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Small Nuclear RNA Genes Transcribed by Either RNA Polymerase II or RNA Polymerase III in Monocot Plants Share Three Promoter Elements and Use a Strategy To Regulate Gene Expression Different from That Used by Their Dicot Plant Counterparts

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RNA polymerase (Pol) II- and RNA Pol III-transcribed small nuclear RNA (snRNA) genes of dicotyledonous plants contain two essential upstream promoter elements, the USE and TATA. The USE is a highly conserved plant snRNA gene-specific element, and its distance from the −30 TATA box, corresponding to approximately three and four helical DNA turns in Pol III and Pol II genes, respectively, is crucial for determining RNA Pol specificity of transcription. Sequences upstream of the USE play no role in snRNA gene transcription in dicot plants. Here we show that for expression of snRNA genes in maize, a monocotyledonous plant, the USE and TATA elements are essential, but not sufficient, for transcription. Efficient expression of both Pol II- and Pol III-specific snRNA genes in transfected maize protoplasts requires an additional element(s) positioned upstream of the USE. This element, named MSP (for monocot-specific promoter; consensus, RGCCCR), is present in one to three copies in monocot snRNA genes and is interchangeable between Pol II- and Pol III-specific genes. The efficiency of snRNA gene expression in maize protoplasts is determined primarily by the strength of the MSP element(s); this contrasts with the situation in protoplasts of a dicot plant, *Nicotiana plumbaginifolia*, where promoter strength is a function of the quality of the USE element. Interestingly, the organization of monocot Pol III-specific snRNA gene promoters closely resembles those of equivalent vertebrate promoters. The data are discussed in the context of the coevolution of Pol II- and Pol III-specific snRNA gene promoters within many eukaryotic organisms.

Eukaryotic cells contain a large population of small nuclear RNAs (snRNAs) which participate in RNA-processing reactions in the nucleus (reviewed in references 12 and 23). Most snRNAs are synthesized from independent transcription units, the snRNA genes, which are either RNA polymerase (Pol) II or RNA Pol III specific (reviewed in reference 16). In many organisms studied to date, particularly in higher eukaryotes, transcription of snRNA genes differs in many aspects from transcription of other gene classes. Firstly, snRNA genes usually contain transcriptional elements which are unique to this gene group (reviewed in references 1, 6, 13, and 16; see also below). Secondly, despite the fact that some snRNA genes are transcribed by Pol II and some are transcribed by Pol III, their promoters are usually structurally related (1, 3, 13, 16, 22, 26, 39, 40). Thirdly, features of snRNA promoters, though highly conserved within one species, vary greatly between different organisms, indicating that snRNA gene promoters evolve quite rapidly (1, 16, 21, 28, 30, 32, 34, 35, 38–42). Investigation of the expression of snRNA genes has contributed significantly to the understanding of eukaryotic transcription. Indeed, the concept of a strong conservation between Pol II and Pol III transcriptional machineries originally emerged from the studies of snRNA genes. In addition, these studies have raised questions about the factors involved in determination of the RNA Pol specificity of genes and have led to speculations regarding eukaryotic promoter diversification during evolution (reviewed in references 1, 13, 16, 17, 24, and 29).

Transcription of snRNA genes has been most extensively studied in vertebrates and in dicotyledonous (dicot) plants (13, 16), as well as in yeasts (9), sea urchins (34, 41), and *Drosophila melanogaster* (42). In vertebrates, two promoter elements, the proximal sequence element (PSE) and the distal sequence element (DSE), are shared between Pol II-specific (e.g., U2) and Pol III-specific (e.g., U6) snRNA genes. The PSE, positioned at the −50 to −60 region, is an essential snRNA gene-specific promoter element that is involved in transcription start site selection. The DSE, positioned at −200 to −250, shares sequence and functional homology with enhancers of mRNA genes, although the mRNA enhancers are usually not interchangeable with the snRNA DSE (reviewed in reference 16). The major determinant conferring Pol III specificity to the snRNA promoter is a −30 TATA box present in Pol III- but not in Pol II-specific genes (16, 22, 26). Additionally, precise positioning of the PSE element relative to the TATA box appears to be crucial for transcription of Pol III-specific genes (20a).

Regulatory sequences in snRNA genes of dicot plants such as *Arabidopsis thaliana* or tomato and determinants of RNA Pol specificity of their transcription are distinct from those of vertebrates. Plant genes contain two promoter elements, the

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upstream sequence element (USE; consensus TCCCACA TCG) and a −30 TATA box (reviewed in references 13 and 33). Detailed analysis of transcription of U2 and U6 genes of *A. thaliana* has revealed that these two elements are necessary and sufficient for transcription initiation in transfected protoplasts (5, 13, 38, 40). The USE is a highly conserved plant snRNA gene-specific element, and its deletion or mutation decreases transcription efficiency 10- to 20-fold (38, 40). The TATA box is structurally and functionally indistinguishable from the TATA elements of protein-coding genes and is recognized by the TATA-binding protein, TBP, in both Pol II- and Pol III-specific genes (15). Unlike in vertebrate snRNA genes, the spacing between the USE and TATA box is the major determinant of Pol specificity of plant snRNA genes. Pol II-specific genes contain the USE and TATA centered approximately four DNA helical turns apart, while in Pol III genes these elements are positioned one DNA helical turn closer (see Fig. 1B). Experiments with *Arabidopsis* U2 and U6 genes and with a tomato U3 gene have revealed that the Pol specificity of the promoter can be altered by either deletion or insertion of 10 bp within the USE-TATA spacer region (18, 39). Interestingly, in plants the U3 snRNA gene is transcribed by Pol III and not by Pol II as in other eukaryotes, and phylogenetic considerations suggested that conversion of promoter specificity of the U3 snRNA gene has occurred during evolution of plants, possibly by a mechanism involving a change in the USE-TATA spacing (18, 25, 39).

In this work we have investigated the requirements for transcription of Pol II- and Pol III-specific snRNA genes in maize, representing monocotyledonous (monocot) plants which are considered to be evolutionarily younger than dicots (8). We have found that, in addition to the USE and TATA, transcription of snRNA genes in maize protoplasts requires an additional element(s) positioned upstream of the USE. Similar to the situation with the USE and TATA boxes of dicot genes (39), the monocot-specific promoter (MSP) elements are interchangeable between Pol II- and Pol III-specific snRNA genes. We also demonstrate that efficiency of snRNA gene expression in maize protoplasts is determined primarily by the strength of the MSP element(s)-containing region, in contrast to the situation in dicot plants, where promoter activity is a function of the quality of the USE element.

