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DcE2F, a Functional Plant E2F-like Transcriptional Activator from *Daucus carota**

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In animal cells the progression of the cell cycle through G_1/S transition and S phase is under the control of the pRB/E2F regulatory pathway. The E2F transcription factors are key activators of genes coding for several regulatory proteins and for enzymes involved in nucleotide and DNA synthesis. In this report we have detected the presence of E2F-like DNA binding activities in carrot nuclear extracts, and we have isolated a carrot cDNA (DcE2F) encoding a plant E2F homologue. The DcE2F gene is expressed in proliferating cells and is induced during the G_1/S transition of the cell cycle. Supershift experiments using anti-DcE2F antiserum have confirmed that the DcE2F protein is a component of the carrot E2F-like nuclear activities. DNA binding assays have demonstrated that the DcE2F protein can recognize a canonical E2F *cis*-element in association with a mammalian DP protein. Furthermore, transactivation assays have revealed that DcE2F is a functional transcription factor that can transactivate, together with a DP partner, an E2F-responsive reporter gene in both plant and mammalian cells.

Cell division in plants is mainly restricted to meristems and is regulated both temporally and spatially in response to plant growth regulators and environmental signals. Meristematic cell division occurs during the entire plant life, and because plant cells cannot migrate, control of cell proliferation is responsible for the formation of plant organs and structures (1). Remarkably, however, plant cells can readily undergo multiple cycles of endoreduplication, and additionally most of them are totipotent and can reprogram cell division even after completing their differentiation. These unique features suggest a unique flexibility in the control of the cell cycle in plants.

In animal cells a pivotal role in the progression of cell cycle is played by the E2F family of transcription factors, which are key components of a critical checkpoint regulating entry of cells into S phase (2, 3). Progression through the G_1 and S phase of the cell cycle ultimately depends on the activation of genes coding for regulatory proteins and for the enzymes involved in nucleotide and DNA synthesis. The expression of many of these genes is largely under the control of the E2F family of transcription factors, which appear to be activated by multiple mitogenic signaling pathways. The E2F proteins bind to specific DNA sequences through a winged helix motif, forming prevalently heterodimers with distantly related partners of the DP (DRTF1 polypeptide) family (4). In mammalian cells six distinct E2F proteins associate with two different DP members and bind to similar DNA elements, which are conserved in the promoters of several genes that are activated in late G_1 and near the boundary G_1/S. E2F gene targets include cell growth regulators such as cyclin A, Cdc2, c-Myc, and proliferating cell nuclear antigen and enzymes such as dihydrofolate reductase, thymidine kinase, ribonucleotide reductase, and DNA polymerase α (5, 6). The importance of the E2F factors for cell cycle progression is further highlighted by the demonstration that transient overexpression of several members of the E2F family is able to induce S phase in quiescent cells in the absence of growth factors (7, 8).

The activity of the E2F factors is in part regulated by members of the pocket protein family, which includes the product of the retinoblastoma tumor suppressor gene (pRB)[1] and the related proteins p107 and p130. These proteins possess a highly conserved A/B pocket domain that is the target of viral transforming proteins such as adenovirus E1A (2). The pocket proteins are also the targets of cyclin-dependent kinase activities, and once hypophosphorylated they can bind the activation domain of the various E2F transcription factors, thereby repressing their transcriptional activity. Furthermore, recent results indicate that the pocket proteins can recruit to the E2F complex a transcriptional repressor such as the HDAC1 histone deacetylase (9, 10). The recruitment of histone deacetylase to promoters containing E2F DNA binding sites is believed to lead to chromatin condensation and to an efficient silencing of transcription. It is therefore now widely believed that the binding of the E2Fs to promoter elements can lead to both repression and activation of transcription, depending on their association with the pocket proteins. Such a concept also provides an explanation for the observation that the E2Fs can act as oncogenes as well as tumor suppressor genes (11, 12).

The mammalian E2F factors have similar primary struc-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ251586.

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[1] The abbreviations used are: pRB, retinoblastoma; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; GUS, β-glucuronidase; CAT, chloramphenicol acetyltransferase; UBI-CEP, ubiquitin-carboxyl extension; CaMV, cauliflower mosaic virus; MES, 4-morpholineethanesulfonic acid; NLS, nuclear localization signal; CDK, cyclin-dependent kinase.

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tures with a highly conserved DNA-binding domain, found near the N terminus, followed by a DP dimerization domain containing a leucine heptad repeat. Next to the dimerization domain is conserved another region, named marked box, which in human E2Fs is the target of the adenovirus E4 protein (13). The C-terminal region contains a transactivation domain that is characterized by the presence of several acidic residues and the presence of a short conserved region involved in the binding to the pocket proteins. E2F-6 lacks the activation domain and the pRB binding region, and it has been shown to function as an inhibitor of E2F-dependent transcriptional activity (14). E2F-1, E2F-2, and E2F-3, but not the other mammalian E2Fs, possess a conserved domain at the N terminus that can bind to cyclin A/CDK2 (15). The DP-1 and DP-2 partners of E2F contain DNA binding and dimerization regions related to the E2F proteins but lack activation domains and other conserved regions (3).

A plant homologue of the retinoblastoma tumor suppressor gene has been discovered and characterized in maize (16–18), suggesting that the transition from G_1 to S phase, a key passage during cell cycle, is regulated similarly in animal and plant cells. The existence of pRB proteins that can interact with plant D-type cyclins and viral replication proteins indicated that homologues of the E2F factors might be present in plant cells and could be involved in the regulation of genes responsible for S phase progression. In this paper we describe the isolation and characterization of DcE2F, a E2F-like gene from carrot cells that is expressed in proliferating cells. We demonstrate that the carrot E2F protein is a functional transcription factor that can bind a canonical E2F *cis*-element in association with a mammalian DP protein and can transactivate through this binding site a reporter gene in both plant and mammalian cells. During the preparation of this manuscript, the isolation of wheat and tobacco E2F homologues was also described (19, 20). Taken together with these results, our data demonstrate that the pRB/E2F pathway is conserved in plants, and the isolation of plant E2F provides a new tool to understand how plant growth and development is controlled.

EXPERIMENTAL PROCEDURES

Plant Materials—Plants of *Daucus carota* L. cv. Lunga di Amsterdam were grown under normal greenhouse conditions. Carrot cell suspension cultures were maintained as described previously (21). For cellular synchronization, quiescent cells from a carrot culture grown to plateau were washed and incubated for 48 h in fresh Muzashige and Skoog liquid medium lacking hormones and sucrose. The cells were released from starvation by dilution in fresh Muzashige and Skoog medium containing growth regulators and sucrose. At different time points after release, small samples were used for DNA synthesis assay by [^{3}H]thymidine pulse labeling experiments, and the remaining aliquots were collected and immediately frozen in liquid nitrogen before isolation of total RNA. For the isolation of carrot nuclei, cells from an actively dividing suspension culture were washed in protoplast isolation buffer (22) and resuspended in approximately 5 volumes of enzyme mixture containing 1% cellulase Onozuka R-10 (Yakult) 0.5% Pectinase (Serva) in protoplast isolation buffer. After incubation for about 6 h at 25 °C, the suspension was centrifuged for 5 min at 200 × *g*, and the pelleted protoplasts were washed three times with protoplast isolation buffer. After resuspension in approximately 10 volumes of ice-cold resuspension buffer (0.4 M sucrose, 25 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.3% Triton X-100, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride), the protoplasts were disrupted in a Teflon homogenizer (1,000 revolutions/min, 5 strokes for three times). The homogenate was then centrifuged for 5 min at 3,000 × *g*, and the pellets were resuspended in two volumes of ice-cold wash buffer (0.4 M sucrose, 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 20% glycerol, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride). The nuclei were finally spun down at 2,000 × *g* for 5 min, and the pellets were stored at −80 °C.

