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DNA methylation has been implicated in mediating several fundamental cellular processes in both prokaryotes and eukaryotes. In higher plants, 5-methylcytosine (m5C) can constitute as much as one-third of the cytosine residues of nuclear DNA and has been found to control transposable element activity (1), genomic imprinting (2), gene silencing (3, 4), inhibition of transcription (3), and inheritance of a variety of epigenetic phenomena (5). More recently, Ronemus et al. (6) showed that DNA methylation is an essential component in establishing or maintaining cellular processes resulting in phase transition and meristem determinacy in transgenic Arabidopsis.

Analysis of the Methylation Pattern of the Maize Opaque-2 (O2) Promoter and in Vitro Binding Studies Indicate That the O2 B-Zip Protein and Other Endosperm Factors Can Bind to Methylated Target Sequences*

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DNA methylation has been implicated in mediating several fundamental cellular processes in both prokaryotes and eukaryotes. In higher plants, 5-methylcytosine (m5C) can constitute as much as one-third of the cytosine residues of nuclear DNA and has been found to control transposable element activity (1), genomic imprinting (2), gene silencing (3, 4), inhibition of transcription (3), and inheritance of a variety of epigenetic phenomena (5). More recently, Ronemus et al. (6) showed that DNA methylation is an essential component in establishing or maintaining cellular processes resulting in phase transition and meristem determinacy in transgenic Arabidopsis plants expressing an antisense cytosine methyltransferase gene. In animals, DNA methylation appears to influence the tissue-specific expression of developmentally regulated genes by affecting the interactions of DNA with chromatin proteins and transcription factors (4). In plants, methylation of the C residue embedded within the consensus sequence for mammalian CREB factors (cAMP response element, TGACGTCA) was found to inhibit in vitro DNA binding activity of CREB-like factors present in nuclear extracts of pea, wheat, soybean, and cauliflower (7). However, binding of plant nuclear proteins to hemimethylated target sequences has been reported for the tobacco CG-1 nuclear factor(s) (8) and the maize ac-encoded transposase (9). Interestingly, binding of the ac transposase occurs only to hemimethylated sequences with a methylated lower strand and not vice versa.

A method based upon chemical treatment of genomic DNA (10) has allowed detailed analysis of the methylation state of several genes in mammals, fungi, and plants and has led to the discovery of m5C outside the canonical symmetrical CpG and CpNpG sites (11–13). In transgenic plants, the application of this method clearly revealed the association between a high level of m5C observed within a silenced CaMV35S promoter and the transcriptional inactivation of the transgene (13, 14). In maize, analysis of the methylation pattern of ac transposable element ends suggested a model to explain the association of element transposition with replication (15). However, at present little is known about the methylation state of endogenous plant promoters, thus correlations between their methylation pattern and gene activity cannot be clearly asserted.

Although a positive correlation between the expression level of the maize Scutellar node (sn) gene and the methylation content of its promoter has been observed (16), no information is available about whether the methylation pattern of a plant promoter may mediate its tissue-specific expression. Similarly, the exact degree of C-methylation of an endogenous plant promoter has never been estimated in a statistically significant manner. To address these questions we have detailed the methylation pattern of the maize opaque-2 (o2) locus in an expressing and non-expressing tissue, endosperm and leaf, respectively.

In maize, o2 regulates the expression of b-32, cyppdk1, and the 22- and 14-kDa zein gene families (17). The o2 locus has been cloned and found to be expressed only in developing endosperm (18, 19). o2 encodes for a member of the basic domain/leucine zipper (b-Zip) class of transcriptional activators (20, 21). Similarly to other plant b-Zip factors (22), the O2 protein target sequence, TGACGTCA, contains an ACGT core. Multiple copies of this sequence are present in the promoter region of the genes that are under o2 transcriptional control. Interestingly, strong in vitro binding of the O2 protein to the odd base palin-
O2 Binding to Methylated Target Sequences

The maize line used throughout this study is the same line used for cloning and sequencing the o2 gene (20). From a single silk-sealed plant, young leaves and immature endosperm (30 days after pollination) were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C.