MATERIALS AND METHODS

Plasmid construction. Plasmids containing wild-type genes used in this work have been previously described. Plasmids AU2 (formerly referred to as pGEM2.U2.2 [37]), AU5 (pGEM1.U5 [36]), and AU6 (pU6.26 [40]) contain *Arabidopsis* snRNA genes. Plasmids MU2 (pMzU2-27.023 [2]) and MU5.1, MU5.2, and MU5.3 (pMzU5.1, pMzU5.2, and pMzU5.3, respectively [20]) contain maize snRNA genes. Plasmid pWU3 (prepared from pWU3-1.3K [25] as described below) contains the wheat U3 gene. Unless indicated otherwise, all techniques used for manipulating DNA were as described by Sambrook et al. (31). The identity of all constructions was verified by sequence analysis.

(i) Pol II-specific chimeras and mutants. MU2/USEsub was generated as follows. The USE was mutated by PCR amplification of MU2 with a 5′ primer complementary to the promoter but containing base changes in the USE and a *Bam*HI site further upstream (for *Bam*HI site location, see Fig. 3B) and a 3′ primer complementary to the 3′ end of MU2 and containing an *Eco*RI site (this site was engineered downstream of the 3′ end formation signal of MU2). The MU2 MSP region was amplified with a 5′ primer complementary to the vector DNA upstream from the promoter and a 3′ primer containing a *Bam*HI site and complementary to the region upstream of the USE. The fragments were digested with the appropriate enzymes and inserted into pSP65 digested with *Hin*dIII and *Eco*RI. MU2/TATAsub was made by using a 5′ primer complementary to the pSP65 polylinker upstream of the MU2 promoter and a 3′ oligonucleotide containing a *Hin*cII site at the 3′ end and the base changes indicated within the MU2 TATA box. The PCR products were digested with *Hin*dIII and *Hin*cII (located at −16 in the MU2 promoter) and ligated to a *Hin*cII-*Eco*RI fragment, isolated from MU2, that contains the MU2 coding and 3′-flanking sequences. The resulting fragment was cloned into pSP65.

The MU2 MSP box deletion and substitution mutants were made in the following way: the box 1 mutant was generated from digestion of MU2 with *Hpa*II (located at position −87 upstream from the MU2 USE) and *Cla*I (located at −77 upstream from the USE) and religated, thereby deleting box 1. Box 2, box 3, and box 2,3 mutants were constructed by PCR amplification of MU2 with a 5′ primer containing the indicated base changes as well as a *Cla*I site and a 3′ oligonucleotide containing an *Eco*RI site and complementary to the 3′ end of the MU2 gene. The PCR products were digested with *Cla*I-*Eco*RI and inserted into the MU2 plasmid digested with *Cla*I-*Eco*RI. Box 1,2, box 1,3, and box 1,2,3 mutants were generated from box 2, box 3, and box 2,3 mutants, respectively, by digestion with *Hpa*II-*Cla*I and religation. MU2ΔMSP was generated by PCR amplification of MU2 with a 5′ primer complementary to the MU2 USE sequence and a *Bam*HI site and a 3′ oligonucleotide complementary to the 3′ end of the MU2 gene containing an *Eco*RI site. The PCR product was cloned into *Bam*HI-*Eco*RI-digested SP65. MU2ΔMSPrep contains the 375-bp *Bam*HI-*Hin*dIII fragment isolated from pBR322 and inserted into the *Bam*HI-*Hin*dIII sites of MU2ΔMSP. MU2/Spe was constructed by PCR amplification of two fragments from MU2 by an approach similar to that used for preparation of MU2/USEsub. The upstream MSP-containing fragment was digested with *Pst*I-*Spe*I, and the downstream fragment was digested with *Spe*I-*Eco*RI, and both were ligated into pGEM2 digested with *Pst*I-*Eco*RI. The downstream *Spe*I-*Eco*RI fragment was also separately inserted into *Spe*I-*Eco*RI-digested pBluescript (KS−) (Stratagene) to yield MU2/SpeΔMSP.

The synthetic MSP regions were assembled from synthetic oligonucleotides containing upstream *Hin*dIII and downstream *Spe*I sites. They were inserted into the MU2/Spe from which the MSP region was removed by *Hin*dIII-*Spe*I digestion, to yield MU2/synMSP and MU2/synMSP*. The sequence of the synMSP region is (*Hin*dIII) CCGGGCCCATGGTACGCATC GATTGAGCCCAGAGCCCACCGCTAACTGG TACCC GTACCTATGGTTTGGCGTGTTGGTGCTTC (*Spe*I) (the MSP boxes are underlined, and sequence changes in MU2/synMSP* are shown in Fig. 3B).

The MU5 promoter-switch constructs, MU5.2/MU5.1p, MU5.3/MU5.1p, and MU5.3/MU5.2p, have been described elsewhere (20). To generate the MU5 MSP switch constructs and the MU5ΔMSP genes, the MU5 genes were first recloned into different vectors. The 590-bp *Eco*RI-*Bam*HI fragment from MU5.1 and the 720-bp *Hin*dIII-*Sac*I fragment from MU5.2 were cloned in pGEM2. The 420-bp *Kpn*I-*Eco*RI fragment from MU5.3 was cloned into pBST(KS−). Each of the resulting recloned genes was used as a template for PCR amplification with four different primers. The MU5.1 MSP region (300 bp) was amplified with a 5′ primer complementary to the SP6 promoter within pGEM2 and a 3′ primer containing an *Spe*I site complementary to the MU5.1 promoter region

upstream from the USE. To generate the MU5.1 promoter and coding region-containing fragment (290 bp), a 5′ primer containing the *Spe*I site and complementary to the MU5.1 USE was used with a 3′ primer complementary to the T7 promoter in pGEM2. MU5.2 and MU5.3 gene fragments were amplified in a similar manner. To generate the MU5/Spe constructs (see Fig. 4B), the MSP and the promoter/coding region fragments originating from the same gene and cut with appropriate enzymes were ligated together and cloned into pGEM2 or pBST(KS−) vectors. Combination of fragments originating from different genes yielded chimeric MU5.x/MU5.yMSP constructs. MU5ΔMSP genes were generated by insertion of only the promoter/coding region-containing fragments into appropriately cut vectors.

The MU2/AU2-USE plasmid was constructed by PCR amplification of MU2 with a 5′ primer complementary to the MU2 promoter region and containing an *Spe*I site and appropriate base changes in the MU2 USE and a 3′ primer complementary to the 3′ end of the MU2 gene. The PCR fragment was digested with *Spe*I-*Eco*RI and inserted into MU2/Spe from which the wild-type *Spe*I-*Eco*RI MU2 fragment had been deleted.

