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Overproduction of Rb Protein after the G_1/S Boundary Causes G_2 Arrest

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The Rb protein is known to exert its activity at decision points in the G_1 phase of the cell cycle. To investigate whether it may also play some role(s) at later points in the cell cycle, we used a system of rapid inducible gene amplification to conditionally overexpress Rb protein during G_2 phase. A cell line expressing a temperature-sensitive simian virus 40 large T antigen (T-Ag) was stably transfected with plasmids containing the *Rb* cDNA linked to the simian virus 40 origin of replication: pRB-wt, pRB-fs, and pRB-Dra, carrying wild-type murine *Rb* cDNA, a frameshift mutation close to the beginning of the *Rb* coding region, and a single-amino-acid deletion in the E1A/T-Ag binding pocket, respectively. Numerous independent cell lines were isolated at the nonpermissive temperature; cell lines displaying a high level of episomal amplification of an intact *Rb* expression cassette following shiftdown to the permissive temperature were chosen for further analysis. Plasmid pRB-fs did not express detectable Rb antigen, while pRB-Dra expressed full-length Rb protein. The Dra mutation has previously been shown to abrogate phosphorylation as well as T-Ag binding. Fluorescence-activated cell sorting (FACS) analysis revealed that cultures induced to overexpress either wild-type or Dra mutant Rb proteins were significantly enriched for cells with a G_2 DNA content. Cultures that amplified pRB-fs or rearranged pRB-wt and did not express Rb protein had normal cell cycle profiles. Double-label FACS analysis showed that cells overexpressing Rb or Rb-Dra proteins were uniformly accumulating in G_2, whereas cells expressing endogenous levels of Rb were found throughout the cell cycle. These results indicate that Rb protein is interacting with some component(s) of the cell cycle-regulatory machinery during G_2 phase.

The *Rb* gene belongs to a growing number of "tumor suppressor" genes; loss of their function is associated with malignant transformation. The *Rb* gene was first cloned through a linkage analysis of familial retinoblastoma (23, 50). The human gene contains 27 exons dispersed over 200 kbp on chromosome 13 (24, 35) and encodes a 105-kDa nuclear phosphoprotein of 928 amino acids (51). The E1A protein of adenovirus (85), the large T antigen (T-Ag) of simian virus 40 (SV40) (15), and the E7 protein of human papillomavirus type 16 (20) physically interact with the Rb protein. T-Ag and E1A mutants unable to bind Rb are transformation defective, indicating that these DNA tumor viruses cause transformation in part by interfering with Rb function. These oncoproteins also bind a diverse assembly of other cellular proteins (19, 22, 27, 33, 58, 66, 83, 86, 88), some of which, for example, p53 and cyclin A, have known cell cycle-regulatory activities. It is thus not surprising that several mutational analyses of the viral oncoproteins indicate that the elimination of Rb activity is a necessary but not sufficient step in the mechanism by which these viruses cause transformation (36, 57, 69, 77, 84, 92). Expression of *Rb* cDNA in tumor cell lines lacking *Rb* expression can elicit a variety of responses, such as morphological reversion, loss of tumorigenicity, growth inhibition, and even senescence (6, 28, 40, 78, 81).

The *Rb* gene is expressed constitutively in most cell types regardless of their proliferation status, and the Rb protein is stable (5, 25, 51). The phosphorylation state of the Rb protein changes during the cell cycle (7, 11, 16, 56, 59). Cells in G_1 and G_0 contain an un- or underphosphorylated form of Rb. At the G_1/S boundary, Rb becomes phosphorylated on multiple sites, remains thus through S and G_2, and is dephosphorylated in M. Upon closer scrutiny, the phosphorylation can be seen to occur in several successive waves during S and G_2 (14). In some cell types, the first phosphorylations become evident at earlier times in G_1 or during the G_0 to G_1 transition (21, 54, 82). Rb phosphorylation can be catalyzed by the $p34^{cdc2}$ kinase in vitro (1, 39, 52–54, 79), but recent evidence suggests the involvement of additional kinases (39, 54).

A large body of evidence indicates that the underphosphorylated form of Rb found during G_1 has growth-suppressing properties. Both the T-Ag of SV40 and the E7 protein of papillomavirus bind exclusively to the under-phosphorylated species (20, 55, 56), and microinjection of excess Rb protein into cells in G_1 prevents entry into S phase (29). The Rb sequences required for interaction with the viral oncoproteins, termed the binding pocket, have been narrowed down to two noncontiguous stretches separated by a spacer in the C-terminal half of the polypeptide (38, 42, 44). Defective *Rb* genes recovered from naturally occurring tumors often contain mutations in the binding pocket sequences. The binding pocket has been shown to also mediate the association of Rb with several cellular proteins, including the transcription factor E2F and related proteins (2–4, 9, 12, 34, 45, 63), the product of the *myc* proto-oncogene (71), and the Cdc2 kinase (39), while the identity of others remains to be established (17, 41, 46).

A number of studies have indicated that Rb can modulate the transcription of several genes implicated in the regulation of proliferation, such as c-*fos* (70), the gene for transforming growth factor β1 and c-*myc* (47, 65), the gene for insulin-like growth factor II (48), the *neu* proto-oncogene (89), and *cdc2*

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kinase (13). To this list can also be added several housekeeping genes with cell cycle-regulated expression, such as those for thymidine kinase, dihydrofolate reductase, thymidylate synthase, DNA polymerase α, and ribonucleotide reductase (18). Many, if not all, of these effects are thought to be mediated by Rb through its physical interaction with cellular transcription factors. For example, E2F is bound and sequestered by Rb in an inactive complex during G_1, and either phosphorylation or E1A binding can release Rb and activate E2F (8, 10, 76, 91). This is currently the best-understood regulatory mechanism involving Rb, but it is already apparent that not all Rb activity is mediated by E2F (48, 49).

The emerging model of Rb function is that of a cell cycle regulator whose inactivation, by either phosphorylation or oncoprotein binding, allows cells to progress from G_1 into S. The simplest version of this model envisions phosphorylated Rb being totally inactive and not participating in functional interactions during S or G_2. Several lines of evidence have, however, recently emerged, suggesting that Rb may also play some role(s) at later points in the cell cycle. First, Rb is phosphorylated in at least three stages during the cell cycle, the last stage occurring in G_2 (14). Second, Rb protein is specifically localized in "replication compartments" during S phase, suggesting that it may be associated with DNA replication complexes (87). This apparent association occurs after the phosphorylation of Rb at the G_1/S boundary. Third, phosphorylation of Rb causes the dissociation of T-Ag but not E1A, which remains bound throughout G_2 (19, 77). Fourth, an E1A mutant that did not bind Rb but bound its other known partners (p400, p300, p107, and cyclin A) was capable of inducing exit from G_0 and DNA synthesis but not mitosis (36). Another E1A mutant capable of binding the underphosphorylated but not the phosphorylated form of Rb was impaired in transformation as well as cooperation with *ras* (77).

In this communication, we report that conditional overproduction of Rb during G_2 leads to cell cycle arrest. This result is not consistent with the simple model that Rb is only active during G_1.

MATERIALS AND METHODS

Cell line construction and culture conditions. Isolation of the BTS-1 cell line has been described before (73). All cell lines described in this communication were constructed by electroporation. Plasmid DNA (50 μg) was linearized with *Xmn*I in the vector backbone, ethanol precipitated, and resuspended in 0.5 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (68). Electroporation was done with a Bio-Rad Gene Pulser apparatus, in a cuvette with a 4-mm gap at a setting of 180 V and 960 μF, as described before (68). Cells were trypsinized after 48 h and reseeded, at various dilutions, into dishes containing 800 μg Geneticin sulfate (GIBCO, Inc.) per ml. All cell lines were cultured in Dulbecco's modified Eagle's medium (supplemented with glutamine, pyruvate, and high glucose; GIBCO, Inc.) containing 3.7 g of sodium bicarbonate per liter, penicillin-streptomycin, and 10% fetal calf serum (GIBCO) in a 5% CO_2 atmosphere at either 33 or 39.5°C, as indicated elsewhere in the text.

