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A conserved region in the sea urchin U1 snRNA promoter interacts with a developmentally regulated factor

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ABSTRACT

The expression of the sea urchin *L. variegatus* U1 snRNA gene is temporally regulated during embryogenesis. Using a microinjection assay we show that a region between 203 and 345 nts 5′ of the gene is required for expression. There are four conserved regions between two sea urchin species in the 345 nts 5′ to the U1 gene. One region, located at about −300, binds a protein factor which is present in blastula but not gastrula nuclei. Three other potential protein binding sites within the first 200 nts 5′ to the gene have been identified using a mobility shift assay and/or DNase I footprinting. Two of these regions bind factors which are not developmentally regulated and one binds a factor which is developmentally regulated. It is likely that the factor which binds at −300 is involved in expression and developmental regulation of the sea urchin U1 snRNA gene.

INTRODUCTION

The small nuclear RNAs U1, U2, U4, U5 and U6 are required for processing of pre-mRNA precursors. In the sea urchin, large amounts of snRNAs are required both during oogenesis and in early embryonic development for synthesis of the maternal and embryonic mRNAs. In a number of different species, including mouse (1,2), *Xenopus* (3,4), *Drosophila* (5) and sea urchin (6), there are changes in the snRNAs synthesized during early embryonic development. In the sea urchin, the large store of maternal snRNAs synthesized during oogenesis may not be utilized by the embryo (7). Rather the sea urchin embryo synthesizes large amounts of snRNAs during early development, starting at about the 32-cell stage (8). The rate of snRNA synthesis declines between blastula and gastrula stage (8,9), as the rate of cell division drops dramatically.

The U1 and U2 snRNAs synthesized in early embryonic development are encoded in tandemly repeated gene sets containing about 60 gene copies (8,10,11). The U1 RNAs found in adult tissues differ in sequence from the U1 RNAs found in the egg and early embryo (6), suggesting that there is a second set of U1 snRNA genes, present in low copy number, which is active in adult tissues. In the sea urchin *L. variegatus*, the tandemly repeated gene set is expressed at a high rate until shortly after hatching. By the gastrula stage the tandemly repeated gene set has been silenced (9) and the embryonic U1 snRNA is gradually replaced by the adult U1 snRNA.

The change in expression of the U1 gene is presumably accomplished by a loss of positive factors necessary for the expression of U1 snRNA and/or by the appearance of negative factors which turn off the gene. Here we present evidence that there are developmentally regulated factors which interact with the U1 snRNA promoter.

MATERIALS AND METHODS

Culture of sea urchin embryos

L. variegatus sea urchins were collected off Turkey Point, FL and maintained at the Florida State University marine laboratory. The embryos were cultured at 28°C. The blastula embryos were harvested within one hour after hatching (8 hrs after fertilization), the time of maximal U1 snRNA synthesis, and gastrula embryos were harvested at 20 hrs after fertilization. Purified nuclei and nuclear extracts were prepared as previously described (12,13). Typical extract concentrations were 5–10 mg/ml protein for extracts from blastula embryos and 8–12 mg/ml protein for extracts from gastrula embryos. Extracts were stored in small aliquots in liquid N_2.

Heparin-agarose Chromatography

For the DNase I footprinting experiments the gastrula extract was fractionated by heparin agarose chromatography. The extract was loaded on the column in 0.1M KCl, 50 mM Tris, pH7.9, 1 mM EDTA, 1mM DTT, 12.5 mM $MgCl_2$, and 20% glycerol and the column washed with the same solution. The DNA-binding proteins were eluted with 0.4M KCl in the same buffer. The DNA binding proteins were quantitatively recovered as judged by recovery of the activity in the mobility shift assay. Seven per cent of the input protein was present in the 0.4M KCl fraction.

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DNA probes

DNA probes were prepared from the intact LvU1.2 gene (14) or from appropriate subclones. The DNA was digested with a restriction enzyme, end-labeled by a fill-in reaction using the Klenow fragment of DNA polymerase and α-$^{32}PO_4$-dATP and/or α-$^{32}PO_4$-dCTP. The fragment was then digested with a second restriction enzyme and the labeled DNA fragment purified by native polyacrylamide gel electrophoresis. The labeled DNA was recovered from the gel by crushing the gel in 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.8M NaCl and soaking overnight at 37°C. The gel was removed by centrifugation, the supernatant extracted successively with phenol and ether and the DNA recovered by precipitation with ethanol.

Complementary oligonucleotides containing cohesive overhangs on the end of each strand were synthesized on an Applied Biosystems Model 381B oligonucleotide synthesizer. The two strands were annealed by heating to 70° for 2 min and then cooling slowly to room temperature. The doubled-stranded oligonucleotides were labeled using the Klenow fragment of DNA polymerase as described above.

