pRB and p107/p130 are required for the regulated expression of different sets of E2F responsive genes

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The activity of the E2F transcription factor is controlled by physical association with the retinoblastoma protein (pRB) and two related proteins, p107 and p130. The pRB family members are thought to control different aspects of E2F activity, but it has been unclear what the respective functions of these proteins might be. To dissect the specific functions of pRB, p107, and p130 we have investigated how the expression of E2F-regulated genes is changed in cultures of primary cells lacking each of these family members. Whereas no changes were found in the expression of E2F-target genes in cells lacking either p107 or p130, deregulated expression of E2F targets was seen in cells lacking pRB and in cells lacking both p107 and p130. Surprisingly, the genes that were dysregulated in these two settings were completely different. These findings show that pRB and p107/p130 indeed provide different functions in E2F regulation and identify target genes that are dependent on pRB family proteins for their normal expression.

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The E2F transcription factor was originally identified as a factor that bound specifically to an element in the adenovirus E2 promoter (Yee et al. 1987). E2F is a heterodimeric factor, containing a subunit encoded by the E2F family of genes and a subunit encoded by the DP family of genes. To date, five E2F genes and two DP genes have been identified in mammalian cells (for review, see Slansky and Farnham 1996), and the E2F-binding activity found in cell extracts is the collective activity of many different forms of E2F.

E2F is a critical regulator of G₁ to S-phase progression and the elevation of E2F activity drives cells from G₀ or G₁ into S-phase (Johnson et al. 1993; Lukas et al. 1996; for review, see Adams and Kaelin 1996). E2F-dependent transcription is repressed by the physical association of E2F with the retinoblastoma protein (pRB) or with two pRB-related proteins, p107 and p130 (for review see Cobrinik 1996). pRB, p107, and p130 are found in independent E2F complexes that exist transiently during the cell cycle. The formation and disruption of these higher order complexes is a major mechanism of E2F regulation and is regulated by phosphorylation. During the G₁ to S-phase progression of the cell cycle, the activation of cyclin-dependent kinases leads to the phosphorylation of pRB family proteins, the release of E2F, and the activation of

E2F-dependent transcription (for review, see Farnham 1996). As the pRB signaling pathway is thought to be inactivated functionally in most human cancers (Weinberg 1995; Harlow 1996; Sherr 1996), E2F-dependent transcription is expected to be deregulated in most tumor cells.

E2F-binding sites have been found in the promoters of many cellular genes whose expression is induced during the G₁ to S-phase progression. Proposed E2F target genes include genes whose functions are involved in DNA synthesis and genes whose functions promote cell cycle progression (for review, see Johnson 1992). Mutational studies have suggested that E2F sites can confer cell cycle regulation in two different ways. Mutation of E2F-binding sites in the dihydrofolate reductase (*DHFR*) promoter resulted in the loss of activation during cell cycle progression (Means et al. 1992; Slansky et al. 1993; Fry et al. 1997), suggesting that these E2F-binding sites mediate transcriptional activation during S-phase entry. In contrast, mutation or deletion of E2F sites in the *B-myb* (Lam and Watson 1993), *cdc2* (Dalton 1992; Tommasi and Pfeifer 1995), *E2F-1* (Hsiao et al. 1994; Johnson et al. 1994), and *cyclin A* (Schultze et al. 1995; Huet et al. 1996) promoters caused derepression of the promoter during G₀ and G₁. In these promoters, the E2F-binding site appears to confer cell cycle regulation by binding a repressor complex in G₀ and G₁ and subsequent activation of gene expression results from release of this re-

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pression. The activity of an E2F site may be greatly influenced by the promoter context of the site. Recent studies of the murine *thymidine kinase* (TK) promoter showed that E2F and Sp-1 bind cooperatively to adjacent sites and that maximal promoter activity requires the correct spacing of these binding sites (Karlseder et al. 1996).

A simple view of pRB/E2F function is complicated by the fact that *Rb*^{-/-} cells contain both p107 and p130 and both of these proteins also regulate E2F. Biochemical studies have suggested potential mechanisms for differential E2F regulation by pRB, p107, and p130. First, pRB, p107, and p130 associate with different subsets of E2F complexes. E2F-1, E2F-2, and E2F-3 bind preferentially to pRB (Lees et al. 1993), E2F-5 binds preferentially to p130 (Hijmans et al. 1995), whereas E2F-4 has been found in complexes with pRB, p107, and p130 (Beijersbergen et al. 1994; Ginsberg et al. 1994; Moberg et al. 1996). The significance of these differences is unclear because it has not yet been possible to show that distinct E2F complexes have different DNA-binding specificity. Second, pRB, p107, and p130 are prevalent in E2F complexes at different times during the cell cycle. p130/E2F complexes are abundant in quiescent or differentiated cells but are less common in populations of cycling cells (Chittenden et al. 1993; Cobrinik et al. 1993; Smith et al. 1996). Conversely, p107/E2F complexes are most readily detected in cycling cells, and especially during S-phase (Devoto et al. 1992; Shirodkar et al. 1992; Cobrinik et al. 1993; Ginsberg et al. 1994). pRB/E2F complexes have been found in extracts of differentiated cells, quiescent cells, G1 phase cells, and possibly even in S-phase cells (Shirodkar et al. 1992; Schwarz et al. 1993; Corbeil et al. 1995). The functional significance of these different complexes for E2F regulation is not known.

It has been difficult to identify the specific roles of pRB, p107, and p130 in E2F regulation because the specificity of interactions between endogenous E2F and pRB family proteins is lost when these proteins are overexpressed. For example, E2F-1 is found primarily in association with pRB in vivo, but both p107 and pRB repress E2F-1-dependent transcription when they are overexpressed (Cress et al. 1993). Similarly, E2F-5 associates with p130 in vivo, but pRB, p107, and p130 all repress E2F-5-dependent transcription in transient assays (Hijmans et al. 1995).

The analysis of mouse cells specifically lacking pRB, p107, or p130 provides an alternative approach. All three pRB family members have been mutated in mice by gene targeting (Clarke et al. 1992; Jacks et al. 1992; E.Y. Lee et al. 1992; Cobrinik et al. 1996; M.H. Lee et al. 1996). *Rb*^{-/-} embryos die in utero between day 13.5 and 15.5 of embryogenesis. *Rb*^{-/-} embryos contain many ectopic S-phase cells and show high levels of apoptosis, particularly in the nervous system (Lee et al. 1994). Mice lacking either p107 or p130 have no overt abnormalities, but *p107*^{-/-}; *p130*^{-/-} animals die within hours of birth, indicating that these proteins share overlapping functions (Cobrinik et al. 1996). In *p107*^{-/-}; *p130*^{-/-} animals the pro-

liferation of chondrocytes is not restricted correctly and limb development is abnormal. Deficiency of p107 also enhances the developmental abnormalities of *Rb*^{-/-} embryos (Lee et al. 1996). As pRB, p107, and p130 all associate with various forms of E2F, functional overlap between these proteins may be attributable, at least in part, to their roles in the regulation of E2F activity.

