MOLECULAR AND CELLULAR BIOLOGY, Dec. 1997, p. 6994–7007 Vol. 17, No. 12
0270-7306/97/$04.00+0

Subunit Composition Determines E2F DNA-Binding Site Specificity

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Received 23 May 1997/Returned for modification 8 July 1997/Accepted 26 August 1997

The product of the retinoblastoma (Rb) susceptibility gene, *Rb-1*, regulates the activity of a wide variety of transcription factors, such as E2F, in a cell cycle-dependent fashion. E2F is a heterodimeric transcription factor composed of two subunits each encoded by one of two related gene families, denoted E2F and DP. Five E2F genes, *E2F-1* through *E2F-5*, and two DP genes, *DP-1* and *DP-2*, have been isolated from mammals, and heterodimeric complexes of these proteins are expressed in most, if not all, vertebrate cells. It is not yet clear whether E2F/DP complexes regulate overlapping and/or specific cellular genes. Moreover, little is known about whether Rb regulates all or a subset of E2F-dependent genes. Using recombinant E2F, DP, and Rb proteins prepared in baculovirus-infected cells and a repetitive immunoprecipitation-PCR procedure (CASTing), we have identified consensus DNA-binding sites for E2F-1/DP-1, E2F-1/DP-2, E2F-4/DP-1, and E2F-4/DP-2 complexes as well as an Rb/E2F-1/DP-1 trimeric complex. Our data indicate that (i) E2F, DP, and Rb proteins each influence the selection of E2F-binding sites; (ii) E2F sites differ with respect to their intrinsic DNA-bending properties; (iii) E2F/DP complexes induce distinct degrees of DNA bending; and (iv) complex-specific E2F sites selected in vitro function distinctly as regulators of cell cycle-dependent transcription in vivo. These data indicate that the specific sequence of an E2F site may determine its role in transcriptional regulation and suggest that Rb/E2F complexes may regulate subsets of E2F-dependent cellular genes.

Functional inactivation of the retinoblastoma (Rb) protein is associated with the genesis of a variety of human cancers, including retinoblastoma, osteosarcoma, and small cell lung, bladder, and breast carcinomas (for reviews, see references 39 and 72). In addition to regulating the rate of cell proliferation, Rb participates in the induction and/or maintenance of cellular differentiation. Rb controls cell proliferation and differentiation, at least in part, via its physical or functional interaction with a bevy of transcription factors (for a review, see reference 27). Interactions of Rb with sequence-specific DNA-binding proteins such as ATF-2, Sp1/Sp3, C/EBP, and myoD can lead to the stimulation of *trans* activation, whereas Rb negatively regulates transcription directed by factors such as E2F, Elf-1, and UBF.

E2F is the first cellular transcription factor that has been shown to bind Rb directly, and it remains the best-characterized target of Rb function (10, 24, 33, 51, 61). Rb forms cell cycle-regulated complexes with E2F via a carboxyl-terminal portion of Rb, the so-called Rb pocket, that is a hot spot for mutational inactivation in human tumors. It is widely suspected that such mutations and their concomitant abrogation of Rb/E2F complexes account at least in part for the unchecked proliferation of tumor cells. In normal cells, E2F and other targets of Rb function are liberated from the Rb pocket via cyclical waves of phosphorylation by the cyclin-dependent kinases (8, 11, 45, 47, 49, 66, 72). Two Rb-related proteins, p107 and p130, form similar cell cycle-regulated complexes with E2F in vivo, and their association with E2F also results in the negative regulation of E2F-mediated transcription (9, 13, 14, 59, 62, 70, 81, 82). Protein complexes composed of Rb family members and E2F can also be disrupted by the binding of their respective pocket regions by several viral oncoproteins, including adenovirus E1A, the simian virus 40 (SV40) virus large-T antigen, and human papillomavirus E7 (2, 4, 17, 19, 30, 78). Transformation by these viral oncoproteins is dependent, at least in part, on their functional inactivation of Rb family members. Thus, complexes of Rb or Rb-related proteins and E2F may be abrogated by mutation, phosphorylation, or physical displacement from the carboxyl-terminal pocket domain.

E2F is an ubiquitously expressed, heterodimeric transcription factor composed of two structurally related subunits, termed E2F and DP (for reviews, see references 43, 51, and 63). Each of these proteins is encoded by gene families: E2F is encoded by at least five genes, denoted *E2F-1* through *E2F-5*, and DP is encoded by at least two mammalian genes, denoted *DP-1* and *DP-2*. Recent evidence indicates that *DP-2* encodes at least three protein isoforms that arise via alternative splicing and internal translational initiation (54, 57). E2F and DP have a number of common structural characteristics. Each carries an amino-terminal DNA-binding domain followed by a central dimerization domain and a region of uncertain function, the so-called marked box, that is well conserved between family members. The E2F *trans*-activation domain is encoded within an acidic carboxy-terminal region that also carries a site for binding by Rb. Rb may be found in association with most if not all E2F family members in vivo, whereas p107 and p130 preferentially associate with heterodimers containing E2F-4 and E2F-5 (5, 23, 26, 50). The formation of heterodimeric protein complexes appears to be essential for the production of high-affinity E2F protein-DNA complexes since E2F homodimers have minimal DNA-binding activity and DP homodimers have little or no affinity for DNA (3, 15, 25, 42). It is not clear whether DP proteins participate in the selection of E2F-binding sites or whether their function is limited to increasing the

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general affinity of transcription factor E2F for DNA. Although functioning as a repressor of E2F-mediated transcription, Rb family members do not in general appear to abrogate the formation of E2F protein-DNA complexes. Instead, such heterotrimeric protein complexes recognize and bind E2F sites, and in this configuration Rb has been shown to block *trans* activation mediated by additional sequence-specific DNA-binding proteins tethered to nearby promoter elements (1, 60, 74, 75). It is not known if the binding of Rb to E2F complexes alters their affinity for particular E2F sites or whether Rb negatively regulates all or a subset of E2F-regulated genes.

Although the abundance of components of transcription factor E2F can vary as a function of cell cycle progression, it is clear that numerous family members are simultaneously expressed in most, if not all, mammalian cells. Given their coexpression and closely related DNA-binding domains, one might predict that E2F complexes govern the activity of completely overlapping sets of cellular genes. However, three recent results suggest that specific subsets of E2F-regulated genes may be bound by particular E2F complexes. First, mice nullizygous for the *E2F-1* gene exhibit hyperplastic cell growth and an increased susceptibility to the development of tumors (20, 73, 79). These results suggest that E2F-1 functions, at least in part, as a tumor suppressor gene and that other E2F family members cannot completely compensate for the loss of E2F-1 activity (72). Second, exogenous expression of particular E2F family members leads to the activation of subsets of endogenous E2F-dependent genes. For example, adenovirus-mediated ectopic expression of E2F-1 stimulates the transcription of endogenous genes such as the thymidylate synthase, ribonucleotide reductase, and cyclin E genes whereas other E2F-regulated genes, such as the dihydrofolate reductase (DHFR) and thymidine kinase genes, are not similarly induced (18). Finally, in vitro protein-DNA-binding assays and genomic footprints of a palindromic E2F-binding site within the hamster DHFR promoter have yielded strong evidence that particular E2F/DP complexes bind this site in a cell cycle-dependent manner (76). Although it is possible that E2F complexes share some overlapping duties, these observations, taken together, indicate that E2F complexes are also quite likely to perform specific functions via their regulation of subsets of cellular genes. This notion is supported by the observation that E2F-binding sites within a cadre of cell cycle-regulated promoters appear to perform distinct functions. For example, E2F sites upstream of genes such as B-*myb*, *E2F-1*, *HsOrc1*, and *cdc2* function as negative regulators of transcription in growth-arrested or pre-S-phase cells whereas E2F-binding sites in the *DHFR* and cyclin E promoters are required for the stimulation of gene expression (7, 21, 22, 29, 32, 44, 52, 68, 84). In contrast, E2F sites within the cyclin A and mouse TK promoters appear to play a dual role, being required for repression of transcription in growth-arrested cells and activation of transcription at the G_1/S transition (34, 58).

To directly assess whether specific E2F/DP and Rb/E2F/DP complexes prefer particular cognate DNA-binding sites, we prepared recombinant heterodimeric and heterotrimeric E2F complexes in baculovirus-infected cells and used a protocol (CASTing) that includes repetitive immunoprecipitation and PCR to identify consensus E2F complex-specific binding sites. Our results indicate that (i) E2F and DP proteins participate in the selection of E2F-binding sites, (ii) E2F/DP complexes favor particular consensus DNA-binding sites, and (iii) the binding of Rb to heterodimeric complexes significantly alters the selection of E2F-binding sites. Additionally, we show that (i) E2F complex-specific binding sites can differ in their intrinsic DNA-bending properties, (ii) E2F/DP complexes induce DNA bending to differing degrees, and (iii) consensus E2F-binding sites selected in vitro direct distinct patterns of cell cycle-regulated transcription in vivo.

MATERIALS AND METHODS

Cell culture. CHO, 293, and COS-1 cells were cultured as previously described (64). Sf9 cells were obtained from Patrick J. Casey (Duke University Medical Center, Durham, NC) and cultured at 27°C in Grace's insect medium (GIBCO/BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Inc., Atlanta, Ga.), penicillin-streptomycin, and 0.1% Pluronic F-68 (GIBCO/BRL).

Construction of recombinant baculovirus stocks. Wild-type and mutated (mutant 9.1, Δ389–580; mutant 12, Δ662–775) human Rb cDNAs that had been tagged at their respective carboxy termini with a 10-amino-acid epitope from influenza virus hemagglutinin (HA) were a kind gift from Dennis J. Templeton (Case Western Reserve University, Cleveland, Ohio). A human E2F-1 cDNA and an HA-tagged DP-1 cDNA were obtained from Joseph R. Nevins (Duke University Medical Center). A human E2F-4 cDNA was obtained from Robert A. Weinberg (Whitehead Institute, Cambridge, Mass.). A human DP-2 cDNA has been described previously (57). Plasmids carrying these cDNAs were combined with specific oligonucleotides and Vent polymerase (New England Biolabs, Inc., Beverly, Mass.), and cDNAs were amplified by PCR. Oligonucleotides used for the amplification of E2F-1 and E2F-4 cDNAs were designed to link an HA epitope tag at their respective carboxy termini. Each amplified cDNA was subcloned into pVL1392/1393 (PharMingen, Inc., San Diego, Calif.), a baculovirus transfer vector, and the integrity of each cDNA was determined by dideoxy sequencing. Sf9 cells were cotransfected with each transfer vector and linearized baculovirus genomic DNA (BaculoGold; PharMingen, Inc.), and viral supernatants were collected and amplified after 5 days of incubation. Expression of recombinant human proteins in Sf9 cells was confirmed by immunoprecipitation.

