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The putative promoter of a Xenopus laevis ribosomal gene is reduplicated

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ABSTRACT

With the aid of a novel poly-dA tailing-partial restriction technique and S1-protection mapping, the 5' terminal coding sequence for the 40S precursor ribosomal RNA of Xenopus laevis has been exactly identified. Since the promoter sequence for the 40S RNA should lie close to its 5' terminal coding sequence, we are able to conclude that the "Bam-Island" sequence reduplication (1) almost certainly represents a promoter reduplication.

INTRODUCTION

The sequence organization of the spacer DNA in a ribosomal gene unit of Xenopus laevis has recently been the subject of a detailed study by our group (1). In particular it was shown that a sequence of 120 bases close to the region coding for the 5' terminus of the 40S precursor ribosomal RNA (pre rRNA) was found duplicated within the non-transcribed spacer at the so called "Bam Islands". Since the 40S pre rRNA is very likely to be a primary transcript (2), it was concluded that these sequence duplications may also represent promoter duplications.

Here we describe the application of a new poly-dA tailing-partial restriction technique to the mapping of the 5' terminus of the 40S pre rRNA. The poly-dA tailing technique, which has also been applied to DNA sequence analyses (3), facilitates the production of a set of overlapping partial restriction fragments all having one poly-dA terminus in common and their other termini staggered across the DNA at the available restriction sites. By hybridization of labelled RNA to such fragments transferred onto nitrocellulose filter (4), it was

immediately possible to determine the position of the coding sequence for the 5' terminus of the 40S pre rRNA to within the nearest restriction sites. Using this information an "S1 protection" experiment (5) was designed from which it was possible to determine the 5' terminal coding sequence of the 40S rRNA. The significance of these data is discussed as are further applications of the poly-dA tailing technique.

MATERIALS AND METHODS

Restriction enzymes were obtained from New England Bio-Labs, terminal transferase from Boehringer Mannheim, bacterial alkaline phosphatase from Worthington Biochemical Co. and T4 poly-nucleotide kinase from P-L Biochemicals. λ exonuclease, prepared essentially as described by Radding (6), was a gift of Drs.H.O. Smith and S.G. Clarkson. S1 nuclease prepared as described by V.M.Vogt (7) was a gift of E. Di Capua. Unlabelled dATP was obtained from Sigma Chemical Co., α(^{32}P)-GTP (300Ci/mmol.), (^{3}H)-dATP (25Ci/mmol.), γ(^{32}P)-ATP (3000Ci/mmol.) and (^{32}P)orthophosphate from Amersham-Searle. Oligo-dT cellulose was grade T3 from Collaborative Research Inc. All reagents were of analytical grade.

Poly-dA tailing

The 6.6 kb rDNA insert of pXl108 was excised and purified by CsCl-actinomycin centrifugation as described previously (8). Endonuclease cleavages were carried out under conditions recommended by the enzyme supplier. The 6.6 kb rDNA fragment, restricted with Bam Hl, was incubated at 0.11 mg/ml with 600 units/ml λ-exonuclease (λ-EXO) in 67mM glycine-KOH pH 9.4, 2.5mM $MgCl_2$ (9) for 6 min. at 14°C. Digestion was terminated by addition of EDTA to 10mM and NaCl to 0.3M, both on ice. The solution was then extracted twice with phenol-chloroform (1:1,v/v), twice with chloroform-isoamylalcohol (24:1,v/v) and the DNA ethanol precipitated. The λ-EXO treated DNA was incubated at 1.2mg/ml with 730 units/ml (~0.1mg/ml) terminal transferase in 100mM Na cacodylate, 8mM $MgCl_2$, 1mM dATP (10,11), 60μCi/ml (^{3}H)-dATP for 30 min at 37°C. The reaction was terminated and

the DNA recovered exactly as described following λ-EXO treatment.

Partial restriction and poly-dT cellulose chromatography

The poly-dA "tailed" DNA fragments were electrophoretically fractionated and recovered as described by Clarkson et al. (12). Partial restriction was allowed to proceed until only a third of the initial DNA fragment was left intact. This extent of restriction was calculated to give the maximum yield of the larger restriction products (3). Restriction was terminated by addition of EDTA to 10mM, the solution was diluted 5 times with 0.4M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA and applied to a 0.4ml column of oligo-dT cellulose equilibrated in the same buffer (13). The column was then washed with several millilitres of application buffer and the poly-dA tailed fragments eluted with one bed volume of 10mM Tris, 1mM EDTA, pH 8.0.

Preparation of RNA

^{32}P labelled X.laevis oocyte RNA was obtained by injecting 0.2μCi α(^{32}P)-GTP into each of 50 oocytes, incubating them for 3 hrs at 18°C and then extracting the total RNA as described by Kressmann et al.(14). ^{32}P labelled RNA was also obtained from a X.laevis kidney cell culture essentially as described by Loening et al. (15).

