The *Drosophila* U1 and U6 Gene Proximal Sequence Elements Act as Important Determinants of the RNA Polymerase Specificity of Small Nuclear RNA Gene Promoters *in Vitro* and *in Vivo*§

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Transcription of genes coding for metazoan splicosomal snRNAs by RNA polymerase II (U1, U2, U4, U5) or RNA polymerase III (U6) is dependent upon a unique, positionally conserved regulatory element referred to as the proximal sequence element (PSE). Previous studies in the organism *Drosophila melanogaster* indicated that as few as three nucleotide differences in the sequences of the U1 and U6 PSEs can play a decisive role in recruiting the different RNA polymerases to transcribe the U1 and U6 snRNAs *in vitro*. Those studies utilized constructs that contained only the minimal promoter elements of the U1 and U6 genes in an artificial context. To overcome the limitations of those earlier studies, we have now performed experiments that demonstrate that the *Drosophila* U1 and U6 PSEs have functionally distinct properties even in the environment of the natural U1 and U6 gene 5′-flanking DNAs. Moreover, assays in cells and in transgenic flies indicate that expression of genes from promoters that contain the "incorrect" PSE is suppressed *in vivo*. The *Drosophila* U6 PSE is incapable of recruiting RNA polymerase II to initiate transcription from the U1 promoter region, and the U1 PSE is unable to recruit RNA polymerase III to transcribe the U6 gene.

In vertebrates, the PSEs of U1 and U2 genes are functionally interchangeable with the PSEs of U6 genes (13, 14). That is, if the PSE of the U6 promoter is replaced with the U1 or U2 PSE, there is no effect on the RNA polymerase III specificity of the U6 promoter. Likewise, the U6 PSE can functionally substitute for the U1 or U2 PSE in the vertebrate U1 and U2 promoters for transcription by RNA polymerase II. In echinoderms and plants, the U1, U2, and U6 PSEs (called USEs in plants) are similarly functionally interchangeable with each other (15–17).

The RNA polymerase III specificity of vertebrate U6 snRNA genes is determined by the presence of a TATA box at a fixed distance downstream of the PSE (2, 13, 18). Paradoxically, the promoters of the vertebrate snRNA genes transcribed by RNA polymerase II lack TATA boxes (Fig. 1A). In plants, on the other hand, snRNA genes transcribed by both RNA polymerases have essential TATA boxes in their promoters. In this case, the choice of RNA polymerase is determined by a 10-base pair difference in the spacing between the TATA and the respective TATA box in the U1 and U6 promoters (Fig. 1A) (16, 17, 19).

In contrast to the organisms described above, *in vitro* experiments carried out in the *Drosophila melanogaster* system indicated that the sequence of the PSE itself plays a major role in determining the RNA polymerase specificity of snRNA gene promoters in insects (20). The basal promoter elements of *Drosophila* snRNA genes are shown in the last two lines of Fig. 1A. The *Drosophila* proximal sequence element A (PSEA) is a 21-base pair conserved sequence that is analogous to the vertebrate U6 TATA sequences (21). The proximal element B (PSEB), which is found in *Drosophila* RNA polymerase II-transcribed snRNA genes, has no obvious counterpart in other organisms. Finally, the spacing of the PSE from the TATA box (8 base pairs) and the spacing of the TATA box (12 base pairs) differs between the two classes of *Drosophila* snRNA genes. Fig. 1B shows typical sequences of the various *Drosophila* U1 and U6 promoter elements.

In a previous study, the cis-acting determinants of RNA polymerase specificity in the *Drosophila* system were studied by transcribing artificial templates that contained all possible combinations of U1 or U6 PSEA, PSEB, or TATA box and 8 or 12 base pair inter-element spacing (20). The results of those *in vitro* transcription experiments indicated that the PSEB and TATA sequences were essentially interchangeable in terms of their effects on RNA polymerase specificity (20). Similarly, the difference in the spacing between the elements affected primarily the efficiency of transcription rather than RNA polymerase specificity. The major determinant of RNA polymerase speci-
RNA Polymerase Selection by the PSE at snRNA Gene Promoters

