

DNA Tumor Virus Oncoproteins and Retinoblastoma Gene Mutations Share the Ability to Relieve the Cell's Requirement for Cyclin D1 Function in G1

Jiri Lukas,* Heiko Müller,** Jirina Bartkova,* Dimitry Spitzkovsky,§ Anne A. Kjerulff,* Pidder Jansen-Dürr,§ Michael Strauss,** and Jiri Bartek*

*Danish Cancer Society, Division for Cancer Biology, DK-2100 Copenhagen, Denmark; **Max-Planck-Gesellschaft, D-13122 Berlin-Buch, Germany; and §Angewandte Tumorstudiologie, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, Germany

Abstract. The retinoblastoma gene product (pRB) participates in the regulation of the cell division cycle through complex formation with numerous cellular regulatory proteins including the potentially oncogenic cyclin D1. Extending the current view of the emerging functional interplay between pRB and D-type cyclins, we now report that cyclin D1 expression is positively regulated by pRB. Cyclin D1 mRNA and protein is specifically downregulated in cells expressing SV40 large T antigen, adenovirus E1A, and papillomavirus E7/E6 oncogene products and this effect requires intact RB-binding, CR2 domain of E1A. Exceptionally low expression of cyclin D1 is also seen in genetically RB-deficient cell lines, in which ectopically expressed wild-type pRB results in specific induction of this G1 cyclin. At the functional level, antibody-mediated cyclin D1 knockout experiments demonstrate that the cyclin D1 protein, normally required for G1 progression,

is dispensable for passage through the cell cycle in cell lines whose pRB is inactivated through complex formation with T antigen, E1A, or E7 oncoproteins as well as in cells which have suffered loss-of-function mutations of the RB gene. The requirement for cyclin D1 function is not regained upon experimental elevation of cyclin D1 expression in cells with mutant RB, while reintroduction of wild-type RB into RB-deficient cells leads to restoration of the cyclin D1 checkpoint. These results strongly suggest that pRB serves as a major target of cyclin D1 whose cell cycle regulatory function becomes dispensable in cells lacking functional RB. Based on available data including this study, we propose a model for an autoregulatory feedback loop mechanism that regulates both the expression of the cyclin D1 gene and the activity of pRB, thereby contributing to a G1 phase checkpoint control in cycling mammalian cells.

THE small DNA viruses, notably the papovaviruses, adenoviruses, and papillomaviruses share several features that reflect the common need to replicate their own genomes free from the restrictions which regulate DNA synthesis of the host cell. These viruses evolved as successful parasites that utilize many of the basic cellular metabolic processes, by intimately interacting with, and subverting the key points of the cell's regulatory controls (reviewed in Dyson and Harlow, 1992; Fanning, 1992; Munger et al., 1992; Nevins, 1992). Owing to the relative simplicity of the DNA tumor viruses and to broad interest in mechanisms of DNA replication, transcription control, RNA metabolism, and tumorigenesis, these viruses have provided very powerful research tools and their relationship with the host cell has become a fruitful field of study. One of the unifying aspects in the field appears to be the ability of the multifunctional viral oncoproteins: the papovavirus large T antigens, adenovirus

E1A proteins, and papillomavirus E7 oncogenes, to induce cellular DNA synthesis and thus act as potent deregulators of cell cycle control (Dobbelstein et al., 1992; Dyson and Harlow, 1992; Fanning, 1992; Munger et al., 1992). These three types of proteins have recently been shown to function, at least in part, through direct physical protein-protein interactions that target a series of key cellular regulators including product of the retinoblastoma tumor suppressor gene (pRB), pRB-related p107, cyclin A, cyclin E, and cdk2 kinase (DeCaprio et al., 1988; Whyte et al., 1988; Dyson et al., 1989; Giordano et al., 1989; Pines and Hunter, 1990; Faha et al., 1993). The SV-40 large T antigen, adenovirus E1A, and papillomavirus E7 proteins bind the underphosphorylated, G0/G1-specific, active form of pRB (Ludlow et al., 1989; Imai et al., 1991; Dyson and Harlow, 1992) thereby inactivating its function in a way equivalent to mutation or loss of the RB gene frequently seen in human neoplasias (reviewed in Cobrinik et al., 1992; Goodrich and Lee, 1993). Mutant DNA virus oncoproteins that have lost their ability to form complexes with pRB have significantly reduced

Address all correspondence to J. Bartek, Danish Cancer Society, Division for Cancer Biology, Strandboulevarden 49, DK-2100 Copenhagen, Denmark.

transforming potential, suggesting that binding of the RB tumor suppressor by large T, E1A, and E7 oncogenes is critical to their oncogenic activity (reviewed in Dyson and Harlow, 1992; Goodrich and Lee, 1993). The identification of sequences within the RB protein, the so-called "RB pocket," responsible for its interaction with T antigen, E1A, and E7 oncogenes stimulated the search for cellular proteins which would bind pRB in a manner analogous to DNA virus oncoproteins (Hu et al., 1990; Huang et al., 1990; Kaelin et al., 1990). Several host proteins have recently been shown to specifically interact with the RB pocket domain, but the functions of most of those remain elusive (Huang et al., 1991; Kaelin et al., 1991). However, there are at least two proteins of known functions among the RB-binding cellular partners and their analysis is beginning to shed some light on the molecular mechanisms underlying the control of cellular proliferation. One of these RB partner proteins is the E2F transcription factor whose target sequence is found in the promoters of a series of cellular genes involved in growth control, including several proto-oncogenes and enzymes essential for DNA replication (Bandara et al., 1991; Chellapan et al., 1991; Chittenden et al., 1991; Helin et al., 1992). This physical interaction with pRB is believed to sequester E2F's transcriptional activity which is restored upon disruption of the pRB-E2F complex through cell cycle-regulated phosphorylation of pRB in late G1 or via direct competition for the RB pocket domain by any of the above mentioned DNA virus oncoproteins (Bandara et al., 1991; Chellapan et al., 1991; Chittenden et al., 1991; Helin et al., 1992). The latter scenario illustrates one of the molecular hallmarks shared by SV-40 T antigen, adenovirus E1A, and papillomavirus E7 proteins, namely their capacity to release the transcriptionally active E2F from its inactive and/or transcriptionally repressive complex with pRB, thereby enhancing the S phase competence of the host cell and either promoting replication of the respective viral genomes (during lytic infection) or contributing to deregulated proliferation of the virally transformed cells (Nevins, 1992). On a similar note, the same RB-binding regions of these viral transforming proteins target the RB-related p107 host protein whose complexes with either E2F alone or E2F, cyclin A and cdk-2 are present during G1 and whose multiprotein interactions involving E2F together with cyclin A and cdk2 kinase are believed to perform a regulatory role during S phase (Bandara et al., 1991; Ewen et al., 1991; Mudryj et al., 1991; Lees et al., 1992; Pagano et al., 1992a; Shirodkar et al., 1992). The G1-specific p107-E2F complex has recently been implicated in regulation of the E2F's transcriptional activity at the G1 > S transition (Schwarz et al., 1993) and it remains to be seen if the respective functions of RB-E2F- and p107-E2F-containing complexes in G1 phase progression are unique or redundant.

The most recent addition to the list of cellular RB-binding proteins appear to be the D-type cyclins (Lew et al., 1991; Matsushime et al., 1991; Motokura et al., 1991; Xiong et al., 1991). Cyclin D1, D2, and D3 have been shown to interact with unphosphorylated and/or underphosphorylated pRB via their NH₂-terminal L-X-C-X-E motifs analogous to those present in homology regions II of the DNA virus oncoproteins (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993). Complexes of D-cyclins with the cdk4 kinase efficiently phosphorylate pRB *in vitro* and in insect cells, but unlike other cyclin-cdk complexes, they are inefficient in

phosphorylating histone H1 (Kato et al., 1993; Matsushime et al., 1992; Xiong et al., 1992). This substrate selectivity may indicate that cyclin D-cdk4 is a relevant pRB kinase *in vivo* and that the physical interaction of D-cyclins with pRB may therefore help to direct cdk4 to this substrate during G1 phase, resulting in pRB phosphorylation and the inactivation of pRB's growth-constraining function. Although provocative, the experimental evidence for functional interaction of pRB and D-type cyclins is largely based on experiments performed *in vitro* and its relevance to regulation of G1 progression and/or G1 > S transition in a living cell remains to be rigorously established.

Given that the important cell cycle control points are frequently targeted by the transforming DNA virus oncoproteins and intrigued by our preliminary observation of strikingly low cyclin D1 protein levels in several human cell lines either transformed by DNA virus oncogenes or harboring RB gene mutations (Bartek et al., 1993a; Lukas et al., 1994), we decided to investigate the potential functional relationship between these viral transforming proteins, pRB and cyclin D1, in more detail. We now report that pRB plays a positive role in regulation of cyclin D1 expression and that SV-40 large T antigen, adenovirus E1A, papillomavirus E7/E6 proteins, and inactivating RB gene mutations share the capacity to eliminate the requirement for functional cyclin D1 in regulation of G1 phase progression.

