The mammalian E2F family of transcription factors plays a crucial role in the regulation of cellular proliferation, apoptosis, and differentiation. Consistent with its biological role in a number of important cellular processes, E2F regulates the expression of genes involved in cell cycle, DNA replication, DNA repair, and mitosis. It has proven difficult, however, to determine the specific roles played by the various known family members in these cellular processes. The work presented here now extends the complexity of this family even further by the identification of a novel E2F family member, which we now term E2F7. Like the expression of the known E2F activators, E2F1, E2F2, and E2F3, the expression of E2F7 is growth-regulated, at least in part, through E2F binding elements on its promoter, and its protein product is localized to the nucleus and associates with DNA E2F recognition sites with high affinity. A number of salient features, however, make this member unique among the E2F family. First, the E2F7 gene encodes a protein that possesses two distinct DNA-binding domains and that lacks a dimerization domain as well as a transcriptional activation and a retinoblastoma-binding domain. In contrast to the E2F activators, E2F7 can block the E2F-dependent activation of a subset of E2F target genes as well as mitigate cellular proliferation of mouse embryo fibroblasts. These findings identify E2F7 as a novel member of the mammalian E2F transcription factor family that has properties of a transcriptional repressor capable of negatively influencing cellular proliferation.

The retinoblastoma (Rb)1 gene was the first tumor suppressor identified in humans some 20 years ago. Studies using tissue culture and in vivo mouse models have led to the identification of the E2F transcription factor family as an important effecter of Rb function impacting cell proliferation, apoptosis, and cellular differentiation. From these studies, a paradigm for Rb action in the control of cellular proliferation has emerged. In this view, cyclin-dependent kinase activation results in the phosphorylation of Rb and the release of E2F family members from Rb-containing complexes, leading to E2F target activation and cell cycle progression (1–3). The regulation of E2F activities is not solely due to the action of Rb but is also subject to control at multiple tiers involving new synthesis, localization, phosphorylation, acetylation, and degradation (2, 3).

The E2F proteins and their heterodimeric DP partners, encoded by eight distinct genes, contain an evolutionary conserved DNA-binding and dimerization domain and, at least for E2F1–5, a conserved transactivation and pocket protein-binding domain. Although heterodimerization with DP proteins is required for E2F DNA binding activity, the specificity of the heterodimer complex is mediated by the E2F subunit (1). Both structural and functional properties have separated the E2F family members into three subclasses. The expression of E2F1, E2F2, and E2F3a, which represent the first subclass, oscillates during the cell cycle, peaks late in G1, and coincides with the activation of G1/S-specific genes (4). During this period, E2F1–3a can be found transiently bound to many E2F target promoters (5). Consistent with an important role for these proteins in cellular proliferation, the combined disruption of E2F1, E2F2, and E2F3 in mouse embryonic fibroblasts (MEF) impedes E2F-target expression and cellular proliferation, indicating that these E2Fs may function as transcriptional activators (6).

In contrast to the E2F activators, the expression of the second E2F subclass composed of E2F3b, E2F4, and E2F5 appears relatively constant in relation to cell growth (4). Several studies have implicated the second E2F subclass in transcriptional repression by recruiting the pocket proteins Rb, p107, and p130 and their associated histone-modifying enzymes to E2F target gene promoters (3). E2F6, the only member of the third subclass, contains domains for DNA binding and dimerization but lacks residues for pocket protein binding or transactivation. Instead, E2F6 is thought to mediate repression either through its direct binding to polycomb group proteins or through the formation of a large multimeric complex containing Mg and Max proteins (7, 8).

The function of E2Fs in regulating cellular proliferation and differentiation has been also investigated in nonmammalian species. Of these, Drosophila melanogaster is the most extensively studied organism, containing at least two E2F genes (dE2F1–2) and one DP gene (dDP). In many ways, dE2F1 appears to be a homolog of the mammalian E2F activator subclass, whereas dE2F2 resembles members of the mammalian repressor subclass (9). E2F related activities have also been described in Caenorhabditis elegans and Xenopus laevis but have not been found in yeast (10, 11).