Cells were grown to 40–60% confluence in Falcon 25-cm² tissue culture flasks and transfected using Promega’s Profection System. Each flask was cotransfected with 6 µg of one of the U6 maxigene plasmids, 2 µg of an internal control plasmid that contained the firefly luciferase gene driven by a Drosophila heat shock promoter, and 12 µg of pBlueScriptSK(+) vector (Stratagene). Cells were harvested 48 h after transfection. A 0.25 µl aliquot of each flask of cells was separated out and used for luciferase assays (see below) to normalize for relative transfection efficiency. RNA was isolated from the remainder of the cells using Promega’s RNAgents Total RNA Isolation System, and samples were assayed for maxigene expression using Promega’s Primer Extension System. The first 13 nucleotides of the maxigene-specific primer (5'-GCGGTATCCTTGGCCGATCC-3’) were complementary to the wild type U6 snRNA sequence, and the last 6 were complementary to the maxigene insertion sequence. Products of the primer extension reactions were resolved on 8% denaturing polyacrylamide gels, and band intensities were quantified by phosphorimager analysis.

Luciferase Gene Expression Driven by the U1 Promoter in Transient Transfection Assays—U1-95.1 gene sequences between positions −381 and +32 were cloned into the promoter-less luciferase vector pGL2-Basic (Promega). Nucleotide substitutions corresponding to those found in the U6 PSEA were introduced into the U1 PSEA at positions 7, 14, 16, 19, and 20 (either as individual substitutions or in groups, see Figs. 1 and 4) by digesting the U1 promoter region with restriction enzymes Mlu I and Xba I that cut on either side of the U1 PSEA, double-stranded synthetic DNA oligonucleotides that contained the desired sequences were inserted to generate seven different chimeric U1-luciferase variants (with U1 or U6 or hybrid U1/U6 PSEAs in the U1 promoter) as indicated in Fig. 4. For assays to measure luciferase activities, S2 cells were transfected in triplicate with 3 µg of one of the chimeric U1/pGL2 constructs, 3 µg of copia-lacZ plasmid (obtained from D. Johnson) as an internal control for transfection efficiency, and 14 µg of pBlueScriptSK(+) plasmid to bring the total amount of DNA to 20 µg. Approximately 48 h after transfection, cells were harvested using Promega’s Reporter Lysis Buffer. Luciferase activities were determined using BD PharMingen’s Enhanced Luciferase Assay kit.

For primer extension assays, S2 cells were transfected in triplicate with 3 µg of one of the chimeric U1/pGL2 constructs, 0.9 µg of a cytomegalovirus-Renilla luciferase construct as an internal control (obtained from R. Tjian, Department of Molecular and Cell Biology, University of California, Berkeley, CA), and 16 µg of pBlueScriptSK(+). Primer extension reactions were carried out as described above using a primer (5'-CTCTCTCCTTTTTACCAACATGATCC-3’) complementary to exon 3 of the U6 gene. The results of the primer extension reactions were analyzed using Promega’s RNAgents Total RNA Isolation System, and samples were assayed for maxigene expression using Promega’s Primer Extension System. The first 13 nucleotides of the maxigene-specific primer (5'-GCGGTATCCTTGGCCGATCC-3’) were complementary to the wild type U6 snRNA sequence, and the last 6 were complementary to the maxigene insertion sequence. Products of the primer extension reactions were resolved on 8% denaturing polyacrylamide gels, and band intensities were quantified by phosphorimager analysis of the primer extension products.

Neon Expression Driven by the U1 Promoter in Transient Transfection Assays—The neon gene was obtained from the pB[neo] P-element vector (20) also known as pUChsneo (24) by polymerase chain reaction with primers that flank the neon gene and contributed an upstream Bocl I site and a downstream BamHI site. This fragment was cloned into the BamHI site of pBlueScriptSK(+) such that the SpeI site of the vector was upstream of the neon gene, producing the construct pSKneo. A fragment containing wild type U1 promoter sequences extending from positions −281 to +32 was recovered from the U1/wild type/pGL2 construct described in the preceding section by digestion with NheI and XbaI. This fragment was cloned in both the forward and reverse orientations into the SpeI site of the pSK/neo construct to generate the constructs pSK/U1(wild type)/neo and pSK/U1(reverse)/neo. The construct pSK/U1(U6PSEA)/neo, in which the U6 PSEA was replaced by the U1 PSEA, was included in a similar manner.