Metabolic labeling of Sf9 cells, antibodies, and immunoprecipitation. Sf9 cells were infected with one or more recombinant baculovirus stocks and cultured for 45 h prior to incubation of cells in methionine-free medium supplemented with 100 μCi of Tran[^{35}S]-label (ICN, Inc., Costa Mesa, Calif.) per ml. Cell extracts were prepared as previously described (64) and incubated with antibodies against the HA epitope tag (12CA5) (69) or the proteins themselves (Rb, rabbit 9300 and XZ77 [64]; DP-1 and DP-2, rabbits 3570 and 3569 [57]; E2F-1 and E2F-4, KH95 and C-108 [Santa Cruz Biotechnology, Santa Cruz, Calif.]). For coprecipitation experiments with viral oncoproteins, radiolabeled Sf9 extracts were mixed with unlabeled 293 or COS-1 cell extracts and Rb proteins were indirectly precipitated with monoclonal antibodies prepared against E1A or large-T antigen (M73 and pAB416, respectively) (78). In vitro dephosphorylation experiments were performed as previously described (57).

Selection of E2F-binding sites via CASTing experiments. PCR primers (ED3, 5′-TCGGTACCTCGAGTGAAGATTGA-3; ED4, 5′-TTACCGCGGATCCGAATTCAT-3′) and an oligonucleotide carrying defined ends and a 16-nucleotide region of degeneracy (ED1, 5′-TCGGTACCTCGAGTGAAGATTGA-N_{16}-ATGAATTCGGATCCGCGGTAA-3′) were synthesized (GIBCO/BRL), and a double-stranded degenerate oligonucleotide was prepared by combining ED1, ED4, deoxynucleotides, and the Klenow fragment of DNA polymerase I (New England Biolabs). Cell extracts prepared as described above from Sf9 cells that had been subjected to a mixed infection with recombinant baculoviruses were incubated for 1 h at 4°C with anti-Rb (rabbit 9300 [64]) or anti-HA (12CA5 [69]) antibodies. Protein A-Sepharose beads were added, and extracts were incubated at 4°C for an additional 1 h. Immunoprecipitates were collected by centrifugation, and pellets were washed three times in cell lysis buffer and twice with ESB buffer (20 mM HEPES [pH 7.9], 40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, 10% glycerol) containing 2% Ficoll, 3 mg of bovine serum albumin per ml, and 50 μg of sonicated salmon sperm DNA per ml and then resuspended in 500 μl of ESB buffer. Double-stranded degenerate oligonucleotides were added (100 μl, 500 pmol of DNA), and the mixture was incubated for 30 min at room temperature. Protein-DNA complexes were recovered by centrifugation and washed three times in ESB buffer, and oligonucleotides were amplified by PCR in a 50-μl reaction mixture containing 200 ng of ED3 and ED4, 50 μCi of [α-^{32}P]dCTP, 20 μM dCTP, 50 μM each dATP, dTTP, dGTP, 1 mg of bovine serum albumin per ml, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), and 1 μl of Amplitaq polymerase (Perkin Elmer, Inc., Foster City, Calif.). DNAs were amplified by 15 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. Amplified DNAs were purified by passage through a Sephadex G-25 spin column and resolution through 8% nondenaturing polyacrylamide gels. Radiolabeled oligonucleotides were excised from gels, eluted overnight, and used in subsequent CASTing cycles and as probes in protein-DNA-binding assays to assess enrichment for oligonucleotides carrying E2F-binding sites.

Protein-DNA-binding (electrophoretic mobility shift) assays. Wild-type and mutated oligonucleotides carrying E2F-binding sites derived from the adenovirus E2 promoter were synthesized on an Applied Biosystems DNA synthesizer (two sites, 5′-GCCGTTTTCGCGCTTAAATTTGAGAAAGGGCGCGAAACTGGA-3′; one site, 5′-GCCGTCGAGCTGCTTAAATTTGAGAAAGGGCGCGAAACTGGA-3′; no site, 5′-GCCGTCGAGCTGCTTAAATTTGAGAAAGGT

CTCTACGCTGGA-3′). Radiolabeled probes of these oligonucleotides and those resulting from CASTing experiments were prepared by PCR as described above or by incubation with T4 polynucleotide kinase (New England Biolabs) and [α-^{32}P]ATP. CHO and Sf9 cell extracts were prepared for protein-DNA-binding assays as previously described (80). Protein/DNA-binding assays were performed for 20 min at room temperature in 10 μl of ESB buffer containing 2% Ficoll, 3 mg of bovine serum albumin per ml, 50 μg of sonicated salmon sperm DNA per ml, 0.5 to 2.0 μl of cell extract, and 10,000 to 20,000 cpm of radiolabeled probe. The binding-reaction mixtures were applied to 5% polyacrylamide gels cast in 1× TBE (50 mM Tris-borate, 1 mM EGTA), and electrophoresis was performed for 3.5 to 4 h at 4°C and 300 V in 0.25× TBE.

Construction of luciferase reporter constructs, transfection, and flow cytometric and luciferase assays. A luciferase reporter construct [pGL2-Xba (+)] (44) prepared from the mouse B-*myb* promoter was a kind gift of Roger J. Watson (Ludwig Institute for Cancer Research, London, United Kingdom). The E2F-binding site (WT, 5′-ACTTGGCGGGAGA-3′) within this construction was mutated (Mut, 5′-ACTTGTATGGAGA-3′) or replaced with E2F sites selected in vitro (E2F-1/DP-1, 5′-ATTTCCCGCCATTATC-3′; E2F-4/DP-1, 5′-CCCTTTTCGCGCGAAAAGGG-3′; Rb/E2F-1/DP-1, 5′-AAATTTTCGCGGGAAAATT-3′) with an in vitro mutagenesis kit (MutaGene; Bio-Rad Laboratories, Inc., Hercules, Calif.). Following mutagenesis, the sequence of each construction was confirmed by dideoxy sequencing. CHO cells were transfected with luciferase reporter constructs with LipofectAMINE (GIBCO/BRL) as specified by the manufacturer. After a 5-h incubation in standard medium, transfected CHO cultures were switched to medium containing 0.2% fetal bovine serum and cultured for 48 h at 37°C. Cell extracts were harvested for luciferase assays after 48 h of incubation or at 4-h intervals following the addition of 10% fetal bovine serum. Luciferase assays were performed by standard procedures with a Berthold AutoLumat LB9501 luminometer. Parallel plates of transfected cells were fixed with 70% ethanol and stained with propidium iodide for flow cytometric analysis.

Protein-DNA off-rate assays and phasing analysis. Off-rate experiments were performed as previously described, except that magnesium was omitted to maximize protein-DNA interactions (31, 80). Phasing analysis was performed as previously described (16, 35, 36) with plasmids pTK-401-26 and pTK-401-28, kindly provided by Tom K. Kerppola (University of Michigan Medical School, Ann Arbor, Mich.). Additional phasing analysis plasmids having spacings of 31, 33, and 36 bp between the intrinsic bend of the vector and a *Sal*I cloning site were also prepared. To construct these additional vectors, three oligonucleotide primers with the sequences 5′-CCCGTCGACACACAGCAAAAACGGGCAAAAACGG-3′, 5′-CCCGTCGACACACACAGCAAAAACGGGCAAAAACGG-3′, and 5′-CCCGTCGACACACACAACAGCAAAAACGGGCAAAAACGG-3′ were used in combination with the oligonucleotide primer 5′-GTTAGCAATTTACTGTGAT-3′ in PCRs with pTK-401-28 as a template. The DNA fragments generated by these PCRs were then used to replace a *Sal*I-*Hin*dIII fragment of pBend2 (the parent vector of the pTK-401 series [provided by Sankar Adhya, National Institutes of Health, Bethesda, Md.]) to generate three novel bending vectors, pTK-401-31, pTK-401-33, and pTK-401-36 (37). Double-stranded oligonucleotides carrying E2F sequences 1.1, 1.2, 4.1, and 4.2 were cloned at the *Xba*I and *Sal*I sites of pTK-401-26 through pTK-401-36 with the following oligonucleotide pairs: 1.1, 5′-TCGACTTATTTTTCCCGCCTTCTC-3′ and 5′-CTAGGAAAGGCGGGAAAAATAAG-3′; 1.2, 5′-TCGACCACTTTTCCCGCCATTC-3′ and 5′-CTAGGAATGGCGGGAAAAGTGG-3′; 4.1, 5′TCGACCCCTTTTGGCGCGAAAC-3′ and 5′-CTAGGTTTCGCGCCAAAAGGGG-3′; and 4.2, 5′-TCGACGCATTTTCCCGCCATTC-3′ and 5′-CTAGGAATGGCGGGAAAATGCG-3′.

DNA probes corresponding to each phasing analysis series were prepared by digestion with *Eco*RI and *Hin*dIII and incorporation of radiolabeled deoxynucleotides with the Klenow fragment of DNA polymerase I. Experiments to determine mean intrinsic DNA bend angles were performed as previously described (35, 36). Experiments to measure protein-induced DNA bends were performed with acrylamide gels under conditions appropriate for slower-moving protein-DNA complexes with an estimated k of 0.72 (35, 36, 80). The intrinsic bends of naked DNAs made it inappropriate to correct protein-DNA complex mobilities for variations in probe mobilities, and thus complex mobilities were normalized to the average mobility of all complexes. Data from at least three individual experiments were then fitted to the phasing function of Kerppola and Curran (35), and the amplitude of the phasing function was then used to estimate the bend angle as previously described (16). The orientation of DNA bending was determined from the minima and maxima of the phasing function, assuming a minor-groove oriented bend of 54° at the center of the AT tract control bend (35, 36, 40, 67).

