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Common Factors Direct Transcription through the Proximal Sequence Elements (PSEs) of the Embryonic Sea Urchin U1, U2, and U6 Genes despite Minimal Sequence Similarity among the PSEs

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The proximal sequence element (PSE) for the sea urchin U6 small nuclear RNA gene has been defined. The most critical nucleotides for expression, located 61 to 64 nucleotides (nt) from the transcription start site, are 4 nt, AACT, at the 5′ end of the PSE. Two nucleotide mutations in this region abolish transcription of the sea urchin U6 gene in vitro. The same two nucleotide mutations greatly reduce the binding of specific factors detected by an electrophoretic mobility shift assay. There is also a conserved AC dinucleotide 57 nt from the start site of the sea urchin U1 and U2 PSEs. The sea urchin U1 and U2 PSEs were substituted for the sea urchin U6 PSE, with the conserved AC sequences aligned with those of the U6 PSE. Both of these genes were expressed at levels higher than those observed with the wild-type U6 gene. Similar complexes are formed on the U1 and U2 PSEs, and formation of the complexes is inhibited efficiently by the U6 PSE. In addition, the E-box sequence present upstream of the PSE enhances U6 transcription from both the U1 and U2 PSEs. Finally, depletion of a nuclear extract with a DNA affinity column containing the U6 PSE sequence reduces expression of the U6 genes driven by the U6, U1, or U2 PSE but does not affect expression of the 5S rRNA gene. These data support the possibility that the same factor(s) interacts with the PSE sequences of the U1, U2, and U6 small nuclear RNA genes expressed in early sea urchin embryogenesis.

The spliceosomal small nuclear RNAs (snRNAs) are among the most abundant transcripts synthesized in metazoans. Most of the spliceosomal snRNAs are synthesized by RNA polymerase II (Pol II), with the exception of the U6 snRNA, which is synthesized by Pol III (7, 20). snRNA promoters differ from promoters of most genes transcribed by Pol II and Pol III (18). Vertebrate snRNA promoters, including the U6 snRNA, contain two major elements: a proximal sequence element (PSE) located at about position −55 which has been loosely conserved and a well-conserved distal sequence element (DSE) located around position −200. The vertebrate snRNA genes transcribed by Pol II lack a TATA box, while the U6 genes contain an essential TATA box (8, 18). There are common factors, including Oct-1, which bind to the DSE and enhance transcription from both the Pol II and Pol III snRNA genes (14, 15, 28). Recently, two active PSE-binding protein complexes, SNAPc (for snRNA-activating protein complex [5]) and PTF (for PSE-binding transcription factor [31]), have been purified from HeLa cells. Both complexes bind to the PSE sequence and direct transcription from U1 and U6 genes, providing direct evidence that the same PSE binding complex is involved in the transcription of both Pol II and Pol III snRNA genes (5, 31, 32). Two subunits of the SNAPc complex have been cloned and shown to be novel proteins (5, 32), although the other factors in this complex have not yet been completely characterized.

In early sea urchin embryogenesis there is a rapid synthesis of snRNAs starting at about the 32-cell stage and continuing through the blastula stage (17). The rate of synthesis of snRNAs declines abruptly between the blastula and gastrula stages (23). The major snRNAs expressed in oogenesis and early embryogenesis are encoded by discrete tandemly repeated gene sets (16, 22, 25), which are active throughout early embryogenesis and then are inactivated. There is also a low-copy-number set of snRNA genes which are constitutively active and encode the snRNAs predominant in later embryos and adult cells (16, 25). We have previously mapped the promoters of the tandemly repeated U1 (30), U2 (26), and U6 (10) snRNA genes and a constitutive U2 snRNA gene (26). As found for the vertebrate snRNA genes, the sea urchin snRNA genes have an important sequence element, termed the sea urchin PSE (suPSE), located at about position −55 relative to the transcription start site, i.e., at a position similar to that of the vertebrate PSE. There is little sequence similarity among the PSEs of the U1, U2, and U6 genes. There are also other elements required for efficient expression of the sea urchin snRNA genes, but these elements are not shared among the U1, U2, and U6 genes (10, 26, 30). In addition, both the embryonic and constitutive sea urchin U2 snRNA genes are unique among metazoan snRNA genes transcribed by Pol II, since they contain a required TATA box (26).

We have previously described the sequence elements required for expression of the sea urchin *Strongylocentrotus purpuratus* U6 snRNA promoter in vitro. There are three important elements: an E box which binds sea urchin USF (10) located at position −80, the suPSE located at about position −55, and a TATA-like element located at position −25 from the start of transcription. All three elements are necessary for efficient expression in vitro (10). We report here additional properties of the U6 PSE and show that the U1 and U2 PSEs can substitute for the U6 PSE despite the fact that there is little sequence similarity between the U6 PSE and the U1 and U2

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PSEs. In addition, we provide evidence that similar factors interact with the diverse suPSE sequences.

MATERIALS AND METHODS

Construction of U6 genes. The construction of the U6 maxigene has been previously described (10). The starting gene for all the constructs contained 856 nucleotides (nt) of 5′ flanking sequence and was cloned in pGEM-5zf(+) (Promega, Madison, Wis.). Site-directed mutations were constructed by the method of Kunkel (9). All mutations were confirmed by sequencing.

