Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 9190–9195, August 1998
Biochemistry

Unusual proliferation arrest and transcriptional control properties of a newly discovered E2F family member, E2F-6

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Contributed by David M. Livingston, June 9, 1998

ABSTRACT **E2F transcription factors play an important role in the regulation of cell cycle progression. We report here the cloning and characterization of an additional member of this family, E2F-6. E2F-6 lacks pocket protein binding and transactivation domains, and it is a potent transcriptional repressor that contains a modular repression domain at its carboxyl terminus. Overproduction of E2F-6 had no specific effect on cell cycle progression in asynchronously growing Saos2 and NIH 3T3 cells, but it inhibited entry into S phase of NIH 3T3 cells stimulated to exit G_0. Taken together, these data suggest that E2F-6 can regulate a subset of E2F-dependent genes whose products are required for entry into the cell cycle but not for normal cell cycle progression.**

The E2F family of transcription factors has been studied in detail as a model system for the link between transcriptional regulation and the cell cycle (for review see refs. 1–3). Binding sites for E2F proteins exist in promoters of certain genes required for cell cycle progression and DNA synthesis and whose expression is induced during the G_1-to-S transition (4, 5). Deregulated expression of E2F can promote cell cycle progression, cellular transformation, and apoptosis (for reviews see refs. 6 and 7). Moreover, E2F function is necessary for normal cell cycle progression (8).

Transcription activation by E2F is regulated by association with pRB, the product of the retinoblastoma susceptibility gene, and by at least two related "pocket proteins," p107 and p130. pRB, a tumor suppressor protein that is inactivated in many human tumors, is a growth suppressor in normal cells (for a review see ref. 9). Complexes of unphosphorylated pocket protein and E2F act as transcriptional repressors with growth-suppressing activity (10–13). The growth-inhibiting properties of pRB are inactivated by phosphorylation catalyzed by certain cyclin-dependent kinases (cdks). When pocket proteins are phosphorylated by cdks, transcriptionally active E2F is released (14, 15), resulting in the activation of certain genes that are essential for G_1 exit (for review see ref. 16). Thus, members of the E2F family can play dual roles as transcriptional repressors and activators, depending upon the stage of the cell cycle.

Each E2F is a heterodimer composed of an E2F-like and a DP-like subunit. Five E2F proteins (E2F-1 through -5) and two different DP subunits (DP-1 and -2) have been isolated (see ref. 2). Conserved E2F domains mediate DNA binding, heterodimerization, pocket protein binding, and transactivation. High-affinity DNA binding requires the formation of E2F–DP heterodimers (17–19). E2F proteins can be subgrouped into two distinct classes based on homology and on functional characteristics. E2F-1, -2, and -3 bind preferentially to pRB. E2F-4 and -5 associate with p107 and p130, although E2F-4 can bind to pRB as well (20). Different E2F–pocket protein complexes are formed at various stages during the cell cycle. Several recent observations suggest that different E2F proteins regulate certain subsets of E2F-dependent genes (e.g., see refs. 21 and 22).

While this work was in progress, two laboratories reported the cloning of a sixth E2F family member, called EMA or E2F-6 (23, 24). EMA/E2F-6 lacks a pocket protein binding and transcriptional activation domain and is a transcriptional repressor. A transcription repression domain has been identified in the amino terminus of the mouse homologue, EMA (23). However, whether this domain is necessary for repression in the context of the EMA protein was not shown. No biological function for EMA/E2F-6 has yet been reported.

We have also identified and cloned E2F-6 on the basis of its homology to the previously described E2F proteins, and we have determined that it lacks a pocket protein and transactivation domain and is a strong transcriptional repressor. Data presented below show that E2F-6 contains a transferable repression domain at its carboxyl terminus that is necessary and sufficient for transcriptional repression. Moreover, in comparison with all other E2F species and most proliferation control proteins, E2F-6 possesses an unusual biological activity.

MATERIALS AND METHODS

Cloning of a Human E2F-6 cDNA. Three partial cDNA clones with significant homology to each other and to the known E2Fs were identified [expressed sequence tag (EST) IMAGE clones 701418, 729316, and 339569]. Additional upstream sequences for this E2F species were obtained by PCR with HeLa Marathon cDNA and the 5′ primer AP1 (CLONTECH) and 3′ primer SG13 (see below for sequences of all oligonucleotides used). PCR products were used in a second PCR with the nested primers SG14 and AP2 (CLONTECH). PCR products were subcloned and sequenced. A full-length E2F-6 clone was obtained by PCR reaction with HeLa cDNA and the primers SG17 and SG16. PCR products were cloned into the expression vector pCDNA3-HA downstream and in the reading frame of the hemagglutinin (HA) tag.

Plasmids. Gal4 E2F-6 was obtained by PCR with pCDNA-HA-E2F-6 as a template and primers SG18 and SG16. The PCR product was cloned in pCMX-Gal. Other Gal4 fusion vectors were generated by PCR using Gal4-E2F-6 as a template and the following primers: Gal-4-E2F-6(1–220): SG18 and SG24; and Gal4-E2F-6(1–62): SG26 and SG16. PCR fragments were subcloned in pCMX-Gal4. To obtain Gal4-E2F-6(173–281), the *Hpa*I–*Xba*I fragment and, for Gal4-E2F-6(1–80), the *Eco*RI–*Bgl*II fragment of E2F-6 were subcloned in pCMX-Gal4.