Materials and Methods

Cell Culture and Synchronization

Human diploid fibroblasts (IMR-90 and MRC-5) were purchased from the American Type Culture Collection. The UMSCC-2 squamous carcinoma cell line was provided by T. Carey (University of Michigan, Ann Arbor, MI), the rat RetA7 cells conditionally transformed by the A7 ts mutant of the SV40 large T antigen gene were obtained from M. Graessmann (Free University, Berlin, Germany); the human skin keratinocyte-derived cell line HaCaT was donated by N. E. Fusenig (German Cancer Research Center, Heidelberg, Germany); human embryonic retina cells (HER)¹ by O. Kranenburg (Sylvius Laboratory, Leiden); and the SW756 cell line, originally derived from a squamous cell carcinoma and shown to display hormone-dependent expression of HPV 18 E7/E6 as a result of integration of the HPV genome in the vicinity of a dexamethasone-responsive cellular sequence (von Knebel Doeberitz et al., 1991), was a gift of M. von Knebel Doeberitz, German Cancer Research Center, Heidelberg, Germany. Primary mammary epithelium (RME), primary human foreskin fibroblasts (HFF), and the human tumor-derived cell lines were obtained from the collections of the Department of Cell Cycle and Cancer (Danish Cancer Society, Copenhagen, Denmark) and the ATV Collection (German Cancer Research Center), and their names and origins are listed in Tables I and II. The cells were cultured in DME supplemented with 10% FCS, 2 mM glutamine, 10 U/ml penicillin, and 10 U/ml streptomycin. The RMT-1 and RMT-2 lines were established in the present study, using the retroviral vector-mediated gene transfer to immortalize the normal human mammary epithelial (RME) cells via expression of the SV-40 large T antigen. The amphotropic retroviral vector and the infection and selection procedures used in this study were essentially as described by Bartek et al. (1991).

Tumor cell lines were synchronized in mitosis by 18 h incubation in the presence of 40 ng/ml nocodazole (Sigma, St. Louis, MO). The rounded cells were subsequently detached from the substrate by gentle shaking and pipetting up and down. After two washes in fresh DME without nocodazole the cells were replated into fresh medium and two hours later, the nonattached cells were washed out. Progression through the cell cycle was monitored by flow cytometry (FACScan; a registered trademark of Becton Dickinson Immunocytometry Sys., Mountain View, CA) after staining DNA with 50 µg/ml propidium iodide (Sigma) in the presence of 10 µg/ml

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CRBP, cellular Rb-binding proteins; HER, human embryonic retina cells; HFF, primary human foreskin fibroblasts; RME, primary mammary epithelium.

DNase-free RNase (Boehringer Mannheim GmbH, Mannheim, Germany). Alternatively, bromodeoxyuridine (BrdU; Sigma) or [^3H]thymidine (Amersham Corp., Arlington Heights, IL) incorporation was monitored (see microinjection paragraph for details).

Antibodies

The DCS-6 mouse monoclonal antibody (IgG2a) specific for cyclin D1 protein, applicable in immunoblotting, immunocytochemistry, and immunoprecipitation, has recently been described by Lukas et al. (1994). The rabbit polyclonal antisera against human cyclin D1 (Baldin et al., 1993) and human cyclin A (Pagano et al., 1992b) were obtained from M. Pagano and G. Draetta (Mitotix, Cambridge, MA), rabbit anti-cdk4 and anti-cdk6 antisera were a kind gift from G. Peters (ICRF, London, UK). Mouse monoclonal antibodies to human cyclin A (Giordano et al., 1989), adenovirus E1A (Harlow et al., 1985), human p107 protein and cyclin E (Lees et al., 1992) were provided by E. Harlow, Kristian Helin, and E. Lees (MGH Cancer Center, Charlestown, MA) and the mouse mAb Pab419 to SV-40 T antigen was obtained from D. Lane (CRC Laboratories, Dundee, Scotland). Anti-BrdU monoclonal antibody was purchased from (Partec Reinach, Switzerland) and diluted 1:50, FITC-conjugated rabbit anti-mouse IgG antibody was obtained from Sigma and diluted 1:100. Biotinylated antiserum to mouse immunoglobulin and Texas red-conjugated streptavidin were from Vector Laboratories (Burlingame, CA).

Immunocytochemistry

Cell monolayers growing on glass coverslips were rinsed in PBS and fixed for 10 min in cold methanol-acetone (1:1) or for 5 min in 100% methanol followed by a 2-min treatment in acetone at -20°C . Fixed cells were rehydrated in PBS at room temperature and processed for cell staining. Anti-cyclin D1 ascitic fluid was diluted 1:200, hybridoma supernatants were used nondiluted. Incubation with primary antibodies was carried out overnight at 4°C in a humidified chamber. After three washes in PBS the coverslips were incubated for 1 h at room temperature with biotinylated horse anti-mouse secondary antibody (dilution 1:150; Vector Laboratories). Cells were washed again three times with PBS and incubated with Texas red-conjugated streptavidin (dilution 1:100; Vector Laboratories). After a final wash with PBS, immunofluorescence samples were directly mounted in Gelvatol. Counterstaining for DNA was performed by adding $1\ \mu\text{g}/\text{ml}$ bis-benzimide (Hoechst 33258; Sigma) into the final PBS wash. The cells in S-phase were detected by thymidine incorporation followed by in situ autoradiography as described by Lukas et al. (1994).

Gel Electrophoresis and Immunoblotting

Total cell extracts were prepared by in situ direct lysis of cell monolayers with hot Laemmli sample buffer (without bromophenol blue). Protein concentration was measured using the Bio-Rad SDS-compatible DC Protein Assay kit. Extracted proteins were electrophoretically separated on 12.5% SDS-PAGE. Gels were either stained with Coomassie blue to control for the balanced loading or blotted onto nitrocellulose (Schleicher and Schuell Inc., Keene, NH) by semi-dry method (Lukas et al., 1994). Filters were subjected to immunoblotting using the ECL (Amersham Corp.) detection system according to the manufacturer's instructions.

Northern Blot Analysis

Total cellular RNA was extracted by the guanidinium thiocyanate/acid phenol method. $10\ \mu\text{g}$ of RNA was electrophoresed on 1% agarose formaldehyde gels and transferred onto nylon membranes. Expression of cyclin D1 was monitored with either the mouse *cyl 1* probe (Matsushime et al., 1991) or the human probe (Xiong et al., 1991), dependent on whether rodent or primate cells were analyzed. Glyceraldehyde-3-phosphate dehydrogenase mRNA expression was analyzed in parallel to control for balanced gel loading.

Microinjection and Electroporation

Cells were synchronized by nocodazole shake-off as described above and microinjected upon replating and attachment, using the automatic microinjection system (AIS; Carl Zeiss, Inc., Thornwood, NY) as described previously (Baldin et al., 1993). Affinity-purified anti-cyclin D1 monoclonal and polyclonal antibodies, or control rabbit or mouse preimmune immunoglobulin ($2\ \text{mg}/\text{ml}$ in PBS) were microinjected into the cytoplasm or directly into the cell nucleus. Immediately after microinjection, the growth medium

was supplemented with BrdU at a final concentration of $100\ \mu\text{M}$ or tritiated thymidine at $1\ \mu\text{Ci}/\text{ml}$. Cells were incubated until they reached the peak of S-phase (80% or more BrdU/thymidine-positive cells; the time varied between 18 and 32 h after replating, depending on the cell line) and fixed and stained for BrdU or developed for autoradiography (see above). Alternatively, the nocodazole-synchronized cells were electroporated by the purified antibodies following the protocol developed by Lukas et al. (1994). The effect of electroporated antibody on the progression of cells into S-phase was analyzed by means of combined immunofluorescence (to identify the immunoglobulin-containing cells) and [^3H]thymidine incorporation/autoradiography at selected time points as indicated in the text. For each time point, 200 immunoglobulin-containing cells per coverslip were scored for evidence of thymidine incorporation. The data are expressed as the mean value of percentage of S-phase inhibition, obtained by comparison of the fractions of S-phase cells in the controls (treated with normal mouse or rabbit Ig, which had no detectable effect on S-phase entry) with S-phase cells in the anti-cyclin D1 antibody-treated cell populations, respectively. This value is close to zero in cells refractory to cyclin D1 knockout while it commonly reaches 50–60% in cells with the preserved cyclin D1 function in G1. Each electroporation and/or microinjection result was verified by three independently performed experiments and the data statistically evaluated by estimating standard deviations. Essentially identical electroporation protocol (Lukas et al., 1994) was employed for the gene transfer experiments using the pSV-neo (a gift from C. Norbury, ICRF, Oxford, UK), pCMV-D1 (Baldin et al., 1993), pCMV and pCMV-RB (Müller et al., 1994), pCMV-neo, pCMV-E1A-neo, and pCMV-E1A ΔCR2 -neo (Fattaey et al., 1993), either individually or as a combination of pCMV-RB or pCMV-D1 with pSV-neo at a 10:1 ratio.

Results

Cyclin D1 Is Downregulated by Several Oncogenes Encoded by the Small DNA Tumor Viruses

Stimulated mainly by the accumulating evidence for the oncogenic potential of cyclin D1, its mRNA levels in human cell lines of various tissue and tumor origin have been investigated by several groups and found to be highly variable (reviewed by Motokura and Arnold, 1993). Our recent immunoblotting analysis of cyclin D1 abundance in a large panel of human cell lines revealed low levels of the cyclin D1 protein in several lines transformed by small DNA tumor viruses (Bartek et al., 1993a; Lukas et al., 1994). To investigate this intriguing correlation in a more systematic way, we have now collected a considerably wider range of cell lines immortalized and/or transformed by SV-40 T antigen, adenovirus E1A, or papillomavirus E7/E6 oncogenes (see Table I), prepared and electrophoretically separated the whole cell lysates from exponentially growing cultures of all the cell types listed in Table I and probed the corresponding blots with both our new mouse monoclonal antibody DCS-6 specific for cyclin D1 (Lukas et al., 1994) and a well characterized rabbit anti-cyclin D1 antiserum (Baldin et al., 1993). The immunoblotting analysis revealed only moderate variation of cyclin D1 abundance among the control cell lysates (see Fig. 1, lanes 1, 3, and 5, for examples). In contrast, we found significantly reduced cyclin D1 levels in all the DNA virus oncogene-expressing cell lines as compared to several control cell types (Fig. 1 and Table I). Quantitative evaluation of the ECL blots by laser densitometry showed this reduction to be in the range of nine times or more, depending on a cell line. The control counterpart cell types included several nonestablished cell strains and/or primary cultures (MRC5, IMR90, HFF, and RME), monkey CV-1, mouse 3T3 cells, and a series of human cancer cell lines, some of which have repeatedly been reported to contain a single copy cyclin D1 gene (Table I). The overall correlation of the low cyclin

