Elimination of Cyclin D1 in Vertebrate Cells Leads to an Altered Cell Cycle Phenotype, Which Is Rescued by Overexpression of Murine Cyclins D1, D2, or D3 but Not by a Mutant Cyclin D1*

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DT40 lymphoma B-cells normally express cyclins D1 and D2 but not D3. When cyclin D1 expression was extinguished in these cells by gene knockout, specific alterations in their ability to transit the cell cycle were observed. These changes are exemplified by a delay of approximately 2 h in their progression through a normal 14-h cell cycle. This delay results in an increase in the number of cells in the G2/M phase population, most likely due to triggering of checkpoints in G2/M. Inability to enter G1 normally, and/or alterations of crucial event(s) in early G1. The defect(s) in the cell cycle of these D1 "knockout" cells can be rescued by overexpression of any normal mouse D-type cyclin but not by a mutant mouse cyclin D1 protein that lacks the LXCE motif at its amino terminus. These data suggest that the cell cycle alterations observed in the D1−/− cells are a direct effect of the absence of the cyclin D1 protein and support the hypothesis that the D-type cyclins have separate, but overlapping, functions. Elimination of cyclin D1 also resulted in enhanced sensitivity to radiation, supporting the hypothesis that the D-type cyclins have separate, but overlapping, functions. Elimination of cyclin D1 also resulted in enhanced sensitivity to radiation, resulting in a significant increase in apoptotic cells. Expression of any normal murine D-type cyclin in the D1−/− cells reversed this phenotype. Intriguingly, expression of the mutant cyclin D1 in the D1−/− cells partially restored resistance to radiation-induced apoptosis. Thus, there may be distinct differences in cyclin D1 complexes and/or its target(s) in proliferating and apoptotic DT40 lymphoma B-cells.

Regulation of the vertebrate cell cycle requires the periodic formation, activation, and inactivation of unique protein kinase complexes that consist of cyclin (regulatory) and cyclin-dependent kinase (CDK; catalytic) subunits. Cell cycle-dependent fluctuations in the levels of many of the cyclin proteins are thought to contribute, at least in part, to the activation of these enzymes (1–4). The cyclins are required to regulate many of the p34cdc2-related cyclin-dependent protein kinases (CDKs), which have diverse functions prominently linked to the control of cell division (5–16). For example, cyclin B participates in the regulation of the G2/M transition by its association with p34cdc2, whereas cyclin A appears to be essential for the completion of S phase and entry into G2 phase in complexes with both p34cdc2 and cdk2 (17–27). In contrast, complexes formed between the D-type cyclins and both cdk4 and cdk6 integrate growth factor signals and the cell cycle, allowing cells to progress normally through G1 phase (10, 12, 28–34). The genetic alterations that occur in this pathway during oncogenesis appear to involve many of its components, including the D-type cyclins, cyclin-dependent protein kinases (cdks), and cyclin-dependent kinase inhibitors (CKIs).

Unlike other G1 cyclins described thus far (e.g. cyclin C and cyclin E), the D-type cyclins are highly homologous to one another and their expression overlaps during G1 phase, suggesting that this group of cyclins may be somewhat redundant in their function. However, it has also been noted that not all D-type cyclins are expressed in each tissue, suggesting that their function may be linked to the specific tissues in which they are expressed. This particular cell cycle pathway is subject to a number of alterations during tumorigenesis, presumably due to its importance in response to mitogenic stimulation (31, 35–43).

The biologic role of multiple D-type cyclins has not yet been resolved. It has been established that each of the D-type cyclins contain a functional pRb-binding motif and that these cyclins are induced in response to mitogens in a cell lineage-specific manner (3, 4). However, cyclins D2 and D3 bind preferentially to pRb, whereas cyclin D1-pRb complexes are less stable (44, 45). Microinjection of cyclin D1 antibodies and/or antisense oligonucleotides into normal human diploid fibroblasts, NIH3T3, and Rat-2 cells revealed that a sub-group of these cells could not enter S phase (46, 47). The ability of these microinjected cells to pass the restriction point and begin DNA replication was directly related to the time that had elapsed between the readdition of serum and microinjection of cyclin D1 antibodies or antisense oligonucleotides. Thus, by decreasing the level of functional cyclin D1 protein prior to the restriction point in late G1 phase, the ability of these cells to progress through the cell cycle was impaired. These agents have no apparent effect on cell cycle once cells have passed the restriction point late in G1 phase of the cell cycle. Finally, it has been shown that ectopic expression of cyclin D1 inhibits MyoD and myogenin-mediated skeletal muscle differentiation indirectly (48). To determine the role of cyclin D1 in muscle differentiation, experiments were designed to determine whether MyoD/myogenin and/or pRb were targets of cyclin D1-CDK phosphorylation. Mutation of CDK phosphorylation sites in
myogenin had no effect on the ability of cyclin D1 to inhibit differentiation. Ectopic MyoD expression in fibroblasts can induce muscle-specific gene expression, as long as wild-type pRB is also expressed (48). In pRB→−/− fibroblasts expressing MyoD and a mutated pRB (which cannot be hyperphosphorylated), ectopic cyclin D1 expression continues to inhibit muscle-specific gene expression, whereas ectopically expressed cyclins A and E have no effect. These data suggest that the initiation of muscle-specific gene expression can be blocked by two distinct pathways, one of which is dependent on pRB hyperphosphorylation and the other is not.

