MOLECULAR AND CELLULAR BIOLOGY, Oct. 1994, p. 6607–6615
0270-7306/94/$04.00+0

Vol. 14, No. 10

Transcription of the E2F-1 Gene Is Rendered Cell Cycle Dependent by E2F DNA-Binding Sites within Its Promoter

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Received 20 May 1994/Returned for modification 8 July 1994/Accepted 18 July 1994

The cell cycle-regulatory transcription factor E2F-1 is regulated by interactions with proteins such as the retinoblastoma gene product and by cell cycle-dependent alterations in E2F-1 mRNA abundance. To better understand this latter phenomenon, we have isolated the human E2F-1 promoter. The human E2F-1 promoter, fused to a luciferase cDNA, gave rise to cell cycle-dependent luciferase activity upon transfection into mammalian cells in a manner which paralleled previously reported changes in E2F-1 mRNA abundance. The E2F-1 promoter contains four potential E2F-binding sites organized as two imperfect palindromes. Gel shift and transactivation studies suggested that these sites can bind to E2F in vitro and in vivo. Mutation of the two E2F palindromes abolished the cell cycle dependence of the E2F-1 promoter. Thus, E2F-1 appears to be regulated at the level of transcription, and this regulation is due, at least in part, to binding of one or more E2F family members to the E2F-1 promoter.

E2F was originally identified as a cellular DNA-binding protein, or proteins, capable of recognizing the sequence TTT CGCGC within the adenovirus E2 promoter (40). A similar, if not identical, activity, called DRTF, was independently identified during studies of embryonic stem cell differentiation (43). Several independent lines of evidence suggest that E2F/DRTF (hereafter referred to as E2F) activity plays a critical role in cell cycle control. For example, the promoters for a number of genes encoding cell cycle-regulatory proteins contain potential E2F recognition sequences [TTT(G/C)(G/C)CG(G/C)]. Among these are the genes encoding dihydrofolate reductase (DHFR), DNA polymerase α, thymidine kinase, thymidylate synthase, c-Myc, c-Myb, cyclin A, Cdc2, and the retinoblastoma gene (RB) product (pRB) (18, 50, 56, 63, 64). In some instances, these E2F sites have been shown to be necessary and sufficient for rendering transcription cell cycle dependent (18, 50). Furthermore, E2F forms protein-protein complexes with cellular proteins which are known or inferred to regulate cell cycle progression. Among these are pRB and the pRB-like proteins p107 and p130 (3, 6, 8, 9, 11, 13, 58). In addition, E2F forms complexes, directly or indirectly, with certain cyclins and their associated kinases (4, 13–16, 29, 44, 46, 48, 54). The proteins which form complexes with E2F are, in turn, cell cycle dependent. For example, E2F appears to bind to p130 in G_0/G_1, to pRB during mid- to late G_1 and possibly into S phase, and to p107 in late G_1 and during S phase (13, 44, 55, 58).

Several unrelated DNA tumor viruses encode transforming proteins which can disrupt the ability of E2F to form complexes with proteins such as pRB. Among these are the simian virus 40 T antigen, the adenovirus E1A protein, and the high-risk human papillomavirus E7 proteins (50). The ability of these viral proteins to transactivate certain E2F-dependent promoters, coupled with the observation that wild-type pRB can suppress the activity of E2F-dependent promoters, has led to the suggestion that at least one species of free E2F is a transcriptional activator and that pRB-E2F complexes are either inactive or transcriptional repressors (1, 33, 50, 62, 65). p107 also represses E2F activity, at least when overexpressed (55, 66). To date all naturally occurring loss-of-function pRB mutants fail to bind to E2F. Furthermore, the smallest fragment of pRB identified to date which retains the ability to suppress the growth of $RB^{-/-}$ cells retains the ability to bind to E2F (26, 51). Thus, the ability of pRB to control cellular proliferation is tightly linked to its ability to bind to, and regulate, E2F.

Using recombinant pRB as a probe, several laboratories have isolated a cDNA encoding a protein with E2F-like properties, called E2F-1 (31, 37, 57). This protein contains an N-terminal DNA-binding domain and a C-terminal transactivation domain. Within the latter is nested an 18-residue RB-binding sequence (31). RB can suppress the ability of E2F-1 to transactivate via direct binding to this sequence (20, 28, 30). To date four additional E2F family members (E2F-2 to -5), at least some of which can also bind to pRB and/or p107, have been identified (7, 24, 35, 45). It now appears that E2F family members bind to DNA in vivo as heterodimers with members of the DP family of proteins (5, 32, 34, 41). DP1 was initially cloned by using microsequence obtained from a polypeptide which bound to E2F DNA affinity columns (25). Heterodimerization with DP1 increases the affinity of E2F-1 for its DNA recognition sequence and for pRB (32, 41).

Overproduction of E2F-1 can overcome the ability of pRB to arrest the growth of $RB^{-/-}$ cells (52, 66). This function is not dependent on the ability to bind to pRB or to induce pRB phosphorylation, in keeping with the view that E2F-1 is a downstream target of pRB action (52). E2F-1 can also induce quiescent fibroblasts to enter S phase, again underscoring that E2F activity likely plays a central role in regulating cell cycle progression (36, 53).

E2F-1 message abundance is cell cycle regulated (37, 59). For example, E2F-1 is not readily detectable in resting peripheral blood T cells, but is readily detectable as T cells enter and traverse S phase (37). Likewise, E2F-1 has features of a late serum response gene in 3T3 fibroblasts (59). In this study, we have cloned the human E2F-1 promoter and find that transcription from this promoter is cell cycle regulated. Further-

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more, this regulation appears to depend upon the ability of one or more E2F family members to bind to four potential E2F DNA-binding sites located immediately upstream of the E2F-1 transcription start site. Thus, in addition to posttranslational control through protein-protein interactions, E2F-1 is subject to transcriptional control, and this transcriptional control depends, at least in part, on the activity of one or more E2F family members.