It is noteworthy that the Arabidopsis thaliana genome contains six E2F genes (AtE2Fa→c) and two DP genes (AtDPa and...
AdDFp). Similar to their mammalian E2F1–3 counterparts, AtE2Fa–c proteins can transactivate E2F targets and are equipped with conserved residues for DNA binding, dimerization, transactivation, and pocket protein binding (12, 13). On the other hand, AtE2Fd–f diverge from the mammalian E2F3b–6 repressors, because they possess two DNA-binding domains (DBD) and lack any of the other conserved regions necessary for independent repression (12, 13).

Comparative analysis of the protein sequences between the human, mouse, fly, nematode, and A. thaliana genomes revealed that the DBD of the E2F proteins is highly conserved. We therefore used the mouse E2F3 DNA-binding amino acid sequence as bait to identify additional putative E2F family members within the recently completed mouse and human genome. We identified a new putative mammalian E2F, which we now designate as E2F7. The salient feature of this member is that it contains two tandem DBDs that are highly related to the DBDs of all other E2Fs and thus has a similar structural organization at AtE2Fd–f from plants. In contrast to most known mammalian E2Fs, this novel member lacks the residues necessary for dimerization, transactivation, and pocket protein binding. We show that this protein can bind to E2F DNA-binding consensus sites, can act as a transcriptional repressor, and can inhibit cellular proliferation.

EXPERIMENTAL PROCEDURES
cDNA Identification and Characterization—The Celera and GenBank databases were searched with the protein sequence of the E2F3 DBD using the PSI-BLAST program. Two overlapping E2F7 mouse expressed sequence tags (EST) were identified and purchased from Research Genetics (BE533279) and RIKEN Yokohama Institute (BB637825). A unique XcmI restriction site within the overlapping region was used to merge the two EST clones to produce a full-length 55-kb-long cDNA. The open reading frame of 2.5 kb was cloned into the expression vector pE3A3-5xMyc, downstream and in the same reading frame of the Myc epitope sequence.

MEF Generation—Primary MEFs were isolated from 13.5-day-old embryos using standard methods (6). The experiments presented here were repeated in at least two different MEF preparations. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 12.5% fetal bovine serum (FBS). Serum Starvation and Serum Stimulation—Subconfluent MEFs were synchronized by incubation in Dulbecco’s modified Eagle’s medium with 0.2% FBS for 48 h and then stimulated to proliferate by the addition of fresh medium with 0.2% FBS for 48 h and then stimulated to proliferate by adding medium with 0.2% FBS for 48 h and then stimulated to proliferate by adding the following medium supplemented with 20% FBS for 48 h.

Northern Blot Analysis—Total RNA for Northern blot analysis was isolated using TRIzol (Invitrogen), and mRNA was subsequently purified using PolyATract mRNA isolation system as described by the manufacturer (Promega). Purified mRNA was separated on a 1% agarose gel containing 6% formaldehyde and transferred onto a GeneScreen membrane (PerkinElmer Life Sciences). The CDNA probe corresponding to the first 1200 bp of the E2F7 open reading frame was labeled using PrimeIt II (Stratagene) with 50 μCi of [α-32P]dCTP. The cell cycle as blot as a commercially purchased mouse tissue Northern blot (Origene) were hybridized overnight under high stringency conditions (5×SSPE, 50% formamide, 5× Denhardt’s solution, 1× SDS at 42°C) and washed several times (0.2× SSC, 0.2% SDS at 65°C) before autoradiography.

Semiquantitative Reverse Transcription PCR—Total RNA was isolated using TRIzol reagent. For the reverse transcription-PCR, oligo(dT)-primed first strand cDNA was synthesized from 5 μg of total RNA using the Superscript Reverse Transcriptase (Invitrogen) following the manufacturer’s protocol, and aliquots of 1 μl were used for each PCR. 45 cycles of PCR amplification were performed in a final volume of 20 μl with 20 ng of the following gene-specific primers mixed together in one PCR: 5′-GGACACACCCCAAGCACCTT and 5′-CGTTGCAACCCACATCAGCTCAGCT and 5′-GCATACCTGTGTGCTTCAC for glycosylphosphatidylinositol.