To measure the relative intrinsic bends of additional E2F sequences we utilized a method described by Koo et al. (40). Briefly, 20-bp double-stranded oligonucleotides carrying E2F-binding sequences were radiolabeled with T4 DNA kinase and [α-^{32}P]ATP. Radiolabeled oligonucleotides were then ligated end-to-end in a forced orientation to generate multimers in which the intrinsic bends of the individual monomers are aligned in phase. Multimeric products were then resolved under gel conditions described by Kerppola and Curran (35, 36). Controls for these experiments included a 10-bp *Bam*HI linker (5′-CGGGATCCCG-3′ and 5′-CGCGGGATCC-3′) that forms linear DNA and a bent control sequence (5′-GGCAAAAACG-3′ and 5′-CCCGTTTTTG-3′) that is estimated to possess an intrinsic bend of 36° for every 20-bp interval (35, 36, 40, 67). The R_L value was determined for each multimer as the distance migrated by the test multimer divided by the distance migrated by a linear *Bam*HI multimer of the same molecular weight. $R_L - 1$ was plotted as a function of the square of the actual molecular weight of the multimers to generate a linear relationship whose slope is proportional to the intrinsic bend of the monomer (65). Intrinsic bends of novel E2F sites were estimated by dividing the slope of this linear relationship by the slope of the curve corresponding to the control bend and multipling this value by 36°, the bend estimated for the control sequence (65). The 20-bp oligonucleotide pairs assayed in this fashion are as follows: 1.1.2, 5′-TCCATTGTTCCCAGCCACTC-3′ and 5′-GAGAGTGGCTGGGAACAATG-3′; 1.1.3, 5′-TCCATGCTTTCCCGCCACTC-3′ and 5′-GAGAGTGGCGGGAAAGCATG-3′; 1.1.4, 5′-TCCATTTTTTGGCGGTTTAT-3′ and 5′-GAATAAACCGCCAAAAAATG-3′; 1.1.5, 5′-TCCATCTTCCCGCCTTATTC-3′ and 5′-GAGAATAAGGCGGGAAGATG-3′; 1.1.6, 5′-TCCTGATTTTGGCGGGATTC-3′ and 5′-GAAGAATCCCGCCAAAATCAG-3′; Rb.1, 5′-TCCATCCTTTGGCGCGAAAA-3′ and 5′-GATTTTCGCGCCAAAGGCTG-3′; Rb.2, 5′-TCCTTTTCGCGCCAAAAGGG-3′ and 5′-GACCCTTTTGGCGCGAAAAG-3′; RB.3, 5′-TCCAGGTTTCCCGCCAAAAA-3′ and 5′-GATTTTTGGCGGGAAACCTG-3′; and RB.4, 5′-TCCAAAAAATTGGCGCGAAA-3′ and 5′-GATTTTCGCGCCAATTTTTTG-3′.

RESULTS

Baculovirus-expressed E2F, DP, and Rb proteins form multimeric complexes that specifically bind E2F sites in vitro. A series of recombinant baculoviruses were prepared that carry human E2F-1, E2F-4, DP-1, or DP-2 cDNAs that had been epitope tagged with a 10-amino-acid sequence derived from the influenza virus HA protein. To ensure that each of these recombinant baculoviruses produced intact proteins, cultures of Sf9 cells were infected with amplified viral stocks and incubated with [^{35}S]methionine and nondenatured cell extracts were immunoprecipitated with antibodies specific for each protein or the epitope tag. As illustrated in Fig. 1, infection of Sf9 cells with baculoviruses carrying E2F-1, E2F-4, DP-1, or DP-2 cDNAs led to the expression of recombinant human proteins with the expected molecular mass. To determine if recombinant E2F and DP proteins formed heterodimeric complexes in vivo, Sf9 cells were subjected to mixed infections with baculoviruses encoding E2F-1 and DP-1 or DP-2 and radiolabeled cell extracts were incubated with a monoclonal E2F-1 antibody. Abundant amounts of DP-1 and DP-2 proteins were noted in E2F-1 precipitates (Fig. 1). Identical results were obtained in immunoprecipitates of Sf9 cells subjected to mixed infections with E2F-4 and DP-1 or DP-2 proteins (data not shown).

To facilitate studies of heterotrimeric E2F complexes, a recombinant baculovirus carrying an epitope-tagged wild-type human Rb cDNA was prepared, amplified, and used to infect Sf9 cells. Infected cultures were incubated with [^{35}S]methionine, and recombinant Rb protein was precipitated with monoclonal antibodies prepared against Rb (XZ77; anti-Rb) or the epitope tag (12CA5; anti-HA). As shown in Fig. 2A, each antibody precipitated copious amounts of protein that comigrates with endogenous Rb protein precipitated from human ML-1 cells. As shown in Fig. 2B, the vast majority of Rb protein synthesized in Sf9 cells appears to be unphosphorylated, since incubation of Rb precipitates with excess amounts of potato acid phosphatase had little or no effect on the apparent molecular mass of recombinant Rb protein. In contrast, a similar treatment of Rb precipitates prepared from ML-1 cells led to a noticeable increase in the electrophoretic migration of endogenous Rb protein. Since we wished to study the functional consequence of heterotrimeric (Rb/E2F/DP) complex formation on DNA-binding site selection, it became important to establish that the carboxy-terminal portion (the so-called Rb pocket) of Rb that physically interacts with proteins such as E2F functioned as expected in Sf9 extracts. To this end, we prepared two additional baculovirus stocks that carry well-characterized Rb cDNAs (Δ389–580 [ΔA] and Δ662–775 [ΔB])

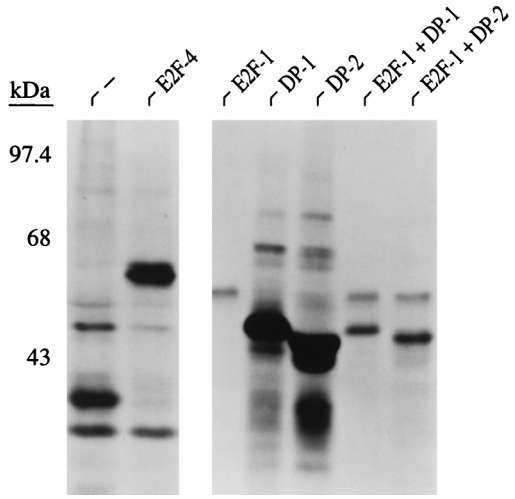


FIG. 1. Expression of E2F/DP family members in baculovirus-infected Sf9 cells. Cultures of uninfected (lane −) or virus-infected (indicated above each lane) cells were incubated with [^{35}S]methionine, and nondenatured cell extracts were immunoprecipitated with antibodies prepared against the proteins themselves (DP-2, E2F-1) or a carboxyl-terminal HA-epitope tag (E2F-4, DP-1). In the rightmost lanes, Sf9 cells coinfected with E2F-1 and DP-1 or DP-2 baculoviruses were extracted and immunoprecipitated with an antibody against E2F-1. Molecular mass markers are indicated on the left.

that had sustained deletions within discrete portions of the Rb pocket. As shown in Fig. 2A, each of these mutated Rb cDNAs was readily expressed in infected Sf9 cells and each was precipitated by anti-Rb and anti-HA antisera. To assess whether the Rb pocket of recombinant Rb proteins functioned as expected, we took advantage of a coprecipitation assay that we and others had previously used to map portions of Rb required for association with viral oncoproteins (17, 28). Sf9 cells were infected with baculoviruses carrying wild-type or mutated Rb cDNAs, nondenatured and radiolabeled cell extracts were prepared, and these extracts were incubated with unlabeled extracts from cells that express adenovirus E1A (293 cells) or SV40 large-T antigen (COS-1 cells). Following incubation of cell extracts, monoclonal antibodies prepared against these viral oncoproteins were used to indirectly precipitate Rb. As expected, wild-type but not mutated Rb proteins were indirectly precipitated by antibodies against E1A and large-T antigen (Fig. 2C and data not shown).

Prior to an analysis of DNA-binding-site selectivity, we wished to establish that baculovirus-expressed E2F, DP, and Rb proteins formed protein-DNA complexes akin to their counterparts in mammalian cell extracts. To address this issue, a radiolabeled oligonucleotide carrying tandem E2F-binding sites from the adenovirus E2 promoter was used in protein-DNA-binding (gel shift) assays with infected Sf9 cell extracts. Incubation of this radiolabeled oligonucleotide with extracts prepared from Sf9 cells subjected to mixed infections with E2F-1 and DP-1 baculovirus stocks led to the formation of a single protein-DNA complex (Fig. 3A). To determine if the formation of this protein-DNA complex was dependent on intact E2F-binding sites within the E2-derived oligonucleotide, competition experiments were performed with an excess of unlabeled oligonucleotides that carry one (Fig. 3A, 1 site), two (2 site), or no (no site) E2F-binding sites. As shown in Fig. 3A, incubation of Sf9 extracts with an excess of oligonucleotides with intact but not mutated E2F-binding sites abolished the formation of radiolabeled protein-DNA complexes. To ensure that the protein-DNA complex detected in these assays was made up of human E2F/DP proteins, protein-DNA-binding assays were incubated with antibodies against E2F-1 or DP-1. As shown in Fig. 3A (lanes αE2F-1 and αDP-1), inclusion of these antisera resulted in a supershifted (anti-E2F-1) protein-DNA complex or abolished (anti-DP-1) the formation of such complexes. With increasing concentrations of baculovirus-in-

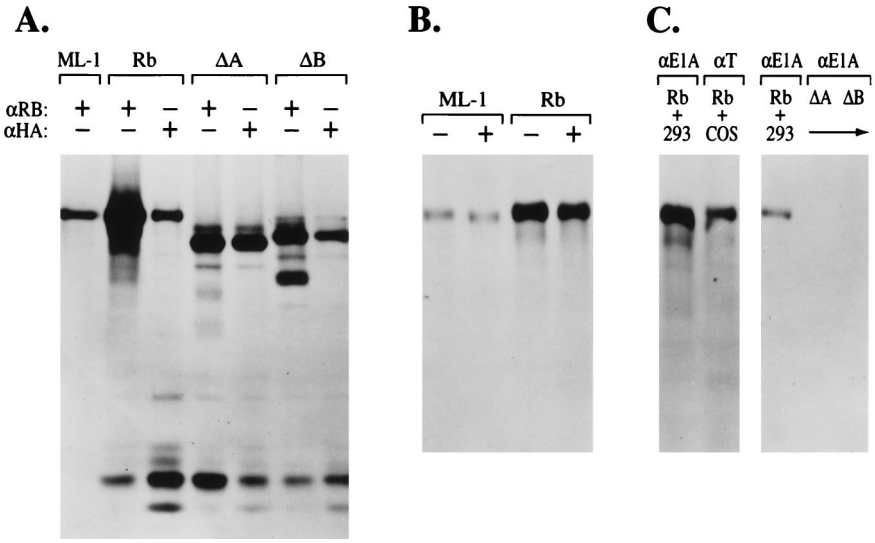


FIG. 2. Expression and function of wild-type and mutated Rb proteins in baculovirus-infected Sf9 cells. (A) Expression of wild-type and mutated Rb proteins. Cultures of human cells (ML-1) or Sf9 cells infected with baculoviruses carrying wild-type (Rb) or mutated (ΔA and ΔB) cDNAs were radiolabeled and immunoprecipitated with antibodies against Rb (αRB) or a carboxy-terminal HA epitope tag (αHA). (B) Phosphorylation state of Rb proteins in human and virus-infected Sf9 cells. Cultures of ML-1 and virus-infected Sf9 cells were radiolabeled, extracted as in Fig. 1, and split in half. Half of each cell extract was treated with potato acid phosphatase (lanes +) and then incubated with antibodies against Rb. (C) Binding of wild-type Rb protein to viral oncoproteins. Sf9 cells were infected with recombinant baculoviruses carrying wild-type (Rb) or mutated (ΔA and ΔB) Rb cDNAs, and radiolabeled extracts were prepared as in Fig. 1. Radiolabeled extracts were incubated with unlabeled cell extracts carrying adenovirus E1A (293) or SV40 large-T antigen (COS), and Rb proteins were indirectly precipitated with monoclonal antibodies against each viral oncoprotein (αE1A, αT).

