis* culture cells. This *in vitro* transcription was insensitive to 100µg/ml  $\alpha$ -amanatin, suggesting that it was mediated by RNA polymerase 1.

**INTRODUCTION**

The genes for the large ribosomal RNA's (rRNA) of the genus *Xenopus* have been well studied. They occur as tandemly repeated units comprising a spacer region, and a region that is transcribed to give the 40S rRNA precursor. The regions that code for 28S, 18S and 5.8S rRNA are very similar in sequence. Much of the spacer, however, has diverged considerably between the species(1,2), though significantly there are spacer elements whose organisation and sequence is conserved (3,4). Previously the origin of the 40S rRNA precursor has been precisely mapped for two species, *Xenopus laevis* (5, 34) and *Xenopus clivii* (3). Sequence homologies were observed, extending from the origins to more than 150bp upstream.

An interesting feature of the spacer regions from *Xenopus laevis* and *Xenopus clivii* is the presence of duplications of the sequence that extends about 150bp upstream from the origin of the 40S precursor (3,6). In the spacer of another species, *Xenopus borealis*, Bach *et al* (3) observed five copies of

a 44bp sequence that had homology with the Xenopus laevis and Xenopus clivii origins. They suggested that one of the homologous sequences corresponded to the origin of the Xenopus borealis 40S precursor. In this work we have mapped precisely the origin of the Xenopus borealis 40S precursor and shown it to be some 390bp downstream of the point previously suggested (3). The sequence that extends upstream of the Xenopus borealis origin shows extensive sequence homology with the same region from Xenopus laevis and Xenopus clivii. Moreover, an in vitro transcription system derived from Xenopus culture cells transcribes cloned Xenopus borealis rDNA from this origin, indicating that RNA synthesis commences at this site in vivo.

#### MATERIALS AND METHODS

Culture cells. The Xenopus borealis cell line XB693, was obtained from Prof. J. Picard (Belgium). XB693 is an epithelioid cell line initiated from stage 35 tadpoles (7), and was cultured in 75%  $\alpha$  modified Eagles medium with 10% foetal calf serum, 10mM Hepes (pH 7.9) and antibiotics at 25°C. The Xenopus laevis cell line, a fibroblastic cell line made by Dr. M. Balls (University of Nottingham, England), was cultured in 50% RPMI with 10% foetal calf serum, 10mM Hepes (pH 7.9) and antibiotics, at 25°C.

Isolation of RNA. Total cellular RNA was prepared from XB693 cells using a modified guanidinium isothiocyanate procedure (12). Total RNA was prepared from Xenopus borealis oocytes by homogenizing the oocytes in 10mM Tris (pH 7.5), 10mM NaCl, 1mM MgCl<sub>2</sub>, 2% SDS, 1mg/ml proteinase K (Sigma). Homogenates were incubated for 30 min at 37°C followed by phenol:chloroform and chloroform extractions. The RNA was stored as an alcohol precipitate.

Sequencing. The fragment that was sequenced was cloned as a TaqI fragment into the AccI site of pUC9 (8) and sequenced without recovery from a gel using the procedure of Ruther et al (9). Briefly, pXboriI was cleaved with BamHI and end labelled with the large fragment of DNA polymerase I and  $\alpha$ -P<sup>32</sup>dGTP (Amersham), or with polynucleotide kinase and  $\alpha$ -P<sup>32</sup>ATP

(Amersham). The labelled molecules were then cleaved with EcoRI and sequenced directly by the method of Maxam and Gilbert (10). Gel plates were treated as described by Garoff and Ansorge (11).