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Abbreviations: HA, hemagglutinin; DHFR, dihydrofolate reductase; BrdU, 5-bromodeoxyuridine; GFP, green fluorescent protein.

Data deposition: The human E2F-6 nucleotide sequence has been deposited in the GenBank database (accession no. AF059292).

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The DNA binding mutant of E2F-6 was generated in two PCRs, using pCDNA-HA-E2F-6 as a template and the following primers: reaction A, SG17 and SG19; and reaction B, SG20 and SG16. The PCR fragments from reaction A and reaction B were ligated into pCDNA3-HA. The amino acids in positions 68 and 69 were changed from Leu-Val to Glu-Ser, and an *Eco*RI site was introduced at this position.

Two deletion mutants of E2F-6 were obtained by PCR using pCDNA3-HA-E2F-6 as a template and the following set of primers: E2F-6(60–281), SG21 and SG16; and E2F-6(1–220), SG17 and SG24. PCR products were subcloned in pCDNA3-HA. The identities of all plasmids were verified by DNA sequencing.

Other plasmids have been described elsewhere: E2F-luc, E2Fmut-luc, and CMV-β-gal in ref. 19; E2F1-luc in ref. 25; pCDNA-E2F-1(1–363) in ref. 26; and pCDNA3-HA-E2F-4 in ref. 27; Gal4-luc and CMV-p21 were a kind gift of J. Chen (Dana–Farber Cancer Institute).

Oligonucleotides. SG13, 5′-GGGAATTCATGAATGTCTTGATAGGTCAC-3′; SG14, 5′-CTTGTTTAAGTCAAGAATACC-3′; SG16, 5′-GGGAATTCGCGTAATTCTCCACGAAGATATTC-3′; SG17, 5′-GGGGATCCATGAGTCAGCAGCGGCCGGCGAGG-3′; SG18, 5′-GGGAATTCATGAGTCAGCAGCGGCCGGCGAGG-3′; SG19, 5′-GGGAATTCCGATACATCAAAACGAGGTCT-3′; SG20, 5′-GGGAATTCTATTTAACTCGAAAATTTTATG-3′; SG21, 5′-GGGGATCCAAGAGACCTCGTTTTGATGTA-3′; SG24, 5′-GGGAATTCTCAGATAGAGTCTTCTCTGGGAGC-3′; SG26, 5′-CCGGATCCAGTAGACTGTTCTAGGTATTTAAAAGC-3′; DHFRwt(s), 5′-CCCGACTGCAATTTCGCGCCAAACTTGGG-3′; DHFRwt(as), 5′-CCCAAGTTTGGCGCGAAATTGCAGTCGGG-3′; DHFRmut(s), 5′-CCCGACTGCAATTTCGATCCAAACTTGGG-3′; and DHFRmut(as), 5′-CCCAAGTTTGGATCGAAATTGCAGTCGGG-3′.

Gel Retardation Assays. One microgram of pcDNA3-HA-E2F-6, pcDNA3-HA-E2F-6E68, pcDNA3-HA-E2F-6(1–220), pcDNA3-HA-E2F-6(60–281), and pcDNA3-DP-2 were translated *in vitro* by using a coupled transcription/translation reticulocyte lysate system according to the manufacturers instructions (Promega). Gel retardation assays were performed as described, using 5 μl of *in vitro* translated proteins and an E2F site derived from the dihydrofolate reductase (DHFR) promoter (19). Competitions were performed with unlabeled double-stranded oligonucleotides corresponding to the E2F site in the DHFR promoter or with a mutated derivative of it. One microliter of antibody [either polyclonal anti-HA-antibody (Babco, Richmond, CA) or anti-myc antibody (9E10)] was added to the binding reaction as indicated.

Cell Culture. U2OS and Saos2 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (HyClone) at 37°C in an atmosphere containing 10% CO_2. NIH 3T3 cells were maintained in DMEM supplemented with 5% bovine calf serum (HyClone).

Reporter Assays. Transfections were performed as described by using a modified calcium phosphate method (28). Briefly, 2×10^5 U2OS cells were plated in 60-mm cell culture dishes. Twenty-four hours later, 2 μg of luciferase reporter plasmid was cotransfected with expression plasmids as indicated in each experiment. Empty vector was added, as needed, to maintain a constant input of pCMV and other vector sequences. Cells were harvested 48 hr after transfection. Luciferase and β-galactosidase assays were performed as described (28). To account for differences in transfection efficiency, 0.2 μg of CMV-β-gal was cotransfected, and luciferase activity was normalized to β-galactosidase activity. Error bars represent the standard deviation within a representative experiment. Each experiment has been repeated at least three times.

