Ribonucleotide reductase (RNR) plays a central role in the formation and control of the optimal levels of deoxyribonucleoside triphosphates, which are required for DNA replication and DNA repair processes. Mammalian RNRs are composed of two nonidentical subunits, proteins R1 and R2. The levels of the limiting R2 protein control overall RNR activity during the mammalian cell cycle, being undetectable in G1 phase and increasing in S phase. We show that in proliferating mammalian cells, the transcription of the R2 gene, once activated in the beginning of S phase, reaches its maximum 6–7 h later and then declines. Surprisingly, DNA damage and replication blocks neither increase nor prolong the R2 promoter activity in S phase. Instead, the cell cycle activity of the mammalian enzyme is controlled by an S phase/DNA damage-specific stabilization of the R2 protein, which is effective until cells pass into mitosis.

Ribonucleotide reductase catalyzes the formation of deoxyribonucleotides from the corresponding ribonucleotides, which is the rate-limiting step in the production of the precursors for DNA synthesis (1). In mammalian cells, the enzyme is composed of two nonidentical subunits, proteins R1 and R2, which are both required for activity. The R1 protein contains redox active disulfides, an active site that binds ribonucleoside diphosphates and also two different allosteric sites binding nucleoside triphosphates (2). Enzyme activity is controlled by binding ATP (stimulation) or dATP (inhibition) to the allosteric activity site, whereas substrate specificity is controlled by binding ATP, dATP, dTTP, or dGTP to the specificity site. A failure in the control of the dNTP levels and/or their relative amounts leads to cell death or genetic abnormalities (3). Protein R2 contributes an essential iron center-generated tyrosyl free radical that can be scavenged by hydroxyurea (4). In mammalian cells, both enzyme activity and the R1 and R2 mRNAs are cell cycle-regulated with maximal levels during S phase (5, 6). The levels of the R1 protein are constant and in excess during the cell cycle, because of a long half-life (more than 20 h). The R2 protein, by contrast, shows an S phase-specific expression, has a half-life of 3 h in logarithmically growing cell cultures, and is limiting for activity (7, 8).

There is no evidence that DNA damaging agents or drugs that block replication activate transcription of the mammalian RNR genes during S phase. Furthermore, it is well established that Escherichia coli and mammalian ribonucleotide reductases control the size of the deoxyribonucleotide pools via dATP inhibition of the enzyme (2). Consequently, overproduction of ribonucleotide reductase in mammalian cells generally does not lead to increased dNTP pools (1), whereas a mutation in the R1 protein destroying dATP feedback inhibition does (9, 10).

In this paper, we present new data showing how ribonucleotide reductase is controlled during the normal mammalian cell cycle and after DNA damage. We demonstrate that transcription of the mouse R2 gene is uncoupled from the S phase progression and is not influenced by DNA damage or replication blocks. Instead, the R2 protein is specifically stabilized during S phase and in response to DNA damage and is rapidly degraded after cells enter into mitosis. In combination with the dATP feedback control, the controlled degradation of the R2 protein directly regulates ribonucleotide reduction in proliferating mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Reagents and Proteins**—[γ-^32P]ATP and [35S]methionine/cysteine (PRO-MIX [35S]cell labeling mix) were obtained from Amersham Pharmacia Biotech; hydroxyurea and roscovitine were from Calbiochem; and aphidicolin, nocodazole, and LLnL were from Sigma. Recombinant Cdc2/Cyclin B complex was obtained from New England Biolabs. Antibodies to R2 protein polyclonal antibodies were developed in rabbits against the peptide acetyl-QLQL(phospho)SPLKRLC-amide, corresponding to mouse R2 protein amino acid residues 16–25, and affinity purified by QCB, Inc. JC4 anti-mouse R2 protein rat monoclonal antibodies were previously developed in our laboratory (11). Mouse recombinant R1 and R2 proteins were prepared from BL21(DE3)pLysS bacteria containing the plasmids pETM1 or pETM2 and purified to homogeneity according to Refs. 12 and 13.