In vitro transcription. The in vitro transcription extract was prepared from Xenopus laevis cells essentially as described by Miller and Sollner-Webb (12) for mouse cells. Briefly,  $5 \times 10^8$ - $10^9$  X.laevis culture cells were harvested, washed twice with cold phosphate buffered saline and twice more with a low salt solution (10mM Hepes (pH 7.9), 10mM KCl, 1.5mM  $MgCl_2$ , 0.5mM dithiothreitol). The cells were resuspended in 4mls of low salt solution and allowed to swell on ice for 15 mins. After swelling the cells were homogenized in a 7ml Wheaton homogenizer, sonicated, and one tenth volume of a high salt solution (300mM Hepes (pH 7.9), 1.4M KCl, 30mM  $MgCl_2$ ) was added. This was then spun at 100,000g in a swing out rotor for 2 hrs at 0°C. The supernatant was removed and dialysed overnight against 20mM Hepes (pH 7.9), 20% glycerol, 100mM KCl, 0.2mM EDTA and 1mM dithiothreitol. After dialysis the extract was stored in 25 $\mu$ l aliquots in Eppendorf tubes at -70°C. The extract was still active after at least 8 months storage under these conditions. The in vitro transcription reactions were made up of 10 $\mu$ l extract and 10 $\mu$ l of a reaction buffer that made the final reaction mix 23mM Hepes (pH 7.9), 6mM  $MgCl_2$ , 10% glycerol, 1mM dithiothreitol, 0.1mM EDTA, 100mM KCl, 0.5mM ATP, GTP, CTP, UTP and 10mM creatine phosphate, and usually, 100 $\mu$ g/ml  $\alpha$  amanatin. Plasmid DNA's were added at varying concentrations, usually 20 $\mu$ g/ml. Incubations were at 25°C for 100 mins. Reactions were terminated by the addition of 180 $\mu$ l of 0.15M NaCl, 50mM Tris (pH 8.0), 0.3M Na Acetate, 0.5% SDS, 6mM EDTA and carrier tRNA, extracted with phenol: chloroform, then ethanol precipitated.

SI nuclease protection analysis. Two different hybridization probes were used during the course of this analysis. (a) The HincII/TaqI fragment which spanned the origin of transcription was purified from a dephosphorylated HincII/Taq digest of pXboril (Fig.1B) and run on a 5% polyacrylamide gel. The fragment was diffusion-eluted and stored in 10mM Tris (pH 8.0), 1mM EDTA. (b) The HinfI fragment spanning the origin of transcription was purified from a dephosphorylated HinfI digest of pXbrOT (Fig.1C)

and run on a 3.5% polyacrylamide gel.

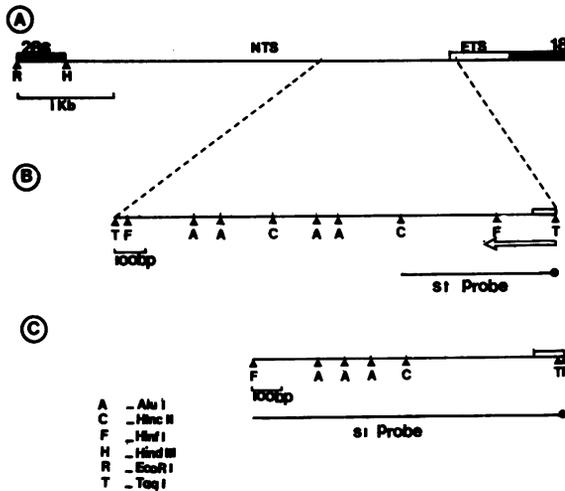
1 picomole of the fragments were labelled at their 5' termini with polynucleotide kinase and  $\gamma$ - $P^{32}$ ATP (5000 Ci/mmol, Amersham) to specific activities of 1 - 3 x 10<sup>6</sup> counts per minute/picomole of ends. The HincII/TaqI fragment was denatured in 0.2N NaOH and the coding strand was separated on a 7% 50:1 acrylamide:bis gel in 100mM Tris Borate (pH 8.3). The HinfI fragment coding strand was purified on a 5% 50:1 acrylamide: bis gel in 50 mM Tris Borate (pH 8.3) after DMSO denaturation as described by Maxam and Gilbert (10).

Probes (2 - 5 x 10<sup>-3</sup> pmoles/hybridisation) were hybridised to RNA samples in 50% formamide, 6 x SSC (40 $\mu$ l) for 15-18 hrs at 65°C. Then 360 $\mu$ l of 0.11M NaCl, 30mM Na acetate (pH 4.5), 5mM ZnSO<sub>4</sub>, was added and incubated with 20 units of SI nuclease (BRL) at 37°C for 1 hour. Digestions were terminated with the addition of 80 $\mu$ l of 1M Na acetate, 330mM Tris (pH 9.5), 67mM EDTA and tRNA, then ethanol precipitated. The pellet was resuspended in 95% formamide with dyes, heat denatured at 100°C for 2 mins and run on an 8% polyacrylamide, 7M urea gel. The fragment size marker was pAT153 digested with HpaII and labelled with the large fragment of DNA polymerase I and  $\alpha$ - $P^{32}$ dCTP (Amersham). It should be noted that in all SI nuclease protection assays in this work the probe was in excess over RNA.

