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p53R2-dependent Ribonucleotide Reduction Provides Deoxyribonucleotides in Quiescent Human Fibroblasts in the Absence of Induced DNA Damage*

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Human fibroblasts in culture obtain deoxynucleotides by *de novo* ribonucleotide reduction or by salvage of deoxynucleosides. In cycling cells the *de novo* pathway dominates, but in quiescent cells the salvage pathway becomes important. Two forms of active mammalian ribonucleotide reductases are known. Each form contains the catalytic R1 protein, but the two differ with respect to the second protein (R2 or p53R2). R2 is cell cycle-regulated, degraded during mitosis, and absent from quiescent cells. The recently discovered p53-inducible p53R2 was proposed to be linked to DNA repair processes. The protein is not cell cycle-regulated and can provide deoxynucleotides to quiescent mouse fibroblasts. Here we investigate the *in situ* activities of the R1-p53R2 complex and two other enzymes of the *de novo* pathway, dCMP deaminase and thymidylate synthase, in confluent quiescent serum-starved human fibroblasts in experiments with [5-^{3}H]cytidine, [6-^{3}H]deoxycytidine, and [C^3H_3]thymidine. These cells had increased their content of p53R2 2-fold and lacked R2. From isotope incorporation, we conclude that they have a complete *de novo* pathway for deoxynucleotide synthesis, including thymidylate synthesis. During quiescence, incorporation of deoxynucleotides into DNA was very low. Deoxynucleotides were instead degraded to deoxynucleosides and exported into the medium as deoxycytidine, deoxyuridine, and thymidine. The rate of export was surprisingly high, 25% of that in cycling cells. Total ribonucleotide reduction in quiescent cells amounted to only 2–3% of cycling cells. We suggest that in quiescent cells an important function of p53R2 is to provide deoxynucleotides for mitochondrial DNA replication.

The synthesis of dNTPs occurs both in the cytosol and in mitochondria. In the cytosol a *de novo* pathway starting from small molecules involves the enzymes ribonucleotide reductase (1), dCMP deaminase (2), and thymidylate synthase (2) as the major players. An auxiliary salvage pathway in the cytosol starting from preformed deoxynucleosides involves thymidine kinase 1 (3) and deoxycytidine kinase (4). Deoxynucleotides formed in the cytosol are imported into mitochondria by specific transporters (5, 6). Salvage of deoxynucleosides occurs also inside mitochondria by two intra-mt[2] enzymes, thymidine kinase 2 (7) and deoxyguanosine kinase (8). These two kinases can between themselves phosphorylate all four canonical deoxyribosides, and because of the presence of appropriate nucleotide kinases the resulting monophosphates are transformed to dNTPs to satisfy the requirement for building blocks for mtDNA replication.

The classical ribonucleotide reductase of the *de novo* pathway consists of two proteins, R1 and R2 (1). Of these, R2 is absent from quiescent cells (9). Also the cytosolic thymidine kinase 1 is S-phase-specific (10). It would therefore appear that the cytosolic pathways cannot provide all required dNTPs for mtDNA replication and repair in quiescent cells. These would therefore be expected to depend on the two intra-mt kinases. In agreement, in humans a genetic deficiency of either kinase (11, 12) depletes mtDNA in organs with terminally differentiated cells causing severe disease. In cultured cells kinetic experiments with labeled thymidine supported the existence of the two pathways for the synthesis of mt dNTPs and the predominance of the salvage pathway in quiescent cells (13).

However, it was recently found (14) that quiescent mouse 3T3 fibroblasts obtained by serum starvation contained p53R2 (15, 16), a protein that can substitute for R2 to form an active enzyme together with R1, the second subunit of ribonucleotide reductase (17). In these cells hydroxyurea, an inhibitor of both R2 and p53R2 (1, 17), decreased the size of dNTP pools suggesting that p53R2 indeed was reducing ribonucleotides. These results strongly suggest that quiescent cells have some capacity to synthesize dNTPs by reduction of ribonucleotides (14).