**Vector Construction**—The R2 promoter-luciferase reporter gene construct was created by ligating a 1517-base pair HindIII fragment containing the entire mouse R2 gene, which was inserted into the pGL3 vector (Promega). The created HindIII fragment was subcloned into the pGL3-basic vector (Promega) to create pGL3-Basic R2 construct. The C-terminal fragment of the Cdc2/Cyclin B complex was obtained from New England Biolabs. Anti-Cdc2/Cyclin B polyclonal antibodies were developed in rabbits against the peptide acetyl-QLQL(phospho)SPLKRLC-amide, corresponding to mouse R2 protein amino acid residues 16–25, and affinity purified by QCB, Inc. JC4 anti-mouse R2 protein rat monoclonal antibodies were previously developed in our laboratory (11). Mouse recombinant R1 and R2 proteins were prepared from BL21(DE3)pLysS bacteria containing the plasmids pETM1 or pETM2 and purified to homogeneity according to Refs. 12 and 13.
FIG. 1. Effects of DNA damage and replication blocks on the activity of the mouse R2 gene promoter in proliferating cells. The luciferase activity was measured in Balb/3T3 cells stably transformed by an R2 promoter-luciferase reporter gene construct and synchronously progressing through the cell cycle. In one set of experiments (solid lines with symbols), 10 J/m² UV light (UV), 2 mM hydroxyurea (HU), or 10 μg/ml aphidicolin (Aph) was applied to the synchronized cells 18 h after the release from serum starvation. Cells were then harvested at 20, 22, 23, 24, and 25 h and analyzed for luciferase activity and flow cytometric profile. In another set of experiments (dashed lines without symbols), 2 mM hydroxyurea or 10 μg/ml aphidicolin were applied to the cells 1 h after release from serum starvation. The treated cells and untreated cells (solid line without symbols) were then harvested every 2 h beginning at 7 h and ending at 25 h after the release from serum starvation and then analyzed for luciferase activity and cell cycle distribution. Flow cytometric histograms for the untreated cells are shown beginning from 18 h; those for the treated cells are not shown, because they are all identical with only the G1 peak.

mutant). All constructs were verified by restriction analysis and sequencing of polymerase chain reaction amplified regions.

Cell Culture—Mouse Balb/3T3 (American Type Culture Collection number CCL-163) cells were cultured in DMEM (Sigma) supplemented with penicillin, streptomycin, and 10% v/v heat-inactivated (56 °C, 30 min) horse serum (Life Technologies, Inc.). Flow cytometry was carried out on a CCA-I flow cytometer, according to the instructions of the manufacturer (Partec GmbH). For synchronization by serum starvation, 3 × 10⁵ cells were plated per 6-cm dish. After 24 h, the medium was replaced with 5 ml of DMEM containing 0.6% heat-inactivated horse serum. At 36 h, the cells were released with 5 ml of DMEM containing 20% heat-inactivated horse serum; the cells were harvested at various intervals as described below. UV irradiation of cells, transfection by electroporation with linearized vectors and selection of stably transformed clones were performed as described earlier (14). The R2 promoter-luciferase reporter gene construct was linearized with XmnI, and the R2 gene constructs were linearized with HindIII prior to electroporation. Luciferase assays were performed on cell lysates obtained by incubating cells with the Cell Culture Lysis Reagent (Promega) according to the instructions of the manufacturer; luciferase activity was measured in a TD-20/20 Luminometer (Turner Designs) using the Luciferase Assay System (Promega). Luciferase activity was expressed in light units normalized against the protein concentration in the lysates, as measured by the Bradford method. Expression of the transfected R2 gene in the stably transformed clones was verified by incubating the cells in increasing concentrations of hydroxyurea and by determining the IC₅₀ value as described before (15).