Transfection of S2 cells was carried out in triplicate using 3 µg of pSK/U1(wild type)/neo, pSK/U1(reverse)/neo, pSK/U1(U6PSEA)/neo, or pB[neo]. The pBlueScriptSK(+) vector was added to bring the total amount of DNA to 20 µg. Thirty hours following transfection, cells were transferred to and subsequently maintained in medium containing 0.3 mg/ml G418 (Genetic, Life Technologies, Inc.). The survival and growth of the cells were monitored by light microscopy over a period of 5 weeks, at which point the experiment was terminated.

P-element-mediated Transformation with U1 Constructs and Determination of G418 Resistance of Transgenic Flies—Chimeric P-element constructs for transformation of flies with the neon gene driven by the U1 promoter were prepared as follows. The constructs pSK/U1(wild type)/neo and pSK/U1(U6PSEA)/neo described above were each di-
FIG. 2. The U1 and U6 PSEAs cannot functionally substitute for each other for transcription in vitro. In vitro transcription reactions were performed in a soluble nuclear protein extract using DNA templates that contained either the wild type U1 or U6 gene promoter or the same promoters with the PSEAs switched. Relevant portions of the templates are shown schematically at the top of the figure. Autoradiograms of primer extension assays of the in vitro transcription products are shown in the lower part of the figure. The primer extension products are located near the top of the portion of the gel shown, and bands near the bottom of the gel represent a 54-mer oligonucleotide that was added as a recovery standard. The RNA polymerase II inhibitor α-amanitin (2 μg/ml) or the RNA polymerase III inhibitor tagetitoxin (1000 units/ml) was included in specified reactions as indicated above the individual lanes.

The U1 and U6 PSEAs are shown in the figure. Autoradiograms of primer extension assays of the in vitro transcription product to tagetitoxin but its resistance to α-amanitin. Thus, a 5-base pair substitution that altered the U1 PSEA to a U6 PSEA was sufficient to switch the promoter specificity from RNA polymerase II to RNA polymerase III, even though the remainder of the promoter and the transcription start site was derived entirely from the U1 gene.

The results of the converse experiment are shown in Fig. 2, lanes 9–16. As observed previously (22), the wild type U6 gene was transcribed exclusively by RNA polymerase III (lanes 9–12). When the U6 PSEA was converted to a U1 PSEA, transcription by RNA polymerase II was activated (compare lanes 11 and 15). However, transcription of this construct by RNA polymerase III was also still observed (lane 14). This is not surprising since the same result was obtained previously from an analogous artificial promoter construct (20). The reason for the RNA polymerase III activity of this template is 2-fold. First, the TATA sequence TTATATA by itself promotes transcription by RNA polymerase III (as well as by RNA polymerase II) that is independent of the PSEA (21). Second, the U1 PSEA functions very ineffectively at a 12-base pair spacing from either the downstream PSEB or TATA box (20). Thus, when a TATA-containing construct is expressed in the Drosophila in vitro transcription system, there often exists a high background of TATA-mediated transcription by both RNA polymerases II and III that is independent of the PSEA. This obscures potential effects of the U1 PSEA when substituted into the U6 promoter.

To overcome this limitation of the in vitro transcription assay, we turned to an in vivo assay. Although a TATA box alone can be sufficient for transcription by RNA polymerases II or III in nuclear extracts, the formation of a stable, active transcription complex in vivo normally requires the presence of additional promoter or activator elements besides the TATA box. Therefore, in the experiments described below we investigated the functional interchangeability of the U1 and U6 PSEAs under the more stringent conditions of transcription in vivo.