Several models of D-type cyclin function have been proposed based on the available data, but they all envision these cyclins as growth sensors (3, 4, 45, 46, 49, 50). The first model suggests that the D-type cyclins are tissue-specific regulatory subunits that are merely redundant in their function. A second model suggests that the functions of D-type cyclins are related to their sequential expression pattern during the cell cycle. More recent studies with cyclin D1 gene “knockouts” in mice have supported the former possibility (49, 50). Specifically, proper development of the retina and breast ductal epithelium was perturbed, suggesting that their normal maturation depends upon the presence of cyclin D1. This mouse model has provided significant insight into the requirements of cyclin D1 for normal development, but a specific requirement for this cyclin during cell cycle progression per se, has not been established.

We now report the successful utilization of the chicken lymphoma B-cell line, DT40, to eliminate the expression of specific G1 cyclins. This particular cell line undergoes gene-specific recombination at frequencies that are ~100-fold higher than those in mouse embryonic stem cells (51). DT40 cells provide a unique reagent to easily and quickly examine the necessity for each of these cyclins individually and collectively, as well as interactions between the various cyclins and other cell cycle proteins. These lymphoma B-cells normally express cyclin D1 and D2 but not cyclin D3. Elimination of cyclin D1 in DT40 cells is not lethal; however, ablation of cyclin D1 gene expression results in marked alterations in the ability of these cells to transit the cell cycle which, in turn, affects their normal growth rate. Furthermore, loss of cyclin D1 did not result in a compensatory increase in the levels of cyclin D2 in these cells. Overexpression of normal mouse cyclin D1, D2, or D3 in the D1−/− cells restored normal growth and cell cycle progression. However, a mutant murine cyclin D1 lacking the LXCXE motif (the so-called retinoblastoma protein (pRB)-binding motif that enhances D-type cyclin interaction with pRB) did not rescue the D1−/− phenotype (44, 45).

Here we show that elimination of cyclin D1 leads to a specific cell cycle phenotype and that these cells can be rescued by expression of any one of the three murine D-type cyclins, but not by a murine cyclin D1 mutant missing the LXCXE motif. The rescue of the D1−/− DT40 cells by murine cyclin D3 is somewhat intriguing, since avians do not express a cyclin D3 homologue in any cell line or tissue examined thus far (52, 53, and this study). Elimination of cyclin D1 in DT40 cells also resulted in a significant increase in apoptotic death following either UV or γ irradiation. Finally, expression of either murine cyclin D1, D2, or D3 in the DT40 D1−/− cells resulted in decreased sensitivity to radiation-induced apoptosis, indistinguishable to what was observed in the parental DT40 cell line. Expression of the mutant cyclin D1 partially rescued cells from radiation-induced apoptosis, suggesting that the mechanism(s) involving cyclin D1 in this pathway is distinct from its role in normal cell cycle progression. Thus, in avian B-lymphoma cells cyclin D1 appears to fully suppress apoptosis, whereas a mutant cyclin D1 partially rescued these cells, suggesting that the targets of cyclin D1-CDK activity in apoptotic cells may be distinct from those in proliferating cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**Agarose was purchased from FMC Bioproducts (Rockland, ME). Restriction and modification enzymes were obtained from New England Biolabs (Beverly, MA) and Boehringer Mannheim. Urea and cell culture reagents, including chicken serum, were purchased from Life Technologies, Inc. Sequenase and DNA sequencing reagents were purchased from U. S. Biochemical Corp. All radiisotopes were purchased from DuPont NEN. All chemicals were purchased from either Sigma or Fisher. The chicken cosmid genomic library was obtained from Clonetech (Palo Alto, CA). The Primelab labeling kit and Durapore membranes were purchased from Stratagene (La Jolla, CA). Selection drugs were purchased from Life Technologies, Inc., Sigma, or Boehringer Mannheim. Fluorescein-conjugated goat anti-mouse and goat anti-rabbit antibodies were obtained from Boehringer Mannheim or Southern Biotechnology. Rabbit polyclonal anti-cyclin D1 antibody was kindly provided by Dr. Charles Sherr (see Ref. 2), and anti-BuDR antibodies were obtained from Boehringer Mannheim. The DNA-specific dye 4′,6-diamidino-2-phenylindole was obtained from Boehringer Mannheim. Oligonucleotides for DNA sequencing were synthesized on an Applied Biosystems 373A DNA/RNA Sequencer by the Molecular Resources Facility of St. Jude Children’s Research Hospital.

Cloning and Sequencing of the Avian Cyclin D1 cDNA, Its Corresponding Gene, and Localization of the Chromosomal Gene—Cyclin D1 cDNAs were isolated from a UG9 T-cell cDNA library (55) by low stringency hybridization with a human cyclin D1 cDNA, kindly provided by Dr. Steve Reed (56). The nucleotide sequence of these cDNAs was determined as described previously (57). Oligonucleotides were spaced approximately 80–100 base pairs apart spanning the entire cDNA. A full-length cyclin D1 cDNA was transcribed and translated in vitro, producing a 36-kDa protein that could be immunoprecipitated with the mouse polyclonal cyclin D1 antibody (see Fig. 4, panel A). The corresponding cyclin D1 (Gallus gallus ccd1) gene was isolated by screening a Cornish White Rock chicken cosmid library (Stratagene) with the cDNA as a DNA probe (58). A 13-kb BamHI restriction fragment containing exons 1–3 of the cyclin D1 gene was subcloned into the pKS plasmid and either this DNA, or cosmIDNA, was used for double-strand DNA sequence analysis of the gene. The same oligonucleotides used for establishing the cDNA sequence were used to establish the molecular organization (intron/exon boundaries, exons) of the gene by sequencing the DNA strands in both directions. Genomic DNA was isolated as described previously (59).