Bisulfite Treatment, PCR, Sequencing, and Statistical Analysis—Maize nuclear genomic DNA was purified from 10 g of leaves or endosperm using a previously described method (26). DNA was cleaved with EcoRI and then subjected to bisulfite treatment as described by Frommer et al. (10) with the following modifications: 2 μg of DNA were denatured with 50 mM NaOH, neutralized by addition of 1 M Tris pH 7.5, and desalted on a Sephadex G-50 spin column. Eluted DNA was immediately denatured at 98 °C for 10 min and processed as described by Meyer et al. (13). At the end of the bisulfite treatment, DNA was recovered using a QIAEX II extraction kit (QIAGEN). In the control reaction, 20 μg of linearized plasmid (pLP20) containing the −1020−58 region of the o2 promoter was methylated in vitro with Sscl CpG-methylease (New England Biolabs) and mixed with 2 μg of salmon sperm DNA. In 50 μl total volume, the first polymerase chain reaction (PCR) amplifications contained 100 ng of bisulfite-treated DNA, 25 pmol of each primer, 200 μM of each dNTP, 1.5 U of Taq polymerase (Promega). PCR products were digested with KpnI and XhoI, gel-purified, and cloned into pBluecript KS+ (Stratagene) plasmid DNA, and sequenced using an ABI373A DNA sequencer (Applied Biosystems). Data were analyzed by standard analysis of variance and Student’s t significant difference was used to compare means. All statistic analyses were performed using the “Statistica Software Package” (Stat Soft Inc., 1996).

Preparation of Substrate DNA Fragments for EMSA—A 188-bp long o2 promoter fragment spanning from −399 to −211 relative to the transcription initiation site of the o2 locus was cloned in the EcoRV site of pBlueScript KS+ to make pLP190. The P188 DNA fragment was excised from pLP190 with HindIII and XhoI, gel-purified, recovered by electroelution, and end-labeled. The P188-G probe was prepared by methylation of Sscl CpG DNA (New England Biolabs) following the manufacturer’s recommendation. Completeness of CpG-methylation was assessed by restriction digestion analysis of the P188-G probe with the methylation-sensitive restriction enzyme HhaI.

The P188–50% probe was prepared by PCR amplification of the P188 DNA fragment cloned in pLP190 using RS and SK primers. PCR reaction mix consisted of 10 ng of pLP190, 1 × Promega buffer, 2.5 mM MgCl2, 200 μM dATP, 200 μM dGTP, 200 μM dTTP, 100 μM dCTP, 2.5 μM each oligonucleotide primer, and 0.5 units of Taq DNA polymerase (Promega). PCR products were digested with XbaI, gel-purified, and spectrophotometrically quantified. Thirty ng of DNA was labeled at one end by end-filling using Klenow DNA polymerase and [α-32P]dCTP; probes were purified from unincorporated nucleotides with Sephadex-G50 spin columns. The P188–100%, P188–30%, and P188–70% probes were produced in the same way. The molar ratios of d32P-dCTP-dCTP were 1:0, 3:7, and 7:3 respectively, in the PCR reaction.

Each hemimethylated fragment was prepared by combining methylated and unmethylated single-stranded complementary DNA. Annealing was achieved by heating the DNA mix at 95 °C for 10 min and allowing by slow cooling to room temperature. Annealed DNA was gel-purified and labeled as indicated above. Single-stranded DNA was prepared as follows. For the upper strand, SsrI-digested pLP190 was subjected to two rounds of 50 endonuclease steps using the KS primer, 1 × Promega buffer, 200 μM of each dNTP, and 1 unit of Taq polymerase. For the lower strand, KpnI-digested pLP190 was used in combination with the
SK primer. A methylated strand was obtained by including m5-dCTP in the reaction mix.