The U1 and U6 PSEAs Are Not Functionally Interchangeable for RNA Polymerases II and III Promoter Activity in Transfected Tissue Culture Cells—We next examined whether the Drosophila U1 PSEA could functionally substitute for the U6 PSEA to promote transcription of the U6 gene in Drosophila S2 cells. For this purpose, the three constructs illustrated at the top of Fig. 3 were prepared. The upper construct contained the wild type U6 promoter and complete U6 gene with a 10-base pair insertion between nucleotides 66 and 67 of the U6 RNA coding region. The insertion permitted the differentiation of the transfected gene product from the endogenous U6 RNA. The second construct was identical to the first except for five nucleotide substitutions that converted the U6 PSEA to a U1 PSEA, and the third construct contained a highly mutated PSEA with 20 of 21 base positions altered. These three constructs were used to transfect S2 cells. Two days after transfection, RNA was isolated from the cells and assayed by primer extension for the presence of the U6 maxigene transcript.

RNA from cells transfected with the construct that contained the wild type U6 promoter yielded a primer extension product of the size expected (89 nucleotides) for initiation of transcription at position +1 of the U6 gene (Fig. 3, lane 2). In contrast,
promote U6 snRNA synthesis by RNA polymerase III. The templates that were used to transfect Drosophila part of the figure shows schematic diagrams of three U6 maxigene constructs that were identical except for substitutions within the PSEA insertion between positions 66 and 67 in the coding region. The other construction levels of the three constructs are shown in the column at the upper right.

Substitution of the U1 PSEA into the U6 promoter reduced transcription activity to almost undetectable levels (Fig. 3, lane 3). Upon prolonged exposure, a very weak band was visible in some experiments, but phosphorimager analysis indicated that transcription was reduced more than 65-fold relative to the wild type U6 promoter. This level was barely above the background obtained either with a mock transfection or with a construct that contained the extensively mutated PSEA (Fig. 3, lanes 1 and 4). From these results it is clear that the U1 PSEA could not effectively substitute for the U6 PSEA as a component of the U6 promoter. The promoter activity of the U1 PSEA was apparently suppressed in the context of the surrounding U6 DNA.

We next performed a reciprocal set of experiments to examine whether the U6 PSEA could functionally substitute for the U1 PSEA in the U1 promoter. Since the U1 promoter normally recruits RNA polymerase II, we used luciferase as a reporter gene for these experiments. The simplicity and sensitivity of the luciferase assay allowed us to examine a greater number of constructs that contained several additional variants of the PSEA. Besides replacing the U1 PSEA with the wild type U6 PSEA, constructs were also prepared using "hybrid" PSEA sequences that contained a mixture of U1 and U6 nucleotides. A generalized schematic illustration of this family of constructs is shown at the top of Fig. 4. For the hybrid PSEAs, we employ a nomenclature that indicates whether a U1 or U6 nucleotide is present at positions 7, 14, 16, 19, or 20 within the PSEA (Fig. 1B). For example, 11166 indicates that U1 nucleotides are present at positions 7, 14, 16, 19 or 20 within the PSEA (Fig. 4C, lanes 5 and 6). Constructs that contained the hybrid 11161 and 11166 PSEAs produced intermediate levels of transcription products (lanes 2 and 3); the 11666 construct (lane 4) was essentially no more active by primer extension analysis than the 66666 construct. For each construct, the relative level of expression was somewhat lower when measured by primer extension (Fig. 4C) than when measured by luciferase activity (Fig. 4B). This may reflect the possibility that the luciferase enzyme assay is more sensitive than the primer extension assay at the lower transcription levels.

Altogether, these results indicate that the U6 PSEA was unable to functionally substitute for the U1 PSEA in the U1 promoter to effectively recruit RNA polymerase II to transcribe the chimeric reporter gene. Furthermore, there was a gradual decrease in transcript accumulation in cells as the U6 character of the PSEA was increased. Thus, the primer extension data provide no evidence for activation of RNA polymerase III transcription in vivo when the U6 PSEA is substituted into the U1 promoter; instead, transcription seems to be suppressed.

The U1 Promoter Efficiently Drives Expression of the Neomycin Resistance Gene in Vivo, but Not Upon Substitution of the U6 PSEA—As an alternative method of examining the properties of the U1 and U6 PSEAs in living cells, the neoR gene, which confers resistance to the drug G418, was cloned downstream of the wild type U1 promoter. A similar construct was made in which the U1 PSEA was replaced with the U6 PSEA (Fig. 5). Two additional constructs were used as controls: the first (negative control) contained the U1 promoter cloned in reverse orientation relative to the neoR gene; the other (positive control) utilized the heat shock promoter to drive neoR gene expression (Fig. 5).

