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Two E2F Sites in the *Arabidopsis MCM3* Promoter Have Different Roles in Cell Cycle Activation and Meristematic Expression*

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The commitment to DNA replication is a key step in cell division control. The *Arabidopsis MCM3* homologue forms part of the mini chromosome maintenance (MCM) complex involved in the initiation of DNA replication at the transition G_1/S. Consistent with its role at the G_1/S transition we show that the *AtMCM3* gene is transcriptionally regulated at S phase. The 5′ region of this gene contains several E2F consensus binding sites, two of which match the human consensus closely and whose roles have been studied here. The identity of the two sequences as E2F binding sites has been confirmed by electrophoretic mobility shift assay analyses. Furthermore the promoter is activated by AtE2F-a and AtDP-a factors in transient expression studies. One of the E2F binding sites is shown to be responsible for the G_2-specific repression of the promoter in synchronized cell suspension cultures. In contrast, the second E2F binding site has a role in meristem-specific expression *in planta* as deletion of this site eliminates the expression of a reporter gene in root and apical meristems. Thus two highly similar E2F binding sites in the promoter of the *MCM3* gene are responsible for different cell cycle regulation or developmental expression patterns depending on the cellular environment.

Cell divisions in plant meristematic regions are necessary for plant differentiation and growth. The control of cell proliferation in these areas is regulated by both environmental signals and plant growth regulators and is key for plant development making cell cycle control and plant development inextricably linked. Cell cycle progression is primarily regulated at the G_1/S transition, prior to DNA replication in S phase, and requires the E2F-initiated activation of a number of genes (reviewed in Ref. 1) notably those involved in cell cycle control, initiation of DNA replication, and DNA synthesis. A group of genes that have an important role in the initiation of replication and are suggested to be under the transcriptional control of E2F factors are the mini chromosome maintenance (MCM)[1] proteins (2). The successful passage through the G_1/S transition into S phase requires that replication is initiated following the formation and activation of prereplicative complexes. The formation of these complexes is initiated by the binding of cdc6 to origin recognition complexes during G_1 allowing the recruitment of the MCM complex of proteins. The MCM complex consists of six related proteins (MCM2–7) that have been shown in yeast to have an important role in replication (3, 4), conditional mutants being defective in initiation at the non-permissive temperature. The recruitment of the MCMs completes the formation of the complex of prereplication, and S phase is triggered by the activation of this complex by cyclin-dependent kinases leading to the switch from the complex of prereplication to a complex of postreplication.

Plants have been shown to have E2F homologues (5–8); six have been recently identified in *Arabidopsis* (9). The AtE2Fs of the first group (E2F-a to c) are functional transcription factors that can specifically recognize an E2F consensus sequence in association with AtDP proteins and can transactivate an E2F-responsive reporter gene in plant cells. The second group of AtE2Fs (E2F-d to f) retain the DNA binding domain but lack any other conserved region, do not require AtDP for DNA binding, and do not act as functional activators of transcription (9, 10). The two distinct groups of E2Fs may therefore have complementary roles in the activation of proliferation, and the second group in particular could be involved in the switch from active division to differentiation. The conservation of the E2F pathway in plants and animals, which does not exists in unicellular organisms such as yeast, may indicate that, as well as its role in cell cycle regulation, it also has a role in the development of multicellular organisms (reviewed in Ref. 11). However, although the factors that associate with E2Fs to regulate their cell cycle activity are known, in plants especially, the mechanisms involved in the switch to differentiation are less well studied.

Arabidopsis E2F factors of the first group have been shown to activate reporter genes in transient expression assays via a consensus E2F binding site repeated six times (7, 8). The ability of the E2F-a and AtDP-a factors to induce S phase in differentiated, non-dividing leaf cells has also recently been shown *in planta* (8). E2F factors thus play an essential role in the activation of S phase and the progression through the cell cycle and therefore may have a role that is linked with the growth and development of a plant via their activity on specific cell cycle gene promoters.

The involvement of E2F binding sites in the promoters of genes involved in the transition from G_1 to S phase has been studied in several cases in plants. The cdc6 promoter contains an E2F consensus site to which E2F factors have been shown to bind (12). The promoter of the ribonucleotide reductase (RNR2) promoter involved in deoxyribonucleotide biosynthesis for DNA replication contains two E2F consensus sites involved in up-

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[1] The abbreviations used are: MCM, mini chromosome maintenance; PCNA, proliferating cell nuclear antigen; GUS, glucuronidase; EMSA, electrophoretic mobility shift assay; DP, Differentiation-regulated transcription factor protein.

regulation of the promoter at the G_1/S transition with one of the elements behaving like a repressor outside S phase (13). The expression of the rice PCNA gene is restricted to meristematic regions, and two promoter elements have been found to be essential for this activity (14); furthermore E2F consensus sequences in the rice and tobacco PCNA promoters are involved in the activation of a reporter gene in both cultured cells and in whole plants (15).

In this report we have analyzed the activation of the promoter of the *Arabidopsis MCM3* gene by E2F transcription factors. The wild type promoter is transcriptionally regulated at S phase, and deletion of the first E2F binding site (D1) leads to loss of the S phase regulation of the promoter by a loss of repression during G_2 in synchronized cell suspensions. *Arabidopsis* E2F factors both bind and activate the wild type promoter in transient expression analyses. Furthermore, interestingly, deletion of the second site (D2) eliminates the expression in meristematic regions *in planta*, and this site with a TATA box is sufficient to induce reporter gene expression in plant meristems.

