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Identification of novel pRb binding sites using CpG microarrays suggests that E2F recruits pRb to specific genomic sites during S phase

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The retinoblastoma (Rb) tumor suppressor protein is an important regulator of cell proliferation and differentiation. Many studies have shown that pRb can negatively regulate the activity of the E2F family of transcription factors during G_0 and G_1 phases of the cell cycle, perhaps by serving as a bridge between the E2Fs and transcriptional repressors such as histone deacetylases and methylases. However, pRb has also been shown to localize to discrete DNA foci during S phase, a time at which pRb is thought to be dissociated from E2F. Numerous other DNA binding proteins have been shown to interact with pRb, suggesting that pRb may control progression through S phase by binding to sites in the genome distinct from E2F target gene promoters. To test this hypothesis, we have identified novel pRb binding sites within the human genome using an unbiased approach which relies upon a combination of chromatin immunoprecipitation and CpG microarray analysis. To provide the greatest opportunity of finding distinct sets of pRb binding sites, we examined pRb binding in chromatin obtained from human Raji cells synchronized in either G_0/G_1 phase or S phase. These experiments have allowed us to identify a large set of new genomic binding sites for the pRb protein. We found that some sites are occupied by pRb only during G_0/G_1 phase, as would be predicted from previous models of pRb function. We also identified sites to which pRb bound only during S phase and other sites which were bound constitutively by pRb. Surprisingly, we found that E2F1 was present at most of the CpG islands bound by pRb, independent of the phase of the cell cycle. Thus, although pRb has the potential to interact with numerous transcription factors, our data suggest that the majority of DNA-bound pRb is recruited to E2F target promoters during both G_0/G_1 and S phases.
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Introduction

The product of the retinoblastoma gene, pRb, is a classic tumor suppressor protein that is mutated in a variety of human tumors (Harbour and Dean, 2000). Additionally, pRb has also been implicated in the regulation of normal differentiation and development (reviewed in Lipinski and Jacks, 1999). pRb is proposed to block cell cycle progression and promote differentiation by negatively regulating the transcription of genes whose products are required for the G_1/S phase transition and DNA replication (reviewed in Farnham *et al*., 1993). However, pRb lacks a DNA binding domain and therefore must be tethered to gene promoters through interaction with DNA binding proteins (reviewed in Morris and Dyson, 2001a). The first such DNA binding protein identified to interact directly with pRb was E2F1 (Helin *et al*., 1992; Kaelin *et al*., 1992; Shan *et al*., 1992). Subsequently, other E2F family members have also been shown to interact with pRb (Ivey-Hoyle *et al*., 1993; Lees *et al*., 1993). Owing to the early discovery of E2F/pRb interactions, many of the studies of pRb function have focused on the ability of pRb to regulate the E2F family of transcription factors. E2F transcriptional activity is inhibited by association with pRb through at least two distinct mechanisms. First, pRb binds to the transactivation domain of E2F, thereby physically blocking activity of this domain. Second, pRb inhibits E2F activity by recruiting chromatin remodeling factors, including histone deacetylases (HDACs), members of the ATP-dependent chromatin remodeling complex SWI/SNF, and methylases, such as DNA methyltransferase 1 (DNMT1) (reviewed in Zhang and Dean, 2001). Interaction between pRb and E2F is thought to be regulated by cell cycle-dependent phosphorylation of pRb. During G_0 and G_1, hypophosphorylated pRb binds to and inhibits the activity of E2F. As cells progress through G_1, cyclin D/cdk4, cyclin D/cdk6, and cyclin E/cdk2 complexes are sequentially activated and phosphorylate pRb. Hyperphosphorylation of pRb results in dissociation from and activation of E2F complexes during late G_1 (reviewed in Trimarchi and Lees, 2001). Therefore, one view is that pRb's primary role in controlling cellular differentiation and proliferation is accomplished during G_0 and G_1 phases by regulating E2F activity.

In contrast, recent evidence indicates that pRb may also function during S and G_2/M phases of the cell cycle.

For example, Kennedy *et al*. (2000) found that pRb, as well as the related family members p107 and p130, can localize to early S phase replication foci in primary cells. However, the mechanism by which pRb was recruited to the DNA during S phase was not investigated in these previous studies. It was not possible to identify the DNA binding factor which recruited pRb to the foci because the visualization technique used (i.e. immunohistochemistry) did not provide any information concerning the sequence to which pRb was bound. Unpublished data mentioned in the manuscript by Kennedy and colleagues suggest that E2F1 can also localize to replication foci, indicating that it is possible that E2F1 may, at least in part, help to recruit Rb to these sites. However, at least 110 unique cellular proteins have been reported to interact with pRb (Morris and Dyson, 2001b). Of these, 72 are thought to be transcriptional regulators. Therefore, a second view of pRb function is that it has a very broad role in gene regulation and is brought to genomic DNA via interaction with a wide number of DNA binding transcription factors. Unfortunately, very few studies have shown direct interaction between pRb and specific transcription factors using endogenously expressed proteins and even fewer studies have demonstrated pRb binding to promoters *in vivo*. However, one convincing demonstration of pRb binding *in vivo* is to the CBFA1 target gene promoter osteopontin (Thomas *et al*., 2001).

To further investigate the role of pRb in regulating cellular proliferation, we have undertaken an unbiased approach to identify novel pRb binding sites, using synchronized human Raji cells collected during either G_0/G_1 or S phase. The pRb binding sites were identified using chromatin immunoprecipitation in conjunction with CpG island microarrays. One advantage of this technique is that it allowed us to demonstrate not only recruitment of pRb to chromatin during S phase but also to identify the specific DNA regions bound by pRb. As reported below, our results support the hypothesis that a primary function of pRb is to control expression of E2F target genes as almost all of the CpG islands that we identified to be bound by pRb were also bound by E2F1. Surprisingly, our results suggest that E2F1 and pRb can bind to chromatin during both G_0/G_1 and S phases, but that individual binding sites display unique cell cycle-stage specific binding patterns.

Results

The identification of novel pRb binding sites using S phase cells

According to the widely accepted model for pRb function, interaction between pRb and E2F is thought to be regulated by cell cycle-dependent phosphorylation of pRb. During G_0 and early G_1, hypophosphorylated pRb binds to and inhibits the activity of E2F. As cells progress through G_1, cyclin D/cdk4, cyclin D/cdk6, and cyclin E/cdk 2 complexes are sequentially activated and phosphorylate pRb. Hyperphosphorylation of pRb is thought to result in dissociation from and activation of E2F complexes during late G_1. To confirm that this model is an accurate portrayal of pRb function in Raji cells, we have examined pRb phosphorylation patterns and used chromatin immunoprecipitation to compare E2F1 and pRb binding patterns on known E2F target genes in both asychronously growing cell populations and in populations enriched for S phase cells. To obtain enriched S phase cell populations, Raji cells were synchronized with a double block of thymidine followed by aphidicolin to create a population of cells synchronized at the G_1/S phase boundary. Cells were then released from the block by removal of the drugs and harvested 5 h later. Owing to the large number of cells required for these experiments, cells from several independent experiments were pooled together. FACS analysis of cells from each independent experiment (Table 1) indicated that the resulting populations were highly enriched for mid-S phase cells which represented from 63 to 87% of the total population of cells. The percentage of cells in S phase is likely an underestimate because of difficulties in distinguishing late G1 cells from very early S phase cells using flow cytometric analysis. Results from a representative FACS experiment are shown in Figure 1a.

As expected, Western blot analysis of synchronized Raji cells showed that although pRb protein was present in G_1 phase cells, it was not phosphorylated (Figure 1b). In contrast, pRb was phosphorylated at several different sites in cells arrested at the G_1/S phase boundary. Phosphorylation of pRb at these four serine residues

Table 1 Flow cytometry analysis of Raji cells used in the microarray experiments

	S phase-enriched populations		
Experiment #	*G_0/G_1 (%)*	*S (%)*	*G_2/M (%)*
1	12.94	87.05	0.00
2	14.23	85.77	0.00
3	12.44	65.40	22.16
4	12.81	66.69	20.51
5	21.86	78.14	0.00
6	13.01	86.99	0.00
7	36.49	63.31	0.20
	G0/G1 phase-enriched populations		
8	94.60	2.17	3.23
9	93.16	5.09	1.75

For experiments 1–7, Raji 525-7 cells were synchronized at the G1/S phase boundary by a thymidine/aphidicolin double block and released into S phase. For analysis of S phase populations by chromatin immunoprecipitation followed by CpG microarray analysis, cells were harvested at 5 h after removal of aphidicolin. At this same time point, aliquots of cells were ethanol-fixed and stained with propidium iodide for subsequent flow cytometry analysis. The percentage of cells in S phase is likely an underestimate because of difficulties in distinguishing very late G1 cells from very early S phase cells using flow cytometric analysis. For experiments 8 and 9, Raji 525-7 cells were synchronized by growth in the presence of DMSO for 20 h. Aliquots were ethanol fixed, propidium iodide stained and analysed by flow cytometry to demonstrate that the majority of cells used in chromatin immunoprecipitation and CpG microarrays were in G_0/G_1 phase