**Gene Constructs—**The constructs containing the inserted drug selection cassettes were made by digestion of the 13-kb BamHI subtype between exons 1–3 of the GgccD1 gene, with the enzyme BstI that produced this fragment at a single site in exon 2 (Fig. 2, panel A). This exon encodes a major portion of the cyclin box region. Approximately 5.5 kb of genomic DNA were cut out on the 5′ side of the restriction site, and approximately 7.5 kb of genomic DNA remained on the 3′ side. Restriction fragments containing either the neo or hyg gene under the control of a β-actin gene promoter (kindly provided by Dr. C. Thompson) were cloned into this BstI site by blunt-end ligating using Klenow enzyme. Further details concerning the selectable marker genes can be found elsewhere (60, 61). The integrity of the constructs was confirmed by DNA sequencing, demonstrating that exon 2 was disrupted and that the remainder of the gene was intact. Homologous recombination of these constructs into the GgccD1 locus of the DT40 cells was demonstrated by the increase in size of a 15-kb XbaI fragment that contains exons 1 and 2 of the gene but that also contains a unique 5′ region (5′-flanking probe) not found in the 13-kb BamHI subtype used for generating the disruption constructs (Fig. 2, panel A). This probe could then be used to analyze genomic DNA from single cells clones after XbaI digestion. Insertion of the neo cassette (2 kb in size) generated a 17-kb XbaI hybridizing fragment (Fig. 2, panels C and D), and insertion of the hyg gene cassette (3 kb in size) generated an 18-kb XbaI hybridizing fragment (Fig. 2, panels C and D).

**Targeted Disruption and Analysis of Cyclin D1 Expression—**DT40 lymphoma B cells (kindly provided by Dr. C.-I. Chen) were grown in suspension cell culture in modified Dulbecco’s Eagle’s medium supple-mented with 10% fetal calf serum, 1% chicken serum, penicillin, streptomycin, and glutamine. pCycD1-neo and pCycD1-hyg were linearized and transfected into DT40 cells by electroporation (550 V, 25 microfarads). Twenty-four hours after DNA transfection, the appropriate concentration of selection drug (2 mg/ml G418 or 1.5 mg/ml hygromycin) was added to the culture medium, and the cells were selected for ~14 days. The correct gene insertion into the chicken cDNA sequence was confirmed by Southern Blot analysis as described previously (60). Essentially all of the antibodies used in this study recognized endogenous chicken cyclin D1.

**Expression of Endogenous Cyclin D1—**Expression of endogenous cyclin D1 in DT40 cells was determined by immunoprecipitation. For each sample, DT40 cells were labeled with 1 μg/ml BrdUrd for 3 h followed by four consecutive washes with DPBS. Cells were then fixed with 2% formaldehyde for 15 min. Cells were harvested and washed twice in DPBS and proteins were extracted with 0.5% NP-40 in DPBS and 0.1 M HCl as described previously (60, 61). For immunoprecipitation, DT40 cell extracts were incubated with pCycD1-neo and pCycD1-hyg were linearized and transfected into DT40 cells by electroporation (550 V, 25 microfarads). Twenty-four hours after DNA transfection, the appropriate concentration of selection drug (2 mg/ml G418 or 1.5 mg/ml hygromycin) was added to the culture medium, and the cells were selected for ~14
days. Single cells, isolated by flow sorting, were expanded into individual clones. Genomic DNA was isolated from multiple single cell-derived clones, digested with the appropriate restriction enzymes and hybridized with the cyclin D1 5′-flanking genomic probe (Fig. 2, panel A) to screen for homologous recombinants. Disruption of one, heterozygote (+/−) or both, D1 cyclin D1 disruptions were examined by both Southern and Northern blotting. RNA was isolated for Northern blotting, and the blots were hybridized to the cyclin D1 cdna probe as described previously (57, 62). Equal loading of RNA samples was verified by reprobing these blots with a human β-actin cdna probe. The cyclin D1 protein expression was examined by immunoprecipitation of [35S]methionine-labeled cell lysates using either mouse polyclonal cyclin D1, D2, or D3 antisera as described previously by others (28). As controls for these experiments, the mouse preimmune sera were used in all experiments, and an appropriate unlabelled cyclin D-GST fusion protein was used as a competitor to demonstrate specificity of the various D-type cyclin immunoprecipitations in DT40 cells (Fig. 4, panel A).

Cell Growth Analysis, Cytod Inclusion, Mitotic Indices, and Flow Cytometric Analysis—Cells were selected in 2 mg/ml G418 or 1.5 mg/ml hygromycin and then maintained in cell culture under continuous drug selection at the same concentrations. For the growth curves, 2 × 105 cells were plated in triplicate and grown in the presence of 10% serum. Each point represents the average of three determinations ± standard error of the mean. Growth curves were obtained for each of the cell lines for 7 days at 1-day intervals. DT40 cells were synchronized by first using centrifugal elutriation to enrich the G1 phase population of cells. These cells were then blocked at the G1/S boundary using 25 mg/ml nocodazole (30). These cells were then irradiated with varying amounts of either UV (UVC, 500, 1000, 1500, and 2000 J/m2). For γ-irradiation (4-Gy or 8-Gy γ-rays), the cells were treated in culture at a density of 5 × 106/ml, as described by others (65, 66). Both the UV and γ irradiated cells were returned to the incubator following addition of fresh media. Cells were then harvested 4–8 h following exposure to radiation, and the percentage of apoptotic cells was determined by TUNEL analysis and/or Hoescht staining of nuclei.