p36wt and p36mt probes were made by annealing 1 μg of primers (p36wt: 5′-atatactaaaagtgttaagttagaagca-3′; 5′-gacagctaacattacaacatg-3′; p36mt: 5′-atatactaaaagtgttaagttagaagca-3′; 5′-gacagctaacattacaacatg-3′), and end-filling of 3 μg of oligonucleotide duplexes with Klenow enzyme in the presence of [32P]dCTP. The radiolabeled probes were purified from unincorporated nucleotides with Sephadex G50 spin columns.

p61wt primers (5′-caggttaatctataacatggaatggctagacaccatgccttaccaacatg-3′; 5′-gacagctaacattacaacatg-3′), and p61mt primers (5′-caggttaatctataacatggaatggctagacaccatgccttaccaacatg-3′; 5′-gacagctaacattacaacatg-3′) were labeled at their 5′ ends with T4 kinase. P61wt and P61mt probes were made by annealing equimolar amounts (1 pmol) of labeled primers and then extended with Klenow enzyme in the presence of cold dNTPs. Radiolabeled probes were purified with Sephadex G50 spin columns.

All oligonucleotides used throughout this study were designed from the genomic sequence of the o2 gene (20) and synthesized by Pharmacia Biotech Inc. Their integrity was verified by gel electrophoresis on acrylamide gels.

Electrophoretic Mobility Shift Assays—Binding reactions were performed in a final volume of 20 μl of binding buffer containing 100 ng/μl poly(dI-C). The amount of E. coli pET3cO2 or endosperm cell extract DNA was varied from 2.5 to 4 μg, whereas 3.2 × 10^6 cpm of end-labeled probe were routinely used. The binding reaction was incubated at 20 °C for 10 min, and 10 μl was then loaded onto a 4–7% Tris borate/EDTA acrylamide gel (29:1). Gels were run at 8 °C at 15 V/cm, dried, and autoradiographed.

RESULTS

Analysis of the Methylation Pattern of the Maize Opaque-2 Promoter in Leaves and Endosperm—Previous analysis of the methylation state of the o2 promoter region in immature endosperm and young leaf tissue with methylation-sensitive restriction enzymes indicated the occurrence of different methylation patterns within the −510, −900 region. Also, in both tissues we observed the presence of m5C residues close to the 5′ binding site.2

To gain more insight into the methylation status of the o2 promoter in these tissues, we undertook a strategy aimed to detail the m5C distribution by means of the bisulfite treatment of maize genomic DNA (10). This method is based upon a chemical treatment that converts unmethylated C residues into U residues that appear as T residues after PCR of specific, unmodified. Separate PCR amplifications were performed for the upper and lower strands to produce DNA fragments that were subsequently cloned and sequenced. CpG-methylated plasmid DNA bearing the o2 promoter was used as a control for complete conversion of unmethylated C residues into U residues (data not shown).

Fig. 1A shows the methylation pattern of a 390-bp region of the o2 promoter (from −436 to −46) in endosperm. We cloned and sequenced 13 upper strand and 16 lower strand PCR products. All clones displayed different methylation patterns and thus were derived from individual genomic O2 sequences. Noteworthy is the considerable level of C-methylation (84%) present in the o2 promoter at the time and place where it leads to gene expression. Moreover, all the C residues embedded within the 5′ binding site (boxed in Fig. 1A), were methylated. The methylation pattern of the lower strand differed markedly from that of the upper strand. Statistical analysis (Student's t test) of the mean methylation values obtained from the collected data indicated that, with p = 1%, the lower strand was significantly less methylated than the upper strand (80% m5C versus 89%). Interestingly, an unequal m5C distribution was observed, and the lower strand was less methylated at the most distal part of the region analyzed. We have also applied the Student's t analysis to compare the methylation level of each C residue within their sequence context. Accordingly, we found that in endosperm, for both strands, CpT and CpG dinucleotides were significantly less methylated than CpA and CpC (p = 1% and 5%, respectively).