(ii) Pol III-specific chimeras and mutants. To create pWU3, the 425-bp *Dde*I (located at −197 in the WU3 gene; filled in with Klenow polymerase)-*Xba*I (located 40 bp downstream from the 3′ end of the WU3 gene) fragment isolated from pWU3-1.3K (25) was inserted into the *Eco*RV-*Xba*I sites of pBST(KS−). WU3/synMSP and WU3/synMSP* genes were constructed by ligating synthetic MSP regions (see above and Fig. 3B) to the WU3 promoter and coding region-containing fragment. The latter fragment was PCR amplified from pWU3ΔDde by using a 5′ primer complementary to the WU3 USE and containing a *Spe*I site and a 3′ primer complementary to the T7 promoter; it was then digested with *Spe*I-*Sac*I. Ligated fragments were inserted into pBST(KS−). Insertion of the *Spe*I-*Sac*I fragment alone into pBST(KS−) yielded WU3/SpeΔMSP. MU2/WU3-MSP and WU3/MU2-MSP were constructed by interchanging the PCR-amplified MSP regions between the WU3 and MU2 genes, using an *Spe*I site introduced directly upstream of the USE (see above). These hybrid genes were inserted into pBST(KS−).

pU6.1, pU6.26, pU6.29, and pU6syn (40) and pU6.35 (unpublished data) contain different U6 genes of *A. thaliana*. The U6 mutant, ΔMSP, was constructed by digestion of pU6.26 with *Hin*dIII-*Bst*XI, blunting of the ends, and religation. Further details of plasmid constructions are available upon request.

Transient expression in protoplasts. Protoplasts (6×10^5) prepared from leaves of *Nicotiana plumbaginifolia* or maize were transfected by the polyethylene glycol method as previously described (14). Fifteen micrograms of the relevant plasmid DNA in addition to 10 μg of pU6.26 used as a transfection efficiency control (with the exception of the experiment shown in Fig. 6, in which AU2 and MU2 were transfected as controls) was used for each transfection reaction mixture. Unless stated otherwise, RNA probes used for RNase A and RNase T_1 mapping were synthesized from the same plasmids used for transfection, linearized appropriately, and transcribed with SP6, T7, or T3 RNA polymerase by using [α-^{32}P]CTP (specific activity, 80 Ci/mmol). A total of 5×10^4 cpm of a gel-purified antisense probe, mixed together with the same amount of the U6 gene-specific probe, was hybridized to 5 μg of RNA from transfected protoplasts (14). RNase A and RNase T_1 mappings were performed as previously described (14). To quantify the expression levels, the protected fragments corresponding to the test and reference gene transcripts were excised from the gel, and radioactivity was determined by scintillation counting. The relative expression of each of the test genes was calculated, taking into account the C content of each probe and averaging the values obtained from three to five experiments.

RESULTS

Differences in expression of Pol II-specific U snRNAs genes between maize and *N. plumbaginifolia* protoplasts. To get some insight into the requirements for snRNA gene transcription in monocot plants, we first compared the expression of Pol II-transcribed monocot and dicot snRNA genes in transfected protoplasts of maize, a monocot plant, and *N. plumbaginifolia*, a dicot plant. The following genes were included in the comparison: three U5 genes (MU5.1, MU5.2, and MU5.3) and one U2 gene (MU2) from maize and *Arabidopsis* U2 (AU2) and U5 (AU5) genes. The *Arabidopsis* genes (36–38) and maize U5 genes (20) were previously shown to be expressed in transfected protoplasts of *N. plumbaginifolia* and maize, respectively.

The genes were introduced into protoplasts by transfection together with a Pol III-transcribed *Arabidopsis* U6 gene (AU6) functioning as a transfection efficiency standard. Expression was analyzed by RNase A and RNase T_1 mapping of RNA isolated from protoplasts, using gene-specific antisense RNA probes. All maize genes were transcribed in *N. plumbaginifolia* protoplasts; however, the efficiencies of their transcription were diametrically different from the efficiencies in homologous maize protoplasts (Fig. 1A). The MU2 and MU5.1 genes, which were the most efficiently expressed in maize protoplasts (lanes 17 and 21) (20) were weakly expressed in *N. plumbaginifolia* protoplasts (lanes 3 and 7). In contrast, the MU5.2 and MU5.3 genes, weakly transcribed in maize (lanes 23 and 25), were strongly expressed in *N. plumbaginifolia* protoplasts (lanes 9 and 11). We have shown previously that the only elements essential for snRNA gene expression in protoplasts of *N. plumbaginifolia* are the USE and TATA box and that point mutations within the USE decrease transcription efficiency up to 20-fold (6, 38). Differences in the activities of the maize genes in transfected *N. plumbaginifolia* protoplasts can be best explained on the basis of the nucleotide changes naturally occurring in the USE sequences of these genes (Fig. 1B; see also below). For example, the $A_7 \rightarrow C$ change which distinguishes MU2 and AU2 USE elements was shown to decrease transcription of the AU2 gene in *N. plumbaginifolia* protoplasts by 70 to 75% (38); similarly, the gene MU5.1, which is significantly less strongly expressed than MU5.2 and MU5.3, deviates from the USE consensus at three positions as opposed to two positions in the genes MU5.2 and MU5.3.

Analysis of the expression of the *Arabidopsis* genes in maize protoplasts has revealed that their transcription is barely detectable (Fig. 1A, lanes 15 and 19) despite the fact that these genes contain USE and TATA elements that conform perfectly with the consensus (Fig. 1B). Inactivity of the *Arabidopsis* genes in maize protoplasts, together with the observations that expression of maize U2 and U5 genes in homologous protoplasts cannot be correlated with the base changes in the USE, suggested that requirements for snRNA gene expression in maize differ substantially from that in *N. plumbaginifolia*.

Transcription of the maize U2 gene in maize protoplasts requires the GC-rich upstream elements in addition to the USE and TATA. We have focused first on expression of the U2 genes. To determine if the differential expression of heterologous snRNA genes in maize and *N. plumbaginifolia* protoplasts was due to differences in transcription initiation and not