Why then do genetic deficiencies of mt deoxynucleoside kinases lead to mtDNA depletion in specific organs? Under these circumstances ribonucleotide reduction clearly does not suffice to supply sufficient dNTPs for mtDNA synthesis. The recent experiments with 3T3 cells demonstrate the potential of quiescent cells for ribonucleotide reduction but do not indicate the relative contribution of the *de novo* synthesis and the salvage of deoxynucleosides. We have previously used isotope

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[2] The abbreviations used are: mt, mitochondrial; FCS, fetal calf serum; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.

techniques to obtain an estimate of the *in situ* activity of enzymes participating in the synthesis of dNTPs in cycling cells and their relation to each other (18–20). Here we apply these techniques to quiescent cells to study some relevant enzymes of the *de novo* pathway. In particular, we ask the question to what extent p53R2 in quiescent cells provides dNTPs, as exemplified by the synthesis of dCTP and dTTP. To this purpose we incubated quiescent and cycling human fibroblasts in culture with labeled thymidine, deoxycytidine, or cytidine and determined the transfer of isotope from each nucleoside to dNTPs and DNA. From the results of these experiments we conclude that ribonucleotide reduction as measured by the reduction of CDP to dCDP is indeed catalyzed by the R1:p53R2 enzyme, proceeding at a rate of 2–3% of the rate in cycling cells. Similarly, dTMP is synthesized at a corresponding rate from dCMP by the combined activities of dCMP deaminase and thymidylate synthase. Our results thus demonstrate the existence of a complete *de novo* pathway of pyrimidine deoxyribonucleotide synthesis in cultured quiescent fibroblasts. Cycling cells use the synthesized deoxyribonucleotides largely for DNA replication, and quiescent cells catabolize essentially all deoxyribonucleotides and excrete them as deoxyribonucleosides into the medium.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Growth—Human skin fibroblasts and an established line of lung fibroblasts (CCD 34Lu) were grown in 10% fetal calf serum (FCS). We routinely seeded 0.5 million cells on a 10-cm dish. After 3–4 days they reached a density of 2.5–3 million cells/dish and were used as cycling cells. After 7–10 days, the cells were contact-inhibited and were thereafter maintained in medium with 0.1% FCS to obtain quiescent cells. As described previously (21) <1% of skin fibroblasts were in S-phase after 1–2 weeks in low serum as judged by flow cytometry, whereas lung fibroblasts required several weeks. Medium changes with 0.1% FCS were made twice a week.

Isotope Experiments—[C^3H_3]thymidine (20,000 cpm/pmol), [6-^{3}H]deoxycytidine (10,000 cpm/pmol), and [5-^{3}H]cytidine (25,000 cpm/pmol) were from Moravek (Brea, CA). We described previously (18–21) the general procedures for labeling and chase experiments. Briefly, at the start of the experiment we fed cells with the isotopic nucleoside in fresh prewarmed medium containing dialyzed FCS (10% for cycling cells and 0.1% for resting cells) and incubated them in a 37 °C incubator for the indicated time. In the chase experiment described in Figs. 4 and 5, we labeled cells first with 1 μM [5-^{3}H]cytidine for 4 h, carefully removed the medium in a 37 °C room, added fresh prewarmed medium with dialyzed FCS at the original concentration, and continued incubation at 37 °C for the indicated times. The chase medium for half of the cultures contained 1 μM nonlabeled cytidine. At each time point we transferred the cultures on ice to a cold room, washed carefully three times with ice-cold phosphate-buffered saline (PBS), extracted the nucleotide pools with 60% methanol, boiled the extract for 3 min, evaporated the methanolic solution to dryness by flash evaporation, dissolved the residue in a small volume of water, and used portions of the solutions for pool analyses by HPLC (6, 22) or by the DNA polymerase assay (23, 24). To determine the excretion of nucleosides, we precipitated 2 ml of medium on ice with 0.25 ml of 4 M $HClO_4$, neutralized with 4 M KOH, and after centrifugation, we determined the amount of radioactive deoxycytidine and deoxyuridine in the supernatant solution by HPLC on a LUNA C18 (Phenomenex, Torrance, CA) column (250 × 4.6 mm) (24).