Microinjection of sea urchin zygotes

The cloned DNA was linearized at the HindIII site 3′ of the U1 gene. The DNA was injected into fertilized eggs of the sea urchin *S. purpuratus* by the procedure of Davidson and coworkers (15) as modified by Colin and coworkers (16). The embryos were allowed to develop to the hatching blastula stage (24 hrs) at 15°C. Total nucleic acid was prepared from the embryos as described by Colin and coworkers (16). The expression of the U1 gene was analyzed using a riboprobe protection assay using the conditions described previously for the sea urchin U1 RNA (6). An aliquot of the nucleic acid was spotted onto nitrocellulose and hybridized to pUC18 labeled by nick-translation to determine the relative amounts of DNA present in the embryos (16).

Mobility shift experiments

The mobility shift assays were performed by incubating 7 ng (0.5 pmoles) of labeled oligonucleotide, 2 μg of poly dIdC (Pharmacia) and 3−40 μg of total nuclear protein in 10 mM Tris·HCl, pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 5% (v/v) glycerol in a total volume of 10 μl, on ice for 20 min. The reactions were loaded onto a 10% non-denaturing polyacrylamide gel and electrophoresis continued at 25−30 ma until the bromphenol blue reached the bottom of the gel. The DNA-protein complexes were detected by autoradiography.

DNase I footprinting

DNase I footprinting reactions (17) were performed by incubating the nuclear extract with 0.1% NP-40 on ice for 15 minutes in the binding buffer used in the mobility shift assays. The nuclear extract was then added to 5−10 ng (0.05 to 0.1 pmoles) of the labeled restriction fragment and incubated at 25°C for 20 minutes under the same conditions used for the mobility shift assays except that the dIdC concentration was reduced to 1 μg/ml. The samples were then chilled on ice and $MgCl_2$ and $CaCl_2$ were added to a final concentration of 5 mM and 2.5 mM, respectively. The samples were then treated with DNase I (final concentration 6.25−50 μg/ml) on ice for 1 min. Digestion was stopped by adding 20 μl of 10 mM EDTA, 0.5% SDS, 0.1M NaCl and 0.5 μg of yeast tRNA. The samples were then extracted with phenol and the DNA recovered by precipitation with ethanol. The DNA was analyzed on an 8% polyacrylamide gel containing 7M urea.

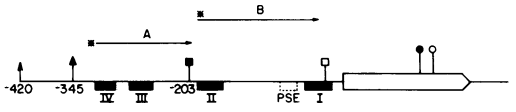

Fig. 1. The *L. variegatus* U1.2 snRNA gene. The structure of the tandemly repeated *L. variegatus* U1 snRNA gene is shown, with the position of the four blocks of sequence (solid boxes I-IV) which are conserved between the *L. variegatus* and *S. purpuratus* gene indicated. The proximal element (PSE) which is not well conserved but which is required for expression of the gene is also indicated by a dashed box. The position of the restriction sites used in constructing the probes and deletions is indicated.
AvaI; BglII; DraI; EagI; HincII; HindIII;

Table I. Homology between the *L. variegatus* and *S. purpuratus* U1 promoters.

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                    **** ****          *  *   **   * *** ****
S. purp.  -350  GATTTTCGTA TGACGCTTTTGTTATTTAAA CCGTGACGTC
L. var.   -329  GCGAGTAAGC TGACGCTTATGTCATTATAA TCTGCAACGT
                                Box IV

                      ******     * **          *  *    * ********
S. purp.  -310  AGTATCTGCG TTGTTATATTCATGAATATGCACCTCT CATCAACAAT
L. var.   -288  AGTAAAGTTA TTGTGAAGTTCATGAATATTCACATCT TACTTCTTCA
                                Box III

                **********      *  *     *  ***       **********
S. purp.  -129  GCAGTCACAC CTCCCAAAGTGGGGCATAGATGAGATT TGAGATTCTG
L. var.   -177  ATTAATGTTG CTCCCAGAGAGGGGCACA  GAGATT GATTTGAGGT
                                Box II

                 **** ***             *  *          *** ** *
S. purp.  -50   CATCCAAGGG  ATTTGATCAAGAACTGCGACCGAT  TCTTTCGAAG
L. var.   -48   CGCAGATAAG  ATTTGATCAAGTACGGCGACCGAT  GGATGGGGAG
                        26  TTGATCAAGAAGGA  39
                                Box I
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The four regions of homology between the *S. purpuratus* and *L. variegatus* U1 promoters are shown and are underlined. Ten nucleotides on either side of the conserved regions are also shown. A portion of the sequence of box I is repeated within the coding regions (10). The numbers are the distance in nucleotides from the start of transcription. The asterisks indicate the differences in sequence between the two species.