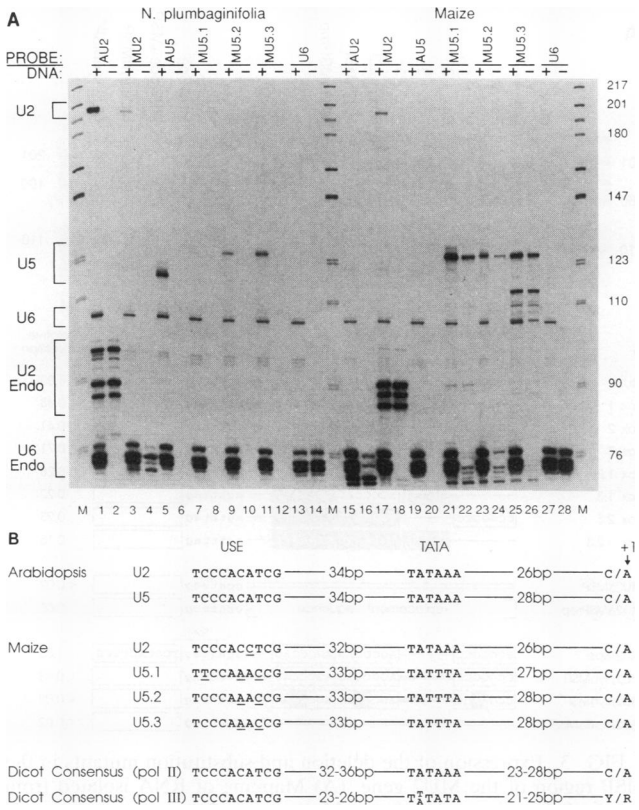

FIG. 1. Comparison of snRNA gene expression in transfected *N. plumbaginifolia* (lanes 1 to 14) and maize (lanes 15 to 28) protoplasts. (A) RNase A and RNase T_1 protection analysis. RNA was isolated from protoplasts cotransfected with plasmids containing *Arabidopsis* U2 (AU2) and U5 (AU5) or maize U2 (MU2) and U5 (MU5.1, MU5.2, and MU5.3) genes in addition to a plasmid containing an *Arabidopsis* U6 gene (U6). Individual genes contain 388 (AU2), 633 (AU5), 187 (MU2), 392 (MU5.1), 259 (MU5.2) and 562 (MU5.3) bp of the upstream promoter sequence. The plasmids used for transfection and probe synthesis are indicated at the top. The + and − indicate that RNA from either transfected protoplasts or nontransfected protoplasts, respectively, was used. RNA from transfected protoplasts was mapped with a mixture of gene-specific and U6-specific RNA probes, while RNA from nontransfected probes was mapped only with gene-specific probes. In lanes 13, 14, 27, and 28, the U6 probe only was used. The positions of U2-, U5-, and U6-specific protected fragments, approximately 200, 120 to 127, and 104 nucleotides in length, respectively, are indicated by brackets. The protected fragments detected with the MU5 gene-specific probes in the nontransfected maize protoplasts (lanes 22, 24, and 26) represent endogenous U5 RNAs that are fully or partially complementary to the MU5 probes; to calculate expression levels of the transfected genes, the full-length endogenous U5 RNA-specific bands were subtracted (see reference 20). The protected fragments labeled U2 Endo and U6 Endo represent endogenous RNAs partially complementary to the U2- and U6-specific probes, respectively. Lanes M, DNA size markers (5′-end-labeled *Hpa*II-digested pBR322). (B) Sequences of the USE, TATA, and −1 to +1 regions, and spacing between them, in transfected snRNA genes. USE nucleotides not conforming with the consensus are underlined. The dicot-derived promoter element consensus for Pol II- and Pol III-specific genes is taken from reference 13.

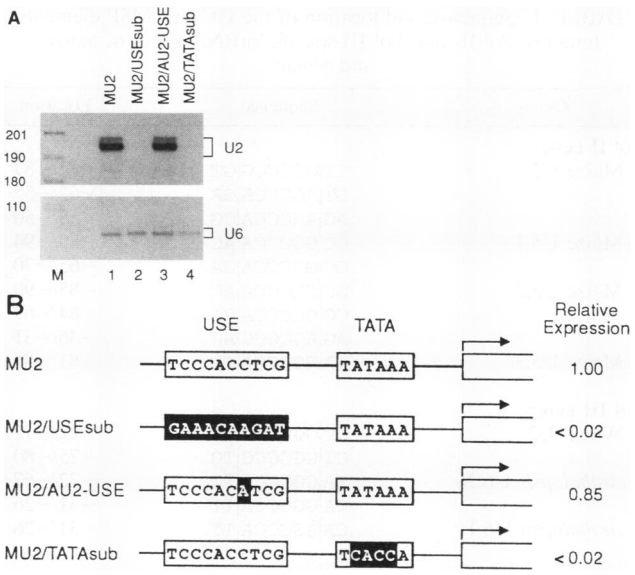

FIG. 2. Expression of MU2 USE and TATA box substitution mutants in maize protoplasts. (A) RNase mapping of RNA isolated from protoplasts cotransfected with pU6.26 and the plasmids indicated at the top and mapped with a mixture of the MU2 and U6 probes. Positions of U2- and U6-specific protected fragments are indicated. Only relevant portions of the gels are shown. (B) Diagram of the promoter regions of the substitution mutants. Base changes in the USE and TATA boxes are indicated in black. The transcription start site is represented by the arrow. Expression of each mutant relative to that of MU2 is indicated.

caused by differential stability or 3′ end processing of the transcripts, chimeric U2 genes were constructed by interchanging the promoter or 3′ end regions of the AU2 and MU2 genes. It was found that expression efficiency of the chimeric genes in either type of protoplast directly corresponded to the expression level of the gene from which the promoter was derived (data not shown), indicating that differential expression of the AU2 and MU2 genes in dicot and monocot protoplasts is caused by differences in transcription initiation.

To check whether the same elements that are necessary for transcription in dicots, the USE and TATA, are also important for transcription in maize, the USE and TATA box substitution mutants were constructed in the promoter region of the MU2 gene (Fig. 2). Substitution of the USE sequence with an unrelated sequence or disruption of the TATA box by four base changes abolished transcription of the MU2 gene, indicating that these elements are necessary for transcription in maize protoplasts. This observation is consistent with the presence of both elements in all monocot snRNA genes isolated to date (see Discussion). We have also tested activity of the mutant MU2/AU2-USE, which contains the $C_7 \rightarrow A$ change in the USE, creating the element with a perfect match to the dicot snRNA gene-derived consensus TCCCACATCG. This base change increases transcription of the AU2 gene in *N. plumbaginifolia* protoplasts fourfold (38) but had no stimulatory effect on transcription of MU2 gene in maize protoplasts (Fig. 2A, lane 3), consistent with the notion that substitutions within the USE are less important for transcription in monocot as compared with dicot protoplasts.

Deletion analysis of the promoter regions of MU2 and MU5 genes indicated that sequences positioned upstream of the USE are important for expression of these genes in maize protoplasts (see below; and data not shown). Inspection of DNA sequences in the region upstream from the USE has revealed the presence of a conserved sequence, having the consensus RGCCCR (Table 1). This GC-rich sequence, termed the MSP element, was present in either orientation, in

TABLE 1. Sequence and location of the GC-rich MSP elements found in Pol II- and Pol III-specific snRNA genes of maize and wheat[a]

Gene	Sequence	Location
Pol II genes		
Maize U2	CT|GGCCCG|GT	−82/−87
	GT|AGCCCA|GA	−62/−57
	AG|AGCCCA|CG	−55/−50
Maize U5.1	CC|GGCCCA|AC	−89/−94
	GC|GGCCCA|GA	−65/−70
Maize U5.2	GC|GGCCCA|AC	−85/−90
	CG|GGCCCA|GA	−64/−69
	AG|AGCCCG|AC	−36/−31
Maize U5.3	AA|GGCCCA|CC	−81/−86
Pol III genes		
Wheat U3	CC|GGCCCA|TC	−122/−127
	CT|GGCCCG|TG	−75/−80
Arabidopsis U6.26	TA|GGCCCA|TT	−72/−67
	CA|GGCCCA|TT	−31/−26
Arabidopsis U6.1	CA|GGCCCA|TT	−31/−26
Consensus	NN|RGCCCR|NN	

[a] MSP elements present in *Arabidopsis* genes U6.26 and U6.1 are also indicated (see text). Location of the elements is given relative to the position of the USE. Some upstream MSP elements in MU2 and MU5.2 genes and the elements in MU5.1, MU5.3, and WU3 genes are present in the reverse orientation. A consensus MSP sequence is indicated at the bottom.