A previous study has shown that *cyclin E* expression is abnormal in *Rb*^{-/-} cells (Herrera et al. 1996a). Here we have extended the analysis of *Rb*^{-/-} cells and have broadened this approach to compare the expression of E2F-target genes in cells deficient for either pRB, p107, or p130, and in cells that lack both p107 and p130. The results reveal that p107 and p130 play overlapping roles in the control of several E2F-regulated promoters, and this overlap closely parallels the functional redundancy between p107 and p130 that was evident in mouse knockout studies (Cobrinik et al. 1996). We observe that pRB has a role in E2F regulation that is functionally distinct from that played by p107 and p130.

Results

Mouse embryo fibroblasts lacking pRB family proteins

Mice carrying mutant alleles of *Rb*, *p107*, or *p130* were interbred and cultures of mouse embryonic fibroblasts (MEFs) deficient in pRB, p107, or p130 were generated from day 13.5 embryos. MEFs lacking both p107 and p130 were also generated and analyzed. In each case the genotype of the culture was established by PCR with sets of primers that were specific for either the wild type or mutant allele. The relative levels of p107, p130, and pRB in the different MEF genotypes were compared by Western blot analysis with specific antibodies using extracts from quiescent and asynchronously growing cultures (Fig. 1A). Individual MEF cultures lacked the appropriate pRB family members. The levels of pRB or p130 did not change appreciably when other pRB family members were missing. However, the level of p107 was increased in both *Rb*^{-/-} and *p130*^{-/-} MEFs relative to wild-type cells, especially when extracts of quiescent cells were compared. Elevated levels of p107 have been reported previously in a pRB-deficient myoblast cell line (Schneider et al. 1994) and are also seen in many tissues of *p130*^{-/-} mice (D. Cobrinik, unpubl.; G. Mulligan and T. Jacks, pers. comm.).

MEF cultures were synchronized by serum starvation and restimulation to analyze the cell cycle changes in E2F activity. Figure 1B shows the profile of [³H]-thymidine incorporation, a marker of DNA synthesis, obtained with representative MEF preparations. Each MEF preparation was effectively rendered quiescent by serum starvation and the cells reentered the cell cycle with similar kinetics after serum restimulation. In most MEF cultures the highest level of [³H]thymidine incorporation was seen at 18 hr after serum addition. Consistent with earlier reports (Almasan et al. 1995; Lukas et al. 1995; Herrera et al. 1996a), *Rb*^{-/-} MEFs entered S-phase ~2 hr earlier than wild-type MEFs. In addition, *p107*^{-/-}; *p130*^{-/-}

Figure 1. MEFs prepared from knockout mice lack the appropriate pRB family members and are effectively synchronized by serum starvation and restimulation. (A) Levels of pRB, p107, and p130 in a panel of MEFs. Western blots of whole cell extracts were probed with antibodies to against p107 (SD-9), p130 (C-20), or pRB (3C8). Seventy micrograms of extract from serum-starved MEFs or 100 μ g of extract from asynchronously growing MEFs was loaded per lane. (B) Synchronization of MEFs by serum starvation and restimulation. MEFs corresponding to the six listed genotypes were serum starved and restimulated. Entry into S-phase was measured by [³H]thymidine incorporation at the nine time points indicated. Data points are shown with standard error from triplicate samples.

MEFs also appeared to reach S-phase slightly earlier than control MEFs (Fig. 1B). This difference was smaller than that seen for *Rb*^{-/-} MEFs but was observed in multiple independent preparations of *p107*^{-/-}; *p130*^{-/-} MEFs (data not shown).

In MEFs lacking pRB, p107, or p130 the expression of most E2F target genes is unchanged

The cell cycle expression patterns of proposed E2F target genes were examined to determine the functional changes in E2F activity that occur when pRB, p107, or p130 are absent. Because E2F-binding sites confer either transcriptional activation or transcriptional repression in different promoter studies we investigated the expression of a broad panel of genes that are either proposed or shown to be regulated by E2F (Table 1). This panel included *cyclin E* (Ohtani et al. 1995; Botz et al. 1996; Geng et al. 1996), *p107* (Zhu et al. 1995), *B-myb* (Lam and Watson 1993), *cdc2* (Dalton 1992; Tommasi and Pfeifer 1995), *E2F-1* (Hsiao et al. 1994; Johnson et al. 1994), *thymidylate synthase* (TS) (Johnson 1992), *cyclin A2* (Huet

et al. 1996; C. Shulze et al. 1995), *DHFR* (Means et al. 1992; Slansky et al. 1993), *DNA polymerase α* (Pearson et al. 1991), TK (Ogris et al. 1993), *ribonucleotide reductase subunit M2* (RRM2) (DeGregori et al. 1995), *topoisomerase I*, proliferating cell nuclear antigen (PCNA) (Lee et al. 1995), insulin-like growth factor-1 (*IGF-1*) (Porcu et al. 1994), *Rb* (Hamel et al. 1992; Gill et al. 1994; Ohtani-Fujita et al. 1994; Shan et al. 1994), *cyclin D1* (Philipp et al. 1994), and *c-myc* (Hiebert et al. 1989; Thalmeier et al. 1989).

MEFs were synchronized by serum starvation and re-stimulation. Total RNA was isolated at the same nine time points indicated in Figure 1, and the expression of proposed E2F responsive genes was determined by Northern analysis. Acidic ribosomal phosphoprotein P0 (ARPP P0) was used as a loading control (Laborda 1991) because, unlike GAPDH and β-actin, levels of ARPP P0 mRNA were unchanged after serum stimulation. As the cell cycle profiles of different MEFs were very similar, the patterns of gene expression could be readily compared between genotypes. The changes in the gene expression patterns are summarized in Table 1.

Table 1. Changes in gene expression in MEFs lacking pRB family proteins

	Wild type ^a	<i>Rb</i> -/-	<i>p107</i> -/-	<i>p130</i> -/-	<i>p107</i> -/-; <i>p130</i> -/-
<i>cyclin E</i>	↑G ₁ -S	derepressed in G ₀ -G ₁	unchanged	unchanged	unchanged
<i>p107</i>	↑G ₁ -S	derepressed in G ₀ -G ₁	not expressed	unchanged	not expressed
<i>B-myb</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	strongly derepressed in G ₀ -G ₁
<i>cdc2</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	derepressed in G ₀ -G ₁
<i>E2F-1</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	derepressed in G ₀ -G ₁
<i>TS</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	derepressed in G ₀ -G ₁
<i>RRM2</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	derepressed in G ₀ -G ₁
<i>cyclin A2</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	derepressed in G ₀ -G ₁
<i>DHFR</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	early induction
<i>TK</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	unchanged ^b
<i>DNA polymerase α</i>	↑G ₁ -S	unchanged ^b	unchanged	unchanged	unchanged ^b
<i>cdc25C</i>	↑S-G ₂ /M	unchanged ^b	unchanged	unchanged	unchanged ^b
<i>cyclin D1</i>	↑G ₀ -G ₁	unchanged	unchanged	unchanged	unchanged
<i>c-myc</i>	↑G ₀ -G ₁	unchanged	unchanged	unchanged	unchanged
<i>E2F-4</i>	↑G ₀ -G ₁	unchanged	unchanged	unchanged	unchanged
<i>E2F-5</i>	↑G ₀ -G ₁	—	—	unchanged	unchanged
<i>DP-1</i>	↑G ₀ -G ₁	—	unchanged	unchanged	unchanged
<i>pRB</i>	↑G ₀ -S	—	unchanged	unchanged	unchanged
<i>topo.1</i>	↑G ₀ -G ₁	—	unchanged	unchanged	unchanged
<i>PCNA</i>	↑G ₀ -S	—	unchanged	unchanged	unchanged ^b
<i>IGF-1</i>	↓G ₀ -S	—	unchanged	unchanged	unchanged