The methylation pattern of the −436, −46 o2 promoter region was also analyzed in leaf by sequencing 10 upper strand and 10 lower strand clones (Fig. 1B). Again, different methylation patterns were observed, indicating that the clones were derived from individual genomic O2 sequences. Analysis (Student's t test) was again used to compare the mean methylation values of DNA obtained from leaf and endosperm. Data indicated that with p = 1% the level of C-methylation in leaf was significantly higher than in endosperm (96% m5C versus 84%). An unequal m5C distribution was also observed in the lower strand of the most distal part (−436, −370) of DNA from leaves. Taken together these findings indicate that the endosperm-specific expression of the o2 gene is not associated with the specific and complete demethylation of any C residue present within the promoter region analyzed.

Recombinant O2 Protein Binds in Vitro to a CpG-methylated o2 Promoter—The expression of the o2 gene appears to be positively autoregulated by its gene product, possibly by binding to the target sequence, TGACGGTTG, which is found in its own promoter and in the promoter of several o2-controlled genes (24, 17). In immature endosperm, genomic sequencing revealed the occurrence of m5C within the CpG site embedded in the AGCT core. Thus, unlike CG-1 and the CREB-like M1-1 transcription factor (8, 7), the O2 protein is likely to bind in vitro to its cognate sequence even if, in both strands, the CpG dinucleotide is methylated. To further confirm this possibility, we performed in vitro gel-shift assays using extracts from E. coli cells expressing a full-length o2 cDNA (pET3cO2; Ref. 27) and in vitro CpG-methylated o2 promoter fragment (P188-CG; spanning from −399 to −211). For the gel-shift analysis a negative control consisted of E. coli cell extracts from cells transformed with pET3c (not expressing O2). Consistent with earlier data (27), the control extracts never resulted in a band shift when tested with the P188-CG probe (not shown). A total of seven methylated CpG sequences are present in the P188-CG probe, one within the 5′ target sequence (see Fig. 1). Under the conditions used, the P188-CG probe resulted in a single retarded band (Fig. 2A, lane 2), and at higher extract concentrations all the probe was shifted (data not shown).

Consistently, a 100:1 molar excess of unlabeled P188-CG was needed to compete with the unmethylated P188 probe and abolish the O2-P188 retarded complex (Fig. 2B, lane 1). Addition of distamycin A, a transcriptional inhibitor that binds to the major groove of DNA (28), did not impair O2 binding to the P188-CG probe; this indicated that the interaction of O2 protein occurs to the major groove of the DNA (Fig. 2B, lanes 3–5). Formation of the O2-P188-CG complex indicated that neither the methylation pattern generated by the SsSI DNA methylase nor the methylation at the C residue located within the O2 binding site were able to impair the in vitro binding activity of the O2 protein to its cognate DNA.

The Level of C-methylation in the o2 Promoter Affects O2 Binding Activity—Because the pattern and level of C-methylation generated by SsSI DNA methylase on the P188 promoter fragment were different from that found in endosperm, we investigated the highest methylation content resulting in a detectable O2 in vitro binding activity. EMSA experiments were performed using o2 promoter fragments generated with different m5C contents. Briefly, partially methylated DNA was prepared by PCR amplification of the P188 promoter fragment by addition of appropriate molar ratios of m5-dCTP and dCTP.
in the reaction mix. Probes with an average \(^\text{mC}\) content of 30, 50, 70, and 100% were generated (named P188–30%, P188–50%, P188–70%, and P188–100%) and used in gel shift and competition experiments with the O2 protein. Assuming an equal rate of nucleotide incorporation, each PCR reaction generates DNA fragments with a C-methylation content that can be plotted as a Gaussian distribution. Accordingly, restriction digestion analyses of the P188–50% probe with a methylation-sensitive enzyme resulted in the digestion of approximately 50% of a labeled PCR product (data not shown). Thus, it is conceivable to assume that, for each partially methylated probe, the most abundant fraction of labeled DNA fragments has an average C-methylation content of 30, 50, and 70%, respectively. However, among these DNA fragments the methylation pattern is stochastically represented.