MATERIALS AND METHODS

Cell culture and transfections. Human SAOS2 ($RB^{-/-}$) and U2OS ($RB^{+/+}$) osteogenic sarcoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal clone (HyClone). Murine NIH 3T3 fibroblasts were grown in DMEM supplemented with 5% defined-supplemented bovine calf serum (HyClone). Akata cells were grown in RPMI 1640 supplemented with 10% fetal clone. All cells were maintained at 37°C in a humidified 10% CO_2-containing atmosphere.

Cells were transfected at 70 to 80% confluence. SAOS2 and U2OS cells were transfected by a modified calcium phosphate protocol (10). Briefly, for each 100-mm-diameter plate of cells, 20 μg of DNA was mixed with 0.45 ml of H_2O, 50 μl of 2.5 M $CaCl_2$, and 0.5 ml of 2× BES [*N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid]-buffered saline (50 mM BES [pH 6.95], 280 mM NaCl, 1.5 mM Na_2HPO_4). pGL2-basic (Promega) was used as carrier DNA. Following a 15-min incubation at room temperature, the calcium phosphate-DNA solution was added dropwise to the cells. The cells were then transferred to a 37°C, 5% CO_2 incubator for 16 h. The following day, the cells were washed with phosphate-buffered saline (PBS), fed with the appropriate medium, and incubated at 37°C in 10% CO_2 for 24 h prior to analysis.

3T3 cells were transfected by the calcium phosphate method as described previously (27). Transfections were carried out in triplicate, with each 100-mm-diameter plate receiving 30 μg of DNA in 0.5 ml of H_2O–0.5 ml of 0.5 M $CaCl_2$–1 ml of 2× *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline (HBS; 280 mM NaCl, 50 mM HEPES [free acid], 1.5 mM Na_2HPO_4). pGL2-basic was used as carrier DNA. Following transfection, cells were glycerol shocked for 4 min in 15% glycerol–1× HBS (vol/vol) and then washed twice with PBS. For synchronization experiments, transfected cells were placed in DMEM–10% serum for 2 to 4 h, after which they were washed twice with DMEM and starved for 48 to 72 h in DMEM–0.5% serum. Following starvation, cells were either harvested or refed with medium containing 10% serum and incubated for up to 24 h. The progression of cells through the cell cycle during these experiments was monitored by flow cytometry of replicate samples. For this analysis, cells were fixed in 80% ethanol, stained with 50 μg of propidium iodide per ml in PBS, and analyzed on a Becton Dickinson flow cytometer.

Genomic cloning and plasmid construction. To isolate E2F-1 genomic clones, a human placental genomic library (constructed by partial digestion with *Sau*3A and cloned into EMBL-3 SP6/T7) (Clontech) was probed with a 250-bp *Nco*I-*Dra*III E2F-1 cDNA fragment which had been radiolabeled with ^{32}P by using a T7 Quick Prime kit (Pharmacia). DNA was purified from positive phage and analyzed by Southern blotting with the same probe. An ~3.3-kb *Eco*RI-*Hin*dIII genomic fragment which hybridized to this probe at high stringency was subcloned into pSP72, using standard techniques, to create pSP72-λ3.3. The authenticity of this clone was confirmed by DNA sequence obtained by using E2F-1 cDNA-specific oligonucleotides and a Sequenase 2.0 kit (U.S. Biochemical) as instructed by the manufacturer. To facilitate sequencing, pSP72-λ3.3 was cut at unique *Dra*III and *Eco*RI sites, blunt ended with Klenow enzyme, and recircularized to create pSP72-λ3.3HD. pSP72-λ3.3HD was cut at unique *Nco*I and *Cla*I sites, blunt ended with Klenow enzyme, and recircularized to create pSP72-λ3.3HN. This plasmid contained an E2F-1 genomic fragment extending ~2.2 kb 5′ of an *Nco*I site corresponding to an *Nco*I in the 5′ untranslated region of the E2F-1 cDNA. pSP72-λ3.3HN was cut at unique *Asp* 718 and *Hin*dIII sites, blunt ended, and recircularized to create pSP72-λ3.3AN, which retained an ~275-base genomic fragment extending 5′ of this *Nco*I site. The *Hin*dIII-*Nco*I and *Asp* 718-*Nco*I genomic fragments from these latter two plasmids were subcloned, using standard techniques, into the luciferase reporter plasmid pGL2-basic, cut with the appropriate enzymes, to create pGL2-HN and pGL2-AN, respectively.

S1 nuclease analysis. Total RNA was isolated by the method of Chomczynski and Sacchi (12) from T cells purified from healthy human donors and incubated for 48 h in the absence (unstimulated) or presence (stimulated) of phorbol myristate acetate (22) or was isolated from asynchronous Akata Burkitt lymphoma cells (60). One hundred-nanogram amounts of two oligonucleotide probes (CTCGGCGAGGGCTCGATCCCG CTCC GCC CCC GGC CGC CCT GCC TG CAA AGT CCC G GCCACT and GCTCCGCCCCCGGCCGCCGCTGCCTG CAAAGTCCCGGCCACTTTTACGCGCCAAATCCTTTT) were end labeled with 60 μCi of [γ-^{32}P]ATP in the presence of 50 mM Tris-Cl (pH 8.0)–7 mM $MgCl_2$–1 mM dithiothreitol (DTT) and T4 polynucleotide kinase for 1 h at 37°C. Unincorporated label was removed by centrifugation on a Sephadex G-25 column.