Table I. Downregulation of Cyclin D1 in Cells Expressing DNA Virus Oncogenes

Cell line	Origin*	Viral oncoprotein	D1 protein [‡]	D1mRNA [‡]
IMR90	Diploid fibroblast	—	+++	ND
MRC5	Diploid fibroblast	—	+++	ND
HFF	Primary fibroblast	—	+++	ND
3T3	Established fibroblast	—	+++	ND
RME	Breast Epithelium	—	+++	ND
CV-1	Kidney epithelium	—	+++	+++
MCF-7	Breast carcinoma	—	+++	+++
T47D	Breast carcinoma	—	+++	+++
SK-LMS-1	Leiomyosarcoma	—	+++	ND
UMSCC-2	Squamous cell ca.	—	+++	ND
HaCaT	Keratinocytes	—	+++	+++
Cos7	Derivative of CV1	SV40 T Ag	±	±
HBL100	Breast epithelium	SV40 T Ag	±	±
RMT-1	Derivative of RME	SV40 T Ag	±	ND
RMT-2	Derivative of RME	Sv40 T Ag	±	ND
Ref A7/32°C	Fibroblast/permissive	SV40 T Ag(+)	+	ND
Ref A7/39°C	Fibroblast/nonpermissive	SV40 T Ag(-)	+++	ND
293	Transformed fibroblast	E1A,E1B	±	±
IREE-1	Fibroblast/oestrogen (-)	E1A(-)	+++	+++
IREE-1/E	Fibroblast/oestrogen (+)	E1A(+)	±	±
M107-Ad5	Infected fibroblast	E1A(+),E1B(+)	±	±
M107-dl312	Infected fibroblast	E1A(-),E1B(+)	+++	+++
HeLa	Cervical carcinoma	HPV18 E7/E6	±	±
C4-1	Cervical carcinoma	HPV18 E7/E6	±	ND
444	HeLa/fibroblast hybrid	HPV18 E7/E6	±	ND
Cg13	HeLa/fibroblast hybrid	HPV18 E7/E6	±	ND
SW 756	Cervical carcinoma	HPV18 (E7/E6)(+)	±	ND
SW756/Dex	Cervical carcinoma	HPV18 (E7/E6)(-)	+++	ND
SiHa	Cervical carcinoma	HPV16 E7/E6	±	±
HPK 1A	Immortal keratinocyte	HPV16 E7/E6	+	±
CaSki	Cervical carcinoma	HPV16 E7/E6	±	±

* All cell types are of human origin, except for CV-1/Cos-7 (green monkey), RefA7 and IREE-1 (both rat), and 3T3 (mouse).

[‡] The immunoblotting and/or Northern blotting data were compared by densitometric scanning and the cyclin D1 expression estimated according to an arbitrary scale, graded as +, ++, +++, and ±. ND, not determined.

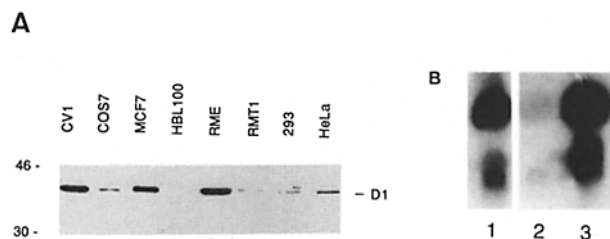


Figure 1. Expression of cyclin D1 protein and mRNA is abnormally low in cell lines transformed by small DNA tumor viruses. (A) Cyclin D1 protein abundance is reduced in cells constitutively expressing the SV40 T antigen (*COS-7*, *HBL100*, *RMT-1*), HPV18 E7/E6 (*HeLa*) and adenovirus 5 E1A/E1B (*293*) oncogenes. The control cell lysates from CV-1, MCF7, and RME show the standard levels of cyclin D1 protein in asynchronously growing cells lacking DNA viral oncogenes. The immunoblotting reactions are visualized by the ECL method and the molecular mass of the markers is given in kD. (B) Northern blot analysis of cyclin D1 mRNA expression. Gel separated RNA, isolated from HaCaT (lane 1), Cos-7 (lane 2), and CV-1 (lane 3) cells, was blotted and probed with the cyclin D1 cDNA probe to reveal the substantial reduction of both cyclin D1 transcripts in the SV-40-transformed Cos-7 cells.

D1 levels with the presence of a DNA tumor virus oncogene was further strengthened by the fact that included in our panel was also a closely matched pair of SV40 T antigen-transformed cell lines and their parental line lacking the viral oncogene (*Cos7* versus *CV-1* cells) (see Fig. 1 and Table I). In addition, two new cell lines (designated RMT-1 and RMT-2) were established as part of this project from the RME normal mammary epithelium via retroviral-mediated introduction of the SV-40 T antigen. Cyclin D1 expression was found to be dramatically reduced in both RMT-1 and RMT-2 as compared to the parental RME cells (Fig. 1 and Table I), and repeated examination of these, and a few other cell lines included in Table I, over the period of more than 12 mo revealed that the expression level of cyclin D1 protein appears to be a relatively stable feature of each cell type (data not shown). To demonstrate the downregulation of cyclin D1 expression by the viral oncoproteins even more directly, we also employed two cellular model systems in which expression of either the papillomavirus E7/E6 proteins or the SV-40 T antigen can be manipulated by culture conditions. First, as can be seen from Fig. 2 A, release from the dexamethasone-mediated suppression of the E7/E6 onco-

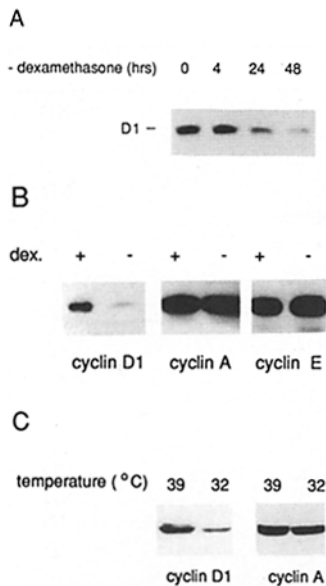


Figure 2. Regulation of cyclin D1 protein levels by conditionally expressed DNA tumor virus oncogenes. (A) Upregulated expression of HPV18 E7/E6 oncogenes leads to decreased accumulation of the cyclin D1 protein. SW756 cell line containing dexamethasone-repressible HPV18 E7/E6 was grown without the presence of dexamethasone and the selected time-points were subsequently analyzed for the cyclin D1 protein levels. (B) Cyclin D1 protein is selectively downregulated by HPV18 E7/E6 since the levels of cyclins A and E remain approximately constant upon E7/E6 induction. SW756 cell line was grown with (positive lanes) and without

(negative lanes) dexamethasone as described in Materials and Methods and the levels of cyclins D1, A, and E were analyzed by ECL immunoblotting. (C) Regulatable expression of SV40 T antigen selectively influences cyclin D1 protein expression. Rat Ref cells (clone A7) expressing the A7 temperature-sensitive mutant of SV40 large T (see Materials and Methods) were grown at permissive temperature (32°C) or at nonpermissive temperature (48 h at 39°C) and the protein levels of cyclin D1 and cyclin A were analyzed by immunoblotting.

gene expression in the SW756 cells resulted in time-dependent stepwise decrease of the cyclin D1 protein abundance. The cyclin downregulation by E7/E6 appears to be specific for cyclin D1 since parallel immunoblots probed with antibodies to cyclin E and A revealed no significant variation dependent on expression of these viral oncogenes (Fig. 2 B). Second, the rat embryo fibroblast cells conditionally transformed by the A7 temperature-sensitive mutant of SV40 T antigen expressed significantly less cyclin D1 protein when grown at 32°C (i.e., the temperature permissive for large T antigen function in these cells) as compared with the cyclin levels at the nonpermissive temperature (Fig. 2 C). Since the expression of both cyclin E and A is tightly regulated during the cell cycle, the observed lack of any appreciable differences in abundance of these two cyclin proteins in virus oncogene-containing cells argues against any viral oncogene-induced gross shift in the relative proportions of cells in various cell cycle phases. Furthermore, to exclude the theoretical possibility that the low level of cyclin D1 in the presence of viral oncoproteins is due to dramatically reduced fraction of cells in the G1 phase, i.e., the period of the cell cycle when cyclin D1 is expressed at the highest level (Motokura et al., 1992; Baldin et al., 1993; Musgrove et al., 1993; Bartkova et al., 1994; Lukas et al., 1994), we explored two additional approaches. First, the FACScan analysis of DNA profiles of exponentially growing cell populations showed only minor differences in proportions of G1 cells, the extent of which cannot account for the pronounced downregulation of cyclin D1 seen in cell lines expressing the DNA virus oncogenes (data not shown). The second approach was based on our earlier immunostaining

data obtained with synchronized normal and tumor cell populations, which demonstrated that cyclin D1 was clearly detectable in G1 nuclei but low or absent in the other cell cycle phases (Baldin et al., 1993; Bartkova et al., 1994; Lukas et al., 1994; see Fig. 3, A, B, and C for example). If the observed low levels of cyclin D1 protein in the viral oncogene-expressing cells were simply due to shortening of the G1 phase, one would predict that there would still remain a detectable albeit smaller proportion of brightly stained, cyclin D1-positive, G1 cell nuclei. The immunofluorescence examination of the viral oncogene-expressing cell lines HeLa, 293, HBL-100, RMT-1, RMT-2, and Cos-7 consistently showed an overall low staining signal, which is consistent with the general inhibition of the cyclin D1 expression rather than selective shortening of the G1 phase (data not shown).

