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Cloning, Chromosomal Location, and Characterization of Mouse *E2F1*

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E2F has been implicated in growth control because of its association with the retinoblastoma protein and the presence of E2F binding sites in the promoters of several growth-regulated genes. Proteins that bind to an E2F site have been cloned from human and mouse cells. However, these two proteins (human E2F1 and mouse DP-1) are quite different in sequence. We have now cloned a mouse cDNA encoding a protein 86% identical to the human E2F1 protein. The mouse *E2F1* cDNA encodes a 430-amino-acid protein with a predicted molecular weight of 46,322 and detects mRNAs of 2.7 and 2.2 kb. Using primers complementary to sequences in the mouse *E2F1* 3′ untranslated region, we mapped the mouse *E2F1* gene to chromosome 2, near the *Agouti* and c-*src* loci. To understand the role of the different E2F family members in the growth of mouse NIH 3T3 cells, we examined the levels of *E2F1* and *DP-1* mRNAs in different stages of the cell cycle. Since the levels of *E2F1* but not *DP-1* mRNA correlated with changes in transcription from the *dhfr* promoter, we examined whether E2F1 could activate various growth-regulated promoters. We found that E2F1 could activate some (*dhfr*, thymidine kinase, and DNA polymerase alpha) but not all (thymidylate synthase, *cad*, and c-*myc*) of these promoters. On the basis of changes in levels of E2F1 and its ability to transactivate growth-regulated promoters, we propose that E2F1 may mediate growth factor-initiated signal transduction.

E2F activity is composed of a collection of cellular proteins that bind to the DNA sequence TTTSSCGC (S = C or G). E2F binding sites are required for a transcriptional increase from the dihydrofolate reductase (*dhfr*), thymidine kinase, c-*myc*, and *cdc2* promoters after serum stimulation of quiescent cells (reviewed in references 13 and 35). We have shown that two overlapping, inverted E2F binding sites at the *dhfr* transcription initiation site are necessary and two copies of these sites are sufficient for regulation of *dhfr* promoter activity in response to growth stimulation of NIH 3T3 cells (31, 46). Furthermore, cotransfection experiments demonstrated that the cloned human E2F1 can transactivate the mouse *dhfr* promoter through these E2F sites (46). However, interpretations of the role of E2F1 in regulation of the murine *dhfr* promoter were difficult since several different human cDNAs that encode proteins that bind to E2F sites have been cloned (17, 18, 22, 43), and the only mouse cDNA (*DP-1*) that has been cloned that encodes a protein that binds to an E2F site is quite different in sequence from human E2F1 (14). Thus, further analysis of the role of E2F in the growth regulation of the mouse *dhfr* promoter required cloning of additional mouse E2F cDNAs. Here we report the cloning and chromosomal location of mouse *E2F1*.

It has been difficult to correlate E2F binding activity with the transcription patterns of the G_1/S-phase-activated *dhfr* promoter because of the existence of the different E2F family members. Proteins having the DNA binding characteristics of E2F can be found in all stages of the cell cycle (7, 28, 34, 42). However, because two mouse E2F family members have now been cloned, it is possible to examine the levels of *E2F1* and *DP-1* mRNAs in different stages of the cell cycle. We show that levels of *E2F1* but not *DP-1* mRNA change in parallel with the levels of *dhfr* mRNA after growth stimulation of quiescent NIH 3T3 cells. Since the pattern of E2F1 expression correlates with transcription from G_1/S-phase-activated promoters, we transfected mouse E2F1 into NIH 3T3 cells and found that transcription from some but not all growth-responsive promoters increased.

MATERIALS AND METHODS

Library screening. A mouse 3T3 fibroblast λgt11 cDNA library (Clontech) in *Escherichia coli* Y1090 was plated on 150-mm-diameter petri dishes at the density of 3×10^4 plaques per plate, and duplicate phage lift filters were made from each plate. The phage DNA was denatured in 1.5 M NaCl–0.2 M NaOH for 2 min and neutralized in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.4 M Tris-Cl (pH 7.6) for 2 min and in 2× SSC for 2 min. The filters were prehybridized for at least 2 h at 42°C in hybridization solution containing 50% formamide, 3× SSC, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid (HEPES; pH 7.4), 0.4% sodium dodecyl sulfate (SDS), 2× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% bovine serum albumin, and 0.02% polyvinylpyrrolidone), and 100 μg of sonicated salmon sperm DNA per ml. Hybridization was carried out overnight at 42°C in the same solution plus 10^6 cpm of [α-^{32}P]dCTP-labeled probe per ml, which was generated by nick translation (39). The filters were then washed at room temperature first in 2× SSC–1× Denhardt's solution for 15 min, then in 2× SSC for 30 min, and finally in 0.1% SDS–0.1% SSC for 1 h at 53°C. The hybridization signals were visualized by autoradiography. Plaques that were positive on duplicate filters were isolated and purified by two additional rounds of screening as described above. The phage DNA was

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purified from plate lysate by using the Magic Lambda DNA purification system (Promega).

A total of 3×10^5 phage were screened by using a 590-bp *Pst*I fragment derived from the carboxyl-terminal portion of the human *E2F1* cDNA, pBKS-RBAP1 (22). A partial mouse cDNA was obtained from this screen and cloned into the *Eco*RI site of pBSM13+ (Stratagene), generating mE2F-i; 6×10^5 phage were then screened by using a 411-bp *Pst*I fragment derived from 5′ end of mE2F-i cDNA as the hybridization probe. A cDNA extending 5′ of the AUG start codon and terminating within the E2F1 coding sequence was obtained from this screen and cloned into the *Eco*RI site of pBSM13+, generating mE2F-p. To construct the entire mouse *E2F1* cDNA, mE2F-p (containing the most 5′-end sequences) and mE2F-i (containing the most 3′-end sequences) were ligated at a unique *Afl*II site present in the overlapping region of the two clones, generating mE2F1.