Analytical Procedures—The size of individual dNTP pools was determined by HPLC on a 100 × 4.6 mm, 3 μm, 1500-A WAX column (PolyLC; Lab Service Analytica, Anzola Emilia, Italy) (6, 22) or by the DNA polymerase assay (23) as modified for the determination of the specific radioactivity of dNTPs (24). The sensitivity of the polymerase assay was at least 10 times higher than HPLC. We used HPLC largely to confirm results from polymerase assays and used the WAX column without pretreatment of the extract on a boronate column (21) to remove ribonucleoside triphosphates. By comparison of peak heights or peak areas of known standards with the corresponding values from extracts, we could analyze 2–3 pmol of dNTPs. In some extracts we could not determine dTTP because of overlap with an unknown peak on the chromatogram. We determined the specific radioactivity of dCTP and dTTP by dividing the total radioactivity of the peak in the HPLC by the determined picomoles or by the modified DNA polymerase assay (24). To measure methylation of dUMP by thymidylate synthase (19, 25) in the chase experiments of Fig. 5, we treated portions of the medium with charcoal and determined the remaining volatile radioactivity. We measured the radioactivity of precipitated DNA at two separate time points of the experiment. To calculate incorporation of a given deoxynucleotide into DNA, expressed as picomoles, we divided the difference between the two values by the average specific radioactivity of the dNTP during the period. In calculations involving incorporation of both dTTP and dCTP we assumed a T/C ratio of 1.3 in DNA.

In an alternative method (26) to analyze the incorporation of cytidine into CTP and dCTP previously used extensively in our laboratories to determine ribonucleotide reductase activity in cell extracts, we added 0.5 ml of 1 M $HClO_4$ to 0.05 ml of the solution containing the radioactive nucleotides after flash evaporation and immersed the tube in a boiling water bath for 10 min to hydrolyze all pyrophosphate bonds of CTP and dCTP. After cooling on ice, we precipitated $KClO_4$ with 4 M KOH and centrifuged and chromatographed the supernatant solution after addition of carrier CMP and dCMP on a 6-ml column of Dowex 50W-X8-H^+ (200–400 mesh) with 0.2 M acetic acid to separate CMP and dCMP. The radioactivity present in each separated peak came from the combined mono-, di-, and triphosphates of cytidine and deoxycytidine and not only from CTP and dCTP. All values in tables and figures are normalized to 1 million cells.

Immunoblotting—We washed the pellet from 2 million cells twice with ice-cold PBS and lysed the cells in 0.2 ml of PBS containing 1.5% SDS and mammalian cell protease inhibitors (Sigma) by aspiration in a syringe through a 30 gauge × 5/16-inch needle. After 30 min we centrifuged the suspension at 19 000 × *g* for 20 min. All manipulations were done at close to + 4 °C. We determined the protein concentration by the BCA protein assay (Pierce) and loaded equal amounts of protein for electrophoresis on a 12% polyacrylamide gel. We ran separate gels for each

subunit of ribonucleotide reductase. After electrophoretic separation, we transferred proteins to a Hybond-C extra membrane (Amersham Biosciences) at 33 mV per gel for 1 h at room temperature in a semi-dry blotter. After transfer, we blocked the membrane for 60 min with 2% ECL blocking agent (Amersham Biosciences) in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20. We incubated the filters overnight at 4 °C with antibodies against the different proteins (anti-p53R2 rabbit polyclonal antibody (Abcam, Cambridge, UK) diluted 1/1000; anti-R2 goat polyclonal antibody diluted 1/2000 (Santa Cruz Biotechnology); or anti-R1 monoclonal antibody AD203 (27), diluted 1/5000), washed the membranes three times, and finally incubated them at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h. After further washing we detected and quantified the stained bands in an ECL-advanced system (Amersham Biosciences) with Kodak one-dimensional image analysis software. For absolute quantification of p53R2, we loaded known amounts of pure recombinant protein on the same gel as the p53R2 sample to provide an appropriate standard curve. Recombinant p53R2 (17), R2 (28), and R1 (29) were preparations available in our laboratories.