Promoter Isolation and E2F Site Mutations—A 8-kbp genomic fragment containing the putative promoter region and the first exon was isolated from BAC clone RP23–80G4 and subcloned into pBluescript SK(−/−). The primers 5′-GAGAGACTCGAGGGGCCAGCTCTAAAT-AACAGT and 5′-GAGAGAAGCTTATATTTTTCTACACAAACAC-GAC containing Xhol and HindIII, respectively, were used to generate a 2-kbp E2F7 promoter fragment via PCR. The PCR product was digested with Xhol and HindIII, subcloned into Xhol/HindIII-digested pGL2Basic, and sequenced.

The three E2F recognition sites in the E2F7 promoter were mutated using the QuikChange site-directed mutagenesis kit (Stratagene) as specified by the manufacturer. The following point mutations were incorporated (the wild-type sequence is followed by the mutant sequence): E2F site 1, 5′–GCCCGGCGGGAAA to 5′–CCCCCCTTGGAAA; E2F site 2, 5′–TTTACCGGGGGA to 5′–TTTAAAGGGGA; and E2F site 3, 5′–TTTCCGGCCAAA to 5′–TTTTGACCCAAA. All of the constructs were confirmed by sequencing.

Reporter Assays—MEFs were grown in triplicate and transfected with the luciferase expression vectors, together with either thymidine kinase (TK) Renilla luciferase or cytomegalovirus (CMV)-β-galactosidase as internal controls, as described previously (14). After transfection, the cells were brought to quiescence by serum starvation and then stimulated to grow by the addition of fresh medium with serum. The cells were harvested at various time points, and luciferase activity was determined by a dual luciferase reporter assay system (Promega). β-Galactosidase assays were performed as described previously (14). After transfection, the cells were brought to quiescence by serum starvation and then stimulated to grow by the addition of fresh medium with serum. The cells were harvested at various time points, and luciferase activity was determined by a dual luciferase reporter assay system (Promega). β-Galactosidase assays were performed as described previously (14).

Immunofluorescence—MEFs were grown in 35-mm dishes and transfected with Myc-tagged E2F7 or control vector. The cells were fixed with 4% paraformaldehyde and methanol/acetone (1:1). The Myc antibody (9E10 (Santa Cruz, SC-40) was incubated for 2 h at a dilution of 1:100 in 1% bovine serum albumin/phosphate-buffered saline. After three washes with phosphate-buffered saline (PBS), the cells were incubated with a rhodamine-conjugated anti-mouse IgG (diluted 1:75; Vector Laboratories). The cells were washed with phosphate-buffered saline, and the nuclei were stained with DAPI.

Western Blot Analysis—Cell protein lysates were separated in SDS acrylamide gels and blotted to polyvinylidene fluoride membranes. The blot was probed by incubation with 15 μl of a 1:500 dilution of the Myc antibody (9E10 (Santa Cruz, SC-40). The blots were then developed in Tris-buffered saline-Tween and subsequently incubated in blocking buffer containing the antibody specific for Myc (SC-40; 1 μg/ml). The primary antibody was then detected using horseradish peroxidase-conjugated secondary antibodies and ECL reagent as described by the manufacturer (Amersham Biosciences).

E2F DNA Binding Assays—E2F assays were performed as described previously (15). Supershift analysis was carried out as previously described using an antibody specific against Myc (SC-40). An end-labeled 130-bp-long plasmid DNA fragment from the dihydrofolate reductase (DHFR) promoter containing two E2F recognition site was used as a probe. The proliferating cell nuclear antigen probe contains the following nucleotide sequence: 5′-TCAAGGTTTTCCCGCTTTCGCTTC and 5′-TCGAGAAGCGCGGAAAAAGCGCGGAAAAAC containing two copies of an E2F sites derived from the tobacco proliferating cell nuclear antigen promoter (13). The E2F probe was annealed oligonucleotide 5′-TCAAGGTTTTCCCGCTTTCGCTTC and 5′-TCGAGAAGCGCGGAAAAAGCGCGGAAAAAC containing two copies of an E2F sites derived from the tobacco proliferating cell nuclear antigen promoter (13). The E2F probe was annealed oligonucleotide 5′-TCAAGGTTTTCCCGCTTTCGCTTC and 5′-TCGAGAAGCGCGGAAAAAGCGCGGAAAAAC containing two copies of an E2F sites derived from the tobacco proliferating cell nuclear antigen promoter (13).