Isolation of Carrot Nuclear Extracts—Pelleted nuclei were resuspended in ice-cold lysis buffer (25 mM Hepes, pH 7.6, 40 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 2 mM dithiothreitol, 20% glycerol, 1 mg/ml antipain, 1 mg/ml leupeptin) and were lysed by adding 0.1 volume of cold 4 M $(NH_4)_2SO_4$. After incubation for 30 min with constant movement at 4 °C, the lysate was centrifuged at 15,000 × *g*, and the nuclear proteins of the supernatant were precipitated by slowly adding 0.5 volume of cold 4 M $(NH_4)_2SO_4$ and incubating 60 min at 4 °C with constant movement. The nuclear proteins were then recovered by centrifugation for 10 min at 4 °C in microcentrifuge and then resuspended in dialysis buffer (same as lysis buffer but without $MgCl_2$) and dialyzed 3 h at 4 °C against the same buffer. The resulting nuclear extracts were divided in small aliquots and stored in liquid nitrogen.

RNA Isolation and Northern Blot Analysis—Poly(A)$^+$ RNA was isolated from carrot cell culture using oligo(dT) cellulose (Roche Molecular Biochemicals) following a standard batch procedure (23). Total RNA was isolated by the hot phenol method (24). For Northern blot analysis, the RNA samples were resolved in formaldehyde gels, transferred to Hybond-N membranes (Amersham Pharmacia Biotech), and hybridized with a DcE2F probe labeled by the random primer method (Amersham Pharmacia Biotech). To verify the level of synchronization, after removal of the DcE2F probe, the filter was subsequently hybridized with a carrot UBI-CEP probe (25).

Isolation of DcE2F cDNAs—To amplify carrot E2F-like cDNAs, 3′-RACE reactions were performed on carrot poly(A)$^+$ RNA using a degenerate primer of sequence *GCGAATTC*MGIMGIATHTAYGA (where I is inosine, M is A/C, H is A/T/C, Y is C/T, and the nucleotides in italics represent the added cloning site) containing all the possible codons for the conserved amino acid sequence RRIYD. Nested PCR reactions were performed with a second primer of sequence *GCGAATTC*GAYATHACIAAYGT containing all the possible codons for the amino acid sequence DITNV. Reverse transcription and PCR reactions were conducted as described previously (26). After electrophoretic analysis of the nested reactions, the major PCR fragment, corresponding to a partial DcE2F cDNA, was isolated and subcloned into the plasmid pBluescriptII KS+ (Stratagene).

To isolate full-length DcE2F clones, approximately 500,000 plaque-forming units from a carrot cell suspension cDNA library (27) constructed in λZAPII (Stratagene) were screened with the partial DcE2F probe. Hybridization and washings were performed as previously reported (26). Two hybridizing plaques were purified and the plasmids containing the DcE2F cDNAs were excised *in vivo* according to the manufacturer's protocol. Sequencing was performed on both strands of the longest cDNA clone (Amersham Pharmacia Biotech sequencing kit).

Production of Recombinant DcE2F and DP-1 Proteins—For the construction of the pRSET-DcE2F expression vector the DcE2F cDNA was digested with the enzymes *Bam*HI and *Hin*dIII, and the resulting DNA fragment, containing the entire DcE2F coding region preceded by 35 base pairs of the upstream untranslated region, was inserted into the corresponding sites of the polylinker of pRSET-A (Invitrogen). The pRSET-DcE2F plasmid was then introduced into *Escherichia coli* BL21(DE3) for the production of a recombinant DcE2F protein carrying a histidine-tagged N-terminal extension of 48 amino acids. The HIS-DcE2F protein was purified under nondenaturing condition by metal affinity chromatography on nickel-nitrilotriacetic acid resin (Qiagen) as previously reported (26). For purification under denaturing conditions, cell lysis and affinity chromatography were performed in phosphate buffer containing 8 M urea. Electrophoretic analysis of the eluted proteins revealed a single polypeptide of the expected dimensions indicating a purification to near homogeneity of the recombinant DcE2F protein. The bacterial GST-DP1 expression vector was introduced into *E. coli* XL-1 blue cells, and the recombinant protein was purified by chromatography on glutathione-agarose (Amersham Pharmacia Biotech) according to manufacturer's instructions.

DcE2F Antiserum and Immunoblotting—The His-DcE2F protein eluted under denaturing conditions was dialyzed and used directly for rabbit immunization. For Western analysis, proteins fractionated by SDS-polyacrylamide gel electrophoresis were transferred to Hybond-C extra membranes (Amersham Pharmacia Biotech) using a semi-dry blotting apparatus (Hoefer Scientific Instruments). The blots were incubated with the anti-DcE2F polyclonal serum, and immunodetection was performed using ECL chemiluminescence detection reagents (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assays—For the electrophoretic mobility shift assays (EMSAs) with carrot nuclear extracts or recombinant DcE2F and DP-1 proteins, the cloned double-stranded oligonucleotides were gel purified and labeled by a fill-in reaction with Klenow DNA polymerase in the presence of [α-^{32}P]dATP (Amersham Pharmacia Biotech). The sequences of the probes were 5′-aattcTTTTCCCGCGCTTTTgaatt-3′ for the canonical E2F binding site (EC) and 5′-aattcTTTTCCATCGCTTTTgaatt-3′ for the mutated E2F binding site (EM). In both sequences the lowercase letters represent the E*co*RI cloning sites. The DNA binding reactions with carrot nuclear extracts were conducted

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incubating 1.5–6 μg of nuclear proteins with 50,000 cpm of radiolabeled oligonucleotide probes and 2 μg of sheared salmon sperm DNA in 15 μl of 25 mM Hepes, pH 7.5, 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, and 10 mM dithiothreitol for 30 min at room temperature. The DNA binding of the recombinant proteins was conducted similarly without adding the salmon sperm DNA. For the competition experiments, increasing amounts of annealed unlabeled oligonucleotides were included in the reactions with the EC probe. The supershift experiments were carried out by preincubating the binding reactions with 1 μl of polyclonal anti-DcE2F serum or preimmune serum for 30 min at room temperature before adding the probe. The protein-DNA complexes were electrophoresed for 3 h at 4 °C on 4% polyacrylamide gels in 0.5× TBE.

The EMSAs in mammalian cells were performed essentially as described (28) using a E2F binding probe of sequence 5′-ATTTAAGTT-TCGCGCCCTTTCTCAA-3′. Extracts were prepared from U2OS cells transfected with each of the indicated expression plasmids (6 μg/100-mm dish). 10 μg of the extracts were used for Western blotting as shown in Fig. 8*A*. To determine the protein composition of the complexes in EMSAs, the following antibodies were used: KH95 (anti E2F-1) (29), TFD10 (anti-DP-1) (8), and 12CA5 (anti-hemagglutinin tag) (30). To generate an expression plasmid for DcE2F, the full open reading frame of DcE2F was amplified by PCR using primers specific for DcE2F and containing *Bam*HI restriction sites in the ends. The PCR product was subsequently cloned into *Bam*HI-digested pCMVHA (29) to generate pCMVHADcE2F. pCMVE2F-1 and pCMVDP-1 have been described previously (29, 31).