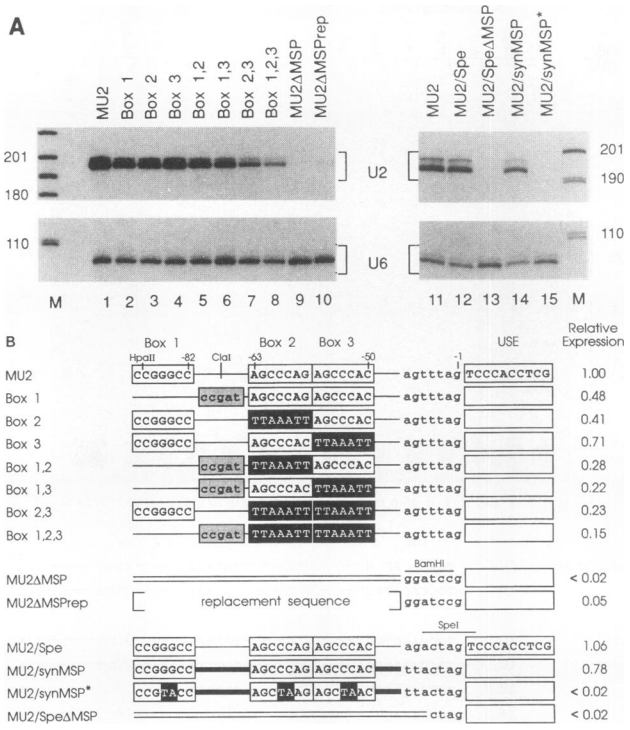

FIG. 3. Expression of the deletion and substitution mutants in the MSP region of the MU2 gene. (A) Mapping of RNA isolated from transfected maize protoplasts. Mutants used for transfection are indicated at the top. (B) Schematic diagram of the MSP regions of deletion and substitution mutants of the MU2 gene. The USE and MSP box 1, box 2, and box 3 sequences are boxed, and the positions of MSP boxes are given relative to the USE. The sequence directly upstream from the USE is in lowercase type. The sequence of the box 1 mutant, generated by deletion, is represented by the shaded box, while substitutions in box 2 and box 3, and in box 1 of the MU2/synMSP*, are shown in black. Note that an additional base pair was included in the MU2 MSP, although the conserved sequence consists of only 6 bp (Table 1). The thick black line between the MSP boxes indicates that these sequences are different from those in the wild-type MU2 gene. Vector sequences are represented by the double line. Replacement sequence in MU2ΔMSPrep originated from pBR322. Expression of each of the mutants relative to MU2 is shown on the right. Inhibition of transcription in the MU2ΔMSP mutants is not due to the introduction of the *Bam*HI site upstream of the USE because a wild-type MU2 gene containing this sequence is transcribed at 75 to 80% of the wild-type efficiency (data not shown); a similar mutation, generating an *Spe*I site (mutant MU2/Spe), also has no effect on transcription of MU2, while deletion from MU2/Spe of the region upstream of the USE (mutant MU2/SpeΔMSP) completely eliminates transcription.

one or more copies, and was located within the range of 100 bp upstream of the USE in each of the maize snRNA genes.

To test directly the possibility that the MSP elements function in transcription of snRNA genes in monocots, a series of deletion and substitution mutants was generated in the three MSP elements (box 1, box 2, and box 3 [Fig. 3B]) of the MU2 gene. It was found (Fig. 3A) that deletion or substitution of each of the MU2 MSP elements had an effect on transcription of the MU2 gene in maize protoplasts. Mutation of box 1 or box 2 had a greater effect (50 to 60% reduction) than mutation of box 3 (30% reduction). Inactivation of the MSP elements in combinations of two reduced transcription efficiency to 20 to 30% (Fig. 3A, lanes 5 to 7) while mutation of all three reduced it to 15% of the wild-type level (lane 8). Deletion of the entire region upstream from the MU2 USE (MU2ΔMSP [lane 9]) abolished transcription completely. This effect was not due to the presence of potentially inhibitory vector polylinker sequences brought into proximity of the USE, because replacement of the upstream sequences with unrelated pBR322-derived sequences also resulted in a 95% reduction of transcription (MU2ΔMSPrep [lane 10]).

To verify that the MU2 MSP boxes were indeed the principal transcriptional elements in the region upstream from the MU2 USE, a MU2 gene containing a synthetic MSP region was constructed (Fig. 3B). MU2/synMSP contains substitutions of all the nucleotides, other than the three MSP boxes, in the region within 100 bp upstream from the MU2 USE. MU2/synMSP* is identical to MU2/synMSP, except that each of the three boxes contains two base changes in the MSP consensus (Table 1). It was found that the MU2/synMSP gene is transcribed at 78% of the level of the wild-type gene (Fig. 3A, lane 14), but transcription of the mutant MU2/synMSP* is reduced by 98% (lane 15). These results confirm that the MSP elements are the sequences responsible for efficient transcription of the MU2 gene in maize protoplasts.

The MSP region is responsible for the differential expression of the maize U5 genes in maize protoplasts. To check whether the MSP element-containing upstream regions are of general importance for transcription of snRNA genes in monocots, we investigated their roles in transcription of the three maize U5 genes. By analysis of a series of chimeric U5 promoter switch constructs, we had shown previously that differences in the expression levels of the maize U5 genes in transfected maize protoplasts (Fig. 1) are due to differences in transcription efficiency (20). To determine if the MSP-containing upstream regions are responsible for this differential expression, a series of U5 chimeric genes was analyzed (Fig. 4). The plasmids, MU5.1ΔMSP, MU5.2ΔMSP, and MU5.3ΔMSP, contain a deletion of all U5-derived sequences upstream of the *Spe*I restriction site introduced next to the USE sequences