Retroviral Infections—Full-length cDNAs for Myc-tagged versions of mouse E2F3a and E2F7 were subcloned into the pBabe retroviral expression vector containing a hygromycin-resistant gene. The retroviral vector was produced by transient transfection of retroviral constructs into the Phoenix-Eco packaging cell line as described previously (17). MEFs were infected with the retrovirus using standard methods and selected for several days in the presence of 400 μg ml−1 hygromycin.

Proliferation Assays—MEFs were seeded at 2 × 104 cells/60-mm dish. Duplicate plates of cells were counted daily using a Beckton Dickson Coulter Counter.

Results

Identification of a Putative Novel E2F Family Member: E2F7—The E2F family of transcription factors is composed of six members. To determine whether additional members exist, we searched the recently completed human and mouse genomes (Celera and GenBank databases) for genes containing amino acid homology to the E2F3 DBD. In addition to the six known E2F genes, this analysis identified an additional
Fig. 1. Genomic structure of E2F7. A, physical map of the murine E2F7 gene. Exons 1–14 are represented by boxes; the introns are represented by lines. The lengths of the exons and introns are indicated. The open reading frame of E2F7 is illustrated by light gray shading; the untranslated region is shown by dark gray shading. The full-length E2F7 cDNA is shown beneath the genomic structure. Arrowheads indicate the position of the nuclear localization signal (NLS); bars illustrate the location of the DBDs; asterisks mark the positions of the polyadenylation signals (pAS). An alignment of EST clones encompassing the E2F7 cDNA are presented at the bottom. B, schematic comparison of the conserved domains between E2F7 and the other E2F/DP family members. CA, cyclin A-binding site; DIM, dimerization domain; Rb, pocket protein-binding domain. The extent of homology between the E2F7 DBDs and the other E2F/DP DBDs is indicated as a percentage of amino acid identity for each of the two E2F7 DBDs (indicated within the DBD boxes). C, phylogenetic relationship of the DBD amino acid sequences between E2F7 and the E2F/DP family (phenogram). The length of each horizontal line represents the evolutionary distance between branching points; the units at the bottom of the tree indicate the number of substitution events. The dotted line on the phenogram indicates a negative branch length.
E2F7 Overexpression Blocks Cellular Proliferation

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**Analysis of Promoter Sequence Controlling the Expression of the E2F7 Locus**—The cell growth-dependent accumulation of E2F7 mRNA suggests that it may be transcriptionally regulated in response to growth stimuli, albeit that other transcriptional-independent mechanisms are also possible. Examination of sequences upstream of the predicted ATG start codon revealed the presence of a number of potential binding sites for a variety of transcription factors (Fig. 3A). Notably, the putative E2F7 promoter contained three potential E2F-binding elements near the 5′ end of the predicted first exon, with Sp1 recognition sites very close to each of the E2F binding elements.

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**Analysis of Tissue-Specific and Cell Cycle-dependent Expression of E2F7**—To determine the patterns of E2F7 expression in adult mice, mRNA levels were measured by Northern blot analysis using an E2F7-specific probe containing the N-terminal 1200 bp of coding sequence. Two specific sized transcripts hybridized to this probe; however, at higher stringencies the slower 5.5-kb migrating transcript was the predominant species that could be detected (data not shown). As shown in Fig. 2A, the highest levels of E2F7 expression were observed in the skin and thymus, with little or no expression in the brain, muscle, and stomach.

Previous studies have detailed the distinct pattern of expression of the various E2F family genes following mitogenic stimulation of cells. To determine whether E2F7 expression was responsive to growth stimuli, E2F7 expression was measured by semi-quantitative reverse transcriptase-PCR and Northern blot analysis in synchronized cell populations. To this end, mouse embryonic fibroblasts were starved in 0.2% FBS for 48 h and subsequently stimulated with 12.5% FBS, and the cells were harvested at different time points for RNA isolation. Cells similarly treated in parallel cultures were incubated with BrdU, fixed at the various time points post-stimulation, and probed for indirect immunofluorescence. This analysis indicated that serum stimulation of quiescent cells results in an increase of E2F7 expression similar to that of E2F1, E2F2, and E2F3 (18), with levels peaking during the S phase, suggesting that the regulation of E2F7 expression is cell growth-dependent (Fig. 2, B and C).

