

# Regulation of Mammalian Ribonucleotide Reduction and dNTP Pools after DNA Damage and in Resting Cells\*

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Ribonucleotide reductase (RNR) provides the cell with a balanced supply of deoxyribonucleoside triphosphates (dNTP) for DNA synthesis. In budding yeast DNA damage leads to an up-regulation of RNR activity and an increase in dNTP pools, which are essential for survival. Mammalian cells contain three non-identical subunits of RNR; that is, one homodimeric large subunit, R1, carrying the catalytic site and two variants of the homodimeric small subunit, R2 and the p53-inducible p53R2, each containing a tyrosyl free radical essential for catalysis. S-phase-specific DNA replication is supported by an RNR consisting of the R1 and R2 subunits. In contrast, DNA damage induces expression of the R1 and the p53R2 subunits. We now show that neither logarithmically growing nor G<sub>0</sub>/G<sub>1</sub>-synchronized mammalian cells show any major increase in their dNTP pools after DNA damage. However, non-dividing fibroblasts expressing the p53R2 protein, but not the R2 protein, have reduced dNTP levels if exposed to the RNR-specific inhibitor hydroxyurea, strongly indicating that there is ribonucleotide reduction in resting cells. The slow, 4-fold increase in p53R2 protein expression after DNA damage results in a less than 2-fold increase in the dNTP pools in G<sub>0</sub>/G<sub>1</sub> cells, where the pools are about 5% that of the size of the pools in S-phase cells. Our results emphasize the importance of the low constitutive levels of p53R2 in mammalian cells, which together with low levels of R1 protein may be essential for the supply of dNTPs for basal levels of DNA repair and mitochondrial DNA synthesis in G<sub>0</sub>/G<sub>1</sub> cells.

Mammalian cells need a balanced supply of deoxyribonucleoside triphosphates (dNTPs)<sup>2</sup> for DNA replication and repair. The rate-limiting step in the formation of DNA precursors is the *de novo* reduction of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates by the enzyme ribonucleotide reductase (RNR) (1). In S phase, the mammalian RNR enzyme is composed of the homodimeric R1 and R2 subunits, which together form a heterotetrameric active enzyme. The large R1 protein (90 kDa) carries the active site, whereas the small R2 protein (45 kDa) contains a diferric iron center generating a tyrosyl free radical necessary for catalysis (1, 2). An additional mammalian RNR protein, p53R2, was identified in 2000 (3, 4). Like the homologous R2 protein, p53R2 contains a tyrosyl free radical and forms an active RNR complex with the R1 protein *in vitro* (5). The tyrosyl free radical of both the R2 and the p53R2 proteins is specifically destroyed by the RNR inhibitor hydroxyurea (5, 6).

Because unbalanced dNTP pools can cause genetic abnormalities and cell death (1), RNR activity is tightly regulated in mammalian cells by S-phase-specific transcription of the R1 and R2 genes (7–9), binding of nucleoside triphosphate allosteric effectors to the R1 protein (10), and anaphase promoting complex-Cdh1-mediated degradation of the R2 protein in late mitosis (11, 12). In cycling cells, the S-phase-dependent activity of the RNR complex is limited by the expression and degradation of R2 protein, whereas the R1 protein is present throughout the cell cycle (13).

One unanswered question is how mammalian cells in G<sub>0</sub>/G<sub>1</sub> phase with no R2 protein obtain dNTPs for DNA repair and mitochondrial DNA replication. One possible answer might be by salvage of deoxyribonucleosides. Mammalian cells have four kinases for salvage of deoxyribonucleosides: thymidine kinase 1 and 2, deoxyguanosine kinase, and deoxycytidine kinase (14). Three of these, thymidine kinase 2 (mitochondrial), deoxyguanosine kinase (mitochondrial), and deoxycytidine kinase (cytoplasmic), are not cell cycle-specific and could theoretically salvage deoxyribonucleosides in a resting cell (14). Another way of obtaining dNTPs is through ribonucleotide reduction. In contrast to the S-phase-specific R2 protein, the R1 protein has a long half-life and is detectable outside the S phase in proliferating cells (13, 15). Furthermore, in addition to the S-phase-specific transcription of the R1 gene, this gene has also been found to be transcribed and translated in response to DNA damage in resting mouse fibroblasts (5). As with the R1 protein, expression of the p53R2 protein is induced by DNA damage (3, 4). The induction is mediated by the tumor suppressor protein p53, a downstream target of the ATM/ATR Chk1/Chk2 kinase pathways (16). The p53 protein homolog p73 has also been shown to induce p53R2 expression after DNA damage (4). Knock-out mice lacking a functional p53R2 gene died from severe renal failure by the age of 14 weeks (17, 18).

It has been shown that DNA damage in budding yeast activates the Mec1/Rad53 kinases (homologs to ATR and Chk1 in mammalian cells (19)), which lead to transcriptional up-regulation of the RNR genes and degradation of the small RNR inhibitor protein Sml1 (20–23). As a consequence, RNR activity increases, and the dNTP pools are elevated 7-fold in logarithmically growing cells, which is about 4-fold higher than the maximal level of dNTP pools during the S phase (24). *Schizosaccharomyces pombe* responds in a similar way to DNA damage, with degradation of the small RNR inhibitor protein Spd1 and a 2-fold increase in the dNTP pools (25, 26).

The p53-dependent induction of the p53R2 protein has been suggested to be important in the cellular response to DNA damage (3, 17), and by analogy with the yeast system, it has been assumed that mammalian cells also increase their ribonucleotide reductase activity and dNTP pools after DNA damage (27, 28). There are conflicting reports in the literature, however, regarding the effects of DNA damage on dNTP pools in mammalian cells (29).

We have studied the levels of R1, R2, and p53R2 proteins and dNTP levels in both logarithmically growing and synchronized cultures of mammalian cells before and after DNA damage and exposure to

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<sup>2</sup> The abbreviations used are: dNTP, deoxyribonucleoside triphosphate; RNR, ribonucleotide reductase; 5-BrdUrd, bromo-2'-deoxyuridine; HPLC, high performance liquid chromatography.

hydroxyurea. In our experiments, cells always show a low, constitutive level of p53R2 protein expression that is independent of the phase of the cell cycle. However, we have never observed any major increase in the dNTP pools in logarithmically growing or resting mammalian cells after DNA damage. These results are in strong contrast to the pronounced increase in dNTP pools observed in yeast after DNA damage. However,  $G_0/G_1$ -phase mammalian cells expressing the p53R2 protein but not the R2 protein were found to show a significant reduction in their dNTP pools after treatment with the specific RNR inhibitor hydroxyurea, indicating that there is indeed ribonucleotide reduction in  $G_0/G_1$ -phase mammalian cells. Our results emphasize the importance of the low constitutive levels of p53R2, which together with low levels of the R1 protein may be essential for the supply of dNTPs for basal DNA repair and mitochondrial DNA synthesis in  $G_0/G_1$  cells.

