The EMBO Journal vol.4 no.6 pp.1537–1543, 1985

Transcription signals in embryonic *Xenopus laevis* U1 RNA genes

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Communicated by L.Philipson

A genomic clone of the most abundant U1 RNA genes from *Xenopus laevis* was isolated from erythrocyte DNA and sequenced. Two different U1 RNA genes, U1A and U1B, are encoded in an *Hind*III 1.5-kb fragment and both are expressed after microinjection in *Xenopus* oocytes. Deletions and site-directed mutagenesis of the clones revealed two promoter elements in the U1B gene; one, located 250–220 nucleotides upstream from the 5′ terminus of mature U1 RNA, functions as an activator, yielding a 10-fold promotion of transcription; the other, located 60–50 nucleotides upstream of the cap site, functions as an essential element for promotion of transcription. The U1A gene contained only the latter element in the cloned fragment. Homologous sequences can be identified in several U RNA genes of *X. laevis*.

Key words: U1 RNA transcription/microinjection/major repeat/promoter elements

Introduction

The small nuclear U1 RNA molecules have been detected in several eucaryotes from insects to mammals but they have not yet been encountered in yeast (for a review, see Busch *et al.*, 1982). Only one U1 RNA species has been detected in humans, chicken and rat but two different variants have been found in mice (Lerner and Steitz, 1979). As many as seven different species of U1 RNA have been encountered in *Xenopus* and their expression appears to be developmentally regulated (Forbes *et al.*, 1984).

The organization of the U1 genes is complex. The human genome contains at least 1000 copies of U1 RNA-related sequences (Denison *et al.*, 1981) whereof only 10% appear to be *bona fide* genes coding for U1 RNA (Manser and Gesteland, 1982; Lund and Dahlberg, 1984); the others being pseudogenes mutated internally or truncated in the 5′ or 3′ end of the flanking sequences. A similar organization of U1-related sequences has been found in the chicken genome (Kristo *et al.*, 1984). Two classes of U1 genes have been identified in the *Xenopus* genome, one present in ~500 copies and consisting of tandemly arranged embryonic U1 RNA genes (Forbes *et al.*, 1984; Lund *et al.*, 1984), the other expressed in oocytes and somatic cells and present in ~50 copies (Zeller *et al.*, 1984).

The U1 RNA genes have generated special interest since this RNA is involved in recognizing the 5′ end of the intron during the splicing reaction (Mount *et al.*, 1983; Keller, 1984). The nucleoprotein particle (U1 RNA) presenting the U1 RNA to the transcript also contains a unique 72-K protein which may be involved in the splicing reaction (Bringmann *et al.*, 1983). There is abundant evidence that U1 RNA is transcribed by RNA polymerase II, that its 5′ nucleotide has a trimethylated cap and several base and ribose modifications and that the transcript is not polyadenylated (Busch *et al.*, 1982).

It has previously been suggested that human U1 RNA could be a processed product after initiation of transcription 183 nucleotides upstream from the U1 RNA sequence (Murphy *et al.*, 1982). Later studies have shown, however, that transcription starts at the level of the trimethylated cap but a region 230–203 bp upstream is necessary for initiation (Skuzeski *et al.*, 1984). In contrast, a minor U1 RNA gene from *X. laevis* only required 150 bp upstream for transcription (Zeller *et al.*, 1984).

To gain further insight into the mechanism of regulation of small nuclear RNA gene transcription, we have chosen to clone and study the expression of the embryonic major U1 RNA genes from *X. laevis*. SnRNA genes can be used as template for transcription in a homologous system by microinjecting them in the nucleus of *Xenopus* oocytes (Mattaj and Zeller, 1983; Zeller *et al.*, 1984; Lund *et al.*, 1984). The introduced genes show a strong promoter activity, and give rise to abundant synthesis of faithful transcripts. The apparent absence of pseudogenes in *Xenopus* provides an additional advantage (Zeller *et al.*, 1984).

We have determined the sequence of the two embryonic major U1 RNA genes from *X. laevis* and started a dissection of the promoter sequence aimed at identifying the essential transcriptional signals in a homologous system. We show by transcriptional analysis that a region 250 nucleotides upstream from the gene product is required for a maximal rate of U1 RNA transcription (see also Krol *et al.*, accompanying paper). A second promoter element is located in close proximity to the coding region between nucleotides −50 and −60 and is probably important for proper initiation.

Results

Cloning of the major repeat of U1 RNA genes

To identify the major repeat of the U1 RNA genes in the *X. laevis* genome, the 535-bp *Bam*HI fragment carrying a human U1 RNA gene in clone pHU1.1D (Murphy *et al.*, 1982) was used as a probe on Southern blots of *Xenopus* DNA from erythrocytes restricted with *Bam*HI or *Hind*III. A predominant 1.5-kb band was revealed after *Hind*III cleavage (Figure 1) which probably corresponds to the major repeat of U1 RNA genes in *X. laevis* (Zeller *et al.*, 1984; Lund *et al.*, 1984). Several minor bands are also seen in the *Bam*HI and *Hind*III digests; they may correspond to less abundant U1 RNA genes interspersed in the *X. laevis* genome.