RESULTS

The tandemly repeated sea urchin U1 snRNA genes have been cloned and sequenced (10,18) from two sea urchin species, *S. purpuratus* and *L. variegatus*. These two species of sea urchin diverged about 35 million years ago (19). We expected that the sequences involved in expression of these two genes would be conserved between the two species since the *L. variegatus* gene is expressed efficiently when it is injected into *S. purpuratus* embryos (see Fig. 2 below). Inspection of the sequences of the U1 genes from the two sea urchin species showed four regions of similarity between the 5′ flanking regions of the U1 gene from the two species (10). Figure 1 shows the structure of one repeat unit of the *L. variegatus* U1 gene, with the four regions of similarity labeled I-IV. In addition there is a sequence at about −50 (the PSE, shown as a dotted box) which is less well conserved, but which is necessary for expression (B. Wendelburg and W.F.M., unpublished results) and plays the same role as the vertebrate proximal element (20). Oligonucleotides corresponding to the sequences in box I-IV were synthesized and used in mobility shift assays. These oligonucleotides are shown in Table II, as are the non-specific oligonucleotides NS I and NS II which were used as competitors in some assays. The sequences of the four conserved elements between the two sea urchin species are also compared in Table I.

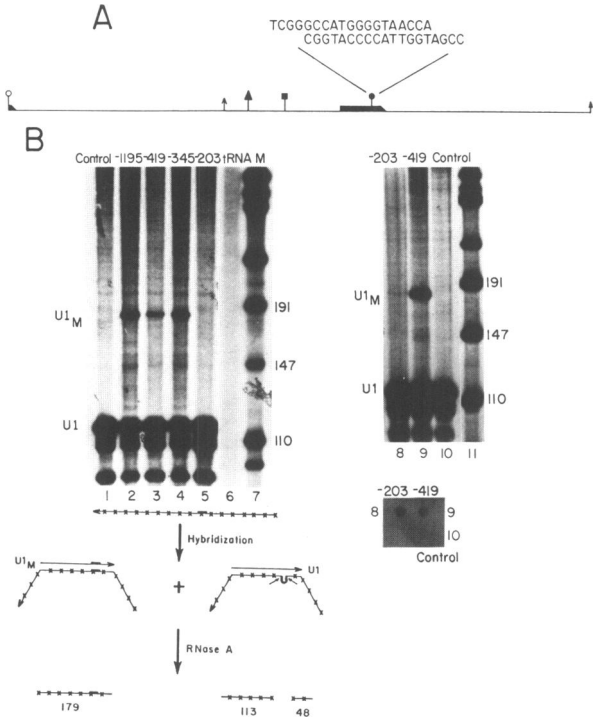

Fig. 2. Expression of the *L. variegatus* gene in *S. purpuratus* embryos. A. A U1 maxigene was constructed by introducing the indicated oligonucleotide at the AvaI site at nt 117 of the U1 coding region. This gene contains 1195 nucleotides 5′ flanking sequence extending from the BglII site at nucleotide 140 in the upstream U1 gene to the HindIII site 3′ of the adjacent gene. The position of the deletion mutations and the restriction enzyme sites used to create U1 genes with truncated promoters are shown and labeled as in Fig. 1. B. U1 maxigenes containing varying amounts of 5′ flanking sequence were injected into fertilized *S. purpuratus* eggs and the embryos developed until the hatching blastula stage (24 hrs). Each lane represents analysis of RNA from 70 embryos. Two independent sets of injections are shown in lanes 1–6 and lanes 8–10. The expression of the injected U1 maxigene was analyzed using the riboprobe assay shown below the figure. The protected fragments resulting from the riboprobe protection assay using a riboprobe derived from the U1 maxigene is also diagramed. This assay protects a 113 nt fragment from the endogenous U1 snRNAs (labeled U1) and a 179 nt fragment (labeled $U1_M$) from the U1 maxigene. Lanes 1 and 10—control uninjected embryos; Lane 2—U1 maxigene with 1195 nts of flanking sequence; Lane 3 and 9—U1 maxigene with 419 nts of 5′ flanking sequence; Lane 4—U1 maxigene with 345 nts of 5′ flanking sequence; Lanes 5 and 8—U1 maxigene with 203 nts of 5′ flanking sequence; Lane 6 is analysis of 10 μg of yeast tRNA. Lanes 7 and 11 are pUC 18 digested with HpaII size markers. DNA from the embryos analyzed in lanes 8–10 was bound to nitrocellulose and hybridized with labeled pUC18 DNA to show that approximately the same amount of injected DNA was present in the two batches of embryos and was absent from the control embryos. The dot blot is shown below the right panel.