## EXPERIMENTAL PROCEDURES

**Cell Culture**—Mouse fibroblast Balb/3T3 cells (American Type Culture Collection, CCL 163) were grown as previously described (5). Human fibroblast WS1 cells (American Type Culture Collection, CCL 1502) and primary mouse fibroblasts were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with penicillin, streptomycin, and 10% (v/v) fetal bovine serum. Where possible, experiments were performed with all three types of cells to exclude cell type-specific results. However, Balb/3T3 cells gave the highest fraction of S-phase cells after synchronization, and these cells were, thus, used preferentially for S-phase studies. Serum synchronization of cells and UV irradiation were performed as described earlier (30). Cell elutriation was performed as described earlier (31), with the following modifications. After trypsinization, Balb/3T3 cells that had been starved of serum for 48 h were resuspended in the spent medium and elutriated in Dulbecco's modified Eagle's medium without serum. After elutriation, the  $G_0/G_1$  cell fraction was seeded in the spent medium containing 0.5% fresh serum. For the generation of primary embryonic mouse fibroblasts, mouse blastocysts were prepared as described (32). Adriamycin and hydroxyurea were added directly to cell cultures to final concentrations of 0.2  $\mu\text{g}/\text{ml}$  and 2 mM, respectively.

**Immunoblotting**—Approximately  $10^6$  cells were washed with ice-cold phosphate-buffered saline, harvested with a rubber policeman, pelleted by centrifugation at  $230 \times g$  for 2 min, and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM  $\text{MgCl}_2$ , 0.5% Nonidet P-40). After freezing and thawing 3 times, cell debris was removed by centrifugation at  $20,000 \times g$  and  $4^\circ\text{C}$  for 20 min, and protein concentration was measured by the Bradford method. From  $10^6$  Balb/3T3 cells, we obtained 0.32 mg of protein. Equal amounts of protein were loaded in separate lanes and electrophoresed on a 10% SDS-polyacrylamide gel. Transfer of proteins to a Hybond-C Extra membrane was made as previously described (11). After transfer, the membrane was blocked in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20 containing 2% dried milk for 30 min before incubation with either a polyclonal rabbit anti-p53R2 antibody (ab2014, Abcam), a polyclonal rabbit anti-R2 antibody (33), or a monoclonal mouse anti-R1 antibody, AD203 (13), for 1 h at room temperature or overnight at  $4^\circ\text{C}$ . A minor cross-reaction was found between the anti-p53R2 and anti-R2 antibodies, but this was not a problem since these two proteins have different electrophoretic mobilities due to their different sizes (5). The primary antibodies were removed by washing the membrane in the same solution as above (but without dried milk) followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The membrane was finally washed as previously described

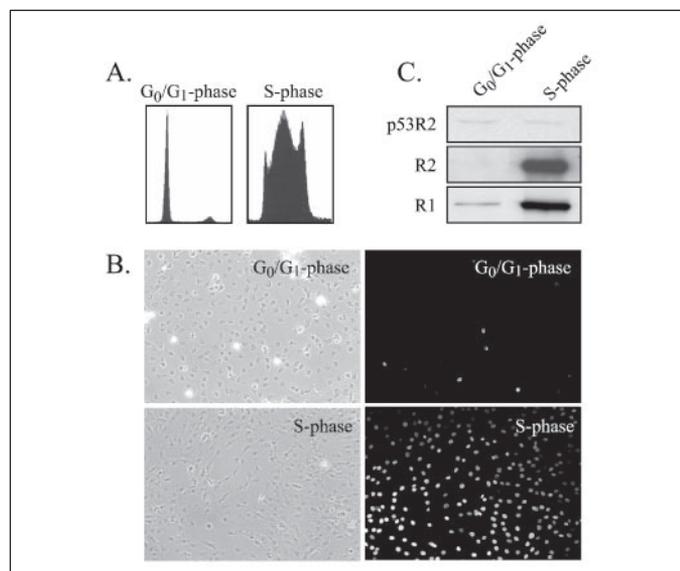
(11), and detection was done using the ECL-plus system according to the instructions of the manufacturer (Amersham Biosciences).

For absolute quantification of p53R2, R2, and R1 protein levels by immunoblotting, known amounts of pure recombinant proteins were loaded in separate lanes on the gel. After detection, the developed film was scanned, and band intensities were measured with Scion image software (Scion Corp.) and plotted against the amount of each recombinant protein to generate a standard curve. Endogenous p53R2, R2, and R1 proteins in cell extracts were quantified by running immunoblots on the same gel as the one used for the standard curve. The intensities of endogenous p53R2, R2, and R1 bands were then compared with the standard curve, and the actual amount of cellular protein was calculated.

**Cell Cycle Analysis**—Flow cytometry analyses were carried out on approximately  $10^6$  cells using a CCA-I flow cytometer according to the instructions of the manufacturer (Partec GmbH, Germany). 5-Bromo-2'-deoxyuridine (BrdUrd) staining was done according to the instructions of the manufacturer using a BrdUrd labeling and detection kit (Roche Applied Science). Briefly, cells were incubated with BrdUrd for 30 min. Incorporation of BrdUrd was then detected by incubating the cells with an anti-BrdUrd fluorescent antibody. The cells were then analyzed using a Zeiss Axiovert 40 CFL microscope, with phase contrast (for total number of cells) or by fluorescence microscopy (for BrdUrd-positive cells).

**Measurement of dNTP Pools**—Approximately  $10^7$  cells were washed twice with cold phosphate-buffered saline and harvested in 0.5 ml of ice-cold 10% trichloroacetic acid supplemented with 15 mM  $\text{MgCl}_2$ . The quantification of nucleotides was done according to Hofer *et al.* (34) with the following modifications. After Freon extraction, 25  $\mu\text{l}$  of the aqueous phase was loaded onto a polyWAX LP 200  $\times$  4.6-mm column for quantification of ribonucleoside triphosphates. The HPLC column was run isocratically in 0.3 M potassium phosphate buffer, pH 5.0, with 2% (v/v) acetonitrile at 1 ml/min, allowing the simultaneous separation of all 8 NTPs and dNTPs from each other. For quantification of deoxyribonucleoside triphosphate, the remaining part of the sample (475  $\mu\text{l}$ ) was passed through a boronate column to remove the ribonucleotides, and 2 ml of the flow-through was collected. 1 ml was loaded onto the HPLC column using the same conditions as described for the ribonucleoside triphosphates. The nucleotides were quantified by measuring the peak heights and comparing them to a standard curve. For quantification of nucleotide pools in mouse skeletal muscle, a male mouse was sacrificed by deceleration of the spinal cord, and a piece of the hind leg muscle was dissected and immediately frozen in liquid nitrogen. The tissue was homogenized in ice-cold 10% trichloroacetic acid with 15 mM  $\text{MgCl}_2$ , and the nucleotides were determined as above. Because we could not determine the number of muscle cells in the sample, we determined each dNTP as the percentage of the total nucleoside triphosphate pool in mouse skeletal muscle cells (CTP + UTP + ATP + GTP). Assuming that the total NTP pool of muscle cells is the same as the total NTP pool in Balb/3T3 cells (reproducibly 10 nmol of NTP/ $10^6$  cells independently of the cell-cycle phase), we could make an approximate calculation of the concentration of each dNTP in pmol/ $10^6$  muscle cells.

**Purification of Recombinant Proteins**—Recombinant human R2, human R1, human p53R2, and mouse p53R2 proteins were expressed and purified as previously described (5). Recombinant mouse R2 and R1 proteins were expressed and purified to homogeneity as previously described (35, 36).

