Properties of a U1 RNA enhancer-like sequence

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ABSTRACT

The properties of a *X.laevis* U1B snRNA gene enhancer have been studied by microinjection in *Xenopus* oocytes. The enhancer-like sequence, defined as a short DNA stretch that is able to activate transcription in an orientation independent manner, is interchangeable between different U snRNA genes. The enhancer sequence alone does not, however, efficiently activate transcription from an SV40 pol II promoter but regains its activity when combined with the U-gene specific proximal sequence element. DNase I protection experiments show that the *X.laevis* U1B enhancer can interact specifically with a nuclear factor present in mammalian cells.

INTRODUCTION

In eukaryotic organisms RNA polymerase II is considered to be responsible not only for the transcription of protein-encoding genes but also for the synthesis of small nuclear RNAs (1). In the last years several U-RNA encoding genes have been isolated and transcriptional analysis has been carried out in various *in vivo* and *in vitro* systems (2-10). At least three distinct elements seem to be responsible for U1 and U2 RNA synthesis and all of them present features that distinguish them from the elements involved in transcription of other pol II genes (reviewed in 11). Two elements are located in the 5' flanking sequence and are required for accurate and efficient U snRNA synthesis. One enhancer-like sequence (2,6,12) is positioned approximately 250 bp from the cap site and activates transcription 10 to 20 fold. This sequence works in both orientations and is well conserved among different U snRNA genes, not only within one species but also between species. Furthermore, the enhancer shows some homology with sequences present in some viral and cellular enhancers.

A second element is located roughly 50 to 60 bp from the cap site. This proximal sequence element is conserved among different U snRNA genes from the same organism and is an essential element for the transcription of *Xenopus* U1 and U2 snRNAs (3,6). It behaves like a TATA-box element in positioning the site of transcription initiation (8,13). However, the TATA box and the proximal sequence element share no sequence homology and are at different positions relative to the cap site. A third conserved sequence, about 14 bp long, is present 10 to 30 bp downstream from the end of the gene in the 3' flanking sequence and is involved in correct 3' end formation (14-18).

In this paper we examine the properties of the enhancer element from an *X.laevis* U1 gene. This element alone is incapable of activating transcription of a pol II transcription unit containing the SV40 21 bp repeats, but the enhancer functions in combination with the proximal sequence element in this transcription unit. This enhancer element is capable of binding a factor present in nuclear extracts from somatic cells.

MATERIALS AND METHODS

General methods.

Bacterial enzymes, T4 DNA ligase, polynucleotide kinase, DNA polymerase I, Klenow fragment and S1 nuclease were purchased from BRL and BioLabs and used following the procedures of Maniatis et al. (19). Transformations and preparations of ds and ss DNA were as described (20). DNA sequence analyses were carried out using the dideoxy-chain-termination method (21).

Microinjections.

Oocytes were injected, fractionated and extracted RNAs were analysed as described (22).

Oligonucleotide synthesis and mutagenesis.

Oligonucleotides were synthesized with the phosphite-amidite method (23). Oligonucleotide-directed mutagenesis on ssDNA templates was performed according to Zoller and Smith (24).

Construction of plasmids.

pUCU1A IDB1 and IDB2: plasmid pUC HindIII-PvuII was linearized with BamHI, the ends repaired with Klenow enzyme and recircu-

larized in the presence of IDB3 and IDB4 oligonucleotides. Clones carrying the insert were selected by hybridization with ^{32}P -kinased IDB3 oligonucleotide and then sequenced (21). pA10 CAT-2 derivatives were all constructed by linearizing pA10 CAT-2 plasmid with BglII (25): blunt ends were generated by treatment with Klenow in the presence of excess deoxynucleotide triphosphates and recircula-rized in the presence of the various UIB segments shown in Fig. 3. R-Alu fragment was purified from deletion Δ -282 (3) making use of the artificial EcoRI site introduced in this position after Bal 31 treatment and of the natural Alu I site in position -84. BglU1.1 and BglU1.2 were constructed making use of the natural PvuII sites at positions -360 and -26.