Preparation of sea urchin nuclear extract. Sea urchins (*S. purpuratus* or *Lytechinus variegatus*) were grown to the hatching blastula stage, and nuclei were prepared from embryos at 1 to 2 h after hatching. Nuclei were prepared exactly as previously described (13) and stored in liquid N_2. Nuclear extract was prepared as previously described (13) except for the final dialysis step, which was done for 4 h against a solution containing 80 mM KCl, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5), 0.1 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride.

In vitro transcription. Transcription reactions were carried out as previously described (10). For each extract, the conditions for transcription were optimized for the expression of the U6 gene with respect to concentrations of protein, DNA template, KCl, and $MgCl_2$. Typically the DNA concentration was 80 μg/ml, the protein concentration was 1 to 2 mg/ml, the KCl concentration was 60 mM, and the $MgCl_2$ concentration was 5 mM. Supercoiled templates were used routinely, although identical results were obtained with linear templates.

The 20-μl transcription reaction mixture contained 20 mM HEPES (pH 7.5), 1 mM dithiothreitol, 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid), 1 mM spermidine, 200 μM (each) unlabeled ribotriphosphates, and 25 μM radiolabeled [α-^{32}P]UTP at 500 μCi/ml (final specific activity, 20 mCi/mmol). All the components were mixed on ice, with the extract added last. The reaction mixtures were incubated at 15°C for 30 min. The reactions were terminated by addition of 100 μl of a solution containing 0.3 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 100 μg of yeast tRNA per ml. Sixty micrograms of proteinase K was added, and the reaction mixture was incubated for an additional 30 min at 37°C. The samples were extracted with phenol-chloroform, and the RNA was recovered from the aqueous phase by precipitation with ethanol. The RNA products were resolved on an 8% polyacrylamide–7 M urea gel. The gel was then dried, and the products were detected by autoradiography.

Mobility shift assay. Mobility shift assays were performed in a solution containing 20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 0.1% Triton X-100, and 10% glycerol with 100 μg of poly(dI-dC) per ml as a nonspecific competitor. Five to 20 μg of nuclear protein was incubated on ice in the binding buffer for 10 min prior to the addition of radiolabeled probe. Different competitor oligonucleotides were added with the probe as indicated in the figures. The reaction mixture was incubated for 30 min at 15°C. The DNA-protein complex was resolved from the free probe on a 4% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA. The gel was dried and visualized by autoradiography.

Depletion of nuclear extract by DNA affinity chromatography. DNA affinity resins containing double-stranded ligated oligonucleotides corresponding to either the U6 PSE or the U1 DSE were constructed as described by Kadonaga (6). For depletion of the extract, 20 μl of the affinity resin was washed with 1 ml of equilibration buffer (0.2 M KCl, 0.1 mM EDTA, 0.125 mM EGTA, 0.025% Nonidet P-40, 500 μg of bovine serum albumin per ml, 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 20% glycerol, 25 mM HEPES [pH 7.5]). Fifty microliters of nuclear extract (5 mg/ml) was adjusted to 0.2 M KCl and applied to the resin. The flowthrough material was collected and passed over the column a second time. The unbound material was then used immediately for in vitro transcription. The sequence of the U6 PSE was the same as that of the oligonucleotide probe used for Fig. 4, and the sequence of the U1 DSE oligonucleotide was AGCTGACGCTTTCTGTCATTATAA.

Materials. Sea urchins were collected from the Gulf of Mexico (*L. variegatus*) or obtained from Marinus (Venice, Calif.) (*S. purpuratus*). Oligodeoxynucleotides were synthesized in the Oligonucleotide Core Facility of the Lineberger Comprehensive Cancer Center at the University of North Carolina at Chapel Hill.

RESULTS

Previously we have demonstrated that 90 nt 5′ of the U6 gene was sufficient for the maximal expression of this gene in vitro (10). Figure 1A shows the first 90 nt of the U6 promoter sequence. In addition to the TATA-like box, two elements are required for U6 expression, an E box which binds sea urchin USF located at position −80 and an suPSE located at about position −55. Mutation of either the E box or the PSE abolished U6 expression in vitro (10). To measure transcription in vitro, we used supercoiled templates containing a U6 maxigene and [α-^{32}P]UTP as a label. [α-^{32}P]UTP also labeled the endogenous U6 snRNA at the 3′ end (12), and this radiolabeled snRNA served as an internal control for RNA recovery. Results of a typical transcription reaction are shown in Fig. 1B, with the transcription product of the maxigene ($U6_M$) and the end-labeled endogenous U6 snRNA ($U6_e$) indicated (Fig. 1B).

