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Interaction of D-Type Cyclins with a Novel myb-Like Transcription Factor, DMP1

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The cyclin D-dependent kinases CDK4 and CDK6 trigger phosphorylation of the retinoblastoma protein (RB) late in G_1 phase, helping to cancel its growth-suppressive function and thereby facilitating S-phase entry. Although specific inhibition of cyclin D-dependent kinase activity in vivo can prevent cells from entering S phase, it does not affect S-phase entry in cells lacking functional RB, implying that RB may be the only substrate of CDK4 and CDK6 whose phosphorylation is necessary for G_1 exit. Using a yeast two-hybrid interactive screen, we have now isolated a novel cyclin D-interacting myb-like protein (designated DMP1), which binds specifically to the nonamer DNA consensus sequences CCCG(G/T)ATGT to activate transcription. A subset of these DMP1 recognition sequences containing a GGA trinucleotide core can also function as Ets-responsive elements. DMP1 mRNA and protein are ubiquitously expressed throughout the cell cycle in mouse tissues and in representative cell lines. DMP1 binds to D-type cyclins directly in vitro and when coexpressed in insect Sf9 cells. In both settings, it can be phosphorylated by cyclin D-dependent kinases, suggesting that its transcriptional activity may normally be regulated through such mechanisms. These results raise the possibility that cyclin D-dependent kinases regulate gene expression in an RB independent manner, thereby serving to link other genetic programs to the cell cycle clock.

The first gap (G_1) phase of the cell cycle represents the interval in which cells respond maximally to extracellular signals, including mitogens, antiproliferative factors, matrix-adhesive substances, and intercellular contacts. Passage through a restriction (R) point late in G_1 phase defines the time at which cells lose their dependency on mitogenic growth factors for their subsequent passage through the cycle and, conversely, become insensitive to antiproliferative signals induced by compounds such as transforming growth factor β, cyclic AMP analogs, and rapamycin. Once past the R point, cells become committed to duplicating their DNA and undergoing mitosis, and the programs governing these processes are largely cell autonomous (reviewed in references 50 and 54).

In mammalian cells, a molecular event that temporally coincides with passage through the R point is the phosphorylation of the retinoblastoma protein (RB) (reviewed in reference 58). In its hypophosphorylated state, RB can prevent G_1 exit by combining with transcription factors such as E2F to actively repress transcription from promoters containing E2F binding sites (7, 17, 22, 31, 59). However, hyperphosphorylation of RB late in G_1 phase prevents its interaction with E2F, so enabling the untethered factor to activate transcription of the same target genes (reviewed in references 32 and 48). As many E2F-regulated genes encode proteins that are essential for DNA synthesis, RB phosphorylation at the R point helps convert cells to a prereplicative state that anticipates the actual G_1/S transition by several hours. Cells that completely lack RB have a markedly reduced dependency on mitogens but remain growth factor dependent, indicating that although RB controls an important aspect of the R-point transition, cancellation of its function is not sufficient for the complete abrogation of all R-point controls (23, 34).

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Phosphorylation of RB is initially triggered by holoenzymes composed of regulatory D-type cyclin subunits and their associated cyclin-dependent kinases, CDK4 and CDK6 (55). The D-type cyclins are induced and assembled into holoenzymes as cells enter the cycle in response to mitogenic stimulation. Acting as growth factor sensors, they are continuously synthesized as long as mitogenic stimulation continues and are rapidly degraded when mitogens are withdrawn (41). In fibroblasts, inhibition of cyclin D-dependent CDK activity prior to the R point, either by microinjection or by scrape loading of antibodies directed against cyclin D1 (4, 34, 35, 51) or by expression of specific CDK4 and CDK6 inhibitors (the so-called INK4 proteins) (21, 24, 30, 36, 44, 53), prevents entry into S phase. However, such manipulations are without effect in cells lacking functional RB, implying that RB may be the only substrate of cyclin D-dependent kinases whose phosphorylation is necessary for G_1 exit.

Given that RB-mediated controls are not essential to the cell cycle per se (10, 26, 33), it seems unlikely that mammalian cells would evolve three distinct D-type cyclins (D1, D2, and D3), at least two cyclin D-dependent kinases (CDK4 and CDK6), and four INK4 proteins, all for the sole purpose of regulating RB phosphorylation. This apparent redundancy might well serve to govern transitions through the R point in different cell types responding to a plethora of distinct extracellular signals, but it is equally possible that cyclin D-dependent kinases also regulate RB-independent events, perhaps linking them temporally to cell cycle controls. Here, we describe a direct interaction between D-type cyclins and a novel myb-like transcription factor which can regulate gene expression in an RB-independent manner.

MATERIALS AND METHODS

Cells and culture conditions. Mouse NIH 3T3 fibroblasts and 293T human embryonic kidney cells (20) were maintained in a 10% CO_2 sterile incubator at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 U each of penicillin and streptomycin (GIBCO/BRL, Gaithersburg, Md.) per ml. Mouse CTLL T lymphocytes were

grown in RPMI 1640 medium containing the same supplements plus 100 U of recombinant mouse interleukin-2 (a generous gift of Peter Ralph, formerly with Cetus Corp. [Cetus is now owned by Chiron]) per ml. *Spodoptera frugiperda* Sf9 cells were maintained at 27°C in Grace's medium containing 10% fetal bovine serum, Yeastolate, lactalbumin hydrolysate, and gentamicin (all from GIBCO/BRL) in 100-ml spinner bottles.

Isolation of DMP1. A yeast two hybrid system (5, 15) as employed previously (24) was used to isolate cDNAs encoding cyclin D2-binding proteins. In brief, a *Bam*HI-*Hin*dIII cDNA fragment encoding mouse cyclin D2 (41, 42) was subcloned into plasmid pAS2 in frame with the yeast GAL4 DNA binding domain to generate the pAS2cycD2 bait plasmid. *Saccharomyces cerevisiae* Y190, whose *HIS3* and *lacZ* genes are induced by GAL4, was transformed with pAScycD2 and then with a pACT library (Clontech, Palo Alto, Calif.) containing cDNAs prepared from mouse T-lymphoma cells fused 3′ to the GAL4 transcription activation domain. Of 6×10^5 colonies screened, 107 grew on SD synthetic medium lacking histidine and expressed β-galactosidase. Colonies that had been induced to segregate the bait plasmid were mated with *S. cerevisiae* Y187 containing either pAS2cycD2 or unrelated control plasmids expressing yeast SNF1 or human lamin fused to the GAL4 DNA binding domain. cDNAs from 36 library-derived plasmids presumed to encode cyclin D2-interacting proteins were sequenced; one encoded a cyclin D-binding myb-like protein, designated DMP1.

Because the recovered DMP1 cDNA (2.6 kb 3′ of GAL4) was shorter than the single mRNA species detected in mouse tissues by Northern (RNA) blotting analysis, plaque lifts representing 4×10^6 phages from a mouse C19 erythroleukemia cell cDNA library (5′ stretch λgt10; Clontech) were screened with a radiolabeled DMP1 probe, and two cDNAs containing additional 5′ sequences were isolated. These contained 200- and 373-bp segments overlapping those at the 5′ end of the probe plus ~800 bp of novel 5′ sequences. The latter sequences were fused within the region of overlap to those in the 2.6-kb DMP1 cDNA to generate a putative full-length cDNA of 3.4 kb.

In vitro binding and protein kinase assays. A *Bgl*II fragment encoding amino acids 176 to 761 of DMP1 (Fig. 1) was subcloned into the *Bam*HI site of plasmid pGEX-3X (Pharmacia, Uppsala, Sweden), and overnight cultures of transformed bacteria were diluted 10-fold with fresh medium, cultured for an additional 2 to 4 h at 37°C, and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h. Induced bacteria were lysed by sonication in phosphate-buffered saline (PBS) containing 1% Triton X-100, and recombinant glutathione *S*-transferase (GST)–DMP1 protein was purified by absorption and elution from glutathione-Sepharose beads as described previously (41). For in vitro binding, 1.5 μg of GST-DMP1 or GST-RB (16) immobilized on glutathione-Sepharose beads was mixed with [^{35}S]methionine-labeled mouse D-type cyclins, prepared by transcription (Stratagene Transcription System; Stratagene, La Jolla, Calif.) and translation (rabbit reticulocyte system from Promega, Madison, Wis.) in vitro as instructed by the manufacturer. Proteins were mixed in 0.5 ml of immunoprecipitation kinase buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.1% Tween 20) containing 10 mg of bovine serum albumin (BSA; Cohn fraction V; Sigma Chemical, St. Louis, Mo.) per ml. After 2 h at 4°C, the beads were collected by centrifugation and washed four times with immunoprecipitation kinase buffer, and the bound proteins were denatured and analyzed by electrophoresis on 11% polyacrylamide gels containing sodium dodecyl sulfate (SDS) (1).