First, we tested O2 binding activity using a fully methylated DNA. Under the conditions tested, no band shift was observed (Fig. 2A, lane 3). Even after the addition of twice the amount of pET3cO2 extract and longer autoradiogram exposures, no band shift was detected (data not shown). As an additional test, we performed competition experiments between a fully methylated probe and a labeled, unmethylated one. A 100-fold molar excess of unlabeled P188–100% did not eliminate formation of the O2 protein-P188 complex (Fig. 2A, lane 4), indicating that, in vitro, complete C-methylation inhibits O2 binding to its cognate DNA. Similar results were obtained using the P188–70% probe (data not shown).

It is noteworthy that we have never observed retardation of the O2 zP188–50% complex as a sharp band but rather as a limited smear (Fig. 3, lane 5). Similar results have also been observed using a P188 probe hemimethylated on either the lower strand (Fig. 5, lane 1) or the upper strand (not shown).

FIG. 1. Methylation map of the -436, -46 o2 promoter region in endosperm and leaf. A, methylation map of the o2 promoter region in endosperm. Data from 13 and 16 clones have been compiled for the upper and lower strand, respectively. B, methylation map of the o2 promoter region in leaf. Data from 12 and 10 clones have been compiled for the upper and lower strand, respectively. Methylated C residues are indicated by black squares, whereas unmethylated C residues are indicated by white squares. CpG dinucleotides are underlined. The O2 consensus sequence is boxed. Short and long arrows indicate the p36 and p61 probe, respectively.
molar excess unlabeled P188–100% competitor O2; 50% probe 100-fold molar excess unlabeled P188–50% competitor; P188–30% probe; lane 4, P36wt probe + 5 μg of F-300 endosperm extracts; lane 5, P36mt probe + 5 μg of F-300 endosperm extracts; lane 6, P36wt probe + 5 μg of F-300 endosperm extracts + 100-fold molar excess unlabeled P36wt; lane 7, P36mt probe + 5 μg of F-300 endosperm extracts + 4 μg of pET3cO2 extract.

O2 Binding to Methylated Target Sequences

We also used the P36wt and P36mt probes (see below) to investigate whether the F-300 could bind to the O2 binding site. Interestingly, we found that both unmethylated probes could efficiently be retarded by F-300 (Fig. 4, lanes 4 and 5). Thus, either the F-300 binding site is adjacent to the O2-box or these proteins share the same target sequence. In the latter case, F-300 binding activity can tolerate the nucleotide substitution which is present in the ACGT core of the O2-box, whereas O2 does not (see below). We then questioned the ability of O2 and F-300 to dimerize. Surprisingly, addition of O2 to the F-300 extracts eliminated the band shift (Fig. 4, lane 7). In rice, occurrence of plant DNA-binding inhibitors has been recently reported. These are b-Zip proteins involved in sequestering a particular group of G-box binding factors, possibly by selective formation of inactive heterodimers (32). Although no direct evidence indicates that F-300 contains a b-Zip protein(s), our results suggest that F-300 might contain a member of this new class of DNA-binding inhibitors, perhaps involved in O2 transcriptional regulation. Finally, a second faint faster-migrating specific retarded complex was also observed using the protein fraction eluted at 150 mM KCl (Fig. 5; data not shown).

O2 Binds In Vitro with Different Affinity to Partially Methylated and Hemimethylated Target DNA—The number of C residues present within the region of the O2 target site is approximately equivalent in each DNA strand. However, in the lower strand, these residues appear much more clustered in the distal part of the O2-box (see Fig. 1). To test the possibility that not all bases in the O2 DNA-binding site are equally important in determining binding affinity, and/or that the distribution of the C residues may also affect O2 binding activity, EMSA experiments were performed with hemimethylated target DNA. Briefly, each hemimethylated probe was prepared by annealing a fully methylated single-stranded DNA to an equimolar amount of the complementary, unmethylated strand (see “Experimental Procedures”), resulting in probes with either the upper (U) or the lower (L) strand fully methylated (M), P188-UM and P188-LM, respectively.