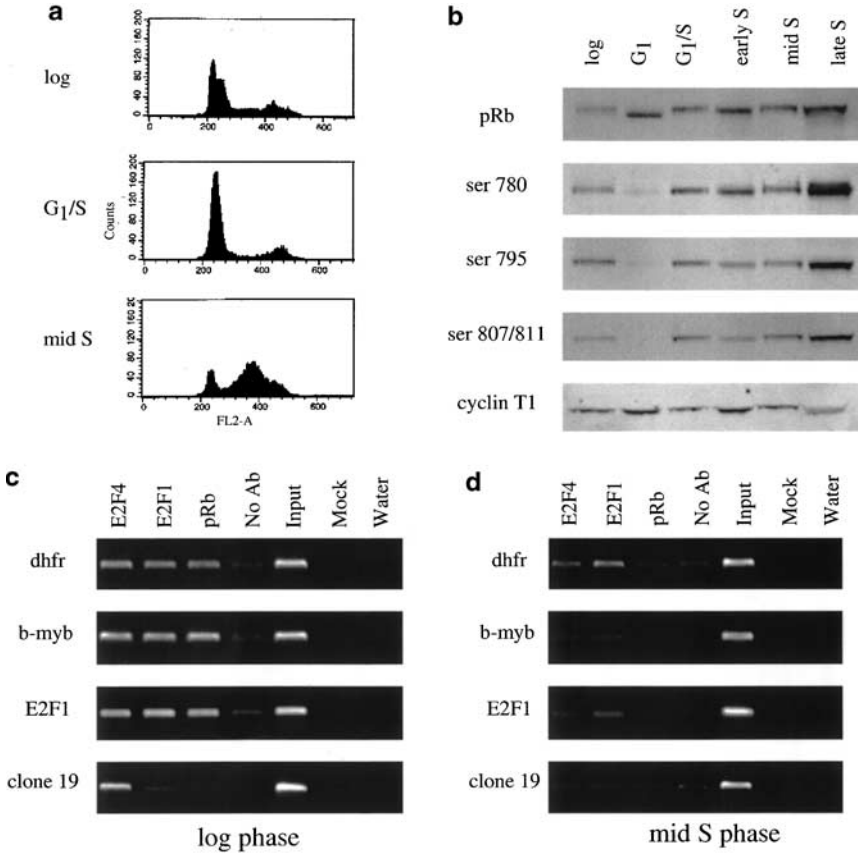

Figure 1 Previously characterized E2F target gene promoters are not bound by pRb during S phase. (**a**) Flow cytometry analysis of Raji 525-7 synchronized at the G_1/S phase boundary by a thymidine/aphidicolin double block and released into S phase. This synchronization technique was used to collect mid-S phase Raji cells throughout this manuscript. (**b**) Western blot analysis of whole cell extracts from synchronized Raji cells collected at the G_1/S phase boundary and at various points after release. The pRb antibody recognizes pRb regardless of phosphorylation status while ser 780, ser 795, and ser 807/811 only recognize pRb when it is phosphorylated at the indicated serine residues. Exposure times with the various antibodies were approximately equivalent except for blot probed with the ser 807/811 antibody which was exposed for almost 10 times as long. This blot was also probed with cyclin T1 as a loading control. ChIP analysis of parallel cultures of Raji cells collected in logarithmic growth (**c**) or in mid-S phase (**d**). Binding to the promoters of the dhfr, B-myb, E2F1, and clone 19, a previously identified E2F4 target gene, was analysed

persisted throughout S phase and increased during late S phase. Cyclin T1 protein levels, which are not known to be cell cycle regulated, remained relatively unchanged throughout this time course. Thus, we have demonstrated that the Raji cell system accurately reproduces the known phosphorylation patterns of the pRb protein throughout the cell cycle.

To determine the influence of pRb phosphorylation on binding to known E2F target genes, chromatin immunoprecipitation analysis using primers specific for the *dhfr*, B-*myb*, and *E2F1* promoters was performed. Previous studies using other cell types have shown that the *dhfr* promoter is constitutively occupied by E2F family members, whereas the E2F site in the B-*myb* promoter is occupied in asynchronously growing cells but is not occupied during S phase (Zwicker *et al*., 1996; Wells *et al*., 2000). Both promoters have been shown to bind to pocket proteins in asynchronously growing cells, but neither promoter is bound by pocket proteins during S phase. In asynchronously growing Raji cells, we found that E2F1, E2F4, and pRb were bound to the *dhfr*, B-*myb*, and *E2F1* promoters (Figure 1c). We have identified clone 19 as an E2F-4-specific promoter (M Oberley and PJ Farnham, unpublished data) which thus serves as a control to show that not all promoters are bound by E2F1 and pRb. As expected, binding of pRb to the *dhfr*, B-*myb*, or *E2F1* promoters during S phase cells was not detected (Figure 1d). Similar to our previous results (Wells *et al*., 2000), we found that E2Fs bound to the *dhfr* promoter, but not to the B-*myb* promoter, during S phase. During S phase, we detected small amounts of E2F1 protein bound to the *E2F1* promoter. We also found that the E2F4-specific clone 19 promoter lost binding of E2F4 during S phase, similar to the B-*myb* and *E2F1* promoters. These results demonstrate that the cell culture model system we have chosen to use provides evidence in support of the currently accepted model for pRb function. Importantly, these results also show the specificity of the antibodies we have used for these studies.

Although our results, and those of others (Wells *et al*., 2000; Ren *et al*., 2002) suggest that pRb may not be

bound to many of the known E2F target gene promoters during S phase, there is evidence to suggest that pRb does play a role during S phase. For example, Kennedy *et al*. (2000) found that pRb, as well as related family members p107 and p130, are specifically associated with early S phase DNA replication foci in primary cells. Therefore, we hypothesized that the sites to which pRb was bound during S phase would be a distinct class of binding sites, separate from known E2F target gene promoters. To identify the specific regions to which pRb binds during S phase, we have used an unbiased method which involves a combination of chromatin immunoprecipitation and CpG microarray analysis (Weinmann *et al*., 2001). CpG islands are GC-rich regions of the genome that often correspond to promoters and origins of replication (Antequera and Bird, 1999). A library of such CpG islands isolated by virtue of binding to methyl-CpG binding protein 2 (MeCP2) was spotted onto glass microscope slides (Cross and Bird, 1995; Yan *et al*., 2001).

Seven different populations of cells were treated with thymidine and aphidicolin and examined by FACS analysis to ensure synchronization had occurred. In all, 50 ChIP reactions using an antibody against pRb, 50 ChIP reactions using an antibody against E2F1, and 50 ChIP reactions using normal rabbit serum were performed. Chromatin that had been immunoprecipitated with the same antibody or control serum was pooled and approximately half of each combined sample was used to probe a CpG microarray. After probing the CpG microarray, we identified 32 clones (out of a possible 7776 CpG islands spotted on the array) that had stronger signals when probed with the chromatin that was precipitated with an antibody against pRb than with the chromatin precipitated using normal rabbit serum. The identity of these clones was determined by sequencing followed by analysis using both the NIH database (BLAST) and the Santa Cruz database (BLAT). These clones included 12 which corresponded to promoters or other regions of characterized genes, eight uncharacterized loci, nine repeat regions and three clones which we were unable to sequence or which did not match any entries in either database (Table 2). The list of characterized clones includes histone H2B (*H2B FR*), a subunit of TFIIH (*GTF2 H4*), *CYP27B1*, and *Myc,* all of which have been previously implicated to be E2F target genes (Albert *et al*., 2001a; Muller *et al*., 2001; Weinmann *et al*., 2002).

To confirm that the sites identified by probing the CpG array with pRb-precipitated chromatin were reproducibly bound by pRb, primers were prepared for PCR analysis of each of the 32 identified clones. Raji cells were again synchronized by a thymidine–aphidicolin block and chromatin was precipitated using an antibody against pRb. As shown in Figure 2, we were able to confirm binding of pRb to all 12 of the sites which corresponded to characterized genes. We also tested seven of the eight uncharacterized loci and found that six of them were bound by pRb in an independent chromatin IP assay (three of these confirmations are shown in Figure 2). Clone MP3B11 did not bind to pRb in subsequent experiments and thus is ruled a false positive (data not shown), whereas clone PY1E6 was not tested due to an inability to optimize primers. Finally, we tested binding of pRb to the repeat regions identified using the CpG array. We found that four of the nine sites confirmed as pRb binding sites, four of the sites were deemed to be identified due to nonspecific hybridization to the array (most likely due to their amplified copy number within the genome), and one site was not tested (data not shown). In all, of the 28 sites we tested, 23 confirmed to bind specifically to pRb in subsequent chromatin immunoprecipitation assays (an 82% confirmation rate). To ensure that the pRb antibody we used was not precipitating chromatin nonspecifically, we also analysed four randomly chosen

Table 2 Identification of clones which are bound by pRb during mid-S phase

S phase pRB clone	*Identity*	*Function*
Known genes/promoters (12)		
SC14 E2	TCP 10	Development
SC5 B9	Myc	Oncogene/tumor marker
SC40 H8	Myc	Oncogene/tumor marker
PY2 H10	SRPR	Signalling
SC71 F12	SRPR	Signalling
SC86 E2	PPT2	Protein modification
SC73 A10	SLC22 AILS	Metabolism
MP2 B9	H2B FR	Chromatin function
SC7 D10	GTF2 H4	Transcription factor
CpG11 B3	SAF B	Chromatin function
CpG5 B6	CYP 27B1	Metabolism
PY2 F7	Sox7	Embryogenesis
Uncharacterized loci (9)		
SC67 E9	9p22.1	
SC7 G4	16p12.1	
MP3 B11	17q23.2	
SC77 A6	2q12.1	
SC74 F8	3q21.2	
CpG12 B3	22q13.31	
CpG12 B9	19q13.32	
PY1 E6	2q22.1	
PY1 E3	1q41	
Repeats (9)		
SC71 D11	Multiple chromosomes	
SC22 B7	Multiple chromosomes	
COMBI E8	Multiple chromosomes	
COMBI F5	Multiple chromosomes	
SC1 F6	Multiple chromosomes	
SC69 B11	Chromosome 1	
PY3 B6	Chromosome 1	
SC67 F7	Chromosomes 1, 16	
CpG15 H12	Chromosome 1	
Unsequenced/no database matches (3)		
CpG79 D12		
SC62 H7		
PY1 H3		

