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A B-myb Promoter Corepressor Site Facilitates in Vivo Occupation of the Adjacent E2F Site by p107-E2F and p130-E2F Complexes*

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Transcription from the B-myb (MybL2 gene) promoter is strictly cell cycle-regulated by repression mediated through an E2F site during G₀/early G₁. We report here the characterization of a corepressor site (downstream repression site (DRS)) required for this activity that is closely linked to the E2F site. Systematic mutagenesis of the DRS enabled a consensus to be derived, and it is notable that this sequence is compatible with cell cycle gene homology region sequences associated with cell cycle-dependent elements in the cyclin A, cdc2, and CDC25C promoters. The B-myb promoter is inappropriately active during G₀ in mouse embryo fibroblasts lacking the p107 and p130 pocket proteins, and we show that the ability of transfected p107 and p130 to re-impose repression on the promoter is dependent on the DRS. In contrast, transfected Rb was unable to repress the B-myb promoter. Consistent with the notion that Rb-E2F complexes are unable to bind the B-myb promoter E2F site in vivo, footprinting showed that this site is unoccupied in cells lacking p107 and p130. Chromatin immunoprecipitation assays showed a requirement for the DRS in recruiting p107 and p130 complexes to the B-myb promoter, indicating that in vivo the DRS governs the occupancy of the adjacent E2F site by transcriptional repressors.

The diversity present within the E2F and pocket protein families suggests that different E2F complexes may play distinct roles in cell cycle regulation of gene transcription. In support of this notion, it was found that “knockouts” of specific pocket protein genes by homologous recombination have quite different effects on mouse embryonic development. Whereas Rb⁻/⁻ mice die in utero with defects in the liver, central nervous system, and ocular lens and a profound reduction in definitive erythropoiesis (9), p107⁻/⁻ and p130⁻/⁻ mice develop normally (10). Inactivation of both p130 and p107 results, however, in neonatal lethality, with defects in bone and cartilage arising from hyperproliferation of chondrocytes (10). Differences in pocket protein function are also manifest at the level of cell cycle-regulated transcription in mouse embryo fibroblasts (MEFs)¹ derived from mutant animals (11). Whereas elimination of either p107 or p130 had no discernible effect on cell cycle-regulated transcription, knockout of both p107 and p130 genes resulted in deregulated expression of a set of genes (B-myb, cdc2, E2F-1, thymidylate synthase, RRMD, and cyclin A2) distinct from those deregulated in Rb⁻/⁻ MEFs (cyclin E and p107). These phenotypic differences between Rb⁻/⁻ and p107⁻/⁻/p130⁻/⁻ MEFs are unlikely to be due to gene dosage effects, as studies with established knockout fibroblasts showed that deregulation of the B-myb promoter in p107⁻/⁻/p130⁻/⁻ cells could not be recapitulated in either p107⁻/⁻/Rb⁻/⁻ or p130⁻/⁻/Rb⁻/⁻ cells (12). It is therefore evident that p107 and p130 have a functionally redundant role in E2F-mediated gene regulation, which is distinct from that of Rb.

It is currently unclear how different pocket protein:E2F complexes discriminate between the genes they regulate. One possibility is that they demonstrate some sequence specificity for the E2F binding site in the promoters of these genes. Although most in vitro binding studies have failed to show this, repetitive selection of redundant binding sites by the CASTing procedure (13) suggests that different E2F complexes do have inherent preferences for particular E2F sites. An additional possibility is that occupation of the E2F site in vivo is influenced by factors binding to adjacent sites or that the ability of E2F complexes to regulate transcription, whether by repression or activation, is dependent upon interactions with these accessory factors. The contribution of a putative accessory binding site to E2F-dependent transcription is exemplified by B-myb (the MybL2 gene), in which it was found that cell cycle regulation is influenced by a distinct promoter site located immediately down-

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1 The abbreviations used are: MEFs, mouse embry fibroblasts; DRS, downstream repression site; CDE, cell cycle-dependent element; CHR, cell cycle gene homology region; mE2F, mutant E2F site; mDRS, mutant downstream repression site; ChIP, chromatin immunoprecipitation; RhNP, non-phosphorylatable Rb; EMSAs, electrophoretic mobility shift assays.
stream of the E2F site (17, 32). Mutations in either the E2F site or the adjacent site (downstream repression site (DRS)) abolish transcriptional repression in G0/G1. Significantly, in vivo footprinting studies revealed that the B-myb promoter E2F site is occupied in quiescent cells, but becomes unoccupied in mid G1 preceding the induction of B-myb transcription (14). As E2F abundance actually increases at the G1/S boundary, it is possible that the role of the DRS is to stabilize interactions of repressor complexes at the adjacent E2F site specifically in G0/early G1.