Fifty micrograms of total RNA from Akata cells or from unstimulated or stimulated T cells or, as a control, yeast tRNA (Sigma) was resuspended in 15 μl of hybridization buffer [40 mM piperazine-*N,N'*-bis(ethanesulfonic acid) (PIPES; pH 6.4), 1 mM EDTA, 0.4 M NaCl, 0.1% sodium dodecyl sulfate] and added to 15 μl of formamide and 2.0×10^5 to 4.0×10^5 cpm of end-labeled oligonucleotide. Samples containing oligonucleotides with a GC content of <60% were incubated for 10 min at 65°C and then overnight at 37°C. Samples with oligonucleotides with a higher GC content were incubated for 10 min at 85°C and then overnight at 50°C. Samples were then digested with S1 nuclease by the addition of 300 μl of S1 buffer (50 mM sodium acetate [pH 4.6], 0.28 M NaCl, 4.5 mM $ZnSO_4$) containing 2,400 U of S1 nuclease (GIBCO/BRL). Following a 30-min incubation at 37°C, samples were phenol-chloroform extracted and ethanol precipitated. The DNA was resuspended in 10 μl of sequencing gel loading buffer (U.S. Biochemical), heated at 68°C for 10 min, and analyzed on an 8% sequencing gel (U.S. Biochemical). The labeled oligonucleotides were also used as templates in Maxam-Gilbert G and A sequencing reactions (47) and used for markers on these gels.

PCR. One nanogram of pGL2-AN was PCR amplified by using *Pfu* DNA polymerase (Stratagene) as instructed by the manufacturer. The program was as follows: denaturation for 1 min at 94°C, annealing for 2 min at 55°C, and primer extension for 3 min at 72°C for 35 cycles, followed by a final primer extension for 10 min at 72°C. The downstream primer for these reactions was 5′-CCGGAATGCCAAGCTTACTTAGATCT CGAGCT-3′. The upstream primers for the creation of plasmids −151-+64, −131-+64, −92-+64, −49-+64, and −24-+64 were 5′-CGCCCCGGTACCCCGCCCCATCTCGCCC CT-3′, 5′-TCTCGCGGTACCGCCAAATCCGGCGCGTTA-3′, 5′-GGAACCGGTACCGTTGTTCGTCACGGCCGG3′,

5′-GGCGGGGGTACCCGGCTCGTGGCTCTTTCG-3′, and 5′-TTTCGCGGTACCAAGGATTTGGCGCGTAAA-3′, respectively. The PCR products were phenol-chloroform extracted, ethanol precipitated, resuspended in water, and digested with *Asp* 718 and *Bgl*II. The digested DNA was resolved in 1% SeaPlaque agarose (FMC) and ligated, using standard techniques, into pGL2-basic (Promega) linearized with these same two enzymes. The sequence of each PCR product was confirmed by double-stranded DNA sequencing using a Sequenase kit (U.S. Biochemical).

In vitro mutagenesis. Two antisense oligonucleotides, 5′-GCCAAATCCTTTTGGTCGAGCAAGAGCCACGAGC-3′ and 5′-TCCCGGCCACTTTGATGCGACCAATCCTTTTT GCC-3′, were synthesized to mutate the 5′ and 3′ E2F palindromic sites, respectively. Mutation of both palindromes was accomplished by using the oligonucleotide 5′-TCCCCGCC ACTTTGATGCGACCAATCCTTTTGGTCGAGCAAGAG CCACGAGC-3′. In vitro mutagenesis was performed essentially as described previously (21), using a Bio-Rad Muta-Gene kit. Sequence analysis was used to confirm the presence of the appropriate mutations.

Transactivation assays. Luciferase assays were performed as described previously (41). Luciferase values were normalized for β-galactosidase activity as previously described (41).

Gel shift assays. Nuclear extracts were prepared as previously described (67), with the following modifications. Cells were isolated and washed with PBS, and the pellet was suspended in 5 volumes of buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT, 0.5 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml). The cells were allowed to swell on ice for 1 h, and lysis was achieved by 20 strokes with a Dounce homogenizer. Nuclei were pelleted for 10 s at maximal speed in an Eppendorf Minifuge, resuspended in 3 volumes of buffer B (20 mM HEPES [pH 7.9], 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 0.5 mM NaF, 0.5 mM $NaVO_4$, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml) and incubated on ice for 30 min. Cellular debris was removed by centrifugation at maximal speed in an Eppendorf Minifuge (at 4°C) for 10 min.

Gel shift assays were performed with the following double-stranded oligonucleotides: E2FDHFRwt (5′-CTAGAGCAA TTTCGCGCCAAACTTG-3′ and 5′-GATCCAAGTTTGGC GCGAAATTGCT-3′), E2FDHFRmut (5′-CTAGAGCAAT TGCTCGASCCAACTTG-3′ and 5′-GATCCAAGTTGGTC GAGCAATTGCT-3′), E2FA (5′-CTAGAGCTCTTTCGCG GCAAAAAGGAG-3′ and 5′-GATCCTCCTTTTTGCCGC GAAAGAGCT-3′), and E2FB (5′-CTAGAGGATTTGGCG CGTAAAAGTGG-3′ and 5′-GATCCCACTTTTACGCGC CAAATCCT-3′). Binding reactions were performed with 3 μl of extract, 7 μl of BFD (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT), 1 μg of sheared salmon sperm DNA, and 4 ng of radiolabeled oligonucleotide in a total volume of 25 μl. Reaction mixtures were incubated for 20 min at room temperature and loaded onto a 4% (30:1 acrylamide/bisacrylamide) polyacrylamide gel containing 0.5× Tris-borate-EDTA. For competition experiments, the reaction mix was incubated with 200 ng of the indicated unlabeled oligonucleotide for 20 min prior to the addition of radiolabeled oligonucleotide.

Database searches. Transcription factor-binding sites were identified by searching the transcription factor database (23) with the FINDPATTERNS program.

