S Phase and Meristem-specific Expression of the Tobacco RNR1b Gene Is Mediated by an E2F Element Located in the 5′ Leader Sequence*

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The RB/E2F pathway is involved in the control of the G1/S transition of the eukaryotic cell cycle where various S phase genes are activated by specific E2F factors. Ribonucleotide reductase (RNR) plays an essential role in the DNA synthesis pathway. Earlier studies showed that there are at least two RNR1 genes (RNR1a and RNR1b) and one RNR2 gene in tobacco. In synchronized tobacco BY2 cells, RNR1b gene expression is at its highest level in S phase. To investigate transcriptional regulation of the RNR1b gene, its promoter region was cloned and sequenced. Unlike its animal counterparts, the tobacco RNR1b promoter contains a consensus E2F-binding site. Surprisingly, this site is found in the leader sequence of the gene. We show here by gel shift analysis and antibody competition that one nuclear complex specifically binds this motif, and an E2F factor is part of this complex. Using reporter gene analysis, tobacco RNR1b promoter activity was detected during S phase in synchronized cells and in plant meristematic tissues. Mutation of the E2F element substantially reduced both activities. For the first time in plants, a single E2F motif found in the leader sequence plays an important role in the meristem and S phase-specific expression of the tobacco RNR1b gene.

Progression of the cell cycle is associated with the phase-specific induction of genes whose products control the cell cycle or are involved in DNA replication (1). Transcription of several genes induced at the G1/S transition is mainly controlled by the E2F pathway (2). E2F complexes can act either as transcription repressors or activators depending on their target genes and the phase of the cell cycle. While transcription repression is generally achieved by binding of retinoblastoma-type proteins (RB) to E2F factors (3), repression of the PAI-1 gene is carried out by direct binding of E2F to its target motif (4). Transcriptional activation is triggered when the E2F factor is associated to its DP partner. E2F-binding sites (TTTC/CCGGC) were identified in numerous gene promoters (5). Some genes, such as cdc2 or B-Myb, appear to be strongly derepressed during the transition from G0 to G1 (i.e. quiescence to growth), while others, such as the p107 and DHRF genes, are up-regulated at the G1/S transition (6).

E2F and DP factors (7–12) as well as RB proteins have been recently identified in plants (13), suggesting that the control of G1/S-phase-specific transcription in plants could be similar to that found in animals. Up to now, a few E2F target genes have been characterized in plants. The two E2F motifs identified in the tobacco RNR2 promoter were shown to specifically interact with a tobacco E2F factor and to mediate up-regulation of the promoter at the G1/S transition of the cell cycle (14). In addition, it was shown that Arabidopsis E2F1 factor binds an E2F motif in the promoter of a S-phase-regulated gene AtCycD6 (10). Alternatively, two E2F cis-elements were found in the promoter of tobacco gene encoding the proliferating cell nuclear antigen (PCNA). Mutation of an E2F consensus element in the PCNA promoter has no clear effect on its activity during the cell cycle but does act as a repressor in differentiated cells (15).

Ribonucleotide reductase (RNR) is a key enzyme in the pathway of DNA synthesis. It catalyzes the reduction of ribonucleoside diphosphates (NDPs) in deoxyribonucleoside diphosphates (dNDPs), a rate-limiting step in DNA synthesis (16). In all eukaryotes, ribonucleotide reductases belong to the RNR Class I in which the active enzyme consists of two large (R1) and two small (R2) subunits encoded by RNR1 and RNR2 genes, respectively. In yeast, two RNR2 (RNR2 and RNR4) genes and two RNR1 (RNR1 and RNR3) genes have been identified (17, 18). In mammals, two RNR2 genes (RNR2 and P53R2) and one RNR1 gene have been isolated (19, 20). RNR genes are differently induced depending on their function: for DNA replication in S phase (RNR2 and RNR1 genes in yeast and human) or for urgent DNA repair (yeast RNR3 and human P53R2 genes) in G1 and G2 arrests of the cell cycle. Interestingly, E2F sites are not involved in S phase-specific expression of RNR genes in mammals: RNR cell cycle regulation is mediated by a TFII-I binding initiator element in the mouse RNR1 promoter (21), whereas a proximal NF-Y binding element is involved in S phase induction of mouse RNR2 gene (22).