^a(↑,↓) Gene expression is increased, or decreased, during cell cycle progression in wild-type MEFs.

^bUnchanged relative to S-phase entry.

Remarkably few differences were found in the expression of E2F target genes in any of the MEF cultures that lacked a single pRB family member. Subtle changes in the expression patterns of a *cyclin E* and *p107* were detected in *Rb*^{-/-} MEFs. The expression of these genes was elevated during G₀ and G₁ by approximately twofold in *Rb*^{-/-} MEFs compared to wild-type MEFs (Fig. 2A,B). As E2F-binding sites are critical for the cell cycle regulation of mammalian *cyclin E* promoters (Ohtani et al. 1995; Botz et al. 1996; Geng et al. 1996), and E2F-binding sites confer repression of synthetic promoters in G₀/G₁, this effect is most likely attributable to the loss of a pRB/E2F repressor complex. In *Rb*^{-/-} MEFs the maximal induction of *cyclin E* expression is similar to wild-type cells but occurs between 2 and 4 hr earlier.

Two p107 transcripts were observed that are similar in size to transcripts reported in earlier studies of p107 expression (Schneider et al. 1994; Kim et al. 1995). The upper transcript, which encodes full-length p107, was approximately twofold higher in *Rb*^{-/-} cells during G₀/G₁ than control cells. The level of the lower transcript, which is predicted to encode an amino terminal-truncated form of p107 (Kim et al. 1995), was unchanged in

Rb^{-/-} cells. Although the change in p107 mRNA seen in *Rb*^{-/-} cells is small, it may be important as the level of p107 protein was clearly elevated in *Rb*^{-/-} MEFs, and this increase was most apparent in serum-starved cells (see Fig. 1A).

The expression patterns of *B-myb*, *cdc2*, *E2F-1*, *TS*, *RRM2*, *cyclin A2*, *DHFR*, *TK*, *DNA polymerase α*, and *Cdc25C* were unchanged in *Rb*^{-/-} cells relative to S-phase entry. Because *Rb*^{-/-} MEFs enter S-phase ~2 hr earlier than wild-type MEFs, the expression of these genes was induced 2 hr earlier in the *Rb*^{-/-} MEFs than in wild-type MEFs. Thus, the expression patterns of these genes were considered normal with respect to cell cycle position.

No changes were found in the expression of any E2F target genes in either *p107*^{-/-} MEFs or in *p130*^{-/-} MEFs. Although this does not rule out the possibility that p107 or p130 may have unique roles in the regulation of some E2F targets, the individual roles of p107 and p130 in E2F regulation are largely nonessential. This observation is consistent with the fact that *p107*^{-/-} and *p130*^{-/-} mice develop normally but is surprising as p107/E2F and p130/E2F complexes are seen largely at different points of the cell cycle. Because p107 and p130 have overlapping functions in mouse development, we investigated whether their roles in E2F regulation were similarly redundant by examining the expression of E2F target genes in cells lacking both p107 and p130.

Figure 2. Altered expression of *cyclin E* and *p107* in *Rb*^{-/-} cells. (A) MEFs corresponding to the three genotypes indicated were arrested by serum starvation and allowed to reenter the cell cycle by the addition of serum. Total RNA was isolated at nine time points and analyzed on Northern blots (20 µg total RNA/lane). The filter was probed sequentially with probes for murine *cyclin E*, *p107*, *B-Myb*, or *ARPP P0* mRNAs. (B) The relative induction of *cyclin E* expression in wild-type and *Rb*^{-/-} MEFs. The cyclin E signal in A was normalized to the internal control (ARPP P0) using the National Institutes of Health (NIH) Image 1.60 program. The normalized expression of *cyclin E* in wild-type MEFs at 0 hr was set as 1.

Abnormal cell cycle expression of many E2F target genes in $p107^{-/-};p130^{-/-}$ cells

Extensive changes were found in the expression of E2F responsive genes in $p107^{-/-};p130^{-/-}$ MEFs (summarized in Table 1). In these cells the expression of six proposed E2F target genes (*B-myb*, *cdc2*, *E2F-1*, *TS*, *RRM2*, and *cyclin A2*) was elevated during G_0/G_1 . An example of the deregulated expression of *B-myb*, *cdc2*, *E2F-1*, *TS*, and *RRM2* is shown in Figure 3. Previous studies have shown that E2F-binding sites in the promoters of *B-myb*, *cdc2*, and *E2F-1* confer repression during G_0/G_1 (Dalton 1992; Lam and Watson 1993; Hsiao et al. 1994; Johnson et al. 1994; Tommasi and Pfeifer 1995), suggesting that the increases in expression seen in $p107^{-/-};p130^{-/-}$ cells are most likely attributable to loss of E2F-mediated repression. The extent of the derepression varied between genes. *B-myb* and *cdc2* were most severely altered by the absence of p107 and p130, whereas *E2F-1*, *TS*, and *RRM2* were changed less dramatically. Quantitation of the Northern blots in Figure 3 showed that *B-myb* expression was 25-fold higher in quiescent $p107^{-/-};p130^{-/-}$ MEFs

compared to control MEFs. After serum stimulation, the level of *B-myb* mRNA was elevated earlier and reached a higher maximal level in $p107^{-/-};p130^{-/-}$ MEFs than in control cells.

MEFs are a mixed population of primary cells and independent preparations often have different growth characteristics. To confirm that the differences in expression patterns of E2F target genes were attributable to the genotype of the cells, the expression of E2F target genes was examined in six additional preparations and compared with control MEFs that were prepared in parallel from the same mother. In each preparation of $p107^{-/-};p130^{-/-}$ MEFs, the expression of *B-myb* was deregulated, whereas the pattern of cyclin E expression was unchanged compared to the control MEFs (two representative lines are shown in Fig. 4). Similarly, derepression of *cdc2*, *E2F-1*, *TS*, and *RRM2* was seen consistently in $p107^{-/-};p130^{-/-}$ MEFs (data not shown).