Sequences 5′ of −200 are required for expression of the U1E gene *in vivo*

A 'maxigene' was constructed from the U1.1 gene by inserting a synthetic linker into the AvaI site at nucleotide 117 of the U1 RNA sequence (Fig. 2A). This gene was injected into *S. purpuratus* zygotes and RNA prepared from blastula stage embryos. Genes containing varying amounts of 5′ flanking sequence were tested for expression in this *in vivo* assay system. A riboprobe synthesized from the U1 maxigene will map both the endogenous U1 snRNAs as well as the U1 snRNAs expressed from the maxigene as separate protected fragments (Fig. 2B). Figure 2B shows the expression of the structure of the U1 maxigene and the results obtained from the microinjection

Table II. Oligonucleotides used in mobility shift assays.

Oligonucleotide I	CCCATTTGATCAAGTACGGCGACCGAT TAAACTAGTTCATGCCGCTGGCTAGGG
Oligonucleotide II	GATCCTCCAGAGAGGGGCACAGA GAGGTCTCTCCCCGTGTCTCTAG
Oligonucleotide III	GATCTTCATGAATATTCACATC AAGTACTTATAAGTGTAGCTAG
Oligonucleotide IV	GATCTGACGCTTATGTCATTATAA ACTGCGAATACAGTAATATTCTAG
NS I	TCGGGCCATGGGGTAACCA CGGTACCCCATTGGTAGCC
NS II	CTAGGATCGTGCTGCACAAACACTAGATTAGTTCTGCCCTG CTAGCACGACGTGTTTGTGATCTAATCAAGACGGCACGATC

The sequences of the synthetic oligonucleotides are shown. Prior to use the oligonucleotides were filled in with the Klenow fragment of DNA polymerase I.

experiments. Genes containing the entire 1135 nt intragenic region, 419, 345 and 203 nts were injected into sea urchin embryos and the embryos harvested at the blastula stage. The three genes containing 345 nts or more of 5′ flanking sequence were expressed at high levels, while the gene with only 203 nts of 5′ flanking sequence was not expressed (Fig. 2B, lanes 6 and 9). Analysis of DNA in the embryos indicated that there was as much, if not more, DNA containing the −203 gene as the −419 gene present in the injected embryos (Fig. 2B, lanes 9 and 10). There is a sequence required for the expression of the U1 gene located between 203 and 345 nts 5′ of the gene. Since the gene from *L. variegatus* is expressed in the *S. purpuratus* embryos, the proteins which interact with the promoter in the *S. purpuratus* embryos must recognize the *L. variegatus* gene. Two of the conserved regions of sequence, box III and box IV, are present in this region.

Specific complexes are formed on the box IV sequence with nuclear proteins from blastula embryos

To detect factors which bind to the box IV sequence, complementary oligonucleotides corresponding to the box IV sequence were synthesized (Table II). The double-stranded oligonucleotide IV was labeled with α-^{32}P-dCTP and α-^{32}P-dATP and incubated with increasing amounts of protein from the blastula nuclear extract. Figure 3A (lanes 1 and 2) shows the incubation of the oligonucleotide alone and with nuclear extract in the absence of non-specific competitor. Lanes 3–6 show that two specific complexes are formed with increasing amounts of nuclear proteins from blastula embryos, in the presence of the non-specific competitor dIdC. The ability of an excess of the homologous box IV oligonucleotide and a heterologous oligonucleotide NS II to compete for formation of the complexes was assessed. These complexes can be efficiently competed by incubating with excess unlabeled oligonucleotide IV (Fig. 3B, lanes 5 and 6), but there was much less competition with an excess of the heterologous unlabeled oligonucleotide NS II (Fig. 3B, lanes 7 and 8).

A DNA restriction fragment extending from −318 to −203 (fragment A in Fig. 1) was labeled at the distal end and incubated with nuclear proteins isolated from blastula embryos. DNAse I footprinting was performed to look for sites on this fragment which interacted with this region of the promoter. Figure 3C shows that there is a clear footprint obtained with extracts from blastula nuclei which protects nucleotides −306 to −294, which includes the 3′ portion of box IV, conserved between *L.*