Recombinant DNA manipulations and Southern hybridization analysis. All recombinant DNA manipulations were performed by established methods (72). The vector used to express the *Rb* cDNA is a derivative of pMAM*neo-Blue* (Clontech, Inc.) containing the cytomegalovirus promoter and enhancer (*Spe*I-*Xba*I fragment from plasmid CDM8; Invitrogen, Inc.) inserted into the *Nhe*I site. The murine *Rb* cDNA was recovered as an *Nae*I-*Bst*XI fragment from plasmid pMRB115 (5), blunt ended with Klenow fragment, and inserted into the blunt-ended *Xba*I site of the vector to construct plasmid pRB-wt. In all cell lines examined, murine Rb protein was not detectable at 39.5°C by Western (immunoblotting) analysis (murine Rb migrates slightly faster than monkey Rb [30]). Plasmid pRB-fs was constructed from pRB-wt by opening with *Not*I, filling in with Klenow fragment, and recircularizing. The *Not*I site interrupts codon 10 of the murine *Rb* gene. The construction inserted 4 bp, as verified by the creation of a new *Nae*I site. Plasmid pRB-Dra was constructed from pRB-wt by opening with *Dra*III, removing 3' overhangs with Klenow fragment, and recircularizing. The construction removed 3 bp, as verified by the creation of a new *Pml*I site. This mutation is identical to the previously described ΔDra mutation (30). Southern blot hybridization was performed by standard methods (72). Probes (as indicated in the text) were gel-purified restriction fragments labeled with [α-^{32}P]dCTP by the random oligonucleotide priming method.

In situ immunofluorescence. Cells were grown on glass coverslips. Coverslips were rinsed in phosphate-buffered saline (PBS) and fixed for 5 min at room temperature in absolute methanol and then for 2 min in acetone at −20°C. Coverslips were incubated in PBS-BSA (PBS containing 0.1% bovine serum albumin [BSA]) for 30 min at room temperature and then in PBS-BSA containing 10 μg of monoclonal anti-Rb antibody Rb-Ab2 (Pharmingen, Inc.) per ml for 1 h at 37°C. After extensive washing in PBS-BSA, coverslips were incubated in PBS-BSA containing the secondary antibody (fluorescein isothiocyanate [FITC]-conjugated rabbit anti-mouse immunoglobulin G [IgG]; Jackson ImmunoResearch, Inc.) for 1 h at 37°C. After extensive washing in PBS, coverslips were mounted for microscopy and observed in a Nikon Diaphot inverted microscope fitted with an epifluorescence attachment. The hypotonic in situ extraction of nuclei was performed as described before (60).

Western blot analysis. Total cellular protein samples were prepared by harvesting cells from a 10-cm dish on ice with a rubber policeman. Cells were pelleted for 15 s in a microcentrifuge, washed twice with 1 ml of PBS, and resuspended in 300 μl of ice-cold lysis buffer (62.5 mM Tris [pH 6.8], 10% [wt/vol] glycerol, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin per ml, 1 μg of pepstatin per ml, 2 μg of leupeptin per ml). Then 15 μl of 20% sodium dodecyl sulfate (SDS) was added and vortexed vigorously, and the viscous solution was immediately transferred to a boiling-water bath for 3 min, chilled on ice, and quickly frozen in aliquots. Protein was quantified with a Micro BCA Protein Assay Kit (Pierce, Inc.). Prior to electrophoresis, the samples were thawed; mercaptoethanol and bromophenol blue were added to 5% (vol/vol) and 0.1% (wt/vol), respectively, the samples were boiled for 3 min and 15 μg of protein was loaded per lane.

Western blotting analysis was performed by standard procedures (32). The Rb-Ab2 antibody was used at 1 μg/ml, and the XZ55 antibody (37) was used at 10 μg/ml. SDS-polyacrylamide gels were transferred onto nitrocellulose, which was blocked with Blotto. The secondary antibody was horseradish peroxidase-conjugated rabbit anti-mouse IgG antiserum (Jackson ImmunoResearch, Inc.). Signals were visualized with the enhanced chemiluminescence System (Amersham, Inc.) according to the manufacturer's instructions.

FACS analysis. Cells were harvested from 10-cm dishes

with trypsin, washed twice in PBS, resuspended in 2 ml of PBS, and fixed by three stepwise additions (2 ml each) of ice-cold absolute ethanol. In single-label experiments, a minimum of 1 h after fixation, cells were resuspended in 1 mg of RNase A per ml for 30 min at 37°C and then stained with 0.05 mg of propidium iodide (PI) per ml for 1 h on ice. In double-label experiments, cells were resuspended after ethanol fixation, incubated for 1 h in PBS-BSA, resuspended, incubated for 1 h at 37°C in PBS-BSA containing 10 μg of antibody Rb-Ab2 per ml, washed extensively with PBS-BSA, resuspended, incubated for 1 h at 37°C in PBS-BSA containing the secondary antibody (FITC-conjugated rabbit anti-mouse IgG; Jackson ImmunoResearch, Inc.), washed extensively with PBS-BSA, washed extensively in PBS, and refixed by three stepwise additions of ice-cold absolute ethanol. After this treatment, the samples were stained with PI as indicated above for the single-label experiment. Fluorescence-activated cell sorting (FACS) analysis was performed with a FACS IV flow cytometer (Becton Dickinson, San Jose, Calif.). For detection of PI, cells were excited at 488 nm and the emission was collected above 590 nm; for detection of FITC, cells were excited at 488 nm and the emission was collected between 515 and 545 nm.

RESULTS

Experimental design and isolation of Rb-overproducing cell lines. We have previously developed an expression system based on rapid inducible gene amplification with the SV40 origin of replication and T-Ag (73, 75). A cell line, designated BTS-1 (BSC-40, temperature-sensitive, SV40) was previously isolated by stably transfecting BSC-40 monkey cells with the *tsA58* allele of the SV40 T-Ag gene (73). The BTS-1 cell line stably expresses the TsA58 T-Ag protein at a low level (73–75). Because the T-Ag is inactive at the nonpermissive temperature (39.5°C), SV40 origins of replication can be maintained stably integrated in the genome. Immediately after a shiftdown to the permissive temperature (33°C), T-Ag is stabilized, and as cells enter S phase, the integrated SV40 origins begin to replicate. The transfected DNA excises and continues to replicate episomally; up to 5,000 to 10,000 copies per cell can accumulate in a single S phase (75). Since the construct containing the *tsA58* T-Ag gene does not contain an SV40 origin, it remains stably integrated, and T-Ag protein expression is thus maintained at a low constitutive level (75).

The structure as well as the copy number of the excised episomes is a reproducible characteristic of individual cell lines. For example, one particular cell line may always produce a unit-size species that amplifies to 5,000 copies per cell, whereas another cell line derived from the same transfection may always generate a rearranged species that amplifies to 500 copies per cell (73). The fraction of cells in a culture that undergo amplification can vary over a broad range, but this is also a reproducible and stable property of individual cell lines. The reasons for these phenomena are not clear, but they may reflect the influence of chromosomal position effects on the excision process combined with differences in T-Ag expression in individual cells in a culture (31). The salient feature of this expression system is that genes closely linked to the SV40 origin can be coamplified, and cells are first exposed to the encoded gene products during the ensuing G_2 phase.

We first demonstrated that the BTS-1 cell line is suitable for the analysis of cell cycle progression. In highly confluent BTS-1 cultures at 39.5°C, approximately 80% of the cells display a G_0/G_1 DNA content (Fig. 1A; serum limitation is not a useful method of synchronizing BTS-1 cells in G_0). Trypsinization, dilution, and shiftdown to 33°C elicit exit from G_0/G_1 and entry into S and G_2 phases some 12 to 16 and 20 to 24 h after release, respectively (Fig. 1B). Significant numbers of mitotic figures begin to appear approximately 32 to 36 h after release, although by this time the synchrony of the culture has deteriorated.