Immunoblotting. Transfected U2OS cells were directly lysed in 1× electrophoresis sample buffer (28). Proteins were transferred to nitrocellulose by standard procedures and detected by using the anti-HA antibody 12CA5. Immunoblots were incubated with horseradish peroxidase-conjugated rabbit-anti-mouse-IgG (Amersham), and proteins were visualized by using enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Microinjection and Transfection of NIH 3T3 Cells. NIH 3T3 cells (4×10^5) were plated onto gelatin-coated coverslips in 60-mm cell culture dishes. Twenty hours later, cells were washed three time with serum-free DMEM and re-fed with DMEM containing 0.1% bovine calf serum. Forty to 48 hr later, cells were microinjected with pcDNA-HA-E2F-6 or -E2F6E68 (25 μg/ml in PBS) at 5–15 hPa. CMV-GFP (10 μg/ml) was coinjected to identify the injected cells. Ten hours after injection, 5% bovine calf serum and 50 μM 5-bromodeoxyuridine (BrdU) were added. After 17.5 hr, cells were fixed and permeabilized as described (27) and then incubated with 10 units/μl DNase I (Boehringer Mannheim) and anti-BrdU antibody (Becton Dickinson) in PBS/10 mM $MgSO_4$ for 1 hr. Secondary antibody conjugated to rhodamine (Boehringer Mannheim) was then applied. Nuclei were counterstained with 1 μg/ml Hoechst 33258 (Sigma). For transient transfections, NIH 3T3 cells were transfected by using Lipofectin with 1 μg of each expression plasmid according to the manufacturers instruction (GIBCO). After 14 hr, 50 μM BrdU was added. Twenty hours later, cells were fixed and stained with anti-BrdU antibody as described above.

Flow Cytometry. Saos2 cells were transfected with 1 μg of CMV-GFP and 5 μg of expression plasmid by using a modified calcium phosphate protocol (28). Forty-eight hours after transfection, cells were harvested and fixed in 3% paraformaldehyde/2% sucrose in PBS for 10 min and than fixed in 70% (vol/vol) ethanol. DNA was stained with 69 μM propidium iodide in 38 mM sodium citrate and 100 μg/ml RNase for 30 min at 37°C. The samples were directly analyzed in a Becton Dickinson FACScan. Transfected cells were identified by the presence of green fluorescence arising from the coexpressed green fluorescent protein (GFP).

RESULTS

Cloning of E2F-6. While searching the EST (Expressed Sequence Tag) database for sequences with homology to E2F, three human EST clones with significant homology to the known E2Fs were identified (IMAGE cDNA clones 701418, 729316, and 339569). These clones appeared to be derived from the same gene, because they share extensive sequence identity at the nucleotide level (not shown). To isolate additional 5′ coding sequences, we performed a 5′ rapid amplification of cDNA ends (RACE) reaction. Several clones that extended the known cDNA by 250–300 nucleotides were isolated. The longest RACE clone contained an ATG upstream of and in frame with the downstream E2F open reading frame (ORF). This clone has high homology on the nucleotide level to two cDNAs in the mouse EST database (IMAGE clones 475364 and 475472). In both mouse clones, a stop codon is present in frame and upstream of the conserved potential translational start site. We concluded that we had, most likely, isolated the complete 5′ coding sequence of a new human E2F species, which we termed E2F-6. A cDNA for this protein, isolated by PCR from HeLa cell cDNA, extended over 909 nucleotides and contained an ORF of 843 nucleotides. It encodes a product of 281 amino acid residues with a predicted molecular mass of 32 kDa (Fig. 1*A*). To determine the expression pattern of E2F-6, we analyzed a panel of tissue-specific cDNAs by semiquantitative PCR. The E2F-6 cDNA was detected in all tissues examined (not shown). The highest