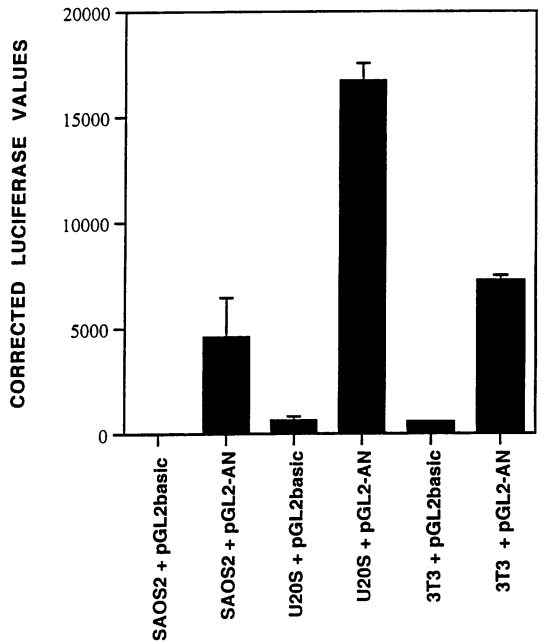

FIG. 1. An *Asp* 718-*Nco*I E2F-1 genomic fragment exhibits promoter activity in multiple cell lines. Ten micrograms of pGL2-basic (Promega) or pGL2-AN, 5 μg of pCMV-βgal, and carrier DNA were transfected in triplicate into the indicated cells grown in 100-mm-diameter dishes (see Materials and Methods); 48 h later, cell extracts were prepared and luciferase and β-galactosidase assays were performed. Data shown are absolute luciferase values (± 1 standard error), normalized for β-galactosidase activity.

RESULTS

We set out to clone and characterize the E2F-1 promoter in order to identify *cis*-acting elements which regulate E2F-1 transcription. To this end, we screened a human genomic library with probes derived from the E2F-1 cDNA. Genomic fragments which hybridized to the E2F-1 cDNA were subcloned into plasmids and sequenced by using E2F-1-specific primers. A detailed analysis of the genomic organization of E2F-1 will be presented elsewhere (49). An ~275-bp *Asp* 718-*Nco*I fragment was chosen for further study, as it contained all of the known 5′ untranslated region of E2F-1 and its 3′ end corresponded to an *Nco*I site found near the 5′ end of the E2F-1 cDNA. This fragment was subcloned upstream of the luciferase cDNA present in the promoterless plasmid pGL2-basic. The resulting plasmid, pGL2-AN, was then transfected into asynchronous SAOS2 osteosarcoma cells ($RB^{-/-}$), U2OS osteosarcoma cells ($RB^{+/+}$), and murine NIH 3T3 fibroblasts in parallel with the backbone plasmid (as a negative control) and pCMV-luciferase (as a positive control). Cell extracts were prepared 48 h following transfection and assayed for luciferase activity. The results of these experiments (Fig. 1 and data not shown) clearly demonstrated that this fragment of the E2F-1 gene contained a promoter capable of directing the transcription of the luciferase cDNA. Similar results were obtained when a genomic fragment which contained an additional ~2 kb of 5′ sequence (pGL2-HN) was similarly assayed (data not shown).

The 275-bp *Asp* 718-*Nco*I E2F-1 fragment was subcloned into pSP72 to create pSP72-λ3.3AN and was partially sequenced in the 3′-to-5′ direction, starting from the 5′ end of the published E2F-1 cDNA sequence, using synthetic oligonu-

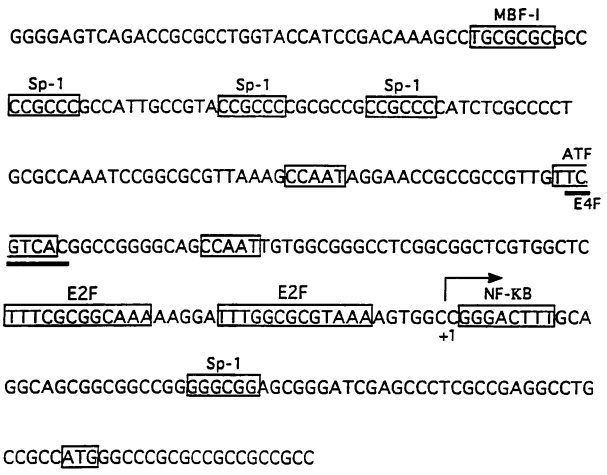

FIG. 2. Sequence of the *Asp* 718-*Nco*I E2F-1 genomic fragment. The E2F-1 translation initiation site and proposed transcription start site (see Fig. 3) are indicated along with the locations of two CCAAT boxes and potential binding sites for MBF-1, Sp-1, ATF, E4F, E2F, and NF-κB.

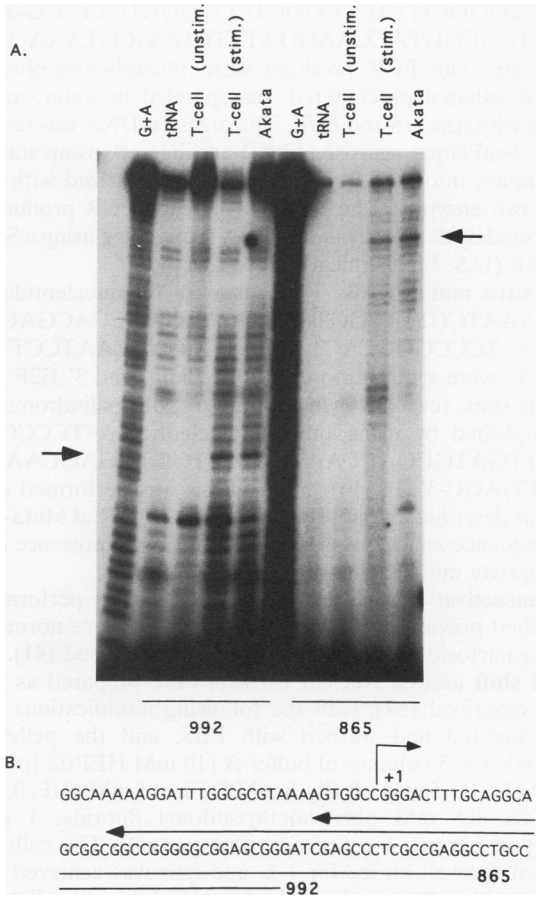

FIG. 3. S1 nuclease mapping of the E2F-1 transcription start site. Total RNA from the indicated sources was hybridized to one of two ^{32}P-end labeled synthetic oligonucleotides (B), digested with S1 nuclease, and resolved in an 8% Tris-borate-EDTA gel. The sizes of the protected fragments (arrows) were determined from their migration relative to Maxam-Gilbert sequencing ladders obtained with the same oligonucleotides (not shown).