Transactivation Assays—For the construction of the DcE2F effector plasmid to be used in plant protoplasts, the cDNA was digested with *Hin*dIII, blunt-ended with Klenow DNA polymerase, and then digested with *Bam*HI yielding a DNA fragment, containing the entire DcE2F coding region. This fragment was inserted into the *Bam*HI and blunt-ended *Sac*I sites found upstream of the terminator sequence of the *nos* gene of *Agrobacterium tumefaciens* cloned as a *Sac*I/*Eco*RI fragment into pUC19. The DcE2F/NOS gene fusion was then digested with *Bam*HI and *Eco*RI, and the resulting DNA fragment was inserted downstream of the cauliflower mosaic virus (CaMV) duplicated 35 S promoter in the plasmid pFF19 (32) to give rise to the p35 S-DcE2F construct. The p35 S-DP1 effector was obtained by cloning into pFF19 a *Bam*HI/*Sal*I-digested DNA fragment from pBSK-DP1 (29). For the construction of the chimeric 6XE2F-minimal 35 S promoter-GUS reporter construct, a DNA fragment containing the six E2F binding sites was isolated from the pGL3TATAbasic-6XE2F vector (8) after digestion with *Asp*I, blunt ending with Klenow DNA polymerase and digestion with *Xho*I. This fragment was then cloned into *Hin*dIII (filled in with Klenow DNA polymerase) and *Sal*I sites of pBI221.9 to give rise to the pBI221-E2F reporter construct.

The transactivation experiments in plant cells were conducted with protoplasts isolated from carrot somatic embryos at the heart torpedo stages of development. The embryos were incubated with 1% cellulase and 0.2% pectinase in protoplast isolation buffer (27.2 mg/liter KH_2PO_4, 101 mg/liter KNO_3, 1.4 g/liter $CaCl_2$, 246 mg/liter $MgSO_4$, 0.16 mg/liter KI, 0.025 mg/liter $CuSO_4$, 10 mM MES, and 0.7 M sorbitol, pH 5.5). The protoplasts were pelleted and washed twice in the same buffer without enzymes and then resuspended at a density of 7.5×10^6 protoplasts ml^{-1} in 10 mM Hepes, 130 mM KCl, 10 mM NaCl, 4 mM $CaCl_2$, 0.2 M mannitol, pH 7.2. For electroporation, aliquots of 0.4 ml of the protoplast suspension were placed in the electroporation cuvettes (ECU-104, Equibio) and then mixed with 10 μg of each test plasmid, 5 μg of CaMV 35 S-CAT plasmid (as internal control) and sonicated calf thymus DNA to a total of 50 μg of DNA. After incubation on ice for 10 min, the electrical pulse was delivered by the Electroporator II (Invitrogen) charged to 330 V electric potential, 500 microfarad capacitance, and 500 Ω resistance. After 10 min on ice, the protoplasts were diluted with 2.1 ml of culture medium (Gamborg's B5 medium supplemented with 300 mg/liter $CaCl_2$-H_2O, 825 mg/liter NH_4NO_3, 100 mg/liter sodium pyruvate, 200 mg/liter malic acid, 200 mg/liter citric acid, 300 mg/liter casamino acids, 200 mg/liter yeast extract, 20 g/liter saccarose, 76 g/liter mannitol, 0.1 mg/liter 2,4-dichlorophenoxyacetic acid, 0.2 mg/liter 6BAP, 10^{-6} M α-naphtaleneacetic acid, 5×10^{-7} M zeatin riboside, pH 5.6) and incubated 40 h in the dark at 25 °C. GUS activity was measured as described by Gallie *et al.* (33). CAT assays was performed using the CAT detection kit (Roche Molecular Biochemicals) as described by the manufacturer.

Transactivation assays in mammalian cells were performed as described previously (8) using pGL3TATAbasic-6xE2F as reporter construct and pCMVβ-gal (CLONTECH) to normalize for transfection efficiency. For a 60-mm tissue culture dish, 30 ng of the indicated expression plasmid was transfected in combination with 1 μg of pGL3TATAbasic-6xE2F and 500 ng of pCMVβ-gal. Cells were harvested 36 h after addition of the DNA-calcium phosphate coprecipitate.

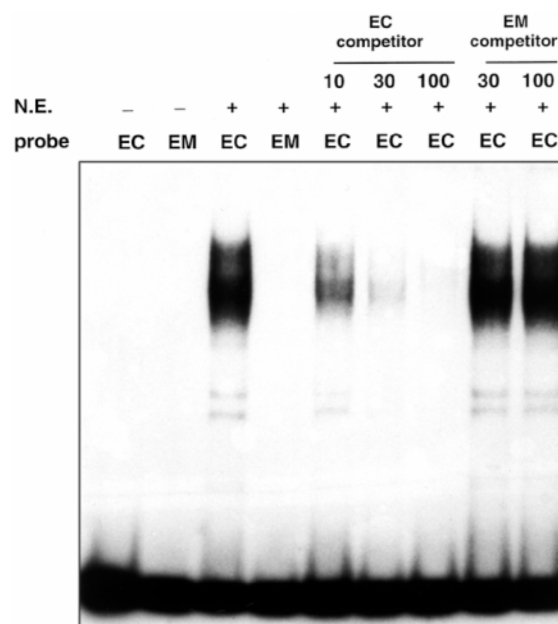

FIG. 1. **Detection of E2F-like DNA binding activities in carrot nuclear extracts.** EMSAs performed with carrot nuclear extracts (6 μg) show the formation of DNA-protein complexes with a probe containing a canonical E2F binding site (*EC*) but not with a probe containing critical mutations of the consensus sequence (*EM*). The binding to the E2F probe is abolished by the addition of increasing amounts of unlabeled EC probe (*EC competitor*) but is unaffected by the addition of similar amounts of unlabeled EM probe (*EM competitor*), confirming the specificity of the carrot E2F-like DNA binding activities. *N.E., nuclear extract.*

RESULTS

Carrot Nuclear Extracts Contain E2F-like DNA Binding Activities—Following the discovery of plant homologues of the retinoblastoma tumor suppressor protein, a yeast two-hybrid assay has shown that the maize pRB protein can bind to human and *Drosophila* E2F transcription factors (34). A transcription interference assay has further shown that maize pRB can inhibit E2F-dependent transcriptional activation in animal cells (34). These remarkable properties of maize pRB strongly indicated that plant homologues of the animal E2F family of transcription factors were likely to exist and to be involved in the regulation of genes responsible for the progression of the cell cycle through the G_1/S transition. To investigate the presumed presence of E2F-like factors in plant cells, DNA binding assays using carrot nuclear extracts were performed (Fig. 1). Animal E2F/DP complexes as well as cellular E2F DNA binding activities have been shown to specifically recognize a consensus sequence TTT(C/G)(G/C)CG(C/G), and mutations of the internal invariable CG doublet have been shown to greatly reduce E2F binding efficiency (4). Accordingly, EMSAs on carrot nuclear extracts were performed with a canonical E2F binding probe of sequence 5′-TTTTCCCGCGCTTTT-3′ and with a mutated probe of sequence 5′-TTTTCCATCGCTTTT-3′. As shown in Fig. 1, carrot nuclear extracts contain DNA binding activities, which, as expected for E2F complexes, recognize the canonical sequence but are unable to bind to the mutated probe. Furthermore, as also shown in Fig. 1, competition experiments with excess of unlabeled probes confirmed the binding specificity for the canonical sequence and demonstrated that E2F-like DNA binding activities exist in carrot cells.