RESULTS

Confluent Quiescent Human Lung Fibroblasts Lack Protein R2 but Contain an Increased Amount of p53R2—Ribonucleotide reductase activity requires the presence of R1 together with either R2 or p53R2. As a background to our kinetic isotope experiments concerning dNTP synthesis in human fibroblasts, we first analyzed by Western blotting the relative amounts of the three proteins in these cells. For the analyses we prepared cell extracts from cycling lung fibroblasts, from cells at confluence at a time when we reduced the concentration of FCS from 10 to 0.1%, and from confluent cells that had been maintained at the low serum concentration up to 64 days. Portions of each extract containing identical amounts of protein were electrophoresed in parallel on the same gel and analyzed by Western blotting. Actin served as loading control. Fig. 1*A* shows the results from one such analysis. As found previously by others, R1 and R2 predominate in cycling cells. After the shift to low serum, R1 decreased but was still faintly visible on the 40th day after the shift. R2 decreased still more, and after 40 days it was no longer detectable. p53R2 instead is present during the whole time period and actually appeared to increase from cycling cells to quiescent cells. In a second experiment (Fig. 1*B*), we compared in more detail the effects of prolonged low serum treatment on the level of R2 and p53R2. Cycling cells again showed a large amount of R2 relative to p53R2. In confluent cells R2 decreased considerably, and after 20 days in low serum had disappeared completely. In contrast, the p53R2 signal did not decrease. When we analyzed p53R2 within the proportionality range of our antibody in repeated blots, we found actually a small increase of the protein from cycling to quiescent cells (Fig. 1*C*). A quantification of the blots of p53R2 in confluent cells and quantification after 64 days of quiescence from a standard curve of pure p53R2 (Fig. 1*D*) gave values of 0.11 ng of p53R2/μg of total protein at contact