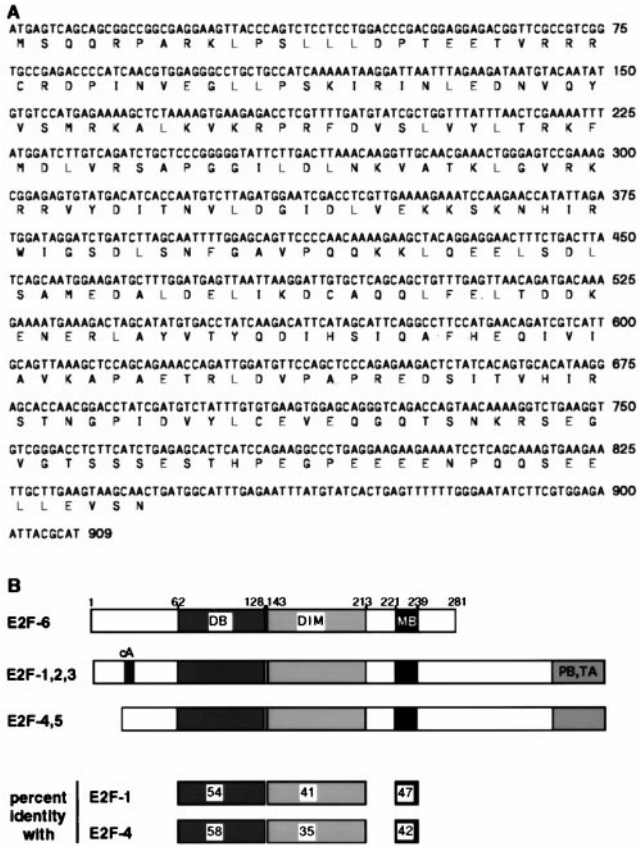

FIG. 1. Human E2F-6 sequence. (*A*) Nucleotide sequence of a cDNA encoding human E2F-6 and the predicted amino acid sequence. The E2F-6 sequence has been submitted to the EMBL/GeneBank database (accession no. AF059292). (*B*) (*Upper*) Schematic comparison of E2F-6 with E2F-1, -2, and -3 and E2F-4 and -5. Shaded boxes indicate homologous regions. cA, cyclin A binding site; DB, DNA-binding domain; DIM, dimerization domain; MB, marked box; PB,TA, pocket protein and transactivation domain. (*Lower*) Homology (as expressed in percent identity in the indicated domains) between E2F-6 and E2F-1 and -4.

levels were noted in placenta, skeletal muscle, heart, and ovary.

Structural Features of E2F-6. E2F-6 shares three regions of homology with the known E2F proteins. These regions correspond to the DNA-binding domain, the dimerization domain, and the marked box, a region of homology between the E2F proteins of unknown function (Fig. 1*B*). Outside of these regions, E2F-6 has little similarity to the other E2F family members. Strikingly, it lacks a pocket protein binding and a recognizable transactivation domain, present at the carboxyl terminus of all other known E2F proteins. In its E2F-homologous regions, E2F-6 is 40–50% identical to the other E2F proteins, but it cannot be grouped into one of the two known E2F subfamilies. Therefore, it may be the first member of a third E2F family or it may be a unique species.

E2F-6 Binds to an E2F Consensus Site. Next we wished to know whether E2F-6 can bind to an authentic E2F DNA recognition sequence. To do so, we analyzed the behavior of *in vitro* translated, HA-tagged E2F-6 (HA-E2F-6) and Myc-tagged DP-2 (myc-DP2) in gel retardation experiments performed with a radiolabeled oligonucleotide corresponding to the E2F site in the DHFR promoter (Fig. 2). Neither *in vitro* translated DP-2 nor E2F-6 bound, individually, to the DHFR site. Cotranslation of E2F-6 with DP-2, however, led to the appearance of a novel retarded complex (filled arrow). The specificity of this interaction was verified by demonstrating that an unlabeled DHFR oligonucleotide competed with the

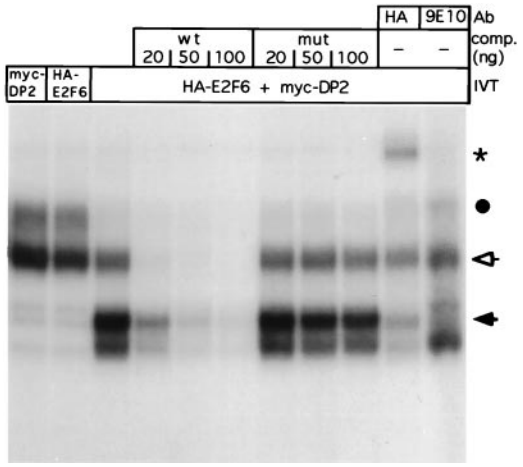

FIG. 2. E2F-6 binds specifically to an E2F site. A labeled oligonucleotide corresponding to the E2F binding site in the DHFR promoter was incubated with *in vitro* translated myc-DP2 or HA-E2F-6 protein (lanes 1 and 2) or cotranslated myc-DP2 and HA-E2F-6 proteins (lanes 3–11). Unlabeled DHFR wild-type (wt) or mutated (mut) oligonucleotides were added in the amounts indicated. In supershift experiments, 1 μl of anti-HA antibody (HA) or anti-Myc antibody (9E10) was added. Filled arrow, position of the specific E2F-6 complex; open arrow, endogenous E2F binding activity in the reticulocyte lysate; *, HA supershifted band; solid circle, 9E10 supershifted band.

radioactive probe for binding to E2F-6, whereas a derivative bearing a mutation in its E2F recognition sequence did not. The presence of both HA-E2F-6 and myc-DP2 in the bound complex was verified by the addition of tag-specific antibodies. A polyclonal anti-HA antibody supershifted (*), as did an anti-Myc (9E10) monoclonal antibody (filled circle). Taken together, these data show that E2F-6 binds specifically to a consensus E2F binding site and that high-affinity DNA binding requires heterodimerization with a DP protein. Thus, the DNA binding and dimerization properties of E2F-6 are similar to those of other E2F proteins.