Hybridization

Hybridization of RNA to "Southern blots" (4) was performed at 70°C for 4 hrs in 1 x SSC, 0.5% SDS. The total RNA concentration was approximately 250μg/ml in the case of oocyte RNA and 25μg/ml in the case of RNA from tissue culture. The hybridized filters were washed for 2 hrs in 1 x SSC. 0.5% SDS at 60°C and then for 2 hrs in 0.1 x SSC, 0.5% SDS at 50°C. Nick translated 6.6 kb rDNA fragment (16) was hybridized to "Southern blots" in the same buffer and at the same temperature as for RNA.

S1 mapping and DNA sequencing

S1 mapping was performed essentially as described by Berck and Sharp. About 0.1mg total oocyte RNA was hybridized for 3 hrs with ~1 pMole of 5' ^{32}P labelled DNA in 50μl of 80%

formamide, 0.4M NaCl, 40mM MOPS pH 6.7, 1mM EDTA. After dilution with 9 volumes of S1 buffer the hybridization solution was incubated with 4u/ml S1 nuclease for 1 hr at 45°C, phenol-chloroform and chloroform-isoamylalcohol extracted and the hybrids ethanol precipitated.

A DNA base sequence ladder was produced according to Maxam and Gilbert (17) as modified by Busslinger et al. (18). Partial cleavage products and S1 protection products were dissolved in 80% formamide, 45mM Tris-Borate pH 8.3, 1mM EDTA and loaded onto an 8% acrylamide, 8M urea sequencing gel in 90mM Tris-Borate pH 8.3, 2.5mM EDTA.

RESULTS AND DISCUSSION

Mapping the 40S transcript using the poly-dA technique

A cloned 6.6 kb X.laevis ribosomal (rDNA) fragment which contains the complete nontranscribed (NTS) and external transcribed (ETS) spacer regions, was excised from the plasmid pXL 108 by restriction with EcoRI as previously described (1). The rDNA fragment was restricted with Bam H1 to produce the three fragments shown in Fig.1. These fragments were incubated with λ(5')-exonuclease (λEXO) to expose single stranded 3' termini which were then used as primers for the addition of poly-dA "tails" by terminal (deoxynucleotidyl) transferase (transferase). The right hand Bam H1 to EcoRI fragment, Fig.1, now poly-dA tailed at both termini was purified by gel electrophoresis, restricted with Xba 1 and the left hand Bam H1 to Xba 1 fragment purified again by gel electrophoresis. The latter fragment, Fig.1, having a single poly-dA tail, was partially restricted with Hinf 1 and the fragments terminating in this poly-dA tail selected by their affinity to poly-dT cellulose, see again Fig.1. The set of overlapping fragments so obtained was then fractionated by gel electrophoresis in 1% agarose, transferred to nitrocellulose filter (4) and hybridized to ^{32}P pulse labelled total RNA from X.laevis oocytes.

The pattern of hybridization obtained from the above experiment is also shown in Fig.1. Here the three larger DNA

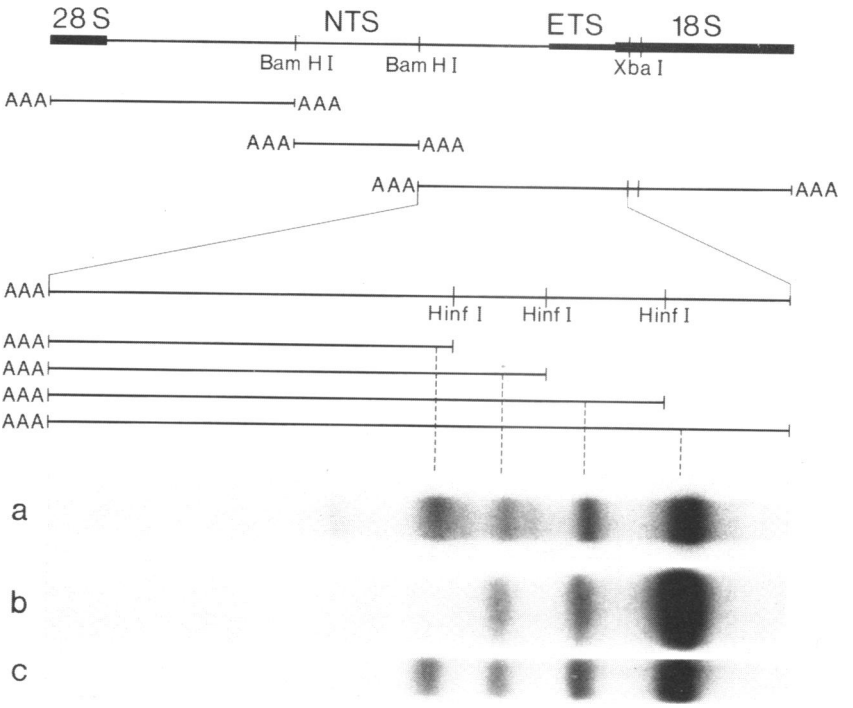

Fig. 1. The poly-dA tailing-partial restriction technique applied to the mapping of the 40S pre rRNA of X.laevis. Hybridization to the poly-dA tailed fragments transferred to nitrocellulose filter; a) nick translated DNA, b) total oocyte RNA and c) total Xenopus kidney cell RNA.