Synchronized Raji cells were collected in mid-S phase and crosslinked with formaldehyde. Chromatin was then immunoprecipitated using an antibody against pRb or normal rabbit serum, the immunoprecipitated chromatin was labeled with cy5 dye and hybridized to a CpG island microarray. Clones which generated a stronger hybridization signal with the pRb precipitated chromatin than with the normal rabbit serum are listed. Positive clones are grouped by function

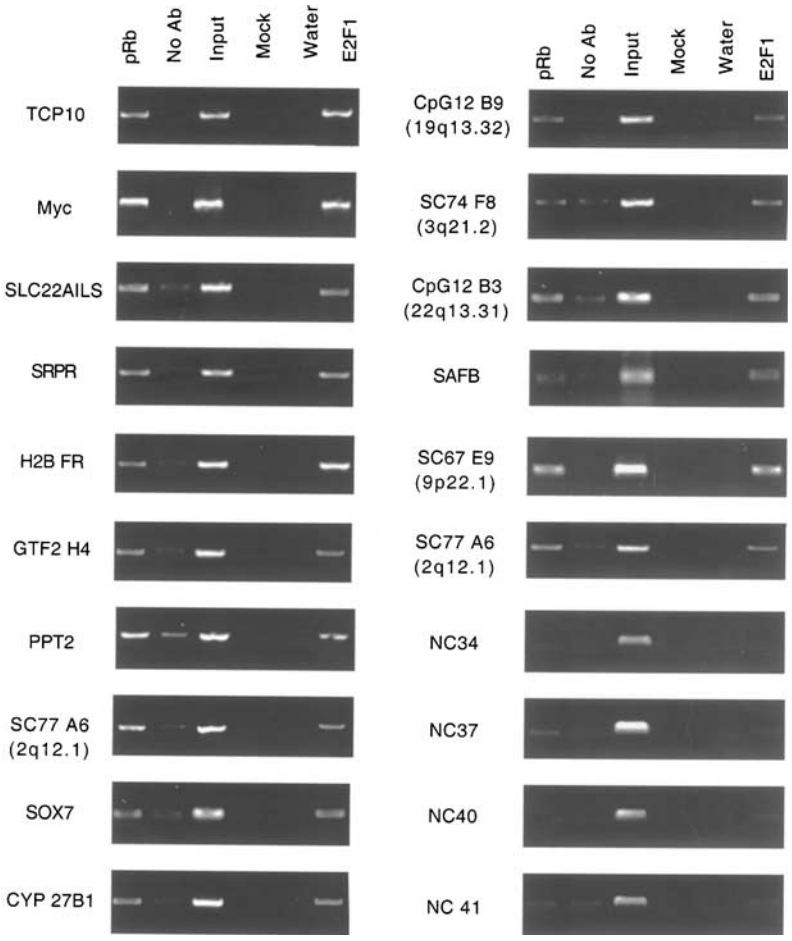

Figure 2 The identification of novel pRb binding sites. Synchronized Raji cells were collected in mid-S phase and crosslinked with formaldehyde. Chromatin immunoprecipitations using antibodies against pRb or E2F1 were performed. As negative controls, one immunoprecipitation reaction did not contain antibody (No Ab) and another immunoprecipitation did not contain chromatin or antibody (Mock). Immunoprecipitated chromatin, 0.3% of the starting chromatin which was not immunoprecipitated (Input), or water was analysed with primers specific to the loci listed in Table 1. Immunoprecipitated chromatin was also analysed with primers specific to four clones (NC34, NC37, NC40, and NC41) that were spotted onto the array, but did not hybridize to chromatin immunoprecipitated by an antibody against pRb

clones from the CpG array that were not identified as pRb targets, NC43, NC37, NC40, and NC41. We did not detect binding of either pRb or E2F1 to any of these clones. Thus, the combination of chromatin immunoprecipitation and CpG microarray analysis can successfully identify specific sites within the genome that were bound by pRb binding during S phase.

E2F1 and pRb colocalize on DNA during S phase

The Rb protein does not contain a DNA binding domain. Rather, it is brought to the DNA by interaction with sequence-specific DNA binding proteins. E2F1 was initially cloned due to its ability to interact with pRb (Helin *et al*., 1992; Kaelin *et al*., 1992; Shan *et al*., 1992) and we have shown that pRb and E2F1 can both bind to specific E2F target genes in asychronously growing cells (Figure 1c), indicating that E2F and pRb can be recruited to E2F target gene promoters *in vivo*. However, one might expect that pRb would be brought to the DNA during S phase via a different DNA binding partner since presently known E2F target gene promoters are not bound by pRb during S phase (Figure 1d). Therefore, we sought to determine if pRb is recruited to the newly identified binding sites via interaction with E2Fs or other DNA binding proteins by asking whether E2F1 binds to the pRb targets identified in the unbiased CpG array experiment. Interestingly, all of the newly identified pRb binding sites which we tested were also bound by E2F1; E2F1 binding to 16 of these sites is shown in Figure 2. Since these pRb binding sites were obtained in an unbiased manner using the CpG array, we were surprised that E2F1 was present at all of these sites. It should be noted that we have shown the specificity of the E2F1 antibody that we have used in our experiments by showing the absence of a signal in E2F1 null cells (Wells *et al*., 2002), as well as in the analysis of negative controls (Figure 2) and the B-myb promoter (Figure 1d). As an additional negative control, we have shown that the CpG islands precipitated by

E2F1 and pRb antibodies are not precipitated when normal rabbit serum from either of two different animals is used (data not shown). Although numerous transcription factors have been shown previously to interact with pRb (Morris and Dyson, 2001a), few studies have been performed to determine if pRb colocalizes with any of these transcription factors at specific DNA sites *in vivo*. Our results suggest that although pRb can interact with a variety of other DNA binding proteins *in vitro* or using overexpression systems, the E2F family may be the major mediator of pRb/chromatin interactions during S phase in Raji cells.

In the experiments described above, we used an antibody against pRb and identified CpG islands that were bound by both pRb and E2F1, suggesting that E2F family members were present at all pRb binding sites. To determine if the opposite conclusion held true, that is, if all CpG islands bound by E2F1 were also bound by pRb, we next probed the CpG microarray with chromatin immunoprecipitated using an antibody against E2F1. This experiment identified 124 clones which were enriched in the anti-E2F1 antibody-precipitated chromatin as compared to the chromatin precipitated with normal rabbit serum. Of these 124 clones, 43 corresponded to known promoters or genes, 30 to uncharacterized loci, 32 to repeat elements and we were unable to sequence or find a database match for 19 clones (Table 3). Clearly, examination of E2F1 and pRb binding to all 124 sites would be a large task. Therefore, we randomly chose 24 of the sites which corresponded to known genes and promoters or single loci for further analysis. First, we wished to confirm that the identified clones were in fact *in vivo* binding sites for E2F1. As shown in Figure 3, all 24 of the selected sites identified by the CpG array were in fact bound by E2F1 in this subsequent, independent chromatin immunoprecipitation experiment. This again indicates that the chromatin immunoprecipitation assay followed by CpG microarray analysis is a reliable way to find new transcription factor binding sites. The next question we asked was whether pRb would be recruited to these newly identified E2F1 binding sites. Interestingly, 23 of the 24 analysed CpG islands which were bound by E2F1 were also bound by pRb with approximately the same signal intensity in the pRb and E2F1 lanes. For example, the *beta lactamase* promoter shows high binding of both proteins whereas the *SMARCA5* promoter shows lower amounts of both proteins. However, E2F1 but not pRb was bound to the *ALG5* promoter. Our data indicate that whereas there are a few CpG islands which can bind E2F1, in the absence of pRb, E2F1, and pRb usually colocalize.

Surprisingly, the data presented in Figures 2 and 3 indicate that E2F1 and pRb colocalize on most of the CpG islands identified even though the experiments were performed using chromatin from S phase cells. Yet the current model predicts that hyperphosphorylated pRb should dissociate from E2F complexes during S phase. Therefore, it seemed possible that the pRb bound to these sites might be protected from phosphorylation. Alternatively, it was possible that these complexes contained phosphorylated pRb protein that remained bound to DNA. To distinguish between these possibilities, we repeated the chromatin immunoprecipitation using antibodies which recognize pRb that has been phosphorylated at four different residues, serine 780, serine 795, or serines 807 and 811. As shown in Figure 4, certain DNA fragments, such as PY1 E3 and SLC22AIL, are precipitated using a general antibody to pRb. However, antibodies which recognize pRb phosphorylated at serines 780, 795, or 807 and 811 cannot immunoprecipitate these fragments. Thus, there must be some pRb that is unphosphorylated or phosphorylated at different residues present in the S phase cells which can bind to these sites. In contrast, phosphorylation of pRb at any of the tested serine residues does not abolish binding to the *beta lactamase* or *Myc* promoters. Binding to other loci, such as the *TCP10* promoter and PY3 H5 clone, is more strongly diminished by phosphorylation at serines 795, 807 and 811 than by phosphorylation at serine 780. Therefore, the effects of phosphorylation on the ability of pRb to bind DNA appear to be binding site dependent. We note that we cannot determine the degree to which the pRb protein is phosphorylated in these experiments, but can only conclude that pRb phosphorylated at these four different sites can, in certain cases, be recruited to DNA.