E2F-6 Is a Transcriptional Repressor. To address the role of E2F-6 in transcription, we performed transient transfection assays (Fig. 3). U2OS cells were transfected with a luciferase reporter plasmid carrying three consensus E2F binding sites in the promoter. Although the basal activity of this reporter plasmid is relatively low, coexpression of E2F-6 further reduced the reporter gene expression by 3- to 4-fold (Fig. 3*A*). Two lines of evidence indicate that repression by E2F-6 depends on sequence-specific DNA binding. First, expression of E2F-6 had no effect on the activity of a reporter plasmid in which the E2F binding sites had been mutated (Fig. 3*A*, right bars). Second, a point mutant of E2F-6 (E2F-6E68) that is defective in DNA binding (see below) only weakly repressed the wild-type luciferase reporter (Fig. 4*D*). E2F-6E68 also had little repressing activity on a different E2F-dependent luciferase reporter gene (driven by the E2F-1-promoter, Fig. 3*B*), confirming that DNA binding is required for transcriptional repression. To achieve the same level of E2F-6E68 repression as was detected with wild-type protein, a 5- to 10-fold higher level of E2F-6E68 protein was necessary (not shown). Western blots confirmed that both proteins were expressed at comparable levels when equivalent amounts of DNA were transfected (Fig. 4*F*).

To determine whether E2F-6 can also block transactivation by another E2F species, we cotransfected expression vectors for E2F-4 and E2F-6 with an E2F-dependent reporter gene (Fig. 3*C*). In the absence of E2F-6, E2F-4 increased reporter gene activity severalfold, as expected. In contrast, E2F-6 abolished transactivation by E2F-4 in a dose-dependent manner. Thus, when the protein concentration is increased, re-

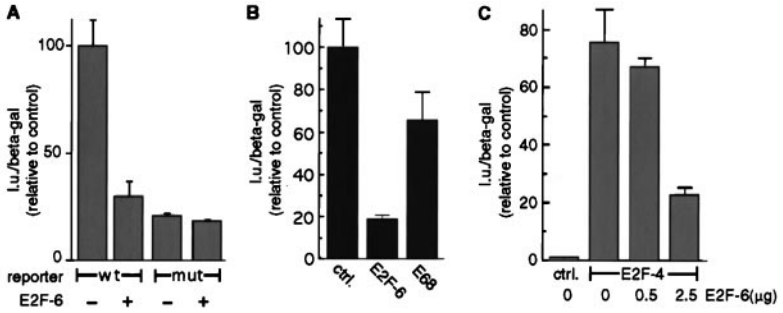

FIG. 3. E2F-6 is a transcriptional repressor. (*A*) A luciferase reporter plasmid with three E2F consensus binding sites (wt) or mutated binding sites (mut) was cotransfected with 1 μg of E2F-6 expression plasmid or empty vector. (*B*) A luciferase reporter with the E2F-1 promoter was cotransfected with 1 μg of E2F-6 expression plasmid, 1 μg of E2F-6E68 (E68, DNA-binding mutant, see Fig. 4), or 1 μg of empty vector (ctrl). (*C*) Five micrograms of E2F-reporter plasmid was cotransfected with empty vector or 1 μg of pCDNA-E2F-4 and 0.5 or 2.5 μg of E2F-6 expression plasmid, as indicated. In all transfections, 0.2 μg of CMV-β-gal was cotransfected as internal control, and luciferase activity was normalized to β-galactosidase activity.

pression by E2F-6 can dominate E2F-4-dependent transactivation, suggesting that E2F-6 can compete with other E2F proteins for the same DNA binding site. Because E2F-6 also reduced the activity of the E2F1 promoter (see Fig. 3*B*), the basal activity of which is not dependent on its E2F binding site (25), it is plausible that E2F-6 can also act, on its own, as an active transcriptional repressor. To test this hypothesis, we generated a chimera of the Gal4 DNA-binding domain and E2F-6, Gal4-E2F-6 (Fig. 4*C*). Gal4-E2F-6 was cotransfected with a GAL4-luciferase reporter plasmid (Fig. 4*A*). Coexpression of Gal4-E2F-6 repressed the activity of this reporter gene, as did Gal4-RB, a known transcriptional repressor. Chimeras of E2F-6 and the Gal4 DNA-binding domain (Fig. 4B) were next used to search for a trans-repression domain in human E2F-6. Coexpression of fusion proteins of Gal4 and the first 220 residues of E2F-6 had little effect on reporter activity. However Gal4-E2F-6(173–281) was a strong repressor, almost as active as intact Gal4-E2F-6. Taken together, these results strongly suggest that E2F-6 can act as an independent transcriptional repressor and that its carboxyl-terminal region contains a modular repression domain.