## RESULTS

### Mapping of 40S precursor origin of transcription

Macleod and Bird (13) showed by an SI nuclease protection assay that the 5' end of the 40S precursor in Xenopus borealis rDNA was about 2.3kb to the 5' side of the EcoRI site in the 18S coding region. In order to identify the true origin of the 40S precursor we sequenced this region of the rDNA. Fig.1A shows the structure of a 6.92kb EcoRI fragment that contains the nontranscribed spacer (NTS), the external transcribed spacer (ETS) as well as the 3' end of the 28S and the 5' end of the 18S coding region. This fragment was cloned into the EcoRI site of pBR325 and was designated pXbrI. A 1.45kb TaqI fragment extending from 2.2kb to 3.5kb upstream from the EcoRI site in the 18S gene was subcloned from pXbrI into the AccI site



**Figure 1** Maps of *Xenopus borealis* plasmids. Solid box indicates 18S and 28S, open box indicates external transcribed spacer (ETS) and thin lines indicate non-transcribed spacer (NTS). Probes used during the SI nuclease protection analysis are shown as thin lines with a solid circle at their 5' labelled ends. Solid vertical arrowheads indicate restriction sites, the letter below indicating the particular enzyme (see list above). The open horizontal arrow indicates the region of DNA that was sequenced on both strands during this work. (A) The 6.9kb EcoRI fragment from *X.borealis* that is the insert of pXbrI. (B) The 1.45kb TaqI fragment subcloned from pXbrI that is the insert of pXBoriI. Below this is shown the HincII/TaqI SI probe derived from this plasmid. The region of DNA that was sequenced is shown as an open arrow. (C) The 1.0kb HinfI insert of pXbrOT. This fragment was subcloned from pXbr11, an independently cloned *X.borealis* rDNA fragment that has previously been described (4). Below this is shown the HinfI probe derived from pXbrOT.

of the plasmid pUC9 (8) to give pXBoriI (Fig.1B). As a first step to locating precisely the 40S precursor origin of transcription, the region of DNA extending 232 nucleotides upstream from the TaqI site was sequenced on both strands using the procedure of Maxam and Gilbert (10) with the modification described by Ruther *et al* (9).

There are strong DNA sequence homologies between this region of DNA and the region surrounding the origin of the 40S precursor from *Xenopus laevis* sequenced by Moss *et al* (14). A region of about 80% homology extends at least 176bp upstream of the StuI site and within this region there are several long stretches of

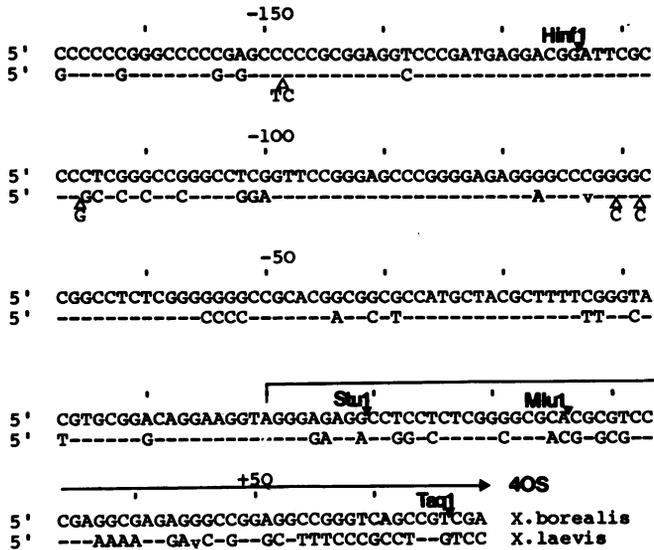


Figure 2 Sequence homology between the region of DNA from X.borealis that was expected to contain the 5' end of the 40S precursor, and that region from X.laevis (14). The region of X.borealis that was sequenced is shown in Fig.1 (B). The complete sequence of one strand of the X.borealis region is shown, with the X.laevis sequence shown schematically beneath it. A dash indicates a nucleotide in X.laevis that is identical to that in X.borealis. A v indicates a nucleotide that is deleted in X.laevis. Where the sequence in X.laevis differs from X.borealis, the sequence is shown. Inserted nucleotides in X.laevis are shown below an upward pointing open arrowhead. The horizontal arrow represents the 40S precursor as mapped in X.laevis. Also shown are the restriction sites for HinfI, TaqI, StuI and MluI. The latter two sites were used in the construction of the deletion mutant pXbrI .