Drosophila S2 cells were transfected with these four constructs, and 30 h later growth medium containing 0.3 mg/ml G418 was added to the cells. The fate of the cells was monitored visually by light microscopy over a period of several weeks. On all plates, the majority of the cells stopped dividing and began to die after a few days. Within 2 weeks, viable colonies of cells were no longer visible in the plates that were transfected with the constructs that contained either the U6 PSEA substitution or the entire U1 promoter in reverse orientation (Fig. 5, right column). In contrast, colonies of cells continued to grow and divide on plates transfected with constructs that contained the neoR gene under the control of either the wild type U1 or heat shock promoters. When grown continuously on medium containing 0.3 mg/ml G418, these latter cells continued to divide and remained robust and healthy until the experiment was terminated 5 weeks after the initial transfection. From these
results we conclude that the wild type U1 promoter can effectively drive expression of the neoR gene. However, the U6 PSEA was unable to substitute for the U1 PSEA for this purpose, presumably because it was unable to recruit the RNA polymerase II required for production of a functional mRNA.

To confirm these results in a truly in vivo situation, similar neoR gene constructs were introduced into the germ line of yw flies by P-element transformation. Homozygous transgenic fly lines were then isolated that contained the neoR gene either under the control of the wild type U1 promoter or under the control of a U1 promoter in which the PSEA had been converted to a U6 PSEA. Several of these transgenic fly lines were then tested for in vivo expression of the neoR gene by allowing flies to lay eggs on food that contained 0.4 mg/ml G418. Significant numbers of G418-resistant offspring were produced from parental flies that contained genomic copies of the neoR gene downstream of the wild type U1 promoter (Table I). In contrast, the flies that contained the substitution of the U6 PSEA in the U1 promoter produced no viable offspring when subjected to the same G418 selection (Table I). These results indicate that the U1 promoter can efficiently drive expression of the neoR gene, but substitution of the U6 PSEA effectively inactivated the RNA polymerase II activity of the U1 promoter in vivo.

**DISCUSSION**

Previous in vitro studies suggested that the RNA polymerase specificity of Drosophila snRNA genes was determined primarily by a few nucleotide differences within the 21-base pair PSEA sequence. That conclusion was based upon transcription of synthetic promoter constructs that contained various mix-and-match features of the U1 and U6 gene promoters. However, it was important to validate those findings both in cells and in the context of the natural promoter environment of the U1 and U6 genes. The data reported here reveal that the Drosophila U1 and U6 PSEAs are functionally distinct in vivo as well as in vitro: the U1 PSEA is compatible with transcription only by RNA polymerase II, and the U6 PSEA supports transcription only by RNA polymerase III. However, there are some differences as discussed below.

Interchanging the U1 and U6 PSEAs Results in RNA Polymerase Switching in Vitro but Suppression of Transcription in Vivo—Earlier published data (20), as well as those presented here (Fig. 2), indicated that the PSEA can act as a dominant element in vitro to determine the RNA polymerase specificity of Drosophila snRNA gene promoters. That is, exchanging the U1 and U6 PSEAs resulted in switching the RNA polymerase
RNA polymerase II does initiate transcription of the U6 maxis as well as to effects on transcription initiation. For example, if contain a switched PSEA may be due to post-initiation events in the absence of detectable transcripts from U1 or U6 promoters that steadied of the classical TATA-binding protein TBP (36).

It is possible that the switching of the PSEAs, the introduced genes may not compete with endogenous genes for available transcription factors.