CAT assays.

CAT assays from injected oocytes were performed as described (26,27).

S1 analysis.

Conditions for formation of DNA-RNA hybrids and for digestion with S1 were as described (28). For the S1 analysis we used as probe a uniformly labelled DNA synthesized on an ssDNA template (29). To this aim we first converted the BglII site in pA10 CAT-2 into a BamHI site and then subcloned the BamHI-EcoRI fragment (Fig. 3) into the M13mp19 vector (20). After elongation with the commercial 17mer (BioLabs) in the presence of α - ^{32}P -dATP and α - ^{32}P -dCTP, BamHI was added. The resulting ssDNA fragment was purified through DNA denaturing gel electrophoresis.

Footprinting analysis.

The probe was the UIB-containing EcoRI-HindIII fragment of Δ 182, end-labelled by kinasing the HindIII site in the presence of γ - ^{32}P -ATP (19). Nuclear extracts were prepared according to Dignam et al. (30) with the modifications introduced by Wildemann et al. (31). 4 μl of nuclear extract from MOLT-4 cells was preincubated with 25 ng of linearized pUC plasmid DNA on ice for 15'. The reaction (10 μl) contained 10% glycerol; 10 mM Hepes-KOH (pH 7.9); 20 mM KCl; 4 mM MgCl₂; 0.1 mM EDTA and 0.25 mM DTT. 10,000 cpm of end labelled probe was added in 2 μl H₂O and incubated for 10 minutes at 20°C. 2 μl DNase I (Worthington DPFF) freshly diluted in the incubation buffer to give final

concentrations of between 7 and 112 $\mu\text{g/ml}$ were added and digestion was carried out for 90 seconds at 20°C . DNase digestion was stopped by addition of 100 μl of phenol/chlorophorm/isoamylalcohol (25:24:1). The aqueous phases were reextracted with chlorophorm/isoamylalcohol (24:1), ethanol precipitated and loaded onto denaturing 6% acrylamide urea sequencing gels.

RESULTS

Partial or complete deletion of the U1B enhancer

We and others (3,4) have recently reported the cloning of two *X.laevis* U1 RNA genes that are expressed at high levels during embryonal development and are called U1A and U1B. The genes are contained in a 1.9 kb DNA segment repeated about 500 times per haploid genome (32). We have cloned a truncated repeat missing about 350 bp at the 5' end (3). Our clone pXU1.1 carries only 223 bp of U1A 5' flanking sequence, the U1A and U1B coding regions, the intergenic region and 536 bp of U1B 3' flanking sequence. In *X.laevis* oocytes this clone gives rise to abundant U1B transcripts, while U1A transcription is ten-fold lower. The U1B enhancer was mapped between nucleotides -256 and -212 by constructing a series of Bal 31 deletions. After comparison with other U snRNA flanking sequences we proposed that the sequence from -237 to -226 is the essential enhancer element. Deletion analysis does not, however, prove conclusively that no other sequences are involved in stimulation of transcription. The results observed might be due to an additive effect of deleting other important upstream sequences or to an inhibitory effect of vector sequences brought closer to the gene. To distinguish between these possibilities we deleted the putative enhancer leaving the sequences upstream and downstream intact. We constructed a 21 base long oligonucleotide (Fig. 1) encompassing the sequence from -237 to -226 and used it to direct mutagenesis on the $\Delta 182$ ss template (see Fig. 1). This clone carries 383 bp of 5' flanking sequence. Several clones were obtained and sequenced. Two were analysed in detail: $\Delta 182$ 2.2 and $\Delta 182$ 2.9 (Fig. 1). The first has the expected deletion, the second has only a deletion of the 3' portion of the enhancer. It probably arose from imperfect annealing of the mutagenizing oligonucleo-

Fig. 1 Schematic diagram of the U1B enhancer insertion and deletion mutants.