Isolation of the Carrot DcE2F cDNA—Although E2F-like DNA binding activities were detected in carrot nuclear ex-

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tracts, it remained uncertain whether they could correspond to a real functional homologue of the E2F transcription factors or could simply represent unrelated nuclear factors that are able to discriminate between the two EMSA probes. To verify the molecular nature of these DNA binding activities, we proceeded to the isolation of a carrot homologue of animal E2Fs. The indication that all the E2F binding activities in animal cells are heterodimers, formed by E2F and DP partners, precluded the possibility to isolate E2F homologues by Southwestern screening procedures. All the animal E2F and DP factors, however, possess highly conserved DNA-binding domains that are found near the N termini of the proteins. In view of the perfect identity of a portion of this domain in the mammalian E2Fs and in a E2F homologue of *Drosophila melanogaster* (35, 36), we proceeded to the isolation of a plant E2F homologue using PCR techniques. A pair of degenerate primers encoding the conserved amino acid sequences RRIYD and DITNV were used in 3′-RACE reactions performed with poly(A)$^+$ RNA isolated from actively dividing carrot cells. Using the same strategy we also attempted the isolation of a DP homologue with a pair of degenerate primers encoding the amino acid sequences RRVYD and DALNV, which are conserved in the human and *Drosophila* DP proteins. Although the 3′-RACE reactions performed with the DP-specific primers were unsuccessful, the reactions performed using the E2F-specific primers led to the isolation of a PCR fragment of about 1.2 kilobases, which was cloned and sequenced. All the clones analyzed corresponded to a unique partial cDNA coding for a carrot E2F-like factor. The subsequent screening of a carrot cDNA library led to the isolation of two longer cDNA clones corresponding to distinct transcripts of the same carrot E2F-like gene, which we called DcE2F. Both cDNAs contain a complete open reading frame and are identical to the RACE products over the overlapping region. In Fig. 2 is shown the sequence of the longest cDNA clone along with the deduced amino acid sequence of the DcE2F protein. The shorter cDNA starts 64 nucleotides downstream of the 5′ end of the longer clone and shows a considerable smaller size resulting from the position of its poly(A) tail, which is located 386 nucleotides upstream of the one of the longer cDNA. The longest DcE2F cDNA is 2191 base pairs long, excluding the poly(A) tail and contains an open reading frame coding for a potential protein of 431 amino acids with a predicted molecular mass of 47.5 kDa. The putative ATG start codon is preceded by a 5′-untranslated region of 258 base pairs containing two in-frame TAA termination triplets and two additional upstream ATGs, both of which are followed shortly by in-frame stop codons.

The DcE2F protein shows extended homology to the recently reported E2F homologues of wheat and tobacco (19, 20), with 50 and 54% amino acid identity, respectively. Compared with animal E2Fs, the carrot protein shows the highest homology to human E2F-2 with 32% amino acid identity. As reported in Fig. 3, a sequence alignment with tobacco NtE2F, wheat TmE2F and human E2F-2 reveals a remarkable conservation of the DNA-binding domain that shows 75% amino acid identity with the corresponding domain in the E2F-2 sequence. The DP dimerization domain and the marked box are also well conserved in the plant E2Fs (Fig. 3) and for the DcE2F protein show, respectively, up to 52 and 62% amino acid identity with human E2Fs. The pRB binding/transcriptional activation domain, which is conserved in five of the human E2Fs, is not well conserved in the plant E2F proteins. A cyclin A-binding domain is also absent in the plant E2F sequences; however, this domain is shared only by human E2F-1, E2F-2, and E2F-3 and could be a specific feature of these tree members because it is not observed in any other animal E2F. Recently, it has been

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ttactaataaatagataacatatagttgtcc(polyA)                       2191
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FIG. 2. **Nucleotide sequence and deduced amino acid sequence of the longest DcE2F cDNA.** The two short upstream open reading frames in the 5′-untranslated leader sequence are *underlined*. The position of the poly(A) tail in the shorter cDNA is indicated by a *black arrowhead*. The conserved RRIYDITNV peptide sequence, encoded by the degenetate primers used for the 3′-RACE reactions, is *underlined* in the DcE2F protein.

demonstrated that the cyclin-binding domain of E2F-1, E2F-2, and E2F-3 contains a nuclear localization signal (NLS) of sequence P(A/V)KR(K/R)L(D/E)L that is able to efficiently direct these E2Fs to the nucleus and to promote their activity. E2F-4 and E2F-5, lacking the cyclin-binding domain, are predominantly localized in the cytosol and are transiently directed to the nucleus only during the G_1 phase of the cell cycle, possibly through interaction with associated factors such as DP-2 (8, 37, 38). Although DcE2F lacks a conserved cyclin-binding domain, it possess a N-terminal NLS of the SV40 large T-antigen type that should be able to direct the protein to the nucleus independently of interactions with other nuclear factors. This NLS of sequence PSKRKP starts at position 35 of the DcE2F protein

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FIG. 3. **Amino acid sequence alignment of DcE2F with tobacco NtE2F, wheat TmE2F, and human E2F-2.** Conserved residues are shown with a *black background*, whereas amino acid similarities have a *gray background*. The conserved functional domains and the putative NLS are indicated.

and is partly similar to the NLS of the three human E2Fs.

Because human cells possess several E2F genes, we performed a Southern blot analysis on carrot DNA using the DcE2F cDNA as a probe to verify whether carrot cells could contain more than one E2F homologue. Although the results of this analysis are consistent with the presence of only one E2F gene in the carrot genome (data not shown), we cannot exclude the possibility that other carrot genes could encode distantly related E2F proteins.