Results shown in Fig. 5 indicated that the binding affinity of the O2 protein for the P188–30% DNA is stronger than for the P188-UM and P188-LM. Retardation of the P188 probe was abolished by a 15-fold molar excess of unlabeled P188–30% competitor (Fig. 5, lane 2) but not from an equal molar excess of unlabeled P188-LM or P188-UM (lanes 3 and 5). Band shifts were instead abolished by 100-fold molar excess of these hemimethylated DNA fragments (lanes 6 and 7). However, a band in lane 7 became visible after longer autoradiogram exposure (lane 8, see upper arrow), therefore indicating that, consistent with the different mC content displayed by the upper and lower strand, O2 binding affinity with hemimethylated target DNA varies depending upon the strand that is methylated.
**Efficient in Vitro Binding of the O2 Protein to an o2 Promoter Fragment Requires the C Residue Embedded within Its Target Site and Extended DNA Flanking Sequences**—An intact ACGT core sequence is required for binding of plant b-Zip factors to their cognate DNA (22). However, in this respect, the O2 protein was shown to tolerate some changes in its recognition sequence. Replacement of ACGT by ACAT in a 22-kDa zein promoter fragment greatly reduced O2 in vitro binding activity (31) and abolished its in vivo transactivation (33). Strong O2 in vitro binding to an odd base C-box, ATGAGTCAT, was reported by de Pater et al. (31) and abolished its efficiency in vitro (32) and abolished its transactivation in vivo (33). Surprisingly, under the conditions tested, neither the P36wt nor the P36mt probe was shifted by the O2 protein (Fig. 6A, lanes 3 and 4), even upon doubling the amount of O2 protein (Fig. 6A, lanes 5 and 6). These observations are at odds with previous findings reporting a shift by the pET3cO2 cell extracts to a 24-bp oligonucleotide duplex containing either a C-box or a G-box core sequence (27). However, it should be pointed out that the sequence of the P36wt has an imperfect G-box, TGACGTTG, and different flanking sequences. Therefore, we concluded that the O2 protein may need either a larger DNA fragment or additional nucleotide sequences, not included in the P36 probe or too close to a DNA end, to efficiently interact with an o2 promoter fragment. To test this possibility, a wild-type and a mutagenized 61-bp long probe were synthesized (P61wt and P61mt, spanning from −325 to −264; see long arrow in Fig. 1) and used in gel shift experiments. Results shown in Fig. 6B indicate that O2 can specifically retard the P61wt probe (lanes 1 and 3). Consistently, 100-fold molar excess of P61wt competed binding of O2 to the P188 probe (not shown). In sharp contrast, O2 did not shift the mutated P61mt probe (Fig. 6B, lane 2) even at higher protein concentration or longer autoradiogram exposure (not shown). Consistently, 100-fold molar excess of unlabeled P36wt did not abolish O2-DNA complex formation with the P61wt probe (Fig. 6B, lane 4). Taken together these findings indicate that, in vitro, efficient sequence-specific binding of the O2 protein depends upon the C residue within the O2 recognition sequence and its flanking sequences.

**DISCUSSION**

We have investigated the methylation pattern of a 390-bp promoter region of the o2 locus in endosperm and leaf cells by genomic sequencing. Our results showed that the pattern and degree of methylation are different in the two tissues. The m5C content in leaf was estimated to be 96%, whereas in the expressing tissue, endosperm, it was 84%. Although an inverse correlation between DNA methylation and gene expression has been reported frequently in animals and plants (5), in endosperm a high level of m5C found in the o2 promoter is in agreement with the high m5C content displayed by a weakly expressed Sn promoter (16). Therefore, the o2 gene was also shown to be weakly expressed in immature endosperm (20), the level of C-methylation of the promoter region of these two genes directly correlates to their expression level.