A) Structure of the w.t. U1B gene (clone Δ182) and of the two oligonucleotide-directed mutants in the enhancer region (2.2 and 2.9). Lines represent U1 5' and 3' flanking regions. Black boxes: coding regions; open boxes: enhancer-like sequences.

B) Structure of the *X.laevis* U1A gene in plasmid pUC HindIII-PvuII and of the two U1B enhancer insertion mutants IDBR and IDBI.

tide. Transcriptional analysis of these two mutants (Fig. 2) shows in both cases a 10- to 20-fold decrease in transcriptional efficiency (Fig. 2, compare lane 1 with lanes 2 and 3).

The U1B enhancer acts on a different U1 RNA gene.

It is likely that the low transcriptional level of U1A in clone pXU1.1 is due to the absence of an enhancer sequence in the 223 bp of 5' flanking sequence. Krol et al. (4) have in fact identified a sequence homologous to the U1B enhancer in the U1A 5' flanking sequence 265 to 255 bp upstream from the cap site, the deletion of which caused a 10 to 20 fold decrease in U1A transcription. We have therefore constructed two complementary oligonucleotides containing the sequence of the U1B 5' flanking region from -238 to -226 and inserted them in both orientations

Fig. 2 Analysis of the expression of the U1B enhancer insertion and deletion mutants. 6% polyacrylamide gel electrophoresis of RNA synthesized in *X.laevis* oocytes: lane 1, $\Delta 182$; lane 2, $\Delta 182$ 2.2; lane 3, $\Delta 182$ 2.9; lane 4, pUC HindIII-PvuII; lane 5, pU1A -IDBR; lane 6, pU1A -IDBI.

in clone pUC HindIII-PvuII upstream of the U1A gene. This clone (Fig. 1) has 212 bp of the U1A 5' flanking region plus 40 bp of 3' flanking sequence. Microinjection of these two plasmids in *Xenopus* oocytes shows that U1A transcription is enhanced in both cases 5 to 10-fold (Fig. 2, compare lane 4 with lanes 5 and 6). It is therefore possible to exchange the U1B enhancer element between two different U-RNA genes without losing the enhancement effect.

The U1 enhancer fails to act on an SV40 promoter

We wanted to test if the U1A transcriptional enhancer is able to work on another pol II transcriptional unit. The SV40 early promoter was selected. Plasmid pA10 CAT-2 carries the SV40 early region including the 21 bp repeats and the early cap site down to the Hind III site at position 5172 (33) fused to the bacterial gene encoding chloroamphenicolacetyltransferase (34). This construct, when injected into *X.laevis* oocytes (27) or transfected into mammalian cells (25), does not give appreciable synthesis of CAT enzyme because the SV40 promoter requires an enhancer in *cis* for activation. This plasmid was used as recipient of different segments of the U1B gene 5' flanking region (Fig. 3A) and these were all cloned upstream of the SV40 early promoter. Plasmids pA10 IDBR and IDBI carry the enhancer sequence alone cloned in the correct and in the inverse orientation respectively. Plasmids pA10 R-Alu 10 and 11 contain a