RESULTS

Isolation and Characterization of the Normal Avian Cyclin D1 cdna and Gene from Avians, and the Construction of Insertion Mutants—The cyclin D1 cdna was isolated by screening a UG-9 T-cell cdna library using the human cyclin D1 cdna as a probe (56). This particular T-cell line expresses cyclin D1 and cyclin D2 but not cyclin D3. Similarly, we found that the DT40 B-cell line expressed cyclins D1 and D2 but not D3 (data not shown; Fig. 3). Mullner and colleagues (53) have suggested that chickens may lack the cyclin D3 gene and the absence of an avian cyclin D3 homologue may indicate an evolutionary divergence in the tissue specificity of D-type cyclin expression. Our results appear to support this hypothesis. Ten positive cyclin D1 cdna clones were isolated and purified. Six of these cdnas had inserts ∼1.3 kb and were used for subsequent determination of the nucleotide sequence and predicted open reading frame (Fig. 1). Comparison of the avian, human, and murine cyclin D1 open reading frames demonstrates a high level of sequence conservation (86% identity with human and mouse) between the proteins, particularly in the conserved “cyclin box” region (1). The corresponding gene was isolated from a chicken cosmid library, and its structure was determined in its entirety by restriction endonuclease and DNA sequence analysis (Fig. 2, panel A). The size, ∼10 kb, and organization of the avian cyclin D1 gene (Ggcnd1) is identical to the human (CCND1) and mouse (ccnd1) homologues (67, 68). A major portion of the Ggcnd1 gene was subcloned for further analysis and construction of insertion mutants to eliminate expression of this gene in DT40 cells. A 13-kb BamHI fragment containing exons 1–3 of the Ggcnd1 gene was used for generating disruption constructs, and a single copy ∼3-kb BamHI fragment located 5′ of the larger BamHI fragment, but outside the region used to disrupt this allele by homologous recombination, was used for genomic DNA analysis (Fig. 2, panel A). We took advantage of a unique BstI restriction site found only in exon 2 of the cyclin D1 gene to generate insertion mutants, containing either neomycin (neo) or hygromycin (hyg) selectable marker genes that inactivate the normal gene (Fig. 2, panel B). Insertion of either of these selectable marker genes cassette into the BstI restriction site would alter the normal mobility of a 15-kb XhoI genomic fragment that is detected by the 5′-flanking probe (Fig. 2, panel A). Introduction of the neo gene cassette by homologous recombination into this region of the cyclin D1 gene generates a 17-kb XbaI fragment when

Quantitation of Cells Undergoing Programmed Cell Death Due to DNA Damage Induced by UV or γ-Irradiation—Normal and D1−/− cells were exposed to varying levels of UV or γ-irradiation to assess whether there are differences in the level of programmed cell death between these different cell lines. Others have previously shown (63, 64) that DT40 cells are more sensitive to UV (UVC) than to γ radiation (63–65). Three different single cell clones were used for this experiment (DT40 cells were pretreated for 16 h with 20 μg/ml UV, 2 mg/ml of 1% Triton X-100, and then irradiated with a range of doses). The UV and γ irradiated cells were returned to the incubator following addition of fresh media. Cells were then harvested 4–8 h following exposure to radiation, and the percentage of apoptotic cells was determined by TUNEL analysis and/or Hoescht staining of nuclei.

2 H. Li, J. M. Lahti, and V. J. Kidd, unpublished observations.
hybridized with the 5’-flanking probe, while the insertion of the *hgy* gene cassette generates an 18-kb *XbaI* fragment using the same probe (Fig. 2, panel C). As shown in Fig. 2, panel D, the wild-type DT40 cells (+/+) contain two copies of the normal 15-kb *XbaI* hybridizing fragment, whereas cells containing one normal allele and one allele disrupted by the introduction of the *neo* gene (+/-, heterozygote DT40 cells) into the cyclin D1 gene contain 15- and 17-kb *XbaI* hybridizing fragments, as expected. In DT40 cells containing both the neo and *hgy* gene cassettes homologously recombined into the cyclin D1 locus (-/-, homozygotes), the wild-type 15-kb *XbaI* hybridizing fragment is completely absent, and it has been replaced by the 17- and 18-kb *XbaI* fragments, respectively (Fig. 2, panel D). Additional proof of cyclin D1 elimination is provided by virtue of Northern blotting (Fig. 3) and protein analysis (Fig. 4).

**Expression of Cyclin D1 mRNA and Protein in +/+ , +/−, and −/− DT40 Cells—Complete elimination of the 15-kb *XbaI* restriction fragment containing the normal Gcccd1 gene was easily obtained in a number of single cell DT40 −/− clones (Fig. 2, panel D, lanes 4–9). When expression of the cognate cyclin D1 mRNA (~1.3 kb in size) was examined by Northern blotting using these same single cell clones, as well as a number of additional clones (15 total), expression of the mRNA was undetectable in −/− homozygotes and barely detectable in +/− heterozygotes (Fig. 3). However, a cross-hybridizing cyclin D2 mRNA (~6 kb) was readily detected in all cell lines (54, 58).**

Consistent with the results of others, we were not able to detect an avian cyclin D3 mRNA species, or a corresponding gene, in these same single cell clones (Fig. 4, panel D). Additional experiments involving the expression of either the wild-type or mutant murine cyclin D1 cDNAs in these same −/− cells (the mutant protein is truncated and no longer contains the pRb-binding motif LXCXE (44, 45)) can easily be detected using this strategy (Fig. 4, panel C, normal murine cyclin D1, clones 1/56 and 1/57; mutant murine cyclin D1, clones 1/2 and 1/17).