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**Analysis of Promoter Sequence Controlling the Expression of the E2F7 Locus**—The cell growth-dependent accumulation of E2F7 mRNA suggests that it may be transcriptionally regulated in response to growth stimuli, albeit that other transcriptional-independent mechanisms are also possible. Examination of sequences upstream of the predicted ATG start codon revealed the presence of a number of potential binding sites for a variety of transcription factors (Fig. 3A). Notably, the putative E2F7 promoter contained three potential E2F-binding elements near the 5′ end of the predicted first exon, with Sp1 recognition sites very close to each of the E2F binding elements.
To explore the role of transcriptional regulation in the control of E2F7 expression, we isolated and cloned a 2-kb genomic DNA fragment containing the 5'-flanking sequences immediately upstream of each exon. The E2F recognition sites within the E2F7 upstream regulatory sequences and the site where the 2-kb promoter fragment was fused to the firefly luciferase reporter is illustrated. The asterisks indicate the positions of the E2F-binding elements where mutagenesis was carried out as outlined under "Experimental Procedures." A, genomic organization of the E2F7 promoter. The genomic region including exons 1 and 2 is shown together with the DNA sequences immediately upstream of each exon. The E2F recognition sites within the E2F7 upstream regulatory sequences and the site where the 2-kb promoter fragment was fused to the firefly luciferase reporter is illustrated. The luciferase activity was normalized to Renilla luciferase activity. B, analysis of the E2F7 wild-type promoter (wt pr-E2F7) and the E2F7 promoter containing mutated E2F binding elements (mut pr-E2F7) following mitogenic stimulation. MEFs were transfected with wt pr-E2F7-luciferase or mut pr-E2F7-luciferase, together with a TK-Renilla luciferase vector as an internal control as described in the text. Asynchronous proliferating (P), density-arrested (D), and synchronized cell populations were analyzed. Firefly luciferase activity was normalized to Renilla luciferase activity. C, analysis of wt pr-E2F7 and mut pr-E2F7 during quiescence. The data represent the average and standard deviation from three independent experiments. D, the E2F7 promoter is activated by E2F1 and E2F3. MEFs were transfected with 2 μg of the wt pr-E2F7-luciferase construct, together with 0.5 μg of either pCDNA3-E2F1 (E2F1), pCDNA3-E2F3 (E2F3), or pCDNA3 control vector along with 2 μg of CMV-b-galactosidase and treated as described under "Experimental Procedures." The luciferase values were first normalized to b-galactosidase activity and reported as fold induction relative to the values obtained from transfections with a control expression vector. E, activation of the E2F7 promoter by E2F1 is independent of the three E2F-binding elements. MEFs were transfected with either 2 μg of wt pr-E2F7 or mut pr-E2F7 and with either pCDNA3-E2F1 (E2F1) or control pCDNA3 vector and with 2 μg of CMV-b-galactosidase. The luciferase values were normalized to b-galactosidase activity and shown as fold induction relative to the values obtained with control vector.

To explore the role of transcriptional regulation in the control of E2F7 expression, we isolated and cloned a 2-kb genomic DNA fragment containing the 5'-sequence flanking the first exon into a luciferase reporter construct (pr-E2F7). To first determine whether the growth-regulated accumulation of E2F7 mRNA could be accounted for a transcriptional mechanism, the activity of the prE2F7 reporter construct was measured in synchronized MEF populations. To this end, MEFs were transfected with the prE2F7 reporter construct along with a TK-driven Renilla luciferase plasmid as an internal control.
control; the cells were then growth-arrested by serum deprivation and subsequently stimulated to reenter the cell cycle by the addition of 12.5% serum. The cells were then harvested at various times after serum addition, and cell extracts were assayed for firefly and Renilla luciferase activity. As shown in Fig. 3B, E2F7 promoter activity was low in quiescent cells and in early G1 cells but increased 8-fold by 24 h post-stimulation, corresponding with the serum-dependent induction of E2F7 mRNA observed previously (Fig. 2, B and C).