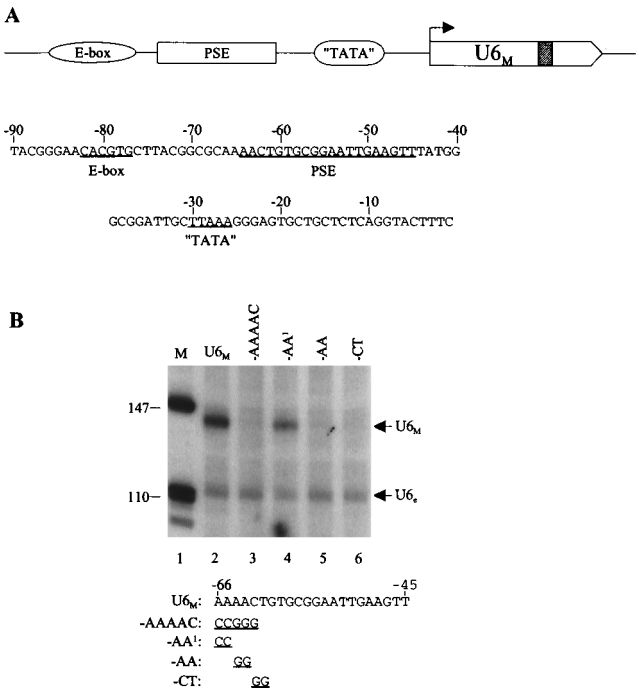

FIG. 1. Effect of point mutations at the 5′ end of the U6 PSE. (A) Schematic representation of the U6 gene promoter with three required sequence elements, an E box located at position −80, a PSE located at about position −55, and a TATA-like element located at about position −25. The first 90 nt of the promoter sequence is shown with the three required elements underlined. $U6_M$ represents the coding sequence for the U6 maxigene, which contains 19 nt of pUC18 polylinker inserted at nt 86 (10). (B) The sequences of the four mutants with mutations in the U6 PSE, −AAAAC, −AA^1, −AA, and −CT, are shown below the gel. These genes were incubated in a nuclear extract prepared from blastula-stage sea urchin embryos in the presence of [α-^{32}P]UTP, and the RNA products were resolved on an 8% polyacrylamide–7 M urea gel. The gel was dried and detected by autoradiography. Since [α-^{32}P]UTP was used as a radiolabel, the endogenous U6 snRNA ($U6_e$) was labeled at the 3′ end. The product of the $U6_M$ gene (127 nt) is indicated. Lane 1 contains a DNA size marker (pUC18 digested with *Hpa*II), with the sizes of the DNA fragments indicated at the left of the gel.

Critical nucleotides in the U6 PSE. The U6 PSE has been defined by a series of scanning mutations as extending from about position −45 to position −66. We previously showed that mutating the 5-nt sequence from position −62 to position −66 abolished U6 expression while changing the 5-nt sequence from position −67 to position −71 stimulated expression (10). Alterations of the PSE sequence in 5-nt blocks 3′ of position −62 reduced but did not abolish expression (10). These results suggested that the 5′ end of the PSE was most critical for PSE function. To further define the 5′ boundary of the U6 PSE, we changed two nucleotides at a time in the AAAACT sequence from position −61 to position −66 (Fig. 1B). Mutation of the sequence AAAAC to CCGGG abolished U6 expression (Fig. 1B, lane 3). Changing the first two A's in this sequence to CC slightly reduced U6 expression (Fig. 1B, lane 4). However, changing the second two A's to GG or the adjacent CT to GG abolished U6 expression (Fig. 1B, lanes 5 and 6). These results

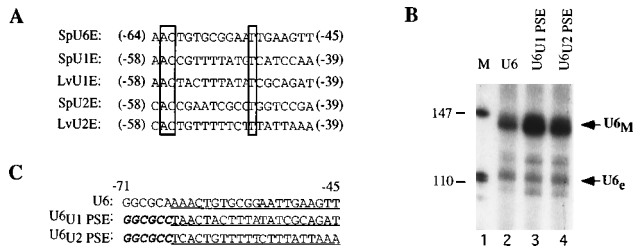

FIG. 2. Transcription of the $U6_{U1PSE}$ and $U6_{U2PSE}$ genes. (A) The sequences of the five PSE sequences from the tandemly repeated U1 (30) and U2 (25, 26) genes of the sea urchins *S. purpuratus* (Sp) and *L. variegatus* (Lv) are aligned with the AC dinucleotide (boxed) in register with the U6 gene. The numbers refer to the distance from the transcription start site. The other invariant nucleotide, the T 10 nt from the end of the AC, is also boxed. (B) The U6 PSE was replaced by the U1 PSE and the U2 PSE by site-directed mutagenesis to create the $U6_{U1PSE}$ and $U6_{U2PSE}$ genes, whose sequences are shown in panel C. Each of these genes was incubated in a nuclear extract prepared from blastula-stage embryos in the presence of [α-^{32}P]UTP, and the RNA products were analyzed as for Fig. 1B. Lane 1 contains DNA size marker pUC18 digested with *Hpa*II. (C) The sequences of the wild-type U6 gene and the $U6_{U1PSE}$ and $U6_{U2PSE}$ genes from position −45 to position −71 are shown.

suggest that the AACT at positions −61 to −64 is the most critical element in the U6 PSE, and mutation of either the AA or the CT abolished the function of the U6 PSE.

The U1 and U2 PSEs can substitute for the U6 PSE. Since the embryonic U1, U2, and U6 genes are all developmentally regulated, we compared all the characterized suPSE sequences from two sea urchin species, *L. variegatus* and *S. purpuratus*. The U1 and U2 PSEs have been mapped previously by site-directed mutagenesis to a region about 55 nt from the transcription start site (26, 30). There is a consensus sequence, (A/C)ACYR, at the 5′ end of all the suPSE sequences. An invariant AC dinucleotide is located precisely 57 nt from the start site in each of the U1 and U2 PSEs, compared with the essential AC located 63 nt from the start site in the U6 PSE (Fig. 2A). All site-directed mutations which changed this AC in the U1 (30) or U2 (26) PSEs abolished the expression of the mutant genes in vivo. Little similarity was observed among the U1, U2, or U6 PSE sequences, and there was not a high degree of similarity between the U1 and U2 PSE sequences from the same species (Fig. 2A). When these AC dinucleotides are aligned, there is only one other invariant nucleotide, a T 10 nt from the AC (Fig. 2A).