Chromatin structure may also play a role in the suppression of transcription in vivo from constructs that contain the wrong PSEA. Positioned nucleosomes have been implicated in both the activation and repression of transcription from snRNA gene promoters (27–30). It is possible that the chromatin environment required for optimal RNA polymerase III transcription is different from that required for optimal RNA polymerase II transcription. The modification of chromatin structure is one of several effects that can be attributed to the binding of transcriptional activator proteins and coactivators to upstream sequence elements. In vertebrates, at least two upstream activator proteins (Oct-1 and SBF/Staf) involved in transcription of the U1, U2, and U4 genes have a well conserved sequence (consensus CATTTATAAATATATNA) in their promoter in the production of luciferase activity but less active than the copia promoter in the production of luciferase activity but less active than the heat shock promoter. In vertebrate systems, an early report indicated that the U1 promoter could not drive the expression of a functional mRNA (37). Later, however, it was demonstrated that sea urchin U1 promoters could be used to produce fully functional histone mRNAs (38). More recent studies have demonstrated that even vertebrate RNA polymerase II snRNA promoters can indeed produce functional mRNAs if care is taken to avoid cryptic 3′ end formation signals within the coding region of the gene (39, 40). We have noticed that Drosophila U1, U2, and U4 genes have a well conserved sequence (consensus CATTATAATATAATNNA) in the 3′ flanking DNA that may act in 3′ end formation of Drosophila snRNAs. Sequences similar to these are not present internal to the luciferase or neoR genes. Thus, it is not surprising that Drosophila U1 promoter can be highly effective for the production of functional mRNAs encoding either luciferase activity or G418 resistance.

Previously published data from our laboratory indicated that the D. melanogaster PSEA-binding protein (DmPBP) interacts differently with the DNA in vitro depending upon whether it is bound to a U1 or U6 PSEA sequence (41, 42). We therefore

### Table I

| Fly line                        | Flies eclosing on food containing 0.4 mg/ml G418 | Flies eclosing on food without G418 | Survival rate on 0.4 mg/ml G418 |
|---------------------------------|--------------------------------------------------|-----------------------------------|---------------------------------|
|                                 | Bottle 1  Bottle 2  Total bottles 1 and 2       | Bottle 3  Bottle 4  Total bottles 3 and 4 | %                               |
|yw (parental line)               | 0  0  0                                         | 397  375  772                      | 0                               |
|U1-Neo(68–18–36–7)               | 430  350  780                                    | 452  447  899                      | 87                              |
|U1-Neo(26–21–56–14)              | 155  140  295                                    | 541  525  1066                     | 28                              |
|U1-Neo(26–8–1–9)                 | 145  91  239                                     | 463  462  925                      | 26                              |
|U1(U6 PSEA)-Neo(8–21–14–8)      | 0  0  0                                         | 424  428  852                      | 0                               |
|U1(U6 PSEA)-Neo(10–34–36–9)     | 0  0  0                                         | 301  337  638                      | 0                               |
|U1(U6 PSEA)-Neo(11–36–66–19)    | 0  0  0                                         | 439  561  1000                     | 0                               |
|U1(U6 PSEA)-Neo(8–7–10–3)       | 0  0  0                                         | 526  412  938                      | 0                               |

**RNA Polymerase Selection by the PSE at snRNA Gene Promoters**

Transgenic fly lines were tested for their ability to produce offspring surviving to adulthood when the larvae were raised on food containing 0.4 mg/ml G418. Two bottles were prepared for each fly line; each bottle was seeded with 8 males and 15 females. Lines designated U1-Neo contained transgenes with the wild type U1 promoter; lines designated U1(U6 PSEA)-Neo contained transgenes with a substitution of the U6 PSEA for the U1 PSEA in the U1 promoter.

Despite the above discussed limitations on the interpretation of the data, the main premise of the findings is clear: the Drosophila U1 and U6 PSEAs are functionally distinct and are not interchangeable either in vivo or in vitro. The U1 PSEA can effectively support only RNA polymerase II transcription, and the U6 PSEA functions effectively only for RNA polymerase III transcription.
hypothesize that DmPBP assumes different conformations upon binding to a U1 or U6 PSEA and that these distinct conformations are each compatible with the downstream recruitment of only RNA polymerase II or RNA polymerase III, respectively. Our current results pave the way toward the use of Drosophila transgenic and genetic technologies to define the molecular interactions that contribute to RNA polymerase specificity. As one example, it may be feasible to obtain suppressor-like mutations in DmPBP that alter the RNA polymerase specificity normally associated with the U1 or U6 PSEAs.

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