Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 2945–2949, April 1994
Cell Biology

Cyclin D1 expression is regulated by the retinoblastoma protein

(cell cycle/promoter/transactivation)

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Communicated by Arthur B. Pardee, December 28, 1993 (received for review October 14, 1993)

ABSTRACT **The product of the retinoblastoma susceptibility gene, pRb, acts as a tumor suppressor and loss of its function is involved in the development of various types of cancer. DNA tumor viruses are supposed to disturb the normal regulation of the cell cycle by inactivating pRb. However, a direct function of pRb in regulation of the cell cycle has hitherto not been shown. We demonstrate here that the cell cycle-dependent expression of one of the G_1-phase cyclins, cyclin D1, is dependent on the presence of a functional Rb protein. Rb-deficient tumor cell lines as well as cells expressing viral oncoproteins (large tumor antigen of simian virus 40, early region 1A of adenovirus, early region 7 of papillomavirus) have low or barely detectable levels of cyclin D1. Expression of cyclin D1, but not of cyclins A and E, is induced by transfection of the *Rb* gene into *Rb*-deficient tumor cells. Cotransfection of a reporter gene under the control of the D1 promoter, together with the *Rb* gene, into *Rb*-deficient cell lines demonstrates stimulation of the D1 promoter by *Rb*, which parallels the stimulation of endogenous cyclin D1 gene expression. Our finding that pRb stimulates expression of a key component of cell cycle control, cyclin D1, suggests the existence of a regulatory loop between pRb and cyclin D1 and extends existing models of tumor suppressor function.**

The retinoblastoma susceptibility gene is a tumor suppressor gene. Loss of its function is involved in cancer development in various types of tissues (see ref. 1 for review). The oncoproteins of DNA tumor viruses were shown to form complexes with pRb and, thereby, most likely disturb the normal regulation of the cell cycle (2–5). Inhibition of synthesis of pRb in normal cells results in stimulation of cell division (6). The activity of pRb is supposed to be regulated by phosphorylation in a cell cycle-dependent manner (7–10). Underphosphorylated or nonphosphorylated pRb species predominate in the G_0 and early G_1 phases. In middle-to-late G_1, pRb becomes phosphorylated on serine and threonine residues and is increasingly phosphorylated during the S and G_2 phases before it undergoes dephosphorylation in mitosis (7–11). Underphosphorylated pRb has been demonstrated to form complexes with transcription factors including E2F (12–18). The interaction with E2F is supposed to result in repression of the activity of this positive transcription factor, which is known to stimulate the expression of genes required for S-phase control, such as c-myc, N-myc, and dihydrofolate reductase (DHFR) (12, 19). Phosphorylation or interaction with viral oncoproteins releases the pRb-imposed block of E2F activity (12). However, this S-phase stimulating activity is not the only one exerted by pRb, because regulation of progression through the G_1 phase is another (20)—probably the major—function of pRb.

The phosphorylated serine and threonine residues of pRb are located in sequence motifs reminiscent of those modified by cyclin-dependent kinases (21, 22). Cyclin D1 (23, 24) is expressed at the highest level in middle and late G_1 (25), suggesting it to be a candidate cyclin for targeting a cdk to pRb. Recently, interaction of pRb with the G_1-specific cyclin D1 has been demonstrated (26) and cyclin D–cdk4 complexes phosphorylate pRb in the insect cell system (27). The complex formation of pRb with cyclin D1 has striking similarities with that observed with viral oncoproteins. It involves the pocket region of pRb and a LXCXE motif of the partner protein (26). Cyclin D1-mediated phosphorylation of pRb by cdk4 could be the mechanism that down-regulates the activity of pRb in late G_1 phase, as suggested by Dowdy *et al.* (26), thereby allowing for $G_1 \rightarrow S$ transition. Thus, cyclin D1 seems to be the natural counterpart of pRb and the cellular "mimic" of the viral oncoproteins.