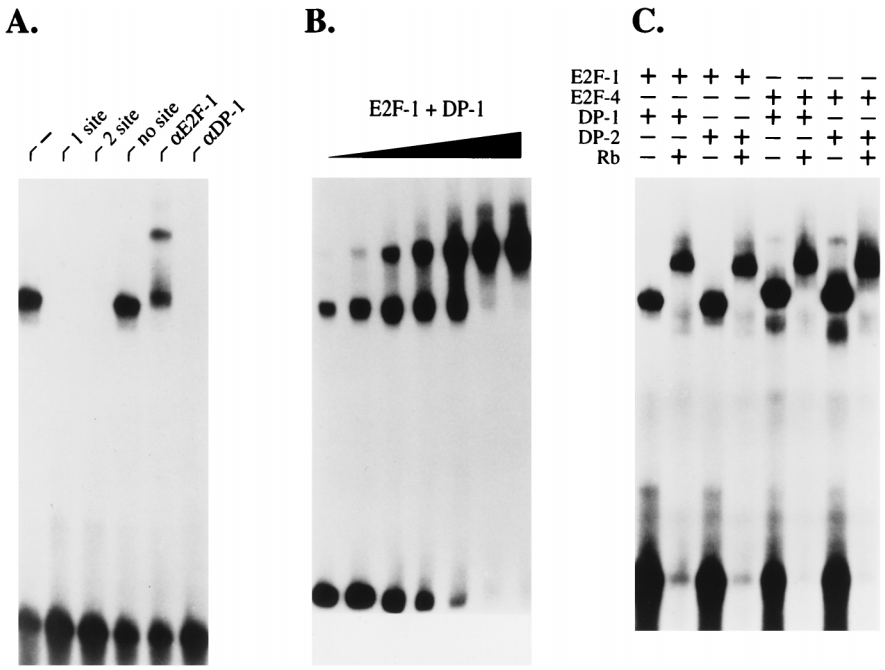


FIG. 3. Protein-DNA-binding assays with extracts prepared from baculovirus-infected Sf9 cells. (A) Protein-DNA-binding assays with wild-type and mutated oligonucleotides derived from the adenovirus E2 promoter. Extracts prepared from Sf9 cells coinfected with E2F-1 and DP-1 baculoviruses were incubated with a radiolabeled wild-type E2 oligonucleotide alone (lane −) or with a 200-fold molar excess of unlabeled wild-type (2 site) or mutated (1 site, no site) E2 oligonucleotides or antibodies against E2F-1 (αE2F-1) or DP-1 (αDP-1). (B) Protein-DNA-binding assays with increasing concentrations of E2F-1/DP-1 cell extracts. (C) Protein-DNA-binding assays with recombinant E2F, DP, and Rb proteins. Protein-DNA-binding assays were performed as in panel A with extracts prepared from baculovirus-infected cells. The extracts used are indicated at the top of each lane.

fected cell extracts, both of the E2F-binding sites within this radiolabeled oligonucleotide could be bound by recombinant E2F-1/DP-1 proteins (Fig. 3B). As shown in Fig. 3C, protein-DNA complexes similar to those formed by E2F-1/DP-1 proteins were formed by E2F-4/DP-1 proteins and mixtures of E2F-1 and E2F-4 proteins with DP-2. Moreover, addition of recombinant wild-type Rb protein to such protein-DNA-binding assays led to the formation of a supershifted heterotrimeric protein-DNA complex (Fig. 3C). As expected, the inclusion of baculovirus-expressed Rb proteins carrying mutations within the Rb pocket did not lead to the formation of heterotrimeric complexes in protein-DNA-binding assays (data not shown).

Taken together, the data presented in Fig. 1 to 3 indicate that recombinant E2F, DP, and Rb proteins produced in baculovirus-infected cells function in much the same way as their counterparts in mammalian cells. E2F and DP proteins form stable heterodimeric complexes that specifically bind DNA, and Rb can physically interact with such complexes to form heterotrimeric protein-DNA complexes.

E2F, DP, and Rb proteins influence the selection of E2F-binding sites in vitro. Satisfied that baculovirus-expressed E2F, DP, and Rb proteins formed protein-DNA complexes as expected, we initiated a series of repetitive immunoprecipitation-PCR (CASTing) experiments to identify preferred DNA-binding sites. These experiments were done with a double-stranded oligonucleotide with defined ends for which PCR primers were available and a degenerate central core region of 16 nucleotides. Baculovirus-infected cell extracts carrying mixtures of E2F and DP proteins or E2F, DP, and Rb proteins were incubated with a monoclonal anti-HA antibody (12CA5) or a polyclonal anti-Rb antibody (rabbit 9300 [64]) and precipitated with protein A-Sepharose beads. Following extensive washes to remove loosely adhering proteins, this antigen-antibody complex was incubated with degenerate double-stranded oligonucleotides, unbound DNA was removed by washing, and bound DNAs were amplified by PCR following the addition of appropriate primers. At least three additional cycles of immunoprecipitation and PCR amplification were used. Following each successive cycle of PCR amplification, the abundance of E2F sites within the degenerate pool was monitored by protein-DNA-binding assays with radiolabeled PCR products. When the abundance of radiolabeled protein-DNA complexes ceased to increase with additional rounds of immunoprecipitation and PCR, the resulting DNAs were cloned and 21 to 26 independent isolates for each protein-DNA-binding reaction were sequenced. The resulting sequences are aligned in Fig. 4A, and relevant features of these sequences are tabulated in Fig. 4B and C and Table 1.

Although consensus E2F sites selected in vitro are clearly similar, several subtle as well as striking differences are also readily apparent. First, it is clear that the DNA-binding domains of E2F and DP proteins contribute to the selection of E2F-binding sites. This is illustrated most clearly when one compares consensus sites recovered from (i) E2F-4/DP-1 and E2F-4/DP-2 and (ii) E2F-1/DP-1 and E2F-4/DP-1 heterodimeric complexes. E2F-binding sites selected by E2F-4/DP-1 and E2F-4/DP-2 complexes are distinguished by several characteristics. E2F-4/DP-1 complexes appear to be significantly more fastidious, holding nearly constant five of eight nucleotides within the E2F core region and having strong preferences for particular nucleotides at 20 of 20 positions. Indeed, 13 of 22 E2F-4/DP-1 clones carried the same sequence (5′-CCTTTTGGCGCGAAA-3′), and only five distinct E2F-4/DP-1 sequences were noted among the isolates examined. In contrast, E2F-4/DP-2-selected sites are significantly more diverse. Preferred nucleotides were noted at only 16 of 20 posi-

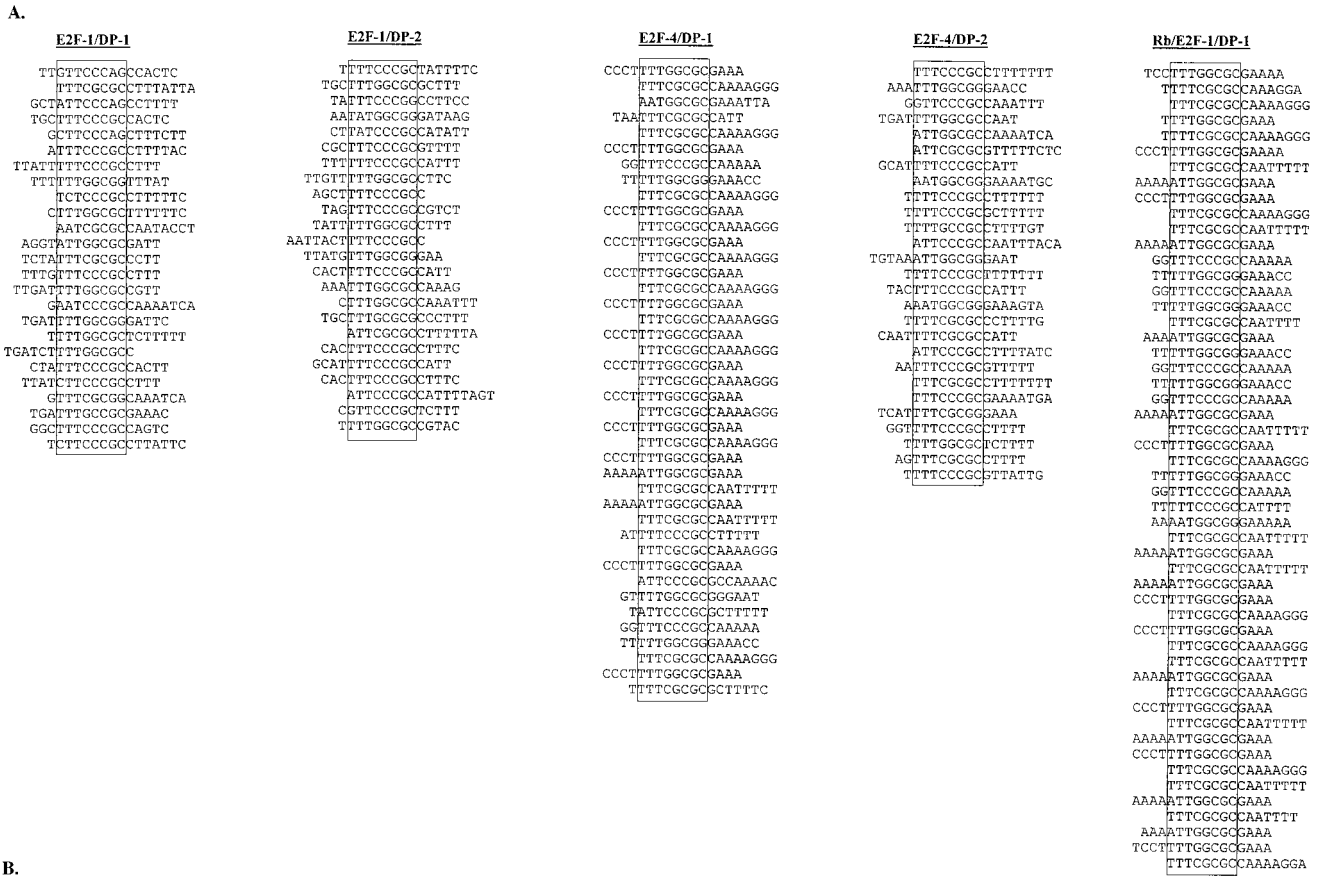


FIG. 4. Sequence alignment of E2F-binding sites recovered in CASTing experiments and derivation of E2F complex-specific consensus sequences. (A) Alignment of E2F-binding sites. E2F-binding sites recovered for each set of CASTing experiments are aligned with respect to a previously defined 8-nucleotide core E2F-binding site (5′-TTTCGCGC-3′) (41, 56). For palindromic E2F-binding sites, each strand of the palindrome is shown. (B) Tabulation of frequencies of recovered nucleotides within degenerate oligonucleotides. The 8-nucleotide core E2F-binding site is indicated by a box. The most frequently recovered residues at each position are indicated, and the percentage of independent clones that carry the indicated nucleotides are shown above and below each position. Boldface letters indicate nucleotides that appear in more than 90% of recovered clones. (C) Consensus complex-specific E2F-binding sites. Nucleotide positions that were not occupied by a particular residue in more than half the sequenced clones are denoted by N.

tions, four of eight core nucleotides were held nearly constant, each of 21 E2F-4/DP-1 clones carried a distinct nucleotide sequence, and a wide variety of GC-rich sequences were recovered within the core region of E2F-binding sites (Table 1). E2F-4/DP-1 and E2F-4/DP-2 complexes were also distinguished by their relative preference for palindromic E2F-binding sites; heterodimeric complexes containing E2F-4 and DP-1 selected palindromic E2F-binding sites three times as frequently (90%) as did complexes containing E2F-4 and DP-2 (30%).