Protein kinase assays (27) were performed with 1.5 μg of GST-DMP1 or GST-RB adsorbed to glutathione-Sepharose as substrates. The beads were suspended in a total volume of 25 μl of kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT) containing 1 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 μM ATP, 1 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham), and 2.5 to 5.0 μl of lysate (corresponding to 5×10^4 cell equivalents) from Sf9 cells coinfected with the indicated cyclins and CDKs. After incubation for 20 min at 30°C (with linear incorporation kinetics), the total proteins in the reaction were denatured and, following centrifugation of the beads, separated on denaturing polyacrylamide gels.

Antisera and immunoblotting. Rabbit antisera to recombinant DMP1 were commercially prepared (Rockland, Gilbertsville, Pa.) by using hexahistidine-tagged fusion proteins produced in bacteria (38) and containing fused DMP1 residues 221 to 439 (serum AJ to the myb-repeat domain) or residues 176 to 761 (serum AH). Antiserum AF was raised against a synthetic peptide representing the nine C-terminal DMP1 residues conjugated to keyhole limpet hemocyanin as described previously (14). All antisera specifically precipitated multiple phosphorylated forms of the full-length DMP1 protein from Sf9 lysates infected with a DMP1-producing baculovirus vector and did not cross-react with mammalian cyclins D, E, A, and B or CDK2, CDK4, and CDK6. To detect DMP1 in cultured mammalian cells, untreated CTLL cells (4×10^7) or transfected 293T cells (1.5×10^6) were suspended and sonicated in 1 ml of radioimmunoprecipitation assay buffer (50 mM Tris HCl [pH 7.5] containing 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and clarified by centrifugation. DMP1 was precipitated with 10 μl of antiserum AJ, denatured and electrophoretically separated on 9% polyacrylamide gels containing SDS, and transferred to nitrocellulose. The filter was incubated with a 1/100 dilution of AJ and AF antisera, and sites of antibody binding were detected by using ^{125}I-protein A (Amersham) as described previously (13).

Expression of recombinant DMP1 in insect cells. *Bam*HI linkers were added to an *Xba*I-*Eco*RV cDNA fragment containing the entire DMP1 coding sequence, and the fragment was inserted into the *Bam*HI site of the pAcYM1 baculovirus vector (43). Production of virus and infection of *S. frugiperda* Sf9 cells were performed as previously described (27). For preparation of radiolabeled cell lysates, cells infected with the indicated recombinant viruses encoding DMP1, CDKs, and/or cyclins were metabolically labeled 40 h postinfection for an additional 8 h with 50 μCi of [^{35}S]methionine (1,000 Ci/mmol; ICN, Irvine, Calif.) per ml in methionine-free medium or for an additional 4 h with 250 μCi of carrier-free $^{32}P_i$ (9,000 Ci/mmol; Amersham) per ml in phosphate-free medium. Cells suspended in 0.25 ml of kinase buffer containing protease and phosphatase inhibitors (2.5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 2% aprotinin, 1 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and 0.1 mM NaF) were lysed by repeated freezing and thawing and clarified by centrifugation. For detection of DMP1 or its complexes with D cyclins, 10 to 20 μl of lysate was diluted to 0.5 ml in EBC buffer (50 mM Tris HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT) containing 2% aprotinin, 1 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and 0.1 mM NaF. Antiserum AF (10 μl adsorbed to protein A-Sepharose beads) directed to the DMP1 C terminus was added, beads were recovered after incubation for 4 h at 4°C, and adsorbed proteins were denatured and resolved on denaturing gels. Where indicated, metabolically labeled Sf9 lysates were treated with calf intestinal phosphatase after immunoprecipitation (27). Determination of CDK activities in the cell extracts was performed by using soluble GST-RB or histone H1 (Boehringer Mannheim, Indianapolis, Ind.) as the substrate.

Selection of DMP1-binding consensus oligonucleotides. Binding site selection and amplification by PCR were performed as described previously (25). Briefly, we prepared single-stranded oligonucleotides containing 30 random bases inter-

A

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MSTVEEDSDT VTVETVNSVT FTQDTDGNLI  30
LHCPQNDPDE VDSEDSTEPP HKRLCLSSED  60
DQSIDDATPC ISVVALPLSE NDQSFEVTMT  90
ATTEVADDEL SEGTVTQIQI LQNDQLDEIS 120
PLGTEEVSAV SQAWFTTKED KDSLTNKGHK 150
WKQGMWSKEE IDILMNNIER YLKARGIKDA 180
TEIIFEMSKD ERKDFYRTIA WGLNRPLFAV 210
YRRVLRMYDD RNHVGKYTPE EIEKLKELRI 240
KHGNDWATIG AALGRSASSV KDRCRLMKDT 270
CNTGKWTEEE EKRLAEVVHE LTSTEPGDIV 300
TQGVSWAAVA ERVGTRSEKQ CRSKWLNYLN 330
WKQSGGTEWT KEDEINLILR IAELDVADEN 360
DINWDLLAEG WSSVRSPQWL RSKWWTIKRQ 390
IANHKDVSFP VLIKGLKQLH ENQKNNPVLL 420
ENKSGSGVPN SNCNSSVQHV QIRVARLEDN 450
TAISPSPMAA LQIPVQITHV SSTDSPAASA 480
DSETITLNSG TLQTFEILPS FPLQPTGTPG 510
TYLLQTSSSQ GLPLTLTTNP TLTLAAAAPA 540
SPEQIIVHAL SPEHLLNTSD NVTVQCHTPR 570
VIIQTVATED ITSSLSQEEL TVDSDLHSSD 600
FPEPPDALEA DTFPDEIPRP KMTIQPSFNN 630
AHVSKFSDQN STELMNSVMV RTEEEIADTD 660
LKQEEPPSDL ASAYVTEDLE SPTIVHQVHQ 690
TIDDETILIV PSPHGFIQAS DVIDTESVLP 720
LTTLTDPIFQ HHQEASNIIG SSLGSPVSED 750
SKDVEDLVNC H                     761
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B

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DMP1   VGK--YTPEEIEKLKELRIKHGN-DWATIGAALGRSASSVKDRCRLMKDTCNT  273
C-MYB  LGKTRWTREEDEKLKKLVEQNGTDDWKVIANYLPNRTD-VQCQHRWQKVLNPE   89

                          11AA                ****  * *
DMP1   ---GKWTEEEEKRLAEVVHEL^GVSWAAVAERVGTRSEKQCRSKWLNYLNWKQ  333
C-MYB  LIKGPWTKEEDQRVIKLVQKYGPKRWSVIAKHLKGRIGKQCRERWHNHLNPE  141

                        6AA
DMP1   SGGTEWTKEDEINLILRIAELDVA^WDLLAEGWSSVRSPQWLRSKWWTIKRQIA 392
C-MYB  VKKTSWTEEEDRIIYQAHKRLGNR-WAEIAKLLPGRTDNAI-KNHWNSTMRRKV 193
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FIG. 1. Amino acid sequence of DMP1. (A) DMP1 protein sequence. The three myb repeats are underlined, with the first (residues 224 to 273) and third (residues 334 to 392) repeats demarcated by italics. Ser-Pro and Thr-Pro doublets are in boldface, and acidic residues clustered at the amino- and carboxyl-terminal ends of the protein are indicated by double underlining. (B) The three myb repeats within mouse DMP1 (top) and c-myb (bottom) are aligned, with identical positions indicated by vertical bars. Three canonically spaced tryptophan residues (W) within each c-myb repeat are double underlined, and sites corresponding to DNA contacts in repeat 2 are indicated by asterisks. Eleven- and six-residue inserts required for maximal alignment of the two sequences are indicated above repeats 2 and 3.

posed between fixed forward (5′-CGCGGATCCTGCAGCTCGAG-3′) and reverse (5′-TGCTCTAGAAGCTTGTCGAC-3′) primers and then generated double-stranded oligonucleotides, using them as templates with the forward and reverse primers. The double-stranded oligonucleotides were mixed with recombinant DMP1 protein immunoprecipitated from Sf9 cells and immobilized to protein A beads. Mixing was performed in 125 μl of binding buffer (25 mM HEPES [pH 7.5], 100 mM KCl, 1 mM EDTA, 1.5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM DTT, 5% glycerol) containing 25 μg of poly(dI-dC) (Boehringer Mannheim) and 25 μg of BSA, followed by incubation with gentle rotation for 30 min at 4°C. Beads were collected by centrifugation, washed three times with binding buffer, and suspended in 50 μl of distilled water. Bound oligomers eluted into the supernatant by boiling were reamplified by PCR using the same primers. After six rounds of binding and amplification, recovered oligonucleotides were subcloned into the *Bam*HI-to-*Hin*dIII sites of pSK Bluescript plasmids (Stratagene), and their sequences were determined by using a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio).