Previous characterization of E2F sites suggests that the different E2F family members readily exchange at a particular site *in vivo*, with little specificity of binding among E2F family members (Takahashi *et al*., 2000; Wells *et al*., 2000). This lack of specificity at well-characterized E2F target promoters has also been observed when analysing E2F-mediated recruitment of pRb, p107, and p130. To determine if similar patterns occurred on the newly identified pRb binding sites, we have further characterized 10 of the novel sites bound by pRb, including four characterized loci, five uncharacterized loci, and one repeat element. In addition to examining binding of E2F1 and E2F4, we also included antibodies to the pocket proteins (to determine if these new sites are pRb-specific) and to RNA Polymerase II (to determine if the sites are in transcriptionally active loci). As shown in Figure 5, ChIP analysis indicated that eight of the 10 islands chosen were bound by E2F4 as well as E2F1. Thus, most of the newly identified islands bind to multiple E2Fs. Nine of the sites bound either or both p107 or p130, as well as RNAP II. However, one of the sites, clone PY1 E3, was bound by only E2F1 and pRb; it was not bound by E2F4, the other pocket proteins, or RNAP II.

Identification of novel G_0/G_1 phase pRb binding sites

As shown above, E2F1 is clearly recruited to the same CpG islands as is pRb during S phase, suggesting that E2F family members are a major mediator of pRb function during S phase. Since pRb has been shown to interact with many other DNA binding proteins, we thought it possible that other transcription factors might play a more important role in recruiting pRb to chromatin during other phases of the cell cycle.

Table 3 Identification of clones which are bound by E2F1 during mid-S phase

S phase E2F1 clones	*Identity*	*Function*
Known genes/promoter (43)		
DL3 A3	RAD51	Replication/repair/ recombination
PY1 E1	H4 FR	Chromatin function
PY1 E2	H4 FR	Chromatin function
PY3 G3	ALG5	Metabolism
MP3 G7	DLEU1/2	Oncogene/tumor marker
MP3 F10	DLEU 1/2	Oncogene/tumor marker
SC69 E7	EIF 358	Replication/repair/ recombination
SC22 G3	Cytochrome *c* oxidase	Electron transfer
PY2 A4	Meis 1	Differentiation/ development
MP3 C2	Meis 1	Differentiation/ development
SC66 C10	UXT	Oncogene/tumor marker
SC62 E6	MFAP 1	Oncogene/tumor marker
SC12 C2	EYA 4	Development
SC21 A8	GRAP	Signalling
DL3 H7	SMARCA 5	Chromatin function
MP2 B3	DCC	Oncogene/tumor marker
MP2 B9	H2B FR	Chromatin function
SC7 D8	ACTRIA	Unknown
SC62 B11	ACTRIA	Unknown
CpG12 D5	NKX6A	Differentiation/ development
MP3 F11	NKX6A	Differentiation/ development
SC70 D1	Diacylglycerol kinase, iota	Signalling
SC5 B9	Myc	Oncogene/tumor marker
SC5 D3	Beta lactamase	Metabolism
COMB1 H12	Beta lactamase	Metabolism
SC22 D7	KCNA3	Potassium ion channel
SC22 D9	Ribosomal protein 516	Oncogene/tumor marker
SC4 B2	PPIR 3A	Metabolism
SC7 D10	GTF 2H4	Replication/repair/ recombination
SC20 H4	B lymphocyte tyrosine kinase	Signalling
SC73 A10	SLC22 AILS	Metabolism
SC74 C12	CBX5	Chromatin function
SC74 F1	CBX5	Chromatin function
SC86 E2	PPT 2	Metabolism
CpG11 G4	H2B FR	Chromatin function
CpG15 C2	EYA2	Development
CpG5 B3	OAZ2	Replication/repair/ recombination
CpG11 B3	SAFB	Chromatin function
CpG11 H7	BMP4	Development
SC77 H6	SNRPC	mRNA splicing
CpG5 B6	CYP27B1	Metabolism
CpG7 B6	NEDD8-conjugating zyme	en-Protein ubiquitination
CpG15 D4	H4FH	Chromatin function
Uncharacterized loci (30)		
PY1 E3	1q41	
MP3 G2	22q11.2	
SC67 E9	9p22.1	
MP3 E2	19p13.13	
SC69 G2	3p25.1	
SC71 G8	7p12.3	
SC87 H9	1q25.1	
SC7 G4	16p12.1	
DL3 D7	3p14.2	
PY3 H5	22p11.23	
MP3 B11	17q23.2	
SC23 B3	15q22.2	
DL3 D6	7q33	
SC41 E3	20p12.2	
CpG21 G11	15q15.3	
SC87 A6	7q22.2	
CpG13 A2	12q24.23	
SC87 H7	14q32.2	
CpG8 B5	2q34	
CpG8 H5	11q24.3	
CpG10 B3	2q33.1	
CpG12 B3	22q13.31	
CpG12 B9	14q13.32	
CpG14 B9	4q13.1	
SC35 B6	17p11.2	
SC87 B6	4q31.21	
CpG6 B6	1p13.3	
CpG79 B10	7p21.2	
SC1 F5	5q31.1	
CpG71 F2	19q13.11	
Repeats (32)		
SC1 G11	Multiple chromosomes	
SC4 G10	Multiple chromosomes	
MP2 F7	Multiple chromosomes	
MP3 F9	Multiple chromosomes	
SC71 D11	Multiple chromosomes	
SC13 H1	Multiple chromosomes	
SC22 B7	Multiple chromosomes	
CpG12 E2	Multiple chromosomes	
SC86 B11	Multiple chromosomes	
SC88 B4	Multiple chromosomes	
CpG5 B2	Multiple chromosomes	
CpG6 F2	Multiple chromosomes	
COMBI E8	Multiple chromosomes	
SC22 E2	Multiple chromosomes	
CpG13 C2	Multiple chromosomes	
MP3 G1	Chromosome 15	
MP3 E10	Chromosome 15	
PY3 B5	Chromosome 10	
SC67 F7	Chromosome 1, 16	
SC17 F7	Chromosome 1	
SC22 D1	Chromosome 17	
PY3 B6	Chromosome 1	
PY2 F8	Chromosome 1	
SC67 F6	Chromosome 1	
SC20 D4	Chromosome 1	
SC43 D11	Chromosome 16	
SC89 F3	Chromosome 1	
SC43 H2	Chromosome 18	
COMB1 F4	Chromosome 17	
SC40 H11	Chromosome 1	
SC69 B11	Chromosome 1	
SC10 B1	Chromosome 17, 19	
Unsequenced/no database matches (19)		
PY2 A6		
SC5 G6		
MP3 E12		
SC70 G4		
PY2 H9		
PY2 B10		
SC15 H6		
SC73 G5		
SC41 H10		
SC76 B4		
SC76 D4		
CpG15 H12		
CpG64 G4		
PY1 H3		
COMB3 F1		
DL3 H5		
SC62 H7		
SC10 F9		
CpG13 E2		

Synchronized Raji cells were collected in mid-S phase and crosslinked with formaldehyde. Chromatin was then immunoprecipitated using an antibody against E2F1 or normal rabbit serum, the immunoprecipitated chromatin was labeled with Cy5 dye and hybridized to a CpG island mircoarray. Clones which generated a stronger hybridization signal with the pRb precipitated chromatin than with the normal rabbit serum are listed. Positive clones are grouped by function

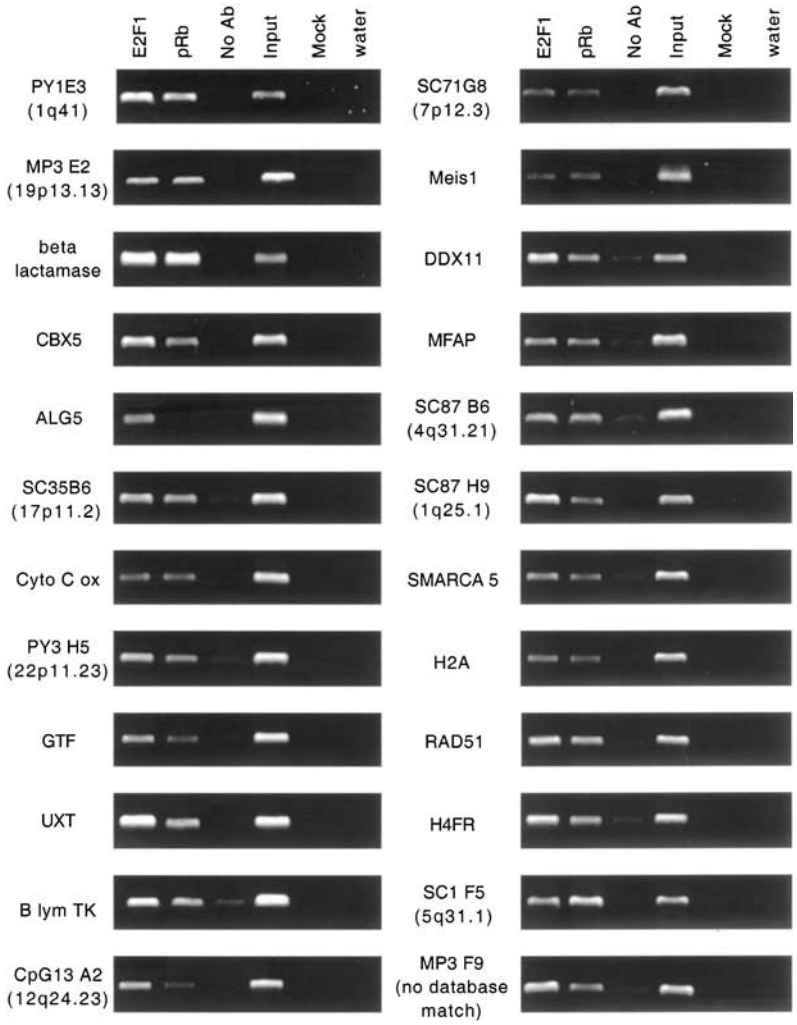

Figure 3 pRb and E2F1 colocalize on loci immunoprecipitated by an antibody against E2F1. Synchronized Raji cells were collected in mid-S phase and crosslinked with formaldehyde. Chromatin immunoprecipitations using antibodies against pRb or E2F1 were performed. As negative controls, one immunoprecipitation reaction did not contain antibody (No Ab) and another immunoprecipitation did not contain chromatin or antibody (Mock). Immunoprecipitated chromatin, 0.3% of the starting chromatin which was not immunoprecipitated (Input), or water was analysed with primers specific to 24 loci randomly chosen from those listed in Table 2