Additional experiments involving the expression of either the wild-type cyclin D2 or D3 cDNA, and its corresponding protein, in the same D1 −/− DT40 cells yielded similar results (Fig. 4, panel D). The murine polyclonal antisera for cyclin D2 was capable of immunoprecipitating both the IVTT-labeled avian cyclin D2 and the metabolically labeled murine cyclin D2 overexpressed in the D1 −/− cells (Fig. 4, panel D). When excess cold murine cyclin D2-GST fusion protein was added to the cyclin D2 antibody, the immunoprecipitation of the [35S]methionine-labeled IVTT protein was effectively competed (data not shown). Since an avian cyclin D3 homologue has not been isolated, a cyclin D3 IVTT control was not available for the corresponding immunoprecipitation of the metabolically labeled murine cyclin D3 protein expressed in the D1 −/− cells (Fig. 4, panel D). Steady-state levels of the various murine cyclins (D1, D2, and D3) were comparable in single cell clones, as judged by [35S]methionine labeling (Fig. 4 and data not shown). These metabolic labeling and immunoprecipitation experiments demonstrate that the cyclin D1 protein is absent in the D1 −/− DT40 cells, as well as confirm the expression of the normal murine cyclin D1, D2, D3, and mutant cyclin D1 in the same D1 −/− DT40 cells.

**Alterations in the Normal Growth of the D1 −/− Cells and Changes in Their Ability to Traverse the Cell Cycle—Cyclin D1 elimination is not lethal, presumably because cyclin D2 continues to be expressed in these cells. This is consistent with results reported in homoyzogous cyclin D1 −/− mice, which are also viable (48, 50). However, alterations in the cellular growth characteristics (Fig. 5) and normal progression through the cell cycle (Table I) were noted by flow cytometric analysis of asynchronously growing cells. The cell cycle phenotype of these cyclin D1 −/− cells, as might be expected, is distinct from that reported for modest overexpression of cyclin D1 in fibroblasts (47, 69, 70). Specifically, the total length of the cell cycle in these DT40 D1 −/− cells is increased by 2–3 h in two separate clones (1 and 3), increasing the cell doubling time from 14 to 16–17 h (Table II and Fig. 5). Additionally, the percentage of
asynchronously growing cells in G$_2$/M phase is increased, and the percentage of cells in S phase is concomitantly decreased (Table I). In one of the mouse cyclin D1 knockout studies, fibroblasts derived from the D1$^{+/+}$ mice, but not normal controls, were also delayed in G$_2$/M phase when quiescent D1$^{+/+}$ cells were restimulated by the addition of serum growth factors (50). Even though the effects of cyclin D1 elimination can be easily seen in asynchronously growing cell populations, they are more dramatic using synchronized cyclin D1$^{+/+}$ DT40 cells (Table II; cells were synchronized at the G$_1$/S boundary as described under “Experimental Procedures”). Once again, two separate cyclin D1$^{-/-}$ clones (1 and 3) were used...
Cyclin D1 Function during Growth and Death

![Diagram of Cyclin D1 expression in DT40 cells and DT40 cyclin D1 +/- or +/- cells. Total RNA (50 μg) from wild-type and cyclin D1 +/- or +/- cells was electrophoresed on an agarose gel, transferred to nitrocellulose, and probed with a 32P-labeled avian cyclin D1 probe. The 1.3-kb band in lanes 1, 2, 14, and 15 represents the avian cyclin D1 transcript. This band is reduced in intensity in clonal cell lines in which one allele of the cyclin D1 locus has been disrupted (lanes 3 and 16), and it is absent in clones that contain two disrupted cyclin D1 alleles (lanes 4–13 and 17–21). The larger 6.0-kb transcript may represent cross-hybridization of the probe to the avian cyclin D2 mRNA.](http://www.jbc.org/)

This suggests that the characteristics of the cyclin D1/–/– phenotype are due to absence of cyclin D1 specifically but that this phenotype can be rescued by expression of any of the normal murine D-type cyclin proteins. It should be noted that cyclins D2 and D3 interact with pRb more efficiently than cyclin D1 (10, 44, 45), suggesting that the LXXCE motif in cyclin D1 may mediate the binding of pRb-related proteins (e.g. p107, p130) or some unknown protein. Apparently normal pRb was detected in DT40 cells; Western blotting of DT40 lysates with a human pRb antibody demonstrated a broad band at ~104 kDa, the reported molecular mass for avian pRb (data not shown) (71, 72). The inability of the mutant murine cyclin D1, but not the normal murine cyclins D1, D2, and D3, to rescue these D1/–/– cells suggests that whatever interacts with the LXXCE motif of cyclin D1, its loss results in an altered cell cycle. These results are also consistent with the studies of Skapek et al. (48) demonstrating that cyclins A and E block muscle gene expression via phosphorylation of pRb but cyclin D1 does not; one interpretation of these data is that a pRb-independent pathway of skeletal muscle differentiation is regulated by cyclin D1. These results are consistent with what we have observed in the DT40 D1/–/– cells. Further biochemical studies of various cell cycle components (i.e. cyclins, CDKs, and CKIs) will require reagents, such as antibodies, that react with the avian proteins, since these proteins do not routinely cross-react with the available human and mouse antisera (data not shown) (52).