Subcloning and sequencing. The inserts from positive phage clones were subcloned into the *Eco*RI sites of pBSM13+ and M13mp19. The sequences were determined by the dideoxynucleotide chain termination method, using Sequenase 2.0 (United States Biochemical Corp.). A combination of both double-stranded and single-stranded DNA templates was used to obtain the complete sequences of both strands. The DNA and derived amino acid sequences were analyzed by the University of Wisconsin Genetics Computer Group software package.

Northern (RNA) blot analyses. Cytoplasmic RNA from growing NIH 3T3 cells or from NIH 3T3 cells at different times after serum stimulation was prepared as described previously (46). Selection of poly(A)$^+$ mRNA was performed according to the manufacturer's instructions, using the PolyATract mRNA isolation system (Promega). Poly(A)$^+$ mRNAs from L cells and macrophages were a gift from Charles Nicolet. Northern analysis was performed as described previously (46). The mouse *E2F1* probe used for Northern blotting was either the full-length cDNA or a partial cDNA (mE2F-i) that contained 73 bp of sequence of unknown origin at the 5′ end. The same bands were detected with either probe. The mouse *DP-1* probe used in the Northern analyses was generated by PCR, using primers corresponding to mouse *DP-1* mRNA positions 749 to 772 and 1093 to 1117 (using the numbering system of Girling et al. [14]). The PCR was carried out for 1 min at 95°C, 1 min at 52°C, and 1 min at 72°C for 35 cycles. Although the PCR product was used directly as a probe for Northern analyses, it was also cloned into the *Sma*I site of pBSM13+ and sequenced to confirm that it corresponded to mouse *DP-1* mRNA.

Gel mobility shifts. Nuclear extracts were prepared from serum-starved NIH 3T3 cells or from cells harvested 4 or 10 h after serum stimulation, using a previously described protocol (1) with a modified buffer system (11). The probes were a double-stranded oligonucleotide corresponding to the −20 to +9 region of the mouse *dhfr* promoter (containing the inverted, overlapping E2F sites shown in Fig. 4A) or a double-stranded oligonucleotide containing three consensus Sp1 binding sites (46). Nine micrograms of nuclear extract was mixed with 1 μg of salmon sperm DNA in 12 μl of 60 mM KCl–24 mM Tris HCl (pH 7.4)–6 mM $MgCl_2$–5% Ficoll–0.12 mM EDTA–0.3 mM dithiothreitol. The volume was adjusted to 19 μl with water, and the mix was incubated for 10 min at room temperature. One nanogram of radiolabeled probe in 1 μl of water was then added, and the incubation continued for 10 min. The probes were prepared by phosphorylating the 5′ ends of the double-stranded oligonucleotides with [γ-^{32}P]dATP, using T4 polynucleotide kinase. The reaction mixtures were electrophoresed for 60 to 120 min at 180 V on a 4% polyacrylamide gel which had been preelectrophoresed for 90 min at 180 V. The gel buffer was 0.25× Tris-borate-EDTA.

Chromosomal location. DNA was isolated from the spleens of C57BL/6J (B6), DBA/2J (D2), C3H/HeJ, and BALB/cByJ inbred mice, (D2 × B6)F_1 × B6 backcross mice, and (D2 × B6)F_2 intercross mice by a proteinase K-phenol method (41) and analyzed by PCR for allelic variation in the size of the d(GT) simple sequence repeat (SSR) identified in the 3′ noncoding region of the *E2F1* cDNA. The primers used for these assays corresponded to nucleotides 1518 to 1538 and 1765 to 1743 of the *E2F1* cDNA. The conditions for PCR amplification were similar to those described previously (10), using *Taq* DNA polymerase from Promega and a Perkin-Elmer Cetus (Norwalk, Conn.) thermocycler, except that the number of cycles of amplication was increased to 50 and the primers were not radioactively labeled. The PCR products were resolved on a 3% Metaphor agarose gel (FMC Bioproducts, Rockland, Maine), and the DNA fragments were visualized under UV light after staining with ethidium bromide. The sizes of the PCR products for B6, C3H/HeJ, and BALB/cByJ mice were identical and consistent with the 257 bp predicted from the cDNA sequence. The product obtained with use of D2 DNA was significantly smaller (approximately 190 bp).

To map the *E2F1* gene, the genotypes for 28 (D2 × B6)F_1 × B6 backcross animals were determined as described above and analyzed for linkage to 74 SSR markers covering the 19 autosomes (27), using Mapmaker 3.0 software (26). In addition, 45 DNA samples from (D2 × B6)F_2 mice were characterized for their genotypes at the *E2F1* locus and for three loci on chromosome 2 (*D2Mit14*, *D2Mit21*, and *D2Mit50*) and analyzed for linkage as described above.

Plasmids. The mouse E2F1 expression construct, CMV-mE2F1, contains the full-length cDNA inserted into the *Eco*RI site of the vector control pcDNA3 (Invitrogen). RB-luc, originally called XRP1 (40), which contains the 5′ flanking sequences from the human retinoblastoma 1 (*RB*) gene, was a gift from Paul Robbins. Myc-luc, which contains human c-*myc* promoter sequences inserted in the *Sma*I site of pGL2Basic (Promega), was a gift from Lynn Allen-Hoffmann. To create TS-luc, the *Pst*I fragment from the mouse thymidylate synthase promoter construct pSphI (8) was inserted into the *Pst*I site of pAAlucA (16). TK-luc was created by amplifying a region of the human thymidine kinase promoter (23) with the primers 5′ CCCGAAGCTTACTCTCCAAGGCCTG 3′ and 5′ AGTC CCTCCCTGCAA 3′. The PCR fragment was digested with *Pst*I and *Hin*dIII and inserted into the same sites of pAAlucA. Pol α-luc, originally called pDPA-BsLΔ5′ (37), which contains the human DNA polymerase alpha promoter, was a gift from Teresa Wang. CAD-luc, originally called pC-81/+83GL (24), and DHFR-luc were previously described (46). The promoter coordinates of each reporter construct (relative to the transcription initiation site at +1) are indicated in Fig. 4A.