The *Hind*III-restricted *Xenopus* DNA was then separated on a sucrose gradient with internal DNA size markers and the fraction enriched for 1.5-kb DNA was cloned in the λ phage vector λ1147 (Murray *et al.*, 1977). Several plaques hybridizing with the pHU1.1D probe were isolated. When DNA isolated from these clones was restricted with *Hind*III all clones carried a 1.5-kb insert which also hybridized strongly with the human U1 gene probe (not shown).

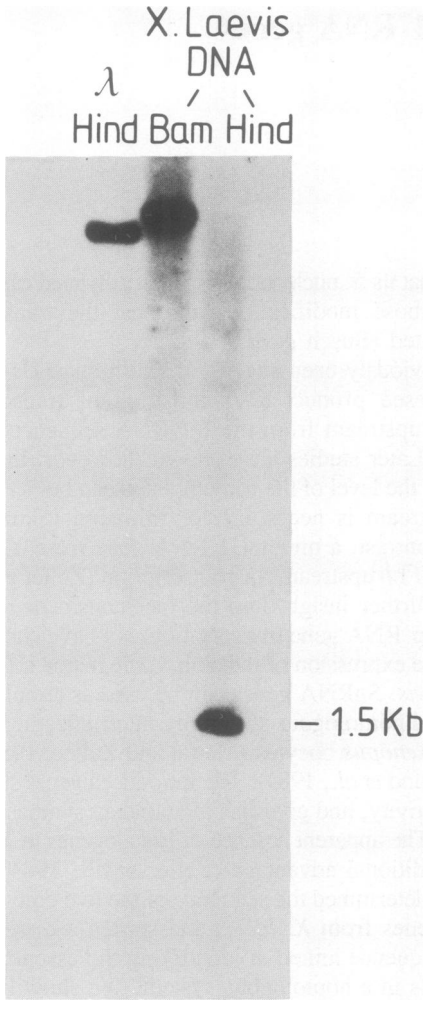

Fig. 1. Southern blot analysis of *X. laevis* DNA. *X. laevis* DNA extracted from erythrocytes was digested with *Bam*HI or *Hind*III. 5 μg of digested DNA was separated on a 1% agarose gel and blotted according to Southern (1975). The nick-translated *Bam*HI-*Bam*HI (10^6 d.p.m./μg) insert from plasmid pHU1.1D carrying a human U1 RNA gene (Murphy *et al.*, 1982) was used as probe. Hybridization was at 65°C for 48 h in 10 x Denhardt, 4 x SET, 0.1% SDS.

Identification of U1 genes by transcription in Xenopus oocytes

Two independent isolates of λ clones (λXU1.1 and λXU1.2) were grown in large quantities and the DNA microinjected into the nuclei of *X. laevis* oocytes together with [α-^{32}P]GTP. Figure 2A shows that the λXU1.1 directs the synthesis of a prominent band in the same size range as the endogenous U1 RNA synthesized in the oocytes. The same results were obtained with the λXU1.2 clone (not shown).

The λXU1.1 also gives rise to a 300 nucleotides long transcript which is not observed in the controls. To identify the U1 RNA-related transcripts, the RNA analyzed in Figure 2A was hybrid selected on a ssM13 phage derivative containing the coding strand of the P*st*I-*Sac*I insert from clone pHU1.6 (Monstein *et al.*, 1983). Figure 2B reveals that only the U1 RNA is selected under these conditions.

Injections of the heterologous human gene pHU1.1D do not give appreciable accumulation of human U1 RNA when total RNA extracted from injected oocytes is examined. The human HU1.1 transcript could only be detected by hybrid selection but the intensity was only 5% of the homologous U1 RNA (not shown).