To determine if U1 and U2 PSE sequences were able to substitute for the U6 PSE, we inserted the U1 and U2 PSEs into the U6 gene by site-directed mutagenesis to create $U6_{U1PSE}$ and $U6_{U2PSE}$ genes (Fig. 2C). The essential AC dinucleotide in the U6 PSE is located 63 nt from the transcription start site, compared with the 57-nt distance in the U1 and U2 genes (Fig. 2A). We reasoned that the difference in the position of the AC between the U6 gene and the other snRNA genes may reflect the fact that these genes are transcribed by different RNA polymerases. Thus, we aligned the PSEs in the $U6_{U1PSE}$ and $U6_{U2PSE}$ genes with the ACs in register at position −63. The sequences of this region of the PSEs in the $U6_{U1PSE}$ and $U6_{U2PSE}$ genes are shown in Fig. 2C. In the creation of these genes a single nucleotide 5′ of the PSE was also changed to create a *Nar*I site (Fig. 2C, bold italics), which aided in identification of the mutant genes.

Genes with either the U1 or the U2 PSE substituted for the U6 PSE were each expressed when they were transcribed in vitro. Strikingly, both genes were expressed at a level higher than the authentic U6 gene (Fig. 2B, lanes 3 and 4). The genes were transcribed by Pol III as judged by sensitivity to α-amanitin (data not shown). Thus, the PSEs of the U1, U2, and U6 genes are functionally interchangeable despite the minimal sequence similarities among them.

The conserved AC dinucleotide is essential for function of the U1 and U2 PSEs. The only feature in common among the different PSEs is a conserved AC dinucleotide, located at position −57 in the U1 and U2 PSEs and at position −63 in the U6 PSE (Fig. 2A). Since mutation of either the AA or the CT in the AACT from nt −64 to −61 of the U6 PSE abolished expression of the U6 gene (Fig. 1B, lanes 5 and 6; Fig. 3A, lane 7), we tested whether this region of the U1 and U2 PSEs was required for expression. The $U6_{U1PSE}$ and $U6_{U2PSE}$ genes were altered by site-directed mutagenesis, changing the ACT at the 5′ end of the PSE to GGG (Fig. 3B). Introduction of this mutation into the $U6_{U1PSE}$ and $U6_{U2PSE}$ genes abolished expression of these genes (Fig. 3A, lanes 3 and 5). Previously we have analyzed a series of scanning mutations in both the U1 (30) and U2 (26) genes. All the scanning mutations which abolished activity in vivo involved mutation of the AC, while mutations of other portions of the U1 and U2 PSEs reduced but did not abolish expression (26, 30). These results demonstrate that the conserved 5′ ends of all the PSE sequences are most critical for the function of these sequences in sea urchin snRNA expression during early embryogenesis.

Factors binding to the U6 PSE. We used the gel mobility shift assay to detect factors which bind to the U6 PSE. Two complexes, designated PBP and PBP′ (for PSE-binding protein complex), were observed by using a radiolabeled oligonucleotide containing the U6 PSE as a probe (10) (Fig. 4A, lane 2). Both complexes were readily inhibited by an excess of the PSE oligonucleotides (Fig. 4A, lanes 3 and 4) but were resistant to competition by a nonspecific oligonucleotide (Fig. 4A, lanes 6 and 7), indicating that they are specific for the U6 PSE sequence. Oligonucleotides containing mutations in the AACT sequence at the 5′ end of the U6 PSE, with either the AA

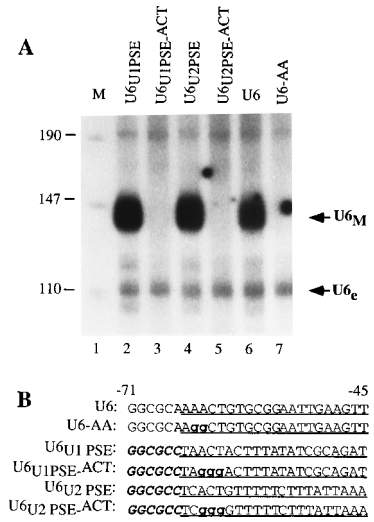

FIG. 3. The AC dinucleotide is important for the function of U1 and U2 PSEs. (A) A 3-nt mutation was made in the PSE region of the $U6_{U1PSE}$ and $U6_{U2PSE}$ genes. These genes were transcribed in a nuclear extract prepared from blastula-stage embryos in the presence of [α-^{32}P]UTP, and the RNA products were analyzed as for Fig. 1B (lanes 2 to 5). The wild-type U6 gene and the U6 −AA mutation (Fig. 1B) were included as controls (lanes 6 and 7). Lane 1 contains DNA size marker pUC18 digested with *Hpa*II. (B) The sequences from position −45 to position −71 of the genes used for panel A are shown. PSEs are underlined, and the PSE mutations are shown in bold lowercase. A *Nar*I site (shown in bold italics) was also created by mutagenesis to aid in identification of mutants.