Electrophoretic mobility shift assay (EMSA). Double-stranded oligonucleotides containing potential DMP1 binding sites (binding site 1 [BS1] and BS2) and mutated versions (M1 to M4) (see Fig. 5B) were end labeled with ^{32}P by using the Klenow fragment of DNA polymerase and [α-^{32}P]dATP (6,000 Ci/mmol; Dupont NEN) (9). Nuclear extracts from mouse NIH 3T3 or CTLL cells were prepared with buffer containing 0.4 M NaCl (2). Mammalian cell extracts (15 μg of protein) or Sf9 lysates (corresponding to 5×10^2 infected cells and containing ~4 ng of recombinant DMP1) were mixed with 3 ng of ^{32}P-labeled probe (10^5 cpm) in 15 μl of binding buffer containing 2.5 μg of poly(dI-dC) and 2.5 μg of BSA and incubated at 4°C for 30 min. For competition experiments, the indicated amounts of unlabeled oligonucleotides were added to the reaction mixtures before addition of the labeled probe. In some experiments, a bacterially produced GST-Ets2 fusion protein containing the complete Ets2 DNA binding domain (11) was used in place of Sf9 extracts containing recombinant DMP1. Protein-DNA complexes were separated on nondenaturing 4% polyacrylamide gels as described previously (9). Where indicated, antiserum to DMP1 together with 2.5 μg of salmon testis DNA (Sigma; used to reduce nonspecific DNA binding activity caused by serum addition) was preincubated with extracts for 30 min at 4°C prior to initiation of binding reactions. Immune complexes were either removed by adsorption to protein A-Sepharose beads (immunodepletion experiments) or allowed to remain (supershift experiments).

Transactivation assay. An *Xba*I-*Eco*RV fragment containing the entire DMP1 coding sequence was subcloned by blunt-end ligation into a *Spe*I-*Xba*I fragment of the Rc/RSV vector (Invitrogen, La Jolla, Calif.) to enable DMP1 expression in mammalian cells. Six-times-concatemerized (6×) BS1, 8× BS2, or 7× M3 oligonucleotides (Fig. 5B) were inserted into the *Xho*I-*Sma*I sites of pGL2 (Promega) 5′ to a minimal simian virus 40 (SV40) early promoter driving firefly luciferase gene expression. After the input dose of plasmid DNA was optimized to achieve acceptable background luciferase levels (Fig. 9A), 1 μg of the latter reporter plasmid together with increasing amounts of pRc/RSV-DMP1 expression plasmid compensated for by decreasing quantities of control pRc/RSV DNA (total of both = 2 μg) were transfected into 293T cells (1.5×10^6 cells per 60-mm-diameter culture dish) by calcium phosphate precipitation (8). Where indicated in Fig. 9C, pRc/RSV plasmids encoding either cyclin D2 or cyclin E and/or CDK4 or CDK2 (51) were included in the transfections. A plasmid encoding secreted alkaline phosphatase from the β-actin promoter was included as an internal control to monitor for transfection efficiency (11), which did not significantly contribute to the results shown. Two days later, cells were harvested, washed three times with PBS, and lysed in 1 ml of 25 mM glycylglycine (pH 7.8; Sigma)–15 mM $MgSO_4$–4 mM EDTA–1 mM DTT–1% Triton X-100. After clearing by centrifugation, 50-μl aliquots were diluted to 350 μl in 15 mM potassium phosphate buffer (pH 7.8) containing 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP, 1 mM DTT, and 67 μM luciferin (Sigma). Total light emission was measured for duplicate samples during the initial 20 s after luciferin injection with an Optocomp I luminometer (MGM Instruments, Hamden, Conn.).

Nucleotide sequence accession number. The GenBank accession number for the mouse DMP1 nucleotide sequence is U70017.

RESULTS

Isolation and molecular features of DMP1. A yeast two-hybrid screen was used to isolate cDNAs encoding proteins able to interact with cyclin D2. Plasmids containing cDNAs prepared from the RNA of mouse T-lymphoma cells and fused 3′ to the GAL4 activation domain were transfected into yeast cells containing a bait plasmid encoding the GAL4 DNA binding domain fused in frame with full-length mouse cyclin D2 coding sequences. From 6×10^5 transformants, we isolated 36 plasmids which, when segregated and mated with a yeast strain containing the cyclin D2 bait plasmid or with control strains expressing unrelated GAL4 fusion proteins, coded for proteins that interacted specifically with D-type cyclins. These cDNAs specified several previously identified cyclin D-interacting proteins (i.e., CDKs and CDK inhibitors) as well as novel polypeptides unrelated to those in searchable databases. Among the latter group was a single clone encoding a protein containing three tandem myb repeats characteristic of the myb family of transcription factors (19, 28, 52). Northern blot analysis revealed that a single ~3.8-kb mRNA related to the cloned sequences was present ubiquitously in adult mouse tissues (i.e., heart, brain, spleen, lung, liver, kidney, and testis) and mouse cell lines (NIH 3T3 fibroblasts, BAC1.2F5 macrophages, CTLL T cells, and mouse erythroleukemia cells); the mRNA was expressed in quiescent cells, and its level did not oscillate throughout the cell cycle in synchronized macrophages and fibroblasts (data not shown). Overlapping cDNAs containing 0.8 kb of additional 5′ sequences were isolated from a mouse erythroleukemia cell library, enabling us to reconstruct a 3.4-kb cDNA which approximates the length of the mRNA detected by Northern blotting. The cyclin D-binding myb-like protein encoded by this clone is designated DMP1.

The DMP1 cDNA contains a long open reading frame that encodes a protein of 761 amino acids with a mass of 84,589 Da (Fig. 1A), but its apparent molecular weight, as determined from its electrophoretic mobility on denaturing polyacrylamide gels, is significantly greater (see below). The initiation codon is the most 5′ AUG in the nucleotide sequence and is preceded by 247 nucleotides that contain termination codons in all three reading frames. DMP1 contains three myb repeats (residues 224 to 392; underlined in Fig. 1A), indicating its role as a transcription factor (6, 29, 60). The clone recovered in the two-hybrid screen lacked the 5′ untranslated region together with sequences encoding amino acids 1 to 175, which were replaced by the GAL4 activation domain. Both the amino-terminal (residues 4 to 169) and carboxyl-terminal (residues 579 to 756) ends of the full-length DMP1 protein are highly acidic. Fourteen SP and TP doublets are distributed throughout the protein, but none represent canonical proline-directed phosphorylation sites for CDKs [SPX(K/R)]. A typical nuclear localization signal was not identified.

Imperfect tandem myb repeats were first identified in the v-*myb* gene product of avian myeloblastosis virus and in its cellular proto-oncogene-coded c-*myb* homologs (Fig. 1B). The prototypic repeat sequence contains three regularly spaced tryptophan residues separated by 18 to 19 amino acids, with the third tryptophan of a repeat separated by 12 amino acids from the first tryptophan of the next (3, 19, 29, 52, 56). Degenerate repeats that contain tyrosine in place of the third tryptophan or isoleucine in place of the first have been identified in other myb-like proteins (56). Authentic myb proteins bind to YAA CNG (Y = pyrimidine) consensus sequences in DNA, with usually two or, rarely, only one of the myb repeats being sufficient to confer binding (6, 18, 46, 47, 60). Scattered amino acid identities enabled us to align the repeat sequences within mouse c-myb with those of DMP1 (Fig. 1B). In particular, there is an exact conservation of KQCR--W-N in repeat 2, which in c-myb contacts the DNA binding site (49). However, the first repeat of DMP1 contains a tyrosine substituted for the first tryptophan and leucine substituted for the third. Moreover, the second and third repeats, which in myb are each required for DNA binding, contain 11- and 6-residue insertions between the first and second tryptophans. These features distinguish the repeats of DMP1 from myb proteins and suggest that if DMP1 binds DNA, its consensus binding site likely differs from the myb recognition sequence.

Interaction of DMP1 with D-type cyclins. Because DMP1 interacted with cyclin D2 in yeast cells, we tested the ability of a GST-DMP1 fusion protein to bind D cyclins in vitro. GST

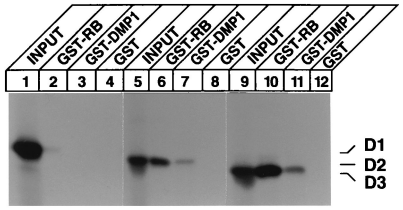

FIG. 2. Binding in vitro of D cyclins to RB and DMP1 fusion proteins. [^{35}S]methionine-labeled D cyclins prepared by in vitro transcription and translation were mixed with the bacterially produced GST fusion proteins or GST controls indicated above the lanes. Proteins bound to glutathione-Sepharose beads were washed, denatured, and separated on gels. Lanes 1, 5, and 9 show aliquots of input radioactive proteins corresponding to 25% of that actually used in each of the subsequent binding reactions. The mobilities of the three different D cyclins are indicated at the right. All protein inputs and exposure times are matched.