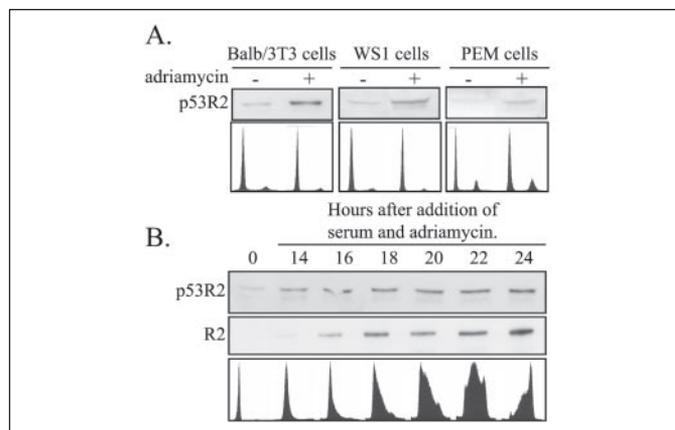


**FIGURE 1. The p53R2 protein is expressed equally throughout the cell cycle of Balb/3T3 cells.** Balb/3T3 cells were synchronized in  $G_0/G_1$  phase by 72 h of serum starvation or in S-phase by serum starvation for 48 h followed by 18 h incubation in fresh medium supplemented with 20% serum. The cells were then analyzed for cell cycle distribution and levels of RNR proteins. *A*, flow cytometry. *B*, BrdUrd staining. The cells were examined using phase contrast (for total number of cells, *left panels*) or fluorescence microscopy (for BrdUrd-positive cells, *right panels*).  $G_0/G_1$ -phase synchronized cells contained 2.6% S-phase cells (total number of cells, 189; BrdUrd-positive cells, 5) and S-phase-synchronized cells contained 91% S-phase cells (total number of cells, 274; BrdUrd-positive cells, 250). *C*, immunoblot analysis of p53R2, R2, and R1. For detection of the R1 and R2 proteins, 1  $\mu$ g of cell extract was loaded in separate lanes. For detection of p53R2, 10  $\mu$ g of cell extract was loaded in separate lanes.

## RESULTS

*The p53R2 Protein Is Expressed at Constant, Low Levels throughout the Cell Cycle, and This Basal Level of Expression Is Increased Both in  $G_0/G_1$ - and S-phase Cells after DNA Damage*—The expression of the p53R2 gene is slowly induced in logarithmically growing cells after DNA damage in a p53/p73-dependent manner (3, 4). However, in these studies the protein also appeared to be present at low levels in undamaged cells, although no special emphasis was placed on this fact. To study the constitutive expression of the p53R2 protein during the cell cycle, mouse Balb/3T3 fibroblast cells were synchronized in  $G_0/G_1$  phase by serum starvation or synchronized in S phase by serum starvation followed by cultivation in fresh medium supplemented with 20% serum. Synchronization was monitored by flow cytometry (Fig. 1*A*) and further confirmed by BrdUrd incorporation (Fig. 1*B*). The levels of the p53R2 protein were the same in both  $G_0/G_1$ - and S-phase-enriched cells as determined by immunoblotting (Fig. 1*C*, *top panel*). In agreement with earlier studies (11), R2 protein was hardly detectable in the  $G_0/G_1$ -phase cells but was strongly expressed in the S-phase cells (Fig. 1*C*, *middle panel*). The levels of the R1 protein were low in the  $G_0/G_1$ -phase cells and strongly elevated in the S-phase cells (Fig. 1*C*, *bottom panel*).

Next, we investigated whether  $G_0/G_1$ -phase cells can induce p53R2 expression after DNA damage. To avoid cell type-specific results, we used three different cell types: mouse Balb/3T3, human WS1, and mouse primary fibroblast cells. All cells were starved of serum for a total of 72 h with or without the addition of the DNA damaging agent adriamycin for the final 24 h, a treatment earlier shown to cause induction of p53R2 in logarithmically growing cells (3). Independent of the addition of adriamycin, this method generated close to pure  $G_0/G_1$ -phase cultures (Fig. 2*A*, *bottom panel*). Analyses of the content of the p53R2 protein by immunoblotting showed that all three  $G_0/G_1$ -phase cell lines



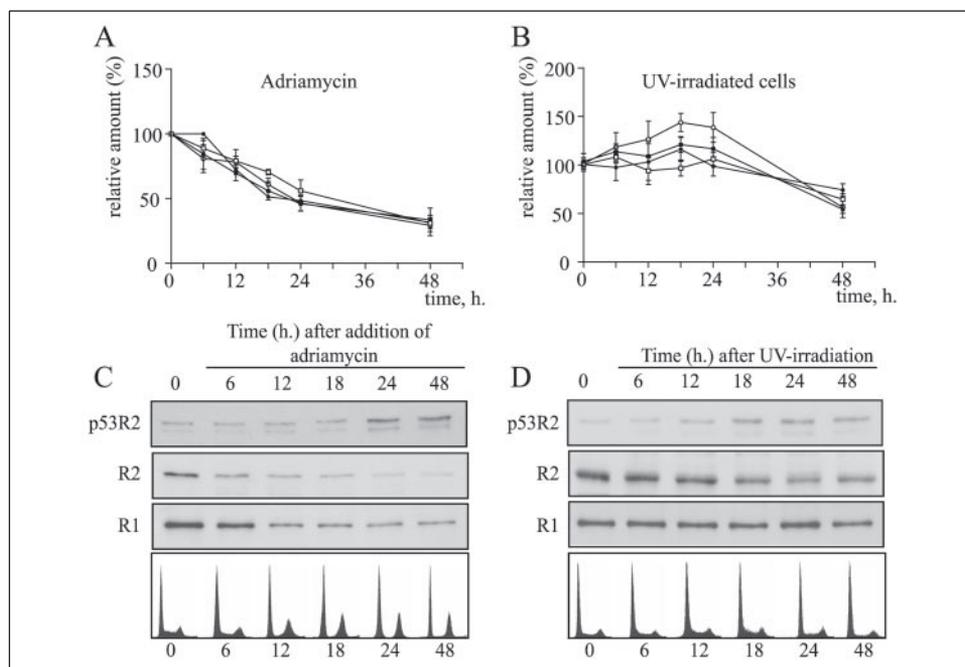
**FIGURE 2. Both  $G_0/G_1$ - and S-phase cells induce expression of the p53R2 protein after DNA damage.** *A*, immunoblot (*top panel*) and flow cytometry (*bottom panel*) analysis of Balb/3T3 cells, WS1 cells, and primary mouse fibroblasts (primary embryonic mouse (PEM) cells). Cells were synchronized in  $G_0/G_1$  phase with (+) or without (-) the addition of adriamycin. *B*, immunoblot (*top panels*) and flow cytometry (*bottom panel*) analysis of Balb/3T3 cells starved of serum and then released into S phase with fresh medium supplemented with 20% serum and adriamycin (time point 0). The same amounts of cell extract as in Fig. 1 were used for immunoblotting.

induced expression of the p53R2 protein in response to adriamycin exposure (Fig. 2*A*, *top panel*).