We were interested in investigating the interaction between pRb and cyclin D1 in more detail and studied the expression of cyclin D1 in Rb-deficient tumor cell lines as well as in cells expressing large tumor antigen (T antigen) of simian virus 40 (SV40), early region 1A (E1A) of adenovirus, or E7 of human papilloma virus (HPV). Here we demonstrate that these cells have dramatically reduced levels of cyclin D1. Upon transfection of a functional *Rb* gene into deficient tumor cells, expression of cyclin D1 is induced. Since these findings suggested that expression of cyclin D1 is directly stimulated by pRb, we carried out cotransfection experiments with the luciferase reporter gene under control of the promoter of the cyclin D1 gene. The results described here clearly show that the expression of cyclin D1 is stimulated by a certain level of pRb. Together with other data, this suggests the existence of a regulatory loop involving pRb and cyclin D1, which controls progression through the G_1 phase and transition into the S phase of the cell cycle.

MATERIALS AND METHODS

Cell Lines. MRC-5 are human diploid fibroblasts; MCF-7 are breast carcinoma cells; U20S are human osteosarcoma cells; Saos-2 are osteosarcoma cells with a deletion of exons 21–27 of the *RB1* gene; BT549 are breast carcinoma cells with gross rearrangement of the *RB1* gene; C33A are cervical carcinoma cells with a partial in-frame deletion of exon 20 in the *RB1* gene; MTSV2-1 are mammary epithelial cells transformed by T antigen of SV40; 293 are embryonic kidney epithelial cells transformed by adenoviral E1A; SiHa are cervical carcinoma cells transformed by HPV16. Characteristics of the cell lines are summarized elsewhere (28).

Recombinant Plasmids. Plasmid pCMVRb was constructed by cloning the *Rb1* cDNA (29) as a 4.9-kb *Asp*718/*Bam*HI

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Abbreviations: T antigen, large tumor antigen; SV40, simian virus 40; HPV, human papilloma virus; CMV, cytomegalovirus.
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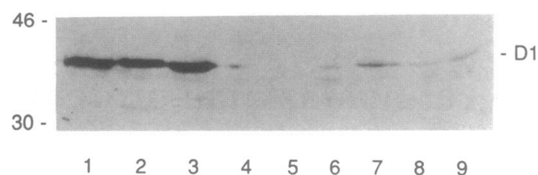


FIG. 1. Accumulation of cyclin D1 protein is significantly reduced in cell lines with defective Rb (BT549, lane 4; Saos-2, lane 5; C33A, lane 6) or in cell lines transformed by a viral oncogene (MTSV-1, lane 7; 293, lane 8; SiHa, lane 9). Normal human diploid fibroblasts (MRC-5, lane 1) and two tumor cell lines containing wild-type Rb (MCF-7, lane 2; U20S, lane 3) show levels of cyclin D1 protein corresponding to those normally present in asynchronously growing cells. Equal amounts of cell extracts were electrophoretically separated on SDS/12.5% polyacrylamide gels and transferred onto nitrocellulose. Cyclin D1 was detected with the monoclonal antibody DCS-6. Numbers on left are kDa.

fragment into the *Bam*HI site of pCMV linking the *Rb* gene to the cytomegalovirus (CMV) promoter/enhancer (30). The promoter of the cyclin D1 gene (31) was amplified by PCR (A.S. and M.E., unpublished data). It was cloned as a 1.5-kb *Eco*RI/*Pvu* II fragment in front of the luciferase gene (32) to give pD1luc. pSVRb was constructed by insertion of the *Asp*718/*Bam*HI Rb fragment (4.9 kb) into the *Hin*dIII site of pSV2neo (33), replacing the *neo* gene. The C-terminal truncation of the *Rb* cDNA was generated by cutting out the internal *Nhe* I fragment (2195 bp) from the *Rb* cDNA, filling in, and religating. This results in a truncation after amino acid 660 (exon 20), whereas the mutant protein in Saos-2 extends into exon 21 (34). A frameshift mutant pCMVRbfs was generated by cutting the *Eag* I site 21 bp downstream of the start codon of *Rb*, filling in, and religating.