Radiation-induced Apoptosis in Normal and D1/–/–, D1/–/+ + Murine D1, D1/–/– + Murine D2, and D1/–/– + Murine D3 DT40 Cells—Considerable controversy exists regarding the possible role(s) of the D-type cyclins, particularly cyclin D1, in programmed cell death (73–78). Evidence from several different cell lines in which cyclin D1 levels are manipulated, either by overexpression or antisense (including rodent neuronal, mouse mammary epithelial, NIH 3T3 mouse fibroblasts, p53/–/– mouse embryo fibroblasts, rat fibroblasts, and human HCE7 esophageal carcinoma), suggests that cyclin D1 expression is required for apoptosis (73, 75–78). Conversely, others (32) have shown that the D-type cyclins function as growth factor sensors that can partially suppress apoptosis in myeloid, interleukin-3-dependent blood cells. Selective tissue atrophy and loss of proliferative capacity have also been observed in the retina and mammary epithelium of cyclin D1/–/– mice, consistent with the hypothesis that cyclin D1 acts as a tissue-specific growth factor sensor (49, 50). In addition, overexpression of cyclin D1 in rodent fibroblasts, or even concomitant expression of both cyclins D1 and E, has not been associated with increased levels of programmed cell death (47, 68, 70). In a separate study, Sofer-Levi and Resnitzky (78) used a tetracycline-responsive promoter to direct expression of cyclin D1 in serum-starved rat fibroblasts. Induction of cyclin D1 protein expression in these serum-starved fibroblasts resulted in significant apoptosis. However, in the presence of normal serum, induction of cyclin D1 did not result in a significant increase in apoptotic cells. The availability of avian D1/–/– B-cells allowed us to examine this question directly.

UV and γ radiation-induced apoptosis occurred in a dose-dependent manner in both DT40 control and DT40 D1/–/– single cell clones (Table III). The percentage of cells undergoing programmed cell death resulting from 500 to 1,500 J/m² of UVC or γ radiation was substantially increased in DT40 D1/–/– cells (10–15% increase after UVC exposure and a 15–25% increase after γ radiation; Table III). To determine whether this increased sensitivity to radiation, reflected as increased programmed cell death, was linked to the expression of D-type cyclins, we examined the effect(s) of γ radiation on D1/–/– cells.
Cyclin D1 Function during Growth and Death

Fig. 4. Analysis of cyclin D1 protein expression in wild-type and cyclin D1 −/− DT40 cells. Panel A, analysis of the ability of the mouse cyclin D1 polyclonal rabbit antisera to detect the avian cyclin D1 protein. The avian cyclin D1 cDNA was in vitro transcribed and translated (IVTT) and then immunoprecipitated using either preimmune sera (pre), mouse cyclin D1 antisera (D1 Ab), or cyclin D1 antisera preincubated with mouse GST-cyclin D1 protein as a competitor. Panel B, analysis of the cyclin D1 protein expressed in wild-type and D1 −/− DT40 cells. Wild-type DT40 cells and clonal cyclin D1 −/− cell lines were [35S]methionine-labeled (200 μCi/ml) for 30 min. The cells were then lysed with RIPA buffer and the cyclin D1 protein immunoprecipitated after the lysates were precleared with an anti-chicken immunoglobulin antibody. The IVTT avian cyclin D1 protein is shown in the first lane as a control. Lanes 2 and 3 represent preimmune (pre) and cyclin D1 (D1 Ab) immunoprecipitates from wild-type DT40 cells. Two independent cyclin D1 −/− clones are shown in lanes 4–7. Panel C, DT40 D1 −/− cells (clone 1 from panel B) were transfected with an expression construct containing the normal mouse cyclin D1 cDNA (D1). As a control, the same parental cells were also transfected with a mouse cyclin D1 cDNA expression construct lacking the retinoblastoma (Rb) interaction domain (Mut D1). Single cell clones were isolated, [35S]methionine-labeled, and immunoprecipitated as described above. Panel D, DT40 D1 −/− cells (clone 1 from panel B) were transfected with an expression construct containing the normal mouse cyclin D2 cDNA (+D2) or cyclin D3 cDNA (+D3). These cells were then [35S]methionine-labeled and immunoprecipitated with either preimmune antisera (pre), cyclin D2 antisera (D2), or cyclin D3 antisera (D3). IVTT murine cyclin D2 or D3 (IVTT control) was used as a positive control for the IPs. The D2 Ab lane is an IP of the D1 −/− clone 1 cell lysate in the absence of exogenously expressed cyclin D2. The +D2, pre lane is a preimmune IP of the murine cyclin D2-transfected and metabolically labeled, D1 −/− clone 1 cells. The +D2, D2 Ab lane is the cyclin D2 IP of the murine cyclin D2-transfected and metabolically labeled, D1 −/− clone 1 cells. The IPs of the murine cyclin D3-transfected D1 −/− clone 1 cells is identical to what was described for the murine cyclin D2-transfected D1 −/− cells. The location of the murine cyclin D2 and D3 proteins are indicated on the left and right sides of the panel, respectively.