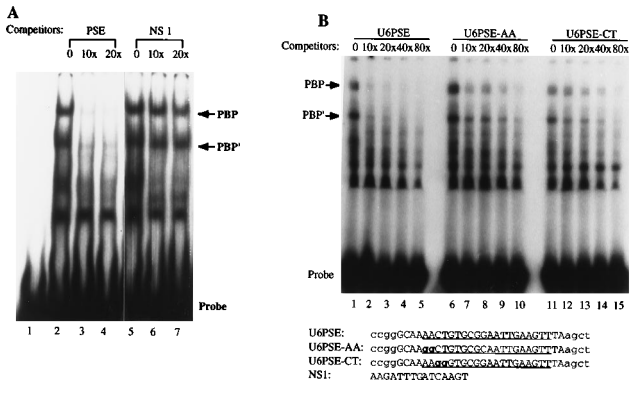

FIG. 4. Specific complexes formed on the U6 PSE. (A) An oligonucleotide corresponding to the U6 PSE was labeled with the Klenow fragment of DNA polymerase I and used as a probe in the gel mobility shift assay. The probe was incubated with (lanes 2 to 7) or without (lane 1) 5 μg of protein from a blastula nuclear extract. Increasing amounts of specific competitor (PSE) or nonspecific competitor (NS1) were added. Two specific PSE binding complexes, PBP and PBP′, are indicated. The sequences of the U6 PSE and the nonspecific oligonucleotide (NS1) are shown in panel B. (B) The U6 PSE probe was used in a gel mobility shift assay as described for panel A. The two specific PSE binding complexes, PBP and PBP′ (lanes 1, 6, and 11), are indicated by arrows. For lanes 2 to 5, a 10-, 20-, 40-, or 80-fold excess of unlabeled U6 PSE oligonucleotide was included in the binding reaction mixture. Two mutant U6 PSE oligonucleotides with the AA-to-GG mutation (lanes 7 to 10) or the CT-to-GG mutation (lanes 12 to 15) were included in binding reaction mixtures as competitors. The sequences of these mutant oligonucleotides are shown below the gel. The additional overhanging nucleotides which were included in the oligonucleotides for labeling are shown in lowercase.

changed to GG (Fig. 4B, lanes 7 to 10) or the CT changed to GG (Fig. 4B, lanes 12 to 15), competed less efficiently than the wild-type U6 PSE sequence (Fig. 4B, lanes 2 to 5) for binding to the U6 PSE. Thus, the specificity of binding to the U6 PSE of the two complexes that we observed correlates with the function of the U6 PSE, since both the AA-to-GG and the CT-to-GG mutations abolished U6 expression (Fig. 1B). Although the mutant PSE sequences still have a measurable affinity for the factors, the reduced affinity must be sufficient to prevent transcription of the U6 genes.

Similar complexes are formed on the U1, U2, and U6 PSEs. Since the U1 and U2 PSEs can function in the place of the U6 PSE in the U6 gene, all three of these elements may interact with the same functionally important factors despite having little sequence similarity. We tested the ability of the U1 and U2 PSE sequences to compete with the U6 PSE sequence for formation of the PBP and PBP′ complexes. Oligonucleotides containing the U1 PSE or U2 PSE sequences competed as effectively as the unlabeled U6 PSE oligonucleotide for the formation of PBP and PBP′ complexes (Fig. 5A; compare lanes 3 and 4 with lane 2). Neither the E box nor a nonspecific oligonucleotide competed for formation of the same complexes (Fig. 5A, lanes 5 and 6).

To verify that PBP complexes could also form on the U1 and U2 PSE sequences, the U1 and U2 PSE oligonucleotides were labeled and used in the gel mobility shift assay. Two complexes with mobilities similar to those of the PBP and PBP′ complexes were observed on the U1 and U2 PSE sequences (Fig. 5A, lanes 7 and 13). The complexes on the U1 PSE sequence were inhibited completely by the U1, U2, or U6 PSE oligonucleotides but not by two nonspecific oligonucleotides (Fig. 5A, lanes 8 to 12). The U2 PSE oligonucleotide also formed two complexes of similar mobilities. In this experiment a higher proportion of the more slowly migrating PBP complex was formed on the U2 PSE than on the U1 and U6 PSE oligonucleotides (Fig. 5A, lane 13). Again, both complexes were inhibited by all three PSE oligonucleotides but not by two nonspecific oligonucleotides (Fig. 5A, lanes 14 to 18). At the concentrations of competitor used, the U2 PSE complexes were not inhibited completely, although the U1 and U6 complexes were (Fig. 5A, lanes 2 to 4 and 8 to 12), suggesting that the PSE factors may bind more tightly to the U2 PSE. Two mutant U6 PSE oligonucleotides, each with two nucleotide mutations which abolished U6 expression, competed less efficiently for the formation of the PBP and PBP′ complexes on the U1 PSE (Fig. 5B, lanes 6 to 15) than did the wild-type U6 PSE oligonucleotide (Fig. 5B, lanes 2 to 5). These same mutant oligonucleotides also competed less efficiently for the formation of the PBP and PBP′ complexes on the U6 PSE (Fig. 4B) and the U2 PSE (data not shown). Taken together, these