100% homology (Fig.2). In particular, there is a 13bp region of perfect homology which spans the mapped origin of 40S transcription in X.laevis (5). A comparable level of homology has been found between X.laevis and X.clivii in this region (3). On the basis of these sequence relationships between X.laevis, X.clivii and X.borealis, we predicted that the origin of the 40S precursor would be at the A residue which is 66 nucleotides to the left of the TaqI site (Fig.2), and to test this we located the 5' end of the 40S precursor by an SI nuclease protection assay (15).

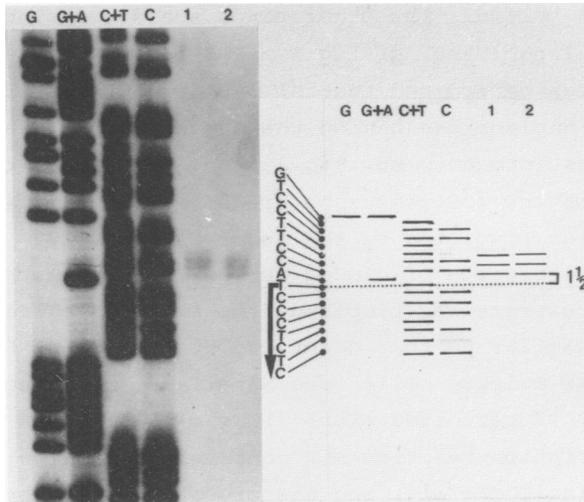


Figure 3 SI nuclease protection assays of *X. borealis* oocyte and culture cell RNA's electrophoresed alongside a sequence ladder of the probe. The 5' end-labelled HincII/TaqI single stranded probe was sequenced by the method of Maxam and Gilbert (10) and run on an 8% polyacrylamide sequencing gel, lanes G, G + A, C + T and C. Lanes 1 and 2 are the protected fragments resulting from the hybridization of 10µg of *X. borealis* oocyte and *X. borealis* culture cell RNA respectively to the HincII/TaqI probe, followed by digestion with 20 units of SI nuclease (see Material and Methods). Beside the gel photograph is a diagrammatic representation of the gel, with the DNA sequence of the probe on the left hand side. The vertical downward pointing arrow represents the mapped transcript and its origin. The bracket on the right shows the 1.5 nucleotide gap between the SI nuclease protected fragments and the mapped origin, shown by dotted line, in the sequencing track (see Text).

As a probe, the coding strand of the HincII/TaqI restriction fragment was labelled at its 5' end. When hybridised to the 40S precursor and trimmed with SI nuclease we expected a protected fragment of 68bp. The probe was hybridised to total *Xenopus borealis* oocyte RNA and *Xenopus borealis* culture cell RNA. The SI nuclease protected fragments were run on an 8% polyacrylamide sequence gel (Fig.3, Lanes 1 and 2 respectively), together with a sequence ladder of the probe fragment. In these hybridisations the protected fragments are in groups of three, each band being separated from the next by one nucleotide. The bottom band represents SI nuclease trimming to yield a flush

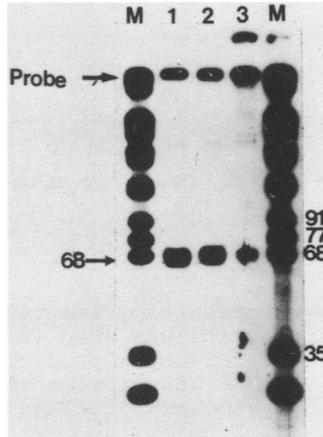
ended RNA/DNA hybrid. The other bands are due to incomplete trimming by SI nuclease, giving 1 and 2 base overlaps. It has previously been determined that SI nuclease protected fragments will run 1.5 nucleotides behind the sequence (5). Taking these considerations into account, Fig.3 shows that the first 4 nucleotides of the 40S precursor are AGGG, as in X.laevis, and X.clivii and as predicted by the sequence homology.