EXPERIMENTAL PROCEDURES

Plant Material—The *Arabidopsis* cell suspension culture derived from *Arabidopsis thaliana* ecotype Columbia was grown at 21 °C in 16 h of light and subcultured every 7 days in Gamborg B5 media (Sigma) supplemented with 0.2 mg/liter α-naphthaleneacetic acid. Tobacco BY2 cell suspension cultures were grown according to a previously described method (16). Plants of *A. thaliana* ecotype Columbia were grown under short day conditions (9 h of light) at 19 °C (day) and 17 °C (night).

Cloning—The *MCM3* promoter fragment was amplified by PCR following standard procedures using a 5′ oligo of sequence: 5′-CATTCCCCGTTTCTTACGGTTGCTGAG-3′ and a 3′ oligo of sequence 5′-CTGGGTTCTTCGTAAGAACTTTTCTTCTTC-3′ (Fig. 1) from *A. thaliana* DNA. The fragment was cloned into pGEM-T, checked by sequencing, and subsequently subcloned into the vector pTAK upstream of the *uidA* reporter gene to create pMCM3-*uidA*. For stable expression, in either *Arabidopsis* plants or tobacco BY2 strains, a *Hin*dIII-*Eco*RI fragment of the cassette containing the *MCM3* promoter, *uidA* gene, and terminator was inserted into the binary vector pPZP111.

The site D2 was cloned into pUC18 by annealing forward and reverse oligos of sequence 5′-GCCTTGAGGAAATCAAAC**GCGCCAAA**CAAGCGCGTAGACG-3′ (site D2 in bold). An *Eco*RI-*Sal*I fragment containing the site D2 was then cloned into the vector pLP140 upstream of the minimal promoter fused to the *uidA* reporter gene to create D2-*uidA*.

Construction of Mutated Promoter Sites and Other Constructions—Site-directed mutagenesis was carried out according to the manufacturer's instructions (Stratagene) on the pMCM3-*uidA* construct in pTAK to create the following mutants: pMCM3d1-*uidA*, primer: 5′-GCCTTGAGGAAATCAAACCAAGCGCGTAGACG-3′ and exact complement; pMCM3d2-*uidA*, primer: 5′-GGCCCAAAATGACCCAAGGGTACAGGTTATC-3′ and exact complement; pMCM3d1-d2-*uidA*, the double mutant was created using the oligos for pMCM3d2-*uidA* on the pMCM3d1-*uidA* clone. All clones were checked by sequencing for the presence of the correct mutations. 35S::AtE2F-a and 35S::AtDP-a for transient expression assays were cloned in the vector pDH51 as previously described (8).

Transformation of BY2 and Cell Synchronization—Transformations of BY2 cell lines was carried out as described previously by co-cultivating with an *Agrobacterium tumefaciens* culture in Petri dishes (17). For S phase synchronization a previously described method was followed (18): 4 ml of stationary phase cells were transferred to 40 ml of fresh medium composed of 4.33g/liter Murashige & Skoog (M-5524, Sigma), 3% sucrose, 200 mg/liter KH_2PO_4, 1 mg/liter thiamine, 100 mg/liter *myo*-inositol, supplemented with 1 μM 2,4-dichlorophenoxyacetic acid (Sigma) and containing aphidicolin 2 μg/ml (Sigma) prepared in dimethyl sulfoxide. Aphidicolin was removed from the culture by centrifuging, and the pelleted cells were washed twice in the same volume of culture medium. The cells were resuspended in the same volume of fresh medium, and G_2-synchronized cells were obtained ~6 h after the synchronization. To determine the metaphasic rate, 100 μl of cell suspension was added to 1 ml of a 2% (w/v) solution of cold paraformaldehyde (dissolved in phosphate buffer pH 7). The cells were stained with Hoechst 33342 (19), and the mitotic index was determined by microscopic observation.

RNA Isolation and Northern Blot Analysis—Total RNA was isolated by grinding tissue in liquid nitrogen in the presence of TRIzol reagent (Invitrogen) following the manufacturer's instructions. After electrophoresis in a 1% agarose gel containing 2% formaldehyde and blotting onto a Hybond N+ membrane (Amersham Biosciences), hybridization was carried out with ^{32}P-labeled probes labeled by the random primer method (Appligene). Hybridizations were performed at 62 °C (20).

Electrophoretic Mobility Shift Assays—EMSAs were carried out as previously described (7). Purified recombinant AtE2F or AtDP factors (9) (50–300 ng) were incubated with 50,000 cpm of annealed, radiolabeled probe in 15 μl of 25 mM Hepes, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 10 mM dithiothreitol for 30 min at room temperature. To show the specificity of binding, wild type or mutated cold annealed probes were included in the reactions. Wild type and mutated oligos for each site were used as follows. The sense oligo only is shown, the antisense oligo being the exact complement on the other strand: wild type site D1, 5′-GCCTTGAGGAAATCAAAC**GCGCCAAA**CAAGCGCGTAGACG-3′; mutated site d1, 5′-GCCTTGAGGAAATCAAACCAAGCGCGTAGACG-3′; wild type site D2, 5′-GGCCCAAAATGACCC**TTCCCGCCAAA**AAGGGTACAGGTTATC-3′; mutated site d2, 5′-GGCCCAAAATGACCCAAGGGTACAGGTTATC-3′. The protein-DNA complexes were electrophoresed for 3 h at 4 °C on 4% native polyacrylamide gels in 0.5× TBE. Gels were dried and exposed to film.

Transient Expression Assays, Arabidopsis Transformation, and Analysis of GUS Activity—Protoplast preparation, transformation (using polyethylene glycol), and GUS activity assays were carried out as previously described (17, 21). *Arabidopsis* were transformed using the floral dip method (22). Analysis of *uidA* expression *in planta* was carried out by incubating plantlets in 1 mM 4-methylumbelliferyl-β-D-glucuronide for 12 h followed by washing in 100% ethanol.