The murine *Rb* cDNA was cloned into an expression vector containing an SV40 origin and the selectable *neo* gene (pRB-wt, Fig. 2). Two mutant *Rb* cDNA genes were also cloned in the same vector: a frameshift mutation leading to premature termination (pRB-fs, created by filling in a *Not*I site early in the coding region), and a single-amino-acid deletion in the T-Ag binding pocket, shown previously (30) to abrogate phosphorylation and T-Ag binding (pRB-Dra, created by blunt-ending a *Dra*III site). The Rb-Dra mutant protein has been shown not to associate with T-Ag even when overproduced in COS cells (30).

BTS-1 cells were electroporated with linearized plasmids, G418-resistant colonies were selected at the nonpermissive temperature, and numerous clonal cell lines were established. Cell lines were screened for the level of amplification and the structure of the excised episomes by Southern blot hybridization 72 h after shiftdown to the permissive temperature. Cell lines displaying a high level of amplification (5,000 to 10,000 copies per cell) of unrearranged *Rb* genes were chosen for further analysis (Fig. 3): cell lines RB-A3, RB-A5, and RB-A14 contain plasmid pRB-wt, cell line FS-B15 contains pRB-fs, and cell line DRA-C26 contains pRB-Dra. Cell line RB-A11, amplifying pRB-wt rearranged in the *Rb* coding region, was chosen as an additional control.

Analysis of Rb protein overproduction. Patterns of Rb protein expression were analyzed by in situ immunofluorescence (Fig. 4). At the nonpermissive temperature, all cell lines displayed low-intensity punctate nuclear staining (RB-A3 is shown in Fig. 4A). This pattern of expression is entirely consistent with previous reports (60) and was identical to that observed with untransfected parental BTS-1 and BSC-40 cells. The transfected murine Rb was not detectable at 39.5°C above the endogenous monkey Rb signal. After shiftdown to 33°C and incubation for 72 h, a very strong nuclear signal was detected. The fraction of nuclei displaying the strong signal was variable and cell line specific: 5 to 10% for RB-A5 (Fig. 4D), 30 to 40% for DRA-C26 (Fig. 4E), and up to 70% for RB-A3 (Fig. 4B). The fraction of nuclei overexpressing Rb varied somewhat from experiment to experiment but generally fell within the range characteristic of a particular cell line. Cell lines such as RB-A5 which induce amplification in only a small fraction of the cells are rarely recovered; typically, cell lines are recovered that induce amplification in the range of 40 to 60% of the cells. These patterns of expression are characteristic of the inducible amplification system and have been documented previously with other gene products (64, 73–75). Cell lines FS-B15 and RB-A11, as expected, did not show Rb overexpression (data not shown).

The level of Rb overproduction was analyzed by quantitative fluorescence microscopy and found to extend over a wide range, with the most intense nuclear signals being approximately 200-fold greater than the endogenous monkey Rb signal. The levels of Rb overproduction were independently confirmed by FACS analysis (see Fig. 7). The time course of Rb induction (Fig. 4F, G, H, and I) correlated very well with the time of entry into S phase (Fig. 1); the first bright nuclei appeared approximately 20 h after shiftdown, and the process was essentially complete after 48 h. The

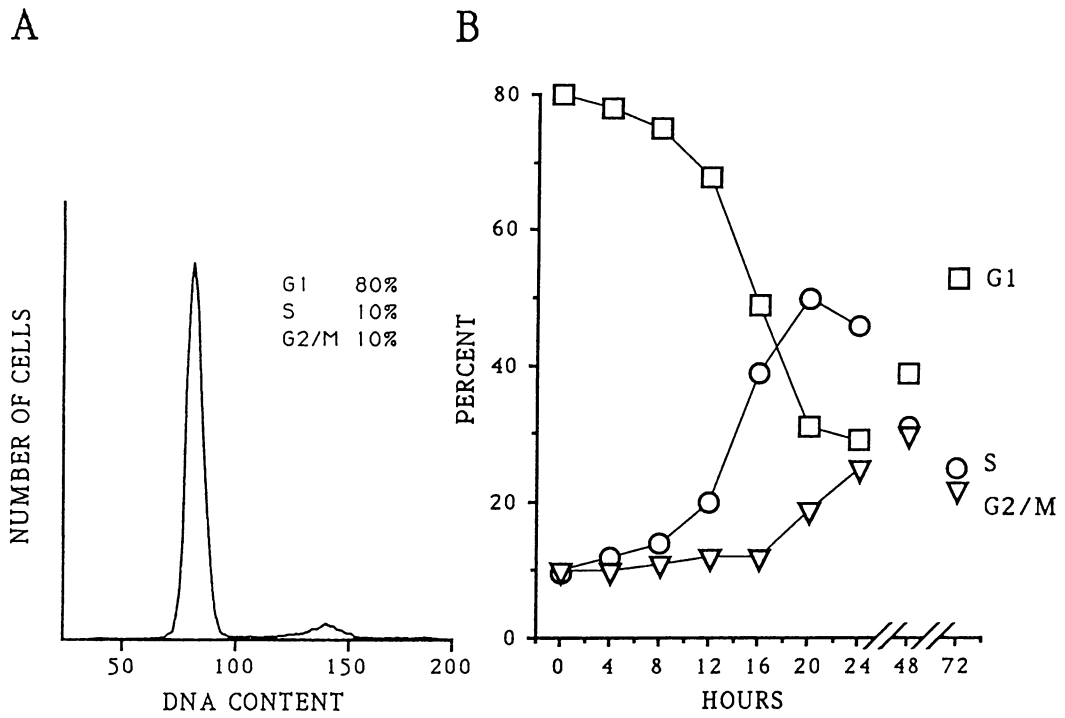

FIG. 1. Capacity of the BTS-1 cell line for withdrawal into G_0 and synchronous reentry into the cell cycle. (A) DNA content of quiescent cells. Cells were rendered quiescent by contact inhibition for 48 h in complete medium supplemented with 10% serum. FACS analysis was performed as described in Materials and Methods. (B) DNA content of cells in a culture at successive times during reentry into the cell cycle. Cells were rendered quiescent as indicated above. At the zero time point, cells were trypsinized and reseeded at 50% confluency in complete medium supplemented with 10% serum. Dishes were harvested at successive time points and analyzed by FACS as described in Materials and Methods. □, G_0/G_1-phase DNA content; ○, S-phase DNA content; ▽, G_2/M-phase DNA content.

overproduced Rb protein was extractable from the bright nuclei in situ under low-salt conditions (Fig. 4C); this property has previously been shown to be characteristic of the phosphorylated Rb form found in cells during the S and G_2 phases of the cell cycle (60).

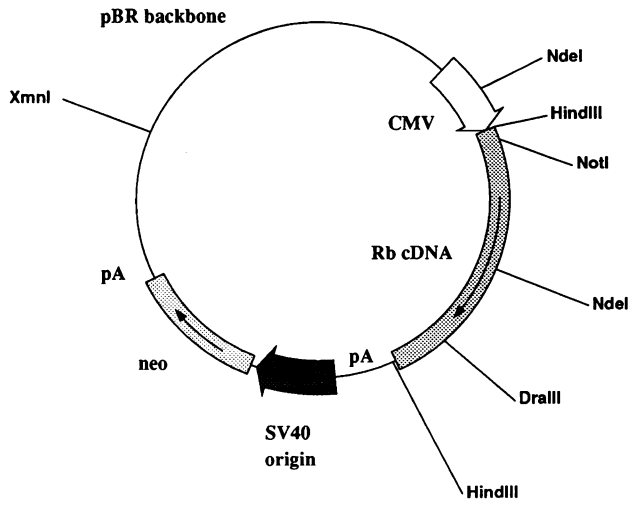

FIG. 2. Structure of plasmid pRB-wt. The constructions of plasmid pRB-wt and its mutant derivatives pRB-fs and pRB-Dra are described in Materials and Methods. Only sites relevant to the discussion in the text are indicated. The diagram is drawn to scale. The plasmid is 11.6 kbp in size. CMV, cytomegalovirus promoter; pA, poly(A) site.