Transcriptional repression is a common mechanism to extinguish expression of periodically regulated genes during cell cycle arrest and quiescence (15–28); however, there is evidence that the regulation of certain of these genes is not directly dependent on E2F. Most notably, transcriptional repression of CDC25C in G0/early G1 involves bipartite elements termed CDE/CHR (23, 29). The CDC25C CDE/CHR elements are partially homologous to the B-myb E2F/DRS sites and, moreover, have an identical spatial relationship (30); however, E2F binds weakly if at all to CDE/CHR. Compared with E2F/DRS, the CDE/CHR elements confer a subtly different cell cycle kinetics on promoters: both sets of elements permit derepression of transcription in the latter part of G1; however, induction is attenuated within the spacer; and mutant CDE/CHR elements confer a subtly different cell cycle kinetics with respect to the start of the protein coding sequence, and these CDE/CHR elements are par-}

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**EXPERIMENTAL PROCEDURES**

**Cell Culture and Flow Cytometry—**MEFs from control p107/+/− and gene knockout p107−/−/p130−/− animals were kindly provided by Dr. Nick Dyson (Massachusetts General Hospital Cancer Center, Charlestown, MA) and were expanded and used at passage 4. MEFs and Swiss 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Established 3T3 cell lines were derived from MEFs by splitting cultures every 3 days according to standard procedures. These established 3T3 and NIH 3T3 cells (obtained from Dr. Rene Bernards) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. Flow cytometry was performed on ethanol-fixed cells stained with propidium iodide as described previously (22).

**B-myb Promoter/Reporter Plasmids and Preparation of Stably Transfected Cell Lines—**The mouse B-myb promoter/reporter cassette was made using PCR with Pfu DNA polymerase (Stratagene) to introduce AatII and BclI sites 5’ and 3’, respectively, of the B-myb promoter E2F/DRS sites in the pGL2−(536) luciferase reporter (15). Double-stranded DNA oligonucleotides containing 5′-AatII and 3′-BclI ends were then inserted using T4 DNA ligase to replace the wild-type sequence with variant transcriptional control sites. The sequences of these oligonucleotides (sense strand only) were as follows: mE2F, GGCGGGAGATTTGAATG; mDRS, AATCCGGCAGATCTTCCTTTT; 3′-AATCCGGCAGATCTTCCTTTT (amplified promoter sequences from −533 to −88 with respect to the start of the protein coding sequence, and these HindIII fragments replaced the SV40 promoter in pGAE/EluW Plasmids. Transfections were performed into NIH 3T3 cells using calcium phosphate coprecipitation, and stable transfectants were selected with 0.5 μg/ml G418 using the conditions described to limit copy number (31). Clones carrying approximately five copies of each transgene were selected for chromatin immunoprecipitation (ChIP) analysis.

**Pocket Protein Expression Plasmids—**An expression plasmid encoding untagged p107, pCMVp107, was kindly provided by Dr. Liang Zhu (Sanofi-Aventis). AatII and BclI site digests containing BclI wild-type Rb and non-phosphorylatable Rb (RbNP; in which 14 of 15 cyclin-dependent kinase sites were mutated to alanine) were kindly provided by Dr. Sybille Mittnacht (33). Expression vectors encoding Myc epitope-tagged p107 and p130 proteins were constructed by fusing p107 and p130 coding sequences lacking the initiating methionine codon, NruI restriction endonuclease digestion, downstream of the Myc epitope coding sequence in pcDNA3(9E10); mutant N417 (amino acids 417–1139) contains the A and B pockets, the spacer region, and the entire C-terminal domain; mutant C1065 (amino acids 2–1065) is C-terminally truncated within the spacer; mutant C1065 (amino acids 2–1065) is C-terminally truncated immediately after the B pocket; and mutant dH228−S18 contains a deletion of most of the spacer. PCR-generated sequences were checked by DNA sequencing.

**Cell Transfection and Reporter Assays—**MEFs and 3T3 derivatives were stably transfected using the manufacturer’s instruction in 60-mm Petri dishes (106 cells) using 20 μl of LipofectAMINE (Invitrogen) and 4 μl of DNA (3 μg of luciferase reporter + 1 μg of β-galactosidase plasmid). After 5 h, the LipofectAMINE was removed, and cells were allowed to recover in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum overnight before recovering the medium with Dulbecco’s modified Eagle’s medium containing 0.5% fetal calf serum. Cell lysates were made 60 h later, and luciferase and β-galactosidase assays were performed as described (15). Where cell extracts were prepared from transfected 3T3 derivatives, cells were transfected in 100-mm dishes using 60 μl of LipofectAMINE and a total of 12 μg of DNA.

**Northern and Western Blots—**RNA was prepared from p107+/+ and p107−/−/p130−/− 3T3 cells using RNAzol B (Biogenex) as described by the manufacturer and resolved on 1 agarose gels containing 2% formaldehyde. Northern blots were probed with a 32P-labeled murine B-myb cDNA probe. Western blotting was performed under standard conditions using anti-c-Myc antibody 9E10 (Santa Cruz Biotechnology sc-40) to detect the tagged pocket proteins.