RESULTS

The MCM3 Promoter Contains E2F Consensus Sites—The *MCM3* gene transcripts are up-regulated at the G_1/S transition[2] consistent with the role of this protein in the formation of the complex of prereplication necessary for progression into S phase. The cloning of seven *MCM3* cDNAs, among which five include two in-frame stop codons upstream of the ATG and were therefore predicted to be full-length,[3] has allowed the transcription start site to be predicted. The 5′-untranslated region, based on the cDNA sequences, extends to the *arrow* marked in Fig. 1 at −60 bp; upstream of this point there are no obvious TATA, CAAT, or GC boxes, but analysis using Markov Chain Promoter Finder McPromoter V3.0 (23) predicted that transcription was most likely to start between −165 and −65 bp relative to the ATG, which correlates with the length of the 5′-untranslated region of five of the *MCM3* cDNAs.

Many of the genes regulated at the G_1/S transition are transcriptionally controlled by E2F factors, and therefore we analyzed, using the Transplorer software (www.developmentontheedge.com/transplorer.shtml), the 715 bps directly upstream of the ATG of the *MCM3* gene for consensus E2F sites. This fragment upstream of the *MCM3* cDNA (GenBank™ accession no. AJ000058) was cloned by PCR (see "Experimental Procedures") from data available from the complete sequencing of the *Arabidopsis* genome (At5g46280). Several binding sites for different transcription factors were found, those presenting the highest homology were (with numbers of putative sites in brackets): MYB.PHY3 [2], E2F [5], AP-1 [3], MADS-B [4], and Sp1 [3] (Fig. 1). Two E2F consensus binding sites matched the human consensus very closely, TTT(C/G)(C/G)CGC, and included the central CG essential for E2F binding (Fig. 1). The first site was at −272 bp from the ATG of sequence CGCGCCAAA, and the second at −99 bp of sequence CCCGCCAAA.

Transcriptional Regulation of the Promoter and Mutants—To analyze the role of these two E2F sites in the transcriptional regulation of the *MCM3* transcripts, the promoter fragment spanning the two sites was cloned upstream of the

[2] R. Stevens and C. Bergounioux, unpublished data.
[3] C. Bergounioux, unpublished data.

CATTCCCCGTTTCTTACGGTTGCTGAGATTTCAGATTTTGTTTGTTCTCTCTCTTGTCTGCGGAAT
GTTGTGTATCTTAGTTCGTTCCATA***TTTGCGT***AATTTATAAAAACAGAAACTGAGAGAATCTTGT
AGTAACGGTGTTATTGTCAGAATAATCCAATTAGGGGATTCTCATCTTTTATTTCTCAACAATTC
TTGTCGTGTTTTTACATTCGAAGAAATTAGATTTATACTGAATTTTTTTTCAGTTATACATATGTT
GTGATACATCCGAAAATGTTCTCAAATCACACAGGTTTATTTTGGTTTTCATATATACATTTTTC
GGTTTACTGGCTTGAAGGCAGTAGCAAAAGTTAAACCGAATTTACAATGAATTGATTTGGTATG
TCTGGTTTTTGGTTTAGAATCTGGGTGTATTATACCGCCTTGAGGAAATCAAA**CGCGCCAAA**CA
AGCGCGTAGACGTAAGTCCAAAATTGTACCGGCCCAAATTTGGTTAATCAAAAAGCACGTAGA
CGAAAGGCCCAACTAATAT***CGGCCCAAA***ATGACCCTT**CCCGCCAAA**AAGGGTACAGGTTAT***CT***
CACGAAATCCCGCCATAAGGAGAAGATCCGCCCTTTATGCCTCTCGAAATCGGAAATCGAAAAT
CTCTAATCAG*TTTTTTTTTTTAAGTAGAAGAAGAAAAGTTCTTACGAAGAACCCAGATTTTGTTAAGAA***ATG**

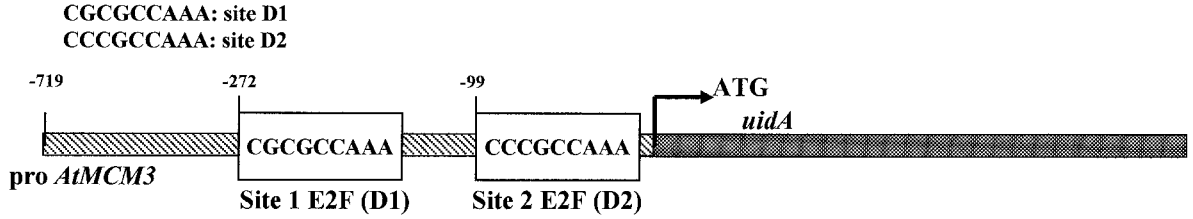

FIG. 1. **Sequence of the *AtMCM3* promoter.** *Top*, the 715-bp promoter sequence upstream of the ATG is shown. Putative sites for the following transcription factors are found (with number of sites in *brackets*) E2F [5]: sites D1 and D2 are *boxed* and shown in *bold*, whereas other sites are marked in *bold-italics*; Sp1 [3]) (*sites underlined*); MYB.PHY3 [2] at −640 bp and −584 bp; AP-1 [3] at −574 bp, −218 bp, −170 bp; and MADS-B [4] at −632 bp, −408 bp, −400bp and −159 bp according to the Transplorer software (see "Results"). An *arrow* marks the start of the *MCM3* cDNA sequences and the approximate transcription start site (see "Results"). *Lower*, the 715-bp *AtMCM3* promoter fragment upstream of the ATG was fused to the *uidA* reporter gene. The positions and sequences of the two E2F binding sites are shown, the first (D1) at −272 bp and the second (D2) at −99 bp from the ATG.