in which murine D-type cyclins were expressed. These experiments involved the same D1 −/− +D-type cyclin(s) cell lines described above, in which cell cycle abnormalities resulting from ablation of cyclin D1 were rescued by expression of any murine D-type cyclin. Expression of mouse cyclin D1 in DT40 −/− cells (Fig. 4, panel C, D1 −/− clone 1/57) eliminated the increased sensitivity to γ irradiation exhibited by the D1 −/− cells (Table III). In fact, the percentage of apoptotic cells in the D1 −/− + murine D1 cells was nearly identical to the parental cells. Similarly, when D1 −/− + murine D2 or D1 −/− + murine D3 cells were used (Fig. 4, panel D, D1 −/− clone 1 + D2/D3), enhanced sensitivity to γ irradiation, as reflected by
the number of apoptotic cells, was apparently eliminated (Table III). Intriguingly, expression of the mouse mutant cyclin D1 (missing the LXXE motif) partially, but not completely, restored cellular resistance to radiation-induced apoptosis (Table III). Since the increased sensitivity of the D1 →− cells to radiation-induced apoptosis can be effectively reversed by expression of any normal D-type cyclin, it is likely that these proteins do, in fact, suppress apoptosis in DT40 cells. Furthermore, the ability of the mutant cyclin D1 protein to partially suppress radiation-induced apoptosis but not cell cycle abnormalities (Tables I and III) suggests that proliferating and apoptotic cells may contain distinct cyclin D1 complexes. Whether these complexes are composed of different polypeptides and/or distinct post-translational modifications is not known at this time. Further studies with the D1 →− DT40 cell lines will help to elucidate whether the observed defect in G1 checkpoint control following radiation-induced DNA damage is due entirely to the disruption of normal p21<sup>wafl/cip1</sup> cyclin D1 interactions or inappropriate interactions between p21<sup>wafl/cip1</sup> and other cell cycle machinery in these cells. However, this response is consistent with data from p21<sup>wafl/cip1</sup> →− mice, demonstrating that these mice are defective in G1 checkpoint control (79), as well as the developmental abnormalities observed in D1 →− mice (49, 50) and the inactivation of cdks following UV irradiation (80).

**DISCUSSION**

The specific biologic functions of the three D-type cyclins remain somewhat of an enigma. Mice lacking cyclin D1 by gene targeting in embryonic stem cells have recently been generated, but the results of this study reflect a more generalized requirement of this particular cyclin during development than a specific requirement during the cell cycle (49, 50). Furthermore, interpretation of the effect(s) of gene “knockouts” in animal models can be complicated by the expression of functionally redundant proteins that may mask the phenotype in certain tissues (81). We have now generated avian B-cell lines that lack cyclin D1 but retain normal cyclin D2 expression, which exhibit defects in normal cell cycle progression. The growth of the D1 →− cells is slightly retarded (~20% slower than wild-type DT40 cells), presumably due to a delay in G<sub>S</sub>/M phase. A similar delay in G<sub>S</sub>/M phase was observed when mouse embryo fibroblasts derived from the D1 →− mice were analyzed (50). The cell cycle “defects” observed in the DT40 D1 →− cells can be specifically rescued by the expression of normal murine cyclin D1, D2, or D3 but not by a mutant cyclin D1 protein that lacks the LXXE pRb-binding motif (44). The fact that overexpression of the murine cyclin D3 protein, for which there is no apparent avian homologue, rescues the D1 →− phenotype in DT40 cells strongly supports the hypothesis that the D-type cyclins have overlapping functions (3, 4, 82). Our results are also consistent with a specific requirement for cyclin D1 for normal transit through the cell cycle and that this requirement cannot be compensated for by the endogenous cyclin D2 protein. A normal cell cycle can only be acquired by exogenous expression of any one of the D-type cyclins. The molecular basis of this rescue is unknown at this time. One can speculate that the observed effect(s) on the cell cycle are due to inappropriate level(s) and/or association(s) between D-type cyclin interactors and other components of the cell cycle machinery (e.g. inappropriate levels of p21<sup>wafl/cip1</sup>, p27<sup>Kip1</sup>, INK4 or cdk4/6 proteins may affect the activity of other cyclins, such as cyclin E). Further biochemical analyses of these cells will require the production of appropriate antibody reagents for avian cyclins, cdks, and ckis.

We would suggest that the cell cycle perturbations observed in the cyclin D1 →− cells might be due to 1) an insufficient “threshold” level of cyclin D1 required for entry into G<sub>S</sub> phase from mitosis or 2) defects in replication resulting from the absence of cyclin D1 that might trigger G<sub>S</sub> phase checkpoints and delay cells at this point until such damage is repaired. These two possible mechanisms are suggested since the most obvious outcome of cyclin D1 elimination, lengthening of G<sub>S</sub> phase, was not observed. The second possible explanation is supported by data from this study in which D1 →− cells, blocked at the G<sub>S</sub>/S boundary by centrifugal elutriation and treatment with aphidicolin, are subject to a 2–3-h delay in progression through the cell cycle when released from this cycle block (Table II and Fig. 6). A delay in G<sub>S</sub> phase due to the activation of checkpoints before mitosis is triggered, possibly involving inappropriately associated cyclin-dependent protein kinase inhibitory (CKI) subunits, could potentially explain these observations. Whether the observed cell cycle phenotype and enhanced programmed cell death associated with these D1 →− B-lymphoma cells will be found in other cell types, or is specific for this cell lineage, is not known at this time. Further study of these cell lines, as well as the murine cyclin D1 knockout models (49, 50), should provide answers to these questions. The ability of any of the D-type cyclins to “rescue” D1 →− DT40 cells suggests that these cyclins are somewhat redundant in function. However, we cannot completely rule out

**FIG. 5.** Analysis of the growth rates of wild-type and cyclin D1 →− clones. Equivalent numbers of cells (20,000) from wild-type DT40 (solid black box), a single cyclin D1 allele disruption, +/+ (top crosshatched box which is superimposed on the solid black box), and three independent cyclin D1 →− clones (three progressively less shaded boxes) were seeded into 12-well cluster dishes. The number of viable cells was determined by using a hemacytometer at 24-h intervals. Each point represents the data averaged from three independent dishes.