In vitro transcription of cloned Xenopus borealis rDNA

An S100 extract was prepared from X.laevis culture cells by a method similar to that described by Miller and Sollner-Webb (12) for mouse culture cells (see Materials and Methods section for details). Supercoiled pXbrI (10µg/ml) was added to an in vitro transcription reaction mix containing the S100 extract, and  $\alpha$  amanatin (100µg/ml), and the products were hybridised with the HincII/TaqI probe (Fig.1B). SI nuclease-resistant fragments were then run on an 8% polyacrylamide, 7M urea gel (Fig.4, lane 3). A protected fragment was observed at the same size ( $\pm 1$  nucleotide) as those protected by X.borealis oocyte and culture cell RNA (Fig.4, lanes 1 and 2 respectively). This result was confirmed (Fig.5, lane 1) using an SI probe derived from an independent X.borealis rDNA clone (Fig.1C) which gives a 95 base protected fragment after hybridisation and SI nuclease digestion. Control experiments showed that the transcription was inhibited by actinomycin D (50µg/ml) (Fig.5, lane 4) and was identical in the presence and absence of  $\alpha$  amanatin (100µg/ml) (Fig.5, lanes 6 and 7 respectively). Thus the transcription that we observed had the characteristics of transcription by RNA polymerase I. Additional controls showed no protected fragments in the absence of added cloned X.borealis rDNA (Fig.5, lane 2) or if RNase was added to the reaction mix along with pXbrI (data not shown).

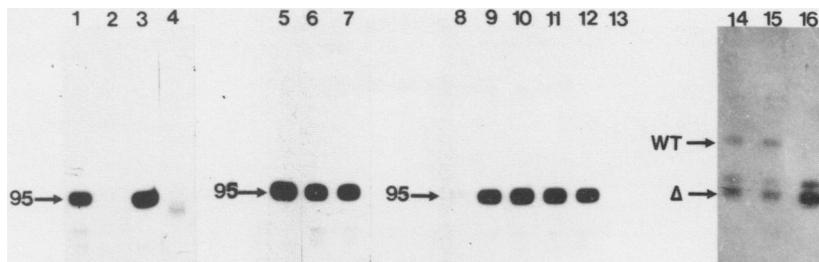
Fig.5 (lanes 8-16) shows the effect of varying the concentration of supercoiled pXbrI on in vitro transcription reactions. The amount of transcription was constant in the range of 5-50µg/ml, but was lower at 75µg/ml (Fig.5, lanes 8-13). At still higher DNA concentrations, the amount of transcription observed was generally lower but variable (data not shown).

It has been shown previously that the level of transcription



**Figure 4** SI nuclease protection analysis of *in vitro* transcribed *X.borealis* RNA, oocyte RNA and culture cell RNA. Track M contained pAT153 digested with HpaII, and end labelled with  $\alpha$ - $^{32}$ P dCTP with the large fragment of DNA polymerase I. Some of the fragment lengths of the marker are shown. Lanes 1 and 2 were SI nuclease protection assays of oocyte and culture cell RNA respectively as in Fig. 3 lanes 1 and 2. Lane 3 was the protected fragment produced by RNA from an *in vitro* transcription reaction that contained pXbr1 (20 $\mu$ g/ml) and  $\alpha$  amanatin (100 $\mu$ g/ml) (see Materials and Methods). 20 units of enzyme were used in each SI nuclease digestion. The protected fragments observed (lanes 1-3) were 68 base in length. Probe that remained full size after digestion is labelled.

from linear rDNA in *Xenopus* oocytes is far less than that from circular rDNA (16). To compare transcription efficiencies of linear and supercoiled templates in the *in vitro* transcription system, a deletion mutant was made by restricting pXbrI at its unique StuI and MluI sites. The MluI site was then filled in with the large fragment of DNA polymerase I, and the molecule religated. The product, pXbrI $\Delta$ , has the region between the MluI and StuI sites of pXbrI deleted (Fig.2). As a result, RNA transcribed from pXbrI $\Delta$  and hybridised to the HinfI probe (Fig.1C) can only protect 69 bases of the probe, compared with 95bp for the undeleted plasmid. This means that transcripts of pXbrI and pXbrI $\Delta$  can be distinguished. Both pXbrI and pXbrI $\Delta$  were linearised by cutting at the unique BamHI site in the vector DNA. *In vitro* transcription reactions were carried out with equal