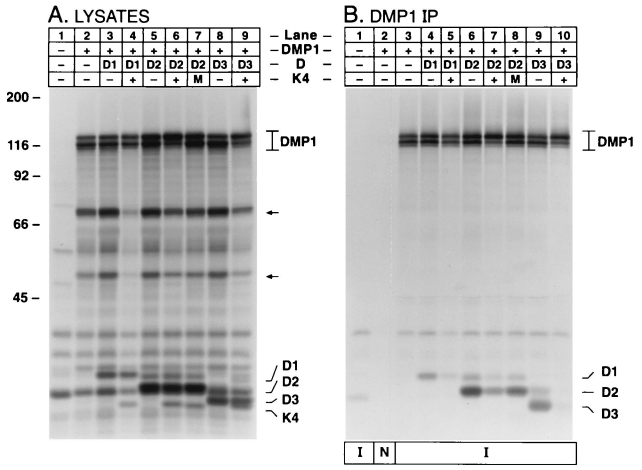

FIG. 3. Binding of D cyclins to DMP1 in insect Sf9 cells. Insect cells coinfected with baculovirus vectors encoding DMP1, D cyclins (D1, D2, D3), wild-type CDK4 (K4), or a catalytically inactive (K35M) CDK4 mutant (M) (27) as indicated at the top were metabolically labeled with [^{35}S]methionine. Lysates were divided in half, and proteins in one aliquot were separated directly on denaturing gels (A). The remaining proteins were immunoprecipitated (IP; B) with immune serum to the DMP1 C terminus (I) or with nonimmune serum (N), and the washed precipitates were electrophoretically separated in parallel. Positions of DMP1 isoforms, 78- and 54-kDa products (arrows; see text), D cyclins, and CDK4 are indicated at the right of each panel, and those of molecular weight markers are shown in kilodaltons at the left of panel A. The exposure time for both panels was 18 h.

was fused to residues 176 to 761 of DMP1 (in lieu of GAL4 in the original cDNA clone), and the bacterially synthesized recombinant protein was incubated with [^{35}S]methionine-labeled D-type cyclins prepared by transcription and translation in vitro. As a positive control, we used GST-RB, which can specifically bind D-type cyclins in this assay (16). Bound cyclins recovered on washed glutathione-Sepharose beads were analyzed by electrophoresis on denaturing gels. Figure 2 (lanes 6 and 10) shows that cyclins D2 and D3 interacted strongly with GST-RB in vitro (~20% of the total input protein was bound; see legend), whereas, as seen previously (16), cyclin D1 bound much less avidly (lane 2). GST-DMP1 was less efficient than GST-RB in binding cyclins D2 and D3 (~4-fold less binding), and under these conditions, an interaction with D1 was not detected (lanes 3, 7, and 11). No labeled proteins bound to GST alone (lanes 4, 8, and 12), and neither cyclin A nor cyclin E bound to GST-RB or to GST-DMP1, although both readily interacted with GST-CDK2 (data not shown). A cyclin D2 mutant disrupted in an amino-terminal Leu-X-Cys-X-Glu pentapeptide that is required for high-efficiency GST-RB binding (12, 16, 27) was not detectably compromised in its interaction with GST-DMP1 (negative data not shown); in agreement, DMP1 bears no apparent homology to RB or to RB-related family members (p107 and p130).

We next coexpressed full-length DMP1 together with D-type cyclins under baculovirus vector control in insect Sf9 cells. After metabolically labeling infected cells with [^{35}S]methionine, we precipitated DMP1 with an antiserum directed to a peptide representing its nine C-terminal residues. Electrophoretic separation of unfractionated metabolically labeled lysates from infected cells enabled direct autoradiographic visualization and relative quantitation of the recombinant mouse proteins (Fig. 3A). Cells infected with a vector containing DMP1 cDNA (lane 2) produced a family of ~125-kDa proteins, as well as smaller species of ~78 and ~54 kDa, which were not synthesized in cells infected with a wild-type baculovirus (lane 1). The proteins in the 125-kDa range represented phosphorylated forms of DMP1 (see below), which were specifically precipitated with three different DMP1 antisera (Fig. 3B, lane 3; see also below) but not with nonimmune serum (lane 2). The 78- and 54-kDa species may represent C-terminally truncated DMP1 products arising from premature termination or proteolysis, because they were not precipitated with the antiserum to the DMP1 C terminus (Fig. 3B). Apart from their phosphorylation, the full-length DMP1 proteins had apparent molecular masses significantly larger than that predicted from the cDNA sequence.

When DMP1 and different D-type cyclins were coexpressed in Sf9 cells (Fig. 3A, lanes 3, 5, and 8), anti-DMP1 coprecipitated cyclins D2 and D3 (Fig. 3B, lanes 6 and 9) and brought down D1 less efficiently (Fig. 3B, lane 4). In analogous experiments using RB in place of DMP1, stronger binding was also observed for D2 or D3 than for D1 (27). Given the preference of both DMP1 and RB for interactions with D2 and D3, it is possible that the observed differences in binding, while experimentally reproducible, are artifacts due to the improper folding or relative instability of the recombinant D1 protein, as also suggested by previous studies involving the abilities of the different cyclins to activate CDK4 in vitro (38). In assays using coinfected Sf9 cells containing approximately equivalent levels of DMP1 and cyclin D2 or D3, only 5 to 15% of the cyclin was stably bound to DMP1, whereas their binding to RB in such experiments is ~1:1 (27). Antisera to D-type cyclins reciprocally precipitated DMP1 without any notable preference for particular DMP1 isoforms. Again, no interactions were detected between DMP1 and cyclins A or E (data not shown). Overall, these results were completely consistent with the in vitro binding data obtained with DMP1 and RB (Fig. 2).

When Sf9 cells producing DMP1 were coinfected with baculoviruses encoding both a D-type cyclin and CDK4 (Fig. 3A, lanes 4, 6, and 9), complex formation between the cyclins and DMP1 was significantly diminished (Fig. 3B, lanes 5, 7, and 10). At least in part, this effect is likely due to competition between CDK4 and DMP1 for binding to D cyclin. Coproduction of a cyclin D-binding but catalytically inactive CDK4 mutant (Fig. 3A, lane 7) partially inhibited the interaction of DMP1 with cyclin D2 (Fig. 3B; compare lane 8 with lane 6). The catalytically inactive CDK4 subunits were not coprecipitated with antiserum to DMP1 (Fig. 3B, lane 8), indicating that CDK4 did not enter into stable ternary complexes with DMP1-bound cyclin D molecules. In agreement, an antiserum to CDK4 which readily precipitates binary cyclin D-CDK4 complexes produced in Sf9 cells (27) failed to coprecipitate DMP1 in the experiments performed here (data not shown). These

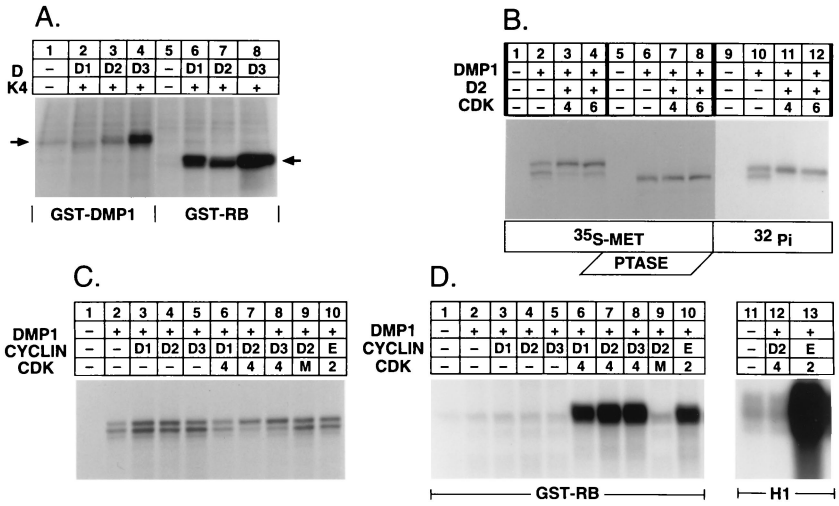

FIG. 4. Phosphorylation of DMP1. (A) Lysates from Sf9 cells coinfected with wild-type baculovirus (lanes 1 and 5) or with vectors encoding the indicated D cyclin and CDK4 (other lanes) were used as sources of kinases to phosphorylate the GST fusion proteins indicated at the bottom. Substrates were in excess, and phosphorylation occurred with linear kinetics. (B) Sf9 cells were coinfected with recombinant baculoviruses encoding DMP1, cyclin D2, and CDK4 (indicated as "4") or CDK6 (indicated as "6") as shown at the top. Cells were metabolically labeled with either [^{35}S]methionine (lanes 1 to 8) or $^{32}P_i$ (lanes 9 to 12), and half of the [^{35}S]methionine-labeled lysates were treated with calf intestinal phosphatase (PTASE; lanes 5 to 9). All lysates were then precipitated with an antiserum to the DMP1 C terminus, and DMP1 was resolved on denaturing gels. (C) Sf9 cells were coinfected with the indicated baculovirus vectors encoding DMP1, D cyclins (D1, D2, D3), cyclin E (E), CDK2 (2), CDK4 (4), or a catalytically inactive CDK4 mutant (M), cells labeled with [^{35}S]methionine were lysed and precipitated with antiserum to DMP1, and the protein was resolved on denaturing gels. (D) Lysates used for the experiment shown in panel C were assayed for protein kinase activity, using either a GST-RB fusion protein (lanes 1 to 10) or histone H1 (lanes 11 to 13) as the substrate. Autoradiographic exposure times were 8 h for panel A and 18 h for panels B to D.