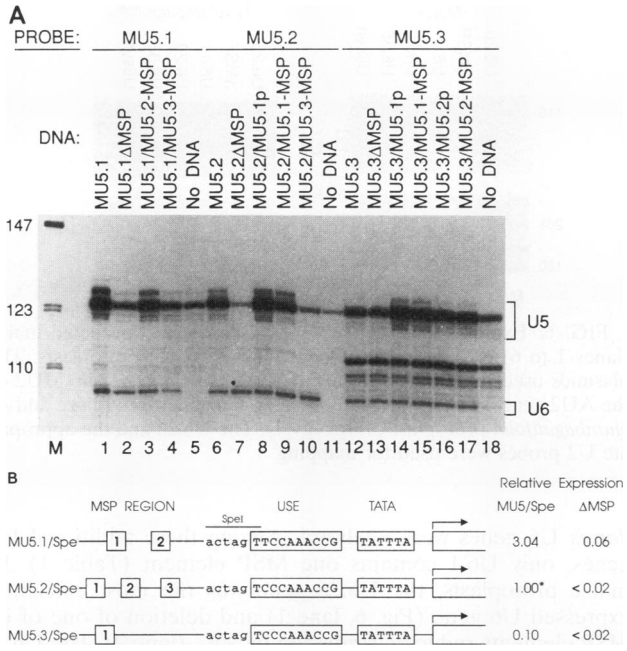

FIG. 4. Expression of MU5 promoter and MSP region switch mutants in maize protoplasts. (A) RNase protection analysis. The plasmids used for transfection are indicated at the top, as are the U5 gene-specific probes utilized for the mapping. No DNA, mappings performed with RNA isolated from nontransfected protoplasts. (B) Structure of the MU5/Spe genes used for construction of the chimeric genes. The MSP elements and USE and TATA sequences are boxed. The sequence upstream of the USE, forming part of the introduced *Spe*I site, used for MSP region switching, is indicated. Relative expression of each of the genes was calculated after subtraction of the background full-length bands protected by endogenous maize protoplast U5 RNA (20). *, expression of MU5.2/Spe was normalized to 1.00, and the expression of other genes was calculated relative to that of MU5.2/Spe. Expression values for the MU5/Spe and the MU5/ΔMSP gene series are indicated. The following values were calculated for the remaining genes: MU5.1/MU5.2-MSP, 1.26; MU5.1/MU5.3-MSP, 0.07; MU5.2/MU5.1p, 2.31; MU5.2/MU5.1-MSP, 2.45; MU5.2/MU5.3-MSP, 0.03; MU5.3/MU5.1p, 2.94; MU5.3/MU5.1-MSP, 2.52; MU5.3/MU5.2p, 1.19; and MU5.3/MU5.2-MSP, 1.10.

(Fig. 4B; introduction of the *Spe*I site has no effect on transcription of the MU5 genes in maize protoplasts [data not shown]). In the MSP switch chimeric genes, this *Spe*I site was used to exchange the MSP containing-regions among the three U5 genes. Finally, the promoter switch chimeric genes, MU5.2/MU5.1p, MU5.3/MU5.1p, and MU5.3/MU5.2p, containing the entire promoter region of the MU5.1 or MU5.2 gene precisely fused to the coding and 3′-flanking regions of the MU5.2 or MU5.3 gene (20), were used as controls. As shown in Fig. 4A, deletion of the MSP regions from the U5 genes resulted in a reduction in transcription to the level of the background endogenous gene expression (compare lanes 2 and 5, 7 and 11, and 13 and 18). In all chimeric genes, the transcription efficiency directly corresponded to the expression level of the gene from which the promoter or MSP region was derived. Interchanging the MSP regions or the entire promoters had a similar effect (Fig. 4A; see legend for the quantitation).

We have also analyzed expression of a series of MU5/MU2 chimeric genes, containing the three maize U5 MSP element-containing regions fused to the MU2 gene upstream from the MU2 USE sequence. Efficiency of expression of different MU2/MU5-MSP genes correlated well with the expression level of the MU5 gene from which the MSP region was derived (data not shown). Taken together, these results reveal that sequences upstream from the USE are necessary for snRNA gene transcription in maize protoplasts and that they are functionally interchangeable between different genes. They also show that efficiency of snRNA gene expression is modulated by the strength of the MSP element-containing upstream regions.

When the MU5 promoter switch constructs described in the legend to Fig. 4 were tested in *N. plumbaginifolia* protoplasts, expression levels also depended on the promoter driving transcription of the gene (data not shown) (20). However, the relative efficiencies of transcription were different from those seen in maize protoplasts, consistent with the results shown in Fig. 1. To assess the importance of the USE for expression in *N. plumbaginifolia* protoplasts, reciprocal USE mutants between MU5.1 and MU5.2 genes and between MU2 and AU2 genes, where the USE sequence of one gene is replaced with the USE of another gene (for sequences of respective USE elements, see Fig. 1B), were tested. It was found that expression levels are directly related to the USE sequence. For example, conversion of the AU2 USE to the MU2 USE resulted in a fivefold decrease in transcription of the AU2 gene; similarly, the $C_7 \rightarrow A$ change in the MU2 USE increased transcription of the MU2 gene fourfold (data not shown). Hence, in marked contrast to the situation in maize (for an example, see Fig. 2), efficient transcription of snRNA genes in *N. plumbaginifolia* protoplasts strictly depends on the quality of the USE (5, 38).

Transcription of the Pol III-specific U3 snRNA gene in maize protoplasts also requires GC-rich MSP elements. We also compared the expression of Pol III-transcribed dicot and monocot snRNA genes, represented by the tomato U3 (TU3) gene (18, 19) and the wheat U3 (WU3) gene (25), in transfected *N. plumbaginifolia* and maize protoplasts. Like the Pol II-specific genes, the monocot WU3 gene was expressed in dicot protoplasts, but expression of the TU3 gene in maize protoplasts was not detectable. Additional experiments, performed with chimeric TU3/WU3 genes, have shown that differences in expression between dicot and monocot protoplasts are due to differences in transcription initiation (data not shown). These findings suggested that transcription of Pol III-specific snRNA genes in monocot plants also requires promoter elements other than the USE and TATA.

Inspection of the sequence upstream from the USE in the WU3 gene revealed the presence of two MSP elements (Table 1). The following constructs (Fig. 5B) were prepared to determine whether MSP elements are important for transcription of the WU3 gene in maize protoplasts and, if so, whether MSP-containing regions found in Pol II- and Pol III-specific U snRNA genes are functionally interchangeable. (i) The MSP-containing region positioned upstream of the USE was removed from the WU3 gene, creating WU3/SpeΔMSP. (ii) The *Spe*I site introduced adjacent to the USE in WU3/SpeΔMSP was utilized to exchange the MSP-containing regions between the WU3 gene and different MU2 gene constructs. Therefore, WU3/MU2-MSP contains the MU2 MSP region fused upstream of the WU3 USE, and MU2/WU3-MSP is the inverse. WU3/synMSP and WU3/synMSP* are WU3 genes containing synthetic and mutated synthetic MSP regions fused upstream of the USE. As shown in Fig. 5A, deletion of the MSP region from WU3 greatly reduced the transcription of WU3 (lane 8) in a manner similar to that seen with deletion of the MSP region from MU2 (lane 2). Replacement of the WU3 MSP region with that of MU2 restored efficient transcription of