**Electrophoretic Mobility Shift Assays—**Nuclear and cytoplasmic protein fractions were prepared from MEFs and transfected 3T3 cell lines containing p107 and p130 as described previously (24). Nuclear and cytoplasmic extracts containing 0.4% Nonidet P-40 essentially as described (34). Equivalent amounts of nuclear and cytoplasmic fractions (~10 μg) were used in electrophoretic mobility shift assays (EMSAs) using an oligonucleotide probe encompassing the mouse B-myb promoter E2F/DRS sites as described previously (15). Where appropriate, antibodies to p107 (monoclonal antibody SD15; a gift of Dr. Nick Dyson), E2F-4 (polyclonal; a gift of Dr. Eric W.-F. Lam), p130 (polyclonal; a gift of Dr. Nick Dyson and Dr. Nick Dyson), were prepared from NIH 3T3 cells stably transfected with the wild-type or mDRS B-myb promoter as described previously (15). These were used in EMSAs as described above; however, the antibodies used in supershifts were the same as those used in ChIP assays (rabbit anti-p107 polyclonal antibody, Santa Cruz Biotechnology sc-318; rabbit anti-p130 polyclonal antibody, Santa Cruz Biotechnology sc-317; and anti-Rb monoclonal antibody 21C9). In Vivo Footprinting and Chromatin Immunoprecipitation—Footprints were obtained by linker-mediated PCR on DNA alkylated in vivo by addition of 0.2% dimethyl sulfate to cell cultures as described previously (22). The B-myb primers used were as published (14).

ChIP assays were performed using essentially the published method (35). Sonicated chromatin from 107 cells treated in vivo with 1% formaldehyde for 10 min at ambient temperature was immunoprecipitated with 2 μg of each antibody (control (normal rabbit serum), rabbit anti-p107 polyclonal, and rabbit anti-p130 polyclonal antibodies and anti-Rb monoclonal antibody 21C9) and collected by mixing for 1 h at 4°C with 15 μl of protein A beads in TE buffer (20 mM Tris-HCl (pH 8.1) and 1 mM EDTA) preadsorbed with sonicated salmon sperm DNA (10 μg/ml). Rabbit anti-mouse IgG (2 μg; Sigma M7023) was added to monocolonal immunoprecipitations for 1 h before addition of protein A beads. The beads were collected by brief microcentrifugation, and the supernatant of the control antibody sample was retained for use as an input control. The beads were washed.
Following transfection, the cells were arrested in G0 by serum-myb-ified reporter plasmid, the so-called B-DRS mutations could then be quickly transferred into the modulation (32). Double-stranded oligonucleotides containing the regions mutated do not influence cell cycle-regulated trans-crease, and cell extracts were made from these cells and measured in cells arrested in G0, and in parallel cultures that were restimulated by re-addition of serum (S phase). Flow cytometry indicated that 62% of the cells were measurably in S phase at the time the serum-induced samples were collected. cation of an E2F Corepressor Site in the B-myb Promoter

RESULTS

Identification of Functionally Critical DRS Nucleotides—To characterize the precise sequence requirement for transcriptional repression of the B-myb promoter, each nucleotide within the previously delimited DRS motif (GGAAA, nucleotides −198 to −194 relative to the start of the coding sequence) was mutated individually to each of the three alternatives. To facilitate this, AatII and BclI restriction sites were first introduced into the B-myb promoter at sequences immediately flanking the E2F/DRS elements by substituting 2 and 3 nucleotides, respectively (Fig. 1A). Previous studies have shown that the regions mutated do not influence cell cycle-regulated transcription (32). Double-stranded oligonucleotides containing DRS mutations could then be quickly transferred into the modified reporter plasmid, the so-called B-myb promoter cassette, by direct ligation (see “Experimental Procedures”). The functions of the wild-type and mutant DRS elements were then assayed in transient transfection assays of NIH 3T3 cells. Following transfection, the cells were arrested in G0, by serum deprivation, and cell extracts were made from these cells and from duplicates that were induced to enter S phase by serum stimulation for 18 h. The results of a typical experiment (Fig. 1B) show that the central 3 nucleotides in the DRS (GGAAA, nucleotides −197 to −195) are particularly critical for E2F-mediated transcriptional repression. Thus, mutation of either A residue to any other nucleotide resulted in a significant increase in promoter activity in both G0 and S phase cells. Mutation of the central G residue to A had no effect upon promoter activity, whereas either C or T at this position resulted in a very significant increase in promoter activity. It is pertinent that the equivalent DRS sequence in the human B-myb promoter is GAAAA, consistent with the evidence that either G or A at nucleotide −197 is permissive for repression function. In contrast, mutation of the first G residue of the DRS (nucleotide −198) had only a marginal effect on transcriptional derepression; mutation to A led to a slight loss of repression, whereas mutation to C actually resulted in a slight increase in repression. Mutation of the ultimate A residue (nucleotide −194) to C or T had little or no effect upon DRS function, whereas substitution by G led to a substantial loss of repression function (Fig. 1B). Comparison of a DRS consensus sequence derived from this analysis with CHR sequences located downstream of the cyclin A2, cdc2, and CDC25C CDE sites (Fig. 1C) revealed that all three CDRs are actually compatible with the DRS sequence requirements.

It is notable that promoters in which the critical GAA core of the DRS is mutated were substantially induced in S phase (Fig. 1B), suggesting that the E2F site can mediate transcriptional activation in S phase when DRS function is compromised. To test this possibility, the activity of a promoter in which both sites have been mutated was compared with those of reporters containing single E2F or DRS mutations, and this assay confirmed that activation of the mDRS promoter was indeed abolished when the adjacent E2F site was also mutated (Fig. 2A). As the mDRS promoter was significantly more active in S phase than the wild-type promoter, it appears that transactivating E2F complexes are able to bind to the B-myb E2F site only in the absence of the DRS. It may therefore be suggested that the DRS specifically prevents activating E2F species from binding to the adjacent E2F site while conversely facilitating binding of repressive E2F complexes.