β-glucuronidase (*uidA*) reporter gene in the plasmid pTAK (see "Experimental Procedures"). Deletion mutants were created in each consensus site separately (pMCM3d1 or pMCM3d2 or together to form the double mutant (pMCM3d1-d2) to study the effect of the two E2F binding sites on the promoter activity. A BY2 culture was stably transformed with the following constructions: pMCM3-*uidA*, pMCM3d1-*uidA*, pMCM3d2-*uidA*, and pMCM3d1-d2-*uidA* (see "Experimental Procedures"). Calli were regenerated on selective media and checked for GUS activity. Once established, each transformed culture was synchronized using aphidicolin treatment to block cells in G_1/S and released from the block by washing in fresh medium. Samples of cells were taken at 0, 2, 4, 6, 8, 10, 12, 14, and 16 h after the release from the block, and RNA was prepared for Northern blot analysis of the *uidA* transcript. The results are shown in Fig. 2 for each transformed line and include Histone H4 as the marker for S phase as well as mitotic index data for G_2 to show that each synchronization had worked efficiently. The *uidA* transcripts appear slightly before the rise in Histone H4 levels and are absent when the mitotic index is high (Fig. 2*A*). Therefore *AtMCM3* is transcriptionally regulated at G_1/S phase (as our unpublished observations have suggested following analysis of the transcripts levels of this gene).

pMCM3d1-*uidA* transcripts are present at all points during the cell cycle and are no longer down-regulated during G_2 (Fig. 2*B*). The culture is shown to be synchronized because of the H4 and mitotic index data. Therefore pMCM3d1 is no longer regulated at S phase compared with the pMCM3-*uidA* expression and becomes constitutively activated during the cell cycle. In contrast, pMCM3d2-*uidA* transcripts show S phase transcriptional regulation similar to the wild type promoter, although transcript levels are much lower being transcriptionally up-regulated at G_1/S and silenced during G_2 (Fig. 2*C*, the blot being exposed to film for twice as long as the other blots). The double mutant pMCM3d1-d2 is no longer cell cycle-regulated having lost the transcriptional down-regulation seen for the wild type promoter during G_2 (Fig. 2*D*). The profile of transcript expression for the double mutant reflects that of the pMCM3d1 mutant, the *uidA* transcript appearing in all cell cycle phases. Results indicate that in cell suspension cultures the site D1 represses the cell cycle G_2 inhibition of pMCM3 activity, whereas site D2 may affect the level of promoter activity in S phase.

E2F Factors Specifically Bind the Two E2F Sites in the MCM3 Promoter—The identity of the two E2F binding sites was studied by electrophoretic mobility shift assays (EMSAs) using available AtE2F factors (selected for ease of purification). E2F factors AtE2F-c, AtE2F-d, and AtDP-b were cloned and purified as previously described (9) and used in EMSA with each of the two wild type putative E2F pMCM3 sites in the form of radioactively labeled probes (see "Experimental Procedures"). Both sites allow the formation of E2F-DP complexes (E2F-c belongs to the first group of AtE2F factors and requires AtDP for DNA binding) as well as allowing binding of the factor AtE2F-d (of the second group of AtE2F factors, which do not require AtDP for DNA binding). To determine that the binding was specific for each site, competition experiments were carried out with either wild type or the mutated fragments. For both sites D1 and D2, cold wild type competitor reduces the quantity of binding to the radioactively labeled probe as the factor is titrated out, whereas addition of excess quantities of cold-mutated probe do not affect the gel shift signal observed (Fig. 3). Thus both sites are characterized as binding E2F factors of both groups 1 and 2 *in vitro*. However, even if EMSA is not quantitative, the signal obtained with D2 is weaker than that obtained with D1 using the same amount of recombinant AtE2Fc. This observation suggests that even *in vitro* the two sites behave differently.