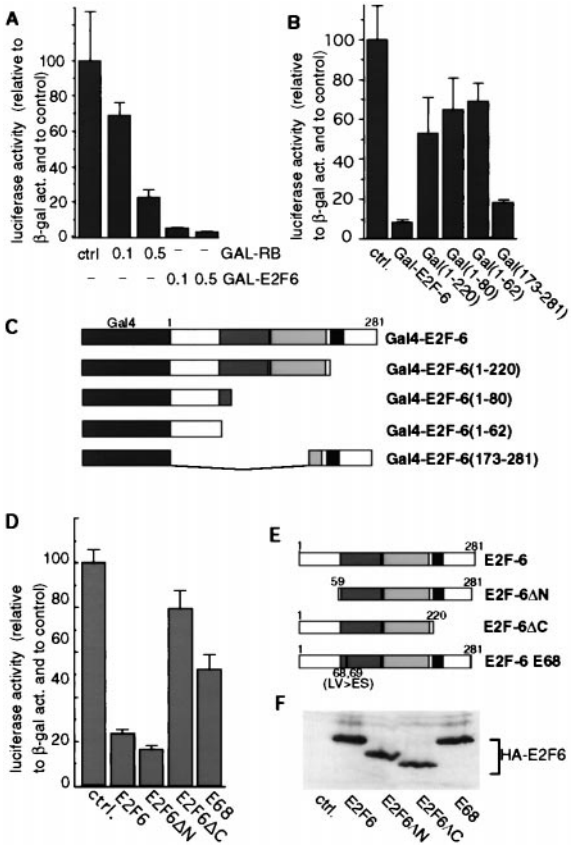

FIG. 4. E2F-6 contains a transferable repression domain. (*A*) Five micrograms of a luciferase reporter gene with multiple Gal4 binding sites was cotransfected with the indicated amounts of Gal4-RB or Gal4-E2F-6. (*B*) Five micrograms of the Gal4 luciferase reporter was cotransfected with 0.1 μg of the indicated Gal4-E2F-6 fusion protein expression vectors. (*C*) Schematic representation of the Gal4 fusion vectors used in *A* and *B*. (*D*) Five micrograms of E2F-luciferase reporter gene was cotransfected with 0.5 μg of empty vector (ctrl) or 0.5 μg of the indicated E2F-6 expression vectors. (*E*) Schematic representation of the E2F-6 mutants used. (*F*) Western blot analysis using an anti-HA antibody, showing that all E2F-6 proteins are expressed at comparable levels. In all reporter assays, 0.2 μg of CMV-β-gal was cotransfected, and luciferase activity was normalized to β-galactosidase activity.

We next wished to test the hypothesis that the repression domain in E2F-6 is normally required for E2F-6 repression of a reporter containing an E2F binding site. Two deletion mutants, E2F-6ΔN and E2F-6ΔC, were generated (Fig. 4*E*). Both retain the domains required for DNA binding and heterodimer formation. We also generated a point mutation in the DNA-binding domain (E2F-6E68) to address the role of DNA binding in transcriptional repression. Initially, we compared the ability of these different proteins (translated *in vitro*) to bind to the E2F site from the DHFR promoter by gel retardation methodology, as described above (not shown). E2F-6ΔC bound to the DHFR probe like wild-type E2F-6, whereas E2F-6ΔN bound with somewhat reduced affinity. As expected, E2F-6E68 was inactive in this assay at the low levels of protein tested (not shown). DNA binding by E2F-6ΔC and by E2F-6ΔN was specific for an E2F site, as demonstrated by competition experiments.

Next, we asked which segments of E2F-6 structure are necessary for transcriptional repression. Coexpression of E2F-6ΔN with an E2F-dependent reporter reduced reporter activity by about 5- to 10-fold, similar to the effect observed with wild-type E2F-6 (Fig. 4*D*). In contrast, E2F-6ΔC was largely inactive in this assay. The DNA binding mutant, E2F-6E68, retained only limited repression activity, confirming the results shown in Fig. 3. Western blot experiments showed that all proteins were expressed at comparable levels (Fig. 4*F*). Immunofluorescence staining with an anti-HA antibody confirmed that all proteins were similarly concentrated in the nucleus (not shown). Thus, transcriptional repression depends on the integrity of a carboxyl-terminal repression domain and upon E2F-6 DNA binding activity. Moreover, repression was not relieved by coexpression of a DP protein, suggesting that it is not a result of E2F-6 sequestering endogenous DP proteins