cleotides (Fig. 2). In an attempt to identify the transcription start site(s) for E2F-1, S1 analysis was performed with synthetic oligonucleotides based on this sequence and its alignment relative to the 5′ ends of the published human E2F-1 cDNA sequences (31, 37, 57). These oligonucleotides were end labeled with ^{32}P, hybridized with mRNA obtained from either resting or stimulated peripheral blood T cells, digested with S1 nuclease, and electrophoresed in acrylamide gels. Resting peripheral blood T cells contain very little if any E2F-1 mRNA, whereas E2F-1 mRNA can be readily detected in T cells that have entered S phase (37). S1 analysis was also performed with tRNA (as a negative control) and mRNA obtained from a Burkitt lymphoma cell line in which E2F-1 mRNA is readily detectable by Northern (RNA) blot analysis (19). The results of these experiments (Fig. 3) suggest that there is a major transcription start site 121 bases upstream of the translation start site. We have thus far been unable to interpret data obtained from primer extension experiments performed with E2F-1 mRNA, possibly because of low message abundance and problems due to secondary structure near the 5′ end of the message. Thus, we cannot at present exclude the possibility that other transcriptional start sites exist 5′ of −121. We note, however, that the transcriptional start site identified by us is in precise agreement with the transcription start site determined for murine E2F-1 (33a).

To more precisely demarcate the promoter region for E2F-1, a series of 5′ truncation mutants of pGL2-AN were generated by PCR. These plasmids were then scored for luciferase activity following transfection into mammalian cells as described above. These experiments, summarized in Fig. 4, suggest that the core E2F-1 promoter is contained within a colinear segment of ~150 bases upstream of the transcription start site. This region, which is highly conserved between the mouse and human E2F-1 genes (33a), contains potential binding sites for E2F, Sp-1, ATF, E4F, and NF-κB, in addition to two CAAT boxes (Fig. 2) (17).

E2F-1 message abundance is low or undetectable during G_0/G_1 and rises as cells enter and traverse S phase (37, 59). In addition, the presence of functional E2F sites is necessary and sufficient, at least in some contexts, to render the activity of certain promoters cell cycle dependent. These observations, coupled with the presence of potential E2F DNA-binding sites within the E2F-1 promoter, suggested that transcription of E2F-1 might be both E2F and cell cycle dependent. We therefore examined whether the wild-type E2F-1 promoter in pGL2-AN gave rise to cell cycle-dependent transcription of the luciferase cDNA. pGL2-AN was introduced into asynchronous NIH 3T3 cells by transfection. The cells were then grown in low serum for 48 to 72 h to induce quiescence. Serum was then readded ($T = 0$), and at various time points thereafter, cells were harvested and analyzed for cell cycle position by fluorescence-activated cell sorting (FACS) (following propidium iodide staining) or lysed and analyzed for luciferase activity. The results of these experiments clearly demonstrated that pGL2-AN (wild type) directed the synthesis of luciferase in a cell cycle-dependent manner (Fig. 5A). The changes in luciferase activity paralleled the changes which are observed in E2F-1 mRNA abundance under similar conditions (59).

To determine whether either or both of the putative E2F-binding-site palindromes were responsible for the cell cycle dependence observed in Fig. 5A, we introduced mutations, singly or in combination, into these two sites and assayed the ability of the resulting promoters to direct the synthesis of luciferase in cells which were rendered quiescent by serum

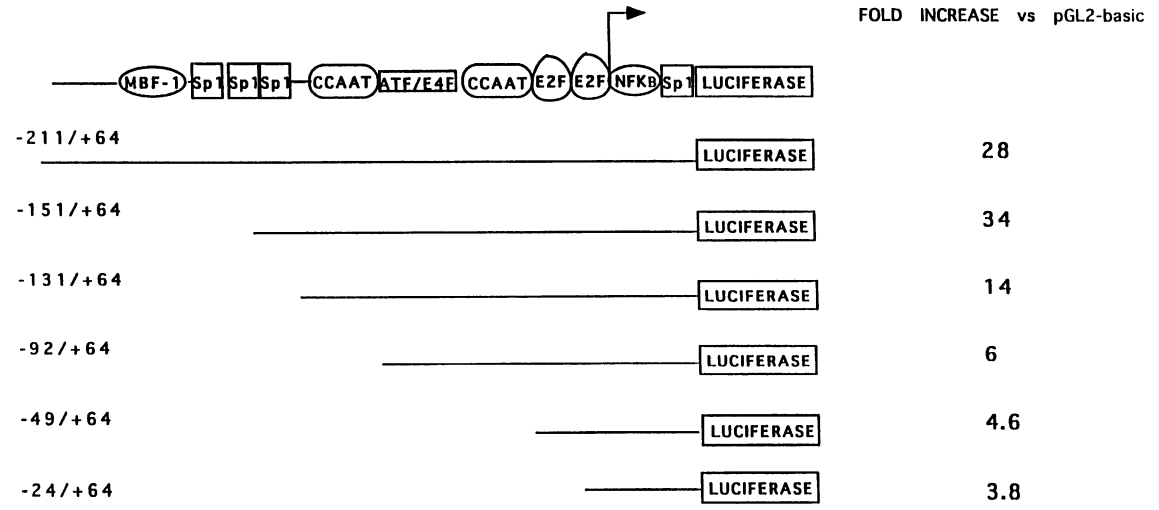

FIG. 4. Deletion analysis of the E2F-1 promoter. U2OS osteosarcoma cells grown in 100-mm-diameter dishes were transfected, in triplicate, with 10 μg of the indicated E2F-1 promoter fragment (cloned upstream of the luciferase cDNA in pGL2-basic), 5 μg of pCMV-βgal, and 5 μg of carrier DNA (see Materials and Methods); 48 h later, cell extracts were prepared and luciferase and β-galactosidase assays were performed. Luciferase values were normalized for β-galactosidase activity and are expressed as fold increase relative to cells transfected with pGL2-basic alone.

starvation ($T = 0$) and in cells which had been induced to enter S phase by serum refeeding ($T = 15$). Mutation of either the upstream palindrome (E2F A) or the downstream palindrome (E2F B) alone was insufficient to completely abrogate cell cycle dependence (Fig. 5B). In contrast, mutation of both sites led to constitutive activity of the promoter which was at least as high as that observed with the wild-type promoter under serum-fed conditions.