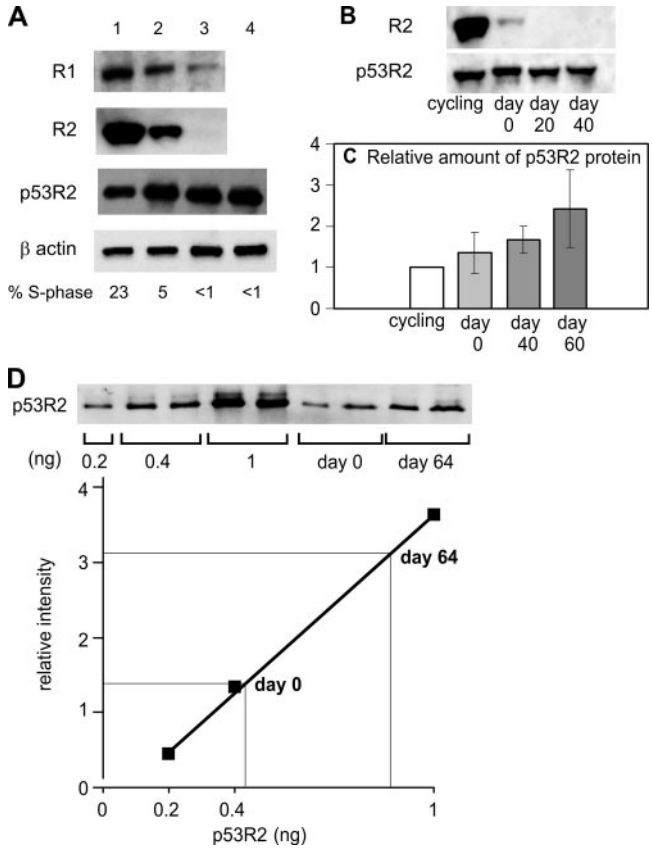

FIGURE 1. **Proteins R1, R2, and p53R2 in cycling and quiescent cells determined by Western blotting.** *A,* relative amounts of each protein in cycling cells (*lane 1*), confluent cells (*lane 2*), and cells maintained 40 (*lane 3*) or 60 (*lane 4*) days with 0.1% FCS after confluence. We blotted 10 μg of extracted cell protein for R1 or R2 and 4 μg for p53R2. R1 and R2 were not analyzed after 60 days. *B,* R2 and p53R2 in 10 μg of protein from cycling cells and confluent cells and after 20 or 40 days in 0.1% serum. *C,* average relative values for p53R2 in 4 μg of extracted cell protein determined in six blots of cycling and quiescent cells from two series of separately grown cultures. For each blot the value for the cycling cells is set as 1. *D,* absolute amounts of p53R2 in confluent cells and in cells 64 days after confluence. Extracts from each time point containing 2 and 4 μg of total protein were electrophoresed in parallel with a standard curve of pure p53R2. From the curve we calculate that the samples contained 0.44 ng of p53R2/4 μg of total protein at confluence and 0.88 ng of p53R2/4 μg of total protein after 64 days. For technical reasons the signal intensities of each protein can be evaluated only within each part of the figure 1 but not between the four parts.

inhibition and 0.22 ng of p53R2/μg of total protein after prolonged quiescence.

General Strategy for Isotope Experiments—We supplied tritiated nucleosides [C^3H_3]thymidine, [6-^{3}H]deoxycytidine, and [5-^{3}H]cytidine to human fibroblasts in culture to label DNA via dTTP and dCTP formed from the nucleosides by the pathways indicated in Fig. 2. Isotope from the three nucleosides reaches the two dNTPs by different metabolic steps. Thymidine presents the simplest case. Its tritium is transferred to dTTP directly in three phosphorylation steps before being incorporated into DNA. dTTP is simultaneously synthesized *de novo* from nonlabeled small molecules, and the tritium in dTTP coming from thymidine is therefore diluted by nonradioactive material. The decrease in the specific radioactivity from thymidine to dTTP gives an estimate of the relative contribution of the *de novo* pathway and the salvage of thymidine. We can cal-

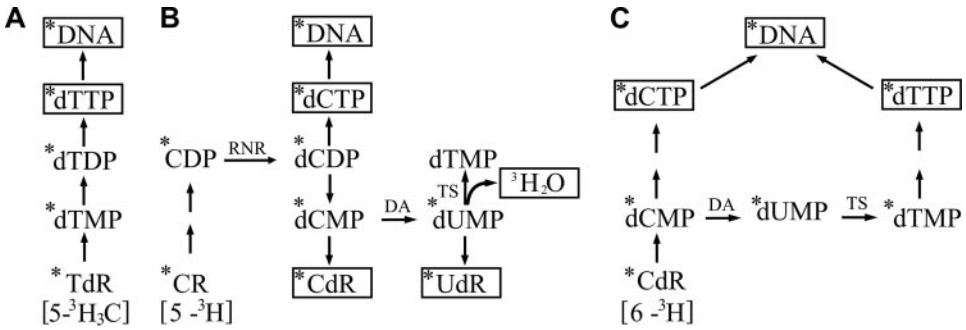

FIGURE 2. **Metabolic pathways for the incorporation of labeled nucleosides into dNTPs and DNA.** *A,* isotope is transferred from [C^3H_3]thymidine to dTTP and DNA. *B,* [5-^{3}H]cytidine is phosphorylated to CDP, reduced to dCDP by ribonucleotide reductase (*RNR*), and after phosphorylation to dCTP incorporated into DNA. dCDP is also dephosphorylated to dCMP, deaminated by dCMP deaminase (*Da*) to dUMP, and further methylated to dTMP by thymidylate synthase (*Ts*). During this reaction the isotope from the 5-position of the pyrimidine ring appears in water and is transferred to the medium. dCMP and dUMP are also dephosphorylated to deoxycytidine and deoxyuridine, respectively, and excreted into the medium. *C,* [6-^{3}H]deoxycytidine after phosphorylation to dCMP is incorporated as dCTP into DNA. dCMP is also deaminated to dUMP, methylated to dTMP, and incorporated as dTTP into DNA. All isotopically labeled compounds are indicated by an *asterisk*. We determined the amount of isotope of all compounds in *boxes*.