Rb expression in cell lines RB-A3, RB-A5, RB-A14, and DRA-C26 was further characterized by Western blot analysis (Fig. 5). At the time of shiftdown, all cell lines contained low levels of predominantly underphosphorylated Rb protein (Fig. 5, lanes 1, 8, 10, and 14), consistent with a G_0/G_1 cell cycle position. At 24 h after shiftdown, the majority of the Rb signal shifted to the hyperphosphorylated position, consistent with an S/G_2 cell cycle position, but the levels were still relatively low (Fig. 5, lanes 2, 11, and 15). All cell lines showed substantial overproduction at 36 to 48 h after shiftdown (Fig. 5, lanes 3, 4, 9, 12, 13, 16, and 17). Significantly shorter exposures were required to unambiguously resolve the major overproduced Rb species which appeared in the hyperphosphorylated position in cell lines RB-A3, RB-A5, and RB-A14 and in the underphosphorylated position in cell line DRA-C26 (Fig. 5, lanes 5, 6, 18, and 19). The latter result was expected, since the Dra mutation has previously been shown to block phosphorylation (30). The accumulation of hyperphosphorylated Rb in cell lines RB-A3, RB-A5, and RB-A14 persisted long after shiftdown, at times when the cells should have been returning to G_1 (Fig. 1).

Overproduction of underphosphorylated Rb was specifically analyzed in RB-A3 cells with an antibody (XZ55 [37]) that preferentially recognizes the underphosphorylated species (Fig. 5, lane 7). In exponentially growing BTS-1 and BSC-40 cells, the XZ55 antibody displays a single band migrating in the underphosphorylated position with a signal strength similar to that produced by the Ab2 antibody (data not shown). The band migrating in the hyperphosphorylated position in Fig. 5, lane 7, is due to residual low-level reactivity with the greatly overproduced hyperphosphory-

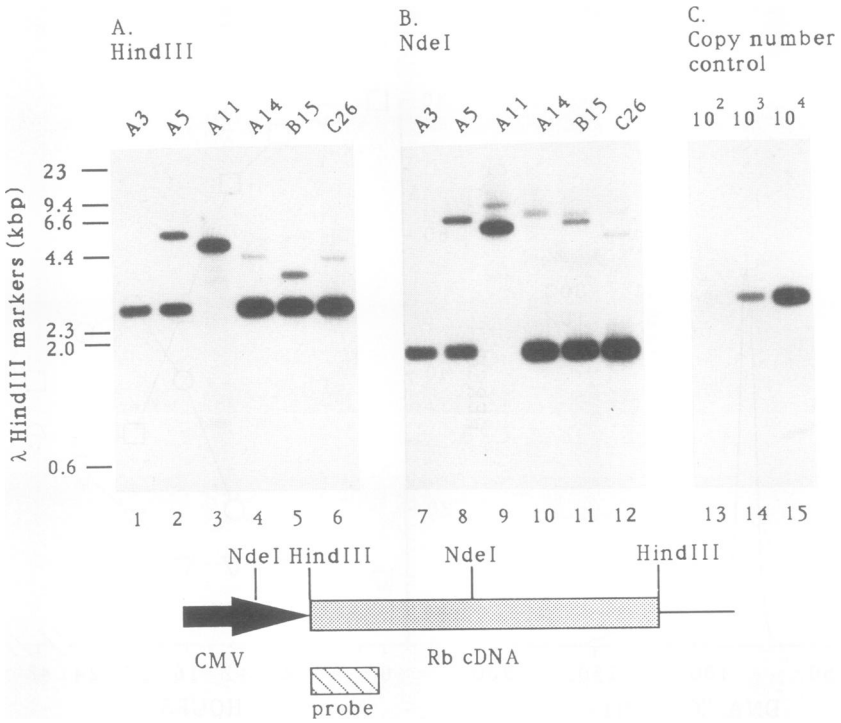

FIG. 3. Southern hybridization analysis of Rb-overproducing cell lines. (A) Digestion with *Hin*dIII. (B) Digestion with *Nde*I. Cell lines are indicated above the lanes. Total cellular DNA was prepared from cells incubated at 33°C for 72 h. Samples were treated with a mixture of RNases A and T_1 and precipitated twice with ethanol, and their DNA content was determined by A_{260} measurements. Each lane was loaded with 1 μg of digested DNA. Southern blot hybridization was performed as described in Materials and Methods. The probe was a 413-bp *Nae*I-*Cla*I fragment from plasmid pMRB115, corresponding to the N-terminal portion of the *Rb* gene, as indicated in the diagram (CMV, cytomegalovirus promoter). The diagram also illustrates the relative positions of the restriction sites used in the analysis; *Hin*dIII should produce a band of 2.8 kbp, and *Nde*I should produce a band of 1.8 kbp. Migration of *Hin*dIII-digested lambda DNA markers is indicated on the left. (C) Copy number control. Plasmid pRB-wt was digested with *Hin*dIII, serially diluted, and loaded in amounts to yield 10^2, 10^3, and 10^4 haploid genome equivalents.

lated murine Rb protein. The very low intensity of the band migrating in the underphosphorylated position indicates that it is largely, if not entirely, due to endogenous monkey Rb produced in cells that have not been induced into amplification.

Cell cycle phenotypes of Rb-overproducing cell lines. Cultures were shifted to 33°C, and 72 h later, the cells were harvested and analyzed for DNA content by FACS. The RB-A3, RB-A14, and, interestingly, DRA-C26 cell lines (Fig. 6B to D) showed a clear overrepresentation of G_2/M-phase cells relative to the BTS-1 parental control (Fig. 6A). Microscopic examination of cultures indicated that all cells had intact nuclei, and mitotic spindles were not visible by fluorescence microscopy with a tubulin antibody (74). We concluded that a significant fraction of the cells were arrested or pausing in G_2 at 72 h after shiftdown.

A more detailed analysis of the kinetics of cell cycle arrest (Fig. 7A) showed that both S phase and G_2 phase occurred at normal times following shiftdown, indicating that Rb does not cause a delayed G_2 but rather inhibits exit from it. Growth curves (Fig. 7B) emphasized that Rb-overproducing cells were arrested in the first G_2 phase following shiftdown. In contrast, the DNA content profiles of the cell lines FS-B15 and RB-A11 (Fig. 6E and F), which amplify episomes to similar levels but do not overproduce Rb protein, more closely resembled that of the BTS-1 control. Cell lines SupD3 and SupD12 (75) (Fig. 6G and H), which overproduce an amber suppressor tRNA to high levels, were also analyzed and found not to display a G_2/M accumulation. Accumulation of amber suppressor tRNA is known to cause cessation of growth (75); this control thus demonstrates that not all deleterious gene products are associated with G_2 arrest when overproduced in the inducible amplification system.