The Expression of the DcE2F Gene Is Induced upon Re-entry into Cell Cycle—Studies on the expression of some of the human E2F genes during progression of the cell cycle have revealed distinct patterns of transcription that are specific for different E2F members. E2F-1 and E2F-2 transcripts are almost undetectable in quiescent cells, and following serum stimulation, their expression is strongly induced as cells re-enter cell cycle and progress from G_1 to S phase. Conversely, E2F-3, E2F-4, and at a lower level also E2F-5 are clearly expressed in quiescent cells, but also their expression increases during re-entry into cell cycle. In cycling cells, the expression of E2F-1, E2F-2, and E2F-3 remains relatively constant, whereas the expression of E2F-4 and E2F-5 fluctuates, reaching a peak in mid-G_1 and returning to the initial levels as cells enter S phase (5, 39). To investigate the expression of the DcE2F gene in carrot cells during re-entry into cell cycle, Northern blot analyses were conducted on total RNA isolated from a carrot cell culture synchronized by starvation (Fig. 4*A*). The efficiency of synchronization was determined by monitoring [^{3}H]thymidine uptake (Fig. 4*B*). The RNA samples were isolated from quiescent cells of a culture at plateau (Fig. 4*A*, *lane pl*) and from cells harvested at various times after the release from starvation (Fig. 4*A*, *lanes 0–15*). Equal amounts of RNA, as verified by staining with ethidium bromide (data not shown), were analyzed for each sample. To further evaluate the efficiency of synchronization, the same blot was also hybridized with a probe for a ubiquitin-carboxyl extension mRNA from carrot (UBI-CEP), which shows a cell cycle-regulated expression restricted to the late G_1 phase (25). The efficiency of synchronization and its extension in time are cell culture dependent, and, as shown by [^{3}H]thymidine uptake and by hybridization with the probe UBI-CEP, our carrot cell culture could sustain only a partial synchronization. Nevertheless, as shown in Fig. 4*A*, the DcE2F transcripts are hardly detectable in quiescent cells, and their expression increases remarkably after release from starvation reaching a peak after 5 h similarly as the UBI-CEP mRNA. Our results therefore suggest that the expression of the DcE2F gene, like in the case of mammalian E2F-1 and E2F-2, is strongly induced during re-entry into cell cycle. Moreover,

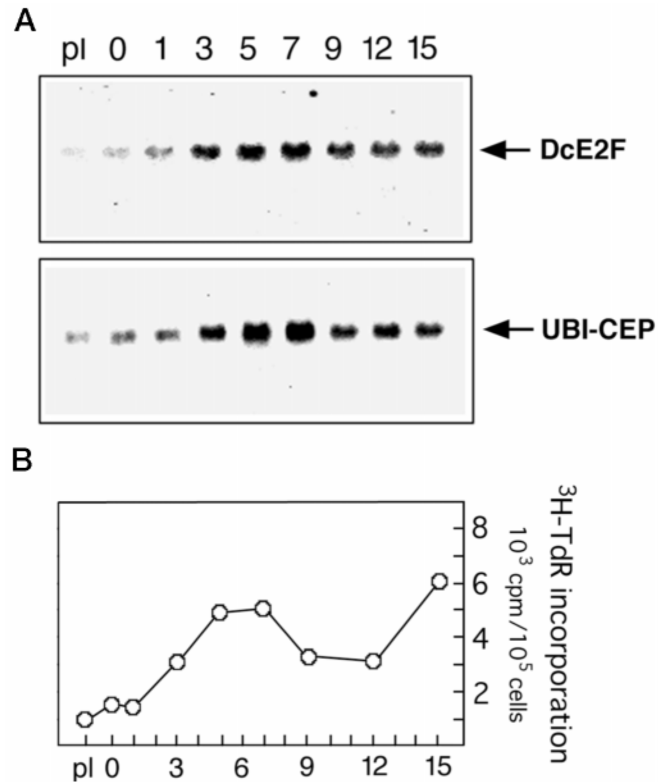

FIG. 4. **Expression of the DcE2F gene is induced in quiescent carrot cells re-entering cell cycle.** *A*, expression of the DcE2F gene in partially synchronized carrot cells. The Northern blot analysis was conducted on total RNA isolated from a cell culture at plateau (*pl*) and from aliquots of a cell culture subjected to starvation (*0*) harvested at different hours after readdition of nutrients (*1*, *3*, *5*, *7*, *9*, *12*, and *15*). Each sample contained equal amounts of RNA as also evaluated by staining with ethidium bromide prior blotting. The blot was first hybridized with the DcE2F probe (DcE2F) and subsequently analyzed with a probe for carrot UBI-CEP. *B*, [^{3}H]thymidine uptake of the partially sychronized carrot cell culture. DNA synthesis was monitored by [^{3}H]thymidine pulse labeling experiments on samples of the same aliquots used for RNA isolation.

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further resembling the expression of UBI-CEP mRNA, DcE2F expression appears to decrease slightly after 9 h from release, suggesting that, as in the case of mammalian E2F-4 and E2F-5, the DcE2F gene could be regulated during cell cycle progression in proliferating cells. In agreement with such a notion, the expression of tobacco NtE2F in synchronized BY-2 cells has been recently shown to fluctuate during cell cycle, peaking in the late G_1 phase (20), and the expression of wheat TmE2F has been shown to be up-regulated during the transition to the S phase in cells partially synchronized by hydroxyurea treatment (19).

Carrot E2F Is a Ubiquitous Protein—To verify the presence and distribution of the DcE2F protein, Western blot analyses were carried out on total extracts from carrot cell culture and from various tissues of 40-day-old plantlets. To obtain specific antibodies, the coding region of the DcE2F cDNA was expressed in *E. coli* to produce a recombinant DcE2F protein carrying a N-terminal extension of 48 amino acids containing six consecutive histidine residues. The His-tagged DcE2F protein was purified to near homogeneity by nickel-chelate chromatography and was used to raise polyclonal antibodies in rabbit. The Western blot analyses revealed that in all the tissue samples tested the anti-DcE2F serum recognize a single protein, which in agreement with the lack of the N-terminal extension is slightly smaller than the purified His-DcE2F protein (Fig. 5). The apparent molecular mass of both recombinant and endogenous DcE2F is slightly larger than predicted, recalling the electrophoretic behavior of wheat TmE2F and human E2F-1 (19, 28). A single endogenous E2F protein of the same size is also recognized in extracts from carrot cell cultures (data not shown).

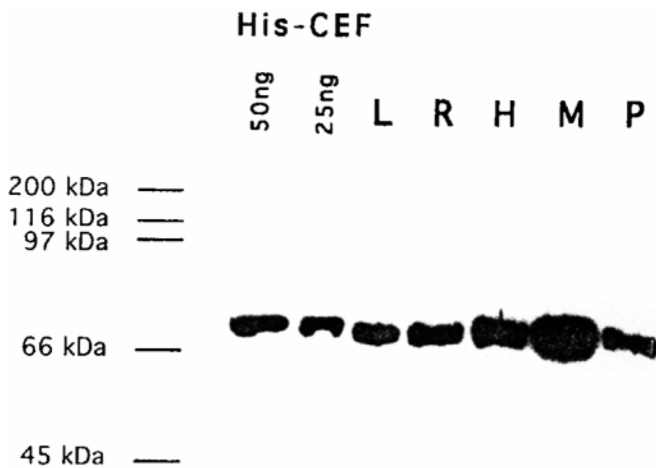

FIG. 5. **Immunodetection of the DcE2F protein.** Western blot analysis with total extracts from carrot leaves (*L*), roots (*R*), hypocotyls (*H*), apical meristems (*M*), and petioles (*P*) isolated from 40-day-old plantlets. 15 μg of each extract was loaded on an 10% SDS-polyacrylamide gel and processed for Western blotting as described under "Experimental Procedures." Two different amounts of recombinant DcE2F protein (*His-CEF*) were included as positive controls.