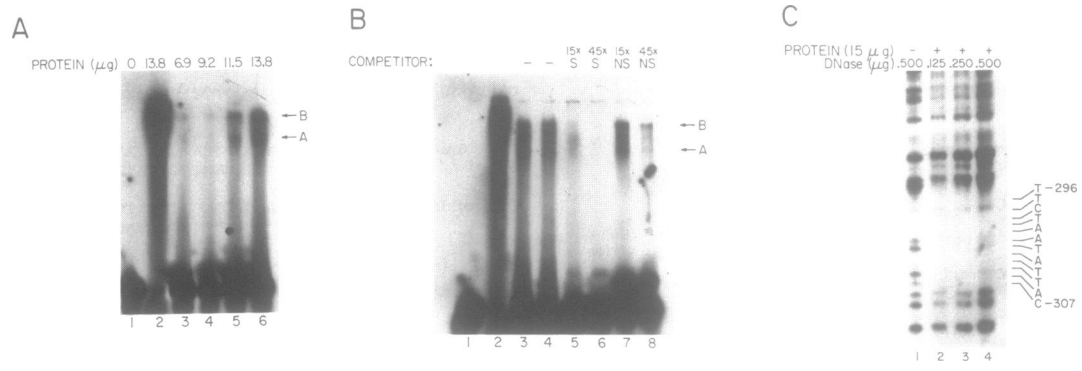

Fig. 3. Binding of a factor in blastula nuclei to box IV sequences. A. The double-stranded oligonucleotide IV (see Table II) was incubated with different amounts of nuclear protein from blastula embryos. Lane 1 is the oligonucleotide incubated in buffer. Lane 2 is the oligonucleotide incubated with 13.8 μg of protein in the absence of dIdC. Lanes 3–6 shows the results of incubating the oligonucleotide with different amounts of blastula protein in the presence of dIdC. The two complexes (A and B) detected are indicated by arrows. B. Oligonucleotide IV was incubated with 15 μg of nuclear protein from blastula embryos (lanes 3 and 4). In lanes 5 and 6, a 15 and 45-fold molar excess of unlabeled box IV oligonucleotide was included and in lanes 7 and 8 a 15 and 45-fold excess of unlabeled heterologous oligonucleotide NS II (see Table I) was included. Lane 1 is the box IV oligonucleotide and lane 2 the results of incubation with blastula nuclear proteins in the absence of dIdC. C. DNase I footprinting of the box IV sequence. 15 μg of nuclear protein from blastula embryos was incubated with the end-labeled restriction fragment from −318 to −203 (fragment A in Fig. 1) containing the box IV sequence. The samples were treated with varying amounts of DNase I and analyzed by gel electrophoresis. Lane 1 is digestion of the box IV fragment in the absence of protein. Lanes 2–4 shows the results of incubation with blastula protein and increasing amounts of DNase I. The sequence of the protected region is indicated on the left (see Fig. 4A).

variegatus and *S. purpuratus*. There were no other protected regions evident on this fragment (not shown). Thus specific protein complexes which interact with box IV can be detected by both DNase I footprinting and the mobility shift assay.

We have not been able to detect specific interactions with the box III sequence and proteins in the nuclear extracts, either by mobility shift assays or DNase I footprinting (not shown).

The binding to the box IV sequence is developmentally regulated

Since the U1 snRNA gene is maximally active in hatching blastula embryos and inactive in gastrula embryos (9), we tested gastrula extracts for the ability to bind to the same sites found bound by proteins from the blastula embryos. Figure 4 shows the footprinting analysis of the box IV region using nuclear proteins from blastula and gastrula embryos. The same blastula and gastrula extracts were used for the footprinting experiments shown in Figs. 4 and 5, and this was a different blastula extract from that used in Fig. 3C. Again there was a clear footprint on the 3′ portion of box IV with the blastula extract (Fig. 4A) but there was no detectable binding of proteins from gastrula embryos to the DNA sequence in box IV, either when assayed by the mobility shift (not shown) or the footprinting assay (Fig. 4B). The gastrula extract was enriched for DNA binding proteins by chromatography on heparin-agarose prior to the DNase I footprinting analysis and was active in DNA binding activity. Thus the activity which binds to the box IV sequence is present in blastula and not in gastrula embryos. These results show that there is a great reduction in the DNA-binding activity for the box IV sequence between the blastula and gastrula stage, and are consistent with the possibility that the factors which bind the box IV sequence may be important in the developmental regulation of the sea urchin U1 snRNA gene.

A second protein-binding site 5′ of the U1 gene detected by DNase I footprinting

We also examined the fragment containing the box II sequences extending from −203 to −19 (fragment B in Fig. 1) for potential