Cell culture and transfections. NIH 3T3 cells (American Type Culture Collection) were maintained as described previously (46). Calcium phosphatase transfections were performed as described previously (46), with the following specifications. CMV-mE2F1 and the vector control, pcDNA3, were used at a 1:0.5 or 1:1 ratio with reporter plasmids; similar results were obtained with either ratio. These conditions were determined to be optimal in titration experiments (45). The amount of reporter plasmid was adjusted according to promoter strength; 5 μg of DHFR-luc, TK-luc, Pol α-luc, and Myc-luc and 2 μg of RB-luc, TS-luc, and CAD-luc were used per transfection reaction. The total DNA transfected per dish was increased to 15 μg with pBSM13+ vector or salmon sperm DNA. Cells were exposed to medium containing 0.5% serum for approxi-

mately 44 h, harvested, and assayed as described previously (46).

Nucleotide sequence accession number. The sequence of the mouse *E2F1* cDNA has been assigned accession number L21973 in the GenBank data base.

RESULTS

Isolation of the mouse *E2F1* cDNA. A λgt11 cDNA library prepared from mouse NIH 3T3 cell mRNA was screened with a probe derived from the carboxyl-terminal portion of the human *E2F1* cDNA (22). From 3×10^5 plaques screened, 10 positive clones were obtained and sequenced. All of the 10 clones were overlapping and contained sequences very similar to that of human *E2F1*, including part of the coding sequence and extending into the 3′ untranslated region (29). However, none of the 10 clones contained sequences similar to the 5′ untranslated sequences of the human *E2F1* cDNA, nor did any of the clones contain an in-frame AUG codon, suggesting that they were incomplete cDNAs. Three clones contained sequence at their 5′ ends that diverged from each other and from the human *E2F1* cDNA sequence. The clone showing the most extensive match to human *E2F1* (1,747 bp) was designated i. This clone diverged from human *E2F1* at nucleotide position 347 (using the numbering of Kaelin et al. [22]) and had 73 bp of unique sequence at its 5′ end. Inspection of the murine *E2F1* genomic sequence revealed that the point of divergence of clone i from human *E2F1* sequence is at the exact point of an exon/intron boundary in the mouse *E2F1* gene (20), suggesting that the unique 5′-end sequence of clone i may result from alternative splicing. We are currently characterizing the genomic structure of mouse *E2F1* to determine whether the divergent sequence at the 5′ end of the i cDNA is due to an alternative splice site. To clone the 5′ end of the mouse *E2F1* cDNA, we next screened 6×10^5 plaques of the same library, using the 5′-most 410-bp *Pst*I fragment of the longest mouse cDNA clone (clone i) as a probe. Ten positive clones were isolated and sequenced. One clone, p, contained sequence very similar to the 5′ end of the human *E2F1* cDNA, spanning the ATG start codon and extending into the 5′ untranslated region. A similar clone has also been obtained by others (17).

The complete nucleotide sequence and derived amino acid sequence of the mouse *E2F1* cDNA are shown in Fig. 1A. The cDNA clone is 1,989 bp long and contains an open reading frame encoding a putative protein of 430 amino acids (nucleotides 90 to 1382) with a predicted molecular weight of 46,322. The putative ATG start codon was chosen on the basis of a match with the Kozak sequence for a translational initiation site (PuCCATGG) (25) and comparison with the human E2F1 amino acid sequence. The coding sequence is flanked at the 5′ end by 89 bp and at the 3′ end by 607 bp. The mouse *E2F1* cDNA exhibited an overall 75% nucleotide identity with the human *E2F1* counterpart. The 5′ untranslated region of mouse *E2F1* is very G/C rich (84%) and is highly conserved (93% nucleotide sequence identity) with that of the human *E2F1*. This high degree of homology may indicate a functional role for the 5′ untranslated region in the regulation of E2F1 transcription, mRNA stability, or translation. The 3′ untranslated region of mouse *E2F1* cDNA has 53% nucleotide identity to the human *E2F1* sequence. There is neither a poly(A) tail nor a consensus poly(A) signal sequence present near the end of the 3′ untranslated region, indicating that the cDNA is partial. A 100-bp-long d(GT) stretch is present in the 3′ untranslated region. This simple repetitive sequence was used as marker for chromosomal mapping (see below).

The amino acid sequence of mouse E2F1 has an overall 86% amino acid identity to human E2F1 (Fig. 1B). The most similar regions are in the previously mapped E2F1 functional domains (18, 22, 43), with a 94% identity between amino acids 57 to 269 containing the DNA binding domain and a 97% identity between amino acids 369 to 430 containing the activation/RB binding domain (including a perfect match to the RB binding sequences). The majority of the amino acid differences between mouse and human E2F1 are conservative substitutions, with the most dramatic differences (60% identity) between the two E2F1 sequences in the region between the DNA binding and activation/RB binding domains. Since this region can apparently tolerate the presence of diverse amino acid residues, it is probably not critical for E2F1 function but instead may serve to link two functional domains.