fragments exhibit strong RNA hybridization while the smallest fragment shows no sign of hybridization. Thus from examination of this hybridization pattern it is clear that the 5' terminus of the 40S pre rRNA lies within, or very close to the DNA interval between the first and second Hinf 1 sites to the right of the Bam H1 site.

Fine mapping of the 5' terminus of the 40S transcript

The Hinf 1 fragment shown in the above experiment to contain the coding sequence for the 40S rRNA 5' terminus, was purified from pXL 108 in two steps; firstly by restriction with Pst 1 and then by double restriction with Alu I and Hinf 1, the fragments being purified at each step by gel electrophoresis.

The Hinf 1 fragment was then 5' ^{32}P labelled (19), hybridized in 80% formamide to unlabelled oocyte total RNA and the hybridized products digested with S1 nuclease. Hybridization was carried out at 55, 60 and 65^{o}C, but only at the lower two temperatures was a protected fragment detectable after gel electrophoresis. At both these temperatures a single protected fragment of about 100 bases was obtained. This was electrophoresed in parallel with the Maxam and Gilbert sequence ladder (17) of the original Hinf 1 fragment asymmetrically restricted with Ava 1, Fig. 2.

The exact extent to which the 40S rRNA protected the Hinf 1 fragment against S1 digestion can be seen in Fig.2. The protected fragment was slightly inhomogeneous in length, terminating at the adjacent bases C, A and T on the coding strand, respectively at sequence positions 4435, 6 and 7 in Fig.3. It should be noted that calibration of an S1 digestion product against the Maxam and Gilbert sequence ladder (17) requires a single base correction since the band read as base "X" in the ladder corresponds to a fragment from which this base has been removed during the cleavage reaction. We have evidence indicating that no further correction is required for the difference of a single 3' terminal phosphate group between the S1 product and its corresponding sequence reaction under the conditions of our experiment. This correction has therefore been neglected.

It is generally accepted that RNA transcription is most probably initiated exclusively with the bases A or G (20). It would therefore be most reasonable to conclude from the experiment of Fig.2, that the 40S transcript is initiated with the base G and/or A at positions 4435 and 4437 (Fig.3). The observation of a protected fragment terminating at the base T, position 4436 (Fig.2 and 3) must then be presumed either to be due to partial terminal degradation of the 40S rRNA or to be an inherent feature of the S1 mapping experiment.

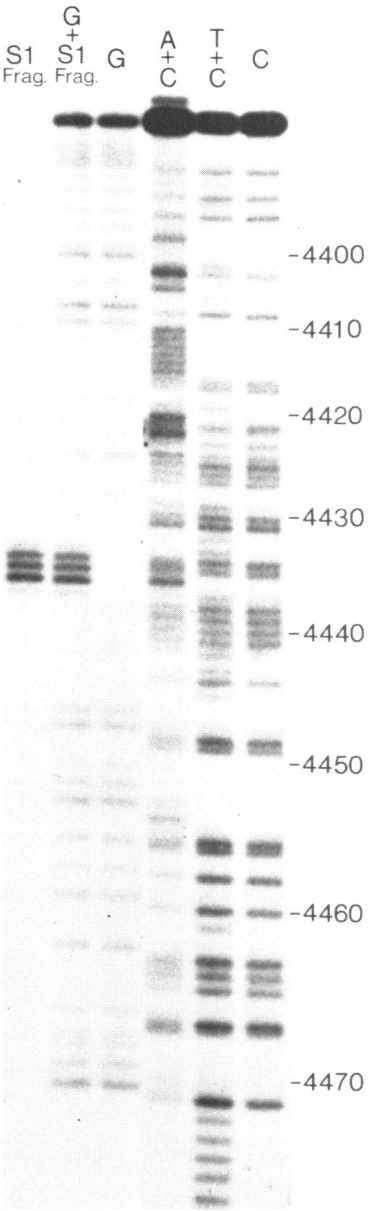

Fig. 2. S1-mapping of the 40S pre rRNA. The protected fragment is shown calibrated against its Maxam and Gilbert (17) sequence ladder. Sequence positions are those given in Fig.3. Note that the coding strand complementary to the pre rRNA is read and that correction by one base is required to arrive at the exact position of the 5' end of the RNA (see text).