From the data described above we conclude that the SV-40 large T antigen, papillomavirus E7/E6, and adenovirus E1A oncoproteins selectively downregulate the expression of cyclin D1 protein in the host cell. The Northern blot analysis with cyclin D1 gene probe of RNAs isolated from several cell lines included in our panel (see Table I) revealed very low abundance of both cyclin D1 gene transcripts in cells expressing the viral oncogenes, when compared with the control group of cell types (Fig. 1 B and Table I). This result indicates that the observed effect of the viral oncoproteins on cyclin D1 expression is primarily caused by a mechanism operating at the mRNA level (inhibition of transcription and/or lower RNA stability) and that the low abundance of the cyclin D1 protein is likely to reflect the level of available transcripts rather than abnormalities at the level of translation or cyclin D1 protein degradation.

DNA Virus Oncoproteins Eliminate the Host Cell's Requirement for Functional Cyclin D1

The experimental knockout of cyclin D1 achieved by microinjection of either affinity-purified rabbit antibodies to cyclin D1 or antisense plasmids into synchronized human diploid fibroblasts in early G1 prevented entry into S-phase (Baldin et al., 1993). Microinjection performed in late G1, however, had no effect on subsequent onset of DNA synthesis, leading to the conclusion that cyclin D1 function is required for G1 progression (Baldin et al., 1993). Using an analogous strategy, we have recently developed an improved functional knockout approach for cyclin D1 based on electroporation of affinity-purified high titre mouse monoclonal antibody DCS-6 and employed this technique to demonstrate that cyclin D1 is required for progression through G1 in several human tumor-derived cell types including the MCF-7 and UMSCC-2 carcinoma lines (Lukas et al., 1994). In this study, we examined whether the observed downregulation of cyclin D1 by DNA tumor virus oncogenes is accompanied by any functional effect and asked whether the remaining low levels of the cyclin D1 protein continue to play an essential role in the cell cycle progression. To assess the requirement for functional cyclin D1 in various cell types, we electroporated the affinity-purified DCS-6 antibody into mitotic cells, allowed the cells to proceed into the cell cycle, and added tritiated thymidine to monitor DNA synthesis, followed by fixation at specified time intervals and processing for immunofluorescence and autoradiography. The proportion of successfully electroporated cells, as indicated by the presence of mouse immuno-

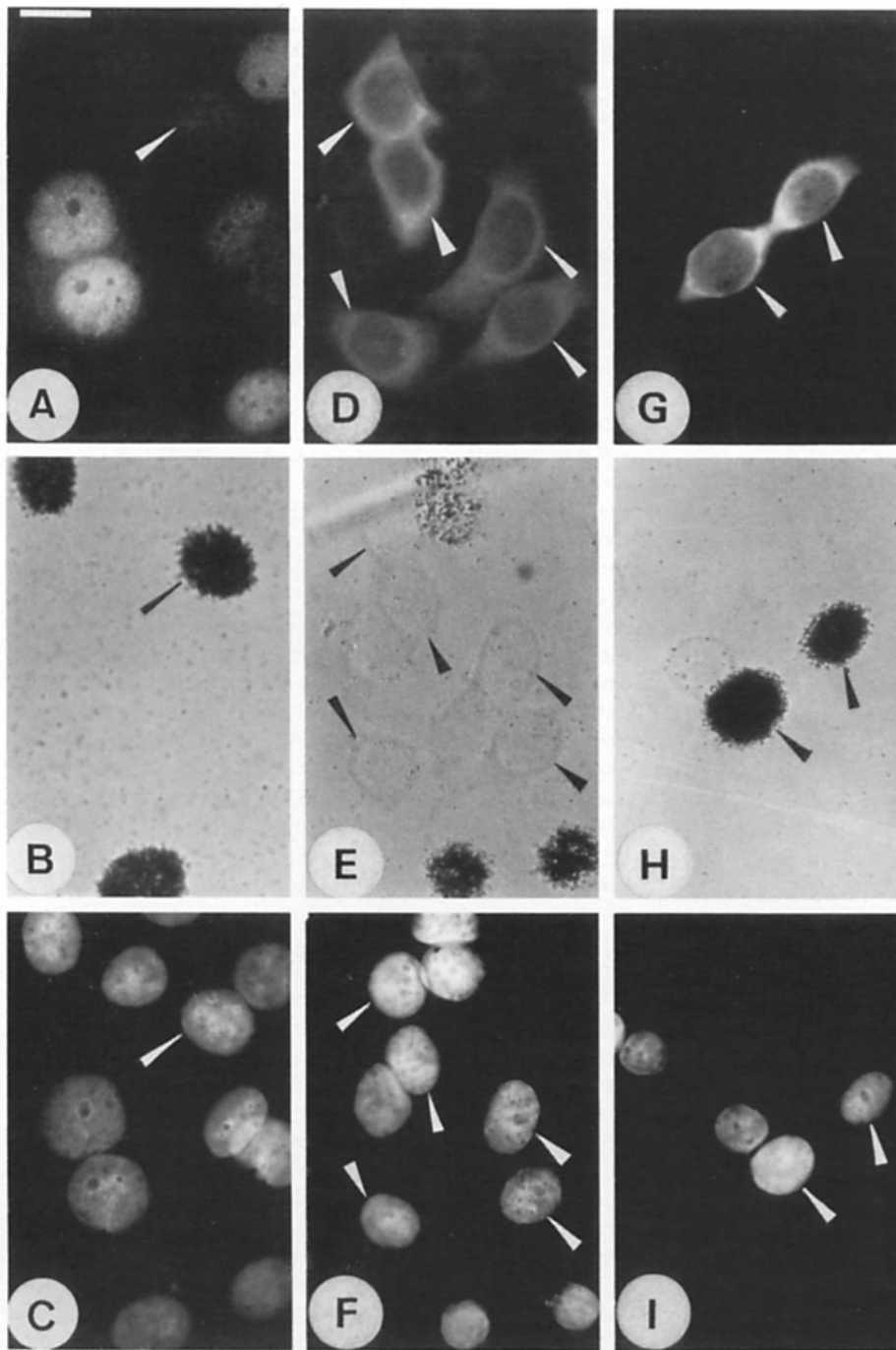


Figure 3. Analysis of cyclin D1 subcellular localization, cell cycle-related variation, and effect of the antibody-mediated cyclin D1 knockout in MCF7 cell line. (A–C) Combined immunofluorescence and autoradiography analysis demonstrating standard levels, nuclear targeting of the cyclin D1 protein and its low abundance in S-phase cells. (D–F) and (G–I) show examples of electroporation into MCF-7 cells of DCS-6 monoclonal antibody and of control mouse IgG, respectively. While the cells treated with DCS-6 are prevented from entering S phase (D–F), the control cells synthesize their DNA irrespective of the antibody treatment (G–I). (A) shows immunostaining with anti-D1 monospecific monoclonal antibody DCS-6; (D and G) show positively electroporated cells revealed by anti-immunoglobulin staining; (B, E, and H) demonstrate thymidine incorporation monitored by in situ autoradiography; (C, F, and I) reveals nuclei counterstained by Hoechst. The arrowheads point to the same nuclei in corresponding triplets of microphotographs. Bar, 10 μ m.

globulin visualized by immunofluorescence in the adherent cell population was reproducibly within the range of 40–80%. Microphotographs in Fig. 3 illustrate the results of a typical electroporation experiment in control MCF-7 cells. Cells electroporated with the anti-cyclin D1 mAb were prevented from entering the S-phase (Fig. 3, D–F) while control antibodies showed no significant inhibition (Fig. 3, G–I). In a control experiment, introduction of the same DCS-6 antibody preparation into late G1 cells was no longer effective. This result suggested that the cells passed the cyclin D1-dependent checkpoint by late G1 and, at the same time, excluded the possibility that the S-phase inhibition was caused by some toxic component in the electroporation solutions

rather than the specific effect of the antibody. Overall, five different cell types from the control series lacking any DNA tumor virus oncoprotein were examined: normal human diploid fibroblasts (IMR90), established but nontransformed CV-1 cells, the cell lines derived from a breast carcinoma (MCF-7), leiomyosarcoma (SK-LMS-1), and the UMSSC-2 squamous cell carcinoma. In every case, electroporation of the anti-cyclin D1 mAb caused a severe delay of DNA synthesis (see Fig. 4 for examples). In contrast to cell cycle inhibition observed for the control normal cells and cell lines derived from tumors of nonviral pathogenesis, electroporation for the DCS-6 antibody into synchronized cell lines expressing SV40 T antigen (HBL-100, Cos-7), adenovirus E1A (293

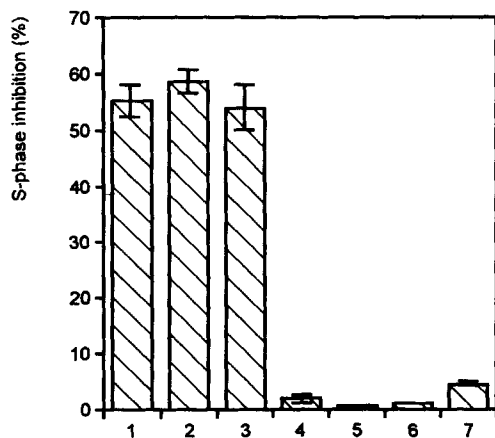


Figure 4. Effects of the anti-cyclin D1 antibody (DCS-6) electroporation into various cell types upon S-phase entry. The cell lines not transformed by DNA virus oncogenes are significantly delayed in their onset of DNA synthesis as shown for 3T3 (1), CV-1 (2), and MCF-7 cells (3), while no obvious effect is seen in case of virally transformed Cos-7 (4), HBL100 (5) HeLa (6), and 293 (7) cell lines. Each experiment was performed three times, the data are expressed as graphs summarizing the mean percentage of S-phase inhibition by DCS-6 and the extent of an experimental error is indicated by standard deviation bars.

cells), and papilloma E6/E7 (HeLa cells), had no detectable effect upon entry into S-phase (Fig. 4).