Therefore, we collected Raji cells synchronized in G_0/G_1 phase using a dimethylsulfoxide (DMSO) block, resulting in greater than 90% of cells in G_0/G_1 phase (Table 1). The collected cells were formaldehyde treated and a ChIP assay was again performed with an antibody against pRb. In total, 50 immunoprecipitation reactions were pooled, labeled and hybridized to a CpG island microarray, identifying 42 clones. Of these 42 clones, 14 corresponded to known genes or promoters, 16 corresponded to uncharacterized loci, nine corresponded to repeat elements and three were unable to be sequenced or did not have a match in either of the two databases tested (Table 4). As for the S phase clones, the G_0/G_1 clones were sequenced, corresponding primers were made, and the sites were tested for binding of pRb in a second, independent chromatin immunoprecipitation assay. Results monitoring binding to 10 sites obtained using the G_0/G_1 chromatin are shown in Figure 6. All 10 sites identified using G_0/G_1 phase chromatin were bound by pRb and E2F1. In all, we have examined 23 sites identified using G_0/G_1 phase chromatin and have found that 19 of the 23 sites were bound by pRb (an 83% confirmation rate). All 19 of these sites that were bound by pRb were also bound by E2F1. Thus, as in S phase, binding of pRb and E2F1 colocalize on the CpG islands identified using the G_0/G_1 phase chromatin.

Cell cycle stage-specific recruitment of pRb to chromatin

In our current experiments, we have used three separate chromatin immunoprecipitation and CpG microarray screens to identify CpG islands which are bound by E2F1 and pRb during either S phase (Tables 2 and 3) or G_0/G_1 phase (Table 4). Comparison of the S phase and G_0/G_1 phase clones identified using an antibody against pRb (Tables 2 and 4) revealed that, with the exception of

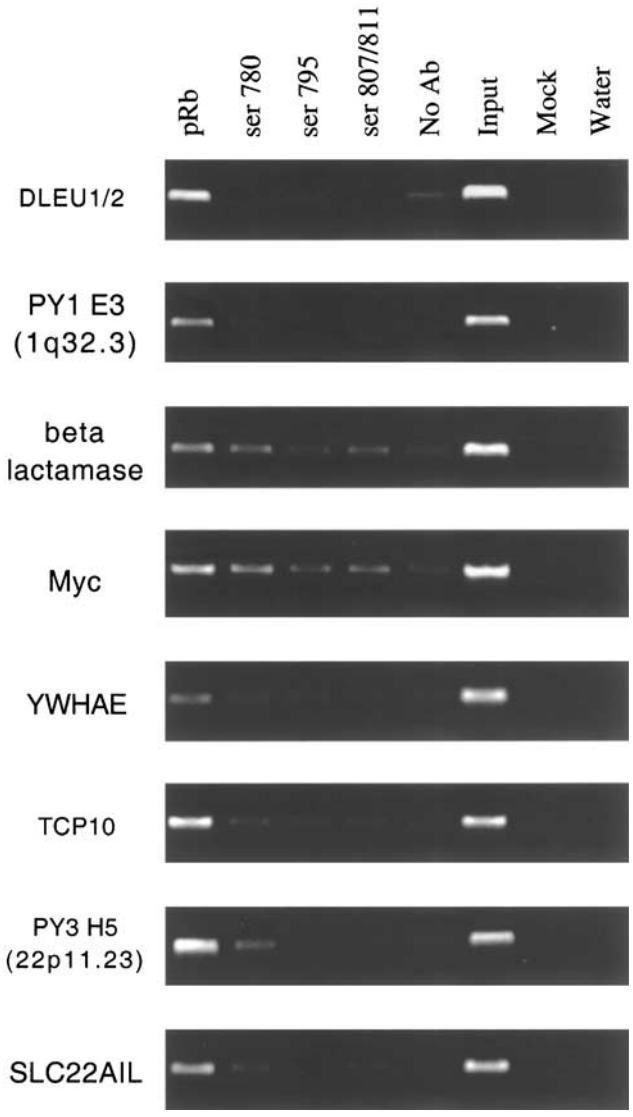

Figure 4 Phosphorylation of pRb at certain residues does not abolish binding to all target sequences. Synchronized Raji cells were collected in mid-S phase and crosslinked with formaldehyde. Chromatin was immunoprecipitated using antibodies against pRb in any phosphorylation state (pRb) or pRb phosphorylated at serine residues 780, 795 or 807 and 811. Immunoprecipitated chromatin was then analysed by PCR with primers specific for loci containing pRb binding sites as described in the legend for Figure 2

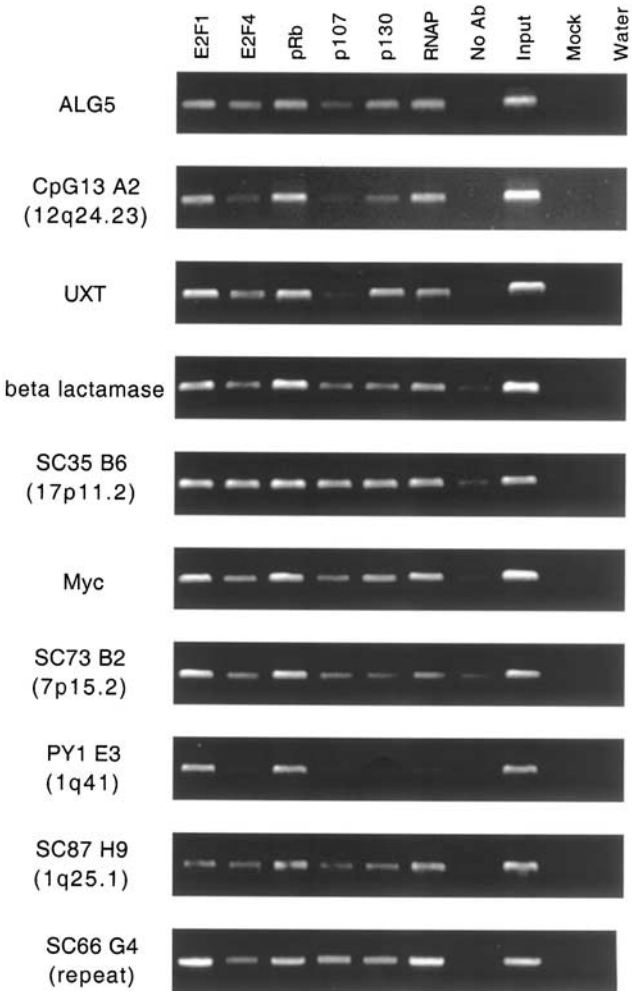

Figure 5 Characterization of novel pRb binding sites. Logarithmically growing Raji cells were crosslinked with formaldehyde and chromatin was immunoprecipitated with antibodies against E2F1, E2F4, pRb, p107, p130, or RNA polymerase II (RNAP). Immunoprecipitated chromatin was then analysed by PCR with primers specific for loci containing pRb binding sites as described in the legend for Figure 2

three uncharacterized loci, the G_0/G_1 phase clones were not identified in the S phase screen. Although this might suggest that the sites were only bound by pRb during G_0 phase, it was also possible that technical concerns did not allow the identification all possible pRb targets in each experiment (see Discussion). Therefore, we determined if any of the sites which were identified on the G_0/G_1 phase array but not on the S phase array were also bound by pRb during S phase. As shown in Figure 7, the examination of five sites originally identified using G_0 phase chromatin (Table 4) revealed that two of them had cell cycle stage-specific binding patterns, binding pRb only during G_0 phase (Figure 6), but not during S phase (Figure 7a). The other three sites were bound by pRb during both G_0 and S phase. All five sites were bound by E2F1 during both G_0 and S phase. We have examined binding to five additional sites obtained using G_0/G_1 phase chromatin and found that all five were bound by E2F1 and pRb during S phase (data not shown). Thus, eight of 10 tested sites identified using G_0/G_1 chromatin showed constitutive binding, whereas two sites showed binding only during the stage of the cell cycle used for the CpG microarray screen.