In transgenic tobacco (13) and petunia (14), genomic sequencing was used to compare the methylation pattern of a transcriptionally active EaMV35S promoter and of a silenced one. Results indicated a correlation between a high level of m5C found in the silenced promoter and its transcriptional inactivation. In animals, there is evidence indicating that DNA methylation affects the binding activity of nuclear factors, although binding of histone H1 to DNA is indifferent to methylation at CpG sequences (35). Similarly, DNA binding of TGA1, a tobacco transcription factor that binds to the A-domain of the CaMV35S promoter, has been shown to be impaired by methylation interference assays (23). This result provides support to the possibility that a methylated CaMV35S promoter is silenced because the transcription factors regulating its expression cannot bind to it. Similarly, CREB-like factors present in nuclear extracts of several plant species have also been shown to be sensitive to m5C residues present within their binding site (7). Evidence that plants’ nuclear proteins may bind to methylated or hemimethylated sequences was also reported for the tobacco CG1 nuclear factor(s) (8) and the maize ac transposase (9). Interestingly, transposase binding to a methylated target sequence was found to be strand-dependent (9). Finally, the maize en/spm-encoded TnpA protein activates an inactive,
methylated spm element in transgenic tobacco (36), thus suggesting its direct interaction to methylated target sequences. In our study we showed that O2 can efficiently retard a CpG-methylated target DNA, in vitro. To our knowledge, this is the first report showing that C-methylation on both strands of the target sequence does not impair in vitro DNA binding of a plant transcriptional activator. However, because the methylation pattern disclosed by genomic sequencing indicates the occurrence of m^5C within an extended o2 promoter region, it is conceivable that the transcription factors that bind within the region analyzed might also bind, in vivo, to methylated DNA sequences. This finding poses the question of how general is this type of interaction. Accordingly, this feature might occur among a small subset of related b-Zip proteins, perhaps involving an unusual structural aspect of the DNA-protein interaction domain. Alternatively, binding affinity with methylated DNA may be shared with other classes of transcriptional activators. Both possibilities are supported by the observation of two specific DNA binding activities, F-150 and F-300, to a 50% methylated o2 promoter fragment in immature endosperm cell extracts. Additional evidence can also be inferred by the high m^5C content disclosed by genomic sequencing of the promoter region of an expressing sn gene in maize (16).

Because maize endosperm is a triploid tissue and all clones sequenced displayed a similar m^5C distribution, all o2 alleles are likely to possess the same methylation pattern. Therefore, because genetic studies indicated that o2 alleles of both maternal and paternal origins can be expressed in the endosperm cells (17), C-methylation unlikely down-regulates o2 gene expression by the specific inactivation of one or more allele of this locus. The unequal distribution of m^5C found, in both endosperm and leaf tissue, in the lower strand of the most distal part of the promoter sequence herein analyzed suggests that the methylation pattern of the o2 promoter might be different in more upstream regions. If so, it suggests the existence of sequence determinants leading to the establishment of the observed methylation patterns. In animals, cis-acting sequences are specifically recognized by nuclear factors that lead to either de novo DNA methylation or demethylation (5). In this respect, it has been shown that in mice the widely present Sp1 element protects from de novo methylation CpG island sequences situated up to 100 bp apart (37). In plants, the existence of similar determinants has been suggested (38). Accordingly, the o2 promoter may be used as a model to investigate the molecular mechanisms that plants use to establish similar methylation patterns.