E2F-binding sites selected by E2F-1/DP-1 and E2F-4/DP-1 complexes are distinguished by several characteristics. E2F-1/DP-1 complexes appear to be among the least fastidious heterodimeric complexes examined in our studies. E2F-1 and DP-1 heterodimers held only two of eight core E2F-binding-site nucleotides nearly constant, exhibited preferences for particular nucleotides at only 15 of 20 positions, and selected the widest variety of GC-rich sequences within the core E2F-binding site (Table 1). Each of the 22 E2F-1/DP-1 clones analyzed also carried a unique nucleotide sequence. As mentioned above, E2F-binding sites selected by E2F-4/DP-1 heterodimers are significantly less diverse, exhibiting strong nucleotide preferences throughout the 20-nucleotide region examined and a

TABLE 1. Frequency of recovery of GC core sequences and palindromic E2F-binding sites[a]

Sequence	Frequency of sequence in:				
	E2F-1/DP-1	Rb/E2F-1/DP-1	E2F-1/DP-2	E2F-4/DP-1	E2F-4/DP-2
CCCAG	3	0	0	0	0
CGCGC	3	20	1	17	5
CCCGC	10	6	13	5	13
GGCGC	5	20	6	17	3
GGCGG	2	6	2	2	4
CGCGG	1	0	0	0	1
GCCGC	1	0	0	0	1
GCGCG	0	0	1	0	0
CCCGG	0	0	1	0	0

[a] The frequencies of palindromic E2F-binding sites in the above complexes are 14, 100, 9, 90, and 30%, respectively.

relatively small subset of GC-rich sequences within the core region of E2F-binding sites (Table 1). When complexed with DP-1, E2F-1 and E2F-4 also exhibit distinct preferences for palindromic or nonpalindromic E2F-binding sites. E2F-1/DP-1 complexes selected palindromic E2F-binding sites relatively infrequently (14%) whereas E2F-4/DP-1 complexes nearly invariably (90%) selected palindromic E2F-binding sites.

Second, the association of Rb with E2F-1/DP-1 complexes results in a profound alteration in the spectrum of E2F-binding sites selected in vitro. In marked contrast to the diversity of sites selected by E2F-1/DP-1 complexes, heterotrimeric Rb/E2F-1/DP-1 complexes exhibited a strikingly strong preference for a small subset of nucleotide sequences. The vast majority of E2F-binding sites (24 of 26) selected by such complexes were represented by three particular sequences (5′-AAAAATTGG CGCGAAA-3′ [10 isolates], 5′-CCCTTTTGGCGCGAAA-3′ [9 isolates], and 5′-TTTTTGGCGGGAAACC-3′ [5 isolates]). Rb/E2F-1/DP-1 complexes strongly preferred a small subset of the GC-rich core sequences selected by E2F-1/DP-1 complexes and selected E2F-binding sites with six nearly invariant nucleotides. In contrast to sites selected by E2F-1/DP-1 complexes (14% palindromic), and reminiscent of sites selected by E2F-4/DP-1 heterodimers (90% palindromic), 26 of 26 sites selected by Rb/E2F-1/DP-1 complexes carry palindromic E2F-binding sites.

In summary, it is apparent from the E2F-binding sites we have selected in vitro that E2F, DP, and Rb proteins each influence the DNA-binding specificity of heterodimeric and heterotrimeric protein complexes. The complexes we have analyzed are distinguished by their relative preference for particular residues at specific nucleotide positions as well as their predilection for palindromic or nonpalindromic E2F-binding sites. It is worth noting that these results may reflect general differences between E2F complexes, e.g., DNA affinity, in addition to differing specificities of their DNA-binding domains.

E2F sites selected by E2F-1/DP-1 and Rb/E2F-1/DP-1 complexes are distinguished by their intrinsic DNA-bending properties, and the net angle of induced DNA bends depends primarily upon the E2F proteins that comprise heterodimeric complexes. We have previously shown that (i) the intrinsic DNA-bending properties of E2F sites can vary and (ii) promoter mutations that perturb the DNA-bending properties of DNAs carrying E2F sites can alter their functional attributes (16). Given the distinct differences in the consensus E2F-binding sites selected by E2F-1/DP-1 and Rb/E2F-1/DP-1 complexes, it became of interest to determine if these sequence variations were associated with significant distinctions in the topological properties of DNA. To quantify the intrinsic DNA-bending properties of DNAs carrying E2F-binding sites, we took advantage of an oligonucleotide ligation method described by Koo et al. (40) that provides for the quantification of the degree of DNA bending as a function of the electrophoretic mobility of ligated oligonucleotides in acrylamide gels. Nine randomly chosen oligonucleotides carrying E2F sites recovered from protein-DNA-binding assays with E2F-1/DP-1 and Rb/E2F-1/DP-1 complexes were examined by this procedure, and the average angle of intrinsic DNA bends was calculated as a function of electrophoretic migration (Fig. 5A). As shown in Fig. 5B, although each set of oligonucleotides exhibits a spectrum of bending properties, the spectrum of intrinsic DNA bends of E2F-binding sites selected by E2F-1/DP-1 complexes is significantly greater than that exhibited by the sites selected by Rb/E2F-1/DP-1 complexes. Specifically, E2F-1/DP-1 selects both flat and bent sequences whereas Rb/E2F-1/DP-1 selects exclusively bent sequences. Scanning the sequences we have examined in this assay, it is also apparent that oligonucleotides carrying E2F-binding sites that are flanked by homopolymeric tracts exhibit the greatest amount of DNA-bending (Table 2).

We and others have previously determined that the binding of heterodimeric and heterotrimeric E2F complexes to DNA induces differential degrees of DNA bending. Our experiments involved a sensitive protein-DNA-binding assay, termed DNA-phasing analysis, to quantify the induction of DNA bends. Since the majority of these experiments relied on semipurified preparations of E2F, the relative impact of particular E2F/DP family members on the bending of DNA has not been established. To determine whether particular E2F/DP complexes induce distinct degrees of DNA bending, we prepared three series of oligonucleotides carrying E2F-binding sites located at different distances from a sequence with a well-characterized intrinsic DNA bend. Each oligonucleotide series carries an E2F site derived from the human E2F-1 promoter or sites recovered from CASTing experiments with E2F-1/DP-1 and E2F-4/DP-1 heterodimeric complexes. A representative phasing analysis with an E2F-binding site from the human E2F-1 promoter and baculovirus-expressed heterodimeric protein complexes is shown in Fig. 6A. The relative electrophoretic mobilities of the protein-DNA complexes were measured and plotted as a function of the center-to-center distance between the E2F site and the flanking intrinsic bend. The resulting data were fitted to a cosine curve, and bend angles were derived from the amplitude of the phasing function (Fig. 6B). The relative bend angles induced by each heterodimeric complex for each E2F-binding site examined are presented in Fig. 6C. Comparing the bend angles induced by each E2F/DP complex, it is apparent that (i) the degree of DNA bending is dependent on the E2F partner present within a given heterodimeric complex and (ii) E2F-4-containing complexes bend DNA to a greater degree than do E2F-1-containing complexes.

The stability of E2F protein-DNA complexes in vitro is determined by the E2F dimerization partner. To determine whether protein-DNA complexes formed by baculovirus-expressed E2F/DP proteins differ with respect to their stability, a series of off-rate experiments were performed with a variety of oligonucleotides carrying E2F sites recovered in CASTing experiments. As shown in Table 2, the stability of protein-DNA complexes depends on the sequence of the oligonucleotide examined and the E2F protein that comprises a given heterodimeric complex. The intrinsic DNA bends of a given E2F site appeared not to influence the stability of protein-DNA complexes. Under the assay conditions we used, the half-life of E2F/DP/DNA complexes varied between 30 and less than 2

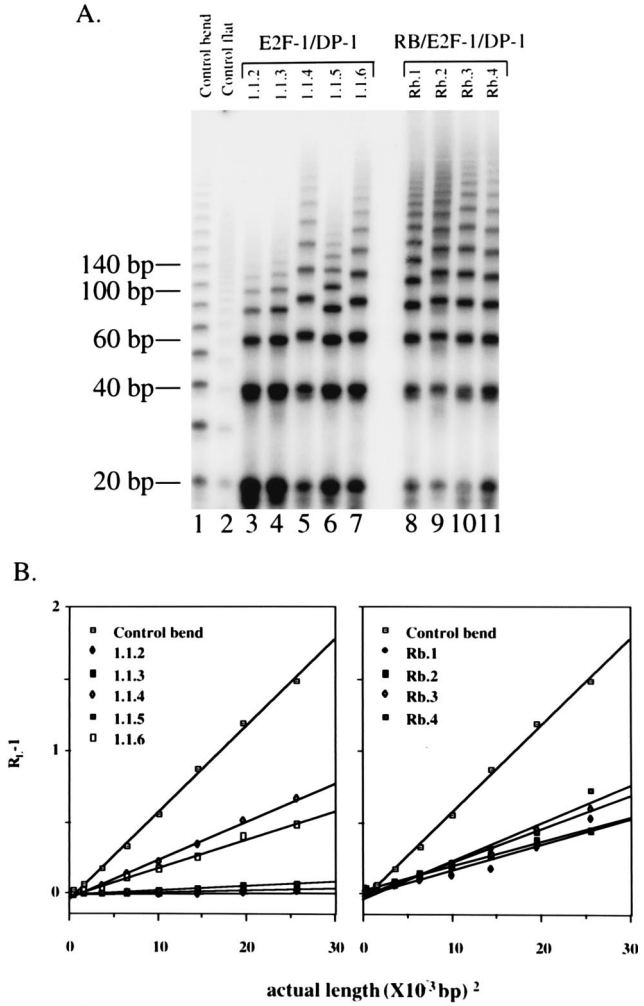


FIG. 5. Relative intrinsic DNA-bending properties of E2F-binding sites. (A) Multimerization assay. Nine randomly selected double-stranded oligonucleotides carrying E2F-binding sites were radiolabeled, multimerized, and resolved on a nondenaturing polyacrylamide gel. Lanes: 1, 10-bp oligonucleotide possessing an 18° bend for every 10-bp increment (control bend); 2, 10-bp *Bam*HI linker previously demonstrated to have no intrinsic bend (control flat); 3 to 7, 20-bp multimers containing the indicated E2F-1/DP-1-selected sequences; 8 to 11, 20-bp multimers containing the indicated Rb/E2F-1/DP-1-selected sequences. (B) Estimation of relative DNA curvature. The value $R_L - 1$ for each multimeric product is plotted as a function of the square of the actual molecular size of the DNA fragment. Estimated bends are summarized in Table 2.

min. Although it is clear from these data that each heterodimeric complex examined is competent to bind a wide variety of E2F sites in vitro, in general heterodimeric complexes containing E2F-1 were significantly more stable (two- to fivefold) than heterodimers containing E2F-4. Additionally, E2F/DP complexes that prefer palindromic E2F sites, such as E2F-4-containing complexes, showed greater stability on oligonucleotides carrying such sites.