To confirm that we had cloned a functional E2F1 protein, we tested the sequence-specific DNA binding properties of mE2F1. A bacterially expressed glutathione *S*-transferase–mouse E2F1 fusion protein was purified through a glutathione affinity column and tested for its ability to bind to E2F sites in gel mobility shift assays. As previously reported for human E2F1 (22), mouse E2F1 could bind with sequence specificity to the *dhfr* E2F sites (29). The DNA binding domain of E2F1 has been proposed to consist of basic, helix-loop-helix, and zipper motifs (22). Proteins having this structure bind to DNA as homo- or heterodimers (38), and recent evidence suggests that E2F1 binds to DNA as a dimer (21). However, others have shown that fragments of human E2F1 lacking the zipper portion (18) or the basic region (43) still possess DNA binding activity, suggesting that neither the basic nor zipper region of E2F1 is required for DNA binding. We note that there are two putative leucine zipper regions in E2F1 (amino acids 148 to 176 and 206 to 234), both preceded by a basic region (amino acids 104 to 122 and 156 to 161). The residues between each basic and leucine zipper region (amino acids 123 to 137 and 167 to 174) are predicted to form helices when analyzed with the Genetics Computer Group software package and the Chou-Fasman or Garnier-Robson structure prediction algorithm (29). Each of the truncated proteins from the previous studies (18, 43) would contain one set of basic, helix-loop-helix/leucine zipper motifs. Thus, E2F1 may have two overlapping DNA binding domains which can function independently.

Northern blot analysis was performed with mRNA from different mouse cell lines, using the *E2F1* cDNA as a probe. As shown in Fig. 1C, two different *E2F1* mRNAs of 2.7 and 2.2 kb were detected with use of mRNA from NIH 3T3, L, and macrophage (RAW 264.1) cell lines. Similar mRNAs were also detected from regenerating mouse liver (3). The detection of two *E2F1* mRNAs differs from the results with use of human cells in which *E2F1* mRNA appears to be a homogeneous population (18, 22, 43). The two mouse *E2F1* mRNAs could be derived from the same gene product as a result of differential promoter utilization and/or differential processing of *E2F1* primary transcripts, namely, alternative splicing (see above) or polyadenylation. The detection of two transcripts could also be due to cross-hybridization with a related mRNA. To date, only one other mouse cDNA (*DP-1*) that is related to mouse *E2F1* has been cloned. Two regions of *DP-1* mRNA are about 55% identical to similar regions of mouse *E2F1* mRNA. The patterns of expression of *DP-1* and *E2F1* mRNAs are distinct (see Fig. 3), suggesting that cross-hybridization with *DP-1* mRNA is not a problem. A probe complementary to the 3′ untranslated region of mouse *E2F1* detects both mRNAs (12), again supporting the hypothesis that both mRNAs seen by Northern analysis are transcribed from the mouse *E2F1* gene. Experiments are in progress to identify the origin of the two mouse *E2F1* mRNAs.