Since not all cells are induced into amplification, cultures after shiftdown are heterogeneous mixtures of cells containing high and normal levels of Rb. To specifically determine the cell cycle distribution of Rb-overproducing cells, a double-label FACS experiment was performed (Fig. 8). Cultures were shifted down to induce amplification, and cells were subsequently harvested, fixed, permeabilized, stained for Rb content with an anti-Rb monoclonal antibody and a FITC-conjugated secondary antibody, stained with PI for DNA content, and analyzed by FACS. BTS-1 cells displayed a monophasic FITC peak of low intensity, indicative of endogenous Rb (Fig. 8A). RB-A3, RB-A5, and DRA-C26 cultures all contained cells displaying significantly enhanced FITC signals (Fig. 8B to D). The RB-A3 FITC profile was biphasic, with almost equal numbers of low- and high-intensity cells. The DRA-C26 FITC profile displayed a large, broad, high-intensity shoulder. The RB-A5 FITC profile contained only a low, long shoulder, stretching into the high-intensity region. These results are consistent with the in situ immunofluorescence analysis (Fig. 4), which showed that the fraction of cells induced into amplification was

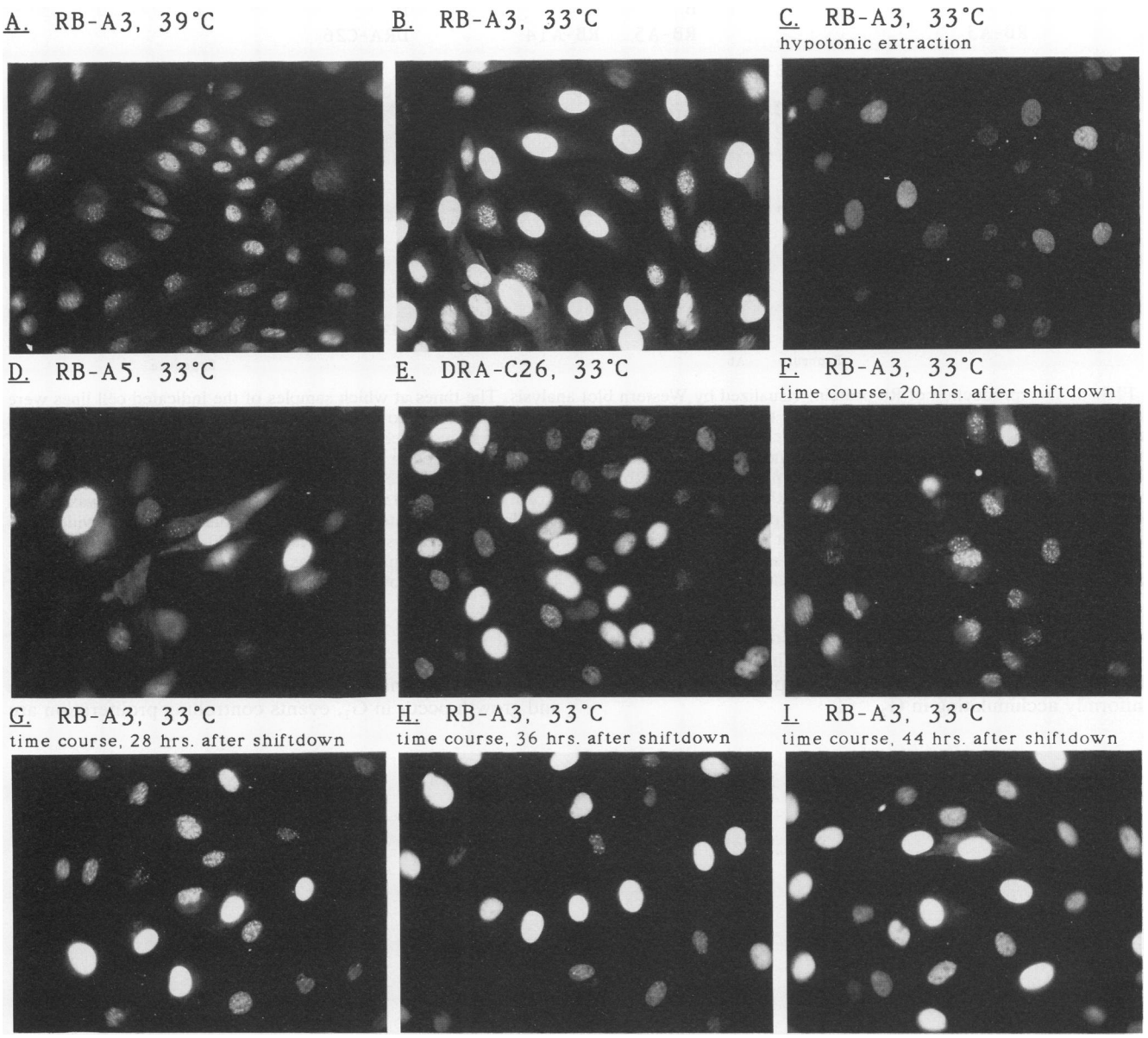

FIG. 4. Overproduction of Rb protein, visualized by in situ immunofluorescence analysis. (A) RB-A3 cell line grown continuously at the nonpermissive temperature. The low-intensity punctate nuclear staining is due to the endogenous monkey Rb protein. Cell lines BTS-1 and BSC-40 grown at either 39.5 or 33°C produce the same staining pattern (data not shown). (B) RB-A3 cell line grown for 72 h at the permissive temperature. (C) Same as panel B except that the cells were subjected to an in situ hypotonic extraction (60). The extractability of the Rb protein indicates that most of the overproducing cells were in G_2. (D) RB-A5 cell line grown for 72 h at the permissive temperature. (E) DRA-C26 cell line grown for 72 h at the permissive temperature. (F to I) Time course of Rb overexpression at the permissive temperature in cells of the RB-A3 line. Samples were harvested at the times indicated above the panels. All samples were processed for fluorescence microscopy as indicated in Materials and Methods.

approximately 70, 30, and 10% in the RB-A3, DRA-C26, and RB-A5 cell lines, respectively.

The PI profile of total cells showed a normal cell cycle distribution in BTS-1 cells and the expected G_2 enrichment in RB-A3 and DRA-C26 cells. The PI profile of total RB-A5 cells did not show significant G_2 enrichment, since only about 10% of the cells were induced to overproduce Rb. Most significantly, when cells with high-intensity FITC signals were analyzed for cell cycle position, the PI profile revealed an essentially pure G_2 DNA content. Cells with FITC signals in the range of endogenous Rb expression were found throughout the cell cycle, and RB-A3 and DRA-C26 cultures were enriched for G_1 relative to the profile of total cells. Even in the case of RB-A5, which induces amplification in only 10% of the cells, essentially all high-FITC signal cells were in G_2. The converse, however, was not the case; not all G_2 cells displayed high FITC signals (data not shown), indicating that the correspondence of high FITC signals with G_2 was not a mere coincidence of cell cycle position due to, for example, the time of harvest after shiftdown. In addition, cell cycle analysis (PI profile) of cells displaying narrow windows of Rb expression (FITC intensity) showed that