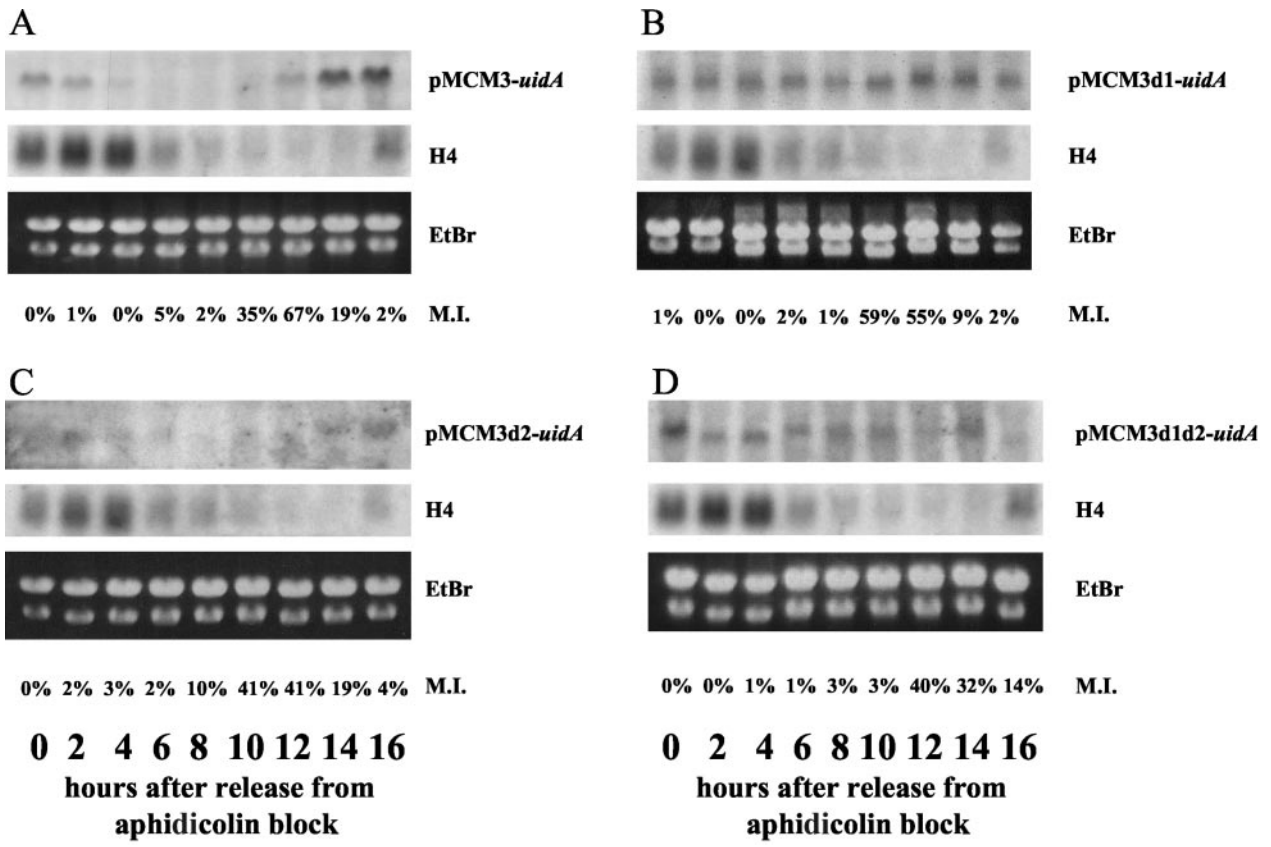

FIG. 2. **Identification of E2F cis-elements involved in the transcriptional regulation of the *MCM3* gene in synchronized BY2 cell cultures**. Northern blots using RNA extracted at 0, 2, 4, 6, 8, 10, 12, 14, and 16 h after an aphidicolin block on BY2 cell cultures transformed with pMCM3-*uidA* (Fig. 2*A*), pMCM3d1-*uidA* (Fig. 2*B*), pMCM3d2-*uidA* (Fig. 2*C*), and pMCM3d1-d2-*uidA* (Fig. 2*D*). Ethidium bromide-stained gels are shown as controls for equal loading. Histone H4 probes were used on each blot as markers for S phase to show effective synchronization as well as the mitotic index, which is expressed as a percentage of mitotic figures seen.

The MCM3 Promoter Is Activated by AtE2F and AtDP Transcription Factors in Transient Expression Studies—We tested the activity of the construct pMCM3-*uidA* in transient expression assays by measuring GUS activity 48 h after transformation of *Arabidopsis* protoplasts. *Arabidopsis* transactivating factors AtE2F-a and AtDP-a under the control of the 35S promoter (8), see "Experimental Procedures," were included in the transient expression assays as shown in Fig. 4. The addition of AtDP-a, AtE2F-a alone, or both factors together increases the GUS activity by over 1.5-fold compared with the control without addition of these transactivating factors. The biggest increase is seen on addition of AtE2F-a alone or both AtE2F-a and AtDP-a together.

Deletion of E2F Binding Sites Reduces the Activity of the MCM3 Promoter in Transient Expression Studies—Having shown that the wild type *MCM3* promoter can activate a reporter gene, the mutants were analyzed for their effect on the total activity of the promoter. Mutating either E2F binding site reduces the GUS activity in transient expression assays (data not shown). Site 2 (D2), under conditions of transient expression, appears to contribute most to the activity of the promoter as the mutated pMCM3d2 shows a greater decrease in GUS activity than the mutation pMCM3d1. Transient expression assays using the double mutant pMCM3d1-d2 show the lowest GUS activity indicating the necessity of the two E2F sites for the activity of this promoter. Thus both E2F sites contribute to the overall activity of the promoter.

The effect of the addition of 35S::E2F-a to the transient expression assays above is shown in Fig. 5. Removal of the site D1 may increase the expression of the GUS reporter gene slightly compared with the wild type promoter. This result shows the promoter activation gained from cells in G_2 where the promoter activity is no longer repressed and shows therefore that the site D1 may act as an E2F-specific repressor of promoter activity. The reverse is true for the site D2, which is necessary for promoter activity, and its removal reduces reporter gene activity on addition of E2F-a transcription factor. The double mutant also shows reduced activity presumably reflecting the loss of the site D2. The results therefore confirm the observations in Fig. 2.