Transfection/Electroporation. Exponentially growing BT549 cells were transfected by electroporation using the Bio-Rad gene pulser with the following settings: 270 V and 125 μF or 230 V and 250 μF in 0.4-cm cuvettes and 50 μl of PBS. After 48 hr, the cells were either harvested and processed for immunoblotting or fixed with methanol/acetone and subjected to indirect immunofluorescence.

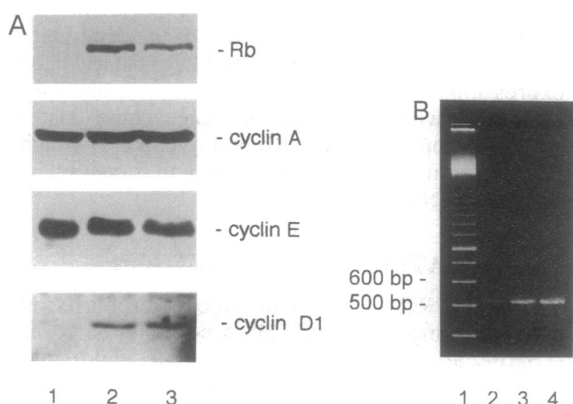


FIG. 2. Synthesis of cyclin D1 protein and RNA is stimulated by transfection with the wild-type *Rb* gene, while expression of cyclins E and A remains unaffected. (*A*) Western blot analysis of mock-transfected BT549 cells (lane 1) or cells transfected with pCMVRb in two independent experiments differing in electroporation settings (lanes 2 and 3). Transfection with pCMVRbΔC had no effect on cyclin expression (data not shown). (*B*) Detection of D1-specific RNA by reverse transcription and PCR. Mock-transfected BT549 cells (lane 2), BT549 cells transfected with pCMVRb (lane 3), and MCF-7 control cells (lane 4). A 100-bp ladder was run for calibration of the gel (lane 1). The amounts of input RNA and of reaction product applied onto the gel were identical for lanes 2 and 3 but were lower for lane 4 (the band from MCF-7 cells served as a marker).

Cotransfection experiments were carried out in triplicate in 2×10^5 cells in 5-cm dishes by the calcium phosphate technique with 3 μg of pD1luc and 6 μg of the respective Rb construct or the corresponding promoter plasmid in the standard experiments and with increasing amounts of Rb plasmids complemented by calf thymus DNA to give a total of 19 μg in the dose–response experiments. Extracts were prepared 36 hr after transfection and were tested for luciferase activity in a luminometer (Berthold, Wildbad, Germany).

RNA Analysis. For detection of D1-specific RNA by PCR, reverse transcription was carried out with purified cytoplasmic RNA (0.1 μg) from each cell type by using an oligo(dT) primer. One-tenth of the cDNA was subjected to PCR for 50 cycles using D1-specific primers corresponding to nt 332–355 (5′-GGATGCTGGAGGTCTGCGAGGAAC-3′) and nt 822–845 (5′-GAGAGGAAGCGTGTGAGGCGGTAG-3′) of the D1 cDNA sequence, and half of the reaction was run on a 3:1 NuSieve agarose gel.

Northern blot hybridization was carried out according to a standard procedure (35) using 10 μg of total RNA and the complete cDNA fragment of human cyclin D1 as a probe labeled by random priming.

Immunoblotting and Immunostaining. A monospecific monoclonal antibody (DCS-6) to cyclin D1 (36) was used for Western blotting and immunostaining. This antibody detects all of D1 in Western blotting and immunoprecipitation when compared with a polyclonal antibody (36).