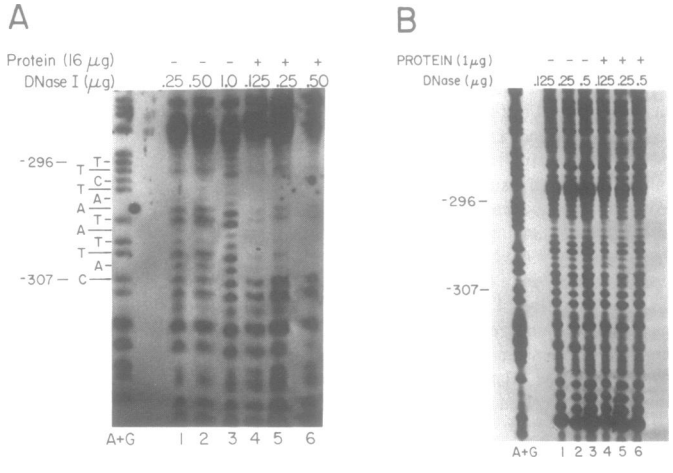

Fig. 4. Developmental regulation of the factor binding to box IV. A. The restriction fragment A (Fig. 1) containing the box IV sequence was labeled at the 5′ end and incubated with buffer (lanes 1–3) or with 16 μgs of nuclear protein from blastula embryos (lanes 4–6). The A + G lane is a Maxam–Gilbert sequencing reaction. The sequence of the protected region is indicated next to the figure. The sequence shown is the complement of the labeled strand. B. The same restriction fragment was incubated in the absence (lanes 1–3) or presence (lanes 4–6) of 1 μg of nuclear protein from gastrula embryos, from the 0.4M NaCl fraction from a heparin-agarose column. The reactions were digested with varying amounts of DNase I and analyzed by gel electrophoresis. The amounts of DNase used are indicated above each lane. Lane A + G is a Maxam-Gilbert sequencing reaction. The region protected in panel A is indicated.

protein binding sites by DNase I footprinting, using the same extracts used in Fig. 4. Incubation of this fragment with nuclear proteins from blastula embryos resulted in complete protection of a region extending from −181 to −168. The 10 nucleotides 5′ of this sequence are also partially protected from DNase I (Fig. 5A). The box II sequences extend from −149 to −168, just 3′ of the protected region and hence the box II sequences are not protected from DNase I. There is no sequence similarity between the two sea urchin species, *S. purpuratus* and *L.*

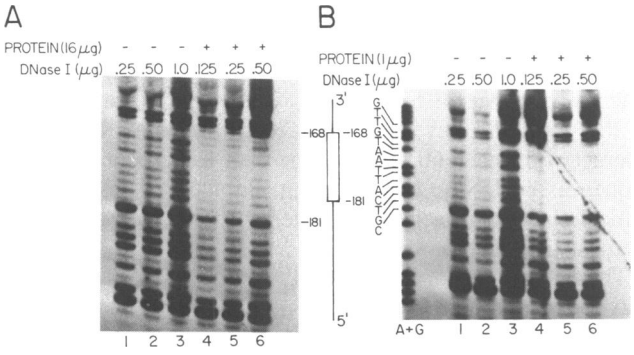

Fig. 5. Footprinting analysis of the region 5′ of box II. A. The end-labeled DNA restriction fragment extending from −203 to −19 (fragment B in Fig. 1) was incubated in the absence (lanes 1−3) or presence (lanes 4−6) of 16 μg of nuclear protein from blastula embryos. The reaction was treated with varying amounts of DNase I (indicated above each lane) and then analyzed by gel electrophoresis. On the right the numbers of the protected region are indicated. B. The same DNA fragment was incubated in the absence (lanes 1−3) or presence (lanes 4−6) of 1 μg of gastrula nuclei extract from the 0.4M fraction after chromatography on heparin agarose. The reactions were treated with increasing amounts of DNase I (indicated above each lane). Lane M is an A + G reaction by Maxam-Gilbert sequencing. The sequence of the protected region, which is the complement of the labeled strand and a schematic of the labeled fragment is shown to the left of the gel. The protein fraction used in this experiment is the same sample that was used in Fig. 4B.

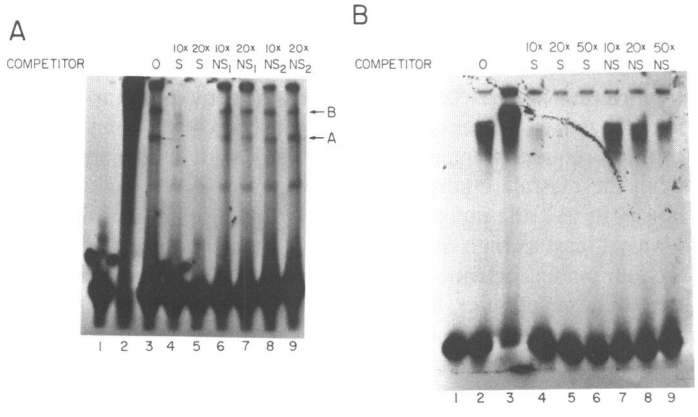

Fig. 6. Detection of factors binding to box I and box II. A. Oligonucleotide I (see Table I) was incubated with 18 μg of blastula nuclear protein in the absence (lane 3) or presence (lanes 4−9) of unlabeled oligonucleotides. In lanes 4 and 5, 10 and 20-fold molar excess of the oligonucleotide I was included; in lanes 6 and 7 a 10 and 20-fold excess of NS I oligonucleotide was included and in lanes 8 and 9 a 10 and 20-fold excess of NS II oligonucleotide was included. The arrows point to the two complexes (A and B) which were reproducibly observed. Lane 1 is oligonucleotide I and lane 2 is incubation in the absence of poly dIdC. B. Oligonucleotide II was incubated in the absence (lane 1) or presence (lanes 2−9) of 15 μgs of nuclear protein from blastula embryos. 2 μgs of poly dIdC was included in all lanes except lane 3. In lanes 4−6 increasing amounts (10-, 20- or 50-fold molar excess) of unlabeled oligonucleotide II was included and in lanes 7−9 increasing amounts of oligonucleotide NS I was included.