To determine whether the CDC25C CHR could indeed substitute for a functional B-myb DBS and, conversely, whether the B-myb DRS could substitute for the CDC25C CHR, the E2F/DRS sites in the B-myb promoter cassette were replaced with the CDC25C CHR, the E2F/DRS sites in the B-myb promoter cassette were replaced...
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Fig. 2. Characterization of DRS function. A, the B-myb promoter/luciferase reporter cassette containing the wild-type (wt) sequence or variants in which the wild-type sequence was replaced by E2F/DRS elements containing mutations in the E2F site (mE2F, CTtGtatG-GAGATAGGAAAAG), the DRS (mDRS, CTTGGCGGAGATAGGAAAAG), or both (mE2F/mDRS, CTTGtatGAGATAGGAAAAG) were transiently transfected into NIH 3T3 cells, and luciferase activities were measured in cells arrested in G0, and in parallel cultures that were restimulated by re-addition of serum for 16 h (S phase). B, the B-myb promoter/luciferase reporter cassette containing the wild-type sequence or variants in which the wild-type sequence was replaced by the CDC25C CDE/CHR sequence (CDE/CHR, GCTGCGCGAGATAGGAAAAG) or combinations of these elements (E2F/CHR, CTTGGCGGAGATAGGAAAAG; CDE/CHR, GCTGCGCGAGATAGGAAAAG) were transiently transfected into NIH 3T3 cells, and luciferase activities were compared as described for A. C, the B-myb promoter/luciferase reporter cassette containing the wild-type sequence or variants in which the wild-type sequence was replaced by E2F/DRS elements containing intervening iterations of either +2 or +4 nucleotides (CTtGCGCGGAGATAGGAAAAG and CTTGGCGGAGATAGGAAAAG, respectively) were transiently transfected into NIH 3T3 cells, and luciferase activities were compared as described for A.

with wild-type CDC25C CDE/CHR or hybrid E2F/CHR and CDE/DRS sites (Fig. 2B). Transfection into NIH 3T3 cells showed that the CDC25C CDE/CHR conferred appropriate cell cycle regulation on the B-myb promoter in that the promoter was repressed in G0 cells and derepressed in S phase (Fig. 2B). Significantly, the hybrid E2F/CHR element conferred more rigorous regulation on the B-myb promoter than either E2F/DRS or CDE/CHR, whereas in contrast, substitution by the hybrid CDE/DRS element led to complete deregelation (Fig. 2B). These findings indicate that the DRS and CHR are not absolutely equivalent. Whereas both elements are functional in the context of the E2F site, the CHR also has a specific function in the context of the CDE, which cannot be substituted by the DRS.

To determine whether the relative positions of the B-myb E2F/DRS sites are important for their functional interaction, small iterations of 2 and 4 nucleotides were introduced between these sites in the reporter cassette, and transient transfections assays were performed as before. Displacing the DRS from the E2F site by 2 nucleotides resulted in significant transcriptional derepression in G0 (Fig. 2C), and this effect was even more pronounced when the sites were displaced by 4 nucleotides. These results indicate that the spatial relationship between the E2F and DRS sites is essential for their cooperation.

The DRS Is Required for p107-mediated Repression of B-myb Transcription—Repression of B-myb transcription is substantially deregulated in quiescent MEFs derived from embryos lacking both the p107 and p130 genes, although it is unaffected in MEFs lacking either of these pocket proteins alone or in MEFs lacking Rb (11). These data suggest that p107-E2F2 and p130-E2F complexes have a redundant function in repressing the B-myb promoter during G0, which cannot be compensated by Rb-E2F2. Consistent with this notion, reintroduction of p107 and, to a lesser extent, p130 into p107−/−/p130−/− MEFs by transient transfection results in repression of a cotransfected B-myb promoter (11), and this activity depends on an intact E2F site. The p107−/−/p130−/− cells therefore provide a convenient system in which to investigate further the interactions between pocket protein-E2F2 complexes and the bipartite E2F/DRS site. To determine first the contribution of the DRS in this system, we performed additional transient transfection experiments in p107−/−/p130−/− and control p107+/+ MEFs. These studies confirmed that the wild-type B-myb promoter is significantly deregulated in quiescent p107−/−/p130−/− MEFs compared with control cells (Fig. 3A). It is notable, however, that derepression of the wild-type promoter was incomplete in the knockout cells compared with a mE2F site promoter, suggesting that quiescent p107−/−/p130−/− MEFs actually contain diminished rather than no E2F repressor activity. It is notable also that the DRS mutation had similar effects in both quiescent p107−/−/p130−/− and control MEFs, being somewhat de-
repressed compared with the wild-type promoter in control
MEFs, but not further deregulated in p107−/−/p130−/− MEFs (Fig. 3A). These findings suggest that residual E2F repressor
activity in p107−/−/p130−/− MEFS is impervious to the influence
of the DRS.