The DcE2F Protein Recognizes a Consensus E2F Binding Site in Association with a DP Protein—Having previously detected DNA binding activities in carrot nuclear extracts that can recognize a canonical E2F binding consensus sequence, we assessed whether recombinant DcE2F can show a similar DNA binding specificity. Furthermore, in view of the fact that the human E2Fs are known to bind to DNA prevalently as heterodimers with a DP protein, we investigated whether DcE2F could also need a partner to recognize E2F *cis*-elements. Because plant DP homologues have not yet been reported, the DNA binding assays were performed using human DP-1 produced in bacteria as a recombinant GST-DP1 fusion protein. The EMSAs were performed incubating various amounts of the His-DcE2F and GST-DP1 proteins with DNA probes containing a canonical (EC) or a mutated (EM) E2F consensus sequence. As shown in Fig. 6*A*, His-DcE2F together with GST-DP1 bind efficiently to the EC probe without recognizing the mutated sequence. Furthermore, DcE2F alone shows only a negligible binding to the EC probe, and its DNA binding capacity increases proportionally to the amount of recombinant DP-1 included in the sample. Because GST-DP1 alone does not recognize the EC probe (data not shown), it is clear that, as reported for the human E2Fs, the high affinity binding of DcE2F to the canonical sequence requires the formation of a heterodimer with a DP partner. Competition experiments with canonical or mutated E2F binding DNA sequences confirmed the binding specificity of the DcE2F/DP-1 complex (Fig. 6*B*). A 100-fold excess of unlabeled canonical sequence can totally abolish the binding of the DcE2F/DP-1 complex to the labeled probe, whereas a similar excess of mutated probe has almost no effects on DNA recognition. The DcE2F/DP-1 complex therefore shows specificity similar to that of the endogenous E2F-like activities (Fig. 1). To determine whether DcE2F is indeed a component of the E2F-like activities detected in carrot nuclear extract, su-

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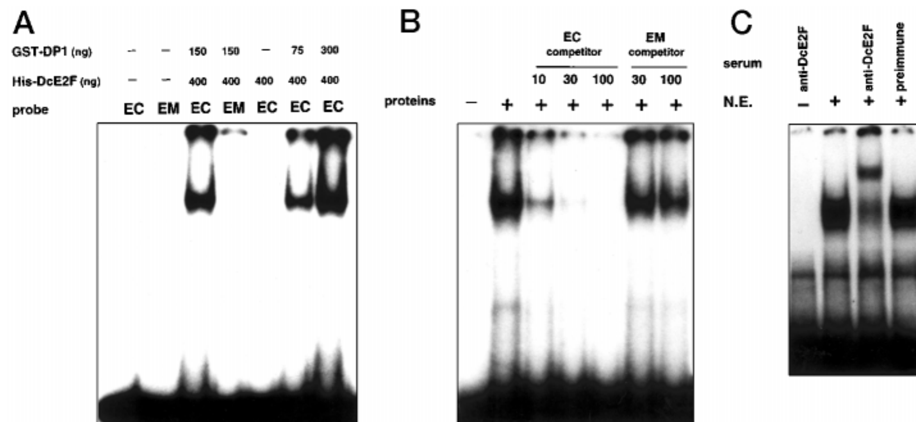

FIG. 6. **DNA binding properties of DcE2F.** *A*, DcE2F binds the DNA in association with DP-1. EMSA showing the binding of the His-DcE2F/GST-DP1 complex to the canonical E2F binding site (*EC*) but not to the mutated site (*EM*). The DNA binding capacity of His-DcE2F is dependent on the amount of GST-DP1 included in the sample. *B*, binding specificity of the His-DcE2F/GST-DP1 complex for the canonical (*EC*) E2F binding probe as shown by competition with 10–100-fold excess of unlabeled EC probe (*EC competitor*) and by lack of competition with 30- and 100-fold excess of unlabeled mutated probe (*EM competitor*). *C*, the DcE2F protein is a component of the E2F-like DNA binding activities contained in carrot nuclear extracts. Polyclonal anti-DcE2F serum (anti-DcE2F) supershifts the protein-DNA complexes formed with the canonical E2F binding probe, whereas preimmune serum has no effects on these complexes.

pershift experiments with anti-DcE2F antibodies were performed. As shown in Fig. 6*C*, the addition of DcE2F antiserum to the nuclear extracts supershifted the binding complex, confirming the molecular nature of the E2F-like activities of carrot cells.

DcE2F Is a Transcriptional Activator—Despite the high conservation of the DNA binding, DP dimerization, and marked box domains, the pRB binding domain is poorly conserved in the DcE2F protein. Furthermore, the region surrounding this putative pRB binding domain is not homologous to the activation domain of animal E2Fs. In consideration of the presence in human cells of several E2F members, one of which lacks an activation domain and functions as a repressor, it was interesting to understand whether DcE2F can act as a transcriptional activator. To address this question we tested the transcriptional activity of DcE2F *in vivo* by performing transactivation experiments in carrot protoplasts (Fig. 7). The reporter gene used for these assays (Fig. 7*A*, pBI221-E2F) consisted of a minimal −67-base pair CaMV 35 S promoter fused to the GUS gene (pBI221.9), upstream of which was inserted a DNA fragment with six consecutive canonical E2F *cis*-elements. The DcE2F effector plasmid (Fig. 7*A*, p35 S-DcE2F) consisted of the entire DcE2F coding region placed under the control of the double CaMV 35 S promoter in the expression plasmid pFF19 (32). The same promoter was also used to drive the expression of human DP-1 (Fig. 7*A*, p35 S-HsDP1). To account for variations in the efficiency of transformation, a CAT reporter construct was included in all the experiments, and in each case the GUS activity was normalized against the CAT activity. Whereas the transactivation with DP-1 as only effector was done twice, each other experiment was performed three times with consistent results, and the averaged normalized GUS activity is reported in Fig. 7*B*. Transient expression of the pBI221-E2F reporter gene in carrot protoplasts yielded a very low GUS activity that is only slightly higher than the one obtained with the pBI221.9 construct. This indicates that endogenous E2F-like binding activities are not sufficient to activate the minimal promoter containing the six E2F binding sites. Furthermore, transient expression of either effector alone together with pBI221-E2F did not increase the GUS activity considerably, but as shown in Fig. 7*B*, co-expression of both DcE2F and DP-1 effectors was able to transactivate efficiently pBI221-E2F via the six E2F binding sites, giving an increase in GUS expression of over 15-fold.