Two additional sets of experiments were subsequently performed to investigate the specificity of this phenomenon. The first set of control experiments confirmed the requirement in control cell types and the lack of dependency on cyclin D1 for G1 progression in the HBL-100, HeLa, and 293 viral oncogene-transformed cell lines using a different set of reagents: the affinity-purified rabbit anti-cyclin D1 polyclonal antibodies and normal rabbit immunoglobulin, for electroporation (data not shown). In addition, the S-phase entry was delayed to a similar extent also upon microinjection, rather than electroporation, of the DCS-6 monoclonal antibody into early G1 cells (an approach control, data not shown).

In conclusion, these data demonstrate a common loss of dependency on the function of cyclin D1 in cell lines transformed by the SV-40 large T antigen, adenovirus E1A, and papillomavirus E7/E6 oncoproteins.

Retinoblastoma Protein Is Involved in Regulation of Cyclin D1 Expression

One hallmark shared by T antigen, E1A, and E7 is their capacity to form complexes with the retinoblastoma protein, thereby preventing its interaction with cellular partner proteins and resulting in functional inactivation of pRB (DeCaprio et al., 1988; Whyte et al., 1988; Dyson et al., 1989). We reasoned that one way to address the question of whether or not the above effects of various oncoproteins on cyclin D1 could be mediated by inactivation of pRB, is to examine the expression and properties of cyclin D1 in cancer cell lines harboring naturally occurring mutations of the RB gene. We therefore performed a comparative immunoblotting and immunofluorescence study of cyclin D1 in a panel of RB-defective cell lines and histogenetically matched con-

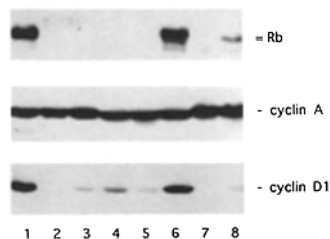


Figure 5. Immunoblotting analysis of pRB, cyclin A, and cyclin D1 abundance in human cancer cell lines. Equal amounts of total cellular lysates prepared from MCF-7 (lane 1), BT-549 (lane 2), MDA-468 (lane 3), Hs913T (lane 4), DU4475 (lane 5), SKLMS-1 (lane 6), Saos-2 (lane 7), and C33-A (lane 8) cells were separated by SDS-PAGE, the corresponding blots probed with the antibodies as indicated on the right, and the reaction visualized by the ECL method. Although not visible on this exposure, the low levels of cyclin D1 in BT-549 and Saos-2 cells, as well as the truncated p95 RB in Saos-2 extract were detectable upon longer exposures.

trol cell types containing normal RB gene and functional RB protein. Of the ten tumor cell lines with RB mutations available for this study, six suffered gross aberrations resulting in a total loss of the RB protein, while four lines carry more subtle RB gene deletions and the corresponding abnormal RB polypeptide is expressed in these cells albeit at lower level (see Table II for details and Fig. 5, top for pRB detection on immunoblots). The control series of cell types with normal RB gene included breast carcinoma, lung carcinoma, and sarcoma lines, as well as normal human retina cells, thus matching most of the RB-defective lines as to their origin (Table II). Apart from their histogenesis and reported lack of RB gene abnormalities, the criteria for selecting these control cell lines were: (a) spontaneous establishment in vitro without any involvement of the DNA tumor viruses; and (b) the expression of functional RB protein as judged from its nuclear localization, apparent molecular mass as predicted for wild-type pRB, phosphorylation status and the capacity to form complexes with SV-40 T antigen upon infection of these cancer cell lines with SV-40 virus (Lee et al., 1988; T'Ang et al., 1988; Horowitz et al., 1990; Shew et al., 1990; Stratton et al., 1990; Bartek et al., 1992). Furthermore, presence of well defined p53 gene mutations in some, and of the wild-type p53 in other cell lines of this control category (Casey et al., 1991; Bartek et al., 1993b) should facilitate detection of any obvious effects of p53 aberrations on cyclin D1 expression in cells expressing normal RB.

To compare cyclin D1 protein abundance in the control and the RB-defective lines, blots of SDS-PAGE-separated whole-cell lysates from exponentially growing cultures of the cell lines listed in Table II were probed with our monoclonal antibody DCS-6 (Lukas et al., 1994) and the rabbit antiserum (Baldin et al., 1993) against human cyclin D1. One blot was cut in half and the upper and lower parts of the membrane were probed with anti-cyclin A and anti-cyclin D1 antibodies, respectively. As can be seen from an example shown in Fig. 5 and from the summary of the immunoblotting data in Table II, all the control cell types expressed easily detectable cyclin D1 protein levels, comparable to those found in cultured normal diploid cells, while strikingly reduced abundance of this cyclin was observed in the cancer cell lines harboring mutant RB. Although not visible on the exposure shown in Fig. 5, low levels of cyclin D1 protein could be detected even in the cell lysates from Saos-2 and BT-549 cells upon longer exposure of the ECL blots. The cyclin repres-

Table II. Cyclin D1 Protein Levels in Cell Lines with Normal and Mutant RB

Cell line	Origin	RB gene*	RB protein*	Cyclin D1†
BTS49	Breast cancer	Gross rearrangement	None	±
MDA-468	Breast cancer	Gross rearrangement	None	±
DU4475	Breast cancer	Gross rearrangement	None	±
Hs913T	Fibrosarcoma	Gross rearrangement	None	+
WERI-1	Retinoblastoma	Gross rearrangement	None	±
Y-79	Retinoblastoma	Deletion of exons 2-6	None	±
Saos-2	Osteosarcoma	Deletion of exons 21-27	p95	±
NCI-H69	Small cell lung cancer	Deletion of exon 22	p100	±
NCI-592	Small cell lung cancer	Deletion of exon 22	p100	±
C33-A	Cervical cancer	Partial deletion in exon 20	p104	±
PMC-42	Breast cancer	Normal	Normal	+++
MCF-7	Breast cancer	Normal	Normal	+++
ZR-75	Breast cancer	Normal	Normal	+++
MDA-231	Breast cancer	Normal	Normal	+++
BT474	Breast cancer	Normal	Normal	+++
Cama 1	Breast cancer	Normal	Normal	+++
MDA-157	Breast cancer	Normal	Normal	+++
SK-BR-3	Breast cancer	Normal	Normal	+++
HER	Normal retina cells	Normal	Normal	+++
U-2-OS	Osteosarcoma	Normal	Normal	+++
RD	Rhabdomyosarcoma	Normal	Normal	+++
SK-LMS-1	Leiomyosarcoma	Normal	Normal	+++
Calu	Lung carcinoma	Normal	Normal	+++
HT 29	Colon cancer	Normal	Normal	++
DLD-1	Colon cancer	Normal	Normal	+++

* The data on RB gene and protein are from: Lee et al., 1988a,b; T-Ang et al., 1988; Horowitz et al., 1990; Shew et al., 1990; Stratton et al., 1990; Scheffner et al., 1991; Bartek et al., 1992.

† The immunoblotting data were compared by densitometric scanning and the cyclin D1 protein level estimated according to an arbitrary scale, graded as + + +, + +, +, and ±.

sion shared by the RB-defective lines appeared to be specific for cyclin D1 since no obvious differences in cyclin A expression could be found when cell lines carrying normal or mutant RB genes were compared (see Fig. 5 for examples). The latter result also implies that all the cell lines examined were proliferating at the time of analysis, the conclusion which was independently confirmed by FACScan analysis of DNA profiles and thymidine incorporation in several cell lines of either category (data not shown). The immunofluorescence analysis of cyclin D1-expression patterns confirmed and extended the above immunoblotting data. Thus, while the exponentially growing cell lines expressing normal RB showed the characteristic nuclear staining of highly variable intensity (see Fig. 3 A for example), very weak to undetectable cyclin D1 staining signal was found in all ten tumor cell lines with RB gene mutations (data not shown).

The above results demonstrate that selective downregulation of cyclin D1 is a phenomenon shared by cell lines transformed by DNA tumor virus oncoproteins known to form complexes with pRB and the tumor-derived cell lines harboring mutations in the RB gene. This analogy suggested that the effect of the viral oncogene products upon cyclin D1 expression could be indirect and possibly mediated through their association with pRB. To investigate this possibility, we tested the relative influence on cyclin D1 protein abundance of ectopically expressed wild type 12S E1A and an E1A mutant with a small deletion within the conserved region 2 domain (E1A-ΔCR2) which is highly homologous to corresponding RB-binding regions in SV-40 T antigen and papilloma E7 protein. The CR2 domain has been shown to

mediate the high-affinity binding of E1A to the pRB's pocket region and the deletion mutant used in the present study failed to form complexes with pRB (Dyson and Harlow, 1992; Fattaey et al., 1993). The SKLMS-1 sarcoma and PMC42 breast cancer cell lines expressing wild type RB, standard levels of cyclin D1 and mutant p53 (to avoid apoptosis upon E1A expression) were electroporated with either the E1A, E1A-ΔCR2, or a control pCMV-neo expression plasmids, the cell populations resistant to G418 were selected over a period of 2-3 wk and the pooled clones lysed and examined for E1A expression and cyclin D1 protein abundance. As seen in Fig. 6 A, introduction of both wild type E1A and E1A-ΔCR2 plasmids resulted in high-level expression of the respective proteins while no E1A protein could be detected in the control cell population electroporated with the pCMV-neo construct. The parallel immunoblotting analysis with DCS-6 antibody revealed a considerably lower level of cyclin D1 protein in the wild type E1A-expressing SKLMS-1 cells as compared with the cell population treated with the control plasmid (Fig. 6 A, bottom). In addition, cyclin D1 level in the cells expressing E1A-ΔCR2 mutant protein was not significantly reduced when compared with the pCMV-neo control (Fig. 6 A), implying that an intact CR2 region is essential for E1A's ability to downregulate cyclin D1 in SKLMS-1 cells. Virtually identical data were obtained using the PMC42 breast cancer cell line as a recipient of the same expression plasmids (results not shown), indicating that the observed dependency of cyclin D1 downregulation on intact CR2 domain represents a common and reproducible phenomenon. We therefore conclude that the ability of E1A to