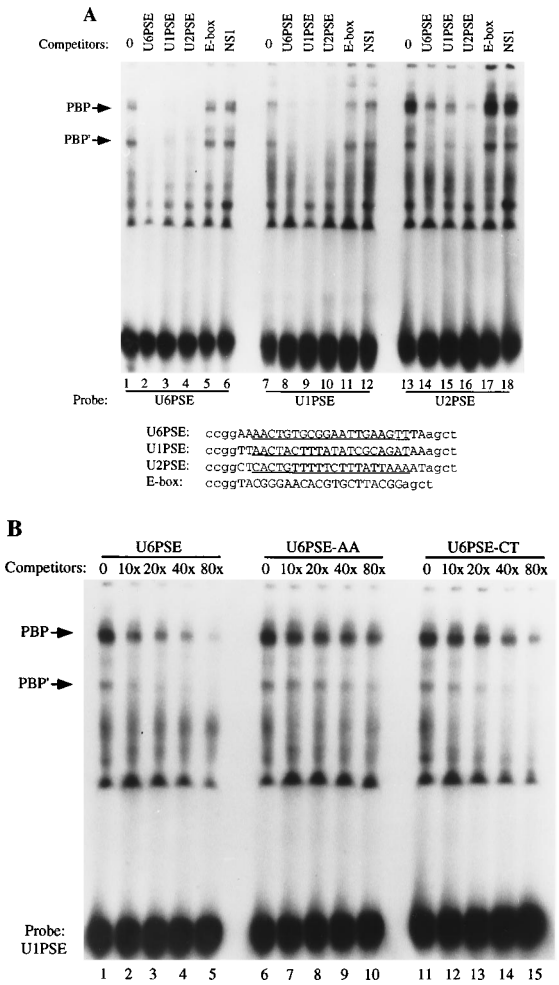

FIG. 5. Similar complexes are formed on the U6 PSE, the U1 PSE, and the U2 PSE. (A) Oligonucleotide probes containing the U6 PSE sequence (lanes 1 to 6), the U1 PSE sequence (lanes 7 to 12), or the U2 PSE sequence (lanes 13 to 18) were incubated with the blastula nuclear extract, and the complexes, PBP and PBP′, were resolved by gel electrophoresis. Unlabeled U6 PSE (lanes 2, 8, and 14), U1 PSE (lanes 3, 9, and 15), and U2 PSE (lanes 4, 10, and 16) were used as specific competitors at 40-fold excess. Two nonspecific competitors, nt −70 to −90 of the U6 promoter containing the E box (lanes 5, 11, and 17) and NS1 (lanes 6, 12, and 18), were also used. The sequences of the oligonucleotides used are shown below the gel. (B) The U1 PSE oligonucleotide probe was incubated with the blastula nuclear extract, and the complexes were resolved by gel electrophoresis. Increasing amounts of the U6 PSE oligonucleotide (lanes 2 to 5) or the two mutant U6 PSE oligonucleotides used for Fig. 4B were added as competitors as described in the legend to Fig. 4B.

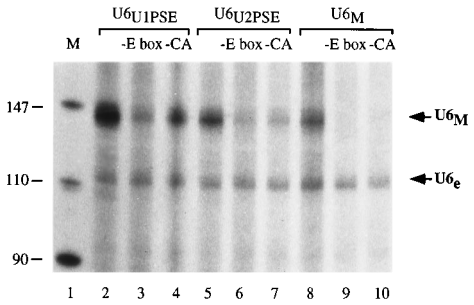

FIG. 6. The E box stimulates U6 expression from U1, U2, and U6 PSEs. The $U6_{U1PSE}$ (lane 2), $U6_{U2PSE}$ (lane 5), and wild-type $U6_M$ genes (lane 8) were transcribed in a blastula nuclear extract as described in the legend to Fig. 2B. Mutants were constructed from the $U6_{U1PSE}$, $U6_{U2PSE}$, and $U6_M$ genes by deletion of the 5′ sequences up to position −80, which removes the first 3 nt of the E box (−E box; lanes 3, 6, and 9), or by mutation of the first 2 nt, CA, of the E box to TT (−CA; lanes 4, 7, and 10). The mutant genes were transcribed in vitro, and the products were resolved by gel electrophoresis and detected by autoradiography. Lane 1 contains a DNA size marker, pUC18 digested with *Hpa*II.

results suggest that the same protein complexes are formed on the U1, U2, and U6 PSE sequences, consistent with the possibility that similar factors are involved in the expression of all three snRNA genes.

The E-box sequence is required for maximal expression of all three PSEs in the expression of U6 gene. Previously we have shown that in addition to the PSE, the U6 gene requires an upstream E box for its expression (10) (Fig. 6, lanes 8 to 10). If similar factors bind to the U1, U2, and U6 PSEs, we would expect the E-box sequence to activate transcription from all three PSE sequences. Two mutations which destroyed the E box were made in the promoters of the U6, $U6_{U1PSE}$, and $U6_{U2PSE}$ genes. The "−E box" mutants have all the sequences 5′ of position −80, including the 5′ half of the E-box sequence, deleted from the promoter, and the "−CA" mutants have the CA at the 5′ end of the E box changed to TT by site-directed mutagenesis. Both of these mutations completely abolished expression of the U6 gene as shown previously (10) (Fig. 6, lanes 9 and 10). Destruction of the E box with either mutation reduced but did not abolish transcription of the $U6_{U1PSE}$ or $U6_{U2PSE}$ gene (Fig. 6, lanes 2 to 7). These results suggest that sea urchin USF, which binds the E-box sequence (10), activated transcription in cooperation with all three suPSEs, providing further evidence that all three suPSEs bind to similar protein factors. The $U6_{U1PSE}$ and $U6_{U2PSE}$ genes are transcribed more efficiently than the U6 gene and do not absolutely require the E box for expression, suggesting that the stronger PSEs support some expression of these genes in the absence of the E box.