From the comparison of multiple MEFs preparations we observed that two additional genes, *cyclin A2* and *DHFR*, were deregulated in $p107^{-/-};p130^{-/-}$ cells. The changes in expression of these genes were subtle but are

Figure 3. Altered expression of many E2F target genes in $p107^{-/-};p130^{-/-}$ MEFs. MEFs corresponding to the three genotypes indicated were serum starved and restimulated. Total RNA was isolated at nine time points and analyzed on Northern blots (20 μ g total RNA/lane). The filter was probed sequentially with probes for murine *cyclin E*, *B-myb*, *cdc2*, *E2F-1*, *TS*, *RRM2*, or *ARPP P0* mRNAs.

Figure 4. The abnormal expression of E2F-target genes in $p107^{-/-};p130^{-/-}$ MEFs is seen consistently in independent MEF cultures. Expression patterns were determined as described in Fig. 3 using six independent preparations of $p107^{-/-};p130^{-/-}$ MEFs and their littermate controls ($p107^{+/+}$ or $p130^{+/+}$). Expression patterns are shown for *B-myb*, *cyclin E*, *cyclin A2*, and *DHFR* on two representative pairs of MEFs.

significant as they were reproduced in each MEF preparation. The expression of *cyclin A2* was slightly elevated during G_0/G_1 in $p107^{-/-};p130^{-/-}$ MEFs when compared with control MEFs (Fig. 4). Of the two murine A-type cyclins that have been identified, *cyclin A2* has the greatest homology to the human *cyclin A* gene and is expressed ubiquitously. Three groups of investigators have identified independently a motif that is essential for cell cycle expression of cyclin A (Huet et al. 1996; Schulze et al. 1996; Zwicker et al. 1996). Although this motif can bind to E2F complexes and can mediate induction of the promoter by E2F (Schulze et al. 1996), it also binds to a factor that is distinct from E2F (Huet et al. 1996; Zwicker et al. 1996). Deregulation of the *cyclin A2* promoter in $p107^{-/-};p130^{-/-}$ MEFs is consistent with E2F regulation of this promoter, but could occur if other proteins that bind to the cell cycle-dependent element/cell cycle response element (CDE/CCRE) are also regulated by p107 or p130.

Expression of the *DHFR* gene was fully repressed in quiescent $p107^{-/-};p130^{-/-}$ MEFs and at early time points after serum stimulation, but the levels of *DHFR* mRNA increased earlier in these cells when compared to control

cells (Fig. 4). Expression of *DHFR* was apparent at 9 hr after serum stimulation of $p107^{-/-};p130^{-/-}$ cells, and in some preparations of $p107^{-/-};p130^{-/-}$ cells the level of *DHFR* mRNA was higher at 9 hr than at peak S-phase. This expression pattern differed from other genes that were expressed abnormally in $p107^{-/-};p130^{-/-}$ in that elevated expression of *DHFR* did not occur in G_0 or early G_1 . Earlier studies have suggested that the E2F sites in the *DHFR* mediate activation of the promoter (Slansky et al. 1993), rather than repression as seen in other promoters, and the change in $p107^{-/-};p130^{-/-}$ MEFs may reflect such a difference.

The expression patterns of *PCNA*, *TK*, *DNA polymerase α* , and *Cdc25C* mRNA were not clearly altered in $p107^{-/-};p130^{-/-}$ MEFs (data not shown). Small changes were observed in the induction of *PCNA* expression but these were no greater than the slight differences in onset of DNA synthesis between $p107^{-/-};p130^{-/-}$ and control MEFs.

The extent of the changes in the cell cycle expression of E2F target genes in $p107^{-/-};p130^{-/-}$ cells suggests that p107 and p130 proteins have significant roles in the regulation of E2F activity but that their roles are functionally redundant. Importantly, the cell cycle-regulated expression of *cyclin E* was unchanged in $p107^{-/-};p130^{-/-}$ MEFs when compared with either $p130^{+/+}$ MEFs and $p130^{-/-}$ MEFs (see Fig. 3). This shows that the changes in expression of other E2F target genes do not simply result from either a wholesale loss of cell cycle control or from a major shift in cell cycle position. It is striking that the set of genes that were deregulated in $p107^{-/-};p130^{-/-}$ MEFs were expressed normally in $Rb^{-/-}$ MEFs, whereas *cyclin E* was normally regulated in $p107^{-/-};p130^{-/-}$ MEFs but was deregulated in $Rb^{-/-}$ MEFs. These results reveal that pRB and p107/p130 are required for the correct expression of different subsets of E2F responsive genes.

Deregulation of the B-myb promoter in $p107^{-/-};p130^{-/-}$ cells is mediated by the E2F-binding site

As p107 and p130 associate with E2F in vivo and the expression patterns of many E2F responsive genes are altered in $p107^{-/-};p130^{-/-}$ cells, it seems most likely that the changes in expression of E2F responsive genes are attributable to the deregulation of E2F. However, pRB, p107, and p130 have the potential to bind to several transcription factors, and it is possible that changes in gene expression could be attributable to factors other than E2F. As *B-myb* expression was most clearly altered in $p107^{-/-};p130^{-/-}$ MEFs, we investigated the role of the E2F-binding site in the *B-myb* promoter in $p107^{-/-};p130^{-/-}$ and control MEFs.

Previous experiments using luciferase reporter constructs fused to a fragment of the *B-myb* promoter, or to a mutant promoter carrying a 3-bp deletion in the E2F binding site, have shown that the E2F-binding site is necessary for repression of *B-myb* promoter in serum-starved NIH-3T3 cells (Lam and Watson 1993). We observed similar derepression of the mutant *B-myb* pro-

motor when the two constructs were transfected into control MEFs (Fig. 5A). In these MEFs expression from the mutant *B-myb* promoter was >20-fold higher than expression from the wild-type promoter in serum-starved control MEFs. If the elevated expression of the endogenous *B-myb* gene in *p107*^{-/-};*p130*^{-/-} MEFs was caused by the loss of an E2F repressor complex, then both the wild-type and mutant promoters are predicted to be derepressed in *p107*^{-/-};*p130*^{-/-} MEFs during G₀/G₁. As shown in Figure 5A, expression from the wild-type *B-myb* promoter was 15-fold higher in the *p107*^{-/-};*p130*^{-/-} MEFs compared to control MEFs. This increase in the activity of the wild-type *B-myb* luciferase in *p107*^{-/-};*p130*^{-/-} MEFs mimics the increase of *B-myb* mRNA seen in the Northern analysis of *p107*^{-/-};*p130*^{-/-} MEFs and suggests that the derepression was attributable to the absence of an E2F repressor complex.