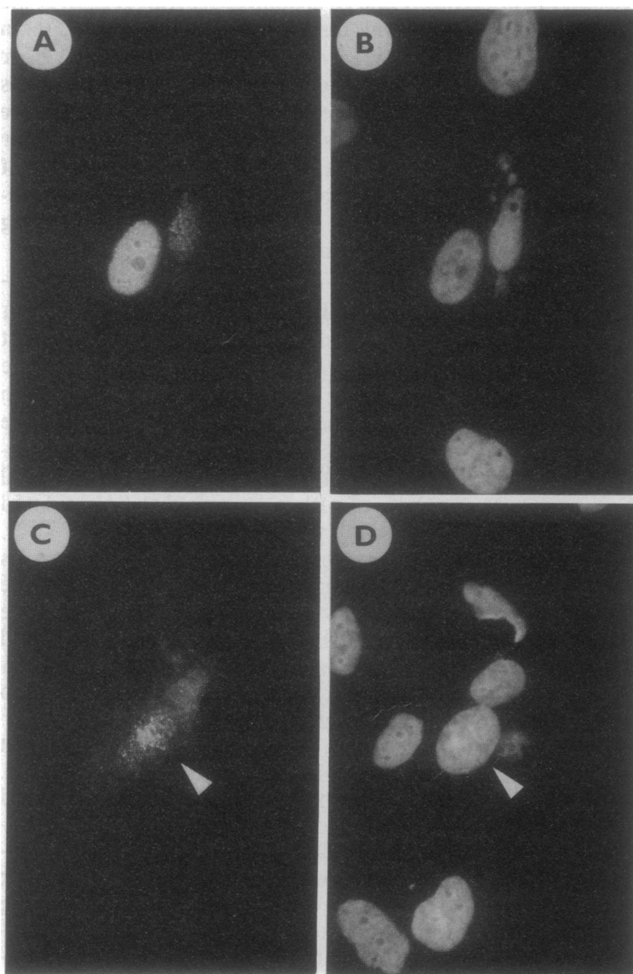


FIG. 3. Immunofluorescence analysis showing effective expression of the wild-type *Rb* gene in BT549 cells (*A*) and localization of the D1 protein in the nucleus of *Rb*-transfected cells (*C*). (*B* and *D*) Nuclei of the corresponding cells stained with Hoechst dye.

Western blotting of 12.5% polyacrylamide gels was done according to the standard semidry method (37) and filters were subjected to immunodetection by the ECL detection system (Amersham). Rabbit antibodies to human cyclin A (38) were obtained from M. Pagano and G. Draetta (Mitotix, Cambridge, MA) and mouse monoclonal antibodies to cyclin E (39) were obtained from E. Lees and E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown). For immunostaining, cells were grown on coverslips and fixed for 10 min in cold methanol/acetone (1:1), rehydrated in PBS at room temperature, and incubated with DCS-6 overnight in the cold in a humidified chamber. After three washes in PBS, coverslips were incubated with biotinylated horse anti-mouse secondary antibody (Vector Laboratories; dilution, 1:150) at room temperature for 1 hr. After three washes with PBS, cells were incubated with Texas Red-conjugated streptavidin (Vector Laboratories; dilution, 1:100). After a final wash with PBS, samples were mounted in Gelvatol. Staining for DNA was performed with 1 μg of bisbenzimide per ml (Hoechst 33258; Sigma) in PBS.

RESULTS

Low Level of Cyclin D1 in Rb-Deficient Cells. Since pRb is known to regulate progression through G_1 phase of the cell cycle, we have tested for a direct link between its function and expression of the G_1-specific cyclin D1. Various tumor cell lines were analyzed by Western blotting. Interestingly, this analysis showed that, in Rb-deficient tumor lines, the level of cyclin D1 was dramatically reduced or barely detectable (Fig. 1). As the transforming proteins of the small DNA tumor viruses are supposed to inactivate some functions of pRb by forming protein complexes, we also tested cell lines expressing T antigen of SV40, E1A protein of adenovirus, or E7 protein of HPV. As shown in Fig. 1, the level of cyclin D1 protein was considerably reduced compared with the corresponding control cell lines. Thus, in cells lacking a functional pRb the expression of cyclin D1 is very low.