results are consistent with the idea that DMP1-bound D cyclins are prevented from interacting efficiently with CDK4 subunits and vice versa. However, in cells coinfected with vectors encoding catalytically active CDK4 (Fig. 3A, lanes 4, 6, and 9), the formation of complexes composed of DMP1 and D-type cyclins was even further reduced (Fig. 3B; compare lanes 5, 7, and 10 with lane 8). Therefore, phosphorylation of DMP1 by the cyclin D-CDK4 holoenzyme (see below) might also limit the ability of DMP1 to bind to D cyclins.

DMP1 is a substrate for cyclin D-dependent kinases. In comparison with many known CDKs, the cyclin D-dependent kinases exhibit an unusual preference for RB over histone H1 as an in vitro substrate (39, 40, 45). To test whether cyclin D-dependent kinases could phosphorylate DMP1, equivalent quantities of GST-DMP1 and GST-RB fusion proteins were compared for the ability to be phosphorylated in vitro by Sf9 lysates containing cyclin D-CDK4. Whereas lysates of Sf9 cells infected with control baculoviruses did not efficiently phosphorylate either fusion protein (Fig. 4A, lanes 1 and 5), lysates containing active cyclin D-CDK4 complexes phosphorylated both (lanes 2 to 4 and 6 to 8). Under equivalent conditions, GST-RB was always a preferred substrate (lanes 6 to 8), and different preparations of cyclin D3-CDK4 were routinely more active than D2- or D1-containing holoenzymes in phosphorylating DMP1 (lanes 2 to 4). Similar results were obtained when immunoprecipitated cyclin D-CDK4 or cyclin D-CDK6 complexes were used in lieu of Sf9 extracts as sources of enzyme, arguing that DMP1 can serve as their direct substrate.

On the basis of data suggesting that DMP1 was posttranslationally modified when expressed in Sf9 cells and that coexpression of cyclin D-dependent kinases could reduce its binding to D cyclins (Fig. 3), we expressed DMP1 in Sf9 cells alone or together with cyclin D2-CDK4 or cyclin D2-CDK6. Infected cells were metabolically labeled with [^{35}S]methionine, and DMP1 was immunoprecipitated from cell lysates and resolved on denaturing gels. By using less radioactive precursor than for the experiments shown in Fig. 3, DMP1 was more easily resolved into two major species (Fig. 4B, lane 2). No protein was precipitated from cells infected with a control baculovirus (lane 1). Coinfection of cells producing DMP1 with cyclin D2-CDK4 or cyclin D2-CDK6 resulted in conversion of the faster-migrating DMP1 species to the slower-mobility form (lanes 3 and 4), whereas treatment of DMP1 immunoprecipitates with alkaline phosphatase converted both species to a single, more rapidly migrating band (lanes 6 to 8). Similar data were obtained when infected cells were labeled with $^{32}P_i$ instead of [^{35}S]methionine (Fig. 4B, lanes 9 to 12). Additional control experiments performed with the ^{32}P-labeled proteins confirmed that the observed effects of alkaline phosphatase on DMP1 mobility were due to removal of phosphate groups and were blocked by 1 mM sodium orthovanadate. Moreover, two-dimensional separation of radiolabeled DMP1 tryptic phosphopeptides revealed complex fingerprint patterns, consistent with multiple phosphorylation sites (data not shown). Therefore, both components of the DMP1 doublet are phosphoproteins. Its basal phosphorylation can be mediated by endogenous kinases present in insect cells (Fig. 4B, lanes 2 and 10), but coexpression of cyclin D-dependent kinases augments accumulation of the hyperphosphorylated, more slowly migrating species.

Hyperphosphorylation of DMP1 was not observed following infection of the cells with vectors producing D-type cyclin regulatory subunits alone (Fig. 4C, lanes 3 to 5). The process depended on a functional catalytic subunit (lanes 6 to 8 versus lanes 3 to 5), and it was unaffected by a catalytically inactive CDK4 mutant (lane 9). DMP1 hyperphosphorylation was not induced by cyclin E-CDK2 (lane 10). This result was confirmed by labeling aliquots of coinfected cells with $^{32}P_i$ in place of [^{35}S]methionine. Kinase assays performed with the same lysates (Fig. 4D) confirmed that the cyclin D-CDK4 complexes were active as RB kinases (lanes 6 to 8), whereas mutant CDK4 was defective (lane 9). Despite its relative inactivity on DMP1 (Fig. 4C, lane 10, and $^{32}P_i$ incorporation experiments not shown), cyclin E-CDK2 readily phosphorylated both RB

A

```
A   04--11--00--03--01--00--00--27--00--02--01--11--10
G   09--06--04--02--02--27--20--00--00--23--04--07--12
C   01--06--21--22--24--00--00--00--00--01--02--03--03
T   13--04--02--00--00--00--07--00--27--01--20--06--02

CONSENSUS:   C   C   C   G  G/T  A   T   G   T
PERCENT:    78  81  89  100     100 100 85  74
```

B

	FLANK	CONSENSUS FLANK	PREDICTED BINDING:
BS1	AATTGA	CCCGGATGT AGGTACGC	DMP1 AND ETS
BS2		CCCGTATGT	DMP1 ONLY
M1		CCCTGCGGT	NONE
M2		TTTGGATGT	
M3		CCCGGAAGT	ETS ONLY
M4		CCAGGATGT	ETS

FIG. 5. DMP1 oligonucleotide binding sequences. (A) The sequences of 27 oligonucleotides selected via repeated rounds of DMP1 binding and PCR amplification were determined. The frequencies of bases at 13 positions are shown at the top, with a 9-base consensus defined below. (B) Six oligonucleotides, all containing identical flanking sequences as indicated, were used as probes or competitors in EMSAs (Fig. 6 to 8) or were concatemerized and inserted 5′ of a minimal promoter used for transactivation assays (Fig. 9).

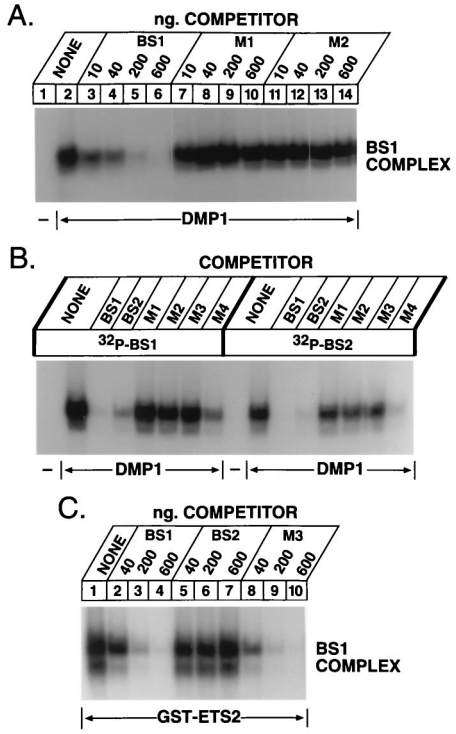

FIG. 6. Oligonucleotide binding specificity of recombinant DMP1 and Ets2 proteins. (A) Sf9 cell lysates containing ~4 ng of recombinant DMP1 were incubated with 3 ng of ^{32}P-BS1 in the absence (lane 2) or presence (other lanes) of the indicated, unlabeled oligonucleotide competitors. The only complex detected on native gels is indicated. (B) Parallel EMSAs were performed as for panel A, using radiolabeled BS1 or BS2 probe and 600 ng of the indicated competing oligonucleotides per lane. (C) Assays were performed as for panel A, using a bacterial GST-Ets2 fusion protein in place of Sf9 lysates containing DMP1. For all panels, the autoradiographic exposure time was 6 h.