Here, we show that RNR1b gene expression is strongly expressed in S phase in synchronized tobacco cells. One E2F site is identified in the RNR1b promoter, downstream from the TATA box and in the 5′-untranslated region. We showed by gel shift assays that an E2F factor-containing nuclear complex binds to this site as does the purified tobacco E2F protein. The promoter drives S phase expression of a luciferase reporter gene and meristem activity of a GUS reporter gene. Mutation of the E2F motif markedly decreases both activities of the

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‡The abbreviations used are: RB, retinoblastoma; PCNA, proliferating cell nuclear antigen; RNR, ribonucleotide reductase; NDPS, ribonucleoside diphosphates; dNDPs, deoxyribonucleoside diphosphates, LUC, luciferase; GUS, β-glucuronidase; HU, hydroxyurea; WT, wild type; MU, mutated; CP, control promoter.
RNR1b promoter. Thus for the first time in plants, a single E2F motif in the leader sequence is shown to act as a transcription activator of the tobacco RNR1b gene in S phase and meristems.

**EXPERIMENTAL PROCEDURES**

**Plant Cell Culture and Synchronization**—The tobacco BY2 cell suspension was maintained by weekly subculture as described previously (23). For synchronization, freshly subcultured stationary phase cells were treated with aphidicolin (3 μg/ml, Sigma) for 24 h and extensively washed. DNA synthesis and mitotic index were monitored as described previously (24).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from frozen BY2 cells according to Goodall et al. (25). 20 μg of purified RNAs were analyzed by formaldehyde-agarose gel electrophoresis and transferred onto a Hybond N+ membrane (Amersham Biosciences). Hybridization was carried out overnight at 42 °C in presence of 50% formamide with 12P-labeled probes corresponding to the coding or the 3’-specific regions of the genes. Transcript levels were quantified using a PhosphorImager (Molecular Dynamics).

**Nuclear Extracts and Electrophoretic Mobility Shift Assays**—Nuclear extracts and purified tobacco E2F factor were prepared as described previously (14). Electrophoretic mobility assays were performed in presence of 6 μg of nuclear extract or 300 ng of purified protein. Protein samples were incubated with 20,000 cpm of radiolabeled probes in 20 μl of binding buffer (25 mM Hepes, pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 250 μg/liter pepstatin, 500 μg/liter leupeptin) in the presence of 0.05% Nonidet P-40 and 1 μg of poly(dI-dC)-poly(dI-dC) (Amersham Biosciences). The double-stranded oligonucleotides used in gel shifts experiments were: wild type E2F (WT) (TATAGCCGGGAAA
ggggggATTTAA, where the boldface letters correspond to the reverse), E2F-
mutated (MU) (TTATTTAAGGTCACTTTTAA) and an unrelated oligo-
ucleotide (TGGCTTACTAAGCTTATGAC). For the competition assays, molar excess of unlabeled double-stranded oligonucleotides or 2–4 μl of the antibody (anti-E2F5 Santa Cruz, α-tubulin, Amersham Biosciences) were included in the reaction. The samples were electrophoresed on 6% polyacrylamide gels in 0.25 TBE (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, Na2HPO4, pH 7.4) and then to the 3’-specific regions of the genes. Transcript levels were quantified using a PhosphorImager (Molecular Dynamics).

**Cloning of the RNR1b Promoter**—RNR1b promoter sequence was amplified by inverse PCR on tobacco genomic DNA. A DNA fragment from −628 bp to the ATG start codon was obtained. The region from −601 bp to −15 bp was subcloned in the XbaI site of the pBluescript (pKS) vector using the XbaI site at −601 bp and the XbaI site introduced by PCR at −15 bp. The resulting construct, called control pro-
motor (CP) was used in the E2F site by PCR-based site-directed mutagenesis (using the same nucleotide changes as in gel-shift assays), giving the mutated construct (MU).

**Promoter-Luciferase Reporter Gene Constructs**—The cloned RNR1b promoter (−601 bp to −15 bp) and its E2F-mutated version were subcloned by PCR in KpnI-NcoI sites of the luciferase (LUC) intron reporter gene plasmid pLuc70 (26), instead of the initial CaMV 35S promoter. Then the KpnI-XbaI fragment carrying the RNR1b promoter-LUC fusion was subcloned in the same nucleotide changes as in gel-shift assays), giving the mutated construct (MU).

**Promoter-GUS Reporter Gene Constructs**—The cloned RNR1b promoter (CP) and its mutated version (MU) were each fused to the GUS reporter gene in the XbaI-SmaI site of the binary vector pBI 101. Plasmid constructs were introduced into A. tumefaciens strain LBA 4404. Tobacco plants (Nicotiana tabacum L. cv SR1) were transformed by the Agrobacterium method (28). Plants were grown in vitro and in the greenhouse. Seeds were collected from 10 independent plant lines and germinated on kanamycin for analysis of the F1 progeny.