Ectopic Rb Induces Cyclin D1 but Not Cyclins A and E. To demonstrate directly a requirement of functional pRb for induction of cyclin D1, we transfected the wild-type *Rb* cDNA under the control of the strong immediate early promoter of CMV into BT549 cells, a Rb-deficient breast cancer line. Direct Western blotting (Fig. 2*A*) demonstrates that expression of cyclin D1 is induced in the transfected cells expressing pRb. No effect on the protein levels of cyclins A and E was detected (Fig. 2*A*), showing the specificity of the D1 stimulation and virtually unaltered distribution of cell

A

CTCGAGGCGGACGGGGCCCCCTGCACCCCTCTTCCCTGGCGGGGAGAAAGGCTGCAGCG

GGGCGATTTGCATTTCTATGAAAACCGGACTACAGGGGCAACTCCGCCGCAGGCAGGCGCG

GCGCCTCAGGGATGGCTTTTGGGCTCTGCCCCTCGCTGCTCCCGGCGTTTGGCGCCCGCG
E2F

CCCCCTCCCCCTGCGCCCGCCCCCGCCCCCCTCCCGCTCCCATTCTCTGCCGGGCTTTGAT
SP-1 SP-1 Inr

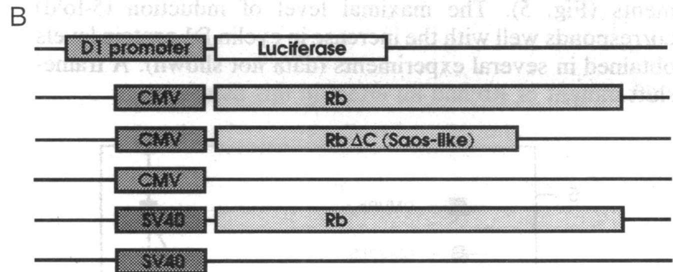
CTTTGCTTAACAACAGTAACGTCACACGGACTACAGGGGAGTTTTGTTGAAGTTGCAAAGTCC

TGGAGCCTCCAGAGGGCTGTCGGCGCAGTAGCAGCGAGCAGCAGAGTCCGCACGCTCCGG

CGAGGGGCAGAAGAGCGCGAGGGAGCGCGGGGCAGCAGAAGCGAGAGCCGAGCGCGGA

CCCAGCCAGGACCCACAGCCCTCCCCAGCTGCCCAGGAAGAGCCCCAGCCATG
Pvu II Met

B



C

cell line	construct	RLU (x10^3) 100 200 300	induction
C33A	pD1 Luc pCMV		—
	pD1 Luc pCMV Rb		2.3
	pD1 Luc pSV40		—
	pD1 Luc pSV40 Rb		2.5
	pD1 Luc pCMV RbΔC		1
BT 549	pD1 Luc pCMV		—
	pD1 Luc pCMV Rb		3
	pD1 Luc pCMV RbΔC		1.5