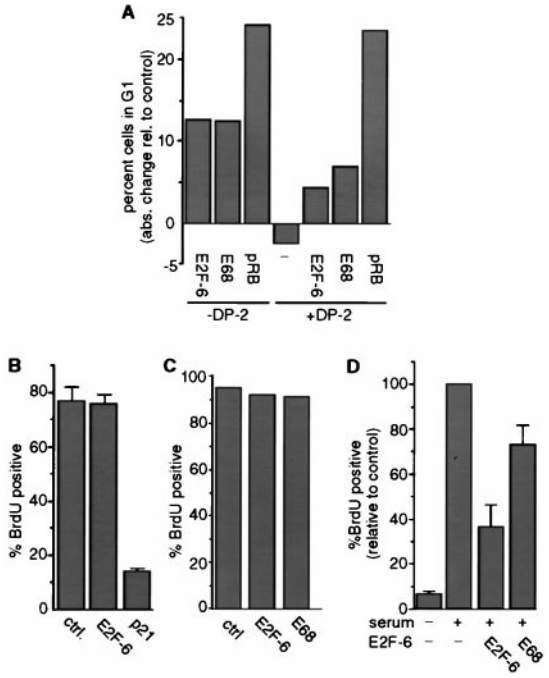

FIG. 5. E2F-6 does inhibit S-phase entry in starved cells but not in asynchronously growing cells. (*A*) Saos2 cells were cotransfected with the indicated expression vectors and CMV-GFP. Transfected cells were identified by their green fluorescence and analyzed by FACScan 2 days after transfection. Shown is the absolute change in the number of G_1 cells compared with a control transfection with empty vector. The experiment was repeated four times. One typical result is shown. (*B*) NIH 3T3 cells were cotransfected with 1 μg of the indicated expression vectors; 0.5 μg of CMV-GFP was cotransfected. Fourteen hours after transfection, cells were labeled with BrdU for 20 hr. Cells were stained with an anti-BrdU antibody and with a rhodamine-coupled secondary antibody. Transfected cells were identified by their green fluorescence, and the number of these cells with coexisting red fluorescence (i.e., the BrdU-positive cells) was determined. The experiment was repeated several times. The mean results from one experiment performed in independent triplicates are shown. Error bars represent standard deviation. (*C*) NIH 3T3 cells were microinjected with 25 μg/ml E2F-6 or E2F-6E68 expression plasmid and CMV-GFP (10 μg/ml). Ten hours after microinjection, 50 μM BrdU was added for 20 hr. DNA synthesis was analyzed as described above with an anti-BrdU antibody and with a rhodamine-coupled secondary antibody. (*D*) E2F-6 inhibits reentry into S phase of serum-starved NIH 3T3 cells. NIH 3T3 cells were serum starved in 0.1% bovine calf serum for 48 hr and than microinjected with 25 μg/ml E2F-6 or E2F-6E68 expression plasmid. In each case CMV-GFP (10 μg/ml) was coinjected. Ten hours after injection, cells were restimulated with 5% serum, and BrdU (50 μg/ml) was added. After 17.5 hr, cells were fixed and stained with an anti-BrdU antibody as described above. The mean results from four independent experiments are shown. Values were normalized to the number of uninjected cells that had entered S phase in each experiment. In a typical experiment, 60% of serum-treated, uninjected cells had entered S phase. Error bars represent standard deviation.

(not shown and ref. 24). Taken together, these data imply that E2F-6 actively represses transcription as opposed to simply competing with other E2F proteins for the same DNA binding site.

E2F-6 Inhibits Reentry into the Cell Cycle in Quiescent Cells. E2F-6 is a transcriptional repressor with DNA binding characteristics similar to those of previously described E2F proteins. Because the ability of Rb to act as a growth suppressor correlates with its ability to bind E2F and to repress transcription (12, 13), one might predict that E2F-6 overproduction has an effect on cell cycle progression analogous to that of Rb overproduction. To test this possibility, we analyzed the effects of ectopically expressed E2F-6 on cell proliferation. An expression plasmid for E2F-6 was transfected into asynchronously growing Saos2 cells. Transfected cells were identified by cotransfecting GFP and analyzing them by FACScan (Fig. 5*A*). Transfection of E2F-6 led to a 10–15% absolute increase in the number of cells in G_1 over the number present when empty vector was transfected. The DNA binding mutant, E2F-6E68, had a similar low effect. In comparison, pRB, the positive control in this assay, was much more active. Thus, there is minimal G_1 accumulation at the hands of ectopically overproduced E2F-6, and what there is does not require specific DNA binding activity. This fact suggests that it might be a result of titrating endogenous DP proteins. In keeping with this idea, G_1 accumulation by E2F-6 in these cycling cells, but not that induced by pRB, was substantially reduced by coexpression of DP-2. We conclude that E2F-6 has no specific effect on the short-term growth characteristics of asynchronously growing Saos2 cells.

We wished to know whether E2F-6 behaved similarly in an untransformed cell line. Therefore, E2F-6 was transiently transfected into asynchronously growing NIH 3T3 cells. As a positive control, we also transfected the cyclin-dependent kinase inhibitor, p21, a potent inhibitor of the G_1 exit. Again, a GFP expression vector was cotransfected to detect acutely transfected cells. Twelve hours after transfection, cells were labeled with BrdU for 20 hours and than stained with an anti-BrdU antibody and with a rhodamine-conjugated secondary antibody. Transfected cells were identified by their green fluorescence, and the number of these cells with coexisting red fluorescence (i.e., the BrdU-positive cells) was determined. (Fig. 5*B*). More than 80% of the cells transfected with E2F-6 incorporated BrdU and, thus, entered S phase. A similar result was obtained in cells transfected with empty vector. By contrast, only 20% of p21 expressing cells incorporated BrdU. Hence, E2F-6 does not interfere with entry into S phase when transiently overexpressed in asynchronously growing NIH 3T3 cells. Similar results were obtained after microinjection of an E2F-6 expression plasmid into asynchronous NIH 3T3 cells (Fig. 5*C*).