To investigate the role of the putative E2F recognition sites in regulating the growth-dependent activation of E2F7, the three E2F binding elements on its promoter were eliminated in combination (Fig. 3A). MEFs were transfected with either the wild-type or each of the mutated E2F7 promoter constructs, as well as with a TK-Renilla luciferase plasmid as an internal control. Transfected cells were growth-arrested in low serum medium and then stimulated to reenter the cell cycle by the addition of 12.5% serum. The promoter with the three E2F sites mutated exhibited a 5-fold increase in activity in quiescent cells (Fig. 3, B and C), suggesting that these E2F-binding elements play a negative regulatory role on the regulation of its expression. In addition, mutation of the E2F-binding sites also resulted in higher E2F7 promoter activity when cells were restimulated with serum, as early as 6 h post-stimulation, indicating that E2F7 expression can also be regulated positively through E2F-binding site-independent transcriptional mechanisms. The fact that wild-type and mutant E2F7 promoter constructs could be activated equally well by the overexpression of E2F1 (Fig. 3, D and E), suggests that E2F activators can regulate the E2F7 promoter independently of these three E2F-binding elements. Together, these data suggest that E2F7 expression is subject to E2F-mediated negative and positive regulation by different mechanisms.

**E2F7 Is a Nuclear Protein, Binds E2F Consensus Sites, and Has Transcriptional Repression Function**—We have also investigated the function of the E2F7 gene product. Because the E2F7 gene contains multiple putative nuclear localization signals (Fig. 1A), we initially evaluated the subcellular localization of a Myc-tagged version of E2F7 (Myc-E2F7). Transient transfection of Myc-E2F7 resulted in its efficient expression as detected by Western blot analysis (Fig. 4A). Cells transfected with Myc-E2F7 were also plated in parallel, fixed, and processed for immunofluorescence with anti-Myc (9E10) antibodies. As shown in Fig. 4B (and data not shown), Myc-E2F7 is localized exclusively in the nucleus regardless of cell cycle position; cells transfected with a control vector did not show any staining, indicating that the antibody did not detect any endogenous Myc protein under these experimental settings.

The ability of E2F7 to bind to E2F DNA recognition sequences was also evaluated. Nuclear or whole cell extracts made from Myc-E2F7 or control vector transfected cells were subjected to electromobility shift assays using three distinct radiolabeled DNA fragments corresponding to the E2F sites in the DHFR, proliferating cell nuclear antigen, and E2 promoters. In each case, a novel complex could be visualized in extracts from Myc-E2F7 transfected cells that migrated faster than the other known E2F family members (Fig. 4C). The presence of the Myc-tagged E2F7 in these fast migrating complexes was verified by supershifting with an anti-Myc (9E10) antibody.

We next examined the possible role of E2F7 in the transcriptional control of E2F-responsive genes. To this end, MEFs were transfected with luciferase reporter constructs carrying a variety of E2F-responsive promoters, including TK, E2F2, E2F3a, polymerase α, and Rb along with increasing amounts of a Myc-E2F7 expressing vector. Standardization with β-galactosidase activity as an internal control revealed that E2F7 expression in cycling population of cells led to a consistent decrease in some of the E2F target genes, with little or no effect in others (Fig. 5A). The inability to activate transcription is consistent with the fact that E2F7 does not contain a transactivation domain.

The inhibition of E2F target genes was accentuated when synchronized population of cells was assayed instead. The
The reporter gene is activated in an E2F-dependent manner during the G1/S transition in cells induced to enter the cell cycle. To determine whether E2F7 can inhibit this G1/S-specific induction, MEFs were transfected with the Myc-E2F7 expression vector and an E2F-reporter construct (prE2F2) as before, but then subsequently arrested in G0 by serum deprivation, and then induced to enter the cell cycle by the addition of serum for 15 h. Expression of Myc-E2F7 inhibited the serum-induced activation of the prE2F2 reporter in a dose-dependent manner (Fig. 5B), suggesting that E2F7 can block the ability of endogenous E2F complexes to activate this reporter.