To answer the question of whether S-phase cells would also induce expression of the p53R2 protein after DNA damage, Balb/3T3 cells were starved of serum for 48 h and then released into S phase by replacing the serum-depleted medium with fresh medium supplemented with 20% serum and adriamycin. Exposure to adriamycin caused a 4-h delay in cell cycle progression compared with cells released in fresh medium without the drug (data not shown). However, 22 h after the re-addition of serum, the majority of drug-treated cells were in mid-S phase (Fig. 2*B*, *bottom panel*). Although the drug-treated cells expressed high levels of R2 protein (Fig. 2*B*, *middle panel*, 22-h lane), they nevertheless induced expression of p53R2 protein (Fig. 2*B*, *top panel*; compare the 0-h lane with the 22-h lane). These results clearly show that p53R2 protein is expressed equally well in undamaged  $G_0/G_1$ - and S-phase cells (Fig. 1) and that both  $G_0/G_1$ - and S-phase cells induce expression of p53R2 protein after DNA damage (Fig. 2).

*Induced Expression of p53R2 Protein after DNA Damage in Logarithmically Growing Mammalian Cells Does Not Result in Elevated dNTP Pools*—Although it is easy to understand the rationale behind induction of p53R2 in resting cells after DNA damage, it is less obvious why an S-phase cell with high levels of R2 protein would do so. Would the induction of p53R2 protein lead to increased dNTP pools? To study the effect of p53R2 induction on intracellular dNTP levels, logarithmically growing Balb/3T3 cells were exposed either to adriamycin (present throughout cultivation) or a pulse of UV light. Cells were harvested at different time points after the addition of adriamycin or UV irradiation, and the intracellular dNTP pools (dTTP, dCTP, dATP, and dGTP) were measured by HPLC. Surprisingly, all four dNTPs decreased with time after the addition of adriamycin (Fig. 3*A*). Furthermore, with the exception of some fluctuations in the dATP pool, the dNTP levels were almost constant in UV-irradiated cells (Fig. 3*B*). A parallel analysis of the levels of the three different RNR proteins by immunoblot showed that p53R2 increased around 24 h after the addition of adriamycin, as observed earlier (3) (Fig. 3*C*, *top panel*). In contrast to the p53R2 protein, both the R2 and R1 protein levels decreased after the addition of adriamycin (Fig. 3*C*, *middle panels*). Analysis of cell cycle distribution by flow cytometry showed that mouse fibroblasts leave S phase in a time-dependent manner after the addition of adriamycin (Fig. 3*C*, *bottom panel*). This

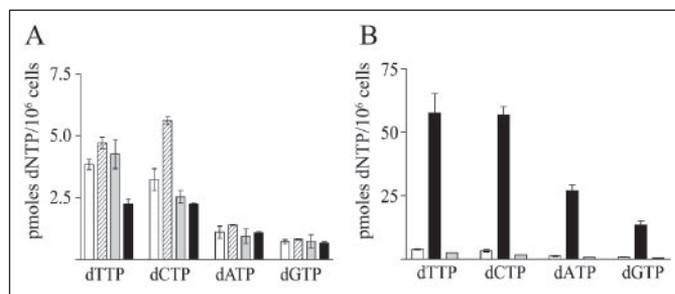
**FIGURE 3. The dNTP pools of logarithmically growing Balb/3T3 cells do not increase after induction of the p53R2 protein.** Time-dependent variations in intracellular dNTP pools, levels of the RNR proteins, and cell cycle profiles of logarithmically growing Balb/3T3 cells after the addition of adriamycin (present throughout cultivation) or a 12.5-s pulse of UV irradiation ( $10 \text{ J/m}^2$ , 254 nm). Cells were harvested at the time points indicated. *A* and *B*, intracellular dNTP pools of adriamycin-exposed (*A*) or UV-irradiated (*B*) cells ( $\square$ , dTTP;  $\blacksquare$ , dCTP;  $\circ$ , dATP;  $\bullet$ , dGTP). The ordinate gives relative dNTP levels compared with untreated logarithmically growing cells, typically 22, 17, 7.5, and 3.6 pmols/ $10^6$  cells of dTTP, dCTP, dATP, and dGTP, respectively. Error bars are  $\pm$ S.D. of at least two independent measurements. *C* and *D*, immunoblot of RNR proteins as in Fig. 1 and flow cytometry (*bottom panels*) of adriamycin-exposed (*C*) or UV-irradiated (*D*) cells.



explains the decrease in the R2 protein level since R2 is S phase-specific. Due to the long half-life of the R1 protein, it decreased in amount more slowly than the R2 protein. Similarly to adriamycin, UV irradiation caused induced expression of p53R2 (Fig. 3*D*, top panel). However, in contrast to adriamycin, this dose of UV radiation only had a small effect on the overall cell cycle distribution (Fig. 3*D*, bottom panel) and, thus, also had only a small effect on the level of R2 and virtually no effect on the level of R1 (Fig. 3*D*, middle panels). Similar results were obtained using the human fibroblast WS1 cell line (data not shown). Taken together, these results clearly show that there is no correlation between induced expression of the p53R2 protein and a general increase in the intracellular dNTP pools in a logarithmically growing cell culture after DNA damage.

**The dNTP Pools of Resting Mammalian Cells Do Not Increase after DNA Damage**—Logarithmically growing mouse and human fibroblasts reduce their dNTP pools and R2 protein levels in a synchronous manner after exposure to adriamycin. Consequently, by measuring the dNTP pools of logarithmically growing cells before and after DNA damage, it is likely that we are only monitoring the R2 protein status of the cell culture. To follow the influence of the p53R2 protein, we decided to measure the dNTP pools of  $G_0/G_1$ -phase-synchronized mammalian cells, with only low levels of R2 (*cf.* Fig. 1*C*) before and after DNA damage. Balb/3T3 cells were serum-starved for a total of 72 h. Before the end of the 72-h starvation period, the cells were either left undamaged, given a pulse of UV radiation 3 h before harvesting, exposed to adriamycin for the last 3 h, or exposed to adriamycin for the last 24 h (which was shown to cause p53R2 induction, *cf.* Fig. 2*A*). With the exception of a small increase in the dCTP pool of UV-irradiated  $G_0/G_1$ -phase Balb/3T3 cells, there was no general increase in the intracellular dNTP pools of damaged  $G_0/G_1$  cells compared with untreated  $G_0/G_1$  cells (Fig. 4*A*). The decrease in the dTTP pool seen in cells exposed to adriamycin for 24 h reflects residual S-phase cells leaving the S phase, similar to the situation seen for logarithmically growing cells exposed to adriamycin (Fig. 3*A*).