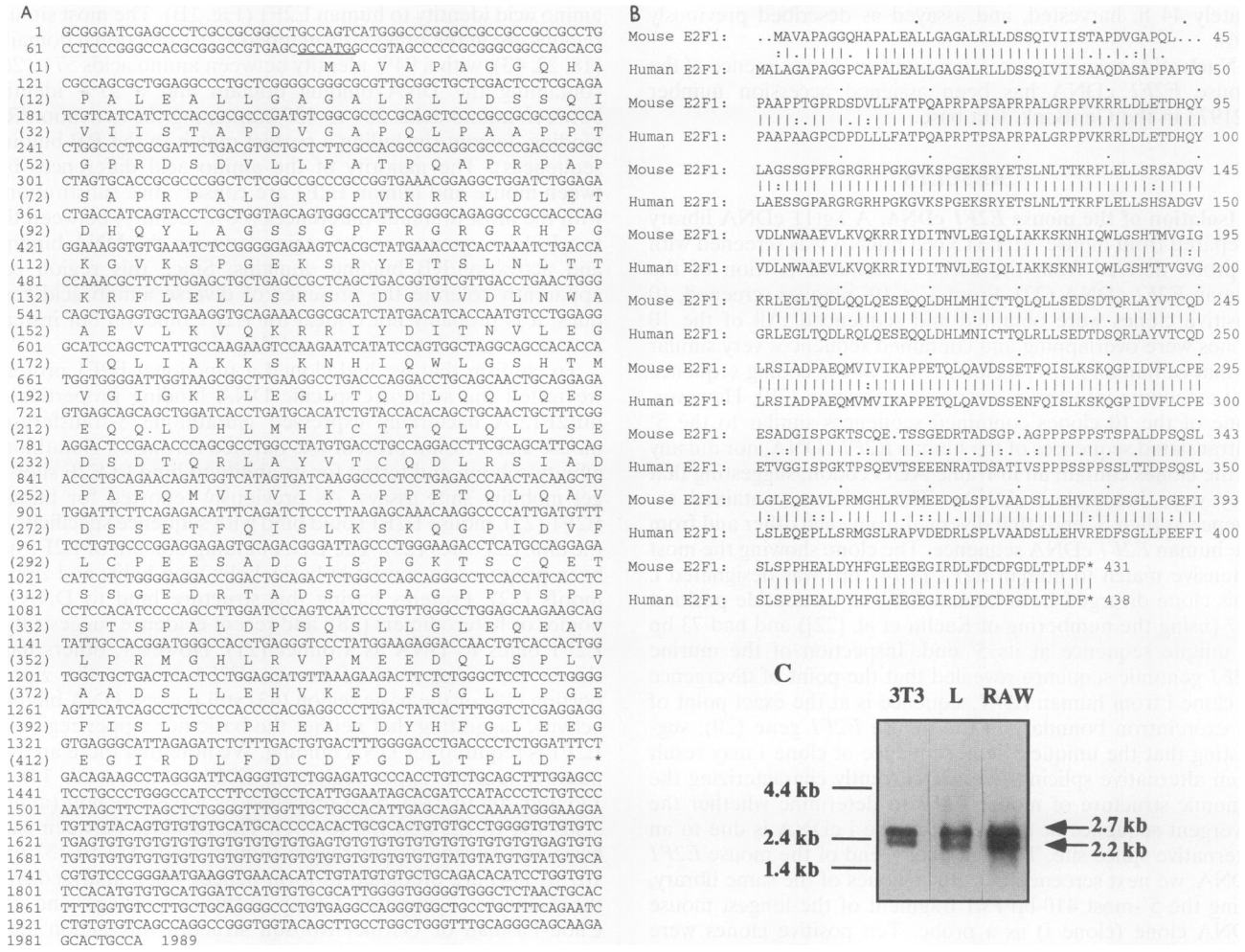

FIG. 1. Characterization of mouse E2F1. (A) Nucleotide sequence of the mouse *E2F1* cDNA clone. The deduced amino acid sequence is indicated in the standard one-letter amino acid code. Nucleotides and amino acids (in parentheses) are numbered at the left. The consensus Kozak sequence in the 5′ untranslated region is underlined, and the termination codon is indicated by an asterisk. (B) Best-fit alignment using the Needleman-Wunsch algorithm with the Genetic Computer Group software package, comparing the amino acid sequences of mouse and human E2F1. Identity is noted by vertical dashes, and similarity is indicated by single or double dots. Periods indicate the gaps that were introduced into the sequences to optimize the alignment. Amino acids are numbered at the right. (C) Northern blot analysis of mouse *E2F1* message. One microgram of poly(A)$^+$ mRNA from proliferating NIH 3T3 cells, L cells, and RAW 264.1 macrophage cells was resolved adjacent to RNA size markers (not shown). The partial mouse *E2F1* cDNA (mE2F-i) was used as a probe. Similar results were obtained when the same RNA sample was probed with the full-length mouse *E2F1* cDNA shown in panel A (12).

Chromosomal location of the mouse *E2F1* gene. The presence of a large d(GT) repeat in the 3′ noncoding region of the *E2F1* cDNA prompted us to search for length polymorphisms in this sequence among inbred mouse strains. Using primers that flanked this SSR, we found that DNA from B6, C3H/HeJ, and BALB/cByJ mice gave rise to a PCR product that was the same size predicted from the 3T3 cDNA, while the product for D2 DNA was approximately 60 bp smaller. We took advantage of this polymorphism to determine the genotypes at the *E2F1* locus of 28 (D2 × B6)F_1 × B6 backcross mice which had been previously typed for 74 SSR markers (27). Multipoint linkage analysis (26) revealed that the *E2F1* locus was on chromosome 2 in the interval between the markers *D2Mit21* and *D2Mit52* (Fig. 2A), with a highly significant logarithmic odds (LOD) score of 6.99. We also determined the genotypes of 45 F_2 mice for the *E2F1* locus and several chromosome 2 markers. A LOD score of 16.1 was obtained for placement of *E2F1* in the interval between *D2Mit21* and *D2Mit50* (Fig. 2B). The most likely location for the *E2F1* locus is 4.8 ± 2.0 centimorgans distal to *D2Mit21*. Thus, the *E2F1* locus is linked closely to the *Agouti* and c-*src* loci (19).

Role of E2F1 in growth regulation. Gel mobility shift experiments using nuclear extracts from serum-starved and stimulated NIH 3T3 cells indicate that the amount of E2F binding activity on the *dhfr* E2F sites increases only slightly as quiescent cells progress into S phase (Fig. 3A). However, the sizes of the DNA-protein complexes detected by gel shifts assays differ in G_0- and S-phase extracts. As a control, gel shift experiments were also performed with an oligonucleotide containing three Sp1 binding sites. No difference in the amount or size of the Sp1 complexes was detected in the different extracts. The different-size complexes formed on the *dhfr* E2F site oligonucleotide are likely due to different protein-protein interactions. Others have shown that E2F proteins from human cells can be found complexed with the RB protein, the RB-related protein p107, cyclin A, cyclin E, and p33^{cdk2} (5–7,

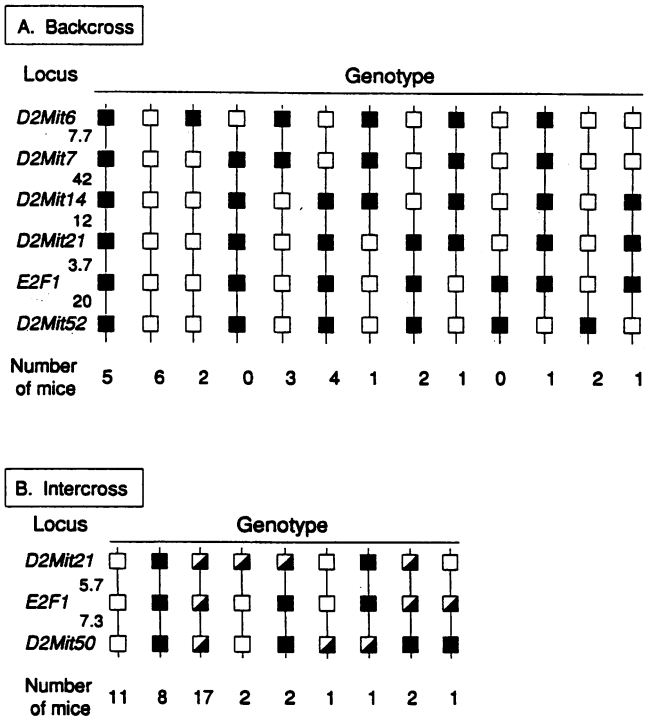

FIG. 2. Chromosomal localization of the mouse *E2F1* gene. The number of animals with each genotype is indicated below the representation of the chromosome. The estimated distances (in centimorgans) between markers are indicated in the spaces between the locus names. (A) Results of genotypic analysis of 28 (D2 × B6)F_1 × B6 backcross mice. The haplotypes shown are those inferred for the chromosome derived from the F_1 parent. Filled symbols represent the D2 allele, and open symbols indicate the B6 allele. The sole, double-recombinant haplotype observed in this collection of animals is shown at the far right. (B) Results of genotypic analysis of 45 (D2 × B6)F_2 intercross mice. For each locus, the symbols indicate the genotype as follows: open square, homozygous for the B6 allele; filled square, homozygous for the D2 allele; half-filled square, heterozygous.