To determine whether the ability of E2F7 to repress E2F target gene expression is E2F-dependent, we asked whether Myc-E2F7 could inhibit the ability of E2Fs to transactivate E2F-responsive reporter constructs. In these experiments, MEFs were transiently transfected with 2 μg of the indicated E2F-responsive reporter constructs along with increasing amounts of pCDNA-MycE2F7 (myc-7) and 2 μg of CMV-β-galactosidase. Extracts from transfected cells were prepared as already described, and the luciferase and β-galactosidase activities were measured. The normalized luciferase activities are presented as the average fold activation relative to the activity in extracts from cells transfected with the reporter construct alone. E2F7 overexpression inhibits the serum-induced activation of the E2F2 promoter. MEFs were transiently transfected with 2 μg of pr-E2F2 along with increasing amounts of pCDNA3-Myc-E2F7 or corresponding doses of the pcDNA3 control vector (con) and 2 μg of the β-galactosidase plasmid as an internal control. The cells were treated as described under "Results," and the extracts were prepared and assayed for luciferase and β-galactosidase activities; the luciferase activity was normalized to the corresponding β-galactosidase activity. E2F7 inhibits the E2F1-induced activation of the E2F2 promoter. MEFs were transiently transfected with the 2 μg of the prE2F2 reporter construct and 0.2 μg of the pCDNA3-E2F1, along with either increasing amounts of pcDNA3-MycE2F7 or pcDNA3 control vector and 2 μg of the CMV-β-galactosidase. The cells were brought to quiescence and then assayed for luciferase and β-galactosidase activity. Luciferase activity was normalized to β-galactosidase activity.

prE2F2 reporter gene is activated in an E2F-dependent manner during the G1/S transition in cells induced to enter the cell cycle. To determine whether E2F7 can inhibit this G1/S-specific induction, MEFs were transfected with the Myc-E2F7 expression vector and an E2F-reporter construct (prE2F2) as before, but where subsequently arrested in G0 by serum deprivation, and then induced to enter the cell cycle by the addition of serum for 15 h. Expression of Myc-E2F7 inhibited the serum-induced activation of the prE2F2 reporter in a dose-dependent manner (Fig. 5B), suggesting that E2F7 can block the ability of endogenous E2F complexes to activate this reporter.

To determine whether the ability of E2F7 to repress E2F target gene expression is E2F-dependent, we asked whether Myc-E2F7 could inhibit the ability of E2Fs to transactivate E2F-responsive reporter constructs. In these experiments,
MEFs were transiently transfected with E2F1 and the prE2F2 reporter construct and increasing amounts of E2F7. As previously shown, E2F1 expression resulted in the activation of reporter gene activity by ~6-fold (Fig. 5C). This E2F1-mediated activation was effectively inhibited by Myc-E2F7 in a dose-dependent manner. These results suggest that E2F7 can act to inhibit E2F-mediated activation of target genes.

**E2F7 Inhibits Cellular Proliferation**—Members of the E2F family play a critical role in the control of cellular proliferation. To explore the potential role of E2F7 in cellular growth, we measured the effect of E2F7 overexpression on cellular proliferation. Wild-type primary MEFs were infected with a retrovirus expressing Myc-E2F7, selected for a brief period with hygromycin, and replated at a concentration of 2 × 10⁵ cells/60-mm tissue culture dish, and viable cells were counted for a period of 7 days. Relative to control cells infected with an E2F3a expressing retrovirus (pBABE-Myc-E2F3a) or an empty pBABE vector, the proliferation rate of Myc-E2F7 expressing cells was decreased (Fig. 6A). To address whether the reduced growth rates of E2F7 overexpressing cells could be attributed to a specific defect in the G₁/S transition, MEFs infected with either a Myc-E2F7 overexpressing or control virus were synchronized by serum deprivation, then stimulated to proliferate by the addition of serum, and assayed for S phase entry by the incorporation of BrdU into replicating DNA. Surprisingly, S phase entry was not delayed in cells overexpressing E2F7 (Fig. 6B). Rather, overexpression of E2F7 led to an accumulation of cells in G₂/M and a decrease in G₁ as determined by flow cytometric analysis (Fig. 6C). This accumulation in G₂/M, although not pronounced, was consistently observed in numerous experiments. Together, these results indicate that E2F7 overexpression can lead to impaired proliferation and accumulation of cells in G₂/M phase. Consistent with the absence of a G₁/S phase-specific defect in E2F7 overexpressing cells, the accumulation of known E2F target transcripts, including Dhrf, Cdc6, cyclin A2, cyclin E, and mcm3 was similar in Myc-E2F7-expressing and control cells (data not shown). These findings suggest that the impaired proliferation in E2F7-overexpressing cells is not related to a defect in the G₁/S transition.