Immunoblotting and Immunoprecipitation—For immunoblotting, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS), pH 7.4, and then lysed for 10 min on ice by the addition of 200 μl of lysis buffer (PBS, pH 7.4, 50 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, pepstatin, and LlnL, 40 mg/ml staurosporine, 0.5% Nonidet P-40). The lysates were collected with a rubber policeman, and debris was centrifuged at 20,000 × g, 4 °C for 20 min. Equal amounts of protein from the lysates were electrophoresed in separate lanes on a 10% SDS-polyacrylamide gel. The proteins were then transferred to Hybond-C extra membranes in a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 200 mA, 4 °C for 1 h. After transfer, the membranes were blocked in PBS containing 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na₃VO₄, and 2% nonfat dry milk for 30 min; then either JC4 anti-R2 rat monoclonal antibodies or anti-pR2 rabbit polyclonal antibodies were added. After 1 h of incubation at room temperature, membranes were washed and further incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. After two washes, detection was done using the ECL-plus system, according to the instructions of the manufacturer (Amersham Pharmacia Biotech).

For pulse labeling with radioactive methionine/cysteine, the cells were washed twice with prewarmed methionine/cysteine-free DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated horse serum and then incubated for 10 min in the same medium. Next, the cells were labeled for 10 min in the same medium, supplemented with 71.5 μCi/ml PRO-MIX [³⁵S]cell labeling mix (Amersham Pharmacia Biotech). For the chase, the cells were washed twice with prewarmed complete DMEM supplemented with 16% heat-inactivated horse serum and further incubated in the same medium.

For immunoprecipitation, cells were rinsed twice with ice-cold PBS, pH 7.4, and then lysed for 10 min on ice by the addition of 1 ml of lysis buffer as above. The lysate was collected with a rubber policeman, and debris was centrifuged at 20,000 × g, 4 °C for 20 min. Supernatant was incubated for 16 h at 4 °C with 5 μg of JC4 antibodies and 15 μl of protein G-agarose beads (Sigma). The beads were washed six times with 1 ml of PBS, pH 7.4, supplemented with 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na₃VO₄, boiled in SDS-polyacrylamide gel electrophoresis loading buffer for 5 min, and loaded directly on the 10% SDS-polyacrylamide gels. After the immune complexes were separated by electrophoresis, the gels were dried, and radioactivity was quantified on a phosphoimager (Bio-Rad).

RESULTS

Effects of DNA Damage and Replication Blocks on the Activity of the Mouse R2 Gene Promoter in Proliferating Cells—The activity of the mouse R2 promoter during S phase was assayed
using an R2 promoter-luciferase reporter gene construct, which was stably transformed into mouse Balb/3T3 fibroblasts. The cells were synchronized by serum starvation and released by serum readdition to progress synchronously through the cell cycle. As seen in Fig. 2, R2 protein accumulates constantly during S phase and reaches its maximum long after the cells pass through the middle of S phase. Interestingly, after slow accumulation, protein R2 seemed to disappear quickly before the next cell cycle. Taken with the observation that the R2 mRNA and R2 protein reaches maximal levels at different times in the cell cycle, this result suggested that the stability of the R2 protein might be regulated during the cell cycle.

Protein R2 Is Highly Stable in S Phase but Degradates Quickly as Cells Undergo Mitosis—The half-life of the R2 protein in logarithmically growing cells is reported to range from 3 to 6 h (7, 20). Our own measurements using pulse-chase and immunoprecipitation techniques showed the same variation, depending on the growth state of the cell culture (data not shown). To compare the half-life of the R2 protein in different phases of the cell cycle, we synchronized mouse Balb/3T3 cells by serum starvation and pulse-labeled them with [35S]methionine/cysteine for 10 min in the early S phase. After a chase in complete medium and immunoprecipitation, we demonstrated that the R2 protein is very stable during S phase (Fig. 3A). In a similar experiment with larger intervals between each time point, we observed that the R2 protein becomes increasingly unstable as cells leave S phase and approach the next cell cycle (Fig. 3B). Plotted on logarithmic graphs, the protein degradation data showed that in S phase the R2 protein disappears exponentially with a half-life of more than 20 h (Fig. 3A), whereas in mitosis the degradation accelerates and is much faster than a calculated exponential degradation or a constant rate degradation.