In Planta Expression of the MCM3 Promoter and Mutants—*Arabidopsis* plants were transformed with the constructs pMCM3-*uidA*, pMCM3d1-*uidA*, pMCM3d2-*uidA*, and pMCM3d1-d2-*uidA* (see "Experimental Procedures") to analyze levels of *uidA* expression *in planta*. Approximately 200 T_1 transformants resistant to kanamycin were obtained. Plantlets from 50 independent transformed lines for each of the four constructions were used for protein extractions and for determination of GUS activity. Compared with untransformed control plantlets with background GUS activity, all the four lines showed increased levels of GUS activity (data not shown). This quantitative measure of total GUS activity in a plant is an average measure of the activity in all plant tissues and does not give any indication of how GUS activity may vary in different cell cycle stages in different plant tissues so *in planta* staining was carried out on 50 independent T_1 lines (Fig. 6). pMCM3-*uidA* showed that the wild type promoter was active in meristematic regions, in particular in secondary root tips and the meristem regions at the base of leaves. The construction pMCM3d1-*uidA* also showed similar expression patterns to that of the wild type promoter with higher activity levels in secondary roots and leaf meristems compared with the rest of

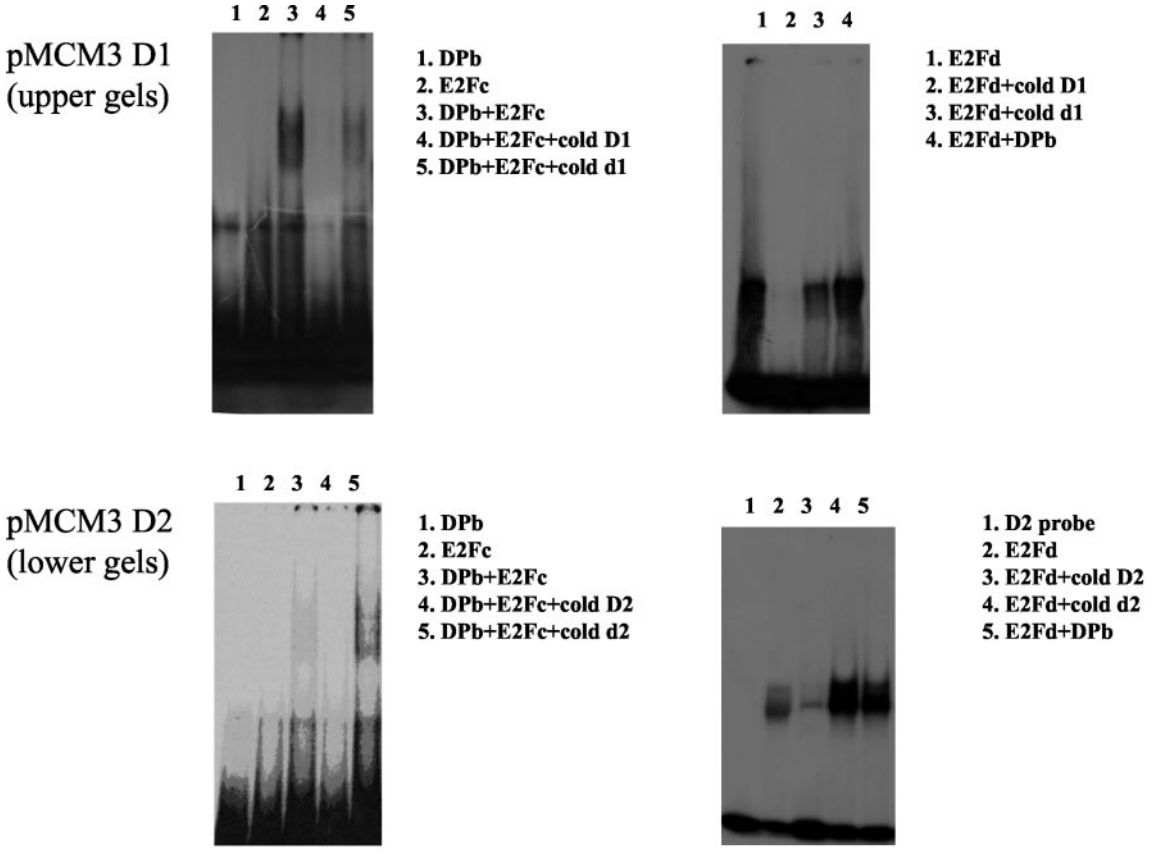

FIG. 3. **Promoter sites D1 and D2 of the *MCM3* promoter specifically bind E2F factors.** EMSA assays were used to show that sites D1 and D2 bind *Arabidopsis* E2F factors. Radioactively labeled pMCM3 D1 or D2 probes were incubated with purified AtE2F-c, AtE2F-d, and AtDPb factors as shown. An excess of wild type (cold D1 or D2 as shown) or mutated probe (cold D1 or D2 as indicated) were added as shown to show that factor binding was specific for the sites.

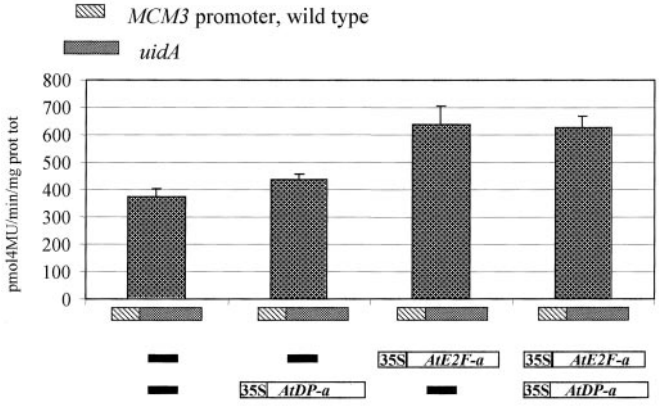

FIG. 4. ***Arabidopsis* transcription factors AtE2F-a and AtDP-a activate the *MCM3* promoter.** GUS activities in *Arabidopsis* protoplasts following transient expression with pMCM3-*uidA* plus the factors shown. *Bars* represent the standard deviation calculated from three replicates.