To test this directly, p107 was reexpressed in *p107*^{-/-};*p130*^{-/-} MEFs and the activity of the wild-type and mutant promoters was measured in serum-starved cells (Fig. 5B). The repression of wild-type *B-myb* luciferase in *p107*^{-/-};*p130*^{-/-} MEFs was restored by the expression of p107 or, to a lesser extent, p130. On the other hand, expression of pRB had no effect on activity of the promoter. Importantly, the p107- and p130-mediated repression of the *B-myb* reporter construct in *p107*^{-/-};*p130*^{-/-} MEFs required a functional E2F site. The activity of the mutant *B-myb* reporter construct was unaffected by the expression of either p107, p130, or pRB. Furthermore, the expression of p107, p130, or pRB had no effect on either the wild-type or mutant *B-myb* promoters in control cells that contained endogenous pRB, p107, and p130 (data not shown). Thus, the ability of p107 and p130 to repress the activity of *B-myb* was dependent on the E2F-binding site and was seen only in

cells that lack endogenous p107 and p130. Taken together, these results demonstrate a high degree of specificity for transcriptional repression, and provide strong evidence that the E2F site mediates p107 and p130 repression of the *B-myb* promoter in serum starved cells.

Altered patterns of E2F complexes in cells lacking pRB family members

The pattern of E2F complexes detected in cell extracts changes during cell cycle progression and it is widely assumed that the individual E2F complexes have specific functions. The lack of a change in the expression of E2F responsive genes in *p107*^{-/-} MEFs and *p130*^{-/-} MEFs suggested instead that the roles of p107 and p130 were redundant. An alternative explanation might be provided if other pRB family proteins were up-regulated in *p107*^{-/-} MEFs and *p130*^{-/-} MEFs allowing them to compensate for the absence of p107 or p130. Other changes in expression of pRB family members might also minimize the changes in E2F-dependent transcription in *Rb*^{-/-} MEFs or *p107*^{-/-};*p130*^{-/-} MEFs. To investigate these possibilities we have characterized the E2F complexes present in cells lacking pRB family members. These experiments provide two examples where E2F complexes are specifically up-regulated in cells deficient for pRB family proteins.

Whole cell extracts were prepared and analyzed with gel mobility-shift assays using an E2F oligonucleotide derived from the adenovirus E2 promoter (Fig. 6A). E2F complexes containing pRB family proteins were identified in extracts of quiescent cells and in extracts of S-phase cells that were made 18 hr after serum restimulation (Fig. 6B,C). All of the complexes detected under these gel shift conditions were specific as they were

Figure 5. Derepression of *B-myb* in *p107*^{-/-};*p130*^{-/-} MEFs is mediated by the E2F-binding site. (A) *p107*^{-/-};*p130*^{-/-} MEFs and a littermate control MEFs (*p130*^{+/-}) were transfected with 1 μ g β -actin- β -Gal and 4 μ g of either wild-type *B-myb* luciferase or a mutant in which the E2F site was mutated. Cells were serum starved for 63 hr after transfection, harvested, and assayed for both luciferase activity and β -galactosidase (β -gal) activity. β -Galactosidase activity was used to correct the luciferase values for transfection efficiency and the level of expression of wild-type *B-myb* luciferase in control MEFs was set to 1. All transfections were done in triplicate and the mean values are shown with standard error indicated. The expression from both the wild-type and mutant *B-myb* promoters is elevated in *p107*^{-/-};*p130*^{-/-} MEFs. (B) The activity of the wild-type but not the mutant *B-myb* promoter is repressed by the reexpression of p107 and p130 in *p107*^{-/-};*p130*^{-/-} MEFs. *p107*^{-/-};*p130*^{-/-} MEFs were transfected with 4 μ g of either wild-type *B-myb* luciferase or mutant *B-myb* luciferase, 1 μ g of β -actin- β -gal, and 0.5 μ g of the indicated expression vectors. Transfections were done in groups of six. Mean values are shown with standard error indicated.

competed by unlabeled wild-type competitor but not by an oligonucleotide in which the E2F site was mutated. Individual complexes were identified by the addition of antibodies against pRB (21C9), p107 (SD-15), or p130 (C-20). Although the monoclonal antibodies against pRB and p107 are specific, the C-20 polyclonal antibody showed a low level of cross-reactivity to p107 in gel shift assays. C-20 has been used to identify p130/E2F complexes in several studies and its ability to disrupt p107/E2F complexes partially was documented previously (Cobrinik et al. 1996). Because of this cross-reactivity, p107/E2F complexes were identified using SD-15, and complexes that were supershifted by C-20 but unaffected SD15 were designated as p130/E2F. The 21C9 antibody enhanced DNA binding by pRB/E2F complexes, and hence the supershifted pRB/E2F complexes were more prominent than the original pRB/E2F complexes.

At all points of the cell cycle, pRB/E2F complexes represented only a minor fraction of the total E2F-binding activity in wild-type MEFs. pRB/E2F, p107/E2F, and p130/E2F complexes were all found in quiescent cells, although, as has been described in other cell types, the p130/E2F complexes represented the most prevalent form. During progression from G₀ to S-phase, the quantity of unbound E2F increased, and both p107/E2F and p130/E2F complexes shifted to slower mobility forms that contain cyclin-dependent kinases (Cao et al. 1992; Devoto et al. 1992; Shirodkar et al. 1992). As reported previously, p107/E2F complexes were the predominant form of bound E2F in S-phase extracts (Fig. 6C).

Although pRB, p107, and p130 have been found to stabilize E2F proteins in transient transfection assays (Hateboer et al. 1996; Hofmann et al. 1996), the total E2F-binding activity found in the panel of MEFs was very similar. Most of the changes seen in the cell cycle profiles of E2F complexes in MEFs lacking pRB family proteins are exactly as would be predicted by simple removal of the protein. For example, the predominant S-phase p107/E2F complex is missing in *p107*^{-/-} MEFs. Although no changes were observed in the expression of E2F target genes in these cells, the absence of the p107/E2F complex does not appear to be accompanied by a clear increase in the abundance of p130/E2F or pRB/E2F complexes that might compensate for its absence. Consistent with previous work (Cobrinik et al. 1996), we failed to observe any dramatic increase in the levels or timing of appearance of p107/E2F or pRB/E2F complexes in *p130*^{-/-} MEFs.

However, in both *Rb*^{-/-} MEFs and *p107*^{-/-};*p130*^{-/-} MEFs the pattern of E2F complexes showed changes in addition to the absence of the respective pRB family proteins. Wild-type quiescent cells contained very little p107/E2F complex; in contrast, p107/E2F complexes were greatly increased in quiescent *Rb*^{-/-} MEFs (Fig. 6B) and were the predominant E2F complex found in nuclear extracts prepared from these serum-starved cells (data not shown). This increase correlated with an increase in both p107 protein (see Fig. 1A) and p107 mRNA (see Fig. 2A) in quiescent *Rb*^{-/-} cells. Although the appearance of p107/E2F complexes might simply be a consequence of

the increased abundance of p107, no increase in p107/E2F complexes was apparent in quiescent *p130*^{-/-} MEFs that also contain elevated levels of p107 (see Fig. 1A). The increase in p107/E2F complexes might allow p107 to compensate for some pRB functions in E2F regulation. It has not been possible to test this hypothesis directly as we have been unable to find a specific difference in the affinities of p107/E2F and pRB/E2F complexes for E2F sites in the cyclin E and B-myb promoters in gel mobility shift assays.