Analyses of R2 Protein Levels during Different Cell Cycle Phases—According to the existing model, levels of mammalian R2 protein follow the levels of the corresponding mRNA, because of the short half-life of the protein. Yet the levels of the R2 mRNA peak in the middle of S phase, at the time of maximal DNA synthesis (5, 16), whereas protein R2 levels and ribonucleotide reductase activity were reported to peak in the G2 phase (7, 17). Contradictory results from other groups demonstrate that maximal activity of ribonucleotide reductase coincides with the middle of S phase (18, 19). To resolve this discrepancy, we did careful immunoblotting measurement of the R2 protein levels in mouse Balb/3T3 cells, which were serum-starved and released to progress synchronously through the cell cycle. As seen in Fig. 2, R2 protein accumulates during the 9-h incubation, as detected by immunoblotting (Fig. 3A). In a contrast, the R2 protein levels in untreated cells decreased dramatically during the same period.
followed by immunoprecipitation confirmed that the R2 protein is not degraded when the cells are cultured in the presence of hydroxyurea (data not shown).

To determine whether this stabilization of R2 protein was hydroxyurea-specific or a consequence of a block in the cell cycle, we utilized agents that stop the cell cycle but do not directly interact with the R2 protein. Aphidicolin, which blocks cell cycle progression by inhibiting the DNA polymerase α (21), had the same stabilizing effect on the R2 protein as hydroxyurea (Fig. 4A). Exposure of mammalian cells to UV light also leads to a block in cell cycle progression as cells carry out repair of damaged DNA (22). When synchronized mouse Balb/3T3 cells were irradiated by UV light in late S phase, they were immediately arrested, with the R2 protein remaining at its maximal levels for at least 5 h (Fig. 4B). In contrast, control cells not arrested by UV light degraded the R2 protein as they progressed through the cell cycle, as described above. Pulse-chase labeling and immunoprecipitation showed that the decay of R2 protein in S phase cells after irradiation by UV light was nearly undetectable (data not shown).

Interestingly, in synchronized cells blocked by hydroxyurea or aphidicolin precisely on entry into S phase, R2 protein started to appear, and the levels increased with time, just as in control cells. Furthermore, the R2 protein was not degraded at the time when untreated cells reached the next cell cycle (Fig. 4C). This result indicates that in S phase-committed cells, the R2 protein is synthesized and stabilized even in the absence of DNA replication.

Protein R2 Degradation Is Activated after the Cells Pass into Mitosis—Hydroxyurea, aphidicolin, and UV light, although acting through different mechanisms, all block the synthesis of DNA. Therefore, the stability of the R2 protein may directly correlate with the presence of unreplicated DNA. Alternatively, R2 protein degradation could be triggered when cells pass a certain cell cycle transition. To distinguish between these possibilities, we made use of drugs that do not directly interfere with DNA synthesis but rather block the cell cycle when the S phase is completed.

Roscovitine is a highly selective inhibitor of the cyclin-dependent kinases Cdc2, Cdk2, and Cdk5 (23). It blocks entry into mitosis by inactivating the Cdc2 kinase, with an average IC_{50} of 16 mM for mammalian cells, but it does not interfere with DNA synthesis at this concentration. S phase synchronized Balb/3T3 cells cultured in the presence of 20 μM roscovitine continued to synthesize DNA and accumulated in late G2 phase (Fig. 4D). The R2 protein levels in the arrested cells remained the same as in S phase cells. These data indicate that R2 degradation is not activated before the cells pass into mitosis and that R2 degradation does not depend directly on the presence of unreplicated DNA. The cells exposed to 10 μM roscovitine were not completely blocked at G2/M, and they partially passed through mitosis. In this case, the R2 levels were lower than in the completely G2/M arrested cells, which indicates normal R2 degradation in the fraction of cells that were capable of passing through mitosis.