Protein phosphorylation may affect either positively or negatively the DNA binding properties of transcription factors (39). In Arabidopsis, phosphorylation of the b-Zip protein GBF1 was observed to stimulate its in vitro binding activity to a G-box target site (40). Interestingly, the O2 protein purified from maize nuclei and the recombinant O2 protein expressed in pET3cO2 E. coli cell extracts were both found to be phosphorylated at various sites, although with a different phosphorylation pattern (41). Thus, in our EMSA experiments, a contribution to the affinity of the recombinant O2 protein to a partially methylated and hemimethylated protein fragment may be derived from its phosphorylated state. We have observed that the P188–50% probe can still be retarded, yet the O2-DNA complex stability was significantly reduced, thus implying a diminished affinity of the protein to a heavily methylated DNA. However, because in endosperm the m^5C content of the o2 promoter is higher than in the P188–50% probe, it might be possible that, in vivo, the O2 phosphorylation pattern increases its binding affinity to form a sufficiently stable complex. Accordingly, the higher stability of the P188–50%–F-300 complex may be conferred by the native phosphorylation state of the endosperm factor(s) that specifically bind to this partially methylated target DNA.

Several studies indicate that the levels of regulatory proteins present in the plant nuclei must be exactly titrated to avoid pleiotropic effects derived from the aspecific expression of unrelated genes. Similar to other classes of b-Zip transcriptional activators (42), the expression of a CaMV35S-O2 construct in tobacco resulted in abnormal plant development. A suggestive hypothesis is that DNA methylation, in addition to the sequence and spacing of the target DNAs, contributes to avoid spurious activation of plant promoters by nonspecific transcription factors. For example, O2 has been shown to bind to sequences with a CATG instead of an ACCT core (24, 33) or an odd base palindromic C-box sequence (23). This possibility is consistent with the loss of meristem determinacy observed in hypomethylated transgenic Arabidopsis plants (6).

The o2 locus is involved in the transcriptional control of different structural genes that are coordinately activated during endosperm development (17). However, the half-life of the O2 transcript is unknown, and no data are available about the actual expression level of this gene. In addition, no information is available about O2 protein turnover. Interestingly, the presence of upstream open reading frames in the O2 mRNA has been shown to mediate a mechanism of post-transcriptional control that reduces the amount of O2 protein translated (43). In this respect, because the O2 mRNA is poorly accumulated (20), another possible strategy to reduce the amount of O2 effectively translated may be to decrease its expression level, possibly by lowering the DNA binding affinity of the regulatory proteins involved in the transcriptional regulation of the o2 gene. Because different binding affinities of the O2 protein to a CpG-methylated, hemimethylated, partially methylated, and fully methylated target DNA have been observed, it might be hypothesized that DNA methylation modulates, in vivo, the response of the promoter to the cognate transcription factors. Different levels of C-methylation of the Sn promoter were found to be positively correlated to different levels of expression of this gene (16). The observation that the tobacco CG-1 nuclear factor(s) binds in vitro with different affinities to an unmethylated and hemimethylated CAGCGTG motif (8) reinforces this hypothesis. It is noteworthy that both naturally occurring hypermethylated O2 and Sn promoters regulate the expression of two classes of transcriptional activators, a b-Zip and a Myc-related helix-loop-helix protein, respectively (24, 44). In contrast, by methylation-sensitive restriction analysis, three maize structural genes, adh1, a1, and sh1, were not found to be methylated at their CpG sites (45), suggesting the occurrence of a m^5C-mediated transcriptional control active only for regulatory genes. In this respect, if the methylation content of O2-controlled promoters, such as b-32, zeins, cypd, was lower than that of the o2 promoter, it might be hypothesized that these unmethylated promoters may act as a “sink” toward a transcription factor that is present in limiting amount. We are currently investigating this possibility by means of a homologous in vitro transcription initiation system.

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3 M. Maddaloni and M. Motto, unpublished results.
Analysis of the Methylation Pattern of the Maize Opaque-2 (O2) Promoter and in Vitro Binding Studies Indicate That the O2 B-Zip Protein and Other Endosperm Factors Can Bind to Methylated Target Sequences
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