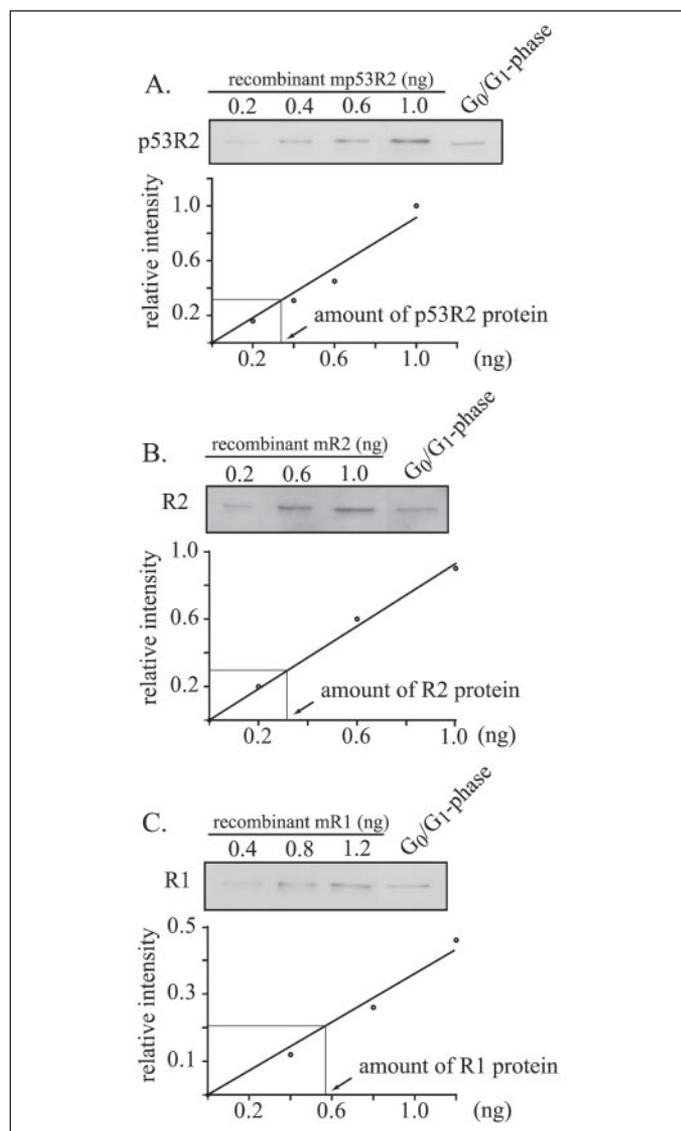
Because fission and budding yeast respond to DNA damage by increasing their dNTP pools to the same and higher levels than in S phase, respectively (24, 26), we wanted to compare the dNTP levels of



**FIGURE 4. Intracellular dNTP pools of  $G_0/G_1$ -phase Balb/3T3 cells measured before and after DNA damage of S-phase-enriched Balb/3T3 cells and in mouse muscle tissue.** *A*, intracellular dNTP levels of  $G_0/G_1$ -phase-synchronized Balb/3T3 cells either left undamaged (*white bars*; 3.8, 3.2, 1.1 and 0.7 pmols/ $10^6$  cells of dTTP, dCTP, dATP, and dGTP, respectively) given a pulse of  $10 \text{ J/m}^2$  UV (254 nm) radiation 3 h before harvesting (*striped bars*), exposed to adriamycin for 3 h before harvesting (*gray bars*), or exposed to adriamycin for the final 24 h before harvesting (*black bars*). *B*, dNTP pools of undamaged Balb/3T3 cells enriched for  $G_0/G_1$  phase (*white bars*; the same values as in *A*) in S-phase-enriched cells (*black bars*; 58, 57, 27, and 13 pmols/ $10^6$  cells of dTTP, dCTP, dATP, and dGTP, respectively) or dNTP levels of mouse skeletal muscle tissue (*gray bars*). For both *A* and *B*, the error bars show  $\pm$ S.D. of at least two independent measurements.

the  $G_0/G_1$ -phase-synchronized mammalian cells to the dNTP levels of S-phase-synchronized mammalian cells. In agreement with earlier studies (1), we found that  $G_0/G_1$ -phase cells had dNTP pools that were  $\sim$ 18 times smaller than those of S-phase-enriched cells (Fig. 4*B*, compare *white* and *black bars*). To ensure that the small dNTP pools in resting cells were not an artifact generated by cultivation of immortal cell lines, we also tried to estimate the dNTP pools of mouse skeletal muscle cells. Only minor differences in the dNTP pools were observed between serum-starved mouse Balb/3T3 fibroblasts and mouse skeletal muscle cells (Fig. 4*B*, compare *white* and *gray bars*). However, an exact comparison is difficult since the dNTPs were determined as the percentage of total NTPs, and there may be differences in the total amount of NTPs per cell between Balb/3T3 cells and muscle cells (see "Experimental Procedure").

**Absolute Intracellular Amounts of p53R2 before and after DNA Damage Compared with the Absolute Amounts of R2 and R1 at Different Phases of the Cell Cycle**—It has been shown that the R2 and p53R2 proteins have similar enzymatic activities together with the R1 protein (5). One



**FIGURE 5. Immunoblots and standard curves used for quantification of the absolute intracellular amounts of the mouse RNR proteins in 10  $\mu$ g of extract from undamaged Balb/3T3 cell cultures serum-starved for 72 h ( $G_0/G_1$ -phase lanes of the immunoblots). The standard curves were generated by plotting the indicated amounts of pure recombinant protein as a function of band intensities from the immunoblots (see "Experimental Procedures"). A, p53R2 protein. B, R2 protein. C, R1 protein.**

possible explanation as to why induced expression of the p53R2 protein does not cause increased dNTP pools may be that the absolute amount of p53R2 in both undamaged and damaged cells is much less than the absolute amount of R2 in S-phase cells. Another possibility might be that active R1 protein is limiting for enzyme activity in  $G_0/G_1$ -phase cells. To measure the absolute amounts of mouse p53R2, R2, and R1 proteins at different phases of the cell cycle before and after DNA damage, we ran immunoblots calibrated with increasing amounts of pure, recombinant p53R2, R2, and R1 proteins. Using this method, we calculated the average cellular content of mouse p53R2 protein in 72-h serum-starved,  $G_0/G_1$ -enriched, undamaged Balb/3T3 cells to be  $34 \pm 3$  ppm (error represents  $\pm$  S.D. of at least three independent measurements) of the total soluble protein content (Fig. 5A). The amount of R2 protein was  $900 \pm 100$  ppm of the total soluble protein content of an undamaged S-phase cell. Consequently, because the p53R2 level is constant throughout the cell cycle (Fig. 1C), an undamaged S-phase cell contains  $\sim 30$  times more R2 protein than p53R2 protein. After 24 h of

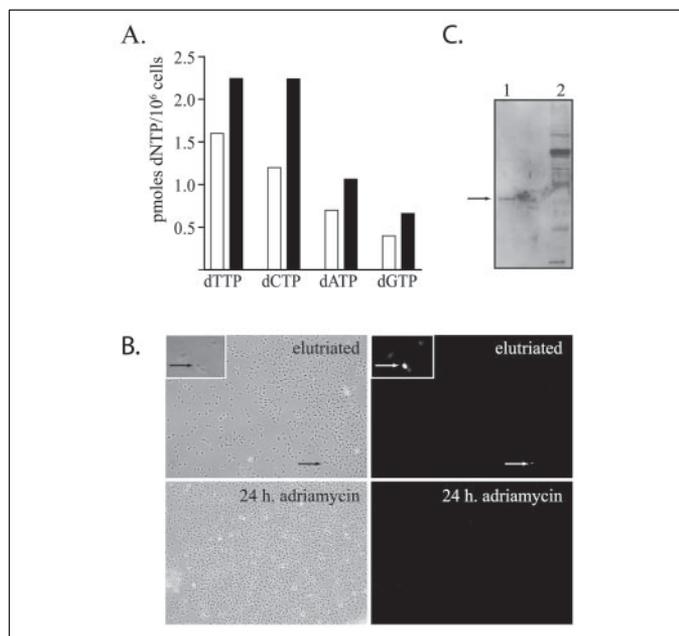
exposure to adriamycin, the intracellular amount of p53R2 increased from  $34 \pm 3$  to  $120 \pm 28$  ppm. Although this corresponds to a 3–4-fold increase in the absolute amount of p53R2 protein, the cellular amount of the p53R2 protein after DNA damage is still only 13% of the amount of R2 protein in an undamaged S-phase cell.