To examine R2 protein stability during mitosis, S phase synchronized cells were cultured in the presence of nocodazole, which blocks the cell cycle in metaphase by interfering with the polymerization of microtubules. Our results show that the levels of R2 protein in the nocodazole-treated cells were very low and comparable with those in untreated cells (Fig. 4D). This is in marked contrast to the high levels in cells arrested in S phase by hydroxyurea. Therefore, we conclude that the degradation of the R2 protein is triggered after the entrance into mitosis, but before or in metaphase. The proteasome inhibitor LLnL, applied to the synchronized cells in the middle of S phase, did not inhibit replication but blocked the cell cycle in mitosis, and it completely abolished the degradation of the R2 protein (data not shown).

Phosphorylation of the R2 Protein by Cell Cycle-dependent Kinases—The mouse R2 protein is uniquely phosphorylated on Ser20 by cell cycle-dependent kinases both in vivo and in vitro, but the function of this phosphorylation has not been determined (24, 25). However, the ribonucleotide reductase activity of in vitro phosphorylated R2 protein, assayed in the presence of an excess of R1 protein, did not differ from nonphosphoryl-
Phosphorylation of the R2 protein does not affect its stability in vitro. A, two plates with Balb/3T3 cells synchronously progressing through the cell cycle were collected every 2 h. One plate was used to prepare a cell lysate and to analyze the levels of phosphorylated R2 protein by immunoblotting with the polyclonal anti-pR2 protein antibodies. The other plate was analyzed by flow cytometry, and the cell cycle distribution is shown above each time point. In the last two lanes, equal amounts of recombinant R2 protein and in vitro phosphorylated recombinant R2 protein were loaded on the same gel to demonstrate the specificity of the antibodies. The asterisk indicates a cross-reactivity band seen only with the polyclonal anti-pR2 protein antibodies used in this experiment. B, two plates with Balb/3T3 cells, stably expressing the Δ20 R2 protein and synchronously progressing through the cell cycle were collected every 2 h. One plate was analyzed for levels of the R2 and Δ20 R2 proteins by immunoblotting with the monoclonal JC-4 antibodies. The other plate was analyzed by flow cytometry, and the cell cycle distribution is shown above each time point. The lower band is specific for the Δ20 R2 protein, and it shows the same cycle dependence as the native R2 protein (upper band). Note that degradation of the Δ20 R2 protein is also blocked by 20 μM roscovitine (R). The data for the transfected native R2 gene and the S20A mutant are similar and are not shown.

First, we determined when R2 protein phosphorylation occurs during the cell cycle. We raised rabbit polyclonal antibodies specific to the phosphorylated form of mouse R2 protein (anti-pR2). Immunoblotting of phosphorylated and nonphosphorylated R2 protein showed no cross-reactivity of the antibodies (Fig. 5A). Immunoblotting of protein extracts from synchronized mouse Balb/3T3 cells showed that the R2 protein becomes phosphorylated in early S phase, and it remains phosphorylated throughout the S and G2 phases until it disappears during mitosis.

To further test the hypothesis that phosphorylation is involved in the regulation of R2 stability, we created R2 proteins with mutations in the phosphorylation site and studied their stability in vivo. We replaced the nucleotides encoding the Ser20 residue with nucleotides coding for Ala (S20A mutant) or removed the nucleotides encoding amino acid residues 2–20 (Δ20 mutant) in a mouse R2 gene including a 1 kilobase promoter fragment. Our results show that both cell clones stably transformed with the native form of the R2 gene and with mutant forms confer resistance to HU and show the same regulation of the R2 protein stability as the nontransformed cells (Fig. 5B).