Next we asked whether E2F-6 has any effect on entry into S phase when ectopically expressed in quiescent cells. To address this possibility, we microinjected an E2F-6 expression plasmid together with a GFP expression plasmid into serum-starved NIH 3T3 cells. The same quantity of DNA was injected here as was injected into cycling NIH cells, where there was no inhibition of DNA synthesis (see Fig. 5*C*). Ten hours after microinjection, cells were re-fed with serum, labeled with BrdU for 17.5 hours, and than analyzed for DNA synthesis (Fig. 5*D*). By comparison with uninjected cells, E2F-6 led to a $\approx$50–60% reduction in the number of cells entering S phase. In contrast, E2F-6E68 had only a weak effect in this assay. Thus, the ability of E2F-6 to inhibit S-phase entry correlates with its ability to bind DNA and repress transcription (see Fig. 3).

DISCUSSION

Members of the E2F family of transcription factors are implicated in the control of cell cycle progression. In this study, we report the isolation and characterization of E2F-6, an additional member of this family. Analogous work has been accomplished by others (23, 24). E2F-6 domains, similar to those present in the previously characterized E2F proteins, mediate DNA binding and heterodimerization. However, E2F-6 is unique among the E2F family of proteins, in that it lacks a pocket protein binding and a transactivation domain. The previously described E2F proteins can behave as transcriptional repressors or activators, depending on whether or not they are bound to a pocket protein. E2F-6, however, is a transcriptional repressor that lacks a transactivation and a recognizable pocket protein binding domain. Transcriptional

repression by E2F-6 was not relieved by coexpression of a DP protein (ref. 24 and data not shown), suggesting that it is not a result of titrating away endogenous DP proteins.

A DNA binding mutant of E2F-6 was defective, albeit not completely so, in transcriptional repression activity compared with the wild-type protein. Although we did not detect any DNA binding activity by this mutant *in vitro*, it is possible that this mutant retains some DNA binding activity *in vivo*, which may account for its limited repression activity. Alternatively, E2F-6 may act through DNA-dependent and -independent mechanisms.

Two lines of evidence suggest that E2F-6 can serve as an active transcriptional repressor. First, E2F-6 reduced the activity of a synthetic E2F reporter and of the E2F-1 promoter. Because the basal activity of the latter is not dependent on the presence of an E2F binding site (25), one might argue that E2F-6, directly or indirectly, inhibits the activity of adjacent transcription factors. Second, E2F-6 contains a repression domain in its carboxyl terminus, and this segment repressed, even when it was fused to the Gal4 DNA binding domain. Hence, it does not mediate repression by competing with other E2F species for DNA binding. Taken together, these data imply that E2F-6 actively represses transcription through the specific action of its dedicated repression domain.

E2F-6 is a homologue of EMA, a recently described mouse E2F-like protein that contains a repression domain in its amino terminus (23). It is surprising that, in our experiments, the carboxyl terminus of E2F-6, but not the amino terminus, has repression activity. However, one amino acid residue, shown to be crucial for transrepression by EMA (Thr at position 6), is not conserved between the murine and human proteins. Moreover, while the amino terminus of EMA was shown to have repression activity, other regions were not tested similarly. Therefore, it remains to be shown whether the amino terminus of EMA is sufficient for EMA repression activity when it is bound at an E2F-binding site and, hence, whether E2F-6 and EMA repress transcription by partially related or wholly different mechanisms. The carboxyl-terminal repression domain of E2F-6 shows no significant homology to any protein in the database. Hence, it does not constitute a known repression domain.

E2F-6 resembles E2F/pocket protein complexes in two ways. First, it recognizes the same DNA binding element, and, second, it acts as a transcriptional repressor (12, 13). However, unlike pocket proteins (9, 29–31), overproduction of E2F-6 had only a weak effect on cell cycle progression in asynchronously growing Saos2 and no effect in NIH 3T3 cells. Indeed, the small G_1 increase associated with E2F-6 overproduction in Saos2 was almost completely nullified by the coexpression of DP-2, suggesting that it resulted from DP protein titration. It was, hence, an indirect effect.

Although E2F-6 failed to induce growth arrest in cycling NIH 3T3 cells, it did inhibit their cell cycle reentry from G_0 under the same experimental conditions. Like transcriptional repression, the ability of E2F-6 to block cell cycle reentry depended on specific DNA binding, suggesting that it is not a result of titrating endogenous DP proteins. It is conceivable that E2F-6 normally recognizes and represses a subset of E2F-dependent target genes, the products of which are required for exit from G_0 but not for normal cell cycle progression.

We do not yet know whether endogenous E2F-6 operates as an inhibitor of G_0 exit. However, unlike all other members of the family, it has both transcription repression and G_0 exit suppression activity, and it seems reasonable to hypothesize that the latter depends upon the former. If this model is valid, its biological function may represent a new element in the interplay between pocket proteins, E2F family members, and the mechanisms that tie them to cellular growth and differentiation control.

We thank our laboratory and divisional colleagues for numerous helpful discussions. This work was supported by grants from the National Institutes of Health/National Cancer Institute to D.M.L., by a grant from the Dana–Farber Cancer Institute–Novartis Drug Development Program, and by a long-term fellowship from the European Molecular Biology Organization to S.G.

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