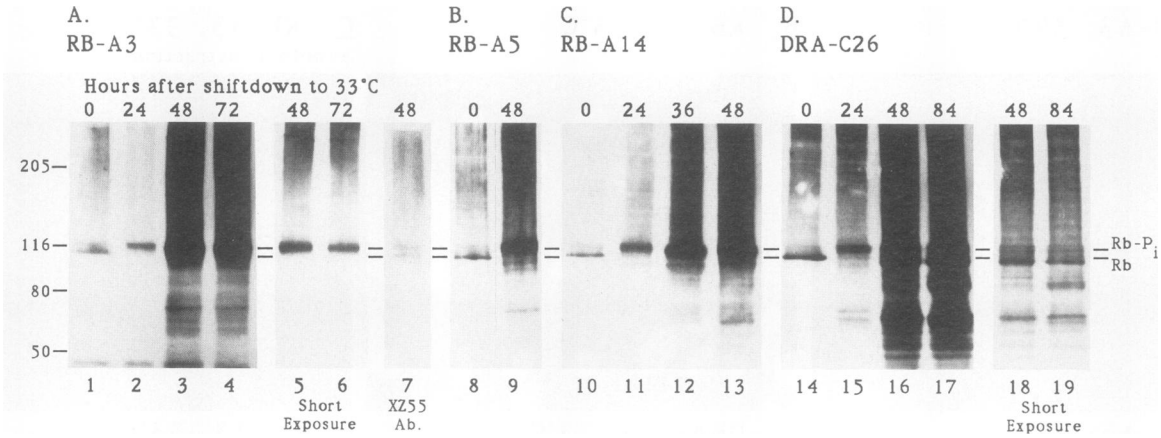

FIG. 5. Overproduction of Rb protein, visualized by Western blot analysis. The times at which samples of the indicated cell lines were harvested after shiftdown to the permissive temperature are shown above the lanes. Zero-time samples correspond to density-arrested cultures at 39.5°C. Samples were prepared and Western blotting was performed as indicated in Materials and Methods. The migration of molecular weight markers (prestained, high-molecular-weight range; Bio-Rad) is indicated in the left margin (in kilodaltons). The migration positions of underphosphorylated and hyperphosphorylated Rb protein species are indicated as Rb and Rb-P_i, respectively, in the right margin. All lanes were probed with antibody Rb-Ab2 (Pharmingen, Inc.) except lane 7 (loaded with same extract as lane 3), which was probed with antibody XZ55 (37). The XZ55 antibody preferentially recognizes the underphosphorylated Rb species. Because of the very wide range of Rb expression, serial exposures of each blot were taken. Lanes 1 to 4 and 7 to 17 were exposed for times (30 to 120 s) optimized to visualize the relatively low level of expression at the 0- and 24-h time points. These exposure times resulted in gross overexposures of lanes containing extracts from later time points. Shorter exposures (3 to 5 s) of lanes 3, 4, 16, and 17 are shown in lanes 5, 6, 18, and 19, respectively.

cells with as little as fivefold overproduction relative to endogenous monkey levels were arresting in G_2 (data not shown). We concluded that cells overproducing Rb were uniformly accumulating in G_2.

DISCUSSION

Although the majority of decisions between quiescence and growth occur in G_1, events controlling proliferation are

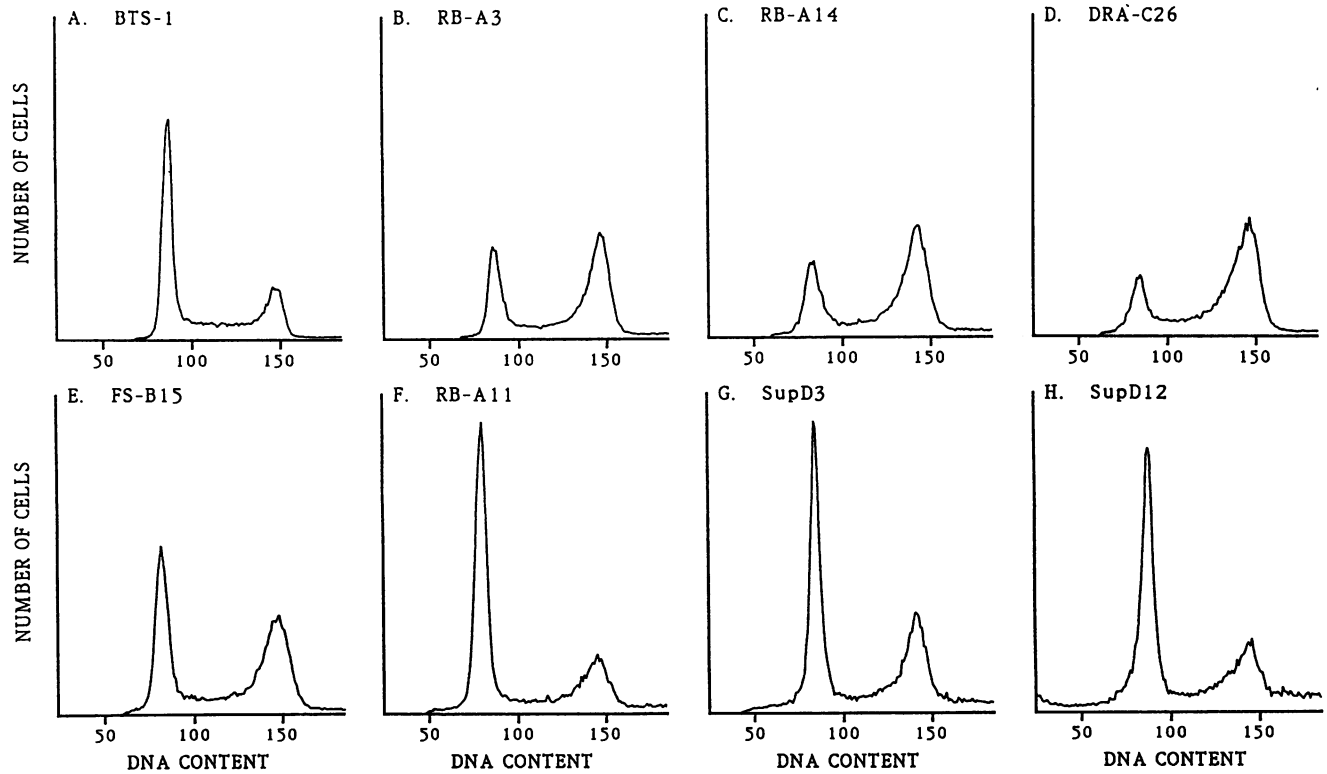

FIG. 6. Cell cycle distribution of cultures overexpressing Rb protein. The cell line is identified in each panel. All cultures were density arrested at 39.5°C, released by dilution and concomitant shiftdown to 33°C, harvested 72 h later, and processed for single-label FACS analysis with PI as indicated in Materials and Methods. The x axis displays fluorescence (PI) intensity in arbitrary units (linear scale).

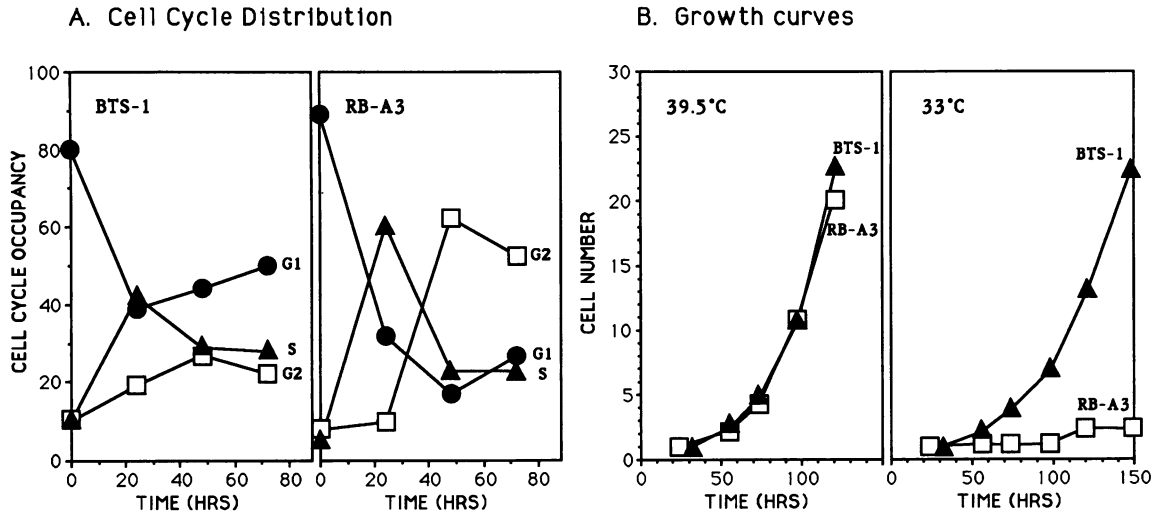

FIG. 7. Kinetic analysis of cell cycle arrest in cultures overexpressing Rb protein. (A) Cell cycle progression determined by FACS analysis. FACS analysis was performed at the indicated time points by the method described in the legend to Fig. 6. The phases of the cell cycle are expressed as a percentage of the total number of cells. (B) Growth curves were performed by seeding 2×10^4 cells per 10-cm culture dish, harvesting duplicate dishes at the indicated times, and counting the cells with a Coulter counter. Cell numbers were normalized to the starting number of cells (zero time point, assigned a value of 1).

also present in G_2 (26, 62). Temperature-sensitive mutant cell lines that arrest in G_2 as well as in G_1 have been isolated (61), and SV40-immortalized cells require T-Ag activity to progress through G_2 (43). Interestingly, SV40 T-Ag can overcome the G_2 arrest in the mutant cell line 3Y1tsF121 (90). It is not known how these phenomena are related to the G_2 checkpoint that monitors completion of DNA synthesis. Some components of the cell cycle regulatory machinery exert their activity at single checkpoints, while others are clearly multifunctional. For example, the $p34^{cdc2}$ kinase is required throughout the cell cycle, and cyclin A is thought to function in the entry into mitosis as well as in promoting DNA synthesis (67).