Luciferase Assays—2 ml of cells were washed twice in PBS buffer (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, Na2HPO4, pH 7.4) and lysed by a 10-min incubation at room temperature in 200 μl of lysis buffer (100 mM potassium phosphate buffer, pH 7.8, 1 mM dithiothreitol, and 0.2% Triton X-100). After centrifugation at 8,000 rpm for 30 min, the supernatant was frozen in liquid nitrogen and stored at −80 °C. Luciferase activity was measured using the Luciferase assay kit (Tropix) in a microplate luminometer (TR 717 Tropix, Applied Biosystems) according to the manufacturer’s instructions.

**GUS Assays**—GUS histochemical staining was carried out as described previously (29) on F1 plants issued from independent transformation events. 8–10 plantlets from each line were tested at the germination stage. Hand-cut sections were viewed by bright- or dark-field microscopy.

**RESULTS**

**Cell Cycle Expression of the RNR1b Gene**—The level of RNR1b transcripts was measured throughout the cell cycle in aphidicolin-synchronized BY2 tobacco cells (Fig. 1). After removal of the drug, cells arrested in S phase progressed synchronously through the cell cycle, reaching a maximal mitotic index at 8 h (Fig. 1A). DNA synthesis and RNR mRNA levels were monitored for 15 h (Fig. 1B). Tobacco RNR1 genes are cell cycle-regulated and show maximum expression in S phase and a significant gene induction at the G2/M transition, compared with the constitutive expression of the EF1α gene (30). Probing with the coding region of tobacco RNR1a cDNA revealed both RNR1 mRNA (RNR1a and RNR1b), since their coding regions share 89.2% sequence identity (31). However, using a 3′-specific probe, we show that the RNR1b gene is strongly expressed in S phase and very weakly at the G2/M transition. Therefore,
the significant level of mRNA detected at G2/M with the coding region probe is mainly due to RNR1a mRNA. 2

Modulation of RNR1b Gene Expression in Response to a Replication Block—To analyze the response of the RNR1b gene to a block in DNA synthesis, we used hydroxyurea (HU). HU directly blocks RNR activity by specifically quenching the tyrosyl radical of the small subunit (32). The cells are deprived of newly synthesized dNTPs, and DNA replication is blocked. HU applied to mid-S phase cycling cells led to rapid decrease in DNA synthesis compared with the control (Fig. 2A). During the first hour of drug treatment, both total RNR1- (Fig. 2A) and RNR1b-specific transcript levels (Fig. 2B) decreased parallel to the control. After 1 h, however, the total RNR1 mRNA level increases in HU-treated cells to reach a maximal level 10 times higher than the control after 6 h (Fig. 2A), in parallel RNR1b-specific mRNA level was restored and stabilized at the level existing when the drug was applied (Fig. 2B). Therefore, the marked increase in RNR1 transcript level after HU treatment may essentially reflect the RNR1a gene response. After a block of DNA synthesis, RNR1a and RNR1b mRNA levels fluctuate differently, suggesting a different regulation in expression of each RNR1 gene.

Sequence Analysis of the Tobacco RNR1b Promoter—Since

the RNR1b gene was specifically induced during S phase, we further investigated the transcriptional regulation of this gene. The 628-bp DNA promoter region was amplified by inverse PCR as described previously (14) by using primers adjacent to the ATG start in the RNR1b cDNA (GenBank™ accession number Y10862). Sequence analysis revealed several potential cis-elements (Fig. 3), notably a reverse E2F-binding site at −177 bp (GCCGGAAA) from the ATG start. The sequence matches perfectly with the consensus sequence found in animals (33) and more recently in plants (10, 14). In the vicinity of the E2F element, a putative MYB-binding site (CAACAG) as well as a telo-box (AAACCCTAA) were found at −204 and −164 bp from the start codon, respectively. Since the E2F element and telo-box are included in the leader sequence of the cloned cDNA, this indicates they are located downstream from the transcription start and the putative TATA box at −405 bp. In mammals, E2F element is also found at a similar location in the cyclin E promoter (34). In plants, teto-boxes, internal telo-meric repeats, are often found downstream from the transcription start in various PCNA promoters (35). Three types of MYB target sequences have been identified in plants, one of which (YAACNG) (36) is similar to those found in animals (37). The MYB-binding site in the RNR1b promoter is similar to this target sequence.