Up-regulation of new E2F-binding activity in p107^{-/-};*p130*^{-/-} MEFs

Intriguingly, an E2F complex was detected in extracts from quiescent *p107*^{-/-};*p130*^{-/-} MEFs that migrated at the same mobility of p107/E2F and p130/E2F complexes (Fig. 7). This complex bound specifically to the E2F site as it was eliminated by competition with wild type but not mutant unlabeled oligonucleotide. The new complex was not recognized by antibodies to pRB, p107, or p130, although these same antibodies eliminated or supershifted all of the major E2F complexes found in extracts of quiescent wild-type MEFs. Because all of the E2F-binding activity in *p107*^{-/-};*p130*^{-/-} MEFs was converted to a faster migrating form by treatment with deoxycholate detergent, these observations indicate that *p107*^{-/-};*p130*^{-/-} MEFs contained a novel E2F-binding protein that was up-regulated in the absence of p107 and p130. The new E2F complex was also disrupted by the addition of E1A (Fig. 7), suggesting that it contains an E2F-binding protein that interacts with E2F in a manner that is similar to pRB, p107, and p130. The properties of this complex suggest that it contains a previously uncharacterized member of the pRB family that is up-regulated in quiescent *p107*^{-/-};*p130*^{-/-} MEFs.

Discussion

The recent analysis of mice deficient for pRB, p107, or p130 proteins has strongly supported the model that pRB family proteins provide regulation that is important during development and lost during tumorigenesis. Because pRB, p107, and p130 share many biochemical properties and interact with similar proteins in vivo, the extent of functional overlap between these proteins has been an important issue.

The phenotypes of knockout mice lacking multiple family members support the idea that the functions of these proteins are connected. Mice lacking two family members (*Rb*^{-/-};*p107*^{-/-} and *p107*^{-/-};*p130*^{-/-}) have developmental phenotypes that are more severe than any of the single mutants, indicating that at some level the functions of these proteins overlap. However, as with any two genes, this type of interaction could be generated in many ways. For example, the double mutants might have a more severe phenotype because two proteins cooperate in the regulation of a common downstream target. Alternatively, the two family members might have different functions, and the interaction may

simply reflect the fact that these separate functions contribute to some stage of tissue development. In general, the source of functional overlap is difficult to determine in the analysis of knockout animals, as the histological appearance of a tissue is far separated from the biochemical activity of the proteins under consideration.

To understand better the functional relationship between pRB family proteins, we have examined the roles that they play in the regulation of E2F-dependent transcription. pRB, p107, and p130 regulate E2F directly and

numerous studies have suggested that E2F is likely to be an important target for pRB family proteins in their control of cell proliferation. We have examined how the expression of E2F target genes is altered when pRB family proteins are specifically absent. As it is the inactivation of pRB family proteins that has been linked to cell proliferation and tumorigenesis, the changes in E2F activity that occur in these loss of function experiments may be particularly significant. The results identify functions in E2F regulation that are redundant between p107 and

Figure 6. (See facing page for C and legend.)

p130, and functions that are distinct between pRB and p107/p130.

Functional differences between pRb and p107/p130 in E2F regulation

Cells lacking pRB or p107/p130 showed elevated expression of E2F target genes. These data provide strong support for the model that pRB/E2F, p107/E2F, and p130/E2F complexes act as active repressors on E2F sites (Weintraub et al. 1992, 1995). Previously, we and others have speculated that the p107/cyclin A/cdk2/E2F complexes that are abundant in S-phase cells may act to promote the expression of E2F target genes. However, we saw no examples of E2F target genes whose expression was decreased in the absence of p107 or p107 and p130.

Strikingly, the sets of genes that are affected in *Rb*^{-/-} and *p107*^{-/-}; *p130*^{-/-} cells were different. In *Rb*^{-/-} cells the expression of two E2F target genes (*p107* and *cyclin E*) was elevated in G₀/G₁, whereas a different and more extensive set of genes was deregulated in *p107*^{-/-}; *p130*^{-/-} cells. Therefore, pRB and p107/p130 have different functions in E2F regulation.

Presently it is unclear what distinguishes E2F sites that require pRB for normal regulation from those that require p107/p130. It is possible that the distinction between classes of E2F-regulated genes stems from the fact that three forms of E2F (E2F-1, E2F-2, and E2F-3) bind

preferentially to pRB (Lees et al. 1993). However, pRB/E2F, p107/E2F, and p130/E2F complexes have not been shown to possess different sequence specificity. Furthermore, using gel mobility-shift assays we have been unable to show that the E2F-binding site in the *cyclin E* promoter, for example, has any preference for pRB/E2F complexes when compared with the E2F site from the *B-myb* promoter, although these promoters are affected differentially by the absence of pRB and p107/p130. Therefore, it seems likely that specificity may depend to a large degree on the context of an E2F-binding site rather than simply on the sequence of the site.

The changes in gene expression in the panel of MEFs highlight the important distinction between a promoter that contains E2F-binding sites and allows transcription when E2F genes are overexpressed, and a promoter that is deregulated in the absence of pRB. As previously reported, the absence of pRB does not result in the constitutive expression of most of the genes that have been proposed to be regulated by E2F (Herrera et al. 1996a). Instead, only a few such targets showed altered expression patterns in *Rb*^{-/-} cells and the changes were subtle. Nevertheless, in both *Rb*^{-/-} cells and *p107*^{-/-}; *p130*^{-/-} cells, the absence of pRB family proteins leads to the inappropriate expression of genes that promote cell cycle progression (*cyclin E* in *Rb*^{-/-} cells and *B-myb*, *cyclin A*, *E2F-1* in *p107*^{-/-}; *p130*^{-/-} cells). Thus, the deregulation of E2F activity is likely to alter the growth properties of

Figure 6. Characterization of E2F complexes in MEFs lacking pRB family proteins. (A) Cells were serum starved and restimulated to enter the cell cycle by the addition of serum. Whole cell extracts were prepared at time points indicated and analyzed by electrophoretic mobility-shift assays (EMSA) using an E2F probe. The forms of E2F indicated in the figure were identified by addition of specific antibodies. (B,C) Composition of E2F complexes in extracts of serum-starved MEFs (B) or MEFs that had been serum starved and restimulated for 18 hr (C). Specific E2F complexes were competed by 100-fold molar excess of unlabeled wild-type E2F competitor but not by 100 mutant competitor. Complexes were identified by that addition of antibodies to pRB (21C9), p107 (SD-15), or p130 (C-20).