variegatus, in the region which is protected from DNase I (10). Fig. 5B shows the DNase I footprint of the sequence from −171 to −180 with the same nuclear extract from gastrula embryos used in Figure 4. There is a clear footprint on this sequence which is identical to the footprint observed with the extract from blastula embryos. Thus the failure of the gastrula extract to bind to the box IV sequence was unlikely to be due to a general deficiency in DNA-binding activity in the gastrula extract.

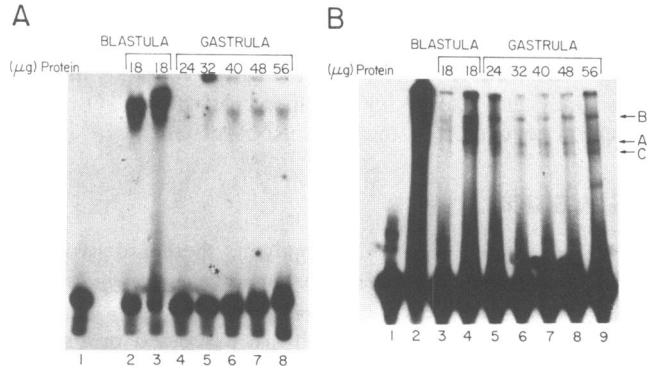

Fig. 7. Developmental regulation of factors binding to box I and box II. A. Oligonucleotide II was incubated with 18 μg of protein from a nuclear extract of blastula embryos in the presence of 2 μg (lane 2) or 1 μg (lane 3) of dIdC. Lanes 4−8 are incubation of the box II oligonucleotide in the presence of 1 μg of dIdC and increasing amounts of nuclear protein from gastrula embryos. The amounts of protein are given above each lane. Lane 1 is the oligonucleotide II. B. Oligonucleotide I was incubated in the presence of the same nuclear extracts used in panel A. Lanes 3 and 4 are incubation with 18 μg of nuclear protein from blastula embryos in the presence of 2 μg or 1 μg of dIdC. Lanes 5−9 are incubation of oligonucleotide 1 with increasing amounts of nuclear protein from gastrula embryos. The amounts of protein used are given above each lane. Lane 1 is oligonucleotide I and lane 2 is incubation with 18 μg of blastula protein in the absence of dIdC. Complexes A and B are the same complexes observed in Fig. 6A. Complex C was observed in gastrula extracts but not in blastula extracts.

Protein-DNA complexes formed on the conserved box I and box II sequences

We also examined the region containing box I and box II for potential DNA-protein interactions by the mobility-shift assay, using oligonucleotides I and II. We detected two protein-DNA complexes (A and B) formed on the box I oligonucleotide (Fig. 6A, arrows). Both of these complexes were competed specifically by homologous (Fig. 6A, lanes 4 and 5) but not by heterologous DNA oligonucleotides (Fig. 6A, lanes 6−9). We detected one specific protein-DNA complex (Fig. 6B, lane 2) which bound to box II oligonucleotide and could be competed by homologous (Fig. 6B, lanes 4−6) but not heterologous sequences (Fig. 6B, lanes 7−9). Using the DNase I footprinting assays we could not detect any footprints over the box I and box II regions or the proximal element (not shown).

Binding to the box II sequence but not the box I sequence is developmentally regulated

We next examined the developmental regulation of these protein-DNA complexes using extracts from blastula and gastrula embryos. The same two nuclear extract preparations, one from blastula embryos and one from gastrula embryos were tested for their ability to specifically bind to box II and box I sequences. Complexes were formed with the box II oligonucleotide in the nuclear extracts from blastula embryos (Fig. 7A, lanes 2 and 3) but not with the nuclear extract from gastrula embryos (Fig. 7A, lanes 4−8). Thus there are also developmentally regulated factor(s) which interact specifically with the box II sequences. In contrast, both of the DNA-protein complexes on box I were formed in similar amounts using the same extracts from blastula (Fig. 7B, lanes 3 and 4) and gastrula embryos (Fig. 7B, lanes 5−9). Thus the factors which interact with the box I oligonucleotide were not temporally regulated during sea urchin development.