Consensus E2F-binding sites derived from CASTing experiments exhibit distinct patterns of cell-cycle-regulated gene expression in vivo. The data thus far indicate that heterodimeric and heterotrimeric E2F complexes show distinct preferences for particular E2F-binding sites, and the binding of E2F/DP complexes to DNA can induce differing degrees of DNA bending. In addition, the intrinsic DNA-bending properties of E2F-binding sites can differ, as can the stability of heterodimeric complexes on DNA. To determine whether these distinctions derived in vitro would be reflected in differential levels of cell cycle-regulated gene expression in vivo, a novel strategy for assessing the functional activity of E2F-binding sites was used. Previous in vivo analyses have largely relied on the overexpression of E2F and/or DP proteins to gauge their relative contribution to the *trans* activation of various artificial and cellular promoters. A serious caveat with such experiments is that excess E2F/DP protein expression can force physical and functional interactions to occur that may not be physiologically relevant. Additionally, given emerging evidence that exogenous E2F/DP proteins can (i) induce the expression of endogenous E2F/DP family members and (ii) otherwise alter levels of transcription factor complexes containing E2F, it is not always obvious which E2F complexes are performing a given function (18, 32). To minimize these concerns, we adopted a strategy whereby a well-characterized site of E2F-mediated transcriptional regulation within the mouse B-*myb* promoter was replaced with consensus E2F-binding sites derived from CASTing experiments. A 600-bp fragment of the mouse B-*myb* promoter had previously been linked upstream of a luciferase reporter gene, and an intact palindromic E2F site at −210 (relative to the start of translation) is required for cell cycle-regulated B-*myb* transcription (6, 44). We predicted that replacement of this endogenous site with consensus E2F sites obtained by CASTing experiments should lead to differential levels of cell cycle-regulated transcription if E2F-binding sites derived in vitro are truly functionally distinct.

To quantify the relative levels of cell cycle-regulated transcription, CHO cells were transiently transfected with luciferase reporter constructs, cultured at low serum levels for 48 h to generate a growth-arrested cell population, and then stimulated to reenter the cell cycle by the addition of serum (44). The cells were then prepared for flow cytometry to gauge the synchronicity of CHO cultures, and extracts were prepared every 4 h for luciferase assays. As previously reported, the wild-type B-*myb* reporter construct elicited low levels of luciferase activity in growth-arrested cells and transcriptional activity increased three- to fourfold as the cells approached the S phase (12 h after serum stimulation in Fig. 7A and C) (44). Also consistent with previous results, a reporter construct (Mut in Fig. 7) that carries a dinucleotide mutation within the B-*myb* promoter that blocks E2F protein-DNA interactions largely eliminated E2F-mediated transcriptional repression in growth-arrested cells (44). A residual increase in gene expression from this reporter at the G_1/S transition is entirely consistent with a recent report that has identified an additional site of negative cell cycle-regulated transcription within this promoter (6). Interestingly, replacement of the endogenous B-*myb* E2F site with consensus sites derived from CASTing experiments with E2F-1/DP-1, Rb/E2F-1/DP-1, and E2F-4/DP-1 complexes resulted in three distinct patterns of gene expression (Fig. 7). Inclusion of a consensus E2F-1/DP-1-binding site led to a generalized increase (twofold) in levels of B-*myb* gene expression throughout the cell cycle yet facilitated the derepression of the promoter at the G_1/S transition (Fig. 7A and C). In marked contrast, inclusion of a consensus Rb/E2F-1/DP-1 site resulted in a pattern of B-*myb* gene expression that was nearly indistinguishable from that of the wild-type promoter (Fig. 7A and C). Finally, replacement of the wild-type E2F site with a consensus E2F-4/DP-1-binding site led to a nearly invariant level of gene expression throughout the cell cycle, eliminating the derepression of the promoter just prior to S phase (Fig. 7A and C). Cell cycle-regulated differences in the transcriptional activities of each of these promoter constructs are also re-

TABLE 2. Intrinsic DNA bends of E2F sites selected in vitro and measurements of protein-DNA complex stability

Oligonucleotide	Sequence	Intrinsic bend (degrees)[a]	Half-lives of heterodimeric protein complexes (min)[b]:			
			E2F-1/DP-1	E2F-1/DP-2	E2F-4/DP-1	E2F-4/DP-2
1.1	5′-TTATTTTTCCCGCCTTT-3′	19 ± 3	27 ± 6	18 ± 1	5 ± 0.1	3 ± 0.1
1.2	5′-CACTTTTCCCGCCATT-3′	20 ± 3	15 ± 2	20 ± 2	6 ± 0.2	3 ± 1
4.1	5′-CCCTTTTGGCGCGAAA-3′	23 ± 3	22 ± 7	30 ± 1	10 ± 2	8 ± 0.2
4.2	5′-GCATTTTCCCGCCATT-3′	26 ± 3	16 ± 1	19 ± 0.1	5 ± 0.4	5 ± 1
1.1.2	5′-TTGTTCCCAGCCACTC-3′	0.2 ± 0.2	<2	<2	<2	<2
1.1.3	5′-TGCTTTCCCGCCACTC-3′	0.5 ± 0.4	18 ± 3	6 ± 1	3 ± 0.2	3 ± 0.4
1.1.4	5′-TTTTTTGGCGGTTTAT-3′	16 ± 0.2	15 ± 1	5 ± 1	3 ± 0.4	<2
1.1.5	5′-TCTTCCCGCCTTATTC-3′	1.0 ± 1.0	29 ± 4	26 ± 5	3 ± 0.4	3 ± 0.4
1.1.6	5′-TGATTTTGGCGGGATTC-3′	12 ± 0.3	16 ± 0.4	10 ± 2	<2	3 ± 0.4
Rb.1	5′-TCCTTTGGCGCGAAAA-3′	9 ± 2	22 ± 5	12 ± 4	2 ± 0.1	3 ± 0.1
Rb.2	5′-TTTTCGCGCCAAAAGGG-3′	15 ± 3	33 ± 6	17 ± 0.4	10 ± 0.4	7 ± 0.1
Rb.3	5′-GGTTTCCCGCCAAAAA-3′	14 ± 1	26 ± 6	17 ± 1	7 ± 1	5 ± 0.4
Rb.4	5′-AAAAATTGGCGCGAAA-3′	15 ± 2	5 ± 0.4	7 ± 1	2 ± 0.1	3 ± 0.1

[a] The intrinsic bends of oligonucleotides 1.1, 1.2, 4.1, and 4.2 were determined by phasing analysis as described in Materials and Methods. The intrinsic bends of the remaining oligonucleotides were determined by a multimerization assay (see Materials and Methods). The results are means ± standard deviations and are based on results from three independent experiments.

[b] The half-lives of protein-DNA complexes were determined by adding a 100-fold excess of unlabeled E2F site-containing oligonucleotides to preestablished protein-radiolabeled DNA complexes. Aliquots of binding-reaction mixtures were then applied to polyacrylamide gels at 0, 2, 5, 10, 20, and 30 min following the addition of cold oligonucleotide for E2F-4-containing complexes or at 0, 5, 10, 20, 30, and 60 min for E2F-1-containing complexes (due to the longer average half-lives of E2F-1-containing complexes). The amount of radiolabeled DNAs that remained associated with protein at different times following addition of unlabeled oligonucleotide was quantitated with a PhosphorImager and ImagQuant software. Means and standard deviations are calculated based on two to four independent experiments.

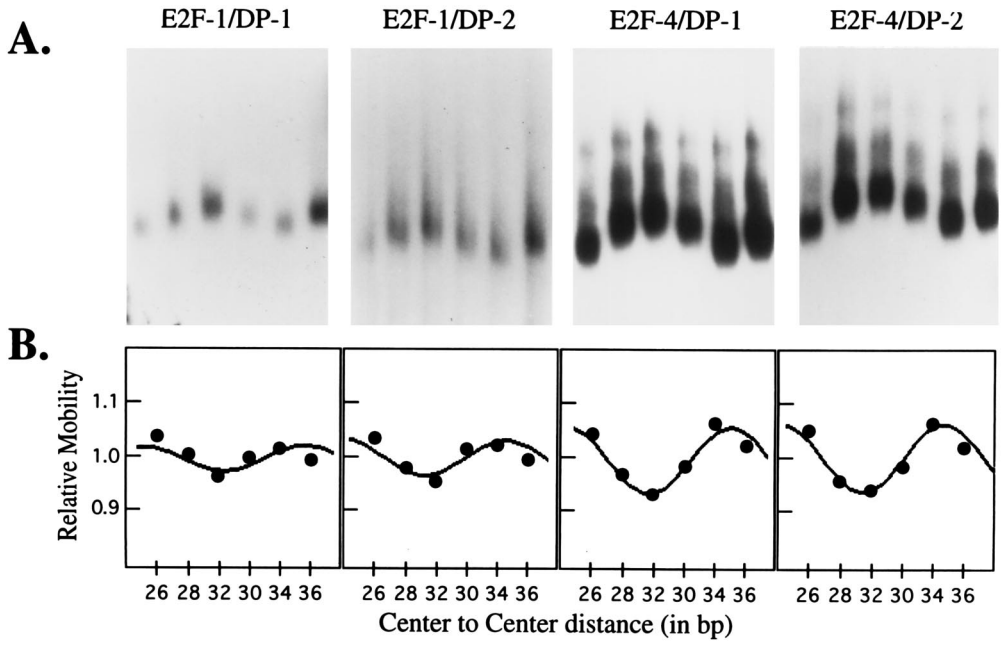
flected in their relative levels of luciferase production in log-phase cells (Fig. 7B). Consistent with results obtained in vitro, we conclude from these in vivo studies that E2F-binding sites are functionally distinct. We presume that these functional distinctions derive from the relative propensity of consensus E2F sites to bind particular E2F complexes in vivo as well as their topological influence on the transcriptional activity of the B-*myb* promoter.