Conversely, we determined if clones identified using S phase chromatin were also bound by pRb during G_0/G_1 phase. As shown in Figure 7b, one site identified using S phase chromatin (TCP10) was bound weakly by pRb in the G_0 phase chromatin but four of the sites were not bound by pRb during G_0 phase. Interestingly, all five of these sites also showed diminished binding of E2F1 during G_0 phase, consistent with an

Table 4 Identification of clones which are bound by pRb in G_0/G_1 phase

G_0/G_1 phase pRB clones	*Identity*	*Function*
Known genes/promoters (14)		
SC22 E5	GMPS	Nucleotide synthesis
SC8 E3	YWHAE	Signalling
SC43 F12	MAGE F1	Oncogene/tumor marker
SC5 H12	PTP N4	Signalling
SC18 H4	HN1	Differentiation/ development
SC5 G5	CMAS1	Polysaccharide biosynthesis
SC18 H2	Nuclear protein 220 (NP220)	Unknown
SC11 A12	SDCCAG10	Oncogene/tumor marker
SC71 D10	GPS2	Signalling
SC62 C3	KPNA4	Protein trafficing
SC66 C10	UXT	Oncogene/tumor marker
SC22 D12	PGRMCI	Differentiation/ development
control 2 C8	ALG5	Metabolism
CpG5 B3	OAZ 2	Replication/repair/ recombination
Uncharacterized loci (16)		
SC62 A5	16p11.2	
SC8 C7	19q13.37	
SC62 C9	7q31.1	
Sc77 A6	2q12.1	
CpG8 C2	20p11.23	
SC20 A7	19p13.13	
CpG71 F2	19p13.11	
SC75 H12	7q32.2	
CpG12B9	19q13.31	
CpG12 B3	22q13.31	
SC73 F3	15q13.2	
SC13 G1	16p11.2	
SC73 B2	7p15.2	
SC73 F5	14q24.3	
SC43 A1	7q22.3	
SC43 E3	2q22.1	
Repeats (9)		
SC1 F5	Multiple chromosomes	
SC66 G9	Multiple chromosomes	
SC66 G4	Multiple chromosomes	
SC66 A9	Multiple chromosomes	
SC43 H4	Multiple chromosomes	
SC67 H12	Multiple chromosomes	
CpG12 B8	Multiple chromosomes	
SC13 G11	Chromosome 16	
SC62 G9	Chromosome 17	
Unsequenced/no database matches (3)		
SC11 B9		
SC43 E1		
CpG66 G4		

Synchronized Raji cells were collected during G_0/G_1 phase and crosslinked with formaldehyde. Chromatin immunoprecipitations with an antibody against pRb or normal rabbit serum and hydridization to the array were performed as described in the legend for Table 1

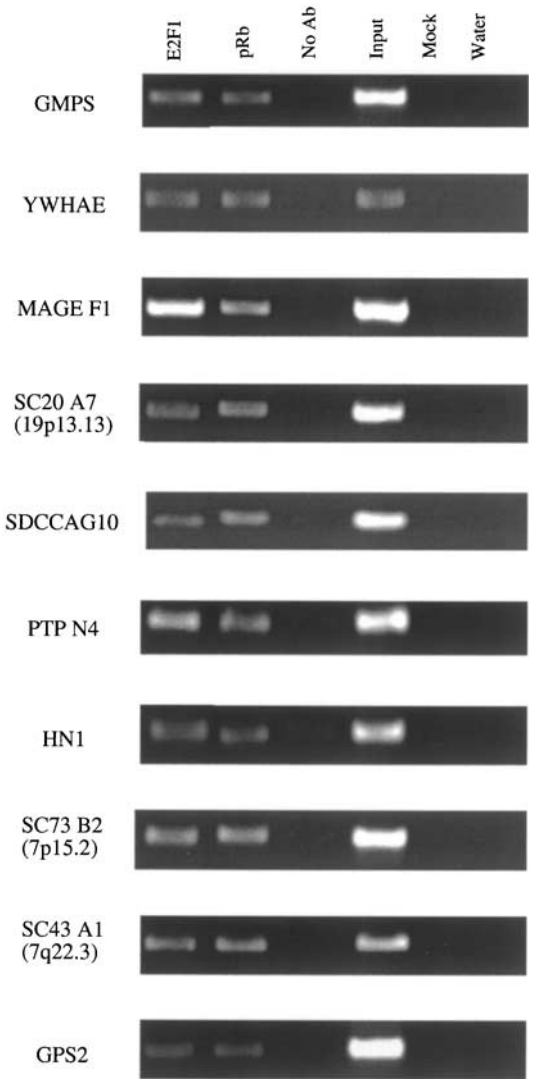

Figure 6 Confirmation of G_0/G_1 phase clones. Raji cells were synchronized in G_0/G_1 phase by treatment with DMSO and formaldehyde crosslinked. Chromatin was immunoprecipitated using antibodies against E2F1, pRb or in the absence of antibody and analysed by PCR with primers specific for 10 of the loci listed in Table 3, as described in the legend for Figure 2

E2F1-mediated recruitment of pRb to these sites during S phase. We have examined five other sites originally identified using S phase chromatin and none of these clones were bound by E2F1 or pRb during G_0 phase (data not shown). Thus, nine of 10 sites originally identified using S phase chromatin were not bound by pRb during Go/G_1 phase. In summary, the sites identified using G_0/G_1 phase chromatin tend to be bound constitutively by pRb whereas the clones identified using S phase chromatin tend to be bound only during S phase. However, we cannot rule out the possibility that the apparent cell cycle stage-specific binding at certain CpG islands was because of the inability of the pRb antibody to gain access to pRb in the chromatin isolated from one of the stages. Such uncertainty is, of course, inherent in all chromatin immunoprecipitation experiments regardless of what antibody is used. However, the fact that at least some CpG islands showed constitutive pRb binding indicates that antibody access is not a problem common to all binding sites.

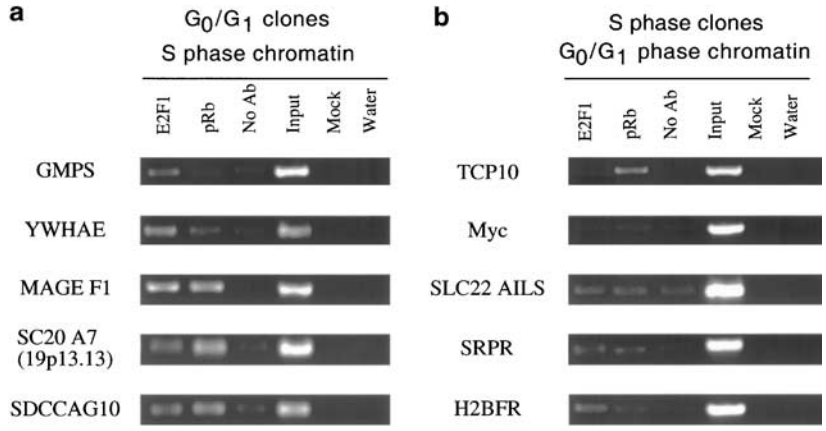

Figure 7 Binding of pRb to some loci is cell cycle regulated. Raji cells were synchronized by DMSO treatment and collected during G_0/G_1 phase or by thymidine/aphidicolin treatment, released and collected in mid-S phase. Collected cells were then formaldehyde treated and crosslinked chromatin was immunoprecipitated with antibodies against E2F1, pRb, or no antibody. (**a**) Chromatin that was immunoprecipitated from cells collected during mid-S phase was analysed by PCR with primers specific for clones isolated from G_0/G_1 phase cells. (**b**) Chromatin that was immunoprecipitated from cells collected during G_0/G_1 phase was analysed by PCR with primers specific for clones isolated from mid-S phase cells, as described in the legend for Figure 2

Recruitment of pRb by E2F

Owing to the strong correlation between binding of pRb and E2F1 to the same CpG islands during both G_0/G_1 and S phases, it seemed likely that E2F family members were the DNA binding proteins responsible for recruiting pRb to the newly identified pRb binding sites. However, due to the nature of chromatin immunoprecipitation experiments, precise localization of the specific site to which a protein binds to chromatin is not possible. However, we did have the ability to perform such experiments on the *Myc* promoter as the Raji cells used for these experiments contain an episome carrying an 8 kb region of the *Myc* gene (Albert *et al*., 2001a). 6 bp of the E2F binding site within the episomal copy of the *Myc* promoter have been mutated, eliminating the binding site for E2F family members. As shown in Figure 8, E2F1, E2F4, pRb, p107, and p130 all bound to the endogenous *Myc* promoter, yet none of these proteins bound to the episomal copy of the mutated *Myc* promoter. Therefore, loss of E2F binding to the *Myc* promoter correlates with loss of pRb binding, indicating that E2F recruits pRb to the *Myc* promoter. Similar experiments placing the newly identified pRb binding sites on an episomal plasmid and then performing a mutational analysis to determine if the exact same sequences are required for pRb binding as for E2F binding (as described in Wells and Farnham (2002)) can be performed for select other pRb binding sites. Clearly, this is beyond the scope of this intial report.