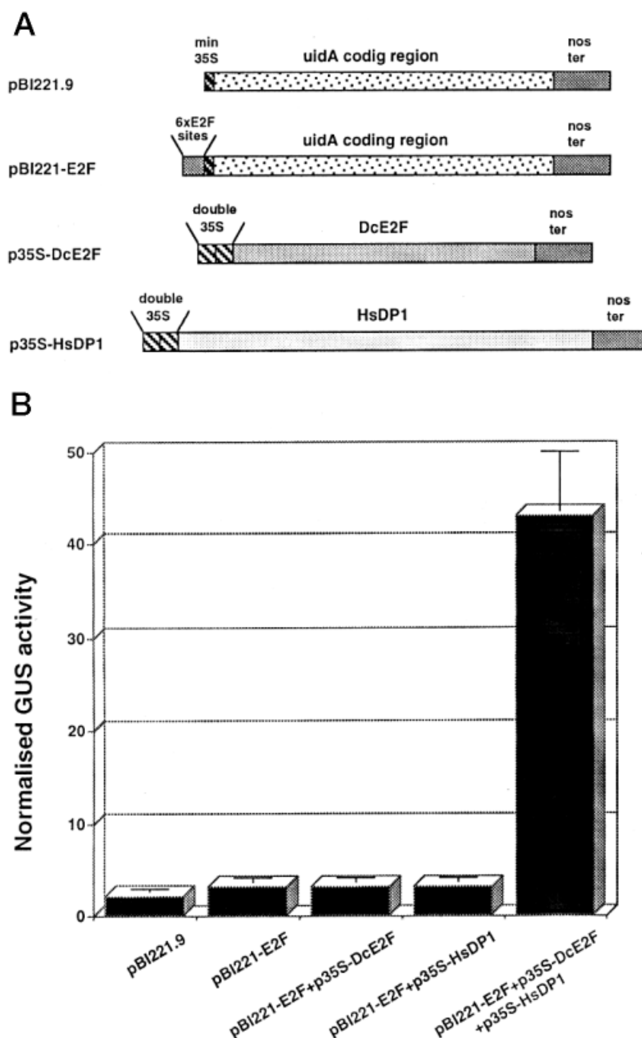

FIG. 7. **DcE2F, together with human DP-1, activates transcription in carrot protoplasts via canonical E2F binding sites.** *A*, structure of GUS (*uidA* gene) reporter constructs and of the DcE2F and DP-1 effector constructs used in this study. *B*, GUS activity in carrot protoplasts following transient expression of the indicated constructs. Cotransfection of a CAT reporter construct was included in all the experiments to normalize for transfection efficiency. The average normalized GUS values obtained in at least two independent transfections are shown. *Bars* represent the standard deviations.

DcE2F Is Able to Activate E2F-dependent Transcription in Animal Cells—To investigate whether DcE2F could have similar activities as the animal E2Fs in mammalian cells, the full-length DcE2F cDNA was inserted into a mammalian expression vector and transfected into mammalian cells with or without a plasmid expressing human DP-1. DcE2F was epitope-tagged at its N terminus to allow the recognition of the expressed protein. As shown in Fig. 8*A*, DcE2F is efficiently expressed in mammalian cells, and the coexpression of human DP-1 does not change the expression of DcE2F. Extracts were prepared for EMSA using a consensus E2F DNA binding site as a probe to evaluate whether ectopically expressed DcE2F formed DNA-binding complexes *in vivo*. As shown in Fig. 8*B*, the expression of DcE2F alone did not lead to any detectable increase in E2F DNA binding activity in mammalian cells; however, when co-expressed with DP-1, a strong increase in DNA binding activity was observed. This DNA binding activity was specific (data not shown), and it was shown to contain the epitope-tagged DcE2F and DP-1 (supershifts with specific antibodies in the right panel of Fig. 8*B*). As a control for these experiments, we used human E2F-1 expressed with and without human DP-1. The expression of human E2F-1 alone led to a slight increase in E2F DNA binding activity of the transfected cells (Fig. 8*B*); however, in agreement with previously published results (29, 40, 41) the coexpression of E2F-1 with DP-1 led to a significant increase in DNA binding activity. To test whether DcE2F has transactivating potential in mammalian cells, the DcE2F expression plasmid was transfected with or without a human DP-1 expression plasmid and a reporter construct containing six E2F DNA binding sites. As shown in Fig. 8*C*, DcE2F is capable of transactivating the E2F-dependent reporter construct in a manner that is strictly dependent on the coexpression of human DP-1. These data are in complete agreement with the transactivation potential of DcE2F in plant cells (Fig. 7), in which the coexpression of DP-1 is also required for transactivation of an E2F-dependent reporter construct. However, these results are in contrast to human E2F-1, which under the conditions shown here efficiently transactivates the E2F reporter construct in the absence of DP-1 coexpression. In summary, our results show that DcE2F has the ability to transactivate an E2F-dependent reporter construct in mammalian cells, and that it is capable of binding human DP-1 *in vivo*. Furthermore, our data show that DcE2F is less potent as a

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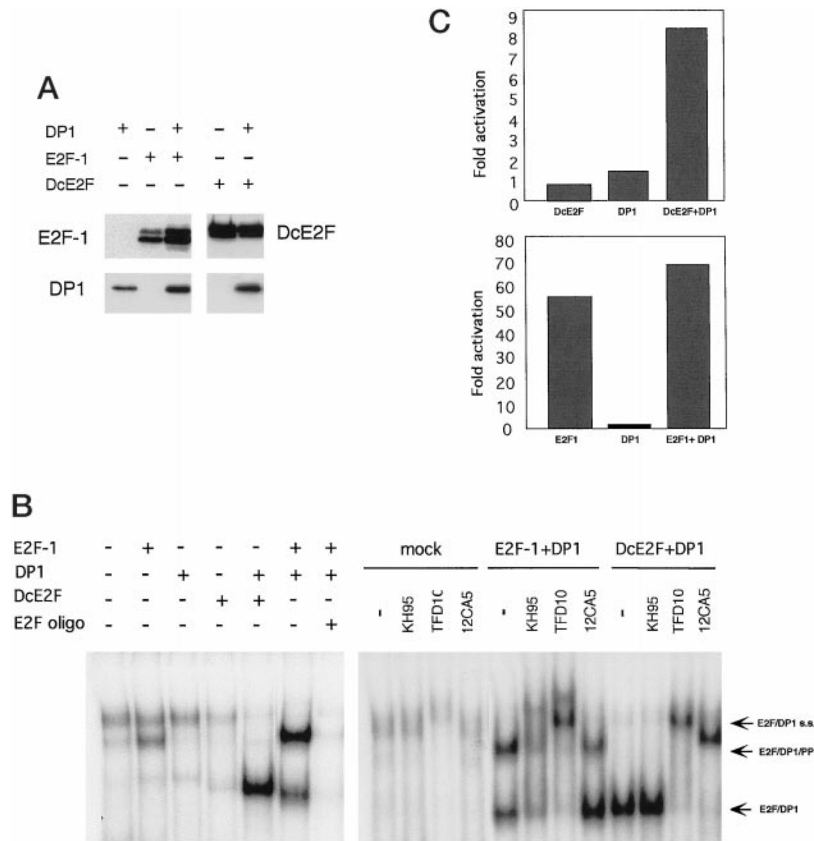

FIG. 8. **The DcE2F/DP-1 complex activates transcription in human cells.** *A*, efficient expression of DcE2F in mammalian cells. Extracts were prepared from U2OS cells transfected with expression plasmids with the indicated genes. 10 μg of each extract was loaded on an 8% SDS-polyacrylamide gel and processed for Western blotting as described under "Experimental Procedures." E2F-1 expression was revealed by probing the blot with a monoclonal antibody to E2F-1 (KH95), DP-1 expression by a DP-1 monoclonal antibody (TFD10), and DcE2F by a monoclonal antibody to the hemagglutinin epitope tag (12CA5). *B*, DcE2F/DP-1 complexes form in mammalian cells and bind the E2F DNA-binding site. EMSA performed with extracts from U2OS cells transfected with the indicated expression plasmids shows the formation of specific DNA-protein complexes with a probe containing a canonical E2F binding site (*left panel*). Supershifts with the specific antibodies described above confirmed the nature of the retarded complex (*right panel*). The E2F/DP-1/PP complex indicates a complex between an E2F/DP-1 and a pocket protein (pRB, p107, or p130), *s.s.* indicates supershift. *C*, transactivation of E2F reporter by DcE2F. Expression plasmids for the indicated proteins were transfected into U2OS cells together with a luciferase reporter construct containing six E2F DNA binding sites upstream of a TATA element. Cotransfection of a plasmid expressing β-galactosidase was performed to normalize for transfection efficiency. Cells were harvested 36 h after transfection and analyzed for luciferase and β-galactosidase activity. Fold activation compared with control is shown.

transcriptional activator than human E2F-1 in mammalian cells, and because it binds human DP-1 and a consensus E2F DNA binding site efficiently, it may suggest that DcE2F interacts less efficiently with the basic transcriptional machinery in mammalian cells than human E2F-1.