DISCUSSION

Combining a sensitive method (CASTing) for the detection of preferred DNA-binding sites with baculovirus-expressed E2F, DP, and Rb proteins, we have developed a series of consensus DNA-binding sites for heterodimeric and heterotrimeric E2F complexes. Although consensus E2F-binding sites have been derived previously, these experiments were not designed to detect differences between heterodimeric complexes or to establish whether the binding of Rb to such complexes alters their DNA-binding specificities (12, 55). Several significant conclusions may be drawn from our results: (i) E2F and DP proteins each play important roles in the selection of DNA-binding sites; (ii) consensus E2F-binding sites may be distinguished by their primary nucleotide sequence, palindromicity, and intrinsic DNA-bending properties; (iii) the relative affinity of E2F complexes for DNA and their relative capacity to bend DNA are determined largely by the E2F dimerization partner; (iv) Rb alters the spectrum of E2F-binding sites selected by heterodimeric protein complexes; and (v) consensus E2F-binding sites direct distinct patterns of cell cycle-regulated transcription in vivo.

Prior to the initiation of our studies, the physiological role(s) of DP family members in E2F-mediated transcriptional regulation was uncertain. Lacking a potent *trans*-activation domain, a site for binding by Rb in vivo, and the capacity to bind DNA as homodimers, it has been suggested that DP proteins may simply function to increase the affinity of E2F family members for DNA and Rb (3, 15, 25, 42). Recent results indicating that (i) DP-2 encodes at least three protein isoforms differing at their respective amino termini due to alternative-splicing and internal translational initiation and (ii) DP proteins can differ in their capacity to shepherd E2F proteins into the nucleus have provided hints that the roles of DP family members may be greater than was initially suspected (48, 54, 57). Since the complex-specific E2F sites we have developed differ depending on the DP partner examined, it is clear that DP proteins actively participate in the selection of E2F-binding sites. Interestingly, the impact that DP proteins have on binding-site selection appears to depend on the E2F dimerization partner examined. That is, the exchange of DP dimerization partners within E2F-4-containing heterodimers results in drastic changes in preferred E2F-binding sites whereas subtle differences distinguish E2F sites resulting from the exchange of DP partners within E2F-1-containing complexes. Thus, E2F/DP proteins appear to interact with each other in a complex-specific fashion that in turn determines their binding-site preferences. We presume that our results reflect, at least in part, significant differences in the relative capacity of the E2F-1 and E2F-4 DNA-binding domains to accommodate particular nucleotides within their cognate binding sites. These results are markedly different from those of studies of other heterodimeric transcription factors, such as those of the b-Zip or basic helix-loop-helix families, where each dimerization partner appears to prefer a particular half-site sequence. Nonetheless, similar to studies on heterodimeric transcription factor families such as *fos* and *jun*, our results indicate that the co-expression of E2F and DP family members within cells leads to a constellation of E2F complexes with partially overlapping DNA-binding activities.

A burgeoning collection of observations indicate that E2F-binding sites are not functionally equivalent. Genomic footprint analyses of E2F sites within the mouse B-*myb* and human *cdc2* promoters have revealed them to be occupied only during portions of the cell cycle (i.e., G_1) where promoter activity is minimal (68, 84). Subtle mutations within such sites that block the formation of E2F protein-DNA complexes in vitro also eliminate site occupancy in vivo and result in the derepression



C.

E2F Site	Sequence	Mean Induced-Bend Angle (degrees +/- S.D.)			
		E2F-1/DP-1	E2F-1/DP-2	E2F-4/DP-1	E2F-4/DP-2
h E2F1	5'-TTTCGCGGCAAA-3'	14 +/- 4	20 +/- 4	29 +/- 3	29 +/- 4
E2F-1/DP-1 1.1	5'-TTATTTTTCCCGCCTTT-3'	19 +/- 2	15 +/- 4	29 +/- 5	28 +/- 4
E2F-4/DP-1 4.1	5'-CCCTTTTGGCGCGAAA-3'	21 +/- 3	18 +/- 4	25 +/- 3	26 +/- 4

FIG. 6. Phasing analysis of E2F-induced DNA bends. (A) Representative phasing analysis experiments with an E2F-binding site from the E2F-1 promoter and the indicated baculovirus-expressed E2F/DP heterodimers (the unbound probe is not shown). The protein-induced bend in DNA is apparent from the phase-dependent differences in mobilities. (B) The relative mobilities of the E2F/DP/DNA complexes from the primary data shown in panel A were measured and plotted as a function of the center-to-center distance between the E2F site and the center of the inherent DNA bend. (C) The relative induced bend angles of the different E2F/DP/DNA complexes were estimated by averaging the individual bend angles calculated from three independent phasing-analysis experiments.

of promoter activity (44, 68). Based on analogous mutational studies, E2F sites within the promoters of the *E2F-1* and *Hs-Orc1* genes are likely to be regulated similarly (29, 32, 52). In contrast, cell cycle-dependent activation of the DHFR and cyclin E promoters appears to require the binding of one or more E2F complexes to their cognate binding sites at the G_1/S transition (22, 53, 76, 77). As might be predicted, mutational inactivation of such E2F sites results in a marked diminution in transcriptional activity as cells approach the S phase. However, other E2F-binding sites appear to function as sites of positive and negative promoter regulation. For example, mutations that disrupt the binding of E2F complexes to sites within the cyclin A and mouse TK promoters result in a derepression of transcriptional activity in growth-arrested cells and a muted induction of transcription at the G_1/S boundary (34, 58). Additional support for the notion that E2F sites are functionally distinct comes from studies of tandem E2F-binding sites within the human p107 promoter (83). Each E2F site contributes to p107 promoter activity; however, a distal E2F site is largely responsible for Rb-mediated transcriptional repression while a proximal site dominates with respect to E2F-mediated transcriptional activation. Taken together, careful analyses of a number of cellular promoters clearly indicate that E2F sites can perform distinct duties. How might these duties be regulated? One possibility is that the nucleotide sequence of E2F sites specifies the binding of particular E2F protein complexes. This notion is strongly supported by our derivation of complex-specific E2F-binding sites and their direction of distinct patterns of cell cycle-regulated transcription in vivo.

Scanning the sequences of well-characterized sites of E2F regulation, it is apparent that such sites can be classified into two groups, palindromic and nonpalindromic. An enduring question in the field has been whether this distinction is functionally significant with respect to (i) the abundance and types of E2F complexes that can physically interact and (ii) the resulting transcriptional activity. Comparing the consensus E2F-binding sites we have obtained, it is quite clear that protein complexes made up of particular E2F and DP dimerization partners exhibit preferences for palindromic or nonpalindromic E2F sites. For example, heterodimeric complexes of E2F-1 and DP-1 or DP-2 showed little predilection for the selection of palindromic E2F sites. In contrast, 90% of E2F-binding sites selected by heterodimeric complexes of E2F-4 with DP-1 are palindromic whereas E2F-4/DP-2 complexes prefer palindromic E2F sites somewhat less frequently (30% of sites). Thus, although heterodimeric complexes containing E2F-1 can bind such sites, E2F-4-containing heterodimers favor palindromic E2F sites in vitro. At least two possible explanations could account for these results: (i) E2F-4-containing heterodimers have an increased affinity for such palindromic sites due to nucleotide and/or topological differences specified by such sites, or (ii) E2F-1- and E2F-4-containing heterodimers differ in their capacity to form higher-order multimeric complexes and/or simultaneously interact with two