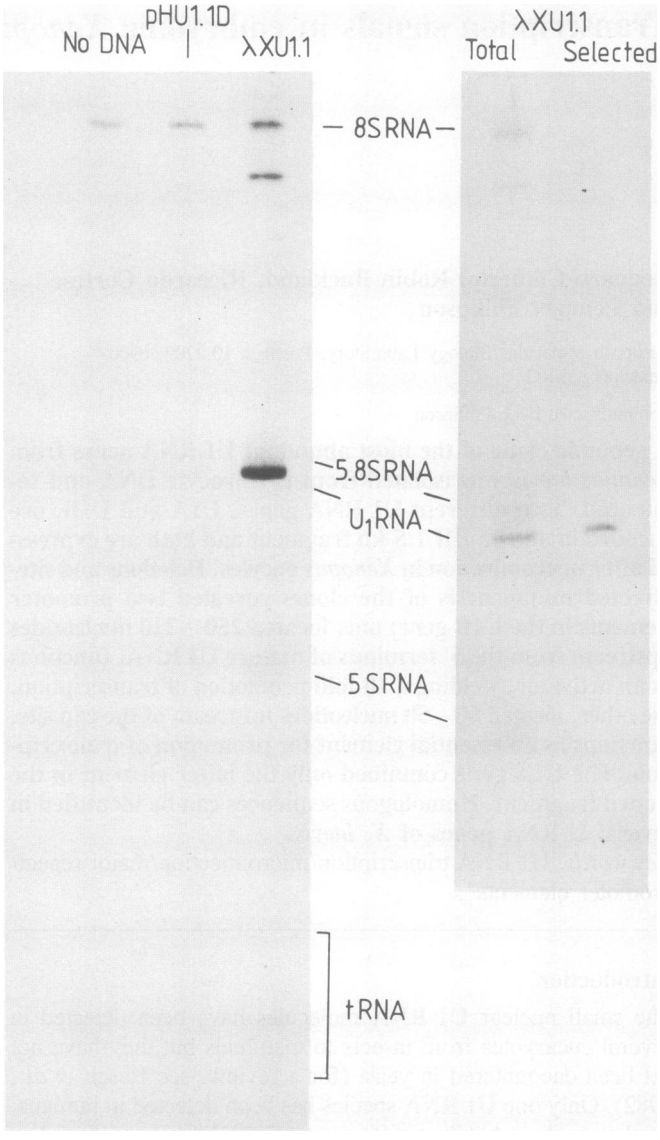

Fig. 2. Transcriptional analysis of λ clones carrying the 1.5-kb repeat. **Left panel.** No DNA: only [α-^{32}P]GTP injected; pHU1.1D: a plasmid carrying one copy of a wild-type human U1 RNA gene (Murphy *et al.*, 1982); λXU1.1: a lambda clone carrying one copy of the *Hind*III-*Hind*III 1.5-kb repeat from *X. laevis*. **Right panel.** Total: same sample as in λXU1.1 lane in the left panel but electrophoresed for longer time. Selected: material coming from 10 oocytes injected with λXU1.1 was hybrid selected according to McGrogan *et al.* (1979) on DNA from clone pHU1.6 (Monstein *et al.*, 1983) carrying one copy of a human U1 RNA gene. Polyacrylamide gels were run at 300 V for 12 h (**left panel**) or 24 h (**right panel**).

Lund *et al.* (1984) have recently shown that the human U1 RNA gene is transcribed at the same efficiency as *Xenopus* U1 RNA genes in the oocyte system. This apparent variability of transcription of the human gene might depend on different experimental conditions: Lund *et al.* (1984) analyze transcripts synthesized in single oocytes, whereas we pooled 10–20 injected oocytes. In this way we observed a preference for transcription of homologous genes in 10 independent experiments.

Sequencing of the embryonic U1 RNA gene

The 1.5-kb *Hind*III fragment in λXU1.1 was subcloned into the plasmid pUC8 in both directions and transferred to the M13 mp9 phage for sequencing. A set of B*al*31 deletions were created from one end and the fragments were subcloned in the appropriate

orientation so that the end of the deletion was inserted next to the region where the primer for sequencing anneals to the single-stranded DNA from M13 in the β-galactosidase coding region. The *Bal*31 deletions were sequenced according to the strategy shown in Figure 3 and the sequence of the entire *Hind*III fragment is shown in Figure 4.

The sequence reveals first that the *Hind*III fragment contains two U1 RNA genes referred to as U1A and U1B, respectively. They correspond to the *X. laevis* embryonic U1A and U1B genes recently identified by Lund *et al.* (1984). The two coding regions contain two base substitutions at nucleotides 78 and 114 from the 5′ end of the mature U1 RNA when compared with each other. In addition, substitutions at nucleotides 33 and 77 were found when the U1A and U1B sequences were compared with that derived from a minor *X. laevis* gene (Zeller *et al.*, 1984).

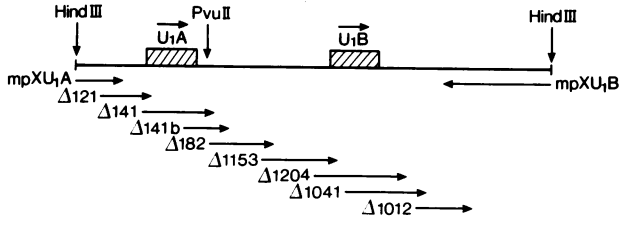

Fig. 3. Schematic map of the 1.5-kb *Hind*III-*Hind*III repeat. Boxes represent U1A and U1B coding sequences. Arrows above them indicate the direction of transcription. mpXU1A and mpXU1B are subclones of the entire repeat in M13 mp9 phage vector in both directions. Δ182 to Δ1012 are *Bal*31 deletions in vector M13 mp9. The length of the arrows indicates the portion of the *Hind*III-*Hind*III segment that has been sequenced in each case.

Both the U1A and U1B genes show structures of homology in the flanking regions. One is located at nucleotide −60 with respect to the cap site and extends over 10 bp with a single substitution. Another homology region is present in the 3′-flanking sequence starting at nucleotide +10 and extending over 13 bp.