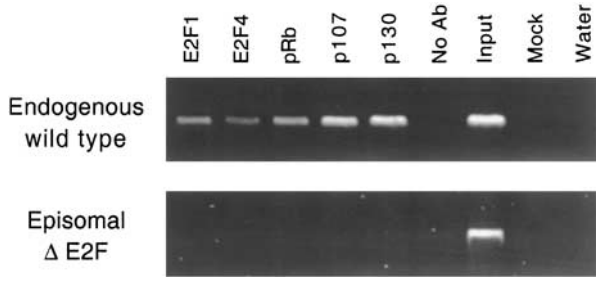

Figure 8 E2F recruits pRb family members to the Myc promoter. Asynchronously growing Raji 525-7 cells were formaldehyde treated and protein–DNA complexes were immunoprecipitated as described in the legend for Figure 2. Immunoprecipitated DNA was analysed by PCR with primers specific for either the endogenous, wild-type Myc promoter or for the episomal Myc promoter that contains a 6 bp substitution within the E2F binding site

Discussion

The Rb protein is an important regulator of transcription, the cell cycle, development and differentiation (Lipinski and Jacks, 1999; Harbour and Dean, 2000). Additionally, pRb functions as a tumor suppressor gene and is one of the most widely mutated genes in human cancers (Weinberg, 1995; Harbour and Dean, 2000). Therefore, it is surprising that despite the obvious importance of understanding the function of pRb, a global screen to identify genes whose expression is regulated by pRb has not been reported to date. A typical target gene screen would compare gene expression profiles in cells containing pRb to cells lacking pRb. This would require high-level expression of pRb in a cell which is functionally null for pRb. However, such overexpression of pRb would alter cell cycle progression and lead to a G_1 phase arrest (Goodrich *et al*., 1991). This perturbation of the cell cycle would result in the identification of many genes that were simply affected by the cell cycle arrest; determining which of the identified genes were directly regulated by Rb would require extensive further characterization. Also, overexpression of a transcriptional regulator such as pRb can result in the inappropriate recruitment of the transcription factor into complexes which would lack the factor under normal physiological conditions. Therefore, in an effort to circumvent all of these

potential difficulties, we have developed a novel method to identify genomic pRb binding sites, using a combination of chromatin immunoprecipitation and CpG island microarray analysis. Our method does not rely upon overexpression or reintroduction of Rb into cells, but rather identifies sites to which pRb is bound under normal physiological conditions. For our screens, we chose to use synchronized human Raji cells collected during G_0/G_1 phase, because pRb is known to be bound to chromatin as a component of E2F complexes at this time. We also used cells collected in mid-S phase, because pRb has been shown to localize to DNA replication foci and to bind to sites near origins of replication during S phase (Kennedy *et al*., 2000; Maser *et al*., 2001). Our analysis using G_0/G_1 phase chromatin identified 42 genomic sites to which Rb bound and our analysis using S phase chromatin identified 32 sites bound by pRb. Analysis of the pRb binding sites has revealed three different classes of pRb binding sites (Figure 9); sites constitutively occupied by pRb (e.g. *MAGE F1*), sites bound by pRb only in $G0/G_1$ phase cells (e.g. *GMPS*), and sites bound by pRb only during S phase (e.g. *Myc*). Our studies have revealed several surprising results including (1) that we did not identify a single CpG island that is bound by pRb that is not also bound by E2F1, (2) that, contrary to the current model, pRb and E2F1 colocalize on chromatin during S phase, as well as during G_0/G_1 phase, and (3) pRb and E2F1 may have a wide variety of functions in the cell.

All genomic sites bound by Rb are also bound by E2F1

We performed two unbiased screens for genomic pRb binding sites, using either G_0/G_1 or S phase cells, by performing chromatin immunoprecipitation with a pRb antibody followed by CpG microarray analysis. Although we expected that a large number of the genomic sites bound by pRb which we identified using the G_0/G_1 phase cells would also be bound by E2F1, we did not necessarily expect that all the newly identified pRb binding sites would show colocalization of the two proteins. Even more surprising was the fact that all of the CpG islands bound by pRb which we identified using S phase chromatin were also bound by E2F1. These results were unexpected for two reasons. First, pRb is known to interact with at least 72 different transcriptional regulators and we expected that we would identify at least some sites to which pRb was recruited in the absence of E2F family members. However, we do note that although our results indicate that the functions of pRb and E2F1 appear to be intimately linked, we have only analysed the portion of pRb that is bound to chromatin. It is possible that pRb which is not bound to chromatin may perform other functions in the cell which do not require interaction with E2F. Second, E2F-regulated genes are, in general, thought to be highly expressed during S phase. The fact that we identified promoters which are bound by E2F1 and pRb during S phase may suggest that pRb may not always function as a transcriptional repressor. After finding that E2F1 was bound to the same newly identified genomic sites as was pRb, we performed a reciprocal screen and identified 124 CpG islands that were bound by E2F1. Further analysis of 24 of these CpG islands showed that all were bound by E2F1 and that 23 of 24 tested islands were also bound by pRb. Thus, three different screens (using an antibody against pRb and G_0/G_1 chromatin, using an antibody against

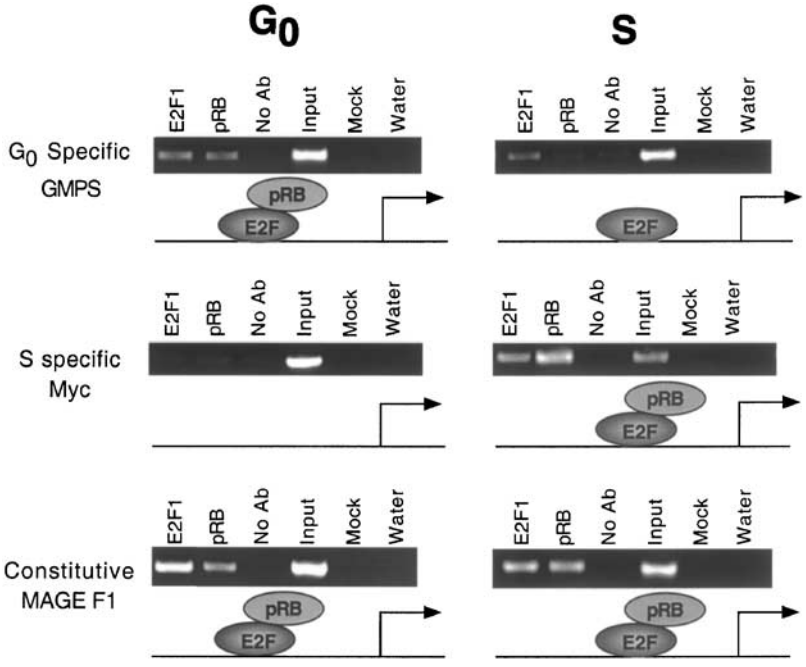

Figure 9 Different types of pRb/E2F binding sites. Schematic representing the three types of pRb/E2F binding sites, those that bind pRb only during S phase, those that bind pRb only during G_0/G_1 phase, those that bind pRb/E2F during both G_0/G_1 and S phase. Results from ChIP analysis of one binding site from each type of site is shown above the schematic

pRb and S phase chromatin, and using an antibody against E2F1 and S phase chromatin) all lead to the same conclusion; that E2F1 and pRb colocalize on chromatin in living cells.

Rb and E2F1 colocalize on chromatin during S phase

One of the most surprising findings of our study is the observation that pRb binds to numerous genomic locations during S phase and that these CpG islands are also bound by E2F1. The current model for E2F-regulated genes predicts that hyperphosphorylation of pRb at the G_1/S phase boundary results in dissociation of pRb from E2F. In fact, we showed this to be true for a set of previously characterized E2F target promoters (Figure 1). If the model held true for all pRb binding sites, we would have expected that genomic sites bound by E2F1 should not be bound by pRb during S phase and that pRb should be recruited to chromatin in an E2F-independent manner during S phase. However, in both the unbiased screen for E2F1 binding sites and in the unbiased screen for pRb binding sites, we found colocalization of both proteins on the same genomic regions during S phase. As expected, we showed by Western blot analysis that a large portion of pRb does become hyperphosphorylated in Raji cells during S phase. It was possible that the pRb bound to chromatin was protected from these phosphorylation events and/or that phosphorylation did not always result in the release of pRb from the chromatin. Further analysis of eight different genomic sites bound by pRb during S phase indicated that some of the chromatin-bound pRb appeared to be protected from phosphorylation and that phosphorylation did not always result in a complete loss of DNA binding activity. Thus, we conclude that the pattern of pRb binding cannot be predicted using a general model; rather, each individual promoter must be analysed.

Rb/E2F1 complexes may have a wide variety of functions in the cell

In both the G_0/G_1 and S phase screens, we identified genomic regions bound by pRb that correspond to promoters and regions which correspond to uncharacterized loci within the genome. The uncharacterized loci represented sites present at one unique location in the genome and also certain classes of repeated elements. We did not anticipate that pRb and E2F1 would bind to such a large number of repeated regions within the genome. At first, we assumed that repeated regions of the genome simply represented chromatin that was immunoprecipitated non-specifically. However, characterization of several of these genomic sites showed that these repeat regions were immunoprecipitated by antibodies against E2F1 or pRb but not in the absence of antibody or with normal rabbit serum (Figure 5 and data not shown). Curiously, several of the identified repeat regions are found only on one or two chromosomes, predominantly chromosomes 1 and 16.

We found that E2F1 and pRb colocalize on promoters of genes involved in cellular functions, such as DNA replication, repair, and recombination, previously known to be regulated by the E2F family (Muller *et al*., 2001; Weinmann *et al*., 2001; Ma *et al*., 2002; Polager *et al*., 2002; Ren *et al*., 2002). We also found that pRb bound to promoters of genes whose products participate in functions known to be regulated by pRb, such as embryogenesis, differentiation, and development. Finally, we found that E2F1 and pRb bind to the promoters of genes involved in metabolism and signaling, as well as to genes which function as oncogenes or tumor markers. In the light of the frequency with which the *Rb* gene is mutated in human cancers, we were not surprised to identify various oncogenes and tumor markers, such as *MAGE F1*, *DLEU1/2*, and *MFAP*, as target genes of pRb. Although no pRb target genes have been identified using DNA-based microarrays, several groups have performed such experiments using overexpression of E2F1 (Muller *et al*., 2001; Ma *et al*., 2002; Polager *et al*., 2002; Ren *et al*., 2002). Owing to the fact that we observed colocalization of pRb and E2F1, one might expect that the same sets of genes should be regulated by both proteins. However, the exact genes identified by our chromatin immunoprecipitation–CpG microarray analyses and the previous E2F1 overexpression cDNA-based microarray experiments are not identical (although the categories are similar). This could be due to the fact that the genes we identified were not on the cDNA arrays and/or regulation by E2F1 and pRb may be cell-type specific. Alternatively, we cannot rule out that binding of E2F1 and pRb to certain promoters does not result in a change in gene expression. Of course, the previous experiments examined only those genes that responded to overexpressed E2F1; it is not known if all of these genes are regulated by E2F1 under normal physiological conditions and few have been examined for their ability to be regulated by pRb.