DISCUSSION

In recent years the study of the plant cell cycle has made considerable progress thanks to the isolation of plant genes coding for cyclins, CDKs, cyclin-dependent kinase-inhibiting proteins, and DNA replication licensing factors (42). These findings suggested that in plants, as in other eukaryotes the CDKs and their associated proteins are key regulators of the cell cycle. However, the nature of the CDK targets and their mode of action remain largely unknown. The discovery of E2F homologues in carrot, wheat (19), and tobacco (20) demonstrates the existence of a pRB/E2F pathway that is likely to control cell proliferation in plants. Although S phase-specific transcription factors unrelated to E2F have been recently involved in the control of proliferating cell nuclear antigen genes in plants (43), other genes that are activated in late G_1 and near the boundary G_1/S could be regulated by plant E2Fs.

Like the wheat and tobacco homologues, the carrot DcE2F protein possesses a similar domain organization as the animal E2Fs. The most strikingly conserved region, as also reported for the animal E2Fs, corresponds to the DNA-binding domain. The extensive homology of this domain, in fact, allowed the isolation of the carrot E2F homologue by PCR techniques. Other highly conserved regions include the dimerization domain and the marked box, whereas the C-terminal pRB binding and activation domains are partly conserved between the plant E2Fs but are very distinct when compared with animal E2Fs. This could explain why the C-terminal region of wheat TmE2F can mediate an efficient binding to maize pRB in a yeast two-hybrid assay, but it is not recognized well by the human pocket proteins (19). In wheat TmE2F a putative pRB binding region of consensus DYX_6DX_4DMWE has been identified upon comparison with animal E2Fs, but this consensus is slightly different in the DcE2F protein where it shows the sequence DYX_4DX_6DMWK. It remains to be verified whether the DcE2F C-terminal region is also able to bind maize pRB and whether these similar consensus sequences are indeed essential for pRB recognition.

The remarkable conservation of the DNA binding and dimerization domain of plant E2Fs predicted that they could recognize a DNA sequence similar to that of the animal E2Fs (19). Although it is known that the DP proteins have an influence on the DNA-binding site specificity of the E2F complexes (44), our analysis indicates that the DcE2F protein has DNA binding properties very similar to those of animal E2Fs. Furthermore, the high affinity binding of DcE2F to the canonical DNA se-

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quence requires the formation of a heterodimer with DP-1. This requirement suggests that functional homologues of the DP proteins exist in plants. Nevertheless, our attempts to isolate a carrot DP-like gene using a similar PCR approach as with DcE2F have been unsuccessful so far. However, the plant functional homologues of DP could be less conserved than the E2Fs in their DNA-binding domain. Moreover, E2F partners not homologous to DP could be involved in the formation of active E2F complexes in plant cells. This hypothesis finds support in a recent report indicating that human cells contain a protein different from DP that can stimulate E2F-dependent transcription by heterodimerizing with E2F-1 through a Myc-type helix-loop-helix motif (45).

In view of the poor conservation of the putative pRB binding and activation domains, it was crucial to verify whether the carrot E2F protein is a functional trancriptional activator. For this analysis the DcE2F cDNA was inserted in an expression vector containing the double 35 S CaMV promoter and directing a constitutive high level of expression in plant cells. Together with human DP-1, transactivation of a GUS reporter gene in plant protoplasts via multimers of the canonical E2F binding DNA sequence confirmed the activation potential of the carrot factor. Remarkably, DcE2F was also able to transactivate, although less efficiently than human E2F-1, an E2F responsive promoter in human cells, suggesting that despite low sequence similarity, the activation domain of plant and human E2Fs are functionally conserved. Consistent with the results of the EMSA analysis, which indicate the necessity of a DP partner for high affinity DNA binding, expression of the DcE2F effector alone was not able to transactivate the 6XE2F-35 S promoter in carrot protoplasts. Furthermore, because it is known that heterodimerization with DP-1 is not sufficient to target E2Fs to the nucleus (8, 38), it can be inferred that DcE2F possesses a functional NLS and that the incapacity of DcE2F to transactivate by itself is not due to its cytosolic localization, as in the case of E2F-4 and E2F-5, but derives from the need of a suitable partner for efficient DNA binding. Interestingly, the DcE2F-dependent transactivation obtained in carrot cells differs with what previously reported for E2F-1 in human cells where, although increased considerably by the co-expression of DP-1, transient expression of E2F-1 alone was able to transactivate via the E2F binding sites (Fig. 8*C*). It is likely, therefore, that both DcE2F and the putative carrot DP-like dimerizing protein are limiting factors for E2F-dependent expression in carrot protoplasts and that overexpression of only one of the two partners is not sufficient to give transactivation.

The DcE2F gene is preferentially expressed in proliferating cultured cells, and Northern blot analyses on partially synchronized carrot cells suggest that, like the human E2F-1 and E2F-2 genes, the DcE2F gene is hardly expressed in G_0 and is induced as cells re-enter cell cycle and progress from G_1 to S phase. This pattern of expression is consistent with an involvement of DcE2F in the induction of cell proliferation. Furthermore, in agreement with similar analyses on the expression of wheat and tobacco E2Fs (19, 20), these results indicate that the transcription of DcE2F could be regulated in a phase-specific manner during carrot cell cycle progression. Nevertheless, although at a very low level, undividing cells are likely to express the DcE2F gene. In this respect, Western blot analyses have shown that the DcE2F protein is distributed ubiquitously in all the plant tissues examined, suggesting a cell division-independent accumulation of the carrot E2F factor. The accumulation of DcE2F protein in differentiated undividing cells could serve important regulatory functions and, as for the human E2Fs, the carrot factor could be subjected to post-translational or targetting control. Furthermore, as shown by the DNA binding and transactivation studies, the activity of the plant E2F proteins is likely to depend on specific interactions with plant DP-like partners and is expected to be strongly regulated by associated factors such as the plant pRB-related proteins. The presence in mammalian cells of many E2Fs suggests that some of the E2F/DP combinations could exert specific or unique functions in particular types of cells or during particular stages of development. Gene targeting in knockout mice has indeed demonstrated that E2F-5 is dispensable for cell proliferation and is involved in the regulation of cell secretion in differentiated neural tissue (46). Whether or not more than one E2F member exists in plant cells, the apparent constitutive and ubiquitous nature of the DcE2F protein in differentiated tissue suggests that, as reported for the mammalian factors, the plant E2Fs are likely to be involved in both induction and repression of gene activity. Because DcE2F can recognize a canonical E2F *cis*-element, the isolation and the analysis of plant promoters containing this conserved consensus sequence will be an important first step toward the definition of plant E2F functions. The discovery of these target genes will demonstrate whether DcE2F is indeed involved in cell cycle control and/or whether it has other important unrelated functions during cell growth and differentiation.

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