Transcriptional analysis of the two U1 RNA genes

To identify the sequences required for faithful transcription of the U1A and U1B genes, we subcloned them separately and analyzed their relative transcriptional efficiency after microinjection in the *X. laevis* oocytes. The U1A gene clone was analyzed by transferring the *Hind*III-*Pvu*III fragment from base 1 to base 523 of the sequence shown in Figure 4 to the plasmid pUC8. Transcription from the U1B gene was analyzed by microinjection of the *Bal*31 deletion clone Δ182 (5′ Δ-383) whose 5′ end point is at nucleotide −383 with respect to the first nucleotide of the U1B coding sequence. The results are shown in Figure 5. The original 1.5-kb *Hind*III fragment gives rise to two RNA species corresponding to U1 RNA (indicated by arrows); a less abundant one migrating above the endogenous 5.8S RNA and a much more abundant one migrating below the endogenous 5.8S RNA. The U1A gene in the *Hind*III-*Pvu*II fragment yields only the slower migrating RNA species, whereas the Δ182 carrying only the U1B gene yields only the faster migrating RNA at the same activity as in the original. Deletion Δ1153 carrying only 212 bp in front of the U1B gene is much less active. We conclude from these experiments that a region 5′ of the coding sequence located between nucleotides −383 (Δ182) and −210 (Δ1153) is required for a maximal rate of transcription of the U1B gene. Similar

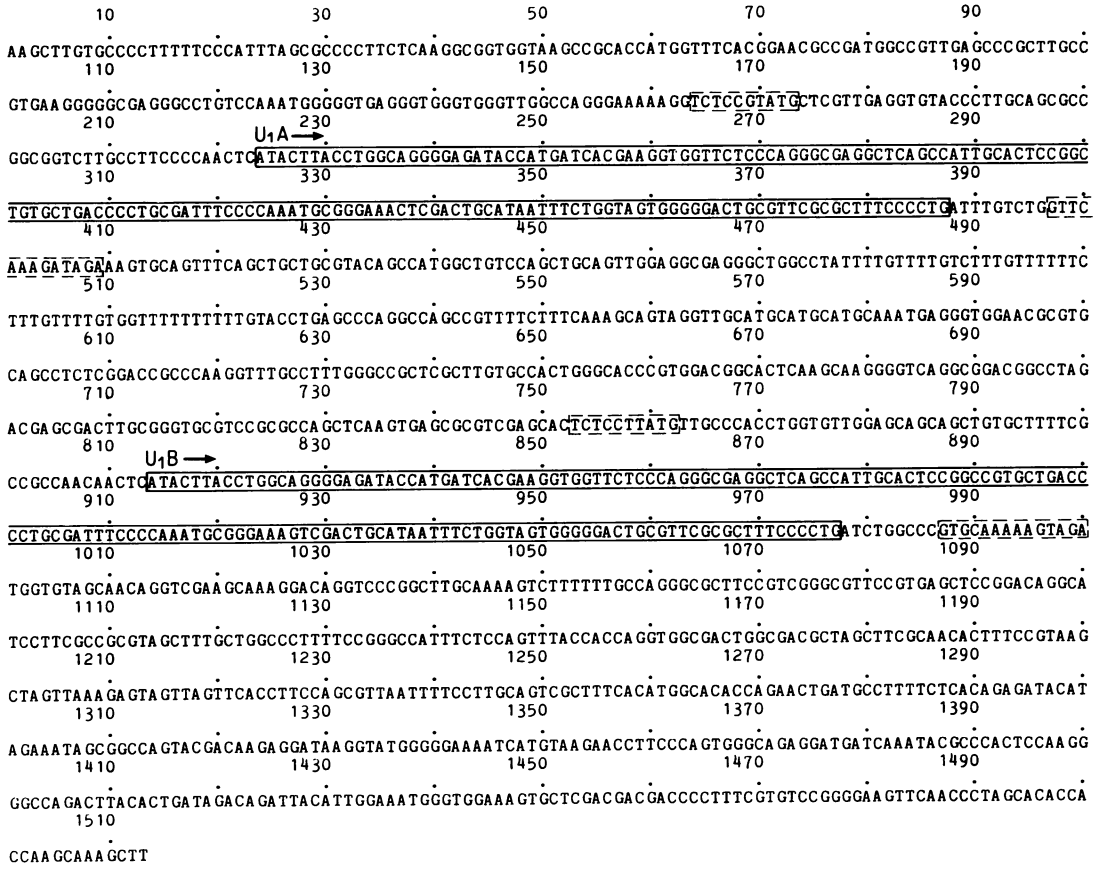

Fig. 4. DNA sequence of the *Hind*III-*Hind*III segment. Continuous boxes represent U1A and U1B coding sequences with the arrows indicating the direction of transcriptions. Dashed boxes represent regions of homology found in the 5′- and 3′-flanking regions (see text).