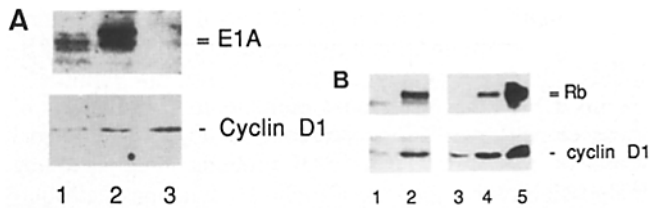


Figure 6. Gene transfer experiments indicating a positive role of pRB in regulation of cyclin D1 expression. (A) Downregulation of cyclin D1 requires intact CR2 domain of adenovirus 5 E1A. The SKLMS-1 cells were electroporated with expression vectors encoding the wild-type 12S E1A (lane 1) or E1A-CR2 mutant missing the L-X-C-X-E motif required for binding to pRB (lane 2). The immunoblot demonstrates cyclin D1 levels in lysates of pooled clones preselected for 2 wk in G418-containing medium; lane 3 shows the data obtained with the control pCMV-neo vector. (B) Reintroduction of wild-type RB cDNA into BT549 cells induces cyclin D1. BT549 cells were transiently electroporated with control pCMV plasmid (lane 1) or human RB cDNA in the same vector (lane 2). 48-h later, the cells were lysed and assayed for RB and cyclin D1 proteins by immunoblotting. Stable clones of BT549 cells expressing pRB were also generated (e.g., lane 4) and found to accumulate higher levels of cyclin D1 as compared to the G-418-resistant control BT549 cells lacking pRB (lane 3). The reference levels of both pRB and cyclin D1 protein are shown in lane 5 (equal loading of CV-1 cell lysate).

downregulate cyclin D1 expression is mediated through its complex formation with, and presumably functional inactivation of, the wild-type retinoblastoma protein. Furthermore, since both SV-40 T antigen and papilloma virus E7 oncoproteins contain pRB-binding regions highly homologous to E1A's CR2 sequence, a similar RB-mediated mechanism is likely to be responsible for cyclin D1 regulation by the latter viral oncogene products as well.

Taken together, the above data suggested that pRB could play a positive role in regulation of cyclin D1 expression. To address directly a requirement of functional pRB for the induction of cyclin D1, the wild type RB cDNA under the control of the CMV promoter was electroporated into two RB-deficient tumor cell lines of different histogenesis (BT549 breast carcinoma and C33A cervical carcinoma lines) and the effect of transient as well as stable expression of pRB on the level of cyclin D1 was investigated by immunoblotting (after cotransfection of pSV-neo and pCMV-RB and selection of G418-resistant, RB-expressing clones). Fig. 6 B, lane 2 demonstrates that the expression of endogenous cyclin D1 is induced in the pooled population of BT549 cells which transiently express wild-type RB, and similarly, cyclin D1 protein accumulates to significantly higher level in the stable clones which proliferate and express moderate levels of the ectopically driven wild-type pRB (see Fig. 6 B, lane 4, for example). No effect on cyclin D1 expression is seen in BT549 cells treated with the control plasmids (Fig. 6 B, lanes 1 and 3). In addition, no detectable alterations of either cyclin A or cyclin E levels were seen upon expression of CMV-driven pRB, thus showing the specificity of the pRB-mediated stimulation for cyclin D1 (not shown). Virtually identical results in terms of specific cyclin D1 stimulation were obtained also with the C33A RB-deficient cell line using the same experimental protocol.

We conclude from this part of our studies that: (a) cyclin

D1 protein is expressed at normal levels in a wide spectrum of human tumor cell lines with normal RB gene and functional RB protein, irrespective of whether or not the p53 gene is mutant in these cells; (b) cyclin D1 but not cyclin A expression is dramatically reduced in the cell lines carrying mutations in the retinoblastoma gene; (c) cyclin D1 downregulation by DNA virus oncoproteins requires intact RB-binding sequences; and (d) ectopic pRB induces cyclin D1 but not cyclins A and E in RB-deficient cells. Therefore, pRB appears to be involved in regulation of cyclin D1 expression in a positive manner.

Cells with Mutant RB Lack the Cyclin D1 Checkpoint in G1 but the Checkpoint Is Restored Upon Reintroduction of RB

The above results showed that cyclin D1 function becomes dispensable in cells expressing the RB-binding DNA virus oncoproteins, and that cyclin D1 is downregulated in cells lacking functional RB. These observations raise two important questions which must be answered to further elucidate the functional interplay between pRB and cyclin D1. Thus, we asked whether cyclin D1 protein is required or dispensable for regulation of G1 phase progression in cell lines carrying mutant RB, and whether the level of cyclin D1 protein is of any functional significance for cell cycle regulation in such cells. To address these issues through cyclin D1 knockout experiments, we first selected three representative model cell lines which carry naturally occurring RB gene mutations (C33A, Saos-2, and BT549) (Table II), and established a series of stable clones ectopically expressing elevated levels of cyclin D1 protein from one of these cell lines (BT549, see Fig. 7 A). The latter clones were prepared by cotransfecting the pCMV-D1 and pSV-neo plasmids at 10:1 ratio into BT549 breast cancer cell line, followed by G418 selection and immunoblotting analysis of cyclin D1 expression in exponentially growing clonal cell populations. In the majority of clones, the cyclin D1 protein abundance was significantly increased as compared to the parental BT549 cells and varied from clone to clone (see Fig. 7 A for examples). The ectopically expressed cyclin D1 protein comigrated with the endogenous protein and could be detected by antibodies to four different epitopes (not shown), thus indicating that it represents a genuine cyclin D1. Two clones with high expression of the cyclin D1 protein approaching the levels seen in cultured diploid cells and in cancer cell lines with wild-type RB, were chosen for subsequent analysis.

In keeping with our previous results, electroporation and/or microinjection of either the mouse monoclonal (clone DCS-6) or rabbit polyclonal antibodies to cyclin D1, but not of normal mouse or rabbit immunoglobulins, resulted in a severe inhibition of S-phase entry in human MCF-7 breast cancer cells (Fig. 7 B) which possess normal RB and p53 (Lee et al., 1988a; T'Ang et al., 1988; Casey et al., 1991; Bartek et al., 1992), single-copy cyclin D1 gene (Lammie et al., 1991) and express an easily detectable cyclin D1 protein level (see Figs. 3 and 5). Essentially the same degree of the S-phase delay was obtained with the SK-LMS-1 cells (Fig. 7 B) expressing functional RB protein (Stratton et al., 1990; Bartek et al., 1992) and similar cyclin D1 level but carrying a mutation in the p53 gene (Stratton et al., 1990) and accumulating the aberrant p53 protein (Bartek et al., 1993b).

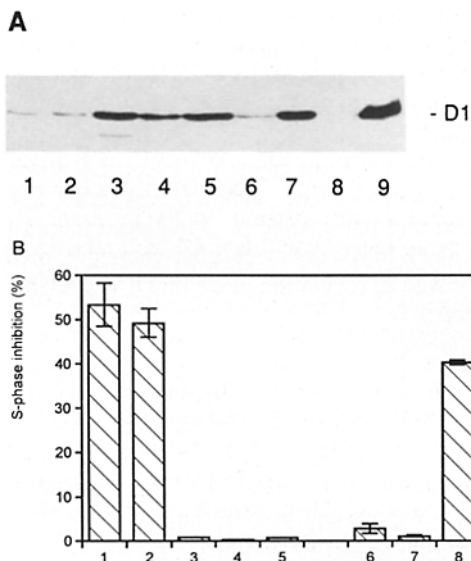


Figure 7. Effect of anti-cyclin D1 antibody knockouts upon onset of DNA synthesis in cell lines with wild-type versus mutant RB. (A) Immunoblotting analysis of cyclin D1 abundance in exponentially growing RB-deficient parental BT549 cells (lane 1) and several clones derived from BT549 cells by coelectroporation of pCMV-D1 and pSV-neo plasmids (lanes 2–8). Lane 9 shows the reference level of cyclin D1 in CV-1 cells expressing wild-type pRB. Stable BT-549–derived clones (designated BT/D1+ series) expressing the cyclin D1 protein abundance approaching the standard levels seen in RB+ cell types were selected for the antibody-mediated knockout analysis. (B) Introduction of anti-cyclin D1 antibodies into synchronized cell populations or inhibits S-phase entry of cells with normal RB: MCF-7 (1), SKLMS-1 (2), and a BT549 clone stably reconstituted with wild-type RB (8), while no effect is observed in cell lines with RB defects: BT-549 (3), Saos-2 (4), C33-A (5), and the two clones of BT549 cells with ectopically expressed elevated levels of cyclin D1: clone BT/D1+ K-C2 (6) and BT/D1+ K-A5 (7). The data are expressed as graphs summarizing the percentage of S-phase inhibition by DCS-6 and the extent of an experimental error is indicated by standard deviation bars.