Figure 7. Appearance of a new E2F complex in $p107^{-/-};p130^{-/-}$ MEFs that is disrupted by E1A. Whole cell extracts of serum starved wild-type or $p107^{-/-};p130^{-/-}$ MEFs were analyzed in E2F EMSA. The E2F complexes found in wild-type extracts were eliminated or supershifted by the addition of a panel of antibodies to pRB, p107, and p130 as described in Fig. 6. This panel included both monoclonal (21C9) and polyclonal antibodies against pRB. The major slow mobility E2F complex found in $p107^{-/-};p130^{-/-}$ extracts was not removed by the same panel of antibodies, but was disrupted by treatment with deoxycholate (DOC). This novel complex was also disrupted by the addition of E1A, which was synthesized by in vitro transcription/translation in wheat germ extracts, but not by the addition of control lysate. A monoclonal antibody to pRB (21C9) was added to the seven lanes on the right to alter the mobility of pRB and to allow the new E2F complex to be seen more clearly.

the cells and render them less susceptible to negative regulation. As different sets of genes are misexpressed in $Rb^{-/-}$ and $p107^{-/-};p130^{-/-}$ cells, the cell cycle appears to be deregulated in different ways in the two cell types. One implication of this finding is that $Rb^{-/-}$ and $p107^{-/-};p130^{-/-}$ cells may respond differentially to specific elements of cell cycle control. Such differences could help to explain why inappropriate proliferation is seen in different cell types in $Rb^{-/-}$ and $p107^{-/-};p130^{-/-}$ embryos.

Unlike the inactivation of pRB, the specific mutation of p107 or p130 has not yet been demonstrated to occur in tumors, and no tumor predisposition has been observed in mice carrying mutant alleles of p107 and p130 (Cobrinik et al. 1996; Lee et al. 1996). Because deregulated E2F activity is thought to play an important role in promoting the proliferation of $Rb^{-/-}$ tumor cells, it is surprising that far more extensive changes in E2F activity were found in $p107^{-/-};p130^{-/-}$ cells than in $Rb^{-/-}$ cells. There are several potential explanations for this paradox. One possibility is that the set of E2F target

genes that are misexpressed in $Rb^{-/-}$ cells might be more important for cell proliferation than the E2F targets that are misexpressed in $p107^{-/-};p130^{-/-}$ cells. Overexpression of cyclin E shortens the length of G_1 (Ohtsubo and Roberts 1993; Resnitzky et al. 1994; Wimmel et al. 1994), and cyclin E may be an example of a pRB/E2F target that is critical. As yet, no systematic screens for E2F-regulated genes have been carried out and the most important targets of pRB/E2F complexes may still be unidentified. An alternative explanation is suggested by mutational studies showing that E2F binding is not sufficient for the cell cycle arrest and growth suppression properties of pRB (Qian et al. 1992; Zhu et al. 1993; Welch and Wang 1995). In addition to E2F at least 39 pRB-associated proteins have been described (for examples, see reviews by Chen et al. 1995; Sanchez and Dynlacht 1996) and it is unclear how many of these interact with p107 and p130. It is possible that the role of pRB in E2F regulation may only be important for tumor suppressor activity in conjunction with other functions that are carried out specifically by pRB and not by p107 or p130.

Functional overlap between pRB family proteins in E2F regulation

E2F activity, as defined by the cell cycle expression of E2F responsive genes, was completely normal in *p107*^{-/-} cells or in *p130*^{-/-} cells. The deregulation of E2F target genes in *p107*^{-/-}; *p130*^{-/-} cells shows that there is extensive functional overlap between p107 and p130 in the regulation of specific E2F targets. Although there may be other unidentified target genes that are uniquely dependent on p107 or p130, it is clear that the functions of these proteins in E2F regulation are largely redundant.

The full extent of functional overlap between pRB family members in E2F regulation is not known. It is possible that there is no further overlap and that pRB and p107/p130 act on completely different sets of genes. Alternatively, most E2F binding sites may be regulated by multiple pRB family proteins and the deregulation of target genes seen here may represent the limited situations in which a specific E2F complex is required. The mutation of E2F-binding sites in the promoters of several E2F responsive genes had a far more severe effect on cell cycle expression of reporter genes than the deregulation seen in *Rb*^{-/-} and *p107*^{-/-}; *p130*^{-/-} cells [for examples, see *E2F-1* and *DHFR* (Slansky et al. 1993; Hsiao et al. 1994; Johnson et al. 1994; Neuman et al. 1994)]. If promoter studies reflect accurately the regulation of endogenous genes, then this would suggest that there is still extensive regulation of E2F activity in *Rb*^{-/-} and *p107*^{-/-}; *p130*^{-/-} cells. Such regulation might be provided by the pRB family members that remain in these cells or may be the result of other types of E2F regulation.

One of the issues raised by the examination of any family of closely related proteins is whether their functions are truly redundant, or whether family members normally have different functions but that in the absence of any one member the other family members compensate by assuming the functions of the missing protein. This issue is difficult to address for E2F/pRB family members as, currently, it is not technically possible to identify the proteins bound to a E2F site in the promoter of an endogenous gene. The MEF cultures used here expressed pRB, p107, and p130. Our experiments indicate that p107 and p130 are equally able to repress the promoters of a set of target genes in quiescent cells, but it is unclear whether or not both proteins would normally carry out this function in wild-type cells.

One potential mechanism for compensation is the up-regulation of remaining pRB family members. We found no overt increase in the abundance of p130/E2F complexes in quiescent *p107*^{-/-} cells or in the level of p107/E2F complexes in *p130*^{-/-} cells. In contrast, upregulation of specific E2F complexes did occur in *Rb*^{-/-} MEFs and *p107*^{-/-}; *p130*^{-/-} MEFs. The level of p107 mRNA was elevated in quiescent cells *Rb*^{-/-} MEFs, an increased amount of protein was found in Western blots of protein from quiescent or asynchronous cells, and immunoprecipitation of ³⁵S-labeled proteins indicated that p107 was more readily labeled in *Rb*^{-/-} MEFs (data not shown). The dramatic increase in p107/E2F complexes in quies-

cent *Rb*^{-/-} cells, particularly when nuclear extracts were compared, could enable p107 to carry out functions normally performed by pRB/E2F. Similarly the appearance of a new E2F complex in *p107*^{-/-}; *p130*^{-/-} cells raises the possibility that a novel member of this family of proteins is up-regulated in these cells and that this unidentified protein might carry out some functions normally performed by p107 and p130. If these changes do represent functional compensation, then this compensation is inefficient, as E2F target genes are deregulated in both *Rb*^{-/-} and *p107*^{-/-}; *p130*^{-/-} cells. We note that there is no direct evidence that the increase in p107 level allows p107/E2F to regulate a broader range of E2F targets in *Rb*^{-/-} cells and this change may simply arise if p107 expression is repressed by pRB/E2F complexes. Analysis of the expression of E2F target genes in *Rb*^{-/-}; *p107*^{-/-} cells may help to resolve this issue. Future studies will also be needed to determine the normal function of the new E2F-binding partner that is up-regulated in *p107*^{-/-}; *p130*^{-/-} cells and to discover how this protein should be incorporated into models for E2F regulation.