The absolute amount of R2 in a 72-h serum-starved  $G_0/G_1$  phase-enriched cell culture containing about 3% S-phase cells (cf. Fig. 1B) was  $\sim 31$  ppm of the total cellular protein content (Fig. 5B). This value is in good agreement with 3% of the S-phase cell content of R2 protein (900 ppm) and is of the same order as the p53R2 content of undamaged cells. After 24 h of exposure to adriamycin, no R2 protein could be detected in 20  $\mu$ g of cell extract from  $G_0/G_1$ -phase-synchronized cell cultures (data not shown). The decrease in the absolute amount of R2 protein after exposure to adriamycin most likely reflects the 3% S-phase cells leaving S-phase, which is similar to the situation seen for logarithmically growing cells exposed to adriamycin (Fig. 3A). The absolute amount of R1 protein was  $520 \pm 170$  ppm irrespective of whether measured in S-phase-enriched or logarithmically growing cells. After 72 h of serum starvation, the R1 protein level decreased to  $57 \pm 20$  ppm of the total protein content of serum-starved cells (Fig. 5C).

Using recombinant human p53R2 protein, we also calculated the cellular content of p53R2 protein in an undamaged human WS1 cell (serum-starved for 72 h) and a damaged human WS1 cell (serum-starved for 72 h with the addition of adriamycin for the last 24 h) to be  $55 \pm 10$  ppm and  $140 \pm 10$  ppm of the total protein, respectively.

**p53R2-dependent Ribonucleotide Reduction in  $G_0/G_1$ -phase Cells after DNA Damage**—Even though we could not detect a general increase in the intracellular dNTP pools of  $G_0/G_1$  phase cells after DNA damage and p53R2 induction, it is possible that a small p53R2-dependent increase in the dNTP pools is counterbalanced by a decrease in the dNTP pools as a consequence of the reduction in R2 protein levels after exposure to adriamycin. If this is the case, resting undamaged mammalian cells with no expression of the R2 protein should have smaller dNTP pools than resting cells exposed to adriamycin for 24 h (and, therefore, containing induced p53R2 protein levels). To test this hypothesis, we starved Balb/3T3 cells of serum for 48 h. To remove the residual S-phase cells, we then elutriated the cells and collected the fractions only containing  $G_0/G_1$ -phase cells. These cells were allowed to re-attach to cell culture dishes for 3 h in the spent medium containing 0.5% fresh serum and were finally tested for BrdUrd incorporation, R2 protein content, and size of the dNTP pools. Compared with the dNTP pools of cells starved of serum for 72 h and exposed to adriamycin for the final 24 h, the undamaged elutriated  $G_0/G_1$  cells had  $\sim 40\%$  lower dNTP levels (Fig. 6A). Only 0.05% of the undamaged elutriated  $G_0/G_1$  cells were BrdUrd-positive (Fig. 6B), and the amount of R2 protein in the cell culture was negligible (Fig. 6C). No BrdUrd-positive cells could be detected after BrdUrd staining of the cells exposed to adriamycin (Fig. 6B).

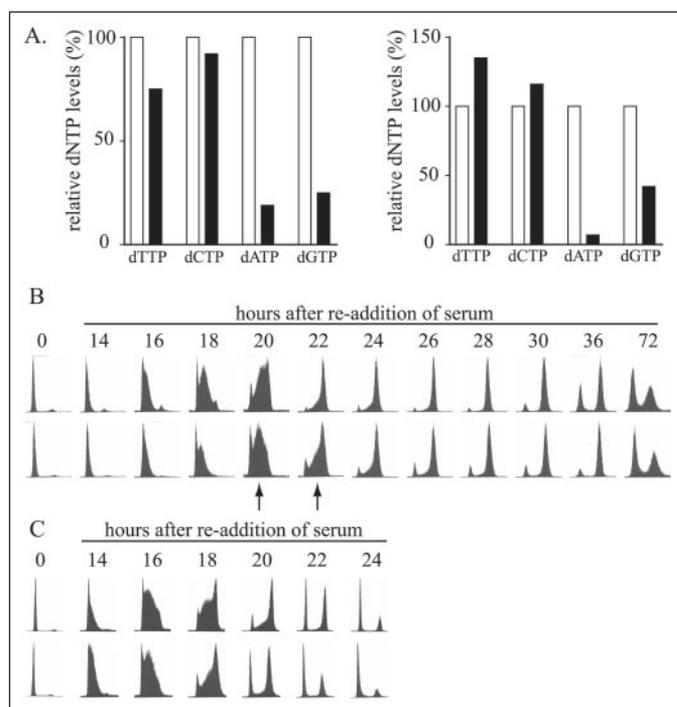
**Exposure to the Specific RNR Inhibitor Hydroxyurea Reduced the dNTP Pools in Resting Mammalian Cells Both with or without DNA Damage**—The results above suggest that there is p53R2-dependent ribonucleotide reduction in  $G_0/G_1$ -phase cells after DNA damage. Because undamaged  $G_0/G_1$ -phase cells also express p53R2, we wanted to know whether such cells have p53R2-dependent RNR activity. If an R1-p53R2 complex supplies non-dividing cells with a low level of dNTPs for basal DNA repair and mitochondrial DNA replication, resting mammalian cells with no expression of the R2 protein should show reduced dNTP pools if treated with hydroxyurea, since this drug destroys the tyrosyl free radical of the p53R2 protein (5). To test this hypothesis, we again used the serum-starved, elutriated  $G_0/G_1$  Balb/3T3 cells described above and allowed the cells to re-attach to cell culture dishes for 2 h in the spent medium containing 0.5% fresh serum. Hydroxyurea was then



**FIGURE 6. p53R2-dependent ribonucleotide reduction in  $G_0/G_1$ -phase cells after DNA damage.** *A*, intracellular dNTP pools of undamaged, elutriated  $G_0/G_1$ -synchronized Balb/3T3 cells (white bars) compared with the intracellular dNTP pools of  $G_0/G_1$ -synchronized Balb/3T3 cells exposed to adriamycin for 24 h (black bars). The dNTP values (given as pmol of dNTP/10<sup>6</sup> cells) for undamaged, elutriated cells are: 1.6 dTTP, 1.2 dCTP, 0.7 dATP and 0.4 dGTP. Those for cells exposed to adriamycin are 2.3 dTTP, 2.2 dCTP, 1.1 dATP, and 0.7 dGTP. *B*, representative panels of BrdUrd staining of the same cells as in *A*. Cells were examined using phase contrast (total number of cells; left panel) or fluorescence microscopy (BrdUrd-positive cells; right panel). The arrows indicate a BrdUrd-positive cell at normal magnification (5 $\times$ ) and at high magnification (20 $\times$ , upper left corner of elutriated cells). The elutriated cells (upper panel) contained ~0.05% S-phase cells (of 3746 cells counted, two BrdUrd-positive cells were found). No BrdUrd-positive cell could be found for the 24-h adriamycin-treated cells (bottom panel, 2070 cells counted). *C*, immunoblot analysis of the R2 protein in undamaged, elutriated  $G_0/G_1$ -synchronized Balb/3T3 cells. Lane 1, 0.2 ng of pure recombinant mouse R2 protein (arrow); lane 2, 10 μg of cell extract.

added to half of the cell cultures dishes. One hour after the addition of hydroxyurea, we harvested the cells, and the dNTP pools were measured. The hydroxyurea treatment caused a more than 50% reduction in purine dNTP pools in the elutriated  $G_0/G_1$ -phase cells (Fig. 7A, left graph). In comparison, only a small effect was seen on the pyrimidine dNTP pools.