longer region from base -282 to -84, thus including not only the enhancer element but also the region downstream of it, but excluding the proximal sequence element. Plasmids pA10 BglU1.1 and BglU1.2 carry the entire U1 promoter from base -360 to base -26 in both orientations. These plasmids were tested for their ability to induce CAT activity after microinjection in *X.laevis* oocytes. The results (Fig. 3B) indicate that only the entire promoter is functional and then only when it is cloned in the correct orientation with respect to the CAT gene. The enhancer sequence by itself or in combination with a segment of the 5' flanking sequence is not able to activate the SV40 21 bp repeats. S1 analysis of RNA from oocytes injected with either pSV2 CAT or pA10 BglU1.1 (which contains the entire U1B 5' flanking sequence in the inverted orientation) shows that the transcription initiation sites are identical (Fig. 3C, lanes 3 and 4, arrowheads); and pSV2 CAT simply produces more transcripts. The transcription pattern of pA10 BglU1.2 is more complex. The U1B promoter construct not only enhances transcription directed by the SV40 21 bp repeats (compare pA10 BglU1.2 and pA10 BglU1.1, Fig. 3C, lanes 4 and 5, arrowheads), but also causes the appearance of several new transcription starts (arrows). The longest of these new transcripts initiates roughly 70 bp downstream of the U1B proximal sequence element. In other words, the U1B sequences are functioning as a complete promoter as well as having a weak activating effect on the SV40 21 bp repeats. Other experiments (I.M., A. Tognoni, P. Matthias, W. Schaffner, E. De Robertis, unpublished) with a *X.laevis* U2 enhancer support this conclusion. The U2 enhancer (6) is capable of stimulating transcription of SV40 T antigen through the 21 bp repeats, or thymidine kinase through the TK promoter only to a very limited extent when tested either in mammalian cell cultures or in *X.laevis* oocytes respectively. In these experiments the weak stimulation only occurs if the enhancer is introduced 5' to the heterologous promoter elements.

A nuclear factor which binds to the U1B enhancer.

Competition experiments (6,9) suggest the existence of a transcription factor which binds to U snRNA gene enhancers. We decided to look directly for the presence of such a factor by foot-

printing. An end-labelled DNA fragment containing the U1B enhancer was incubated with nuclear extract from human MOLT-4 cells which had previously been shown to contain enhancer binding factors (35). After digestion with various concentrations of DNase I, the resulting fragments were resolved on a denaturing acrylamide gel in order to look for regions protected by nuclear factors against DNase digestion. Comparing the lanes in which naked DNA (Fig. 4A, lanes 3 and 4) was digested with DNase I with those in which DNA had been allowed to bind to nuclear factors before digestion (Fig. 4A, lane 5) one obvious region of protection is observed which is indicated by a square bracket. Making use of the size markers (lane 1) and a G+A sequence of the U1B fragment (lane 2) we mapped the protected region, and showed that it coincides with the U1B enhancer sequence (see diagram in Fig. 4B).

DISCUSSION

Efficient transcription of U snRNA genes is strictly dependent on a sequence in the 5' flanking region centered 280 to 220 bp from the cap site (2,3,4,6,7,8,9). Peculiar features of this sequence are the extreme conservation among members of the same snRNA gene family and a striking homology with sequences contained in several viral and tissue-specific cellular enhancers (6,12). This last observation led to the idea, which soon gained experimental support (2,6,12), that this promoter element might act as an enhancer, enabling the formation of a stable transcription com-

Fig. 3 U1B promoter-CAT gene fusions: structure and expression. A: upper part: plasmid pA10 CAT-2 has been described (14). lower part: all U1 inserts were cloned into the unique BglII site of plasmid pA10 CAT-2 as described in the materials and methods section. Open boxes represent the enhancer sequence and black boxes represent the proximal sequence element. B: CAT analysis from oocytes injected with U1B promoter-CAT fusions. Lane 1, pSV2 CAT; lane 2, pA10 CAT-2; lane 3, pA10 BglU1.1; lane 4, pA10 BglU1.2; lane 5, pA10 R-Alu10; lane 6 pA10 R-Alu11; lane 7, pA10 IDBI; lane 8, pA10 IDBR. C: S1 analysis of RNA from oocytes. Lane 1, HpaII fragments of pBR322; lane 2, probe DNA. The next lanes show the probe fragments protected against S1 digestion by RNA from oocytes injected with; lane 3, pSV2 CAT; lane 4 pA10 BglU1.1; lane 5, pA10 BglU1.2.

Fig. 4. A. Protection of the U1B enhancer from DNaseI digestion by a nuclear factor.