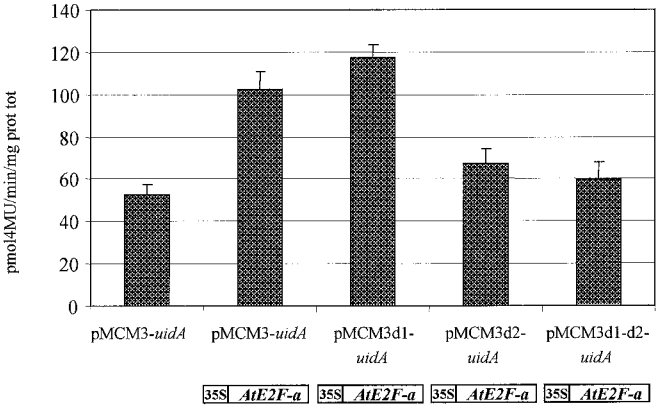

FIG. 5. **Mutation of E2F consensus sites in the *MCM3* promoter affects reporter gene expression on addition of E2F-a.** GUS activities in *Arabidopsis* protoplasts following transient expression with 35S::E2F-a and one of the following constructs: pMCM3-*uidA*, pMCM3d1-*uidA* (site D1 mutated), pMCM3d2-*uidA* (site D2 mutated), and pMCM3d1-d2-*uidA* (both sites D1 and D2 mutated). *Bars* represent the standard deviation calculated from three replicates.

the plant. However the construction pMCM3d2-*uidA* gave altered *uidA* expression patterns, plantlets no longer showed expression in root meristems; these areas appeared white. However, in other areas of the plant GUS activity was observed. Similarly the double mutant pMCM3d1-d2-*uidA* had also lost meristematic expression in the same way as did the pMCM3d2-*uidA* presumably due to the absence in these transformants of the site D2.

Furthermore, we have shown that the site D2 placed in front of a minimal promoter (TATA box; see "Experimental Procedures") is sufficient to activate reporter gene expression in plant meristems (Fig. 7, D2-*uidA*). Therefore the site D2, out of the context of the *MCM3* promoter, is responsible for the specificity of meristematic expression via factors that bind this site. We conclude that D1 has little role in the regulation of *MCM3* in meristems, in contrast D2 is essential for regulation of *pMCM3* in plant meristematic regions.

DISCUSSION

The promoter of the *AtMCM3* homologue, a gene implicated in the initiation of replication and the transition G_1/S, contains

several E2F consensus binding sites, two of which match the human consensus closely: TTT(C/G)(C/G)CGC (24). The mechanisms by which the transcription rate of the *MCM3* gene is controlled will aid our understanding of the processes involved in DNA replication.

The identity of the two sequences as E2F binding sites has been confirmed by EMSA analyses: *Arabidopsis* E2F transcription factors of both group 1 (transcriptional activators requiring DP for DNA binding) and group 2 (transcriptional repressors that do not require DP) (9, 10) bind both sites D1 and D2. This is consistent with previous results showing that a consensus E2F site will bind all AtE2F factors *in vitro* (9). Even though in this study we have not used every factor in our experiments, we would expect to see that *in vitro* all AtE2F factors are capable of binding the two sites as both fit the consensus E2F sequence well. However, these *in vitro* assays do not enable us to determine the activating or repressing roles that these different factors may have *in planta* in the context of the whole promoter or whether different E2Fs compete for the two sites in different ways (25).

Further characterization of the promoter *in vivo* shows that its activity is cell cycle-regulated at G_1/S. The promoter pMCM3 is also activated by AtE2F-a and AtDP-a transcription factors in transient expression studies. The transient expression analyses in Fig. 5 together with the Northern blot analyses would indicate that D1 is responsible for the G_2-specific repression of the promoter seen in a cell suspension culture and that D2 is required for the level of S phase activity. We have previously shown that addition of E2F factors to a cell population increases the proportion of S phase (8); other transcription factors expressed during S phase may therefore be responsible for activation of S phase-specific promoters such as pMCM3. However, it has been shown here that mutating E2F sites within the promoter alters the promoter activity, and we can therefore conclude that the *MCM3* promoter is specifically activated by E2Fs.

It has already been shown that the tobacco RNR2 promoter has two E2F binding sites matching the consensus perfectly but each has a different function in terms of cell cycle regulation (13). One site acts as an S phase activator while the other acts as a repressor outside S phase *in vitro*. Furthermore, a variant E2F site in the promoters of the cdc2, cyclin A, and cdc25 genes contributes to the cell cycle-dependent timing of transcription of these genes; mutation of this site and the overlapping CDF-1 binding site removes the transcriptional repression in G_1 (26) illustrating that adjacent sequences may interact with E2F binding sites. In the human Ran-binding protein 1 promoter, two E2F sites act as activating or repressing elements depending on the neighboring Sp1 element (27).

The most interesting results from this study concern the role of the two sites in different cellular environments: a cell suspension culture and during plant development. Two highly similar E2F binding sites in the promoter of the *MCM3* gene are responsible for different cell cycle regulation or developmental expression patterns depending on the cellular environment.