We next examined which if any of the known E2F species might serve to regulate the E2F-1 promoter. In initial pilot experiments, we cotransfected pGL2-AN or pGL2-AN (ΔE2FA+B) either with a backbone plasmid containing the cytomegalovirus promoter or with mammalian expression plasmids in which the cytomegalovirus promoter was cloned upstream of cDNAs for either E2F-1, E2F-2, or E2F-3 into asynchronous U2OS osteosarcoma cells. Cells were lysed 48 h following transfection and analyzed for luciferase activity. As can be seen from Fig. 6, all of the E2Fs studied were able to transactivate the wild-type E2F-1 promoter. In contrast, no significant effect was observed when these E2Fs were assayed by using the altered E2F-1 promoter in which both E2F palindromes were mutated (data not shown). Furthermore, a DNA-binding defective E2F-1 point mutant (E2F E138) failed to transactivate in these assays (Fig. 6).

We next examined whether an E2F species could bind specifically to the E2F-1 promoter by performing gel shift experiments with synthetic probes corresponding to either the upstream or downstream E2F site (E2F A or B probe, respectively). In parallel, gel shift experiments were performed with a synthetic probe corresponding to the E2F double site in the DHFR promoter. As can be seen from Fig. 7, both the A and B probes can form a number of specific DNA-protein complexes. The specificity of these complexes was determined by competition experiments with each of the three unlabeled probes, as well as with a mutant DHFR probe which fails to bind to E2F. The complexes indicated by the arrows (open and closed) were competed for by the wild-type but not the mutant DHFR probe, suggesting that these complexes contain an E2F species. It seems likely, given what is known of E2F binding to other canonical E2F DNA-binding sites (such as those found in the DHFR probe), that the more slowly migrating E2F-containing complexes forming on the E2F A and E2F B probes (for example, the complexes indicated by closed arrows) contain pRB, or pRB-like proteins, and/or cyclins and their associated kinases, although this remains to be formally proven and is the subject of ongoing experiments. Likewise, experiments designed to identify the E2F or E2Fs which participate in these complexes are under way. Nonetheless, our biochemical and functional data suggest that E2F-1 transcription is cell cycle dependent and that this form of regulation depends upon the binding of one or more E2F family members to its promoter.

DISCUSSION

Previous studies have shown that E2F-1 mRNA abundance is cell cycle regulated, raising the possibility that E2F-1 activity is regulated, at least in part, by alterations in E2F-1 transcription. In this study, we used an E2F-1 genomic clone and S1 analysis to identify a potential transcriptional start site for E2F-1. Furthermore, we showed that a genomic clone extending ~150 bases 5′ of the predicted major transcriptional start site possessed strong promoter activity when it was fused to a heterologous reporter cDNA and transfected into asynchronous cells. Finally, we showed that the human E2F-1 promoter, fused to a luciferase cDNA, gave rise to cell cycle-dependent luciferase activity. In particular, luciferase activity was low in quiescent cells and achieved maximal levels as cells entered S phase, in keeping with what was observed previously for E2F-1 mRNA abundance. We conclude that we have identified the E2F-1 promoter and that E2F-1 transcription is cell cycle regulated. We cannot, at present, exclude the possibility that differences in E2F-1 mRNA half-life also contribute to the cell cycle differences which have been observed previously when E2F-1 mRNA abundance was measured.

Inspection of the sequence of the cloned E2F-1 promoter led to the identification of potential binding sites for E2F, Sp-1, ATF, E4F, and NF-κB. The presence of Sp-1 and ATF sites, and the absence of a TATA box, is fairly typical of known E2F-responsive promoters (2). While the functional signifi-

