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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, RM22, 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 embryo 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 weakly if at all to CDE/CHR. Compared with E2F/DRS, the CDE/CHR elements have an identical spatial relationship (30); however, E2F binds to these elements and, moreover, E2F/CHR sites are partially homologous to the B-myb E2F/DRS sites and, moreover, E2F binds weakly if at all to CDE/CHR. Compared with E2F/DRS, the CDE/CHR elements confer a subtly different cell cycle kinetics weakly if at all to CDE/CHR. Compared with E2F/DRS, the CDE/CHR elements have an identical spatial relationship (30); however, E2F binds to E2F/CHR sites and, moreover, E2F/CHR sites are partially homologous to the B-myb E2F/DRS sites and, moreover, E2F binds weakly if at all to CDE/CHR. Compared with E2F/DRS, the CDE/CHR elements confer a subtly different cell cycle kinetics weakly if at all to CDE/CHR. Compared with E2F/DRS, the CDE/CHR elements have an identical spatial relationship (30); however, E2F binds to E2F/CHR sites and, moreover, E2F/CHR sites are partially homologous to the B-myb E2F/DRS sites and, moreover, E2F binds weakly if at all to CDE/CHR.

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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 O. Ebstein, Charles Town, 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% 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 pGL-2 (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 sequences with variant transcriptional control sites. The sequences of these oligonucleotides (sense strand only) were as follows: mE2F, CCT-TGTATGAGAGATAGGAAGTTT; mDRS, CCTTGGCCGGAGATATAGGAAGTTT; and pGL-2−<5′-AatII>−3′<BclI> as described previously (15). These 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), Rb (monoclonal antibody 21C9; a gift of Dr. Sybille Mittnacht), or c-Myc (monoclonal 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 and used as described previously (31). Western blots were probed with a 125I-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.

Northern and Western Blots—Northern blots were probed with a 125I-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.

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 antibody, rabbit anti-p130 polyclonal antibody, rabbit anti-Rb monoclonal antibody 21C9) and collected by mixing for 1 h at 4°C with 5 μg 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 immunoprecipitations for 1 h. The immunoprecipitations were washed 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.
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successively for 10 min at 4°C with TSE buffer (1% Triton X-100, 0.1% SDS, 2 mM EDTA, and 20 mM Tris-HCl (pH 8.1)). TSE buffer containing 150 mM NaCl, TSE buffer containing 500 mM NaCl, buffer containing 250 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1), and TE buffer. Immunoprecipitated DNA was eluted by washing the beads three times with 150 μl of 1% SDS and 100 mM NaHCO3, and then 10 μg of salmon sperm DNA was added to the pooled eluates. The cross-links were reversed by heating at 65°C for 4 h, and then the DNA was phenol-extracted twice and ethanol-precipitated. DNA was recovered by centrifugation and dissolved in 350 μl of buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 8.1), and 1 mM EDTA. After reprecipitation with ethanol, the final pellet was dissolved in 30 μl of TE buffer. PCR amplification was carried out for 25 or 26 cycles on 2-μl amounts using Taq polymerase. Separate reactions were carried out to detect the transgene and the endogenous B-myb gene; transgene reactions used a 5′-B-myb primer (CTCTCCGTATGTCCGC-CCC) and a 3′-primer to the luciferase gene (TCTAGCCTATGCGAGTTGCTTCTCC), whereas endogenous reactions used 5′- and 3′-B-myb primers (ATTGAATCCCTAAGGTAGGTGTATCTG and TGGTCGCACGTTCCCAAG, respectively). PCR products were resolved on 1.5% agarose gels, Southern-blotted, and detected by hybridization with a 5′-32P-labeled oligonucleotide probe (TTCTGTGCGCTCCCTAGGCAGTGTTCCCAG, respectively). Hybridization was quantitated by phosphorimaging.

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, AarII 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 16 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 transcriptional repression (Fig. 1B). It may therefore be suggested that the DRS is mutated were substantially induced in S phase (Fig. 1C).