**TABLE I**

| Cell cycle position | DT40 | DT40 →− clone 1 | DT40 →− clone 1/56 + D1 | DT40 →− clone 1/2 + mutant D1 | DT40 →− clone 1 + D2 | DT40 →− clone 1 + D3 |
|---------------------|------|-----------------|------------------------|-----------------------------|--------------------|---------------------|
| G<sub>s</sub>        | 27   | 23              | 25                     | 24                          | 23                 | 23                  |
| S                   | 59   | 50              | 59                     | 50                          | 60                 | 61                  |
| G<sub>S</sub>/M      | 14   | 27              | 16                     | 26                          | 17                 | 16                  |
no statistically significant differences were observed between these experiments. No statistically significant differences were observed between these experiments. Tumor cells express very high levels of apparently normal cyclin D1 in parathyroid adenomas (85). As a consequence, these tumor cells may trigger cell death by default. Independent studies of cells but whose inappropriate expression in factor-deprived cells but not when normal growth factor levels are maintained (85, 86).

A separate, but perhaps equally important issue, has arisen regarding the possible role of the D-type cyclins in apoptosis. The availability of D1−/− cells has allowed us to examine the possible relationship between cyclin D1 expression and the induction of apoptosis. Several studies have linked induction of cyclin D1 gene expression in post-mitotic neurons and senescent fibroblasts to programmed cell death (73, 75). In addition, cyclin D1 overexpression in specific tumor cell lines results in enhanced apoptosis by selective agents (77). Finally, two different studies have been published concerning cyclin D1-induced apoptosis in cultured cells (75, 76). In cultured neuronal cells, cyclin D1 overexpression, in the presence of normal growth factors (10% serum), was capable of inducing apoptosis (75). However, in cultured fibroblasts, cyclin D1 overexpression induced apoptosis only in the absence of growth factors (0.1% serum) (78). It should be noted that the human cyclin D1 gene, CCND1, was originally identified by one group as the PRA1 oncogene residing at the breakpoint of a chromosome 11 inversion in parathyroid adenomas (85). As a consequence, these tumor cells express very high levels of apparently normal cyclin D1 mRNA and protein. Thus, it is somewhat paradoxical that elevated expression of cyclin D1 can be so well tolerated in these tumor cells, whereas its overexpression in some transformed cell lines enhances apoptosis (73, 75, 85). In fact, these results parallel what has been observed with c-myc, an oncogene that also induces apoptosis when overexpressed in serum-deprived cells but not when normal growth factor levels are maintained (85, 86). Our results strongly support the role of cyclin D1 as a positive growth regulator that suppresses apoptosis in proliferating cells but whose inappropriate expression in factor-deprived cells may trigger cell death by default. Independent studies of the effects of DNA damage (e.g. UV and/or irradiation) on the cell cycle machinery support the notion that D-type cyclins normally suppress apoptosis (80). In response to UV irradiation, the activity of various CDKs was inhibited in a p53-dependent manner by enhanced expression of CDK inhibitors (e.g. p21Waf1/Cip1), as well as phosphorylation of Thr-14 and Tyr-15 of p34cdc2. Inhibition of CDK activities in response to radiation apparently allows cells to either repair their DNA damage, by suspending progress through the cell cycle until the damage is repaired, or, alternatively, leads to their elimination by apoptosis if the damage is too extensive. The enhanced level of programmed cell death observed in the DT40 D1−/− cell lines in response to radiation-induced DNA damage, as well as the ability of exogenously expressed murine D-type cyclins to desensitize these cells, suggests that cyclin D1 may suppress

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### Table II

**Cell cycle synchrony of various cell lines**

| Cell line | %G1, DT40 | %S, DT40 | %G2/M, DT40 | %G1, Clone 1 | %S, Clone 1 | %G2/M, Clone 1 | %G1, Clone 3 | %S, Clone 3 | %G2/M, Clone 3 |
|-----------|-----------|----------|-----------|-------------|----------|-----------|-------------|----------|-----------|
| DT40      | 1.2       | 13.7     | 27.1      | 30.7        |          |            |              |          |            |
| Clone 1   | 2.9       | 19.8     | 36.8      | 44.5        |          |            |              |          |            |
| Clone 3   | 1.8       | 20.1     | 37.3      | 42.6        |          |            |              |          |            |

### Table III

**Affect of OV and γ irradiation on cell survival**

| Cell line | Control (0 Gy) | 500 J/m² | 1,000 J/m² | 1,500 J/m² |
|-----------|---------------|----------|------------|------------|
| DT40      | 2.5           | 7.7      | 33.2       |            |
| D1−/−, Clone 1 | 2.8       | 22.1     | 49.4       |            |
| D1−/−, Clone 3 | 2.4       | 27.7     | 56.5       |            |
| D1−/−, + mD1 | 2.3       | 8.1      | 34.1       |            |
| D1−/−, + mD2 | 2.5       | 7.8      | 30.9       |            |
| D1−/−, + mD3 | 2.4       | 7.9      | 33.3       |            |
| D1−/−, + nutD1 | 2.7      | 14.4     | 23.4       |            |

* a UV irradiation dose.
* b Results of TUNEL analysis are representative of two separate experiments. No statistically significant differences were observed between these experiments.
* c Results of TUNEL analysis from one of three separate experiments. No statistically significant differences were observed between these experiments.
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 apoptosis. Once again, we do not know whether this is a cell type-specific effect. Further studies of the DT40 D1−/− cells could provide valuable insight into how cyclin D1 functions as a mediator of apoptosis and help determine whether this reflects changes in cyclin D1 complexes with other proteins, such as CDKs, CDKIs, and pRB/p107/p130, as cells transit the cell cycle. In addition, it will now be of interest to determine whether elimination of cyclin D2, on its own or concomitantly with cyclin D1, has a similar effect(s) on the cell cycle and/or programmed cell death.

Finally, these studies demonstrate, persuasively, that the DT40 cell line is amenable to easy and rapid manipulation of type-specific effect. Further studies of the DT40 D1 cells

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