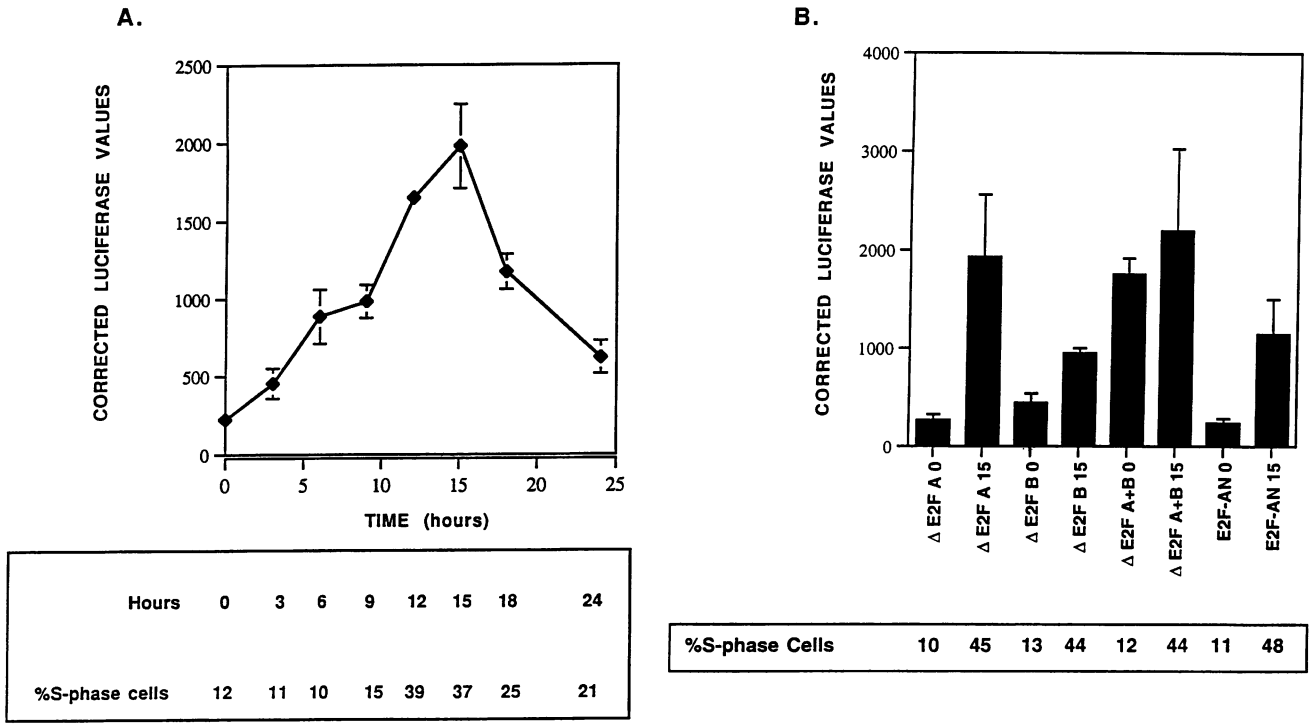

FIG. 5. Cell cycle dependence of the E2F-1 promoter. (A) NIH 3T3 fibroblasts grown in 100-mm-diameter dishes were transfected, in triplicate, with 10 μg of pGL2-AN, 5 μg of pCMV-βgal, and 15 μg of carrier DNA. The cells were placed in low serum (0.5%) 4 h after the removal of the calcium phosphate precipitates. The cells remained in low serum for >48 h to induce quiescence, at which point serum was added ($T = 0$). At the indicated time points, cells were removed for cell cycle FACS analysis (following propidium iodide staining for total DNA content) and for determination of luciferase and β-galactosidase activities. Luciferase values (±1 standard error) were normalized for β-galactosidase activity. (B) NIH 3T3 fibroblasts grown in 100-mm-diameter dishes were transfected, in triplicate, with 10 μg of either pGL2-AN, pGL2-AN(ΔE2FA), pGL2-AN(ΔE2FB), or pGL2-AN(ΔE2FA+B) plus 5 μg of pCMV-βgal, and 5 μg of carrier DNA (see Materials and Methods). Induction of quiescence and determination of β-galactosidase activity were as in panel A. Cells were removed for cell cycle FACS analysis (following propidium iodide staining for total DNA content) and for determination of luciferase activity at $T = 0$ (G_0/G_1) and $T = 15$ (S). Luciferase values (±1 standard error) were normalized for β-galactosidase activity.

cance of this organization is unclear, it is perhaps noteworthy that pRB may, in addition to regulating E2F activity, directly or indirectly interact with Sp-1 and ATF-2 (38, 39, 61).

The four E2F sites in the E2F-1 promoter are organized as two imperfect palindromes. E2F sites, in at least some promoter contexts, are necessary and sufficient to render transcription cell cycle dependent. We found that mutation of both E2F palindromes led to constitutive activation of the E2F-1 promoter. In particular, it appeared that loss of E2F binding to the E2F-1 promoter led to derepression of the promoter during G_1 (Fig. 5B). This observation is consistent with recent studies which suggest that RB-E2F complexes, rather than being inert, may bind to, and actively repress, certain E2F responsive promoters (42, 52, 62).

We found that E2F-1, -2, and -3 were all capable of activating the E2F-1 promoter provided that the E2F-binding sites in the latter were intact. Furthermore, an E2F species bearing a subtle mutation within its DNA-binding domain failed to transactivate. Thus, all of the E2Fs for which cDNAs are currently available can bind and transactivate the E2F-1 promoter. No attempt was made in these experiments to normalize for E2F protein expression. Thus, no significance can yet be attributed to the differences in transactivation observed between the E2F family members tested here. Furthermore, it is possible that differences among the E2F family members with respect to binding site preference and transactivation function in vivo might be obscured by overproduction. Thus, these transactivation studies did not address which E2Fs regulate the E2F-1 promoter under physiologic conditions.

In addition, gel shift analyses performed with synthetic oligonucleotides corresponding to either the upstream or downstream E2F-binding-site palindrome revealed that both palindromes could bind specifically to cellular proteins present in extracts prepared from untransfected cells. That at least some of these cellular binding activities were E2F-like was confirmed by performing competition experiments with unlabeled competitor oligonucleotides corresponding to wild-type or altered versions of a canonical E2F DNA-binding site. Thus, using both biochemical and functional approaches, we conclude that E2F can interact with the E2F-1 promoter.

The existence of functional E2F sites within the E2F-1 promoter raises at least two, nonmutually exclusive, possibilities. The first is that E2F-1 serves to positively autoregulate its own transcription as cells enter S phase. The second is that the activity of another member, or members, of the E2F family is responsible for the orderly activation of the E2F-1 promoter. It is perhaps noteworthy that specific E2F-containing complexes do occur in a seemingly precise temporal order during the cell cycle. For example, p130-containing E2F complexes appear to predominate in G_0/G_1 and pRB-containing E2F complexes arise in G_1 and perhaps persist into S phase, whereas p107-containing E2F complexes arise in late G_1 and S phase (13, 44, 55, 58). Which E2F family members participate in these complexes is currently being investigated, al-

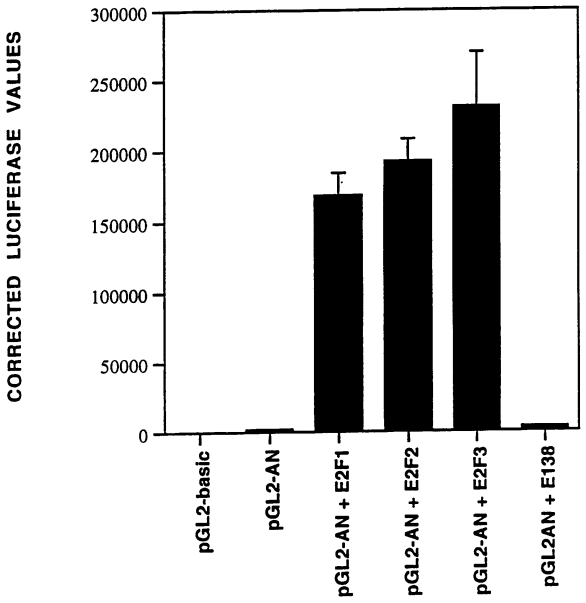

FIG. 6. E2F species can transactivate the E2F-1 promoter. U2OS osteosarcoma cells grown in 100-mm-diameter dishes were transfected, in triplicate, with 10 μg of pGL2-AN, 5 μg of pCMV-βgal, 1 μg of the indicated E2F expression plasmids (or the backbone plasmid), and 4 μg of carrier DNA (see Materials and Methods); 48 h later, cell extracts were prepared and luciferase and β-galactosidase assays were performed. Luciferase values were normalized for β-galactosidase activity.