Several other cell types with normal RB, which we examined previously, showed very similar response to the cyclin D1 knockout (Baldin et al., 1993; Lukas et al., 1994; Fig. 4). In contrast to cell lines with wild-type RB, electroporation and/or microinjection of anti-cyclin D1 antibodies had no obvious effect on G1 progression and S-phase entry in any of the three tumor cell lines with mutations in the RB gene (Fig. 7B). Since each of them shows a different degree of RB aberration, ranging from a homozygous loss in BT-549 (T'Ang et al., 1988), through a severe COOH-terminal deletion in Saos-2 cells (Shew et al., 1990), up to a subtle in-frame deletion of just four amino acid residues of exon 20 in C33-A cells (Scheffner et al., 1991), the lack of requirement for cyclin D1 function appears to be a common feature of the cancer cell lines with mutated RB gene. Furthermore, we argued that the restoration of the normal cyclin D1 protein level in RB-deficient cells could restore the cyclin D1 checkpoint, provided this cyclin D1 function is either independent of pRB or operates downstream of pRB along the same pathway. The experiments aimed at elucidating this issue revealed that the BT549-derived clones stably expressing cyclin D1 levels similar to those found in RB-positive cells remain refractory

to cyclin D1 knockout (see Fig. 7B, bars 6 and 7) indicating that the loss of cyclin D1 cell cycle regulatory function in RB-minus cells is not simply a consequence of low expression of this G1 cyclin. Additional immunoblotting analysis of these clones revealed the presence of apparently normal levels of cdk4, as well as p107 proteins, thus excluding the possibility that the loss of cyclin D1 function is attributable to the lack of these partner proteins rather than pRB (data not shown).

The lack of the cyclin D1 checkpoint in the cyclin D1-reconstituted BT549 clones was consistent with our view of pRB as a major target of cyclin D1 function in G1. On the other hand, the above data could also be explained if BT549 cells lost this checkpoint control through another, unidentified mutation, independent of RB and cyclin D1 level. To exclude the latter possibility, we performed a crucial control experiment by addressing the question whether the cyclin D1 checkpoint would be restored upon reintroduction of wild-type RB into RB-deficient cells. Antibody-mediated cyclin D1 neutralization experiments were performed in two independent clones of the BT549 cells stably expressing ectopically driven wild-type RB (see e.g., Fig. 6B, lane 4 for RB and cyclin D1 protein levels in one of these clones), and in each case, a significant inhibition of S-phase entry was detected (see Fig. 7B, lane 8 for example). A similar degree of S-phase inhibition in the RB-reconstituted clones was obtained using either electroporation or microinjection of the antibodies, while control BT549 clones resistant to G418 but lacking RB remained refractory to the antibody-mediated cyclin D1 knockout (not shown).

In summary, the results of the antibody knockout experiments are consistent with the notion that cyclin D1 protein's function is essential for cell cycle progression in cells with normal RB, regardless of whether wild-type or mutant p53 protein is expressed in such cells. More importantly, our data strongly indicate that loss of the functional RB protein is accompanied by loss of the cell's requirement for the cell cycle regulatory function of cyclin D1, and that the cyclin D1 checkpoint in G1 remains dispensable in the RB-deficient cells even upon restoration of normal cyclin D1 protein level. Upon RB reintroduction, however, the cyclin D1 checkpoint is restored, strongly supporting the notion that pRB functions downstream of cyclin D1 along the G1 phase regulatory pathway.

Discussion

The control of proliferation in mammalian cells depends on mitogenic signals and other factors and is primarily concerned with a decision whether to withdraw from the cell cycle into quiescence (G0 state) or to remain in the cell cycle and divide. This decision to commit to another round of cell division occurs during the G1 phase and represents one of the key cell cycle checkpoints, often called the restriction point (Pardee, 1989). Aberrations in these decision-making processes can result in pathological proliferation which represents one of the hallmarks of cellular transformation. The transforming DNA viruses such as SV-40, adenovirus, and the human papillomaviruses evolved molecular mechanisms which allow them to override both the G0>G1 and the restriction point controls of the host cell. These cell growth-regulating activities are carried out mainly by the viral early

genes including SV40 large T antigen, adeno type 5 E1A, and HPV16 and HPV18 E7 oncogenes (Dyson and Harlow, 1992; Fanning, 1992; Munger et al., 1992). These DNA virus-transforming gene products alter the transcription pattern of the cell, most likely through protein-protein targeting of the pRB-E2F and other regulatory complexes (Nevins, 1992). Despite the recent significant advances in our understanding of cell cycle regulation (Norbury and Nurse, 1992; Sherr, 1993) and cellular transformation (Nevins, 1992; Motokura and Arnold, 1993), a great deal remains to be learned about these fundamental biological processes and the DNA virus oncogenes promise to provide invaluable tools in this field of research.

The present study was designed to shed some light on the functional relationship between the product of the retinoblastoma gene (reviewed by Cobrinik et al., 1992; Goodrich and Lee, 1993), the RB-binding DNA virus oncoproteins, and one of the G1 cyclins, cyclin D1 (Lew et al., 1991; Matsushime et al., 1991; Motokura et al., 1991; Xiong et al., 1991). Here we report that: (a) the SV-40 large T antigen, adenovirus E1A, and papillomavirus E7/E6 oncogenes downregulate expression of cyclin D1 in a fashion apparently dependent on intact RB-interacting sequences; this downregulation is not shared by other cyclins and appears to be exerted primarily at the mRNA level; (b) the retinoblastoma protein is involved in regulation of cyclin D1 expression in a positive manner as suggested by very low abundance of this G1 cyclin in cancer cell lines containing mutated RB gene and by gene transfer experiments in which ectopically expressed wild-type RB leads to specific induction of cyclin D1; and (c) the cyclin D1 protein, normally required for G1 progression, is dispensable for passage through the cell cycle in cell lines whose pRB is inactivated through complex formation with T antigen, E1A, or E7 oncoproteins as well as in cells which have suffered loss-of-function mutations of the RB gene. This G1 phase checkpoint is not regained upon experimental restoration of the normal cyclin D1 expression level, suggesting that pRB may serve as a major downstream target of cyclin D1 whose cell cycle regulatory function becomes dispensable in cells lacking functional RB. The latter view of the cyclin D1/pRB interplay is strongly supported by our present finding that the cyclin D1 checkpoint is restored upon reintroduction of wild-type RB into RB-deficient cell lines.

In an attempt to integrate our new findings with the available data in the field, we now propose a model for a potential mechanism of concerted action of pRB and cyclin D1 in regulation of G1 phase of the mammalian cell division cycle. Most recently, two hypotheses have been put forward by other investigators, in an attempt to explain the functional relationship between pRB and D-type cyclins. Thus, Dowdy, Weinberg, and colleagues have proposed that cyclin D1 protein is downstream of RB along the G1 regulatory pathway and is an object of negative regulation by pRB, mediated via physical protein-protein sequestration (Dowdy et al., 1993). An alternative explanation has been offered by Ewen et al. (1993) who suggested that the pRB-cyclin D complex formation can transiently target a pRB-phosphorylating cyclin-dependent kinase, preferentially cdk4, to the pRB substrate in G1 phase, thereby inactivating its growth-suppressive function. The salient and novel features of our present model include the concept that pRB both regulates cyclin D1 and, in

turn is an object of regulation by cyclin D1, thus creating a kind of an autoregulatory loop operating in G1. Inherent in our model is also a key proposition that pRB serves as a major target of the cell cycle regulatory function of cyclin D1 (Fig. 8). We propose that in normal cells, as in tumor cells with preserved normal RB gene and functional RB protein, cyclin D1 protein (Fig. 8, *pD1*) abundance oscillates in a cell cycle-dependent manner, starting from low level in early G1, raising up to its peak level in mid/late G1, followed by a gradual decay in late G1 and through G1/S and early S-phase, eventually reaching its lowest level in advanced S-phase (Fig. 8A). Consistent with our proposal, such a cell cycle-related fluctuation profile of cyclin D1 levels has been reported for several normal and tumor cell types containing wild-type RB (Motokura et al., 1992; Baldin et al., 1993; Musgrove et al., 1993; Bartkova et al., 1994; Lukas et al., 1994) while it was less obvious in cells transformed by DNA tumor virus oncogenes (Matsushime et al., 1991; Motokura et al., 1991). The transcriptional activation from the cyclin D1 promoter would be driven, directly or indirectly, by the underphosphorylated, active form of pRB, present in early and mid G1 (Buchkovich et al., 1989; reviewed in Cobrinik et al., 1992; Goodrich and Lee, 1993). The positive role of pRB in regulation of cyclin D1 expression is supported by this study as well as by our results (Müller et al., 1994) demonstrating induction of cyclin D1 mRNA by transfection of the RB gene into RB-deficient cells and by dose-dependent stimulation of a cyclin D1 promoter-driven reporter gene expression by cotransfected wild-type but not mutant RB gene. This transactivating form of pRB is likely to include a protein-protein complex of hypophosphorylated pRB with either a positive or inhibitory transcription factor. While the former complex could either stimulate the transcription of the cyclin D1 gene promoter directly or through activation of another intermediate, the latter complex could serve to sequester a relevant transcriptional inhibitor, thereby allowing the cyclin D1 transcription to proceed. Upon inactivation of pRB through phosphorylation in late G1 (reviewed by Cobrinik et al., 1992; Goodrich and Lee, 1993), the specific transcription of cyclin D1 would be shut off or decreased, correlating with the above oscillation of cyclin D1 protein and reflecting the reported short half life of both cyclin D1 mRNA and protein (Matsushime et al., 1991; Sewing et al., 1993). During early G1, the pocket region of hypophosphorylated pRB species interact with numerous cellular Rb-binding proteins' (CRBP) (Fig. 8), which include the E2F transcription factor, other reported pRB-binding cellular partners (see Huang et al., 1991; Kaelin et al., 1991; Helin et al., 1992), and potentially the factor involved in the transcriptional regulation of cyclin D1 (see above). With the increasing cyclin D1 protein nuclear abundance in advanced G1 phase, more and more pRB pocket space would be occupied by cyclin D1, possibly competing out and releasing other CRBPs and, at the same time, transiently targeting a pRB-phosphorylating kinase (most likely cdk4) to this substrate. Consistent with this part of the model is the accumulating evidence for direct physical interaction of D-type cyclins with unphosphorylated and/or underphosphorylated pRB via their NH₂-terminal L-X-C-X-E motifs analogous to those present in homology regions II of the DNA virus oncoproteins (Dowdy et al., 1993; Ewen et al., 1993; Kato et al., 1993), the ability of D-type cyclins to relieve the pRB's cell growth-constraining function in RB-minus