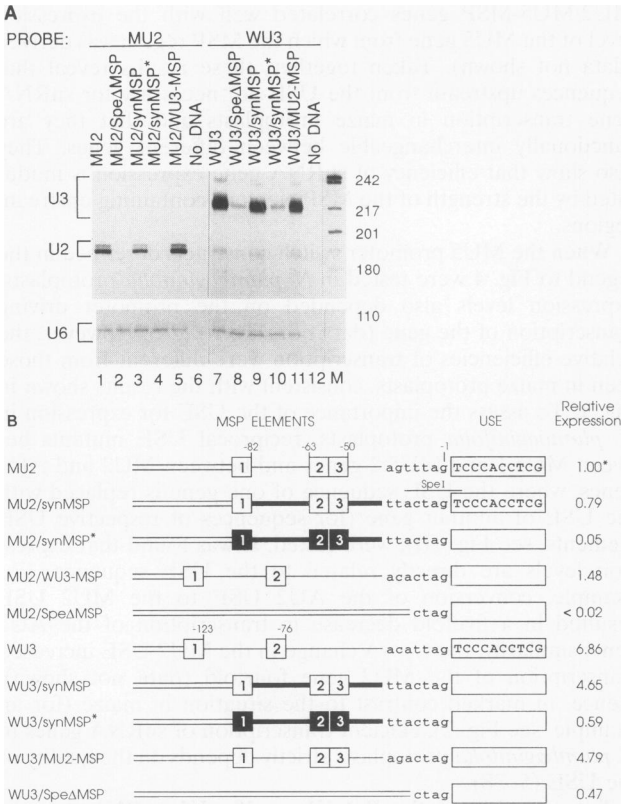

FIG. 5. Expression of WU3 and MU2 MSP region switch mutants in transfected maize protoplasts. (A) RNase protection analysis. Plasmids used for transfection and probes utilized for mapping are indicated at the top. (B) Diagram depicting MSP regions of the MU2 and WU3 genes and of the MSP deletion and switch mutants. The USE and MSP sequences are represented by boxes. The positions of the MSP boxes are given relative to that of the USE. The sequence directly upstream from the USE is indicated in lowercase letters for each of the genes. MSP boxes containing base changes are shown in black. The thick black line between the MSP boxes indicates that these sequences are different from those of the wild-type genes. Vector sequences are represented by the double line. The expression of each of the mutants relative to MU2 is indicated.

WU3 (lane 11). Similarly, replacement of the MU2 MSP with the WU3 MSP restored transcription of MU2 (lane 5). Most importantly, replacement of the WU3 MSP with synthetic MSP (lane 9), but not with its mutated variant (lane 10), restored active WU3 RNA synthesis. We conclude that efficient transcription of the Pol III-specific snRNA gene in maize protoplasts requires MSP elements and that these elements are functionally interchangeable between Pol II- and Pol III-specific snRNA genes.

Arabidopsis U6 snRNA genes which contain MSP-like elements are transcribed in maize protoplasts. It was surprising that in contrast to the tomato U3 gene, another dicot Pol III-specific gene, the *Arabidopsis* U6 (AU6), used as a transfection efficiency control, was expressed in maize protoplasts (e.g., see Fig. 1 to 4). The upstream region of this particular AU6 gene (U6.26) was examined and found to contain two copies of the MSP sequence (Table 1). To show more directly that MSP elements present fortuitously in dicot snRNA genes may contribute to their transcription in maize, activities of U6.26ΔMSP, which is a variant of U6.26 with the more upstream MSP element deleted, and of several other *Arabidopsis* U6 genes were analyzed. Among these additional U6 genes, only U6.1 contains one MSP element (Table 1). In maize protoplasts, the U6.26 gene was the most efficiently expressed U6 gene (Fig. 6, lane 1) and deletion of one of its MSP elements reduced expression by 90% (lane 2). The U6.1, containing one MSP element, was the only additional U6 gene expressed in maize protoplasts (lane 3); expression of other genes was undetectable (lanes 4 to 6). In *N. plumbaginifolia* protoplasts (lanes 7 to 12), all U6 genes were expressed at comparable levels, consistent with the previous findings (18, 40) that regions upstream of the USE do not significantly contribute to transcription initiation in dicot plants.

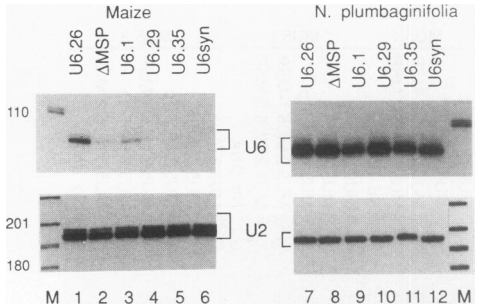

FIG. 6. Expression of *Arabidopsis* U6 genes in transfected maize (lanes 1 to 6) or *N. plumbaginifolia* (lanes 7 to 12) protoplasts. The plasmids used for transfection are indicated at the top. The MU2 or the AU2 gene was cotransfected with the U6 genes into maize and *N. plumbaginifolia* protoplasts, respectively. The U6.26 and the appropriate U2 probes were used for mapping.

DISCUSSION

We have shown previously that transcription of snRNA genes, both Pol II and Pol III specific, in protoplasts of the dicot plant *N. plumbaginifolia* requires two upstream promoter elements, the USE and TATA, with sequences upstream of the USE not significantly contributing to transcription efficiency (38, 40). We have also demonstrated that the spacing between the USE and TATA, which is approximately three and four helical DNA turns in Pol III- and Pol II-specific genes, respectively, determines RNA Pol specificity of the gene (18, 39) (see introduction). The USE and TATA elements are also present in all monocot Pol II- and Pol III-transcribed snRNA genes studied to date (references 2, 20, and 25 and our unpublished data) (see also Fig. 1 and 5), and as shown in this work for the Pol II-specific U2 gene, these two elements are indispensable for transcription in transfected protoplasts of maize, a monocot plant. Since positioning of the elements in monocot snRNA genes is identical to that of the dicot plant genes, it is likely that the difference in spacing of the USE and TATA observed between Pol II- and Pol III-transcribed genes is responsible for RNA Pol selection also in monocot plants. This is further supported by the finding that transcription of U3 snRNA genes in monocots, like that in dicots, but in contrast to that in other eukaryotes, is catalyzed by Pol III and not by Pol II (25) and that U3 genes from both dicots and monocots have the USE-to-TATA spacing diagnostic of Pol III transcription (18, 19, 25, 39).