Depletion of the PSE factor(s) selectively inhibits transcription from genes containing the U6, U1, and U2 PSEs. To demonstrate that factors which bound to the U6 PSE were essential for the transcription of the U6 snRNA gene, we passed the extract over a DNA affinity column with the U6 PSE coupled to agarose resin. Passage of the extract through the U6 PSE affinity column depleted both the PBP and PBP′ complexes as judged from the gel mobility shift assay (data not shown). This extract was then tested for its ability to transcribe the U6, $U6_{U1PSE}$, and $U6_{U2PSE}$ genes. Transcription of all three genes was significantly reduced by passage of the extract over the U6 PSE column (Fig. 7, lane 2). As a control for the specificity of binding, a separate aliquot of the extract was passed over a column containing the U1 DSE sequence. Passage of the extract over the U1 DSE column reduced transcription of these genes by less than 50% (Fig. 7, lane 3). To demonstrate that factors necessary for general transcription by Pol III had not been depleted from the extract, we analyzed the transcription of the sea urchin *L. variegatus* 5S rRNA gene (11). Under our assay conditions, which were optimized for the transcription of the snRNA genes, we routinely observed lower levels of activity for the transcription of the 5S rRNA gene than for the transcription of the U6 gene. The transcription of the 5S rRNA gene was reduced by less than 50% by passage of the extract over either of the columns (Fig. 7, panel 5S). Thus, the U6 PSE column selectively depleted a factor(s) required for U6 snRNA transcription but not for 5S rRNA transcription. These results provide further evidence that the same protein factors bind to the U1, U2, and U6 PSE sequences and could be depleted by the U6 PSE DNA affinity resin.

DISCUSSION

A characteristic of vertebrate snRNA genes is the sharing of factors involved in expression of the nucleoplasmic snRNAs (1, 18, 21). There is a common distal element which has been conserved among different snRNAs. There is also a relatively conserved PSE. Vertebrate snRNA genes transcribed by Pol II lack a TATA box, while the U6 snRNA gene transcribed by Pol III contains a TATA box. Analysis of the sea urchin U1, U2, and U6 snRNA promoters has shown that there are not any shared DSEs (10, 22, 26, 30). Indeed, the sea urchin U2 snRNA promoters contain a required TATA-like box, which is unique among metazoan snRNA genes transcribed by Pol II (26). The U7 genes from sea urchins have also been characterized (3). In vertebrates the U7 gene has a promoter similar to those of the other spliceosomal snRNAs (4, 19). There are no obvious sequences present in the sea urchin U7 snRNA promoters that are shared with the U1, U2, and U6 promoters. The sequence requirements for expression of a minor sea urchin snRNA, the U7 snRNA, in vitro have been determined (24). All the sequences necessary for full expression in vitro

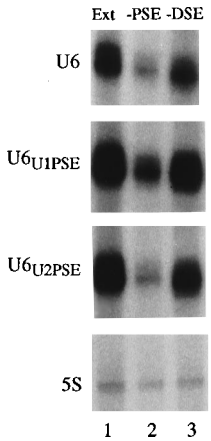

FIG. 7. Depletion by U6 PSE DNA affinity chromatography. Aliquots of a nuclear extract from blastula-stage embryos were passed over DNA affinity columns containing either the U6 PSE oligonucleotide (−PSE) (lane 2) or the U1 DSE oligonucleotide (−DSE) (lane 3) as described in Materials and Methods. The initial extract (Ext) (lane 1) or the two depleted extracts (lanes 2 and 3) were incubated with the U6 maxigene (U6 panel), the $U6_{U1PSE}$ gene (panel $U6_{U1PSE}$), the $U6_{U2PSE}$ gene (panel $U6_{U2PSE}$), or the *L. variegatus* 5S rRNA gene (panel 5S) in the presence of [α-^{32}P]UTP, and the radiolabeled RNA products were analyzed by gel electrophoresis. The 5S rRNA gene has lower-level activity under our assay conditions, which were optimized for U6 snRNA transcription (10). The sequence of the DSE oligonucleotide is given in Materials and Methods.

reside in the first 85 nt, and there is an element at about position −55 that is required for expression. Although this element was not precisely mapped, there is an AC dinucleotide at position −58, and mutations which disrupt this sequence abolish expression (24), consistent with the possibility that the same factors interact with the U7 promoter. Thus, each of the sea urchin snRNA genes transcribed by RNA polymerase II contains an essential element at about position −55, similar to the position of the vertebrate PSE. There is very limited similarity among these elements, either for different snRNA genes from the same species or for the same snRNA genes from different species.

This study more precisely defines the U6 proximal sequence element as a 20-nt sequence extending from position −64 to position −45 (Fig. 1A). The 5′ boundary of the PSE has been defined by the effect of mutations on the expression of the U6 snRNA gene in vitro. A 4-nt sequence, AACT, located at position −61 to −64 is absolutely required for expression. Two separate dinucleotide changes in this 4-nt sequence abolish U6 expression (Fig. 1B). In contrast, changing the 5′ adjacent 2 nt does not affect expression, defining the 5′ boundary of the PSE as the AACT from nt −64 to −61. The 3′ boundary was tentatively defined as position −45, since sequences from position −64 to position −45 were required for the formation of stable PSE binding complexes in the gel mobility shift assay (reference 10 and data not shown). The 5′ AACT is the most critical element for U6 expression. Changes of multiple nucleotides in the 20-nt PSE region adjacent to these critical 4 nt reduced but did not abolish expression (10).