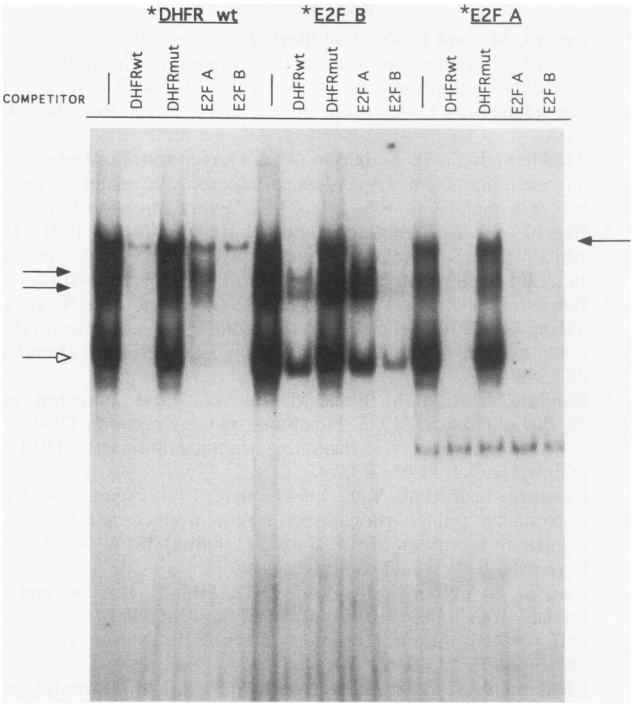

FIG. 7. Two putative E2F elements from the E2F-1 promoter bind to E2F activities from cell extracts. Synthetic oligonucleotides corresponding to the E2F double site found in the wild-type (wt) DHFR promoter or to the two putative E2F-binding-site palindromes found in the E2F-1 promoter (A, upstream palindrome; B, downstream palindrome) were labeled (*) and incubated with nuclear extracts prepared from the Rael Burkitt lymphoma cell line. Complexes were separated by electrophoresis in a 4% nondenaturing acrylamide gel and detected by autoradiography. Reaction mixtures were incubated in the absence or presence of a 50-fold molar excess of the indicated unlabeled competitor oligonucleotides. Specific complexes are indicated by arrows. DHFRwt and DHFRmut, wild-type and mutant DHFR probes, respectively.

though it appears that E2F-1, -2, and -3 are capable of interacting with pRB in vivo, whereas E2F-4 may bind preferentially to p107 (7, 24, 35, 45). From the cotransfection data cited above, we cannot conclude that E2F-1, -2, or -3 interacts with the E2F-1 promoter under physiologic conditions, since as stated above, binding site preference differences in vivo between E2F family members might be obscured if they were overproduced.

E2F-1 production is sufficient to induce quiescent fibroblasts to enter S phase (36, 53). Thus, regulation of E2F-1 mRNA at the transcriptional level may be a critical control with respect to the ability of resting cells to proceed through the cell cycle. A potential conundrum, however, is that E2F activity appears to be repressed during G_0/G_1 by virtue of its interaction with unphosphorylated pRB (reviewed in reference 50). If so, why might there be a need to control E2F-1 at the level of transcription? One possibility is that redundant control mechanisms have evolved to prevent untimely S-phase entry by E2F-1 which, at least under experimental conditions, is followed by apoptosis (53). A second possibility, for which there is now some experimental evidence, is that the ability of pRB to regulate E2F-1 can be overcome by overproduction of the latter (52, 66). Finally, our data, as stated above, might suggest that pRB-E2F complexes, rather than being inert, serve as active transcriptional repressors, as has been suggested by others (42, 52, 62). Indirect evidence suggests that the ability of pRB to induce a G_1 blockade may be due specifically to the activities of these repressor complexes (52). Thus, absence of E2F-1 production may have different functional consequences than being in a state wherein all E2F-1 is bound to proteins such as pRB. In particular, it is formally possible that cells which are entering the cell cycle from G_0 (as opposed to continuously cycling cells) initially lack a kinase activity required for inactivating the transcriptional repression function(s) performed by E2F-1 when bound to pRB-like proteins.

ACKNOWLEDGMENTS

We thank Myles Brown for the human genomic library, J. Lees for the E2F-2 and E2F-3 cDNAs, W. Krek for pRC-E2F-1, -2, and -3, G. McMahon for the β-galactosidase plasmid pCMV-βgal, and J. Nevins for the E2F-1 point mutant E138. We also thank P. Farnham for sharing unpublished data regarding the murine E2F-1 promoter, Tom Graf for assistance with computer database searches, Jennifer Rogers for excellent technical assistance, Suman Shirodkar for advice concerning the design of cell cycle experiments, our colleagues in the Kaelin and Livingston laboratories for helpful discussions, and David Livingston for critical reading of the manuscript and for continued advice and support.

E.N. is supported by the Sandoz Research Institute, and W.R.S. is supported by an NIH National Research Scientist Award. W.G.K. is supported by an NIH Physician-Scientist Award and by a grant from the Sandoz Research Institute. W.G.K. is a McDonnell Foundation Scholar.

ADDENDUM IN PROOF

A paper on E2F-1 expression similar to this one has recently been published (D. G. Johnson, K. Ohtani, and J. R. Nevins, Genes Dev. **8**:1514–1525, 1994).

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