(Fig. 4D, lane 10) and histone H1 (lane 13), but cyclin D2-CDK4 failed to phosphorylate the latter (lane 12). Thus, cyclin D-CDK4 and cyclin E-CDK2 differed in their relative substrate specificities for both histone H1 and DMP1.

Recombinant DMP1 binds to specific DNA sequences. To determine whether DMP1 would bind specifically to DNA, we prepared 30-bp random oligonucleotides flanked by PCR primers and incubated them with Sf9 cell lysates containing the full-length DMP1 protein. Oligonucleotides bound to washed DMP1 immunoprecipitates were amplified by PCR, and after six rounds of reprecipitation and reamplification, the final products were recloned and their sequences were determined. From 27 sets of sequences, we derived the consensus CCCG (G/T)ATGT (Fig. 5A). Repeating the experiment with a histidine-tagged DMP1 polypeptide produced in bacteria in place of the baculovirus-encoded protein, we again isolated oligonucleotides containing GGATG, but the preference for the 5′ CCC triplet was less pronounced. Computer searches indicated that the DMP1 oligonucleotide consensus also represents a binding site for the Ets1 and Ets2 transcription factors [namely, (G/C)(A/C)GGA(A/T)G(T/C)]. All Ets family proteins bind to sequences with a GGA core, with their individual binding specificities determined by adjacent flanking sequences (37, 57). Because the selected DMP1 binding site included either GGA or, less frequently, GTA in the corresponding position (Fig. 5A), we synthesized two oligonucleotides (BS1 and BS2 in Fig. 5B) that differed only in this manner. We also prepared four mutant oligonucleotides (M1 to M4 in Fig. 5B), at least one of which (M1) was predicted to bind neither DMP1 nor Ets proteins and another (M3) that, in contradistinction to BS2, should interact with Ets1 or Ets2 but not DMP1.

Using EMSAs performed after mixing a titrated excess (3 ng) of ^{32}P-end-labeled BS1 probe with Sf9 lysates producing DMP1 (~4 ng of recombinant protein per reaction), we detected a BS1-containing protein complex that was competed for with an excess of unlabeled BS1 oligonucleotide but not with mutant oligonucleotides M1 and M2 (Fig. 6A). Because M1 is disrupted in the three most highly conserved residues (Fig. 5B), its failure to compete was not surprising, but the inability of M2 to compete indicates that CCC sequences 5′ of the G(G/T)A core are also important for DMP1 binding. More subtle mutations within this region may be tolerated, because high concentrations of M4 (Fig. 5B) competed for BS1 binding to both DMP1 and Ets2 in subsequent studies (Fig. 6B; see also below). DMP1 also bound a BS2 probe, and the binding was competed for by excess BS2 or BS1 (Fig. 6B). In agreement with the site selection frequencies (Fig. 5A), binding of ^{32}P-BS1 under equivalent conditions was competed for more efficiently by excess unlabeled BS1 than by BS2 (Fig. 6B). M3, which was predicted to interact only with Ets proteins, did not compete with the BS1 or BS2 probe for binding to DMP1 (Fig. 6B). In contrast, a bacterially produced GST-Ets2 fusion protein did not bind detectably to a labeled BS2 oligonucleotide (not shown) under conditions in which BS1 binding was readily detected (Fig. 6C). In agreement, Ets2 binding to BS1 could be competed for with excess unlabeled BS1 and M3 but not by BS2 (Fig. 6C). Therefore, although both DMP1 and Ets2 can each bind to BS1 sequences, their exclusive interactions with BS2 and M3, respectively, help to distinguish DMP1 and Ets binding activities (as predicted by Fig. 5B).

Under identical EMSA conditions, use of extracts from Sf9 cells coexpressing cyclin D-CDK4 complexes (and containing predominantly hyperphosphorylated forms of DMP1) did not affect the efficiencies or patterns of DMP1 binding to a radiolabeled BS1 or BS2 probe, nor were there apparent differences in the recovery of DMP1-probe complexes between lysates lacking and containing cyclin D. Addition of excess BS1 and BS2 oligonucleotides together with cyclin D did not affect its binding in vitro to unphosphorylated GST-DMP1 produced in bacteria, nor did these oligonucleotides dissociate cyclin D-

DMP1 complexes formed in coinfected Sf9 cells. However, while as much as 15% of DMP1 molecules formed stable complexes with D-type cyclins when the two were coexpressed (Fig. 3, lanes 4, 6, and 9), both polyvalent and monoclonal antibodies to the different D cyclins were unable to supershift DMP1-oligonucleotide complexes formed with the same Sf9 extracts, formally leaving open the possibility that the interaction of DMP1 with cyclin D can inhibit DNA binding.

DMP1 expression and DNA binding activity in mammalian cells. Using an antiserum directed against either a DMP1 C-terminal peptide (serum AF; Fig. 3 and 4), the GST-DMP1 fusion protein (serum AH, residues 176 to 761), or its putative DNA binding domain (serum AJ, residues 221 to 439), we have so far been unable to detect DMP1 in mammalian cells by immunoprecipitation of the protein from metabolically labeled cell lysates. However, sequential immunoprecipitation (with serum AJ) and immunoblotting (with sera AJ and AH) revealed low levels of DMP1 in lysates of proliferating NIH 3T3 fibroblasts (Fig. 7A, lane 3). Most of the protein had a mobility corresponding to that of the hyperphosphorylated form synthesized in Sf9 cells (lane 1). (The baculovirus-encoded protein was separated on the same gel as immunoprecipitates from NIH 3T3 cells, and their relative positions were aligned after multiple autoradiographic exposures.)

Using the non-Ets-interacting ^{32}P-labeled BS2 probe to screen for DNA binding activity in mammalian cells by EMSA, complexes with mobilities indistinguishable from those generated with the recombinant protein in Sf9 lysates (Fig. 8A, lane 1, complex A) were detected with lysates from NIH 3T3 fibroblasts (lanes 2 to 8) and CTLL T cells (lanes 9 to 15). Faster-migrating complexes which lacked DMP1 were also seen (B complexes; see below). As predicted, A complexes containing bound ^{32}P-BS2 were competed for by both unlabeled BS1 (lanes 3 and 10) and BS2 (lanes 4 and 11) but not by the M3 Ets-specific recognition sequence (lanes 7 and 14). In the same lysates, more total binding activity was detected with a BS1 probe (Fig. 8B; note the shorter autoradiographic exposure time for Fig. 8B than for Fig. 8A), the vast majority of which was competed for by M3 (Fig. 8B, lanes 7 and 14) but not by BS2 (lanes 4 and 11). Therefore, the EMSAs performed with ^{32}P-BS1 primarily detected Ets-type DNA binding activity in mammalian cells, whereas assays performed with ^{32}P-BS2 detected an activity indistinguishable from that of bona fide DMP1.

To confirm that DMP1 activity was responsible for the A complexes observed in EMSAs done with the BS2 probe, antiserum to the DMP1 C terminus (AF) was added to the binding reactions (Fig. 7B). This generated a supershifted complex of slower mobility (S; lane 3) which was eliminated by competition with the cognate DMP1 peptide (P1; lane 4) but not with an unrelated control peptide (P2; lane 5). Formation of the A and S complexes was blocked by competition with the unlabeled BS2 oligonucleotide (lane 6) but not with M3 (lane 7), whereas B complexes remained and must therefore contain protein(s) other than DMP1 or Ets1/Ets2. Consistent with these findings, preincubation of NIH 3T3 or CTLL extracts with any of three different antisera to DMP1 (AF, AJ, or AH) but not with nonimmune serum eliminated the formation of A, but not B, complexes in EMSAs (Fig. 7C). Therefore, the BS2-containing A complex formed with extracts of mammalian cells contained authentic DMP1.