A restriction fragment containing the U1B enhancer was generated by HindIII digestion, end-labelling with polynucleotide kinase, then digestion with EcoRI. The G + A sequence of this fragment is shown in lane 2. Lanes 3 and 4 are DNase digestions of the naked fragment (DNase concentrations of 7 and 14 $\mu\text{g/ml}$ respectively). Lane 5 is a DNase digestion (112 $\mu\text{g/ml}$) of the fragment in the presence of nuclear extract made from the MOLT 4 cell line. The protected region is indicated by a bracket. Lane 1, HpaII fragments of pBR322.

B. The position of the protected region on the DNA sequence. The protected region, indicated by a bracket, spans the sequences shown to be important for enhancer activity. The arrow points to a position of extract-induced DNase hypersensitivity.

plex (6), and thus increasing initiation by RNA polymerase II (36,37). A short oligonucleotide containing sequences from the *Xenopus* U2 upstream element is able to enhance transcription in an orientation independent manner at its natural distance from the cap site (6). The same is true for a 72 bp restriction

fragment containing the human U2 enhancer when tested in HeLa cells. In this case the upstream element is able to induce transcription from an enhancerless human U2 gene even when located in the 3' flanking region (12). We show here that the upstream element from the *X.laevis* U1B gene, as a short 14 bp long DNA stretch, can enhance transcription of another U snRNA gene. Ares et al. (2) have previously shown that a 4 bp deletion of the 5' portion of the human U2 enhancer is sufficient to destroy enhancing activity. Our mutant $\Delta 182$ 2.9 in which only 9 bp in the 3' moiety of the enhancer have been deleted also behaves like a complete deletion of the element ($\Delta 182$ 2.2). These two results taken together indicate that the U snRNA enhancer may be considered as a compact structure, from which removal of a relatively short sequence results in the complete loss of enhancing activity. U snRNA gene enhancer-like sequences thus appear to be much shorter and less redundant than other enhancers. As an example the SV40 enhancer is present as a duplicated repeat of a 72 bp sequence which also requires for full activity sequences outside of these repeats (41). Deletions from either side or internal base pair substitutions in this region provoke a more or less pronounced decline in enhancing activity (38,39,40,41). Mutational analyses on several enhancers have led to the idea that cellular or viral enhancers are complex structures composed of several distinct modules, each contributing to the enhancing activity, and each presumably being a binding site for transacting positive or negative regulatory proteins. These modules may individually retain part of the enhancing activity (41,42). Full activity is, however, seen as requiring not only protein-DNA, but also protein-protein interactions. In this view the U snRNA gene enhancers are simplified enhancer sequences in that they may be composed of only one such module.

The U1B enhancer is able to bind a nuclear factor present in limiting amounts in *X.laevis* oocytes, as demonstrated by competition experiments (6). The same factor presumably interacts with all U snRNA enhancer-like sequences since U2 transcription can be competed out by a 5- to 10-fold molar excess of coinjected human U1 gene (9). Our footprinting data show the existence of such a

factor in a nuclear extract from MOLT-4 cells. This result is not surprising if one considers the extreme sequence conservation among U snRNA gene enhancer-like sequences among different species (4,6,12) and the fact that human U snRNA genes are readily expressed in *Xenopus* oocytes (2,7,9). It is possible therefore that the binding protein has not undergone structural changes in the DNA-binding region during evolution. It must also be ubiquitous, since transcription of snRNAs occurs in all cells, and it is probably a general factor necessary for the transcription of several other class II genes. This conclusion is based on the observation that the same U enhancer-like module is present in the middle of other enhancers. Binding studies have shown that apparently the same factor binds to this conserved sequence in the case of *Xenopus* U2, the SV40, and the IgG enhancers (35).

It is not surprising therefore that, due to its simplified structure, the U1 enhancer is not able alone to induce transcription from a viral pol II transcriptional unit. One can envisage that U snRNA gene promoters have evolved their signals in such a way as to require a specific interaction between the distal and proximal sequence elements. The proximal element presumably binds a transcription factor that is responsive to interaction with the nuclear factor binding to the upstream element. The lack of sequences similar to the proximal element in other pol II promoters indicates that this factor may be U snRNA gene specific.

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