As reported previously (11), we found that reintroducing
p107 and p130 by transfection into quiescent p107−/−/p130−/−
MEFs resulted in repression of the wild-type B-myb promoter (Fig. 3B). Consistent with the previous study (11), we found
that p107 repressed the wild-type B-myb promoter more strongly than p130, although this may simply reflect the relative
expression levels of these proteins in these cells. Signifi-
cantly, the ability of p107 and p130 to repress B-myb promoter
activity was dependent upon both an intact E2F site and DRS
(Fig. 3B). Stimulation by p107 of both the mE2F site and mDRS
promoters observed in this experiment presumably reflects an
effect on constitutive activators binding to sites upstream of
the cell cycle control elements. Overall, it is clear that the DRS is
essential for the imposition of transcriptional repression mediated by p107–E2F complexes through the adjacent E2F site.

The DRS Is Required for in Vivo Interactions with p130–E2F and
p107–E2F Complexes—Although it is clear from this study
that the DRS is required for B-myb transcriptional repression
by p130 and p107, previous in vitro binding assays did not
dicate any influence of the DRS on binding of E2F complexes
containing these pocket proteins to the B-myb promoter site
(32). To explain this conundrum, we set out to determine
whether the DRS influences occupation of the adjacent E2F
site in vivo. To facilitate this analysis, the wild-type and mu-
tant B-myb promoters were cloned into a luciferase reporter
gene that incorporates scaffold/matrix attachment sites to
attenuate positional effects on the chromosomally integrated
promoter (31). Stably transfected NIH 3T3 cells expressing
these transgenes were then established using low DNA inputs
to reduce plasmid copy number, and clones containing approx-
imately five copies were selected for analysis. It is evident that
the wild-type and mutant promoters were regulated in these
clones in a manner similar to that in transient transfections
(Fig. 4A). Thus, the wild-type B-myb promoter was activated
8.5-fold as serum-arrested cells entered S phase, whereas in
contrast, the mE2F site promoter was completely derepressed
in G0. Significantly, the mDRS promoter was substantially
deregulated in G0, but was induced −1.5-fold in S phase (Fig.
4A). EMSAs using extracts prepared from wild-type and mDRS
promoter-transfected NIH 3T3 cell lines showed a predomi-
nance of p130–E2F complexes during G0 (Fig. 4B). Lesser
amounts of p107 complexes were also present, but no Rb
complexes were detectable (Fig. 4B).

Previous studies of these cell lines using ChIP assays have
shown that the wild-type promoter transgene is bound by
E2F–4 complexes in quiescent cells and that this association
is abrogated by mutation of the E2F site (37). We adopted a
similar approach to study whether the DRS influences occupa-
tion of the adjacent E2F site. Protein–DNA complexes were
formaldehyde-cross-linked in cells stably expressing the wild-
type and mDRS promoter/luciferase reporters, and sites bound
by pocket protein–E2F complexes were immunoprecipitated
with control, anti-p107, anti-p130, or anti-Rb antibodies. PCR
primer pairs were designed to detect selectively either the
B-myb transgene (the 3′-primer is homologous to luciferase
sequences) or the endogenous B-myb gene. We found that the
transfected wild-type B-myb promoter was bound predomi-

FIG. 4. The DRS is required for occupation of the B-myb E2F site with p120 and p107 complexes. A, stable NIH 3T3 transfectants were
established with luciferase reporter genes driven by either the wild-type (wt) B-myb promoter or mutant B-myb promoters with nonfunctional E2F
(mE2F) or DRS (mDRS) sites. The reporter genes were in each case flanked by scaffold/matrix attachment regions from the human interferon-β
gene to attenuate positional effects, and clones were selected with approximately five transgene copies. Luciferase activities were assayed in cells
arrested in G0 by serum deprivation for 60 h and after restimulation with serum for 16 h (S phase). B, EMSA was performed with whole cell extracts
prepared from NIH 3T3 cell clones stably transfected with the wild-type and mDRS B-myb promoter/luciferase reporters. Pocket protein–E2F
complexes were supershifted with antibodies (Ab) against p107, p130, or Rb as indicated. Control reactions (Con) contained preimmune rabbit
serum. C, NIH 3T3 cell clones stably transfected with the wild-type and mDRS B-myb promoter/luciferase reporters were arrested in G0 by serum
depression, and then protein–DNA complexes were formaldehyde-cross-linked in vivo. Chromatin fragments from these cells were subjected to
immunoprecipitation (IP) with control serum or with antibodies to p107, p130, or Rb as indicated. After cross-link reversal, the co-immunopre-
icipitated DNA was amplified by PCR and detected by Southern blotting. Separate primers were used to detect the transgene and the endogenous
gene. D, shown is the quantitation by phosphorimaging of the results shown in C for the wild-type promoter-transfected cell transgene (wt/Tr) and
endogenous gene (wt/En) and the mDRS transgene (mDRS/Tr) and endogenous gene (mDRS/En).
assays described above suggest that Rb complexes in p107/H11002 presses the B-myb promoter in vitro—found exclusively in the nuclear fraction (Fig. 5, p130 species. Control EMSAs using extracts from quiescent p107 likely that Rb is complexed predominantly with other E2F species. Control EMSAs using extracts from quiescent p107/E2F complexes, which repressed B-myb transcription in control p107/cells (Fig. 5B), it remains possible that their levels are insufficient. To address this question, we wished to compare the activities of Rb and p107 when these proteins were reintroduced into p107/p130 cells by transfection. As primary MEFs are poorly transfectable, we first established immortalized p107/p130 3T3 lines to facilitate the analysis. Northern blotting demonstrated that B-myb was abnormally expressed in the p107/p130 3T3 cells during G0 compared with control p107 3T3 cells (Fig. 6A). Some further increases in B-myb mRNA levels were seen when p107/p130 3T3 cells were induced to enter S phase, similar to findings with primary p107/p130 fibroblasts (11), presumably reflecting the increased metabolism of these cells when serum-stimulated. Nonetheless, it is clear that even after establishment of an immortalized line, the dependence upon p107 and p130 for repression of the B-myb promoter in G0 is maintained (Fig. 6A).