DMP1 can activate transcription. To determine if DMP1 has the capacity to activate transcription, we inserted tandem BS1, BS2, or M3 consensus sites 5′ to an SV40 minimal promoter and fused these control elements to a luciferase reporter gene. Reporter plasmids containing either BS1 or M3 binding

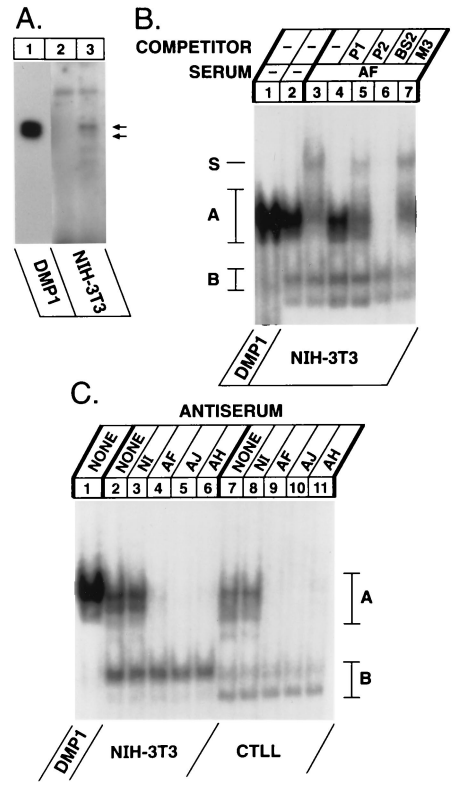

FIG. 7. Expression of DMP1 in mammalian cells. (A) Lysates of NIH 3T3 cells prepared in radioimmunoprecipitation assay buffer were precipitated with antiserum to DMP1 (serum AJ; lane 3) or with nonimmune serum (lane 2), and denatured immunoprecipitates were electrophoretically separated on gels. Lane 1 was loaded with Sf9 lysate containing recombinant DMP1. Proteins transferred to nitrocellulose were detected with a 1:1 mixture of antisera AJ and AF at 1/100 dilution followed by ^{125}I-protein A. Lane 1 was exposed for various times (18 h shown) to position the hypo- and hyperphosphorylated forms of recombinant DMP1 relative to the protein recovered from NIH 3T3 cells (lanes 2 and 3) detected on the same blot after much longer exposure (9 days). (B) Lysates from Sf9 cells containing DMP1 (lane 1) or from NIH 3T3 cells (lanes 2 to 7) were incubated with a ^{32}P-labeled BS2 probe plus antiserum AF (lanes 3 to 7), together with a cognate (lane 4) or irrelevant (lane 5) peptide or with 600 ng of competing BS2 (lane 6) or M3 (lane 7) oligonucleotide. Complexes resolved on nondenaturing gels include those designated A and B in Fig. 7A and a supershifted complex designated S in the left margin. Exposure time was 18 h. (C) EMSA performed with a radiolabeled BS2 probe and extracts from NIH 3T3 (lanes 2 to 6) or CTLL (lanes 7 to 12) cells. The extracts were either left untreated (NONE), precleared with nonimmune serum (NI), or immunodepleted with the indicated antisera to DMP1 (AF, AJ, or AH) prior to incubation with the probe. Exposure time was 18 h.

sites were themselves highly active in a dose-dependent fashion when transfected into 293T kidney cells, most likely as a result of expression of endogenous Ets factors, but the reporter plasmid containing BS2 sites generated even less background activity than one containing only a minimal SV40 promoter (Fig. 9A). When the cDNA encoding DMP1 was cloned into a pRc/RSV mammalian expression plasmid and cotransfected with limiting amounts (1 μg) of the BS2-driven reporter plasmid into 293T cells, we observed significant transactivation of luciferase activity at levels ~20-fold that seen with the BS2 reporter plasmid alone (Fig. 9B). A sevenfold activation of the BS1-driven reporter in response to DMP1 (Fig. 9B) was of even greater absolute magnitude but was initiated from a four- to fivefold-higher basal level (Fig. 9A and B). Use of a BS1-driven reporter containing three rather than six reiterated binding sites reduced the overall magnitude of both basal and DMP1-dependent transactivation by ~60%. In contrast, with

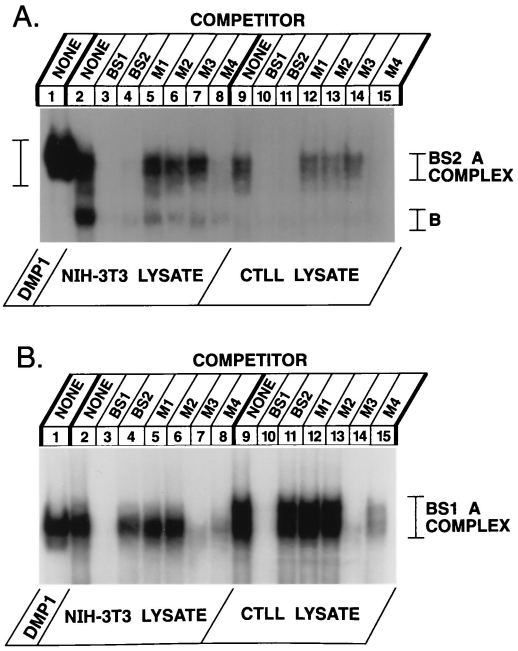

FIG. 8. Binding of radiolabeled BS2 and BS1 oligonucleotides to proteins in mammalian cells. Lysates of Sf9 cells containing recombinant DMP1 (lanes 1), mouse NIH 3T3 fibroblasts (lanes 2 to 8), or mouse CTLL lymphocytes (lanes 9 to 15) were incubated with radiolabeled BS2 (A) or BS1 (B) probe, either in the absence (lanes 2 and 9) or in the presence (other lanes) of the indicated competing oligonucleotides (600 ng). Two distinct sets of BS2-containing complexes (labeled A complex and B at the right of panel A) were detected, only the first of which corresponded in mobility to that formed with recombinant DMP1 (lane 1). Autoradiographic exposure times were 18 h for panel A and 6 h for panel B.

promoters lacking BS2 sites or containing Ets-specific M3 sites, transactivation by DMP1 was not observed. Gross overexpression of DMP1 in these experiments was documented by immunoprecipitation and immunoblotting, and the majority of the ectopically produced protein was localized to the cell nucleus (data not shown). It is also noteworthy that 293T cells lack a functional RB protein, implying that DMP1-mediated transactivation is RB independent. Similar data could also be obtained in transfected NIH 3T3 cells which retain RB function (data not shown).

In a separate series of experiments, cotransfection of a cyclin D2 expression plasmid with DMP1 (with or without additional CDK4) partially blocked its ability to transactivate the BS2-driven reporter gene (Fig. 9C). Similar results were observed with a BS1-driven reporter, while expression of the Ets-responsive M3 reporter used as a negative control (Fig. 9A and B) was completely unaffected (data not shown). Cotransfection of pRc/RSV plasmids expressing cyclin E or A or combinations of these cyclins with CDK2 had no significant effects on DMP1-induced activation of the BS2-driven reporter gene (Fig. 9C). At face value, these results raise the possibility that D-type cyclins negatively regulate DMP1-dependent gene expression, although the mechanism underlying the latter effects remains unclear.

DISCUSSION

DMP1, isolated in a yeast two-hybrid screen using cyclin D2 as bait, is a novel transcription factor containing three tandem myb repeats flanked by highly acidic segments at its termini. Recombinant DMP1 specifically binds to oligonucleotides con-