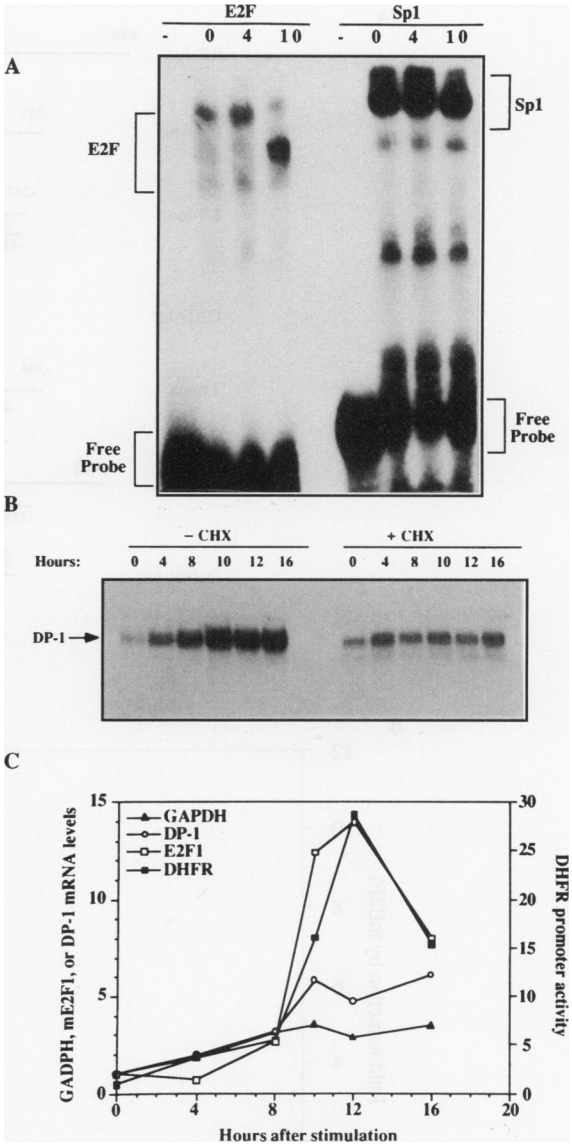

FIG. 3. Cell cycle analysis of E2F DNA binding activity and mRNAs. (A) Gel shift analysis of E2F and Sp1 proteins. Nuclear extracts prepared from serum-starved cells (G_0) or cells stimulated with serum for 4 (G_1) or 10 (G_1/S) h were used in a gel shift experiment with an oligonucleotide spanning from −20 to +9 of the *dhfr* promoter (E2F probe) or with an oligonucleotide containing three Sp1 sites. (B) Serum-starved NIH 3T3 cells were serum stimulated for the indicated times in the absence (−) or presence (+) of 10 μg of cycloheximide (CHX) per ml. Cytoplasmic RNA was prepared for Northern blotting analyses (5 μg per lane) and probed with a portion of the murine *DP-1* cDNA in this representative overnight exposure. (C) Comparison of the increases in *E2F1* mRNA, *DP-1* mRNA, and *GAPDH* mRNA and *dhfr* promoter activity after serum stimulation of quiescent NIH 3T3 cells. S phase begins 10 h after serum stimulation (46). All mRNAs were measured by using the same samples. The results for *dhfr* promoter activity and for *E2F1* and *GAPDH* mRNAs are reprinted from reference 46 with permission.

9, 28, 33, 42, 44). Thus, the gel shift patterns represent a complex combination of different E2F family members and different E2F protein-protein interactions. In contrast to the small changes in E2F DNA binding activity between G_0- and S-phase cells, we have reported previously that the levels of *E2F1* mRNA are very low in quiescent cells and increase at the G_1/S-phase boundary (46). We have now compared the changes in levels of mRNAs of two different members of the E2F family after serum stimulation of quiescent NIH 3T3 cells (Fig. 3). We found that the amount of *DP-1* mRNA increased about fivefold in a pattern similar to the increase in *GAPDH* mRNA. In contrast, in the same RNA samples, levels of *E2F1* mRNA increased 15-fold. Although it is difficult to directly compare levels of *DP-1* and *E2F1* mRNAs, it was very easy to detect *DP-1* mRNA in quiescent cells, both in the presence and in the absence of cycloheximide (Fig. 3B), whereas *E2F1* mRNA was almost undetectable under these same conditions (46). Therefore, a predominant E2F protein in G_0 extracts (Fig. 3A) may be DP-1.