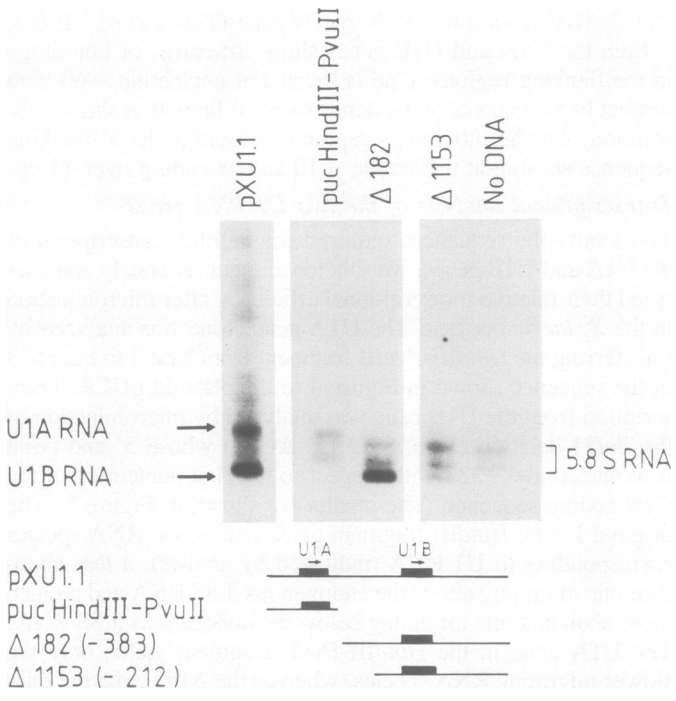

Fig. 5. Expression of U1A and U1B genes. **Upper part:** 6% polyacrylamide gel electrophoresis of RNA synthesized in *Xenopus* oocytes. Arrows point to U1A and U1B RNAs. **Lower part:** schematic structure of the U1A and U1B subclones (see text). The gel was run as in Figure 2, right panel. These conditions give, in our hands, the best separation of the U1 RNA from the endogenous 5.8S RNA transcripts.

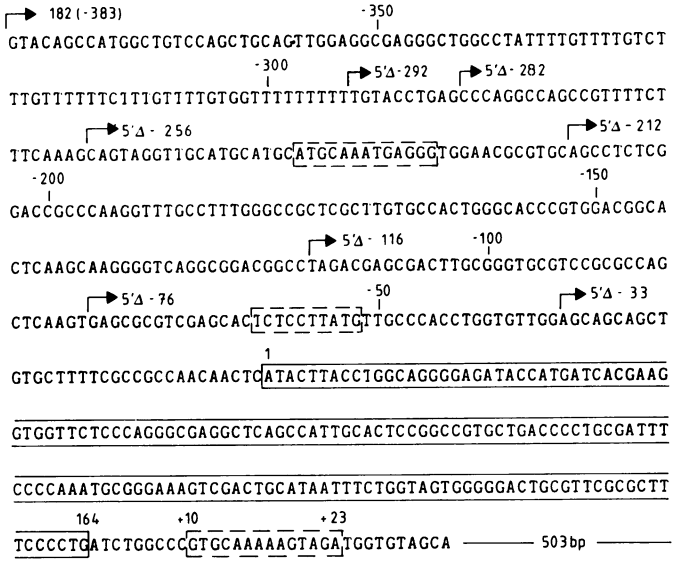

Fig. 6. End point of the *Bal*31 deletions in the 5'-flanking region of the U1B gene. The U1B coding region is boxed starting at nucleotide 1 and ending at nucleotide 164. Dashed boxes represent regions of homology with other U RNA genes and their description is in the text. Arrows indicate the end point of each deletion. 503 bp is the distance from the last nucleotide of the represented sequence and the next *Hind*III site. All *Bal*31 deletions contain this portion of the repeat.

observations were made by Krol *et al.* (accompanying paper). This interpretation might explain the finding that the U1A gene is less active since we have only studied a fragment from position −224. If the two genes have the same requirement for promotion these results suggest that the region −383 to −224 contains an essential promoter element.

More precise mapping of this element in the U1B gene required additional *Bal* deletion mutants. A new set was therefore made using Δ182 as the starting material. The end points of the deletions are indicated in Figure 6. The new deletions were injected into oocytes and the results of the transcriptional analysis are as shown in Figure 7. A first drop in the transcriptional efficiency of the U1B gene is achieved going from deletion 5'Δ-256 to deletion 5'Δ-212. An upstream promoter element is therefore located between position −256 and −212, whose equivalent is missing from our U1A subclone. The transcriptional efficiency of clone 5'Δ-212 is ~1/10 of that obtained with longer 5'-flanking regions. The same reduction is maintained in the further deletions 5'Δ-116 and 5'Δ-76, but transcription is completely lost when clone 5'Δ-33 is analyzed. This last result favours the assumption that a second proximal element exists between positions −76 and −33.