Significantly, transfection of p107/p130 3T3 cells with a p107 expression plasmid resulted in repression of the B-myb promoter (Fig. 6B), whereas in contrast, wild-type Rb and a mutant Rb (RbNP, in which the major cyclin-dependent kinase phosphorylation sites have been eliminated) demonstrated little activity in this assay. Notably, the ability of p107 to repress the B-myb promoter in these cells was largely dependent upon an intact DRS (Fig. 6B).

Cell extracts made from parallel transfections were assayed by EMSAs. Antibody supershifts showed that similar levels of E2F complexes were formed with transfected p107 and Rb (Rb and RbNP were Myc epitope-tagged to distinguish these complexes from endogenous Rb complexes) (Fig. 6C). These experiments therefore indicate that p107 is intrinsically more active than Rb in repressing the B-myb promoter. Moreover, the fact that RbNP is also a weak repressor of B-myb transcription indicates that this differential activity cannot be explained by differences in the sensitivity of p107 and Rb to inactivation by cyclin-dependent kinases.

Repression of B-myb Transcription by p130 Requires Specific Protein Domains—To enable a more systematic comparison between the transcriptional repressive properties of the individual Rb family members on the B-myb promoter, further experiments were performed using Myc epitope-tagged derivatives of all three pocket proteins. By probing Western blots using the common epitope tag, it was evident that Rb was the most highly expressed of these proteins in transfected p107/p130 3T3 cells (Fig. 7B), whereas p107 was relatively poorly expressed (a faint p107 band was detectable just below the background cellular band in Fig. 7B). Additional
experiments (data not shown) showed that the levels of expression that could be obtained with Myc epitope-tagged p107 were severalfold lower than those obtained with untagged p107 used in previous experiments (Figs. 3 and 6). Despite this, tagged p107 was more active than Rb in repressing the B-myb promoter in quiescent p107^{-/-}/p130^{-/-} 3T3 cells, and notably, p130 had the greatest activity in this assay (Fig. 7A). These findings therefore emphasize that E2F complexes containing Rb are weakly active in transcriptional repression of B-myb in comparison with p107 and p130 complexes.

To define the domains of p130 required for transcriptional repression of B-myb, a number of deletion mutants were employed. These experiments showed that deletion of virtually the entire spacer region (as in mutant d622–818) had no effect upon the ability of p130 to repress B-myb transcription, whereas in contrast, removal of the B pocket and C-terminal sequences (as in mutant C793) completely abolished activity. The lack of mutant C793 activity can clearly be attributed to its inability to bind E2F. The activity of a less extensively deleted C-terminal mutant (mutant C1065) was impaired to a lesser extent (Fig. 7C), and this defect may be attributed to loss of a potential C-terminal nuclear localization signal or its inability to bind histone deacetylase-1 (36). Most notably, deletion of sequences N-terminal to the pocket domain (as in mutant N417) significantly reduced transcriptional repression (Fig. 7C), although the truncated protein was expressed at much higher levels than wild-type p130 (Fig. 7D). The N-terminal domain is not required for interaction with E2F, and the reduced activity of mutant N417 indicates that this region contributes a novel functional requirement for transcriptional repression. It may be significant that p130 and p107 display significant homology in this N-terminal sequence, but little homology to Rb.