The pattern of *E2F1* but not *DP-1* mRNA levels correlates with changes in *dhfr* promoter activity (Fig. 3C), which suggests that E2F1 may be the E2F family member that increases transcription from promoters that are activated at the G_1/S-phase boundary. Therefore, we tested a number of different growth-regulated promoters for the ability to be activated by mouse E2F1 in a transient transfection assay. We chose several promoters (*dhfr*, thymidine kinase, thymidylate synthase, *cad*, and DNA polymerase alpha) that are known to be activated at the G1/S-phase boundary, as well as two other promoters

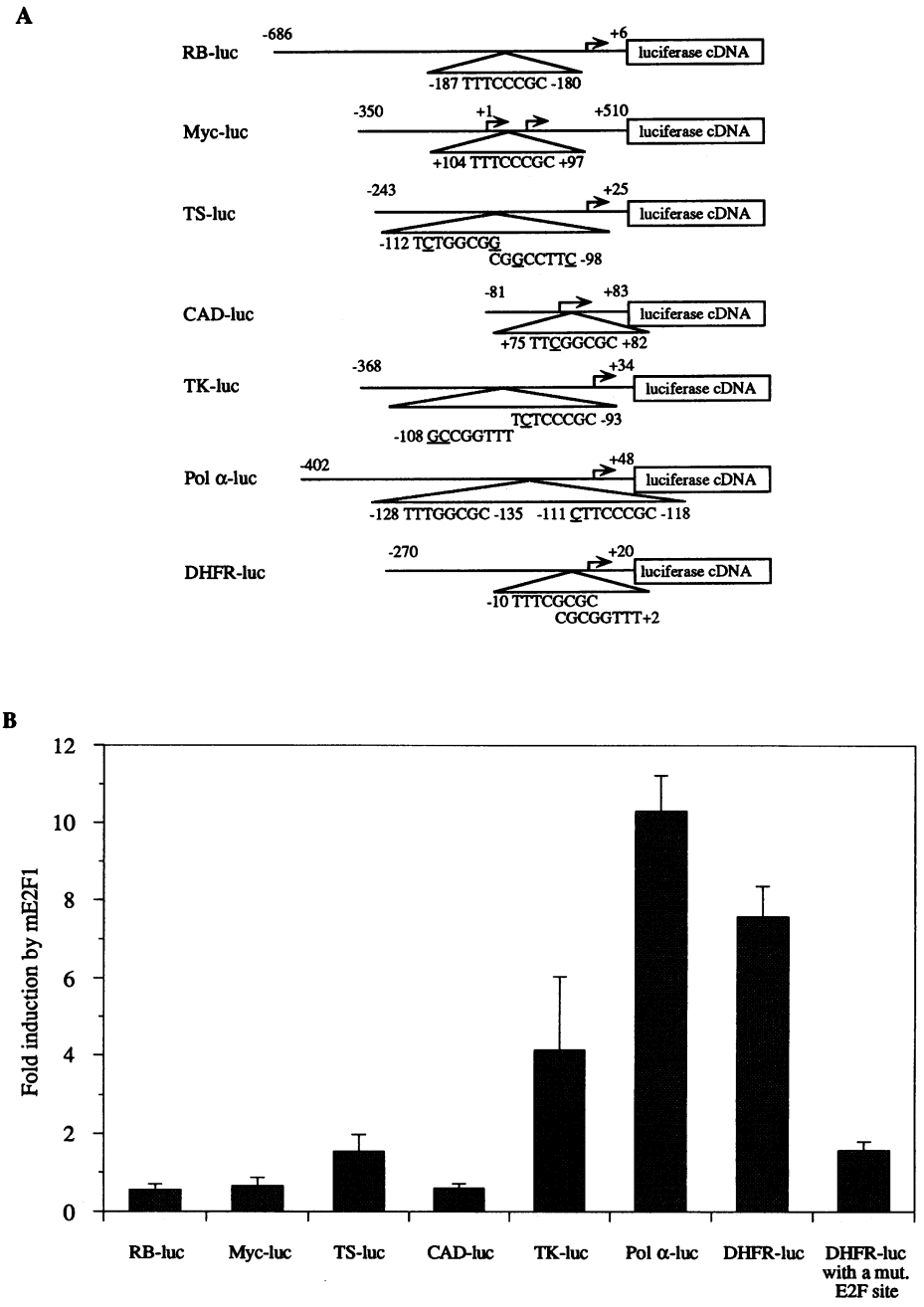

FIG. 4. Mouse E2F1 can activate some but not all growth-regulated promoters. (A) The coordinates and E2F sites in the various promoter constructs (not drawn to scale) are shown. Underlines indicate a mismatch to a consensus E2F site (TTTSSCGC; S = C or G). (B) The promoter-luciferase constructs and the CMV-mE2F1 construct or vector control were cotransfected into NIH 3T3 cells. After the transfection, the cells were starved in medium containing 0.5% calf bovine serum for 48 h before harvesting for luciferase assays. Promoter activity is reported as the activity of a promoter in the presence of CMV-mE2F1 divided by the activity of the same promoter in the presence of the vector control. Values are averages ± the standard errors of at least three independent experiments with at least two different DNA preparations.

(c-*myc* and *RB*) that have been suggested to be regulated by E2F (reviewed in references 13 and 35). The best fit to a consensus E2F site that can be found in each promoter is shown in Fig. 4A. Each of these sites has been shown to bind to E2F (29, 35, 36), with the exception of the site in the *cad* promoter; competition experiments suggest that this site does not bind E2F (4). Transient transfection analyses were performed with each promoter reporter plasmid plus either the constitutively expressed mouse E2F1 or the vector control. After transfection, the cells were placed in medium containing low serum to reduce the levels of endogenous E2F1. The cells were harvested approximately 44 h later and analyzed for luciferase activity (Fig. 4B). Although E2F sites are implicated in the transcription of the c-*myc* and *RB* promoters (15, 34), we found that expression of E2F1 in serum-starved cells did not increase transcriptional activity from either of these two pro-

moters. Also, expression of E2F1 in serum-starved cells was not sufficient to increase transcription from the *cad* or thymidylate synthase promoter. We found that expression of E2F1 in serum-starved cells was sufficient to increase transcription from the *dhfr* and DNA polymerase alpha promoters and, to a lesser extent, from the thymidine kinase promoter. Mutation of the E2F sites in the *dhfr* promoter abolishes growth regulation (31) and reduces the response of the promoter to mouse E2F1 (Fig. 4B). Similarly, deletion of the consensus E2F site in the DNA polymerase alpha promoter (37) abolishes growth regulation (2) and reduces the response of the promoter to E2F1 (45). Thus, E2F1 transactivation of the *dhfr* and DNA polymerase alpha promoters is specifically dependent on the presence of a consensus E2F site. These results, in combination with data indicating that E2F1 cannot transactivate the growth-regulated *cad* promoter fragment (32), suggest that E2F1 may be involved in the transcriptional increase of some but not all G_1/S-phase-activated promoters. We found that a DP-1 expression plasmid did not significantly affect the activity of any of the promoters listed in Fig. 4A (45). It should be noted that cloned DP-1 has not yet been shown to transactivate any promoter; therefore, we could not include a positive control for DP-1 activity in our transient transfection assays.