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 DRS 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

FIG. 1. Characterization of nucleotides required for DRS function. A, nucleotides flanking the E2F/DRS sites in the B-myb promoter/reporter pGL2(−536) were mutated as indicated in lowercase to generate AarII and BclI restriction sites. B, double-stranded oligonucleotides containing the DRS mutations indicated in lowercase were introduced into the pGL2(−536) cassette by replacement of the AarII/BclI fragment. The wild-type and mutant promoter/reporters 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 (S phase). Flow cytometry indicated that 62% of the cells were measurably in S phase at the time the serum-induced samples were collected. C, shown is a comparison of the mouse B-myb E2F/DRS sequences (underlined) with the bipartite CDE/CHR sequences (also underlined) in the human cyclin A (CCNA), CDC2, and CDC25C genes. With respect to the major transcription start sites, the sequences presented are B-myb (−49/−31), CCNA (−35/−20), CDC2 (−25/−7), and CDC25C (−19/−1). Nucleotide changes permissible for DRS function are indicated above the DRS sequence.
cycle regulation on the B-myb promoter in that the promoter myb was repressed in G0 cells and derepressed in S phase (Fig. 2). CDC25C showed that the promoter was not absolutely equivalent. Whereas both elements are functional 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 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 derepression (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.

Transcriptional repression of the B-myb promoter by p107/E2F complexes requires the DRS. A, the wild-type (wt) B-myb promoter/reporter pGL2(-536) or derivatives containing mutations in either the E2F (mE2F) or DRS (mDRS) site were transfected into primary MEFs derived from control p107+/− or knockout p107−/− p130−/− animals. Luciferase was assayed in transfected cells deprived of serum for 60 h. B, knockout p107−/− p130−/− MEFs were transfected with the B-myb promoter/reporters described for A together with either the empty pCMV expression vector (control (Con)) or vector encoding p107 or Myc epitope-tagged p130. Luciferase was assayed in transfected cells deprived of serum for 60 h. Data are presented as -fold repression, calculated by dividing the average luciferase value obtained with the control empty vector by the average value obtained for that reporter with the p107 or p130 expression vector. The control for each reporter was assigned a value of unity.
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. Significantly, 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 p107 and p130 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 indicate 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 mutant 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 approximately 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 derepressed 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 predominance 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 occupation 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-
nantly by p130 complexes and, to a lesser extent, by p107 complexes, but little Rb was cross-linked to this site (Fig. 4, C and D). The endogenous promoter in these cells showed a very similar pattern of occupancy. The relative levels of p130 and p107 binding to the B-myb promoter detected in the ChIP assays therefore reflected their levels associated with E2F complexes as detected by EMSAs (Fig. 4B). Significantly, p130 and p107 complexes were only weakly associated with the mDRS promoter, although interactions of these proteins with the endogenous promoter were still detected in these cells (Fig. 4, C and D). These experiments therefore demonstrate that the DRS has an important role in promoting binding of p130-E2F and p107-E2F complexes to the adjacent E2F site.

In Vitro Analysis of E2F Complexes in p107−/−/p130−/−MEFs—In contrast to NIH 3T3 cells, quiescent p107−/−/p130−/−MEFs contain abundant Rb-E2F complexes, which are readily detectable by EMSAs using an adenovirus E2A promoter probe (11). It is therefore unclear why these species are unable to fully repress the B-myb promoter in p107−/−/p130−/−cells. To begin to address this issue, it was important to determine whether Rb-E2F complexes in quiescent p107−/−/p130−/−MEFs are located in the nucleus, as at least certain E2F complexes are found sequestered in the cytoplasm (4). Moreover, it was necessary to confirm that these Rb-E2F complexes are able to recognize the distinctive B-myb-E2F site. To these ends, nuclear and cytoplasmic extracts were prepared from quiescent p107−/−/p130−/−MEFs, and EMSAs were performed using a B-myb E2F site oligonucleotide. The addition of specific antibody to the EMSAs revealed that the nuclei of p107−/−/p130−/−MEFs contained Rb-E2F complexes, which were able to bind the B-myb E2F site in vitro (Fig. 5B); however, these higher order complexes were absent from the cytoplasmic fractions (Fig. 5A). It is notable that the cytoplasmic species in these extracts was composed exclusively of free E2F-4; however, the anti-E2F-4 antibody failed to recognize the majority of the nuclear Rb complex (Fig. 5B). Although it is possible that the E2F-4 epitope is masked by association with Rb, it is more likely that Rb is complexed predominantly with other E2F species. Control EMSAs using extracts from quiescent p107−/−/p130−/−MEFs showed that both cytoplasmic and nuclear fractions contained p130-E2F species, whereas Rb-E2F complexes were again found exclusively in the nuclear fraction (Fig. 5, C and D).