Importance of the −60 to −50 bp box of homology

The results presented above point to the existence of two distinct elements in the 5'-flanking region of the U1B gene responsible for promotion of transcription in the *X. laevis* oocytes. However, the *Bal*31 deletion analysis does not allow a conclusion about the relative role exerted by each component. Transcription is still observed albeit at a 10-fold lower level when only the distal element has been deleted; but how does the proximal element influence transcription? We hypothesized that the proximal element might reside in the region of homology between nucleotide −61 and −51 and therefore synthesized, by the phosphoamidite method, a 20-mer oligonucleotide containing the sequence corresponding to 10 nucleotides 5' and 10 nucleotides 3' of the homology box. This oligomer was used to remove the homologous sequence in the U1B gene by annealing it to a single-stranded DNA from the Δ182 (5'Δ-383) followed by primer extension with DNA polymerase and ligation (Zoller and Smith, 1983). Isolated clones were controlled by restriction mapping of the plasmid DNA and sequence analysis. One of them (Δ182/OL6) was chosen for microinjection into *X. laevis* oocytes. The results shown in Figure 7, panel B, established that deletion of this box abolishes U1B transcription.

Discussion

This paper identifies a repeat structure in *Xenopus* DNA which contains two different U1 RNA genes referred to as U1A and U1B, respectively. U1A and U1B genes encode the embryonic U1 RNA species in *X. laevis* (Forbes *et al.*, 1984; Lund *et al.*, 1984). The two U1 RNAs only differ in two internal positions but are very different in their 5'- and 3'-flanking sequences, with the exception of two homology regions, one 5'-terminal relative to the mature U1 RNAs over 10 bp and the other 3'-terminal over 13 bp (Figure 4). This amplified cluster of U1 RNA genes has independently been characterized by Krol *et al.*, (accompanying paper), who have also determined the sequence of the genes and studied their transcription after microinjection in *X. laevis* oocytes.

The microinjection of the embryonic U1 RNA genes from *Xenopus* into *Xenopus* oocytes appears to give faithful products in high yield, as previously established for a minor U1 gene (Zeller *et al.*, 1984).

The transcription of the embryonic U1B gene requires a region from 256 to 212 nucleotides upstream from the initiation site for efficient transcription (Figures 5 and 7). Within this region a