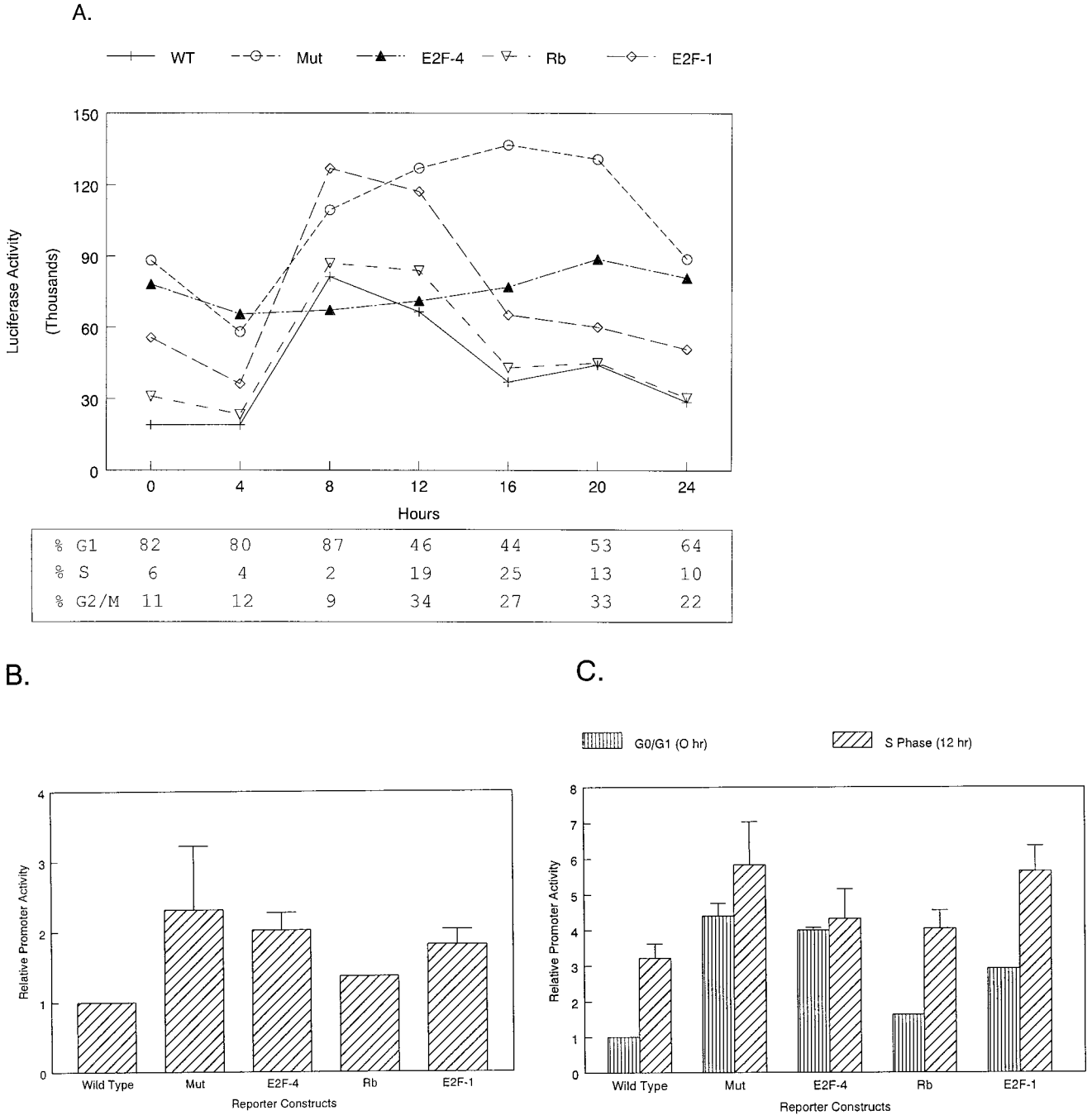


FIG. 7. Cell cycle-regulated transcription of B-*myb* promoter constructs carrying wild-type, inactive, and complex-specific E2F-binding sites. (A) Luciferase reporter constructs carrying the mouse B-*myb* promoter (WT) or derivatives that lack E2F-binding sites (Mut) or carry complex-specific binding sites (E2F-4, Rb, and E2F-1) were analyzed in transiently transfected CHO cells for cell cycle-dependent transcription. Luciferase results are derived from a single cell cycle experiment in which three independent plates of transfected cells were analyzed for each construction. The mean values obtained for each construct are plotted, and differences between constructions were shown to be statistically significant ($P < 0.001$) by analysis of variance. Identical results were obtained in two additional cell cycle experiments. Cell cycle progression of transfected cultures was monitored by flow cytometry, and the fraction of cells within each cell cycle phase is indicated at the bottom. (B) Luciferase activity of B-*myb* constructs in exponentially growing CHO cells. Mean values and standard deviations are plotted for each construct. The values indicated are derived from five independent experiments in which 6 to 12 plates of transfected cells were analyzed for each construct. (C) Comparison of luciferase activity in growth-arrested and S-phase cells. Mean values and standard deviations are plotted for each construct. Values indicated are derived from three independent cell cycle experiments in which nine plates of transfected cells were analyzed for each construct.

closely juxtaposed E2F sites. To address these issues, we have performed two additional experiments. First, cell extracts prepared from Sf9 cells coinfected with various heterodimeric partners were fractionated over glycerol gradients, with and without the inclusion of oligonucleotides carrying palindromic E2F-binding sites, and the distribution of E2F/DP proteins throughout the gradients was established by Western blotting. These experiments showed no obvious differences in the dis-

tribution of E2F-1- and E2F-4-containing complexes in vitro (65a). Second, to directly determine whether multiple heterodimers can simultaneously bind to each half-site of a palindrome, we have performed a variety of protein-DNA-binding assays that involve excess amounts of coinfected Sf9 cell extracts. None of these experiments have indicated that more than one E2F heterodimer can occupy simultaneously a given palindromic binding site in vitro (65a). Although we are well aware of the limitations inherent in these assays, we interpret the results to indicate that the preference of E2F-4-containing complexes for palindromic sites does not reflect their propensity to form higher-order multimeric complexes or for E2F-4 heterodimers to bind closely juxtaposed sites. Instead, we speculate that the preference of E2F-4-containing heterodimers for such sites reflects their proclivity for the nucleotide and/or topological features of such sites. Similar results have previously been reported for recombinant E2F/DP complexes and E2F complexes harvested from nuclei (71).

Perhaps the most significant findings reported here spring from a comparison of complex-specific sites selected by E2F-1/DP-1 heterodimers and Rb/E2F-1/DP-1 heterotrimeric complexes. It is apparent from a comparison of consensus E2F-binding sites that Rb significantly alters the spectrum of sites selected by E2F-1/DP-1 complexes in vitro. In contrast to the wide variety of largely nonpalindromic E2F sites selected by E2F-1/DP-1 complexes, heterotrimeric Rb/E2F-1/DP-1 complexes selected a tightly restricted subset of palindromic E2F-binding sites. These results indicate that the sequestration of transcription factor E2F within the Rb pocket results in a profound qualitative alteration of E2F/DP DNA-binding activity in addition to masking the E2F *trans*-activation domain from the basal transcription complex. Should these results translate to the occupancy of E2F sites in chromatin, we would predict that Rb negatively regulates a subset of E2F-1-dependent cellular genes. We presume that this subset of Rb-regulated cellular genes includes those whose deregulation in E2F-1 knockout mice leads to hyperplastic cell growth and tumorigenesis (20, 79). It is worth noting that several sites of E2F regulation, including E2F-binding sites within the human and mouse *E2F-1*, hamster DHFR, and mouse TK promoters, bear a striking resemblance to consensus Rb/E2F-1/DP-1 sites. Based on the consensus Rb/E2F-1/DP-1 site we have developed, additional predicted sites of regulation by this complex include an E2F-regulated site in the adenovirus E1A promoter and a putative site of E2F regulation upstream of the human cdc21/MCM4 gene (X = C or G):

Rb/E2F-1/DP-1 consensus	5′-AANT**TTTXGCGC**XAAAANTN-3′
human/mouse *E2F-1*	5′-GCTC**TTTCGCGG**CAAAAAGG-3′
hamster *DHFR*	5′-GCAA**TTTCGCGC**CAAACTTG-3′
mouse *TK*	5′-TTGA**GTTCGCGG**GCAAATGC-3′
adenovirus *E1A*	5′-CCAT**TTTCGCGG**GAAAACTG-3′
hcdc21/MCM4	5′-GCGT**TTTCGCGG**GAAAACTG-3′

It will be of interest to determine if Rb alters the DNA-binding site preference of other E2F/DP complexes and if Rb-related proteins also have the potential to regulate E2F DNA-binding activity. The former possibility appears quite likely since previous CASTing experiments with bacterially expressed glutathione *S*-transferase–Rb proteins bound to E2F harvested from cell nuclei revealed consensus sequences that are distinct from the Rb/E2F-1/DP-1 consensus sequence described here (12, 55).

Distinctions in the sequence of sites selected by E2F-1/DP-1 and Rb/E2F-1/DP-1 complexes are clearly reflected in their relative activity within the mouse B-*myb* promoter. Replacement of the native B-*myb* E2F site with a consensus E2F-1/DP-1 site led to a higher overall level of B-*myb* transcription and a modest level of repression in pre-S-phase cells. In contrast, replacement of the endogenous palindromic E2F site with a consensus Rb/E2F-1/DP-1 palindrome resulted in a pattern of cell cycle-regulated gene expression that is almost indistinguishable from that of the wild-type promoter. This result underlines the physiological relevance of the consensus E2F sites we have selected in vitro, since current models of B-*myb* transcriptional regulation include repression of promoter activity via one or more Rb family members tethered to DNA by E2F (44, 84). Surprisingly, replacement of the native B-*myb* E2F site with another palindromic E2F-binding site, the one preferred by E2F-4/DP-1 complexes, led to an entirely distinct pattern of gene expression. B-*myb* transcription in early cell cycle stages was derepressed, a minimal increase in promoter activity was noted at the G_1/S transition, and the overall promoter activity was higher than in the wild type. Thus, the E2F-4/DP-1 consensus site does not function as a site of E2F-mediated repression when localized at a native site of E2F regulation within the B-*myb* promoter. Given that E2F-4 and DP-1 proteins predominate in CHO cell extracts (65a, 76, 77) and bind the E2F-4/DP-1 consensus site with high affinity in protein-DNA-binding assays (65a), these results suggest that the local architecture of the B-*myb* promoter selectively precludes the activity of the E2F-4/DP-1 site as a node of E2F-mediated transcriptional repression. An equally plausible possibility is that E2F-4/DP-1 complexes fulfill a more circumscribed role in gene expression than do other E2F complexes. Interestingly, we note that the E2F-4/DP-1 consensus site we have examined is closely related in sequence to the cell cycle-regulated E2F-binding site within the DHFR promoter. DHFR transcription is directly correlated with an increase in the binding of E2F-4/DP-1 complexes to this site, and recent results suggest that this site functions only when the appropriate E2F *trans*-activation domain is tethered to it in close proximity to the site of transcriptional initiation (21, 77). Since the native B-*myb* E2F site is itself closely juxtaposed to sites of transcriptional initiation, it is unlikely that E2F-4/DP-1 proteins bound to this site would be unable to communicate with the basal transcription complex (44). Why, then, is an E2F site that directs cell cycle-regulated transcription within the DHFR promoter incapable of doing so within the B-*myb* promoter? These results led us to conclude that although the nucleotide sequences of E2F-binding sites favor the binding of particular E2F complexes, the functional consequence of these interactions is also determined by the promoter context within which they occur.

Our results and others suggest that at least four features may contribute to the function of E2F sites. (i) The primary sequence of E2F sites specifies the binding of particular heterodimeric or heterotrimeric complexes. These protein-DNA complexes may differ in their ability to activate transcription or mediate transcriptional repression. (ii) The intrinsic topological structure of E2F sites may affect promoter architecture. Some E2F sites, such as many selected by E2F-1/DP-1 complexes, are linear, whereas others, such as a site within the hE2F-1 promoter and those selected by Rb/E2F-1/DP-1 complexes, are highly bent (16). These intrinsic topological differences may dramatically affect how regulatory or basal transcription factors assemble on an E2F-dependent promoter. (iii) The linear relationship of E2F sites to other transcription factor-binding sites and/or the transcriptional start site may affect how multiple factors assemble on a given promoter. For example, E2F-1, E2F-2, E2F-3, and Rb physically or functionally interact with Sp1 whereas E2F-4 and E2F-5 do not, and

Sp1-binding sites are commonly located in close proximity to E2F-binding sites (34, 38, 46, 69). Thus, the juxtaposition of E2F sites and those for other transcription factors may contribute to cooperative functional interactions. (iv) Three-dimensional spatial interactions between proteins bound to E2F sites and neighboring elements may affect promoter activity. In addition to the spacing between factor-binding sites within a promoter, the degree of DNA bending induced by particular E2F complexes may have significant functional consequences. It is highly likely that some or all of these four architectural attributes play a pivotal role in the regulation of E2F-dependent promoters.

ACKNOWLEDGMENTS

We are indebted to members of the Horowitz laboratory, Tom K. Kerppola, and Michael C. Ostrowski for helpful discussions and to Joseph R. Nevins and Robert A. Weinberg for cDNAs used in this study.

This work was supported in part by National Cancer Institute grant CA53248 and Faculty Research Award A-73970 from the American Cancer Society to J.M.H., by a University of South Florida Research and Creative Scholarship Award to W.D.C., and by National Institutes of Health training grant CA09111. J.M.H. is a member of the Pew Scholars Program in the Biomedical Sciences.

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