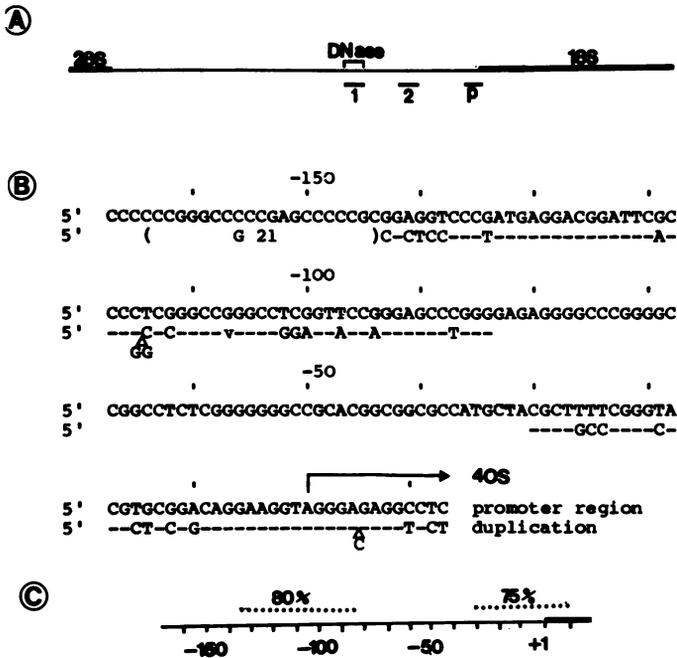
**Figure 5** *In vitro* transcription experiments. In these experiments the 1 kb *Hinf*I probe was used (Fig.1C) in the SI nuclease protection assays, giving a predicted 94 base protected fragment. Lane 1, *in vitro* transcription reaction with pXbrI (20 µg/ml) plus α amanatin (100 µg/ml). Lane 2, as lane 1 but no plasmid added. Lane 3, *X.borealis* oocyte RNA (10 µg). Lane 4, as in lane 1 but with actinomycin D (50 µg/ml). Lane 5, *X.borealis* oocyte RNA (10 µg). Lane 6, *in vitro* transcription of pXbrI (20 µg/ml) + α amanatin (100 µg/ml). Lane 7, as lane 6 but no α amanatin present. Lanes 8-13, *in vitro* transcription reactions of pXbrI at varying plasmid concentrations with α amanatin (100 µg/ml). Lane 8, 75 µg/ml; lane 9, 50 µg/ml; lane 10, 25 µg/ml; lane 11, 10 µg/ml; lane 12, 5 µg/ml; lane 13, no plasmid added. Lanes 14-16 *in vitro* transcription of linear molecules versus supercoiled molecules. Lane 14, supercoiled pXbrI (10 µg/ml) + BamHI digested pXbrIΔ (10 µg/ml); lane 15, BamHI digested pXbrI (10 µg/ml) + supercoiled pXbrIΔ (10 µg/ml); lane 16, supercoiled pXbrIΔ (20 µg/ml). Lanes 14-16 had α amanatin (100 µg/ml) present.

concentrations of supercoiled pXbrI plus linearised pXbrIΔ (Fig.5, lane 14) and with linearised pXbrI plus supercoiled pXbrIΔ (Fig.5, lane 15). The results of these reactions show that linearised and supercoiled templates are transcribed with equal efficiency *in vitro*.

## DISCUSSION

### Mapping the *Xenopus borealis* 40S precursor and sequence homologies

The major transcripts of *Xenopus laevis* (5) and *Xenopus clivii* (3) rDNA have previously been mapped by an SI nuclease protection assay. Furthermore, it has been shown in the case of *X.laevis* that the major transcript is the primary transcript, because of the presence of a 5' polyphosphorylated end (5). In this work we have used SI-nuclease protection to map the origin of the major transcript of *Xenopus borealis* rDNA. Several



**Figure 6** Sequence homologies between the *X. borealis* promoter region and an *X. borealis* "Bam-island" like promoter duplication. (A) The EcoRI fragment, containing the spacer region from *X. borealis*, showing the location of the promoter region, P, and the two "Bam island" like promoter duplications, 1 and 2. The DNaseI hypersensitive site is shown as a bracket over promoter duplication 1. (B) Sequence comparison between the promoter region and a duplication (1 in Fig.6A). The scheme is the same as in Fig.2. The gaps in the duplication sequence represent regions that have poor homology with the promoter region. (G21) represents 21 consecutive G residues. (C) A diagrammatic representation of the sequence comparison above (Fig. 6B). Regions of strong homology are shown as dotted lines with percentage homology above.

lines of evidence indicate that this origin is the true origin of rRNA precursor transcription in this species. (i) The transcript we have mapped is the only transcript in RNA from cultured cells or oocytes that hybridises to the probes used in this study. (ii) The region of DNA surrounding the 5' end of the major transcript has strong DNA sequence homology to the region of DNA surrounding the origin of the *Xenopus laevis* 40S primary transcript. (iii) The *in vitro* transcription system described in this work gave rise to transcripts originating at the same point as the

transcripts observed in vivo.