E2F Transcription Factor Is Part of the Nuclear Complex Associated with the RNR1b E2F Element—In animals, E2F motifs are important to mediate G1/S induction of cell cycle-regulated genes. We therefore investigated the role of the E2F site in the RNR1b promoter. First we verified the capacity of the E2F element to bind nuclear complexes. Gel retardation assays were performed with nuclear extracts prepared from exponentially growing BY2 cells and 32P-labeled double-stranded oligonucleotides carrying either the E2F motif (WT) or its mutated version (MU). In the presence of nuclear extract, two retarded complexes (NC, I and II) were detected and titrated out by an excess of unlabeled wild type E2F oligonucleotide (Fig. 4A, WT). Neither the oligonucleotide mutated in the E2F site (MU) nor an unrelated oligonucleotide (U) were able to displace the complex I, indicating that this complex has a specific binding activity for the E2F motif. To show that an E2F transcription factor is part of the complex I, antibody competi-

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tion was performed in gel-shift experiments. Since the DNA binding domain of E2F factors is conserved between tobacco and mammals (75% amino acid identity) (8), we used a polyclonal antibody raised against the DNA binding domain of the human E2F5 factor. This antibody was previously shown to specifically recognize a purified tobacco E2F factor (14). Increasing the concentration of E2F5 antibody (2–4 μl) (Fig. 4B, αE2F5) dramatically decreased the E2F motif binding activity of complex f compared with an unrelated antibody used as a control (UA). The purified tobacco E2F factor was able to specifically interact with the E2F probe (Fig. 4C, tE2F), since the protein-DNA complex formation was competed out by an excess of unlabeled WT probe but not by the E2F-mutated oligonucleotide (MU). Moreover, the E2F5 antibody prevents binding of the E2F factor to the WT probe, whereas an unrelated antibody has no effect. It therefore appears that the tobacco E2F factor (14) or a E2F-containing nuclear complex specifically binds to the E2F motif of the RNR1b promoter.

The E2F Site Is Necessary for S Phase Induction of the RNR1b Promoter—To investigate the role of the E2F site in the activity of the RNR1b promoter, the control promoter (CP) or the E2F-mutated promoter (MU) were fused to the luciferase reporter gene. BY2 mid-log phase cells were stably transformed by the different constructs in Agrobacterium. Pools of 1000 individual clones were cultured as cell suspensions and analyzed. After synchronisation of the transgenic lines by aphidicholin, DNA synthesis and the mitotic index were monitored throughout the cell cycle progression (Fig. 5A). In both transgenic lines (CP and MU), a maximal mitotic index of 40% was reached at 9 h, and DNA synthesis was maximal at 19 h. Luciferase activity was monitored in parallel. The activity of the control construct was low in G2 (4–6 h), M (6–14 h), and G1 (14–17 h) phases and increased concurrently with DNA synthesis in S phase (17–21 h). The residual activity detected could be due to incomplete cell synchronisation. Alternatively, it is also possible that a second promoter element, responding to the cell cycle, acts in synergy with the E2F element. The Myb element could be a possible candidate. However, we can conclude that the E2F motif is important for
In the transgenic plants, GUS staining was observed in young cotyledons (panel a), but as the plants grew, staining in the cotyledons became weak and speckled (panel b). Such GUS expression may be due to DNA endoreduplication frequently observed in young cotyledons of Arabidopsis seedlings (38). Significant GUS staining was also observed in young leaves (panel c) but not in the apical meristem (panel d). Primary root tips (panel g) were labeled, but the signal decreased as plantlets grew (panel h). Higher GUS activity was detected in axillary meristems, in leaf primordia (panels e and f), and in the meristematic zone of lateral root primordia: first, in dividing cells in the pericycle (panels i and j), then, in lateral root primordia before root formation (panels k and l), and finally, in emerging root tip (panels m–o). In contrast, no GUS activity was found in plants carrying the mutated E2F promoter–GUS fusion (panels p). Consequently, it appears that the control promoter (−601 to −15 bp) is primarily active in axillary meristems and lateral root primordia in growing plantlets. The E2F motif in the leader sequence of the RNR1b gene is shown to be required for such activity.