E2F target genes were deregulated to varying extents in *p107*^{-/-}; *p130*^{-/-} cells. There are many potential reasons for this variability, but two factors may be particularly relevant. First, the importance of an E2F site in regulating the cell cycle expression of a gene will depend on whether other repressor elements are present and whether any other factors that are necessary for full activation of the promoter are also cell cycle regulated. Second, because the occupancy of an E2F site can require interaction with surrounding factors (Karlseder et al. 1996), the ability of pRB family proteins to compensate for one another may vary depending on the context of the E2F-binding site. Potentially, the ability of E2F complexes to substitute for one another on a promoter may vary between cell types and stages of development.

Importantly, there are striking parallels between the overall pattern of E2F regulation in the panel of MEFs and the appearance of abnormal cell proliferation in the corresponding knockout mice. The absence of either p107 or p130 had no effect on E2F regulation or on mouse development. E2F was deregulated in different ways in *Rb*^{-/-} and *p107*^{-/-}; *p130*^{-/-} MEFs, and different patterns of abnormal cell proliferation were seen in *Rb*^{-/-} and *p107*^{-/-}; *p130*^{-/-} embryo. These parallels provide support for the idea that E2F is an important target of pRB family proteins and suggest that the future analysis of E2F target genes in *Rb*^{-/-}; *p107*^{-/-}, *Rb*^{-/-}; *p130*^{-/-}, and *Rb*^{-/-}; *p107*^{-/-}; *p130*^{-/-} cells will provide further insights into the functional relationships within this family of proteins.

Materials and methods*MEF preparation*

MEF cultures were prepared from day 13.5 embryos according to standard techniques. After removal of the head and internal organs, dissected embryos were minced, digested in trypsin for 45 min at 37°C, and resuspended in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 50

U/ml of penicillin, 50 µg/ml of streptomycin, and 2 mM L-glutamine. Yolk sacs from the dissected embryos were genotyped by PCR using primer sets that have been described before, *Rb* (Jacks et al. 1992), *p107* (Lee et al. 1996), and *p130* (Cobrinik 1996).

Serum starvation and restimulation

Passage 4 MEFs were washed twice with 1× PBS and serum starved in DMEM with 0.1% serum for 62–65 hr. MEFs were stimulated by the addition of media containing 10% serum. The 0-hr time points were processed before the addition of 10% DMEM. [³H]thymidine incorporation assays were performed in triplicate using passage 4 MEFs labeled with 5 µCi of [³H]thymidine (ICN) for 1 hr at 37°C. Fluorescence-activated cell sorter (FACS) analysis and bromodeoxyuridine (BrdU)-labeling experiments showed that ~60% of the cells entered S-phase.

Western blot analysis

Cell pellets were lysed in RIPA (Harlow and Lane, 1988) containing protease and phosphatase inhibitors (1 mM AEBSF, 1 µg/ml of aprotinin, 1 µg/ml of leupeptin, 0.5 mM NaF, 0.1 mM Na₃VO₄, 0.5 mM DTT). One hundred micrograms of extract from asynchronously growing MEFs and 70 µg of extract from serum-starved MEFs were resolved in an 8% SDS-polyacrylamide gel, and transferred to Immobilon P (Millipore) according to standard procedures (Harlow and Lane 1988). The following antibodies were used for Western blot analysis: SD-9 for p107 (Dyson et al. 1993), C-20 for p130 (Santa Cruz, lot no. B206), and 3C8 (Canji) for pRB.

Northern blot analysis

Total RNA was prepared from passage 4 MEFs. Cells were lysed into 2 ml of RNA STAT-60 total RNA isolation reagent (TEL-TEST "B"), frozen on dry ice, and stored at -70°C for batch processing later according to the manufacturer's instructions. For each time point 20 µg of total RNA was denatured, separated in a 1% agarose gel containing 2.2 M formaldehyde, and transferred to Hybond-N+ (Amersham). Formamide hybridization solution (Dyson 1991) was used for both prehybridization and hybridization. All cDNA probes were labeled by random oligonucleotide-primed synthesis with 50 µCi [α -³²P] dCTP (Tabor and Struhl 1994). The following full-length mouse cDNAs were used as probes for Northern analysis: Cyclin E, p107, B-Myb, cdc2, RRM2, cyclin A2, DHFR, TK, Cyclin D1, c-Myc, E2F-5, DP-1, PCNA, IGF-1, and ARPP P0. Partial mouse cDNAs were used to make probes for E2F-1 (nucleotides 524–1388), TS, DNA polymerase α (nucleotides 43–1639), Cdc25C (1.4 kb 5' fragment), E2F-4 (700 nucleotides 3' noncoding sequence), Rb (1.9 kb 5' fragment), and topoisomerase I (nucleotides 1046–2934).

Transfections and luciferase assays

Passage 4 MEFs were transfected with lipofectamine according to the manufacturer's instructions. Transfections with the control MEFs were done in triplicate, and transfections with the *p107*^{-/-}; *p130*^{-/-} MEFs were done in groups of six. DNA (5 µg or 5.5 µg) and 15 µl of lipofectamine were used per well. Cells were incubated with the DNA liposome mix for 5 hr. DMEM containing 10% serum was then added for 12 hr. Cells were washed twice with 1 × PBS, incubated in media containing 0.1% serum for 63 hr, then lysed by the addition of 300 µl of 1× cell culture

lysis reagent (Promega). Luciferase and β -gal assays were carried out by standard techniques.

E2F electrophoretic mobility-shift assays

Whole cell extracts were prepared according to standard procedures. Cell pellets were lysed in 300 µl extraction buffer [20 mM HEPES (pH 7.6), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% of glycerol, 50 mg/ml of leupeptin, 50 mg/ml of aprotinin, 0.6 mM Na₃VO₄, 0.5 mM NaF, 0.5 mM DTT] and samples were centrifuged at 4°C for 20 min at 42,000 rpm in a TLA45 rotor (Beckman) to remove cell debris. A 43-bp oligonucleotide from the adenovirus E2 promoter (Lam et al. 1994) was used for E2F bandshifts. Binding reactions (20 µl) used a binding buffer described previously (Cobrinik et al. 1993). For competition experiments, 40 ng of oligonucleotide containing either a wild-type E2F site or a mutant E2F site (Cao et al. 1992) was added to the binding reaction before addition of the extract. For antibody supershift reactions, 2 µl of 21C9 [α pRB (Cobrinik et al. 1993)], 1 µl of SD-15 [α p107 (Dyson et al. 1993)], or 0.5 µl of C-20 [α p130, Santa Cruz, lot E055] was added. All samples were separated on a 4% polyacrylamide gel that contained 2.5% glycerol at 4°C.

Adenovirus 13S E1A was synthesized in the TNT SP6 coupled wheat germ extract system (Promega). Both programmed and unprogrammed lysates (0.1, 0.5, and 1 µl) were used in gel mobility-shift reactions.

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