Results from a previous study using human cells (Kennedy *et al*., 2000) suggested that pRb bound to sites of DNA replication during S phase. Studies of the pRb and E2F homologs from Drosophila suggest that dE2F1 and dRb function together at replication origins (Royzman *et al*., 1999; Bosco *et al*., 2001). Finally, we have shown that E2F1 binds to a viral origin of replication (Maser *et al*., 2001) and to the origin of replication located 30 kb downstream of the *dhfr* gene in hamster cells (Wells, Bartley, and Farnham, unpublished). As a result of the previous studies suggesting that S phase-specific pRb binding sites might correspond to sites of DNA replication, we were surprised that almost one-third of the sites we identified using S phase-specific chromatin are within the promoters of previously characterized genes, such as *Myc*. Although the *Myc* promoter is located within a few kb of a characterized origin of replication, additional origins of replication have not yet been localized near other promoters identified in our screen. However, it is clear that transcription factor binding sites can play important roles in enhancing DNA replication (Turner and Woodworth, 2001) and that origins are characterized

by the high frequency of consensus binding sites for transcription factors. Therefore, it is not surprising that promoters and origins will overlap to some extent. The previous studies implicating pRb in origin recognition found that pRb was not constitutively bound to origins and/or sites of DNA replication, but rather showed increased localization to these sites during S phase (Kennedy *et al*., 2000; Maser *et al*., 2001). Perhaps the sites identified by CpG microarray analysis, to which pRb binds only during S phase, would be good candidates as possible origins of replication. However, we do note that the previous results were performed using mainly primary cells, and we have used immortalized Raji cells.

In summary, our data suggest that pRb may play novel roles in the cell, perhaps by regulating the expression of target genes during both G_0/G_1 and S phases, by binding to repeat elements within the genome, and by binding to unique sites which may correspond to origins of replication rather than promoters. Of course, we do not yet know if pRb plays an important regulatory role at each of the newly identified genomic binding sites. Future studies addressing this issue are clearly needed. Surprisingly, each of the newly identified CpG islands bound by pRb is also bound by E2F1. This result suggests that interaction with E2F family members may be the primary method by which pRb is recruited to the chromatin, regardless of the stage of the cell cycle or the particular cellular process being regulated by pRb. However, even if recruited to the chromatin via an E2F family member, pRb may function to alter the activity of numerous other transcription factors bound nearby (Weintraub *et al*., 1995).

As can be seen by comparing the list of CpG islands identified by hybridizing S phase chromatin isolated using E2F1 versus Rb antibodies (Table 2 *versus* Table 4), the same clones were not identified in the two experiments. However, we found that, when examined in subsequent confirmatory chromatin immunoprecipitation experiments, almost all CpG islands identified using an antibody to E2F1 were also bound by Rb and *vice versa*. We now believe that this apparent discrepancy was due to inefficiencies during hybridization of the arrays with small amounts of immunoprecipitated chromatin. This resulted in the detection of only a subset of the positive clones and thus our results represent an underestimate of the true number of pRb and E2F binding sites in the genome. We have recently performed an additional CpG array screen using the pRb antibody (results not shown). In this second screen, stronger overall hybridization signals were obtained and 166 clones were identified. Importantly, most of the sites identified in the first screen were included in the larger number of sites obtained in the second screen. We are currently modifying the hybridization protocol to include a signal amplification step, which we hope will then allow detection of all positive CpG islands bound by a particular transcription factor. We also note that it is possible that the almost complete overlap between genomic regions bound by E2F1 and pRb may be due to the fact that we screened CpG islands. It remains possible that some of the approximately 50% of human genes that are not regulated by GC-rich promoter regions would show binding of pRb, but not E2F1. However, this analysis awaits the development of a comprehensive promoter array for the human genome.

Materials and methods

Cell culture and synchronization

Raji 525-7 cells (Albert *et al*., 2001b) were grown in RPMI-1640 medium (BRL-Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Nova-Tech, Grand Island, NE, USA), 1% penicillin–streptomycin (BRL-Life Technologies, Grand Island, NY, USA) and 200 μg of hygromycin B/ml (Calbiochem, San Diego, CA, USA) and grown in a 5% CO_2 incubator. Raji cells were synchronized by incubation in the presence of 2 mM thymidine (Sigma, St Louis, MO, USA) for 14 h, released into drug-free medium for 11 h, and incubated in the presence of 1 mg of aphidicolin/ml (Sigma, St Louis, MO, USA) for 14 h as previously described (Maser *et al*., 2001). Cells were then released into drug-free medium and harvested at 0 h (G_1/S phase), 3 h (early S phase), 5 h (mid-S phase), or 7 h (late S phase) after removal of aphidicolin. To synchronize cells in G_0/G_1 phase, Raji 525-7 cells were grown for 15 h in RPMI-1640 medium supplemented with 1% fetal calf serum (BRL-Life Technologies, Grand Island, NY, USA), 1% penicillin–streptomycin (BRL-Life Technologies, Grand Island, NY, USA) and 1.5% dimethylsulfoxide (DMSO; Sigma, St Louis, MO, USA) and harvested (Sawai *et al*., 1990; Ponzio *et al*., 1998). Replicate cultures of cells were trypsinized, fixed in ethanol, and stained with propidium iodide. Stained cells were analysed on FACS Caliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest acquisition and analysis software. Pulse width and area allowed exclusion of doublets. Cell cycle percentages were calculated with ModFit 2.0 software (Verity Software House, Topsham, ME, USA).

Western blot analysis

Raji cells (1×10^6), which had been synchronized by either thymidine/aphidicolin or DMSO treatment, were harvested by centrifugation at time points corresponding to G_0/G_1, G_1/S, early S, mid-S and late S phases. The degree of synchronization was measured by flow cytometry. Cell pellets were washed twice with $1 \times$ PBS and lysed by the addition of 100 μl of $3 \times$ SDS–PAGE buffer (1.3 M beta-mercaptoethanol, 9% SDS, 0.1% bromophenol blue, 30% glycerol). The resulting lysate was sheared by 10 passages through a 22 gauge needle and incubation at 95°C for 5 min. In all, 2 μl of each extract was diluted with water to a final concentration of $1 \times$ SDS–PAGE buffer and separated by electrophoresis on a 10% polyacrylamide gel, transferred onto a nitrocellulose membrane (Osmonics. Inc., Westborough, MA, USA), probed with an antibody against either pRb, phosphorylated pRb (serine 780, serine 795, or serines 807/811) or cyclin T1 and visualized by ECL (Amersham, Piscataway, NJ, USA). The antibody which recognizes pRb regardless of phosphorylation state (G3-245) was purchased from BD Pharmingen (San Diego, CA, USA), whereas a kit of antibodies against different phosphorylated states of pRb (9300) was purchased from Cell Signaling Technology (Beverly, MA, USA). The cyclin T1 (sc-8128) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Formaldehyde crosslinking and immunoprecipitation

Cells were crosslinked with formaldehyde and desired protein–DNA complexes were immunoprecipitated as previously described (Boyd *et al*., 1998) with the modifications described in Maser *et al*. (2001); a complete protocol can be found on our website at http://mcardle.oncology.wisc.edu/farnham/. A mixed monoclonal antibody against E2F1(KH20/KH95) was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA) and polyclonal antibodies against E2F4 (sc-866) and RNA polymerase II (sc-899) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies to pRb were as described for Western blot analysis.

Immunoprecipitates were dissolved in 30 μl of water, except for input samples which were dissolved in 100 μl. Each PCR reaction mixture contained 2 μl of immunoprecipitated DNA, 1 × Taq reaction buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 50 ng of each primer, 1.7 U of Taq polymerase (Promega, Madison, WI, USA), 200 μM deoxynucleotide triphosphates (Promega, Madison, WI, USA) and 1 M betaine (Sigma, St Louis, MO, USA) in a final reaction volume of 20 μl. PCR mixtures were amplified for one cycle of 95°C for 5 min, annealing temperature of the primers for 5 min, and 72°C for 3 min followed by 31–33 cycles of 95°C for 1 min, annealing temperature of the primers for 2 min, and 72°C for 1.5 min and one cycle of 72°C for 7 min. PCR products were separated by electrophoresis through 1.5% agarose gels and visualized by ethidium bromide intercalation. The sequences of the individual primers are available from our website.

CpG island microarray analysis

Raji cells were collected in mid-S or G_0/G_1 phase and a total of 50 individual chromatin immunoprecipitations per antibody were performed, using 1×10^7 cells per immunoprecipitation. In addition to immunoprecipitations with antibodies against pRb and E2F1, 50 immunoprecipitations were also performed with antiserum from a normal rabbit (Carney *et al*., 1998) as a measure of nonspecific background binding. Immunoprecipitations performed with the same antibody or preimmune sera were combined and labeled with Cy5 fluorescent dye as previously described (Yan *et al*., 2001). The labeled DNA fragments were then used to probe two human CpG island microarrays (Yan *et al*., 2001) containing 7776 clones. The ratio between the average signal intensities of known repeat sequences was used as a normalization factor for the two images. Clones hybridized to pRb and E2F1 chromatin having a normalized intensity at least twofolds above their preimmune sera counterparts were selected as positive clones. Individual positive clones were then sequenced by standard methods using primers corresponding to vector sequences.

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