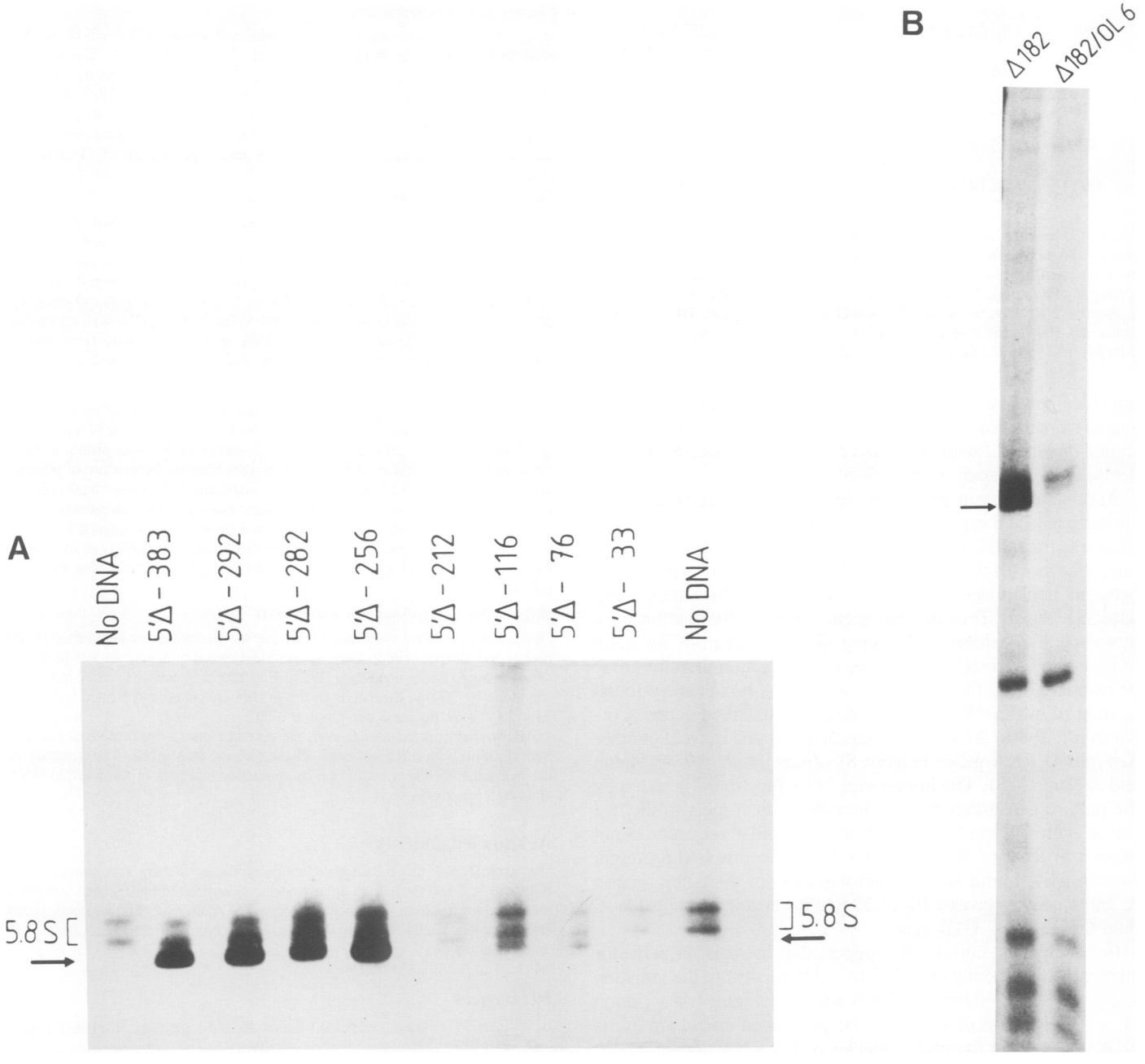

Fig. 7. Expression analysis of U1B deletions. **Panel A.** Progressive *Bal*31 deletions in the 5′-flanking region of the U1B gene were microinjected in *X. laevis* oocytes and the RNA products were displayed on a 6% polyacrylamide gel. Arrows indicate U1B RNA related products. **Panel B.** Transcriptional analysis of the −60 to −50 deletion (182/OL6).

sequence can be identified, from nucleotide −237 to −226 (boxed in Figure 6), that is homologous to the upstream element promoting human U1 RNA transcription (Skuzeski *et al.*, 1984). The homologous sequence starts in the human clone HU1.1D in position −219 and shows a 10 out of 12 bp fit with the putative upstream element found in front of the U1B gene (Figure 8). A similar sequence could not be found in the 223 nucleotides preceding the U1A sequence in our clone (Figure 3) which could explain why microinjection of this gene gives rise to U1A RNA transcripts at 10-fold lower levels than those observed with the U1B-carrying templates. This element for the U1A gene may, however, be located at a larger distance from the gene within the 1.9-kb repeat fragment (Lund *et al.*, 1984) and thereby it is not included in our *X. laevis* 1.5-kb λ clones because of our cloning strategy. Krol *et al.* (accompanying paper) have, in fact, independently determined the sequence of the 1.9-kb major repeat from *X. laevis* and similar sequences partially homologous to the U1B putative upstream element can be identified starting at nucleotides −262 of the U1A gene. (Figure 8)

A similar consensus sequence was identified in the minor U1 RNA gene between nucleotides −34 and −23 (Zeller *et al.*, 1984) (Figure 8) relative to the start of transcription. This would explain the efficient expression of the microinjected gene when only 150 bp of the 5′-flanking region are present.

More recently, Mattaj *et al.* (1985), in the analysis of the promoter of a *X. laevis* U2 RNA gene, have identified an upstream element located at approximately the same location as in the U1B gene and showing a long stretch of homology with the U1B gene

U1B	-237 ATGCAAATGAGG	-61 TCTCCTTATG
U1A	-262 ATGTAAACGCGC	-61 TCTCCGTATG
U1MINOR	-34 ATGTAG-TGGGG	-60 TCTCCGTATG
XU2.5	-269 ATGCAAATAGGG	-60 TCTCCCCATG
HU1.1	-219 ATGTAGATGAGG	——

Fig. 8. Comparison of putative promoter elements in U1 and U2 RNA genes. U1A and U1B sequences are from this paper with the exception of the U1A putative upstream element (which is from Krol *et al.*, accompanying paper). U1 minor is from Zeller *et al.* (1984). XU2.5 is a *X. laevis* U2 RNA gene isolated by Mattaj and Zeller (1983). HU1.1 is a human U1 RNA gene directing U1 RNA transcription in *Xenopus* oocytes (Murphy *et al.*, 1982; Skuzeski *et al.*, 1984).

sequence between positions −237 and −226 (Figure 8) that is able to increase promoter activity by ~20-fold. They also demonstrated that the U2 sequence behaves like an orientation-independent element (Mattaj *et al.*, 1985).

Another important promoter element is located in a region closer to the U1B cap site. This is supported both by the analyses of the shorter *Bal*31 deletions 5′Δ-76 and 5′Δ-33 and by the microinjection of the Δ182OL6 deletion that removes the region between nucleotides −61 and −51 leaving the surrounding sequence intact. This element seems to exert its function in a more specific fashion, influencing the mechanism of initiation of transcription by RNA polymerase II. Its action therefore may be compared with that exerted by the TATA-box element found in front of most of RNA polymerase II transcribed genes (Corden *et al.*, 1980). An identical sequence is found in front of other *Xenopus* U RNA genes (Figure 8) (Zeller *et al.*, 1984; Mattaj and Zeller, 1983). The importance of its function is stressed by the precise conservation of its location with respect to the cap site of both U1 and U2 RNA genes. A similar sequence is not present in front of the human HU1.1 gene; this finding might explain why, in our hands, microinjection of the latter gene in *X. laevis* oocytes gives a 10- to 20-fold lower transcriptional level than the *Xenopus* U1B gene.