The Rb protein is known to exert its activity at decision points in G_1. Rb mutants with specific G_2 phenotypes have not been isolated to date, but such mutants would be expected to be rare because any gross perturbation of Rb function is known to produce an overriding G_1 phenotype. To investigate whether Rb may also play some role(s) at later points in the cell cycle, we used a system of rapid inducible gene amplification (73, 75) to conditionally overexpress Rb protein during G_2 phase.

The parental BTS-1 cell line displays some differences from the familiar BALB/c-3T3 model system. Only contact inhibition can be used to induce quiescence, since serum deprivation elicits a rapid decrease in viability. The growth rate in 10% serum decreases sharply after confluence is reached, and 80% of the cells contain a 2N complement of DNA. Release from G_0 was accomplished by reseeding at subconfluent densities, and T-Ag was activated by a concomitant shift to 33°C. Approximately 12 h after this treatment, significant numbers of cells started entering the S phase. Maximum S-phase content (50% of the culture) was seen at approximately 20 h, at which time the first cells entered G_2. The first mitotic figures were seen at approximately 32 to 36 h, but significant mitoses could be seen well past 48 h, indicating deterioration of synchrony. Inducible DNA amplification is a very rapid process which is initiated and completed within one S and G_2 phase, as evidenced by the observations that the onset of replication is correlated with entry into S and that the episome copy number reaches a plateau within 48 h (73–75). T-Ag-catalyzed replication of SV40 origins occurs in S and G_2 nuclei but not in G_1 nuclei.

The expression of Rb protein closely follows the amplification of the gene. At 24 h, increased expression was not detectable by Western blotting, but a few very intense nuclei could be seen by immunofluorescence; by 36 h, expression was clearly detectable by Western blotting, and maximal levels of expression were reached by 48 h. The fraction of cells that are induced into amplification is a characteristic property of individual cell lines and can vary from 70% (RB-A3) to 5% (RB-A5). Double-label immunofluorescence microscopy revealed a very close correlation between nuclei expressing T-Ag and Rb proteins (74). The parental BTS-1 cell line displays approximately 70% T-Ag-positive nuclei at 33°C. The heterogeneity of T-Ag expression is not due to simple clonal heterogeneity, since repeated cycles of subcloning do not yield cell lines with 100% expression (74). Subclones of the BTS-1 cell line typically display 30 to 70% T-Ag-positive nuclei, although occasional clones with much lower fractions are also recovered. Subclones tend to be quite stable and continue to display their characteristic patterns of expression over many passages. These properties of T-Ag-transfected cell lines, although not understood in detail, have also been documented by others (31). The level of Rb protein in individual cells induced into amplification was determined by both FACS analysis and quantitative fluorescence microscopy. Both methods revealed a broad range of overproduction, with an average value of 50-fold over the endogenous Rb level.

The detailed analysis of the amplification process revealed the following sequence of events: (i) cells are released from G_0 by dilution, and T-Ag is activated by temperature shift-down; (ii) chromosomal SV40 origins remain silent until cells enter S; (iii) as cells enter S phase, SV40 origins begin to replicate rapidly, leading to excision and continued episomal replication; (iv) Rb protein expression, driven by gene amplification, increases rapidly during S and G_2; and (v) cells

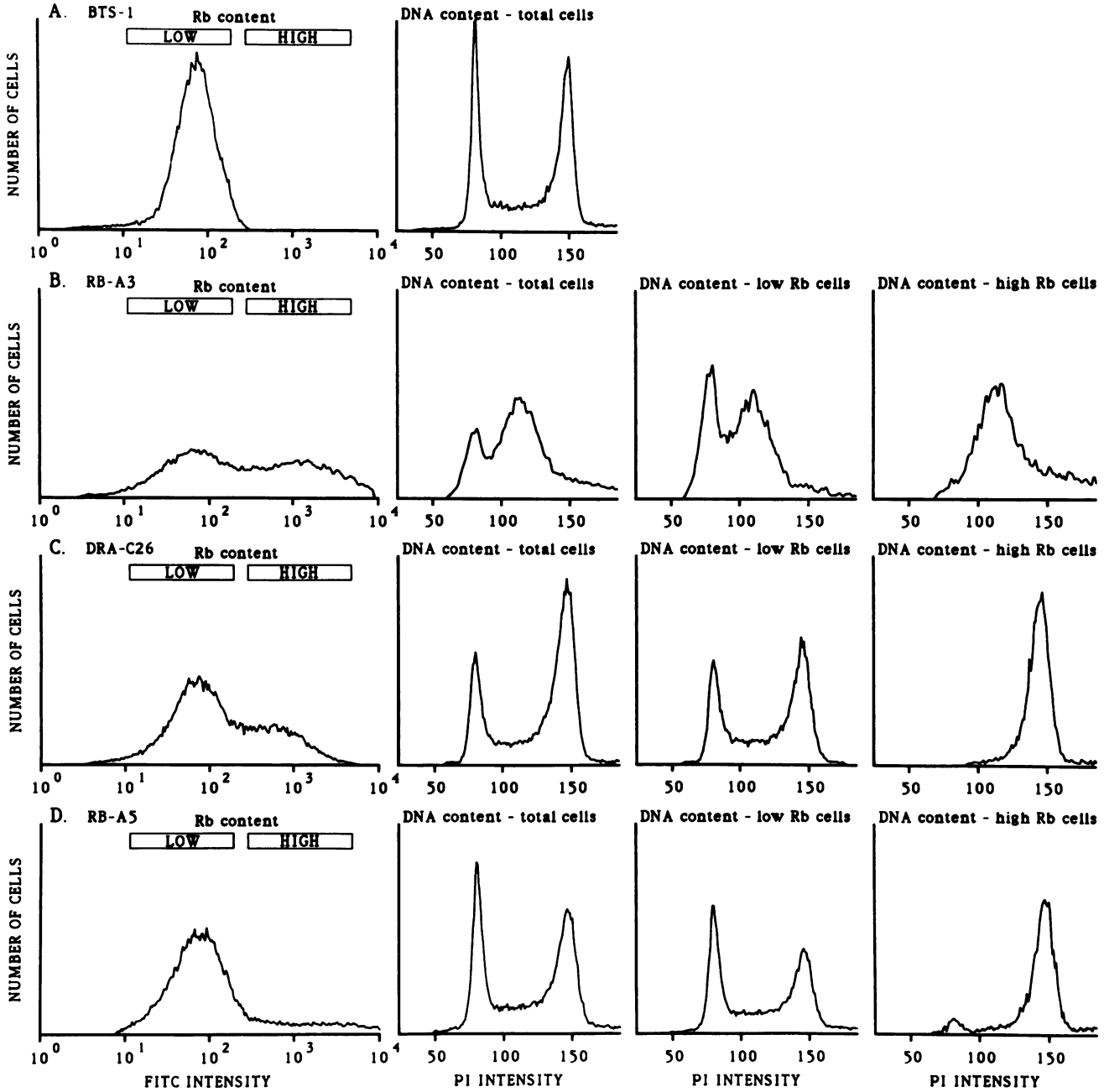

FIG. 8. Cell cycle position of cells expressing high and normal levels of Rb protein. All cultures were density arrested at 39.5°C, released by dilution and concomitant shiftdown to 33°C, harvested 48 h later, and processed for double-label FACS analysis. Rb protein was detected with antibody Rb-Ab2 and FITC-conjugated rabbit anti-mouse IgG; DNA content was analyzed with PI, as indicated in Materials and Methods. The leftmost panels of each row show the distribution of Rb expression in the indicated cell line, displayed as FITC intensity. Note that the x axis (FITC intensity) is plotted on a logarithmic scale. The remaining panels display cell cycle distribution determined by PI fluorescence, either in the total population of cells or in selected subpopulations expressing either normal levels of Rb (low FITC signal) or high levels of Rb (high FITC signal). The positions of the PI peaks of cell line RB-A3 are shifted because the experiment was run on a separate occasion under somewhat compromised conditions.