The B-myb Promoter E2F Site Is Unbound in p107^{-/-} p130^{-/-} Cells—A number of explanations could account for the inability of Rb-E2F complexes to repress B-myb transcription in p107^{-/-}/p130^{-/-} MEFs: (i) the conformation of the promoter in vivo may preclude strong interactions with Rb-E2F complexes while permitting binding of p107-E2F and p130-E2F complexes; (ii) binding of Rb-E2F complexes may be competed by higher levels of free E2F/DP heterodimers resulting from deregulation of E2F gene transcription (11); and (iii) Rb-E2F complexes may bind the site, but be unable to repress transcription. To help distinguish between these possibilities, we used the in vivo footprinting technique to study whether complexes are bound to the E2F site in quiescent p107^{-/-}/p130^{-/-} MEFs as would be predicted from Explanations ii and iii. Previous studies in NIH 3T3 cells demonstrated that the B-myb promoter E2F site is occupied in G0, but becomes unoccupied in G1 concurrently with derepression of transcription (14). Using Swiss 3T3 cells initially, we confirmed that the E2F site was partially protected when intact cells were treated with dimethyl sulfoxide (Fig. 8A), but this degree of protection was lost when cells were stimulated by serum to reenter the cell cycle (16- and 24-h time points). Incomplete protection is a characteristic of this procedure and does not necessarily imply that the site is incompletely occupied. No protection of the DRS was observed, consistent with previous results (14), suggesting that interactions at this site are not made in the major groove. We next compared occupation of the E2F site in serum-starved MEFs derived from p107^{-/-}/p130^{-/-} and control (wild-type) embryos; as a control, in vivo footprints were also obtained with quiescent Rb^{-/-} MEFs. It was apparent that the E2F site was partially protected in both wild-type and Rb^{-/-} MEFs (Fig. 8C); however, this site was completely unprotected in the p107^{-/-}/p130^{-/-} MEFs. These findings are therefore inconsistent with occupation of the E2F site by free E2F species or inactive Rb-E2F complexes and strongly imply that only p107-E2F and p130-E2F complexes are able to make stable interactions in vivo.

**DISCUSSION**

We have demonstrated here that the sequence requirements for B-myb promoter DRS corepressor function overlap with that of the CHR site, which is part of a bipartite element required for cell cycle regulation of the CDC25C, cyclin A, and cdc2 promoters (38). Notably, the CDE/CHR element was capable of regulating cell cycle transcription in place of the E2F/DRS sites in the B-myb promoter; however, the DRS was un-
able to substitute for the CHR in this context (Fig. 2B). This suggests that, although the E2F/DRS and CDE/CHR elements are similar in many respects, there is some homology between the respective elements, and their relative spacing is identical, there are certain functional differences between them. In the case of the cyclin A and cdc2 CDE/CHR elements, where the CDE can clearly double as an E2F site, these bipartite elements may act analogously to the B-myb E2F/DRS elements in binding p107-E2F and p130-E2F complexes in G0 and early G1. Potentially, the cyclin A and cdc2 elements may then have a dual role, acting analogously to the CDC25C CDE/CHR elements to prolong active transcription into S.

E2F complexed to each of the three pocket proteins can bind the B-myb E2F site in vitro binding assays, and this is not influenced by the presence of the DRS (32). These assays clearly do not reflect the specificity of the B-myb promoter for binding p107-E2F and p130-E2F complexes as evidenced in this study by in vivo footprinting (Fig. 8) and repression assays (Figs. 6 and 7). Notably, previous ChIP assays have also led to the conclusion that the B-myb promoter is bound specifically by p107-E2F and p130-E2F complexes in vivo and that whereas Rb binds to other E2F-regulated promoters, these complexes are absent from B-myb (39). Indeed, this study suggested that E2F-4 complexes exclusively regulate B-myb in cells synchronized by serum starvation. In further distinction to other E2F-regulated promoters, the B-myb promoter is not occupied by E2F complexes during S phase (39, 40). Altogether, published evidence and our current findings suggest that B-myb displays a distinctive mode of regulation that utilizes a closely linked DRS corepressor site to selectively bind transcriptionally repressive p130-E2F and p107-E2F complexes. A role for the DRS in excluding binding of transcriptionally active "free" E2F complexes is also suggested by our finding that mutation of the DRS led to activation of the promoter through the adjacent site in S phase (Fig. 2A).

Clearly, a full understanding of the way in which DRSs and CHR elements co-regulate transcription during the cell cycle requires identification of the factors that bind these sites. We
In this respect, it is of interest that the DRS sequence (GGAAA) in vitro treated cells were compared with those of Swiss 3T3 DNA methylated linker-mediated PCR on DNA extracted from the dimethyl sulfate-serum. Cells taken at 0, 16, and 24 h after restimulation as indicated 60 h and then restimulated to reenter the cell cycle by addition of can bind the B-myb promoter E2F/DRS sites in a 5' to 3' direction, top to bottom. B, shown are the results from flow cytometry of propidium iodide-stained Swiss 3T3 cells used in A at 0, 16, and 24 h after serum stimulation. C, MEFs obtained from wild-type (WT), Rb−/−, and p107−/−/p130−/− embryos were arrested in G0 by serum deprivation for 72 h and then methylated in vivo with dimethyl sulfate. Footprints obtained by linker-mediated PCR on DNA extracted from the dimethyl sulfate-treated cells were compared with those of wild-type MEF DNA methylated in vitro (IV). The sequence of the (+)-strand shown spans the B-myb promoter E2F/DRS sites in a 5' to 3' direction, top to bottom. B, shown are the results from flow cytometry of propidium iodide-stained Swiss 3T3 cells used in A at 0, 16, and 24 h after serum stimulation. C, MEFs obtained from wild-type (WT), Rb−/−, and p107−/−/p130−/− embryos were arrested in G0 by serum deprivation for 72 h and then methylated in vivo with dimethyl sulfate. Footprints obtained by linker-mediated PCR on DNA extracted from the dimethyl sulfate-treated cells were compared with those of wild-type MEF DNA methylated in vitro (IV). The sequence of the (+)-strand shown spans the B-myb promoter E2F/DRS sites and a putative SP-1 site (which was occupied in vivo in all the cells tested) located 5' to the regulatory elements.