In conclusion, our results suggest that there is an activator element within the region −237 to −226 relative to the initiation site for the U1B embryonic RNA which increases transcription of ~10-fold. This element may be positioned elsewhere in the U1A gene and in the minor species of U1 RNA (Zeller *et al.*, 1984). The box of homology with other *Xenopus* snRNA genes positioned between nucleotides −60 and −50 relative to the cap site of the U1 RNAs appears to be another important promoter element with a TATA box-like function.

Materials and methods

Bacterial strains, plasmids and phage vectors

Escherichia coli K12 (strain 71/18) was used for transformation (Gronenborn and Messing, 1978). The M13 derivatives mp8, mp9, mp18 and mp19 and the plasmid vector pUC8 (Messing, 1983) were used as vectors for subcloning and sequencing. Transformations and preparations of ds and ss DNAs were as described (Cortese *et al.*, 1978; Messing, 1983). DNA sequence analyses were carried out using the dideoxy chain termination method (Sanger *et al.*, 1977).

Enzymes

Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, DNA polymerase I holoenzyme and Klenow fragment were purchased from BRL and Biolabs. The ^{32}P-labelled compounds were purchased from Amersham Buchler. AMV reverse transcriptase was from Boehringer, Mannheim. *Bal*31 nuclease was purchased from BRL.

Construction of phage λ library

50 μg of *X. laevis* were digested with *Hind*III and applied on a 5−30% sucrose gradient in 1 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA. The gradient was centrifuged for 22 h at 26 K at +5°C. Fractions containing the *Hind*III-*Hind*III 1.5-kb fragment (analyzed by dot-spot hybridization to the pHU1.1D probe) were pooled and cloned into lambda vector λ1147 using a host *E. coli* strain POP136 (Murray *et al.*, 1977). 50 000 plaques were obtained and subsequently screened using as probe the human U1 RNA gene in plasmid pHU1.1D (Murphy *et al.*, 1982).

Microinjections and RNA gel electrophoresis

Microinjections in *X. laevis* oocytes were performed as described (Cortese *et al.*, 1978) using 30 nl solution of DNA (200−300 μg/ml) and [α-^{32}P]GTP as RNA precursor (410 Ci/mmol, 10 mCi/ml). Usually at least 20 oocytes/sample were injected. RNA was extracted and analyzed by gel electrophoresis in TBE, 7 M urea, 6% acrylamide (Acryl:bis, 20:1). Polyacrylamide gels (40 cm long, 20 cm wide, 1 mm thick) were run at 300 V for 24 h. λ DNA was injected at the concentration 200 μg/ml, plasmid DNA at a concentration from 300 to 500 μg/ml. Selection of the hybrids was according to McGrogan *et al.* (1979).

Construction of Bal31 deletion mutants

Clone pUC *Hind-Hind* (Figure 5) was linearized with *Bam*HI and 10 μg of linearized plasmid were subjected to treatment with *Bal*31 (0.2 U/μg) at 30°C for variable incubation times (1−20 min). The mixture was extracted with phenol and the DNA ethanol precipitated. After digestion with *Hind*III, the mixture of plasmid and *Bal*31-deleted 1.5-kb insert was shotgunned into M13 vector mp9 digested with *Hind*III-*Sma*I. Several white plaques were analyzed. They all contained deletion subclones of the original 1.5-kb insert and we never found deleted fragments of the original pUC8 plasmid. This was probably due to instability of M13-pUC recombinants. *Bal* deletions of clone Δ182 were generated following the same protocol.

Oligonucleotide synthesis and mutagenesis

Oligonucleotides were synthesized following the phosphite-amidite method (Winnacker and Dorper, 1982). Oligonucleotide-directed mutagenesis was performed according to Zoller and Smith (1983). Enrichment of double-stranded molecules was done by 30 min digestions of the primer elongated and ligated molecules with 5 U of S1 nuclease per pmol at 37°C.

After transformation into *E. coli*, the correct clones were detected by colony hybridization with the 5′-labelled oligonucleotide as a probe. The structure of the isolated clones was confirmed by restriction mapping of the plasmid DNA and sequence analysis.

Acknowledgements

We thank Dr R.Frank for oligonucleotide synthesis. We are also grateful to Dr Maurizio Sollazzo for help in the oligonucleotide-directed mutagenesis and Petra Stevenson for technical assistance. We thank Drs Alain Krol, Elsebet Lund, James Dahlberg, Iain Mattaj and Eddy De Robertis for communicating their results prior to publication.

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Received on 1 April 1985