To investigate any possible influence of DNA damage and induction of the p53R2 protein on how the dNTPs of resting cells respond to hydroxyurea, both mouse Balb/3T3 and human WS1 cells were synchronized in  $G_0/G_1$  phase by serum starvation for a total of 72 h. 24 h before harvesting, adriamycin was added to all cell cultures (which was shown to cause p53R2 induction, cf. Fig. 2A). Three hours before harvesting, hydroxyurea was added to half of the cell cultures, and dNTP levels were measured as before. As for the situation with the undamaged elutriated  $G_0/G_1$  cells, we found that Balb/3T3 cells exposed to a combination of adriamycin and hydroxyurea showed significantly reduced purine dNTP pools compared with control cells that had only been exposed to adriamycin (Fig. 7A, right graph). In addition to reduced purine dNTP pools, the WS1 cells showed a 71% reduction in the dCTP pool (data not shown). There was no significant reduction in the pyrimidine pool of the Balb/3T3 cells. About 90% of the treated cells were capable of attachment to a tissue culture dish and of growth in fresh medium supplemented with 10% serum, showing that they were viable. Taken together these results indicate that there is p53R2-dependent ribonucleotide reduction in  $G_0/G_1$  phase cells both before and after DNA damage.



**FIGURE 7. Exposure of resting mammalian cells to hydroxyurea caused reduced dNTP pools and delayed cell cycle progression.** *A*, effects of hydroxyurea on the dNTP pools of undamaged, elutriated  $G_0/G_1$ -synchronized Balb/3T3 cells (left) and of  $G_0/G_1$ -synchronized Balb/3T3 cells exposed to adriamycin for the last 24 h (right). Before harvesting, the cells were exposed to hydroxyurea (black bars) or were left untreated (white bars). The ordinate gives relative dNTP levels compared with cells not exposed to hydroxyurea, typically 1.6 pmol of dTTP, 1.2 pmol of dCTP, 0.7 pmol of dATP, and 0.4 pmol of dGTP/10<sup>6</sup> cells for undamaged, elutriated  $G_0/G_1$ -synchronized Balb/3T3 cells and 2.3 pmol of dTTP, 2.2 pmol of dCTP, 1.1 pmol of dATP, and 0.7 pmol of dGTP/10<sup>6</sup> cells for  $G_0/G_1$ -synchronized Balb/3T3 cells exposed to adriamycin for the last 24 h. *B* and *C*, flow cytometry profiles (at indicated time points) of Balb/3T3 cells synchronized in  $G_0/G_1$  phase by 72 h of serum starvation and subsequently released into S phase by re-addition of serum. In *B*, cells were exposed to adriamycin alone (upper panel) or to a combination of adriamycin and hydroxyurea (bottom panel) for the last 24 h before re-addition of serum (time point 0). The arrows mark clear differences in S-phase progression. In *C* (control experiment), serum-synchronized Balb/3T3 cells were cultivated in the absence of drug (upper panel) or in the presence of hydroxyurea for the last 24 h before the re-addition of serum (bottom panel).

*Cells Exposed to a Combination of Adriamycin and Hydroxyurea Have Delayed Entry into S-phase Compared with Cells Exposed to Adriamycin Alone*—If repair of DNA damage in  $G_0/G_1$ -phase cells is dependent on p53R2 activity, exposure of such cells to hydroxyurea should impair this process. If on the other hand the nucleotides required for repair are obtained solely by salvage, there should be no effect of hydroxyurea. To study the effect of hydroxyurea on DNA repair, we starved Balb/3T3 cells of serum for 48 h. Subsequently, either adriamycin or a combination of adriamycin and hydroxyurea was added to the cells, which were left for an additional 24-h period. The drugs were then removed by washing with phosphate-buffered saline, and finally the cells were released into S-phase by re-addition of serum. Cells exposed to a combination of adriamycin and hydroxyurea before the release showed a delayed entry into S-phase as compared with cells exposed to adriamycin alone (Fig. 7B). A 24-h incubation of the cells with hydroxyurea alone had no effect on the cell cycle progression compared with untreated control cells (Fig. 7C). These results suggest that ribonucleotide reduction is important for the supply of dNTPs for DNA repair in resting mammalian cells.

## DISCUSSION

Our data show conclusively that, unlike budding and fission yeast, mammalian cells do not show any major increase in the size of dNTP pools in response to DNA damage. Expression of p53R2 induced by

## Ribonucleotide Reductase and dNTP Pools

DNA damage appears to give a less than 2-fold increase in dNTP levels in resting cells, but even after this increase the dNTP pools of such cells are only 5–10% that of the size of the dNTP pools in S-phase cells. Our data agree with earlier observations that the pyrimidine dNTP pools become smaller in mouse embryonic fibroblast cells after oxidative stress (17). These findings show that mammalian cells and yeast cells regulate their dNTP pools quite differently after DNA damage. Budding yeast cells elevate their dNTP pools in an RNR-dependent manner after DNA damage, and 2 h after DNA damage the dNTP pools are higher than in S-phase cells (24).

Our results and those of other laboratories make us question the importance of the p53-induced expression of the p53R2 protein in dNTP synthesis and DNA repair. First, resting mouse fibroblasts with 15–20 times lower dNTP pools than S-phase cells only marginally increase their dNTP pools at the time of increased p53R2 expression. Second, like resting cells, S-phase cells induce increased expression of the p53R2 protein, although they already contain ~30-fold higher levels of the R2 protein and maximal dNTP pools. Third, DNA repair is generally thought to be accomplished within hours of DNA damage (37, 38), whereas maximal induction of p53R2 expression is, paradoxically, reached no earlier than 24 h after DNA damage (3).

If the slow 4-fold induction of p53R2 expression by p53 after DNA damage is of less significance, what function does the p53R2 protein have in the cell? It must be essential, because knock-out mice die at an early age. Is it the low constitutive expression of p53R2 that is important? Another question is how  $G_0/G_1$ -phase mammalian cells generate dNTPs for “everyday” DNA repair. It has been estimated that  $\sim 2 \times 10^4$  DNA-damaging events occur in every cell of the human body every day as a result of oxidative damage and depurination (39). One possible answer to the second question could be through a low, constant ribonucleotide reduction by a non-cell cycle-specific RNR complex. Because no alternative form of the mammalian R1 protein has been detected, such a complex would have to consist of the R1 protein and a radical-supplying small RNR protein expressed outside the S phase. Transcription of the R2 gene is S-phase-specific, and the protein has a KEN box at the amino-terminal end that targets it to degradation in late mitosis (11, 12). Thus, the R2 protein is undetectable in  $G_0/G_1$ -phase cells and cannot contribute to DNA repair in resting mammalian cells. In contrast, the mouse p53R2 protein, which shows 81% identity with the mouse R2 protein at the amino acid level, lacks the KEN box (12). The p53R2 cDNA was originally cloned from a mouse skeletal muscle cDNA library (3), which might argue that the gene is transcribed in undamaged, resting mammalian cells.