DISCUSSION

We have cloned a mouse cDNA encoding a protein that binds to and activates transcription from E2F sites. The sequences of two other proteins that can bind to E2F sites, human E2F1 (18, 22, 43) and mouse DP-1 (14), have been reported. The predicted protein sequence from our cDNA has an overall 86% amino acid identity with the sequence of human E2F1 but only a 42% identity with a 55-amino-acid region of the DP-1 DNA binding domain. Thus, we believe that our cDNA encodes mouse E2F1.

Although mouse E2F1 and DP-1 are clearly distinct proteins, they each encode a 430-amino-acid protein that binds to a consensus E2F site. Their similar sizes and DNA binding specificities, and the fact that both are expressed in NIH 3T3 cells, make it difficult to determine which, if either, of the two proteins is responsible for the growth regulation of various promoters containing E2F sites. To address this problem, we compared the levels of *E2F1* and *DP-1* mRNAs in NIH 3T3 cells from different stages of the cell cycle. Northern analyses of *E2F1* and *DP-1* mRNAs indicate that DP-1 can be easily detected in G_1-phase cells and suggest that DP-1 is more abundant than E2F1 in all stages of the cell cycle. Thus, a predominant E2F binding activity in G_1 cells in probably due to DP-1. This conclusion is supported by the ability of DP-1 antiserum to affect most of the E2F gel shift activity in extracts from mouse F9 cells (14) and by the inability of a human E2F1 antiserum to recognize the predominant gel shift bands in extracts from human T cells (22). We found that the levels of *E2F1* mRNA increase 20-fold from 4 to 12 h after serum stimulation, whereas levels of *DP-1* mRNA increase only 2.4-fold during the same interval. A recent study using gel shift analysis to examine different E2F binding activities in human T cells suggests that an abundant E2F protein (that is not E2F1) is found in G_1 cells and increases slightly in S-phase cells (7). Our RNA analyses suggest that this protein may be human DP-1. In agreement with our Northern analysis, E2F1 antiserum detects gel shift complexes only in late G_1- and S-phase cells (7).

Since changes in the levels of *E2F1* but not *DP-1* mRNA parallel the increase in transcription from late-response promoters, we examined whether E2F1 could transactivate the promoters of several genes that are regulated in response to changes in cell growth. We found that although both the c-*myc* and *RB* promoters contain E2F sites that have been implicated in transcriptional regulation (15, 34), expression of E2F1 does not activate these promoters. Also, E2F1 cannot activate transcription from the thymidylate synthase or *cad* promoter. There are several possible reasons for failure of E2F1 to activate these promoters. For example, E2F1 may require heterodimer formation to activate some but not all promoters. It is possible that the levels of DP-1 or a different protein are not sufficient in serum-starved cells for efficient heterodimer formation. Alternatively, another E2F family member may activate these promoters. Perhaps sequences flanking the E2F sites or other transcription factors binding nearby influence which E2F protein can activate a promoter. Although the thymidylate synthase promoter can bind affinity-purified E2F (29), the thymidylate synthase promoter is not sufficient for growth regulation (30). Therefore, E2F1 may be necessary but not sufficient for regulation of the thymidylate synthase gene. The *cad* promoter fragment used in our studies can confer late serum response regulation on the luciferase reporter (32). However, gel shift competition experiments suggest that this fragment of the *cad* promoter does not contain a high-affinity E2F site (4). This finding, in combination with the inability of E2F1 to transactivate the *cad* promoter, suggests that E2F1 may not be involved in the G_1/S-phase activation of the *cad* promoter.

The patterns of E2F1 expression are similar to the changes in thymidine kinase, *dhfr*, and DNA polymerase alpha mRNAs, and expression of E2F1 in serum-starved cells increased transcription from these promoters. We suggest that E2F1 is the limiting component for the transcriptional regulation of these three G_1/S-phase-activated promoters. Transfected DP-1 did not have a significant effect on the activity of any of the promoters listed in Fig. 4, suggesting that DP-1 levels are not limiting in quiescent cells or that DP-1 transactivates a different subset of E2F-driven promoters than those tested. We cannot rule out that transfected E2F1 activated transcription by releasing the normal activator from an inhibitory complex. Both E2F1 and DP-1 can bind to p107 and RB (14, 18, 22), the two known inhibitors of E2F activity. However, DP-1 did not increase activity of the *dhfr*, thymidine kinase, and DNA polymerase alpha promoters, suggesting that the activation of transcription by E2F1 from these three promoters was specific.

In summary, we have cloned a cDNA for mouse E2F1, a growth-regulated protein that can bind to and transactivate several G_1/S-phase-regulated promoters. We suggest that E2F1 plays an important role in the regulation of transcription at the G_1/S-phase boundary. It remains possible that E2F1 requires interaction with a constitutively present DP-1 via heterodimer formation for its function. It is also possible that other E2F family members, such as E2F2 and E2F3, that have been identified in human cells (17) are also present in mouse cells. Further understanding of the exact role of E2F1, E2F2, E2F3, and DP-1 in the regulation of the mammalian cell cycle requires the specific inactivation of these different family members.

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