**DISCUSSION**

How important is the control of R2 protein levels, which determine the overall RNR activity, during the mammalian cell cycle? Constitutively expressed R2 protein in Balb/3T3 cells stably transformed with a retroviral expression vector containing mouse R2 cDNA led to a greatly increased frequency of focus formation in cooperation with Ha-ras transformation (27). Therefore, it was suggested that a deregulated R2 protein might be a novel tumor progression determinant, cooperating with a variety of oncogenes to determine transformation and tumorigenic potential.

The presence of the R2 protein during the G1 phase theoretically would lead to an increase of dNTP pools to the S phase levels, because the R1 protein is available throughout the cell cycle. Because high dNTP pools are a prerequisite of an untimely replication, there should be a reliable mechanism to eliminate the R2 protein after the completion of DNA synthesis.

Previously, it was believed that the cell cycle control of the R2 protein is achieved at the level of transcription in combination with an inherited short half-life of the R2 protein. Here, instead of a transcriptional control we found that differential protein stability tunes the R2 protein levels both during the mammalian cell cycle and, indirectly, after DNA damage or a replication block. Protein R2 slowly accumulates during S phase or in S phase arrested cells, and it does not undergo noticeable degradation before the cells enter mitosis. Degradation appears to correlate with passage into mitosis rather than being controlled by the presence of unreplicated DNA, as indicated by the results with roscovitine and nocodazole (Fig. 6). That the ubiquitin-proteasome-dependent pathway is involved is suggested by the prevention of R2 protein degradation by the proteasome inhibitor LLnL.

Another level of the mammalian RNR regulation is the feedback inhibition of the holoenzyme by dATP. This mechanism, along with an elaborate mammalian dNTP excretion system (reviewed in Ref. 3), keeps the S phase dNTP pools at a level that is optimal for replication, which does not increase even when the limiting R2 protein is overproduced. For instance, in hydroxyurea-resistant, protein R2-overproducing mouse cells
having 3–15 times higher RNR activity than the parent cells, all dNTP pools were close to normal, except for a 3–4 times higher dATP pool, as expected from the allosteric mechanism (28). Furthermore, hydroxyurea-resistant, protein R2-overproducing mouse mammary tumour TA 3 cells, containing about 40-fold more of R2 protein than the parent cells (7), had the same dNTP pools as the parent cells.5 In both of these cases the cell cycle regulation of the R2 protein is not disturbed. In contrast, the pyrimidine dNTP pools are increased 3-fold, and the purine dNTPs are increased 9-fold, in comparison with the parental cell line in mouse cells containing an R1 protein with a D57N mutation in the allosteric activity site, which abolishes dATP feedback inhibition (10). The spontaneous mutation rate is about 100 times higher in this mutant cell line compared with the parental cells with a normal RNR, most probably reflecting both the dNTP pool unbalance and the overall increased pools.

Our data demonstrate that DNA damage or replication blocks do not additionally induce the expression of the limiting R2 protein during the S phase. This suggests that the S phase levels of the R2 protein are sufficient for the maximal biochemical activity of the RNR holoenzyme allowed by the dATP feedback inhibition mechanism.

Earlier, we reported that expression of the mouse R2 protein was regulated by an S phase-specific release from a transcriptional block (29). This model was based on reporter gene studies and in vitro nuclear run-on experiments. However, we have not been able to verify our model in vivo; furthermore, our new R2 promoter-luciferase reporter gene constructs, such as the one used in this work, show a very good correlation between luciferase activity and R2 mRNA levels, which indicates a pure transcriptional control of cell cycle regulated R2 gene expression. Using cells stably transfected with the R2 promoter-luciferase reporter gene construct, we found that R2 promoter activity is neither increased nor prolonged in response to DNA damage or replication blocks after the cells enter the S phase. In contrast to earlier models, our new data suggest that transcription of the mouse R2 gene is turned on in the beginning of S phase, reaches its maximum 6–7 h later, and then decreases. This process, once initiated in the beginning of S phase, seems to be not affected by cell cycle progression or DNA damage.