D

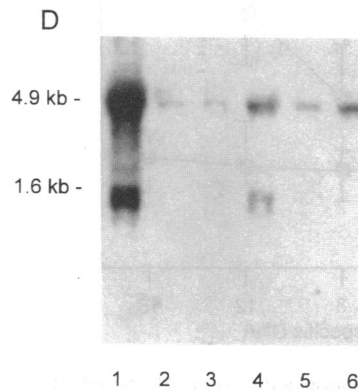


FIG. 4. Cyclin D1 promoter is transactivated by cotransfection with the *Rb* gene. (*A*) Partial sequence of the cyclin D1 promoter. The *Pvu* II site was used for ligation to the luciferase gene. The sequence contains an initiator element, an E2F consensus binding site, at least two Sp1 binding sites, and several other potential factor binding sites, which are not indicated. (*B*) Schematic maps of the plasmids used. pCMVRb, pSVRb, and pD1luc are described in *Materials and Methods*. pCMV and pSV are the promoter control plasmids without the *Rb* gene. pSVRbΔC contains a C-terminally truncated Rb gene. (*C*) Reporter gene assays were carried out in C33A and BT549 cells, which have defective *Rb* genes. Luciferase activities are given as relative light units (RLU) per 5 × 10^5 cells. Background level of mock-transfected cells (320 RLU) was subtracted in all cases. (*D*) Northern blot analysis of cyclin D1-specific mRNA in control MCF-7 (lane 1), Saos-2 (lane 2), BT549 (lane 3), BT549 + pCMVRb (lane 4), C33A (lane 5), and C33A + pCMVRb (lane 6) cells. Note that both cyclin D1-specific RNA species are induced by transfection of the *Rb* gene.

cycle phases after pRb expression. The induction of cyclin D1 upon transfection with pCMVRb is also demonstrated by immunofluorescence studies (Fig. 3). In addition, analysis of D1-specific RNA by reverse transcription and PCR revealed an increase in the D1-specific band in transfected cells (Fig. 2*B*).

Activation of the D1 Promoter by pRb. The results described above suggest that pRb stimulates expression of cyclin D1. To test for this direct link, we investigated activation of the promoter of the D1 gene by pRb. The human cyclin D1 gene promoter was cloned (A.S. and M.E., unpublished data), sequenced (Fig. 4*A*), and linked to the luciferase reporter gene (pD1luc). When the reporter plasmid was cotransfected together with pCMVRb or pSVRb (Fig. 4*B*) into Rb-deficient C33A or BT549 cells, the expression of luciferase from the D1 promoter was stimulated 2.3- to 3-fold (Fig. 4*C*), indicating that pRb can indeed activate the cyclin D1 promoter. This factor of stimulation is highly reproducible for the given ratio of the two plasmids. The stimulation of luciferase expression from the ectopic cyclin D1 promoter corresponds to an increase in the endogenous D1-specific mRNA as detected by Northern blot analysis (Fig. 4*D*). However, the levels of induction are lower in the transient reporter gene assay as compared with stimulation of the endogenous cyclin D1 gene. In the same reporter gene assay, a C-terminal truncation mutant of pRb, which corresponds to the Saos-2 mutant protein (34), has little if any effect on the D1 promoter-dependent luciferase activity (Fig. 4*C*), confirming the requirement for a functional Rb protein. This conclusion was also supported by dose-response experiments (Fig. 5). The maximal level of induction (5-fold) corresponds well with the increase in cyclin D1 protein levels obtained in several experiments (data not shown). A frameshift mutant of Rb had no effect in this assay.