These results demonstrate that there are at least two regions of the temporally regulated U1 snRNA promoter which form complexes with proteins which are also temporally regulated. It is likely that one or both of these activities plays a role in temporal regulation of the U1 snRNA gene.

DISCUSSION

During early sea urchin development, there are two sets of snRNA genes used; a tandemly repeated gene set which is active in oogenesis and in early embryos and a low copy number set which are the only genes which are expressed in adults (6). Transcription of the tandemly repeated gene set is abolished between blastula and gastrula (9). The change in activity of the tandemly repeated gene may be due to a loss of necessary transcription factors.

In order to help define the sequences and factors which might be involved in expression of the U1 snRNA gene, we have compared the promoters of two sea urchins, *S. purpuratus* and *L. variegatus* to define conserved elements in the 5′ flanking region (10). There are four regions of extensive homology in the 320 nts 5′ to the gene. The *L. variegatus* promoter functions in *S. purpuratus* embryos since the *L. variegatus* U1 gene is expressed well when it is introduced into *S. purpuratus* by microinjection. Deletion analysis revealed that there is a region essential for transcription of the LvU1 gene located between nucleotides 203 and 345 5′ of the U1 gene (Fig. 2).

There are two regions of homology between the two species in this 140 nt region. One of these, box IV, located between −299 and −318, specifically binds a protein which is present in blastula extracts as judged by a mobility shift assay and DNase I footprinting. The protein protects a region from −294 to −306, which represents about 50%, the 3′ portion, of the box IV sequence conserved between the two species. This portion of box IV, but not the 5′ portion of the sequence, is also found in the 5′ flanking region of the tandemly repeated U6 gene from *S. purpuratus* (S. Sakallah and W.F.M., unpublished results). It is not present in either the *S. purpuratus* or *L. variegatus* U2 snRNA genes (11). Thus it is possible that this sequence is involved in the expression of several, but not all, sea urchin snRNA genes.

The snRNA promoters from vertebrates have been extensively studied. The snRNA promoters from vertebrates are characterized by a distal element (DSE) which contains an octamer motif and a proximal element (PSE) which determines the start site of transcription (20). The DSE binds to the general transcription factor, oct1, and has many properties of an enhancer (21). Nothing is known about the sequences involved in developmental regulation of the vertebrate snRNA genes. We have recently shown that the sea urchin U1 and U2 promoters are organized similarly to the vertebrate promoters, with a sequence at about −60 (22, and B. Wendelburg, unpublished results) and a distal element which is absolutely required (U1 genes) or enhances the expression (U2 genes). The sea urchin sequences have no similarity to the vertebrate sequences, which is not surprising given the great evolutionary distance between the species and the fact the sea urchin snRNA genes are not expressed when they are injected into *Xenopus* oocytes (23).

It is likely that box IV is the analog of the vertebrate distal element for the sea urchin U1 snRNA genes. Recently it has been shown that the region between −310 and −290 (encompassing box IV) is absolutely required for expression of the LvU1 gene, while the other sequences conserved between the two species are not required for expression (Wendelburg, unpublished results). This suggests that the protein-DNA interaction which we have detected by DNase I footprinting is involved in the expression of the gene. We have not been able to detect proteins which interact with the −60 sequence or with the conserved box III sequences by DNase I footprinting. Similarly it has been very difficult to detect proteins which interact specifically with the PSE element of vertebrates (20).

The inactivation of the tandemly repeated U1 snRNA genes may be due to the destruction of a necessary factor(s) for expression after the blastula stage and/or the expression of a negative factor(s) after the blastula stage. Our results suggest that one factor which might be involved in the shut-off of the U1 genes after the blastula stage is the disappearance (or inactivation) of the factor which binds to the 3′ end of box IV. The other protein binding site we have detected by DNase I footprinting (from −180 to −171) does not show a developmental change between blastula and gastrula, demonstrating that the gastrula extract was not generally deficient in DNA-binding proteins.

Another tandemly repeated gene set in the sea urchin, the α-histone genes, is also active in early embryogenesis and then silenced. There are both positive and negative elements involved in regulating the transcription of these genes (24−26). There is also a set of low copy number late histone genes which are activated after the blastula stage and are active in adult tissues. Specific temporally regulated DNA-binding proteins are involved in the activation of these genes (27).

In addition to the protein binding sites defined by DNase I footprinting we detected specific binding by proteins which interact with two other conserved regions, box I and box II. Each of these sequences interacted specifically with nuclear proteins from the blastula embryos. There was no binding activity for box II in gastrula embryos, while the binding activity for box I was at least as high in blastula and gastrula embryos. Thus of the four binding activities detected on the sea urchin U1 promoter, two of these activities, box IV and box II were developmentally regulated, while the binding activities which interacted with box I and with the −171 to −180 sequence were present in both blastula and gastrula embryos.

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