In support of our results with the reporter gene construct, it is demonstrated that the levels of the mouse R2 mRNA are not increased in logarithmically growing mouse SC 2 cells after addition of hydroxyurea, whereas R2 protein levels are elevated (30). The increase in the R2 protein levels can be explained by accumulation of the S phase cells that cannot pass through mitosis and therefore cannot degrade the R2 protein. Furthermore, we observed no increase in R2 protein levels during a 9-h period after the addition of hydroxyurea to cells synchronized in the middle of S phase, which also indicated that the transcription of the R2 gene was neither increased nor prolonged. Pulse-chase experiments showed that there was no degradation of R2 protein during the same period.

There is another example of an uncoupling of transcription of S phase genes (DNA polymerase α, proliferating cell nuclear antigen, and the two RNR subunits) from cell cycle progression, which is described in early Drosophila embryos (31). Here, the coordinate program of transcription operates normally after cell cycle arrest in G2 phase of string mutant embryos; the program is controlled by developmental signals rather than by the cell cycle.

The phosphorylation of the R2 protein by a cell cycle-dependent kinase was an obvious candidate for a regulatory signal triggering ubiquitin-mediated proteolysis. We determined that the earlier reported R2 protein phosphorylation occurs uniquely on a Ser20 residue as soon as newly synthesized R2 protein can be detected, and it remains until the R2 protein disappears during the mitosis. However, neither the in vitro activity nor the in vivo stability of the R2 protein seemed to be affected by this phosphorylation.

The involvement of the anaphase-promoting complex/cyclosome in R2 protein degradation appears less likely because this E3 ubiquitin protein ligase is not activated until cells are released from a nocodazole-induced mitotic arrest (32). We show that the R2 protein is already degraded in nocodazole arrested cells. Furthermore, cyclosome-mediated degradation is dependent on “destruction boxes,” which are N-terminally located 9-amino acid regions containing two invariant residues RXXL (33). In mammalian R2 proteins, such a conserved sequence is present between amino acids 5 and 13. However, our Δ20 R2 protein mutant, lacking this putative destruction box, showed the same stability as native R2 protein (Fig. 5B).

Therefore, the signal for the regulated R2 proteinolyse remains to be elucidated.

Mammalian thymidine kinase (TK) is another example of a cell cycle-regulated protein, where enzyme activity is controlled by protein stabilization. In cycling cells, TK levels increase at the G1/S border and remain stable during S phase. The transcription of the TK gene is turned off at about mid-S phase; the protein is degraded rapidly during and after mitosis (34). The signal for degradation appears to reside in the C terminus, but the details of the mechanisms involved in regulation of TK stability are not known.

Finally, there might be an interesting link between the prolonged activity of the mammalian RNR, in response to replication blocks, and the requirement for dATP to trigger apoptosis. It has been shown that one way to initiate the apoptotic protease cascade requires the cytochrome c/dATP-dependent formation of an Apaf-1/caspase-9 complex (35). Here, dATP has a 1000-fold higher affinity for the Apaf-1 than does ATP. A sustained ribonucleotide reduction because of prolonged stability of the R2 protein in arrested cells might lead to a progressive increase in dATP levels, contributing to triggering apoptosis.

In conclusion, we propose a new model for the cell cycle-dependent regulation of mammalian RNR during the normal cell cycle and after DNA damage/replication blocks (Fig. 6). The overall activity of the mammalian RNR during the cell cycle is controlled by the regulated stability of the limiting R2 protein.

Acknowledgments—We thank Dr. Stefan Björklund, Dr. Sven Carlsson, Anders Hofer, Anna Lena Hofslagare, and David Modjeska for helpful discussion and advice.

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