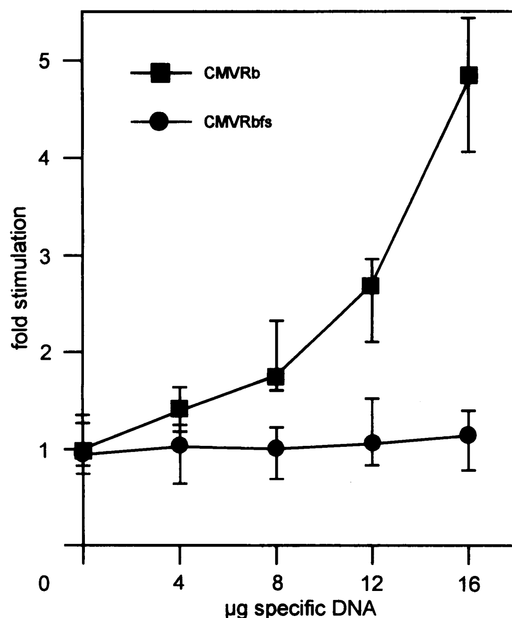


FIG. 5. Dose-dependent stimulation of the cyclin D1 promoter by pRb. C33A cells were transfected with 3 μg of pD1luc together with the indicated amounts of either pCMVRb or pCMVRbfs plus calf thymus DNA to give a final amount of 19 μg of DNA. A frameshift mutant of Rb was used as the control in order to avoid unspecific effects of a mutant protein and to show that the DNA of the CMVRb construct does not exert any effect. Relative light units were measured after 36 hr and individual numbers were divided by the values obtained from control transfections with pD1luc and calf thymus DNA only to give the factor of stimulation.

DISCUSSION

Our results show that expression of the G_1-specific cyclin D1 is positively regulated by pRb at the transcriptional level, providing evidence for direct regulation of the expression of a cell cycle protein by this tumor suppressor. Expression of cyclin D1 is restricted to the G_1 phase of the cell cycle in both normal (23, 40, 41) and tumor (36) cells. The activity of pRb is assumed to be regulated by phosphorylation/dephosphorylation during the cell cycle, with the underphosphorylated species being the active form in G_1 phase (7–11, 20). Stimulation of cyclin D1 expression by pRb suggests that the cyclin D1 gene is a downstream target of Rb. Cyclin D1, in turn, would regulate G_1 progression. Cyclin D1 forms a complex with pRb (26) and phosphorylation of pRb seems to result from the complex-mediated targeting of CDK4 (27). The phosphorylated pRb would no longer be able to stimulate D1 expression. This would suggest that in Rb-positive cells, cyclin D1 is an essential counterpart for pRb by participating in its inactivation and thereby regulating the G_1 to S transition. Inactivation of the D1 protein would lead to cell cycle arrest (23, 36). According to this model of a regulatory loop, cyclin D1 appears to function as a key regulator of G_1 phase progression at the point that is reminiscent of the restriction point suggested by Pardee (42).

Hinds *et al.* (43) have shown that overexpression of cyclins A and E but not of cyclin D1 stimulates phosphorylation of pRb in Saos-2 cells. However, all three cyclins could override the growth-inhibiting effect of pRb to some extent. This result suggests the existence of an alternative mechanism for reducing the amount of functional pRb, at least in Saos-2 cells. Other authors could demonstrate cyclin D1-dependent phosphorylation of pRb in diploid cells (50). Thus, it remains to be clarified by which mechanism D1 downmodulates the function of pRb in different cell types.

The positive correlation between pRb and cyclin D1 was detected in cycling cells expressing ectopic Rb. It has been shown by several investigators that overexpression of pRb in Rb-deficient tumor cells (ref. 44 and references therein) and in Rb-positive cells (44) can reduce their cell cycle activity. It was also suggested recently that elevated amounts of pRb might be involved in regulation of differentiation (45, 46). Thus, it will be interesting to determine whether the growth-inhibiting effect of high levels of pRb corresponds to the induction of differentiation and whether this effect is related to changes in the expression of cyclin D1.

Our data provide clear evidence for a gene-activating function of pRb. This stimulatory function was previously detected only in the cases of the TGFβ1 and IGF-II promoters (47, 48) and was attributed to stimulation of Sp1 by pRb (48). Indeed, the D1 promoter contains at least two perfect Sp1 consensus binding sites in close vicinity to the initiator element (Fig. 4*A*). On the other hand, it also contains an E2F consensus binding site, which would be regarded as a potential response element for negative regulation by pRb (12–15, 49). However, a negative effect of pRb on cyclin D1 expression has not yet been detected. Our findings suggest that the current view of pRb being only a repressor of positive transcription factors like E2F/DRTF1 acting on promoters that are regulated in a cell cycle-dependent manner must be modified. The molecular mechanisms underlying the dual function of pRb as a repressor or activator of genes remains an intriguing puzzle for future studies.

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