At the whole plant level, the mutation of the site D1 has no effect on expression in plant meristems. However, the site D2 has a role in the meristem-specific expression of the *MCM3* promoter. Deletion of this site clearly removes expression from root meristems and the apical meristem while maintaining the level of GUS activity elsewhere in the plant. Furthermore this site, with only a minimal promoter, is sufficient for activation of a reporter gene in plant meristems (and could be a useful tool for meristem-targeted expression). This site could therefore play several roles in the plant. First, it could bind specific E2Fs and E2F-associated factors, which within the meristems activate expression. Alternatively, the existence of E2Fs or associated factors that bind to the site D2 and act as repressors in non-meristematic regions could be envisaged. Other results *in*

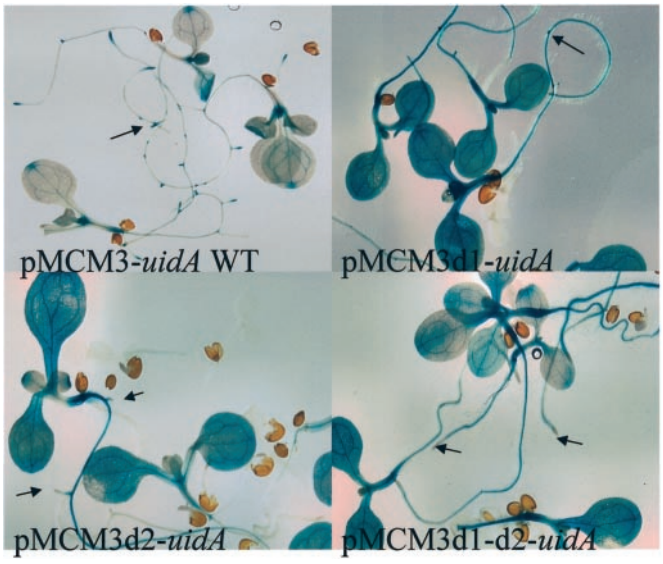

FIG. 6. **Identification of E2F cis-elements involved in the transcriptional regulation of the *MCM3* gene in developing plantlets.** Plantlets transformed with pMCM3-*uidA*, pMCM3d1-*uidA*, pMCM3d2-*uidA*, and pMCM3d1-d2-*uidA* were grown on selective media on agar plates and were stained for GUS activity before being photographed under the microscope.

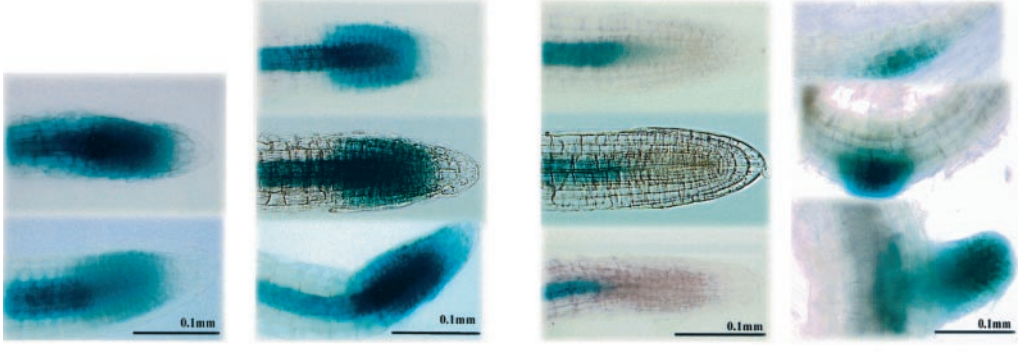

FIG. 7. **The site D2 is sufficient to activate reporter gene expression in the meristem.** Plantlets transformed with pMCM3-*uidA*, pMCM3d1-*uidA*, pMCM3d1-d2-*uidA*, and D2-*uidA*, were grown on selective media and stained for GUS activity before being photographed under the microscope.

planta have shown that an E2F site in the PCNA promoter is responsible for the repression of transcriptional activity of this gene in mature leaves (28). Also, mutation of a single E2F site in the RNR1b 5′-untranslated region is sufficient to eliminate all reporter gene expression from tobacco plantlets (29).

It is clear that differences exist between meristematic (dividing) and other (differentiated) tissues that are linked to a complex of proteins including E2Fs involved in transcriptional activation. In our study, results from meristematic expression show that additional levels of regulation exist for the site D2 in meristems compared with cell suspension cultures. Mutation of the site D2 completely eliminates reporter gene expression in meristems, in all transgenic lines analyzed, and not other parts of the plant, whereas in cell suspension cultures a low level of activity is maintained in S phase. This suggests that cell suspension cultures cannot be used as a model for plant meristematic regions.

It is possible that the two E2F groups or the two AtDPs have different roles *in planta* and are expressed in different tissues to enable the form of regulation seen for the site D2 to occur. Results leading to a similar idea have been shown in certain tissues during *Drosophila* oogenesis. DP is used uniquely for cell cycle arrest rather than cell cycle progression and is required for the successful development of the dorso-ventral axis (30). Other non-identified factors involved in differentiation could be responsible for the meristem-specific expression in complex with E2F proteins. In our case the factor involved may be an Sp1-like transcription factor (as yet unidentified in *Arabidopsis*) because the site D2 overlaps a putative Sp1 binding site (human consensus sequence, Fig. 1). Sp1 factors have been shown to interact with E2F, an interaction that is essential for the regulation of certain promoters (31, 32).

In this study we have shown the role of two E2F sites in control of the cell cycle and also the role of one site in meristematic cells *in planta.* It is interesting to note in our study that two E2F binding sites can have very different roles depending on the context of transcriptional regulation (reviewed in Ref. 33). This phenomenon can occur by several mechanisms involving action at a distance, overlapping binding sites, protein-protein interactions, and the cellular environment. Thus the role or activity of a site is influenced by the surrounding promoter structure and the cellular environment or tissue type. Factors such as Sp1, as mentioned above, can function as basal promoter elements or upstream activators by bending the DNA through protein-protein interactions and thus can function at a distance. Thus the context of the two sites in the *MCM3* promoter depends on the cellular environment as we have shown plus as yet unknown protein-protein interactions that must now be identified and may be responsible for their different roles.

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Two E2F Sites in the *Arabidopsis MCM3* Promoter Have Different Roles in Cell Cycle Activation and Meristematic Expression

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