Transfected Rb Forms E2F Complexes, but Inefficiently Represses the B-myb Promoter—Although the in vitro binding assays described above suggest that Rb complexes in p107−/−/p130−/−MEFs are at least as abundant as the p130-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 transfected, 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 lines 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 proteins expressed in the p107−/−/p130−/−/p130−/−MEF cytoplasmic extracts; D, p107−/−MEF nuclear extracts.
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-
The p130 N terminus is required for transcriptional repression of the B-myb promoter. A, knockout p107\(^{+/-}\)/p130\(^{+/-}\) 3T3 cells were transfected in triplicate with the wild-type B-myb promoter/luciferase reporter pGL2(−536) together with 1- and 2-μg amounts as indicated of a control pcDNA3 vector (CON) or pcDNA3 encoding Myc epitope-tagged Rb, p107, or p130. Cells were arrested in G\(_0\) by serum deprivation for 60 h, and luciferase activities were assayed in cell extracts. The results are presented as -fold repression by these proteins on the reporter, where the average luciferase activity obtained with the control transfection was given a value of unity. B, Western blotting was carried out using antibody 9E10 as the primary antibody, and the results show the relative levels of expression of Rb, p107, and p130 in transfected p107\(^{+/-}\)/p130\(^{+/-}\) 3T3 cells at inputs of 1 and 2 μg of expression vector. Note that p107 appears as a faint band immediately below the major background band obtained in all lanes. The background band is indicated with an asterisk. C, transcriptional repression of the B-myb promoter by p130 requires the E2F binding domain and an N-terminal domain. Knockout p107\(^{+/-}\)/p130\(^{+/-}\) 3T3 cells were transfected in triplicate with the wild-type B-myb promoter/luciferase reporter pGL2(−536) together with 0.2-, 0.5-, and 1-μg amounts as indicated of a control pcDNA3 vector or pcDNA3 encoding Myc-epitope-tagged p130 and p130 mutants N417 (amino acids 417–1139), C793 (amino acids 2–60), C1065 (amino acids 2–1065), and dl622–818 (deletion of amino acids 622–818 within the spacer). Cells were arrested in G\(_0\) by serum deprivation for 60 h, and luciferase activities were assayed in cell extracts. The results are presented as -fold repression by these proteins on the reporter, where the average luciferase activity obtained with the control transfection was given a value of unity. D, Western blotting was performed using antibody 9E10 as the primary antibody, and the results show the relative levels of expression of wild-type and mutant p130 proteins in transfected p107\(^{+/-}\)/p130\(^{+/-}\) 3T3 cells at 1-μg input of expression vector. Lane 1, wild-type p130; lane 2, mutant N417; lane 3, mutant C793; lane 4, mutant C1065; lane 5, mutant dl622–818. The major background band is indicated with an asterisk. E, shown is a schematic representation of Myc epitope-tagged p130 and p130 deletion mutants. Myc epitope-tagged p130 contains amino acids 2–1139 of wild-type p130, and the amino acid numbers defining the boundaries of the A and B pockets and the intervening spacer are indicated. Sequences retained in the p130 deletion mutants are indicated below.

Able to substitute for the CHR in this context (Fig. 2B). This suggests that, although the E2F/DRSs 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 G\(_0\) and early G\(_1\). Potentially, the cyclin A and cdc2 elements may then have a dual role, acting analogously to the CDC28C CDE/CHR elements to prolong active transcription into G\(_1\).

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 vitro 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 DRS 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) promoter E2F/DRS sites in a 5′ to 3′ direction, top to bottom. A, 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. B, shown is the sequence of the (+)-strand shown spans the B-myb promoter E2F/DRS sites in a 5′ to 3′ direction, top to bottom. B and C, shown are the experiment of fluorescence-activated cell sorting (FACS) analysis of propidium iodide-stained Swiss 3T3 cells used in A at 0, 16, and 24 h after serum stimulation. C, shown is 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 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. 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−/− MEFs. A, Swiss 3T3 cells were arrested in G0 by serum deprivation for 60 h and then restimulated to reenter the cell cycle by addition of serum. Cells taken at 0, 16, and 24 h after restimulation as indicated were 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 Swiss 3T3 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.

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 is insufficient for the loss of p107 and p130 function in these cells (39), possibly reflecting the absolute dependence of 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 DRS corepressor site. To date, we have been unable to identify proteins binding to the DRS, 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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REFERENCES