accumulate high levels of Rb protein and arrest in G_2. The data concerning the point in G_2 at which arrest is occurring can be summarized as follows: (i) chromosomal DNA was fully replicated (determined by FACS analysis); (ii) chromatin was not condensed (determined by fluorescence microscopy of 4′,6-diamidino-2-phenylindole-stained nuclei); (iii) nuclei displayed four to five distinct nucleoli and an intact nuclear membrane (determined by light microscopy); and (iv) centrioles had divided and migrated to opposite poles of the cell, but the mitotic spindle was not formed (determined by fluorescence microscopy with antitubulin antibodies [74]). These observations are consistent with a mid- to late G_2 arrest.

Rb protein overproduced in the BTS-1 system was found

in its hyperphosphorylated form, as determined by mobility during SDS gel electrophoresis. No underphosphorylated Rb other than the low levels of endogenous Rb contributed by the nonarrested cells in the cultures was found by an antibody specific for the underphosphorylated form of the protein. Most importantly, very high levels of Rb overproduction were not required for cell cycle arrest, since the double-label FACS data revealed that individual cells along the whole overproduction spectrum (from 5- to 200-fold overproduction relative to normal endogenous levels) were all uniformly arrested in G_2. These observations thus argue against two explanations for the G_2 arrest: (i) saturation of the Rb kinase(s) with excess substrate and (ii) accumulation of un- or underphosphorylated Rb at the wrong time in the cell cycle. The simplest explanation is that Rb physically interacts with some component(s) of the cell cycle regulatory machinery during G_2 and that fully phosphorylated Rb can participate in this interaction.

Several lines of evidence show that the observed G_2 arrest is due specifically to overexpression of Rb protein and not to other aspects of the inducible amplification system. First, expression of T-Ag is not deleterious, since BTS-1 cells grow at 33°C at the same rate as the parental BSC-40 cell line and T-Ag expression is stable for many generations (73). Second, several heterologous proteins have been overexpressed to date with this system without encountering cell cycle arrest phenotypes. For example, cell lines overexpressing blood clotting protein factor VIII grew at normal rates at 33°C, and production and secretion of factor VIII were constant for many generations (64, 73). In contrast, cell lines overexpressing deleterious gene products, such as amber suppressor tRNA and Rb protein, display transient growth arrest after a shiftdown to 33°C (74, 75). The duration of the growth arrest depends on the fraction of cells induced to overexpress the deleterious gene product, and the cultures are eventually overtaken by cells that have not been induced into amplification. Third, three independent Rb-overexpressing cell lines displayed G_2 arrest, indicating that the phenotype is not cell line specific (due to, for example, excision from a particular chromosomal location). Fourth, DNA amplification in the absence of Rb protein expression did not elicit G_2 arrest, indicating that amplification per se of the specific vector used in these experiments was not deleterious. Finally, amplification of amber suppressor tRNA elicited random cell cycle arrest, indicating that not all instances of deleterious gene product overexpression result in G_2 arrest.

One formal possibility is that the arrest is an artifact elicited by the presence of T-Ag. This is unlikely for the following reasons. First, T-Ag is known to have positive effects on cell cycle progression, including G_2 progression (43, 90). In this context, it should be noted that neither exit of density-arrested BTS-1 cells from G_0 nor the subsequent cell cycle traversal, including transition through G_2 into M and G_1, is dependent on T-Ag activity (the cell cycle transition profiles shown in Fig. 1 are identical whether the experiment is performed at 33 or 39.5°C). Second, the levels of T-Ag are low and remain unchanged during the amplification process, while Rb levels rapidly build up to a large excess. Third, T-Ag has been shown not to associate with either phosphorylated Rb or the ΔDra mutant Rb protein, even under conditions of overexpression (30). Fourth, removal of T-Ag from G_2-arrested RB-A3 cells by temperature shiftup failed to overcome the arrest (74). The TsA58 T-Ag protein is unstable at 39.5°C and is turned over with a half-life of 1 to 2 h (80). RB-A3 cells arrested in G_2 are metabolically active and indistinguishable from control cells, as evidenced by, for example, glucose consumption and incorporation of [^{35}S]methionine into cellular proteins (74). Most important, even if the presence of T-Ag somehow exacerbated the effects of Rb overproduction, an interaction between Rb and a cellular target would still have to be postulated.

The major decision point between quiescence and proliferation in mammalian cells is executed in G_1. Normal cells under exponential growth conditions continuously traverse the cell cycle, but several factors, such as serum limitation, contact inhibition, and damage to DNA, can activate one or several checkpoints and arrest cells in G_1/G_0. The principal evidence that Rb is functionally involved in G_1 checkpoint regulation and/or execution is the phenotype of *Rb* mutants: loss-of-function mutants lose the ability to arrest, and gain-of-function (overproduction) mutants arrest inappropriately (29). The second major checkpoint is found at the G_2/M boundary, at which the readiness to enter mitosis is monitored (for example, completion of DNA synthesis). The finding that Rb gain of function in G_2 elicits growth arrest exactly parallels the results of studies in which Rb was overproduced in G_1 (29).

Interpretation of gain-of-function phenotypes is often complicated by the possibility that an interaction that does not normally take place under physiological conditions is being forced by the overproduction. That the G_2 arrest elicited by Rb falls in this category is unlikely since (i) a very modest level of overproduction (fivefold over the endogenous level) was sufficient to trigger arrest and (ii) the phenotype (point at which cells were arrested) was the same over a very broad range of Rb expression. The specific function of Rb in G_2 can only be determined from further biochemical experiments, but the genetic analysis presented here indicates that it interacts with some component of the molecular machinery involved in G_2 checkpoint execution.

At least two proteins are known to associate with Rb in G_2, E1A and the kinase responsible for the last wave of phosphorylation. By analogy with the situation found in G_1, the G_2 arrest phenotype would argue for the existence of an additional cellular G_2 Rb-binding protein(s). Since the titration of this putative G_2 Rb-binding protein with excess Rb has a growth-suppressive effect, it seems likely that the G_2 Rb-binding protein is performing some functional role(s) necessary for successful G_2 progression. The functional role of Rb in the regulation of G_2 progression under physiological conditions and whether this process is regulated by the last wave of Rb phosphorylation remain to be determined.

One surprising finding was that the Dra mutant Rb protein was effective in eliciting G_2 arrest. The mutant protein is known to be defective for its G_1 functions, such as binding to T-Ag, and cannot be phosphorylated at later points in the cell cycle (30). Since the G_1 and G_2 phenotypes of *Rb* can be differentially affected by this mutation, the simplest mechanistic explanation is that the protein(s) that interacts with Rb in G_2 does so via contacts that are distinct from those important for recognition during G_1. Whether this is indeed the case should be resolved by the overproduction of other Rb mutants. In this context, it would also be interesting to determine the effects of mutations preventing the last wave of Rb phosphorylation.

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