Given that the average volume of a fibroblast is 3.4 pl (40) and that we could extract 0.32 mg of total soluble protein per  $10^6$  cells, our immunoblot data show that the concentration of the p53R2 protein in an undamaged  $G_0/G_1$ - or S-phase cell is  $\sim 3.2 \mu\text{g/ml}$ . Although it is straightforward to determine the amount of p53R2 in a resting cell, it is much more difficult to evaluate the amount of the R1 protein in such a cell due to the long half-life. Solution hybridization assays using Balb/3T3 cells starved of serum for 42 h indicated that these cells contained less than 2 molecules of R1 mRNA per cell, as compared with a value of 98 molecules of R1 mRNA per S-phase cell (7). Our finding that the concentration of R1 protein is  $\sim 48 \mu\text{g/ml}$  in both logarithmically growing and S-phase Balb/3T3 cells is in good agreement with an earlier determination using a method based on radioactively labeled proteins (41). The long half-life of the R1 protein ( $>24$  h) makes the levels almost constant during the cell cycle in proliferating cells (13, 15). However, induced differentiation of cycling human promyelocytic leukemia HL-60 cells led to a slow decline in R1 protein to levels less than 10%

after 6 days (15). The concentration of R1 in Balb/3T3 cells starved of serum for 72 h was  $5.3 \mu\text{g/ml}$ .

The R1 and the p53R2 proteins can form an active RNR complex *in vitro* (5), and our results indicate that both proteins are expressed in  $G_0/G_1$ -phase cells. However, do resting cells form an active p53R2/R1 complex also *in vivo*? Hydroxyurea is a radical scavenger that destroys the tyrosyl free radical of both R2 and p53R2, thereby inhibiting mammalian RNR complexes (5, 6). If an R1-p53R2 RNR complex supplies resting cells with dNTPs for DNA repair, exposing such cells to hydroxyurea would result in reduced dNTP pools. Because S-phase cells have 15–20 times higher dNTP pools than resting cells, only a small number of S-phase cells in a cell culture would have a major impact on the average dNTP pools measured. To measure possible fluctuations in dNTP pools caused by inhibition of p53R2 by hydroxyurea, it is, therefore, vital to use absolutely pure resting  $G_0/G_1$ -phase cell cultures. Even a few percent of S-phase cells would make it impossible to distinguish the effect of hydroxyurea on the p53R2 protein from the effect on the R2 protein. Although we synchronized mouse and human fibroblasts by serum starvation for 72 h, there were still around 3% of S-phase cells in the cultures, as seen by BrdUrd staining. However, by elutriation of serum-starved cells, we were able to generate cell cultures in which less than one cell of 1800 showed BrdUrd incorporation. Such cultures had at least a 50% reduction in their purine dNTP pools after hydroxyurea treatment. The results with the pyrimidine dNTPs are always more difficult to interpret due to a more efficient salvage of pyrimidine deoxyribonucleosides than purine deoxyribonucleosides (1). Because mammalian cells in  $G_0/G_1$  do not express the R2 protein but low levels of p53R2 and most likely also R1, the most plausible explanation as to why we detect reduced dNTP pools after hydroxyurea treatment would be that hydroxyurea inhibits a p53R2-containing RNR complex. Mammalian cells in  $G_0/G_1$  with induced expression of the p53R2 protein also had reduced purine dNTP pools after exposure to hydroxyurea, similar to undamaged mammalian  $G_0/G_1$  cells. These results indicate that there is indeed an RNR-dependent reduction in ribonucleotides in resting cells both before and after DNA damage.

An alternative to the ribonucleotide reduction pathway for resting mammalian cells to obtain dNTPs for DNA repair and mitochondrial DNA replication is through salvage of deoxyribonucleosides. These deoxyribonucleosides can subsequently be phosphorylated to dNMPs by thymidine kinase 2, deoxyguanosine kinase, and deoxycytidine kinase inside the cell (14). Salvage of deoxyribonucleosides into mitochondria is important for mitochondrial DNA replication (42), and it may also be of significance for DNA repair. It is unlikely, however, that salvage is the only way to obtain dNTPs for DNA repair in non-dividing cells. It has been shown that cultivation of non-dividing lymphocytes in a medium supplemented with deoxyadenosine has toxic effects on the cells. Accumulation of deoxyadenosine by salvage progressively retarded the DNA repair pathway, presumably by allosteric inhibition (by dATP) of an RNR enzyme that is not S-phase-specific (43). Furthermore, it was reported in same paper that the RNR-specific inhibitor hydroxyurea negatively affected DNA repair in non-dividing lymphocytes. We observed a delayed progression of the S-phase in DNA-damaged,  $G_0/G_1$ -synchronized cells treated with hydroxyurea when they were compared with DNA-damaged cells without hydroxyurea, which is in line with the published results.

If an R1-p53R2 protein complex supplies dNTPs in resting cells, an increase in the amount of dNTPs would be expected after the induction of the p53R2 protein. Surprisingly, the slow 2–4-fold induction of the R1 (5) and p53R2 proteins after DNA damage did not result in increased dNTP pools. This may be explained by the fact that  $\sim 3\%$  of our undam-

aged 72-h serum-starved  $G_0/G_1$  cell cultures were in the S-phase and, thus, expressed R2. After exposure to adriamycin, the number of S-phase cells, and as a direct consequence, the amount of R2 protein decreased in a time-dependent manner. This decrease in R2 protein levels in the cell culture seemed to balance the induced p53R2 protein-dependent increase in ribonucleotide reduction. This hypothesis is supported by the finding that undamaged, elutriated  $G_0/G_1$  cells, with only 0.05% S-phase cells, had ~40% lower dNTP levels than  $G_0/G_1$  cells with induced expression of p53R2. A contributory explanation to why we cannot observe a more pronounced increase in the dNTP pools after p53R2 induction may be that the induction of p53R2 expression increases the flow through the dNTP pool. If the dNTPs are used at the same rate as they are synthesized, our static method would not detect such an increased flow.

An intriguing question is how DNA synthesis can function in a proper way both in repair and replication, with a 15-fold difference in substrate concentration. Even though the actual number of dNTPs required for DNA repair is small, the concentration of dNTPs during DNA replication seems to be critical since altered dNTP pools have been repeatedly shown to be mutagenic (1). However, the dNTPs might be compartmentalized close to the site of DNA damage, giving a high local concentration. Earlier data indicated a cytoplasmic localization of the R1 and R2 proteins in undamaged mouse tissues as well as in tissue culture cells (44, 45). In contrast, p53R2 has been reported to be relocalized from the cytoplasm to the nucleus after DNA damage (3). This situation would be quite the opposite to the situation in budding yeast, where DNA damage elevates the dNTP pools to levels higher than in the S-phase (24), and even higher dNTP levels increase the survival after DNA damage but at the price of an increase in mutation frequency.

In addition to DNA repair, non-dividing cells need dNTPs for replication of mitochondrial DNA. Whether p53R2 has any function in providing dNTPs for this process remains an important unanswered question. Based on our results, we propose a model in which an R1-p53R2 complex, in combination with salvage, supplies cells outside of S-phase with dNTPs for everyday repair and for mitochondrial DNA replication.

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