**DISCUSSION**

In a previous report, we identified two RNR1 cDNAs and one RNR2 cDNA (31). Since the coding regions of both RNR1 cDNAs are closely related, 3′-specific probes were necessary to distinguish the expression pattern of each cDNA. Interestingly, RNR1b expression is mainly observed in the S phase of the cell cycle, whereas overall RNR1 gene expression is detected both in the S phase and at the G2/M transition, suggesting that the two RNR1 genes are differentially regulated during the cell cycle. Plant histone and PCNA genes (8, 24) are expressed in the S phase but 1 h later than the RNR1b gene. When DNA replication is blocked in mid-S phase, after RNR gene induction, the RNR1b mRNA level first decreases then increases to its initial level and finally remains constant. In contrast, total RNR1 transcript levels sharply increased, suggesting that this overall response is mainly due to the RNR1a gene induction.2 These results indicate that different pathways regulate the RNR1 genes, leading to a different cell cycle expression. In yeast, the two RNR1 genes (RNR1 and RNR3) are differently regulated during the cell cycle and in response to a DNA synthesis block (39). On the other hand, in mammals, one RNR2 gene is induced in S phase and the other (P53RNR2 gene) in response to urgent repair of DNA damage (20). It has been shown recently that the S phase RNR2 gene is not transcriptionally regulated in response to a replication block, but the R2 protein level is stabilized by a controlled protein degradation (40). It appears, therefore, that different modes of regulation of the RNR1 and/or RNR2 genes have evolved. In plants, further investigation is necessary to obtain an overview of the RNR regulation pathway.

Transcriptional regulation of the RNR1b gene has revealed several interesting features. The RNR1b promoter sequence is the first to be cloned in plants and unlike its animal counterparts contains a single E2F site, TTTCCTGC, located in the leader sequence at −177 bp from ATG. The same motif was previously described in the tobacco RNR2 promoter (E2Fa, −355 bp from the ATG) and was identified in a reverse orientation in Arabidopsis (−258 from ATG) and rice (−279 bp) RNR1 promoters (GenBankTM accession numbers AC007019 and AB023482). An identical motif was also found in various promoters of S phase-regulated genes such as the tobacco PCNA gene (15), Arabidopsis, genes encoding CDC6, minichromosome maintenance, DNA polymerase α, or origin recognition complex component proteins (10). In plants, the position of the E2F site is not conserved in the different promoters as in
mammals (33). However, the presence of a functional E2F motif in the leader sequence is not usual, since only human cyclin E promoter presents such features (34). We show by gel-shift assays that in exponentially growing cells, a nuclear complex associates specifically with the E2F site in the RNR1b promoter. In addition, the purified tobacco E2F factor interacts with this E2F motif. From reporter gene expression, we conclude that the E2F element plays an important role in the specific S phase induction of the RNR1b promoter. In the tobacco RNR2 promoter, the E2Fa site was previously described to bind the E2F factor and acts as an activator element (14). Similarly in Arabidopsis, the E2F site of the CDC6 promoter binds the AtE2F1 trans-activating factor in presence of DP (10). In plants, the E2F element is directly involved in specific RNR gene induction, while this is not the case for yeast or mammal RNR genes (18, 21, 22). In animals, expression of E2F-regulated genes mostly results from the concerted action of two E2F motifs throughout the cell cycle (41, 42). In a few cases, such as the H2A gene, S phase induction is mediated by a single E2F site (43).

When fused to the GUS reporter gene, the cloned RNR1b promoter (–601 to –15 bp) showed active in primary and secondary root meristems as well as in axillary meristems of developing plantlets, but not in the apical meristem. No GUS activity was detected in transgenic seedlings harboring the mutated E2F construct. These results indicate that the E2F element drives meristic activity of the RNR1b promoter, especially in the axillary buds and lateral roots, which underlines the importance of the E2F motif in the S phase-specific induction of the RNR1b gene. Up to now in plants, specific GC-rich elements, such as octamer and nonamer or specific GC-rich elements, such as octamer and nonamer or in cell growth and differentiation (46). There are several lines of evidence suggesting that the c-MYB factor regulates transcription by activation or by repression (47). Telo-boxes were previously identified in plant genes encoding components of the translational apparatus or genes induced in cycling cells and were shown to act as co-regulators of transcription factors during the G₁/S transition (35). Since a Puro-like DNA-binding protein binds to a plant telo-box (48), it is tempting to postulate that Puro could modulate the E2F activity as described in mammals (49). In several cases, E2F-mediated activation does not simply result from the E2F site function but also from the cooperative effect of YY1, SP1, or NF-Y-binding sites (50). Additionally, the functional role of E2F is also dependent on its position relative to other regulatory elements in the promoter (41).

In conclusion, we report for the first time in plants that a single E2F site located in the leader sequence of the RNR1b gene is involved in both cell cycle and plant developmental regulation. Further investigation will be necessary to identify the components of the E2F complex and to determine whether the MYB and telo elements play a role as E2F co-regulators in plants.

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