In Xenopus laevis rDNA, the region between - 145 and +6 has been shown to be important in the transcription of cloned rDNA. If deletions are made within this region, the transcription observed in oocyte injection experiments is greatly reduced or abolished (17-19). This region has therefore been termed the promoter region. In Xenopus laevis there are two or more duplications, of the promoter region (the so-called "Bam islands") in the spacer (6). Five copies of a 44 bp sequence that has homology with the region immediately surrounding the origin of the 40S precursor (-22 - +22) from X.laevis have been observed in the spacer of X.borealis (3). It was previously suggested (3) that the most righthand copy corresponded to the origin of the X.borealis 40S precursor. We have shown this suggestion to be incorrect, since the actual origin is about 390bp to the right of this.

Two promoter duplications of the "Bam island" type have been found in the spacer of X.borealis (4). We compared the sequence of the X.borealis promoter region with the sequence of one of these duplications (Fig.6A, duplication 1) (4). Two regions of strong homology were observed (Fig.6B and C). The first of these stretches from about -140 to -84 with 80% homology, and the second from about -30 to +9 with 75% homology. Between these two regions, from -83 to -31, little homology was observed. The situation in X.borealis is therefore slightly different from X.laevis where there is good homology across the entire promoter region and its duplication (6). A faithful promoter duplication has also been observed in Xenopus clivii (4).

It is probable that such promoter duplications are of biological significance since they have been found in the rDNA of three Xenopus species and of Drosophila melanogaster. A DNaseI hypersensitive site that correlates with expression is associated with the most leftward promoter duplication in X.laevis and X.borealis (Fig.6A) (4). Furthermore, in X.laevis it has been observed that these promoter duplications can give rise to short spacer transcripts (19). In Drosophila also, promoter duplications give rise to spacer transcripts (20-

23).

### In vitro transcription

We have shown in this work that an S100 extract prepared from Xenopus laevis culture cells will support accurate transcription of X.borealis rDNA. The extract transcribes linear and supercoiled molecules with equal efficiency. This contrasts with the oocyte assay system, in which linear genes are transcribed at a much lower efficiency than circular genes (16, and cited in 33). A possible explanation for this difference lies in the ability of oocytes, but not extract, to assemble chromatin. Consistent with this explanation, Mertz (33) has shown that linear molecules are not assembled into chromatin in oocyte nuclei, and she suggests that the low level of transcription of linear molecules in oocytes is equivalent to that observed for linear and circular molecules in cell extracts. We have also noted that the efficiency of transcription of circular Xenopus rDNA injected into oocytes is orders of magnitude greater than the transcription of circular or linear molecules incubated in the extract (data not shown).

The fact that a Xenopus laevis extract transcribes X.borealis rDNA is of interest since, it has been observed that a human cell extract does not transcribe mouse rDNA (24-26) and a mouse extract does not transcribe human rDNA (25,26). Similarly an extract from Drosophila melanogaster cells will transcribe D.melanogaster but not D.virilis rDNA (27). The species specificity that is observed in mouse and human cell extracts is paralleled in mouse/human cell hybrids. Cell lines that are segregating human chromosomes synthesize only mouse rRNA, even when human rDNA is present (28,29), while, hybrids that are segregating mouse chromosomes synthesize only human rRNA (30,31). Nucleolar dominance is also observed in Xenopus species hybrids since only laevis rRNA is synthesised in Xenopus laevis/Xenopus borealis hybrid embryos (32). In this case, however, the dominance of laevis over borealis occurs whether the maternal cytoplasm is of X.laevis or X.borealis origin. This would suggest that the mechanism of dominance in Xenopus species hybrid embryos is different from that observed in mouse/human hybrid cells. The ability of an X.laevis cell

extract to transcribe X.borealis rDNA supports this idea.

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#### NOTE

We have learned recently that the equivalent region of an independent X.borealis clone has been sequenced in the laboratory of Dr. B. E. H. Maden. This sequence is closely related but not identical to our sequence.