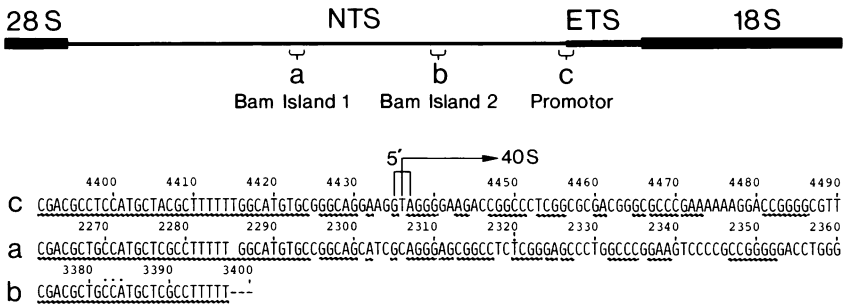

Fig. 3. Comparison of the DNA sequence at the 5' terminus of the 40S pre rRNA (c), with those of the "Bam Islands" (a) and (b). The sequence data is taken from Boseley et al. (1) and Moss and Birnstiel (3).

Relationship between the 40S 5' terminal coding sequence and the "Bam Island" homologies

The sequence homologies observed in X.laevis rDNA between the external transcribed/non transcribed spacer (ETS/NTS) boundary and the so-called "Bam Islands" of the non transcribed spacer (NTS) are shown in Fig.3. From the sequence of Bam Island I, Fig.3, it is clear that near sequence identity extends to within $3^{+}_{-}1$ nucleotides of the 40S 5' terminal coding sequence. However, it is also clear that significant sequence homology persists for about 20 nucleotides into the 40S coding sequence.

As mentioned above the 40S rRNA has been shown to have an oligo phosphate 5' terminus and is thus most probably a primary transcript (2). By analogy to bacterial systems the DNA sequence to the immediate 5' of the 40S terminal coding sequence should therefore contain an RNA polymerase promoter site. From the data of Fig.3 this promoter site would then necessarily occur in the Bam Island 1 sequence (and probably in Bam Island 2). It is therefore important to ask if the Bam Islands are active in promoting transcription.

Certainly, the absence of hybridization in the poly-dA experiment of Fig. 1 and the absence of multiple protected fragments in the S1 mapping experiment of Fig.2 would argue

against any gross level of initiation in the Bam Island sequences, unless these transcripts were subject to very rapid processing. However, several investigators have noted transcription complexes in, or transcription of, the NTS both under normal conditions and conditions thought to inhibit RNA processing (21,22). These latter observations are certainly consistent with initiation of transcription in the Bam Islands.

We have made a preliminary investigation of the possibility of NTS transcription using the mapping experiment of Fig.1. This time ^{32}P labelled total RNA from a X.laevis tissue culture was hybridized to the poly-dA fragments bound on nitrocellulose filter. Fig.1c shows that now RNA hybridized strongly not only to the larger three fragments, but also to the smallest fragment which contains no 40S sequences. The reason that we observe such hybridization with tissue culture RNA, but not with oocyte RNA is unclear. Initial calculations suggest that it is not simply due to the higher specific activity of tissue culture RNA ($\sim$100,000cpm/μg against $\sim$ 1000cpm/μg) but that it may be a factor of the relative concentrations of rDNA spacer transcripts.

The data of Fig.1c of course only indicate the possibility of initiation in the Bam Island sequences. In order to investigate this possibility further, it will be necessary either to identify *in vivo* transcripts mapping to the Bam Islands or to demonstrate by gene manipulation that the Bam Islands can be used as functional promoters.

CONCLUSION

With the aid of a novel poly-dA tailing-partial restriction technique we have been able to identify the 5' terminal coding sequence for the 40S pre rRNA of *Xenopus laevis*.

Since the 40S pre rRNA is most probably a primary transcript, the DNA sequences controlling initiation of transcription in the ribosomal genes (i.e. promoter sequences) should lie close to this 5' terminal coding sequence. We have previously noted that the rDNA spacer of X.laevis contains in particular a

reduplicated sequence referred to as the "Bam Island" (1). It is now possible to conclude that the reduplicated sequence almost certainly contains the promoter sequence of the 40S rRNA and possibly also its initiation site. Thus the evolution of the X.laevis rDNA spacer has, very likely, involved promoter duplication events.

The poly-dA tailing partial restriction technique used here for transcript mapping, has also been extensively applied to the preparation of DNA fragments (3) for sequence analysis by the Maxam and Gilbert procedure (17). However, the poly-dA technique may have several other applications. For example, it can be adapted to the in vitro deletion or rearrangement of specific sequences in cloned DNA. The technique may thus be used for gene manipulation in order to identify biologically active sequences.

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