have been unable to detect proteins binding to the DRS in in vitro binding assays, although in the presence of spermine, a novel lower mobility p130-E2F complex was detected in G0 extracts from various rodent fibroblasts that was absent on probes in which the DRS was mutated.2 A protein (CHF) that can bind the B-myb DRS has been purified (41); however, evidence that this binding correlates with the sequence requirements for transcriptional corepression is lacking. Evidence from in vivo footprinting that protein contacts are made with the CDC25C CHR in the minor groove suggests that minor groove contacts are also made with the B-myb DRS. The CHR and DRS may therefore act in some respects analogously to high mobility group I(Y) binding sites for assembly of an NF-κB complex on the human interferon-β gene promoter (42). In this respect, it is of interest that the DRS sequence (GGAAA) is present within the PRDII site, which binds high mobility group I(Y) (42); however, the sequence specificity for high mobility group I(Y) binding within the interferon-β site is inconsistent with sequence requirements within the B-myb DRS.

Rb-E2F complexes were abundant in p107−/−/p130−/− cells (Fig. 5), yet were unable to repress efficiently the B-myb promoter. Moreover, transected Rb was unable to substitute efficiently for the loss of p107 and p130 function in these cells (Figs. 6 and 7). The latter deficiency cannot be ascribed to hyperphosphorylation of Rb in the p107−/−/p130−/− cells, as RbNP was similarly inactive in repressing B-myb transcription (Fig. 6). It is also apparent that Rb-E2F complexes could be formed in the transfected p107−/−/p130−/− cells (Fig. 6). It can be concluded that Rb-E2F complexes have relatively low affinity for the B-myb promoter E2F site in vivo in comparison with p107-E2F and p130-E2F complexes. This conclusion is consistent with a previous ChIP analysis of E2F target gene specificity in NIH 3T3 cells (39), in which Rb could be detected on the promoters of a number of E2F-regulated genes (dihydrofolate reductase, thymidine kinase, cdc2, and cyclin E), albeit at different stages of the cell cycle, whereas in contrast, only p130 and p107 occupied the B-myb promoter. It is also notable that B-myb was found to have a singular mode of regulation by E2F in that it was the only promoter not to bind E2F complexes in S phase (39). As DRS mutations enabled B-myb transcription to be activated through the adjacent E2F site in S phase (Fig. 2A), it may be suggested that the DRS promotes binding of transcriptionally repressive p107-E2F and p130-E2F complexes while preventing binding of transactivating E2F species.

Although corepressor CHR sites have been described in the promoters of several genes that are maximally activated during S/G2, such as CDC25C, cyclin A, polo-like kinase, cyclin B2, and survivin (38, 43–45), sites directly analogous to the B-myb DRS have not been described in other G1/S-regulated genes. This again points to the singular nature of B-myb regulation and illustrates how the precise positioning and context of an E2F site can influence cell cycle transcriptional control. It is notable that B-myb transcription was found to be the most strongly deregulated of ~20 genes tested in p107−/−/p130−/− MEFs (11), possibly reflecting the absolute dependence of p107-E2F and p130-E2F complexes, under the influence of the DRS corepressor, for transcriptional repression of B-myb.

In view of the significant role played by transcriptional repression in regulating B-myb mRNA levels, it is surprising that induction of B-myb expression is severely impaired in MEFs derived from E2F-3−/− mouse embryos (46), implying that E2F-3 function is required to transcriptionally induce B-myb at the G1/S transition. This conclusion is not consistent with our finding that mutation of the B-myb promoter E2F site resulted in constitutive highly active transcription in stably transfected cells that contained a low copy number of the transgene (Fig. 4A), indicating that in this context, at least the E2F site is not required for activation by E2F-3. Moreover, ChIP data indicate that this promoter is not bound by E2F-3 or other E2F species under these conditions or, alternatively, a role for E2F-3 transactivation through distal promoter sites that are not retained in the B-myb promoter/reporters used. Analysis of B-myb promoter occupancy in E2F-3−/− MEFs using the ChIP assay should help to resolve this issue.

The B-myb promoter has been used in many studies as a model for E2F regulation. Our current findings indicate that occupancy of this E2F site in vivo by transcriptionally repres-
sive complexes is governed by the adjacent DR5 corepressor site. To date, we have been unable to identify proteins binding to the DR5, and we can only speculate on the mechanism whereby it acts to promote binding of complexes to the E2F site. Recent studies of the E2F-1 promoter have identified a nucleosome proximal to the E2F binding site (37), which may provide a target for histone acetylases and deacetylases to modulate transcription initiation. Possibly, the DRS is involved in nucleosome positioning, thereby influencing the ability of repressive E2F complexes to interact with the adjacent E2F site. The recognition that this corepressor site plays a prominent role in occupancy of the B-myb promoter by transcriptionally repressive p107-E2F and p130-E2F complexes will add significantly to future studies using this system as a model for E2F regulation.

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