Comparison of the U6 PSE with the U1 and U2 PSE sequences (Fig. 2A) from two different species revealed that there is an AC sequence present in each of the PSEs. The A in the conserved AC in the U6 gene is located 63 nt from the start site and is positioned 33 nt from the TATA-like box. In the U2 gene, the conserved A is 57 nt from the start site and 27 nt from the TATA-like box. In the U1 gene, the conserved A is also 57 nt from the start site, i.e., at a position identical to the location in the U2 gene, even though the U1 gene lacks a TATA-like box. All mutations that we have tested which disrupted the AC dinucleotide in the U1 and U2 PSEs abolished expression (26, 30). The sea urchin U7 snRNA genes have an AC located at position −58, also in a region which is essential for expression in vitro (24). When the 20-nt sequence containing the U6 PSE is exchanged for the 20-nt U1 or U2 PSE sequence, expression of the U6 gene by Pol III is enhanced, although there are only 3 nt in common among these sequences (Fig. 2A). Thus, the U1 and U2 PSEs, although very different in sequence from the U6 PSE, are able to substitute for the U6 PSE sequence.

The U6 snRNA gene has an absolute requirement for the E box located at position −80 (10), while the $U6_{U2PSE}$ and $U6_{U1PSE}$ genes do not absolutely require the E box, although their expression was reduced at least 80% when the E box was mutated (Fig. 6). The function of the E box is to bind sea urchin USF (10), and USF likely assists in the PSE function in the U6 gene. The $U6_{U1PSE}$ and $U6_{U2PSE}$ genes were both expressed at a level higher than that at which the U6 gene was (Fig. 6B). This result suggests that the U1 and U2 PSEs are stronger than the U6 PSE. There are no required E-box sequences in the U1 and U2 genes. The U1 and U2 genes differ in that the U2 genes have a required TATA-like box (26) and the U1 genes have a required DSE (30). It is not clear why there are different promoter elements in the U1 and U2 genes transcribed by Pol II if they both share the same PSE. Perhaps the PSE factor cannot function well autonomously and must interact with other factors and these factors vary from gene to gene.

We have detected two specific complexes, which we term PBP and PBP′ according to the initial nomenclature for the human PSE binding factors (29), which bind to the U6 PSE sequence. Similar complexes are also formed on the U1 and U2 PSE sequences. Most importantly, these complexes are readily inhibited by oligonucleotides containing either U1, U2, or U6 PSE sequences, providing strong evidence that U1, U2, and U6 PSEs bind to the same factors. In addition, mutations in the AC at the 5′ end of the PSE which abolished the function of all three PSEs also reduced the affinity for PSE binding complexes (Fig. 4B and 5B). These results suggest that the same factors bind to the U1, U2, and U6 PSEs and that these factors are involved in the expression of the sea urchin snRNA genes.

The consensus sequence at the 5′ end of the sea urchin PSEs (A/CACYR) is similar to the consensus sequence of the vertebrate snRNA genes (G/CACCG) (18), suggesting that the PSEs may have retained some similarity during metazoan evolution (2). Recently, two PSE binding activities, SNAPc (for snRNA-activating protein complex) (5) and PTF (for PSE-binding transcription factor) (31), have been purified from HeLa cells. These factors also bind to multiple PSE sequences, which often are quite different from one another (15). Both SNAPc and PTF complexes contain multiple polypeptides and are capable of binding to the PSE sequence to direct transcription from either Pol II (U1) or Pol III (U6 and 7SK) snRNA genes (5, 31). Therefore, common factors can interact with the PSE sequences from different snRNA genes in mammalian cells. Whether SNAPc and PTF represent the same functional complex is not known at present, although both complexes appear to contain at least four polypeptides with similar molecular masses, i.e., 180, 54, 45, and 43 kDa (5, 31). In addition, SNAPc contains TATA-binding protein (5), which is absent from the PTF complex (31). The cDNA clones for the 43-kDa (5) and 45-kDa (32) factors have recently been isolated, and these factors are not similar to other proteins present in the protein databases.

There are at least two sets of sea urchin snRNA genes. In early embryogenesis and in oogenesis the tandemly repeated gene sets which express the majority of the snRNAs present in eggs and early embryos are active (16, 25). These tandemly repeated gene sets are silenced between the blastula and gastrula stages (23) and are not expressed in adult cells (16, 25). After the gastrula stage and in adult sea urchins, the snRNAs are expressed from a low-copy-number gene set. Thus, it seems likely that the embryonic tandemly repeated gene sets utilize common factors for expression and that the constitutive gene set utilizes at least one different factor. We have previously shown that the PSE of the tandemly repeated U2 genes is capable of conferring temporal regulation characteristic of the early U2 gene when placed in the constitutive U2 gene promoter (27). Since the evidence reported here suggests that a common factor interacts with all three PSEs, it is very possible that temporal regulation is accomplished by regulating the activity of the PSE factor(s). Purification and characterization of the polypeptides present in PBP and/or PBP′ should allow us to understand the mechanism of the temporal regulation in sea urchin early development.

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