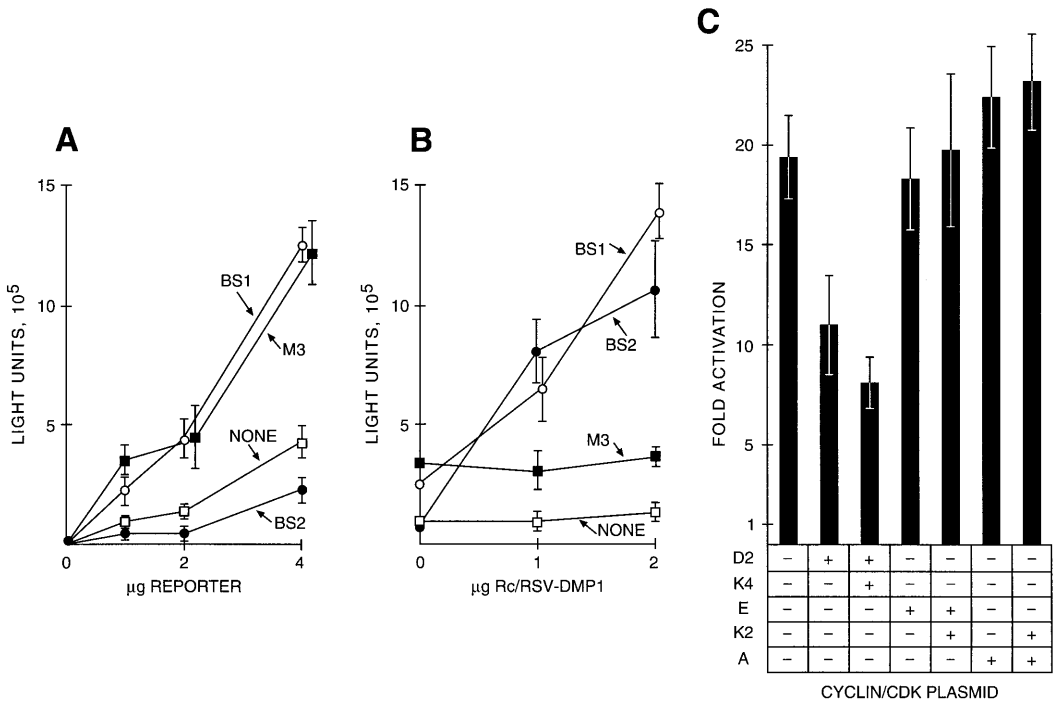

FIG. 9. Transactivation of reporter plasmids in 293T cells transfected with recombinant DMP1. (A) Increasing concentrations of reporter plasmids containing a luciferase gene driven by a minimal SV40 promoter with 5′ concatemerized BS1 (open circles), BS2 (closed circles), or M3 (closed squares) sequences or no additions (open squares) were transfected into 293T cells, and luciferase activity was determined 48 h later. (B) Reporter plasmids (same as in panel A; 1 μg of each) were cotransfected with increasing quantities of DMP1 expression plasmid, and luciferase activity was measured 48 h later. (C) The BS2-containing reporter plasmid (1 μg) was cotransfected with the DMP1 expression vector (1 μg) together with pRc/RSV expression plasmids (0.5 μg) encoding the indicated cyclins and CDKs. Background luciferase activity for the BS2 reporter plasmid in the absence of DMP1 (B, 0 input) was set to 1.0 arbitrary activation units. The fold activation relative to this value is plotted on the *y* axis. For each set of experiments, the total input DNA concentrations were adjusted where necessary by addition of parental pRc/RSV plasmid DNA lacking inserts to yield 4 μg (A) or 3 μg (B and C) for each transfection. The error bars indicate standard deviations from the mean.

taining the nonamer consensus sequence CCCG(G/T)ATGT and, when transfected into mammalian cells, activates transcription of a reporter gene driven by a minimal promoter containing concatemerized DMP1 binding sites. Low levels of DMP1 mRNA are normally expressed, albeit ubiquitously, in mouse tissues and cell lines and were detected in both quiescent and proliferating macrophages and fibroblasts without significant oscillation throughout the cell cycle. Correspondingly low levels of DMP1 protein were detected in cell lysates by sequential immunoprecipitation and immunoblotting, and in assays using GTA core-containing consensus oligonucleotides as probes, these extracts contained EMSA activity with antigenic and oligonucleotide binding specificities indistinguishable from those of the recombinant DMP1 protein.

Ets family transcription factors, including Ets1 and Ets2, can bind to and activate transcription from those DMP1 consensus recognition sites that contain a GGA core. Promoter-reporter plasmids containing consensus binding sites with either a central GGA or GTA trinucleotide could each respond to overexpressed, recombinant DMP1 in transactivation assays. However, in the absence of ectopically expressed DMP1, background levels of reporter gene activity were significantly higher with the Ets-responsive promoters, implying that endogenous Ets activity greatly exceeded that of endogenous DMP1, at least in the cell types used here. Similarly, when the GGA-containing consensus oligonucleotide probe was used for EMSA, competition studies indicated that Ets family members predominated in complexes resolved from lysates of NIH 3T3 and CTLL cells. Particularly in cases such as these, in which total Ets binding activity greatly exceeds that of DMP1, the use of oligonucleotide probes containing the GTA core is essential for unambiguously demonstrating endogenous DMP1 DNA binding activity by EMSA. Only under such circumstances could complexes be depleted or supershifted with antisera to DMP1 and not be competed for by the unlabeled Ets-binding M3 oligonucleotide.

Not only does DMP1 specifically interact with cyclin D2 when overexpressed in yeast cells, but translated, radiolabeled D-type cyclins bind directly to GST-DMP1 fusion proteins in vitro, and complexes between full-length DMP1 and D-type cyclins readily form in intact Sf9 insect cells engineered to coexpress both proteins under baculovirus vector control. DMP1 undergoes basal phosphorylation when synthesized in Sf9 cells and is further hyperphosphorylated in cells coexpressing catalytically active, but not dysfunctional, cyclin D-CDK4 complexes. Immune complexes containing cyclin D-CDK4 can also phosphorylate DMP1 in vitro. However, other kinases must also contribute to DMP1 phosphorylation in insect cells, given the accumulation of multiply phosphorylated forms of the protein even in cells not engineered to coexpress recombinant cyclin-CDK complexes. Although most of the endogenous DMP1 detected in proliferating NIH 3T3 cells appeared to correspond in mass to the hyperphosphorylated form, we as yet have no direct evidence that DMP1 interacts with D-type cyclins or undergoes phosphorylation by cyclin D-dependent kinases in mammalian cells.

At first glance, the observed interactions of DMP1 and D-type cyclins seemed reminiscent of those previously observed with RB, but there are important differences. First, side-by-side comparisons indicated that D-type cyclins bind less avidly to DMP1 than to RB, both in vitro and in Sf9 cells. Second, the efficiency of RB binding to D-type cyclins is strongly influenced by a Leu-X-Cys-X-Glu pentapeptide sequence that D-type cyclins share with certain RB-binding oncoproteins (12, 16, 27), whereas a cyclin D2 mutant containing substitutions in this region remained fully able to interact with DMP1. Third, RB was phosphorylated to much higher stoichiometry than DMP1 by cyclin D-CDK4 complexes. CDK4-mediated phosphorylation of RB in vitro or in Sf9 cells can occur at multiple canonical CDK sites (27). However, even though there are 14 Ser-Pro and Thr-Pro doublets distributed throughout the DMP1 protein, none of these represents a typical CDK consensus, indicating that cyclin D-dependent kinases phosphorylate atypical recognition sequences in this protein. Conversely, cyclin E-CDK2 complexes did not detectably phosphorylate DMP1, and no physical interactions between DMP1 and cyclin E or cyclin A were detected. Finally, phosphorylation of RB by cyclin D-CDK4 complexes cancels its ability to bind D-type cyclins, so that in coinfected Sf9 cells, stable ternary complexes could be generated only between RB, D-type cyclin, and catalytically inactive CDK4 subunits (27). However, catalytically inactive CDK4 could not enter into stable ternary complexes with DMP1 and cyclin D. This finding again suggests that cyclin D contacts DMP1 and RB via different residues (see above) and raises the possibility that DMP1 and CDK4 interact with overlapping binding sites on cyclin D, being able to compete with one another for cyclin D binding. In agreement, introduction of catalytically inactive CDK4 into cells expressing both cyclin D2 and DMP1 reduced the extent of D2 binding to DMP1, although to a lesser extent than wild-type CDK4. Therefore, the effect, if any, of cyclin D binding on DMP1 function may not necessarily be to trigger CDK4-mediated phosphorylation, even though hyperphosphorylation of DMP1 by the holoenzyme can decrease its ability to bind cyclin D.

Together, these findings provide circumstantial evidence that cyclin D may influence gene expression via its binding and/or phosphorylation of DMP1. Enforced transient expression of cyclin D2 in mammalian cells, alone or in combination with CDK4, appeared to negatively regulate DMP1's ability to transactivate reporter gene expression. The fact that cyclin D2 alone could mediate this effect does not preclude that it acts via a CDK partner, since it is the cyclins that are generally rate limiting in the formation of the active holoenzymes (4, 40, 51). Under similar experimental conditions, cyclin E or A, alone or together with CDK2, was without effect. An important caveat in interpreting the results of the latter experiments concerns the impracticality of controlling the levels of expression of each of the cotransfected proteins and, hence, the formation of distinct complexes between potential partners, the levels of induced kinase activity that might result, and the degree of DMP1 phosphorylation. Therefore, the mechanistic basis for the observed inhibitory effects of cyclin D2 and CDK4 remains unresolved. Other preliminary experiments suggest that enforced expression of cyclin D-CDK4 influences neither the stability of overexpressed DMP1 nor its ability to preferentially localize to the nucleus of transfected mammalian cells. Coexpression of cyclin D or cyclin D-CDK4 together with DMP1 in Sf9 cells also had no apparent effect on the ability of DMP1 to form EMSA complexes with consensus oligonucleotide probes. However, the majority of DMP1 molecules in such extracts do not contain stably bound cyclin, and their extent and sites of phosphorylation are unknown. Oligonucleotide-bound proteins from such extracts or from mammalian cells could be supershifted in EMSAs performed with antisera to DMP1, but polyvalent antisera or monoclonal antibodies to D cyclins were without detectable effect on their electrophoretic mobility, leaving open the possibility that cyclin D binding and/or cyclin D-CDK4-mediated phosphorylation might potentially interfere with the ability of DMP1 to bind to DNA. Direct effects of cyclin D with or without CDK4 on DMP1-mediated transactivation potential are equally plausible. While we are a long way from demonstrating that cyclin D can physiologically regulate

DMP1's activity, these observations nonetheless underscore a potential for RB-independent control of gene expression by D-type cyclins.

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