**DISCUSSION**

E2F plays an important role in the control of cell proliferation in many different species, including mammals, flies, nematodes, amphibians, and plants. Comparative evaluation of amino acid sequences reveal that the DBD of E2Fs are evolutionary conserved and therefore represent an essential feature that serves to identify E2F family members. Herein we describe the cloning and characterization of a novel murine E2F member that serves to identify E2F family members. Herein we describe the cloning and characterization of a novel murine E2F member that serves to identify E2F family members. Like E2F6, E2F7 also lacks the C-terminal domains necessary for transactivation and pocket protein binding, consistent with its inability to activate known E2F-responsive genes. Instead, E2F7 is able to block the endogenous E2F-dependent transcriptional activity as well as the transactivating activity of overexpressed E2F1. The mechanism of transcriptional repression is unclear but might involve competition between E2F7 and other E2Fs for the same DNA target sites. E2F7 has been shown to actively repress gene expression by interacting directly with polycomb group proteins through their association via its marked box domain (8). Although E2F7 does not possess a similar marked box domain, it might contain a functionally related domain(s) that could potentially recruit co-repressors to E2F-regulated promoters. Interestingly, E2F7-mediated transcriptional repression appears to be target gene-dependent because only a subset of known E2F-regulated promoters can be efficiently repressed by E2F7 overexpression, indicating functional specificity for distinct E2F targets. Whether this specificity is dictated by the selectivity of binding to different E2F DNA recognition sites, by the recruitment of co-factors, or through other mechanisms is not known.

The identification of E2F7 as a novel E2F family member adds a further level of complexity to the control of E2F-dependent gene expression. It is possible that by inhibiting the DNA binding capacity of the other known E2Fs during the cell cycle, it might modulate cell cycle progression or might provide cells with the ability to respond to additional external cues. In this respect, our data show that the regulation of E2F7 expression is cell growth-dependent, suggesting that E2F7 may play an important role in counter balancing E2F-induced gene expression, as has been demonstrated for the antagonistic relationship between the Drosophila E2Fs (9). Although measurement of E2F7 expression by either Northern, reverse transcriptase-PCR, or reporter assays indicate that its regulation is cell growth-dependent, analysis of the E2F7 gene product has yet to be carried out and must await the generation of E2F7-specific antibodies. In contrast to other E2Fs, E2F7 overexpression led to an inhibition of cellular growth in primary MEFs, accompanied by a moderate but reproducible accumulation of cells in the G₂ phase. The E2F7-mediated accumulation of cells in the G₂ phase may arise from a delay in G₂ progression, a delay in the exit of cells from G₂, or an inability of cells to efficiently enter mitosis. Further investigation will be required to identify the exact mechanism of cell growth inhibition mediated by E2F7 and to identify the specific targets regulated by E2F7 that might mediate this effect.

Because E2F7 overexpression has the capacity to slow down cellular growth, one could envision that E2F7 may function as a tumor suppressor gene and that mutations within this gene might result in cancer formation. Although we have yet to establish the true physiological function of E2F7, its broad expression pattern suggests that it may provide an important contribution to the regulation of E2F activities in multiple tissues during development and/or tumorigenesis.

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Identification and Characterization of *E2F7*, a Novel Mammalian E2F Family Member Capable of Blocking Cellular Proliferation
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