However, in marked contrast to the situation in dicots, transcription of Pol II- and Pol III-specific snRNA genes in maize protoplasts requires, in addition to the USE and TATA, sequences positioned upstream of the USE. Deletion of upstream regions in maize U2 and U5 genes or the wheat U3

gene resulted in inhibition of transcription by more than 90%. The following evidence indicates that the positive effect of upstream regions on gene expression in maize is mediated by the GC-rich MSP elements having a consensus RGCCCR and located within −30 to −130 bp upstream of the USE. (i) One to three copies of the MSP element are present, in either orientation, in all monocot snRNA genes studied in this work (Table 1) and also in other recently characterized genes such as wheat U6 (25) and four additional U2 genes and one U3 gene of maize (our unpublished data). (ii) Mutations of individual MSP elements, or combinations thereof, in the maize U2 gene decrease its transcription by up to 85% (Fig. 3). (iii) Activity of the maize U2 or the wheat U3 gene having the MSP region deleted is rescued by addition of the synthetic 100-bp-long upstream region (synMSP) which contains substitutions of all the nucleotides other than the three MSP boxes of the maize U2 gene; the synMSP variant (synMSP*) having two point mutations in each of the three MSP boxes does not rescue transcription (Fig. 3 and 5). (iv) Among five different *Arabidopsis* U6 genes, only the two which fortuitously contain one or two MSP elements (genes U6.26 and U6.1) are expressed in maize protoplasts, and expression of the gene U6.26 is significantly decreased when the upstream MSP element is deleted (Fig. 6).

We have demonstrated that the MSP element(s)-containing upstream regions are exchangeable between different U5 snRNA genes of maize and between the U2 and U5 genes of maize. These experiments have also revealed that efficiency of snRNA gene expression in maize protoplasts is determined primarily, if not exclusively, by the strength of the MSP regions (Fig. 4 and data not shown). This contrasts with the situation in dicot plant protoplasts, in which activity of the promoter is a function of the quality of the USE element. In dicot plant genes, the USE sequence (consensus TCCCACATCG) is very highly conserved, and as expected, point mutations in the element usually decrease gene transcription by as much as 75 to 95% (5, 13, 38). On the other hand, USE elements in all monocot snRNA genes deviate in at least one and often in two or three positions from the TCCCACATCG sequence (Fig. 1B). Some of the base substitutions naturally occurring in monocot USE sequences had been tested for the effect on snRNA gene expression in *N. plumbaginifolia* protoplasts and were found to reduce transcription efficiency by up to 95% (38). As found in this work, these substitutions have little or no effect on transcription in maize protoplasts. (Inspection of the USE region in cloned monocot snRNA genes revealed additional conserved nucleotides [sequence TAR] immediately upstream of TCCCACATCG. However, mutation of the 5′-proximal TA to CC in the context of the maize U2 gene had only a marginal effect on transcription in maize protoplasts [our unpublished result]. Additional experiments are needed to better characterize the USE element of monocot genes.) Altogether, these results indicate that dicot and monocot plants use different strategies to regulate efficiency of snRNA gene transcription. While quality of the USE plays a key role in dicots, the newly described MSP element is responsible for the promoter strength in monocots.

The MSP regions are also interchangeable between Pol II- and Pol III-transcribed genes of monocots, and experiments with the synthetic upstream regions indicated that the same MSP elements participate in transcription of both classes of genes (Fig. 5). Therefore, it is apparent that Pol II- and Pol III-specific genes of monocot plants share as many as three different promoter elements: the TATA, USE, and MSP. It is not known at present whether the TATA, USE, and MSP are the only promoter elements important for transcription of snRNA genes in monocot plants. *Arabidopsis* U5 gene (AU5) is not efficiently expressed in maize protoplasts (Fig. 1), although it contains two MSP-like elements positioned upstream of the USE (36). Furthermore, we have found that fusion of the MU2 MSP region upstream of the *Arabidopsis* U2 (AU2) gene results in only weak activation of expression of this gene in maize protoplasts (our unpublished data). It is possible that in addition to the USE and TATA, other sequences in the −1 to −80 promoter region also contribute to transcription of snRNA genes in monocot plants.

The Pol II and Pol III snRNA gene promoters of monocots represent, in addition to those of mammals, frogs, dicot plants, and possibly nematodes (1, 13, 16, 35) (see the introduction), one more example of promoters which are remarkably similar despite being recognized by two different RNA Pols. The findings concerning the structure of snRNA gene promoters in monocot plants add further support to the strong conservation of Pol II and Pol III transcription machineries (1, 16, 17, 27). It is not clear at present why the Pol II- and Pol III-specific snRNA gene promoters in higher eukaryotes tend to evolve together. The fact that U snRNA gene promoters have been rather poorly conserved in evolution (1, 16, 21, 28, 32, 34, 35, 38–42) makes this phenomenon even more intriguing. It is possible that sharing transcriptional elements and factors between Pol II- and Pol III-specific snRNA genes helps to coordinate synthesis of different snRNAs. snRNAs encoded by this group of genes are all involved in RNA-processing reactions in the nucleus (12, 16, 23), and coordination of their expression may be beneficial for the cell.

We have noted previously that dicot plant snRNA promoters closely resemble eubacterial promoters which also contain two conservatively spaced upstream elements. This observation, and the findings about Pol specificity changes of plant snRNA genes in vitro and during evolution, led us to speculate that promoter diversification during eukaryotic evolution might have involved changes in the spacing of ancient promoter elements (13, 18, 39). How can the findings about the snRNA gene promoters of monocots, which are considered to be evolutionarily younger plants than dicots (8), be accomodated into the evolutionary schemes? It is possible that it was advantageous to separate the two functions that the USE element performs in dicot plants: contribution to RNA Pol specificity determination and control of the promoter strength. Taking over of the latter role by the MSP elements in monocot plants offers more straightforward ways to regulate efficiency of snRNA gene transcription. Such an event would also remove the pressure to maintain the high conservation of the USE element observed in dicot plants.

Interestingly, organization of the Pol III-specific snRNA gene promoters of monocots closely resembles that of vertebrate counterparts. Pol III-transcribed vertebrate genes contain the TATA and the PSE elements at positions which are equivalent to the positions of the TATA and USE elements in plants (reviewed in references 13 and 16). The upstream part of the PSE sequence which is more conserved among different vertebrates (16) is located at a distance from the TATA box almost identical to that of the USE in plant snRNA genes, suggesting that PSE and USE elements perform equivalent functions. In the −200 to −250 region, vertebrate genes contain the DSE, which is usually composed of several different enhancer elements including the octamer motif (16). Although the DSE and mRNA gene enhancers are structurally homologous, they are usually not functionally interchangeable between two classes of genes (4, 7). It will be interesting to establish whether MSP elements can function as mRNA gene enhancers and, vice versa, whether upstream promoter ele-

ments of mRNA genes can functionally replace the MSP signals in snRNA genes. By searching through databases, we have identified sequences corresponding to MSP elements which were present in the upstream regions of some mRNA coding genes of monocots (unpublished data); however, it is not known whether any of these MSP-like signals are important for transcription. Interestingly, the GC-rich sequence present in the intergenic region of maize streak virus, shown to be essential for viral gene transcription, contains three perfect copies of the MSP element (10, 11). It will be important to investigate whether the MSP and GC-rich elements of mRNA coding genes have similar properties.

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