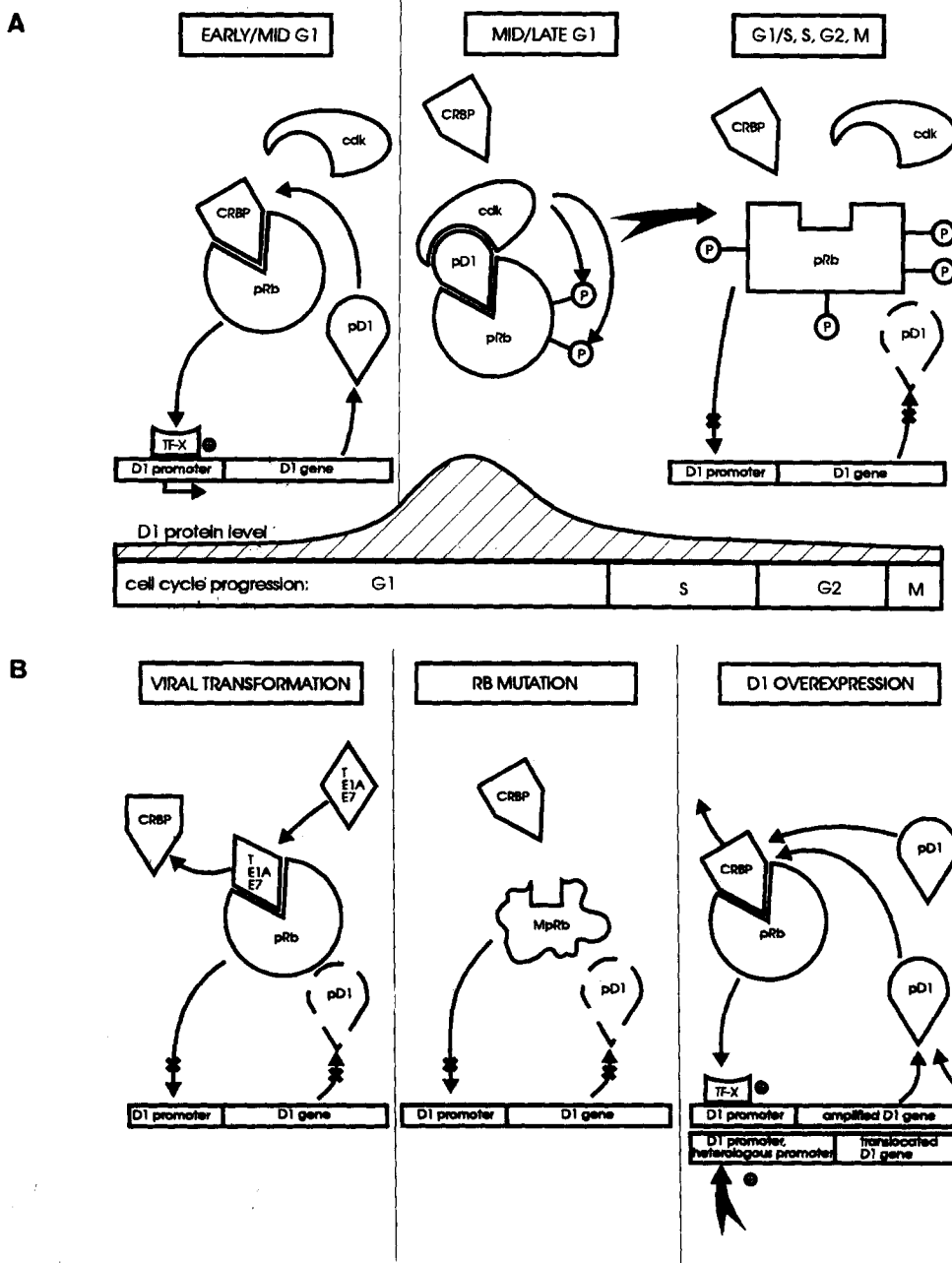


Figure 8. Schematic representation of the proposed autoregulatory loop interaction between pRB and cyclin D1, involved in regulation of G1 phase progression. (A) Represents a situation applicable for normal cycling cells and tumor cells which possess wild-type RB and single-copy cyclin D1 gene. (B) Shows aberrations of the RB/cyclin D1 regulatory mechanism caused by DNA tumor virus oncoproteins, RB gene mutations, and cyclin D1 overexpression resulting from either gene amplification or translocation. For detailed explanation of this model, see text in the Discussion section. Symbols used in this figure: pRB, retinoblastoma protein; pD1, cyclin D1 protein; CRBP, cellular Rb-binding protein; cdk, cyclin-dependent kinase; MpRB, mutant RB protein; T, E1A, E7, respective DNA virus oncoproteins; P, phosphate group; TF-X, postulated transcription factor involved (potentially complexed to pRB) in regulation of the cyclin D1 gene promoter.

cells (Hinds et al., 1991; Dowdy et al., 1993; Ewen et al., 1993), and the capacity of the cyclin D-associated kinase(s) to selectively phosphorylate the RB protein (Kato et al., 1993; Matsushime et al., 1992). Both the competition for available pRB pockets and the pRB phosphorylation with the concomitant conformational change of pRB would result in free CRBPs, released from their captivity and ready to trigger S-phase entry. The major task of cyclin D1 cell cycle regulatory mission would thus be completed by late G1 and its abundance would drop due to the lack of transcriptional stimulation by underphosphorylated pRB, thereby closing one circle of the proposed autoregulatory loop (Fig. 8 A). The decision whether to enter another loop cycle or withdrawal into quiescence is likely to be influenced by available mitogens and other factors involved in the regulatory recep-

tor-mediated network controlling cell proliferation, which eventually passes the message on the RB/cyclin D1 loop in G1.

This normal, physiological mechanism can potentially become disregulated in various ways but at least three biologically relevant scenarios can easily be documented using our model as a mechanistic basis. First, the transforming oncogenes of several DNA tumor viruses, including SV-40 T antigen, adenovirus E1A, and papillomavirus E7 oncoproteins are known to subvert the cell cycle regulation of the target cells, at least in part, by complexing to hypophosphorylated pRB (Ludlow et al., 1989; Imai et al., 1991; Dyson and Harlow, 1992). We report here that cyclin D1 protein level is very low in cells transformed by the above viral oncogenes and, in addition, that the remaining cyclin D1 protein is no longer required for G1 progression in these cells, and down-

regulation of cyclin D by adenovirus E1A has also been observed by Buchou et al., 1993. According to our model (Fig. 8 B), tight complex formation with the respective viral oncoproteins would occupy the available pRB pockets in G1, thereby preventing pRB's normal transactivating role in stimulation of cyclin D1 transcription. At the same time, since pRB's growth-suppressive function is already neutralized by the viral oncoproteins in such cells, cyclin D1 protein loses its major target and is dispensable for the aberrant cell cycling under these conditions. Second, a situation analogous to that just described for the virally transformed cells can be envisaged for cancer cells which have suffered loss of function mutations in the RB gene (reviewed by Cobrinik et al., 1992; Goodrich and Lee, 1993) or cells with experimentally repressed RB expression (Strauss et al., 1992). The mutilated pRB pockets would be unable to complex with either CRBPs or cyclin D1, accounting for unconstrained progression into S-phase due to free CRBPs, lack of phosphorylation typical for the mutant RB gene products, low levels of cyclin D1, and loss of requirement for cyclin D1 function, consistent with our experimental data reported here and with the proposed model (Fig. 8 B). Third, cyclin D1 gene is rearranged and/or amplified in several types of human malignancies, resulting in overexpression and consistent with the current opinion classifying this cell cycle regulator as a candidate proto-oncogene (e.g. Lammie et al., 1991; Motokura et al., 1991; reviewed by Motokura and Arnold, 1993). When considering this situation in the framework of our model, abnormally high level of cyclin D1 in G1 phase of the cell cycle would lead to a more efficient occupation of the pRB pockets, faster inactivation of pRB through phosphorylation and premature S phase entry due to release of free CRBPs (Fig. 8 B). In fact, this prediction of our model is also consistent with a recent report that moderate overexpression of D-type cyclins leads to shortening of G1 phase and correspondingly faster cell division cycles in mouse fibroblasts (Quelle et al., 1993).

As any other model, the proposed mechanism summarized in Fig. 8 should be regarded as a working hypothesis aimed to stimulate further research to elucidate the as yet uncertain structural and functional aspects of the RB-cyclin D1 interaction. The results summarized in the present report, together with the known involvement of both RB and cyclin D1 in molecular oncogenesis (reviewed by Cobrinik et al., 1992; Goodrich and Lee, 1993; Motokura and Arnold, 1993) suggest a central role of this regulatory interplay between the products of a cellular tumor suppressor gene and a cell cycle regulatory proto-oncogene, in control of the G1 phase progression at the point highly reminiscent of the restriction point proposed by Pardee (1989).

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