We conducted our experiments with cultured cells of either secondary skin fibroblasts or a line of human lung fibroblasts. In both cases we compared data from cycling (~20–30% in S-phase) and quiescent cells. We obtained the latter by first growing cycling cells to confluence in 10% FCS and then changing the medium to 0.1% dialyzed FCS. They were then maintained in 0.1% FCS for further 10 days (skin fibroblasts) or 20–40 days (lung fibroblasts) before isotope experiments. In all experiments we supplied fresh medium with dialyzed serum to the cells before addition of isotope to ensure the absence of nucleosides as substrates for the salvage pathways. As far as possible we used simultaneously maintained cultures in parallel experiments with the three labeled nucleosides.

TABLE 1

Utilization of [C^3H_3]thymidine for the synthesis of dTTP and DNA by cycling and resting skin and lung fibroblasts

Cells were incubated with 0.3 (cycling cells) or 1 μM (quiescent cells) labeled thymidine (20,000 cpm/pmol) for the indicated time. Quiescent cells had been maintained for 11 (skin fibroblasts) or 39 days (lung fibroblasts) with 0.1% FCS. The amount and radioactivity of the dTTP pool were determined by the polymerase assay. DNA synthesis was determined from the incorporation of isotope and the average specific radioactivity of the dTTP pool. The data are normalized for 1 million cells.

Fibroblasts	Incubation	dTTP pool	Specific radioactivity	DNA synthesis
	min	*pmol*	*cpm/pmol*	*pmol dTMP incorporated/min*
Cycling				
Skin	20	110	2,500	
	60	120	4,600	0.7
Lung	60	60	5,400	
	120	61	5,500	0.9
Resting				
Skin	20	3.0	12,000	
	60	2.9	12,000	0.01
Lung	60	1.0	11,000	
	120	1.3	13,000	0.003

Experiments with Labeled Thymidine—In both skin and lung fibroblasts the dTTP pool was labeled rapidly from thymidine (Table 1). In cycling cells the specific radioactivity of dTTP increased between 20 and 60 min with a final value of one-fourth that of the precursor thymidine, demonstrating that three-fourths of the dTTP was synthesized by the *de novo* pathway from nonlabeled small molecules. Quiescent cells contained much smaller dTTP pools, and the specific radioactivity of dTTP was closer to that of the precursor thymidine already after 20 min, with no further increase with time, suggesting a more limited competition from *de novo* synthesis. When we calculated the rate of DNA synthesis (pmol of dTMP incorporated per min) from the incorporation of isotope into DNA and the specific radioactivity of dTTP, we found that cycling cells had incorporated 0.7 (skin fibroblasts) or 0.9 pmol/min (lung fibroblasts). The corresponding value for the quiescent cells was ~0.01 pmol/min, indicating a very low level of DNA synthesis.

culate the rate of DNA synthesis from the incorporation of isotope into DNA and the specific radioactivity of dTTP.

Similar principles apply to the experiments with the other two nucleosides. [5-^{3}H]Cytidine provides the tool for an assay of the *in situ* activity of ribonucleotide reduction. The substrate for the reductase is CDP, formed by phosphorylation of labeled cytidine. CDP is also synthesized from small, nonradioactive molecules, giving a dilution of radioactivity of the substrate for the reductase. To assess ribonucleotide reduction we must know the specific radioactivity of CDP (or CTP). Isotope from [5-^{3}H]cytidine is not incorporated into dTTP as the hydrogen at position 5 of cytosine is lost from the base and appears in water during the transformation of dUMP (formed by deamination of dCMP) to dTMP (see Fig. 2). The amount of ^{3}H in water thus measures the combined activities of dCMP deaminase and thymidylate synthase. We also employed [6-^{3}H]deoxycytidine to measure these two steps. ^{3}H is not lost from the 6-position during the methylation of dUMP, and its appearance in dTTP monitors directly the synthesis of dTMP.

Experiments with [5-^{3}H]Cytidine—We incubated cycling or resting lung fibroblasts with [5-^{3}H]cytidine and determined after 60 and 120 min the specific radioactivities and sizes of the CTP and dCTP pools and isotope incorporation into DNA (Table 2). In comparison to the efficient incorporation of labeled thymidine into dTTP and DNA (Table 1), we found only a limited transfer of isotope from labeled cytidine in both cycling and quiescent cells. The specific radioactivities of both CTP and dCTP was less than 5% of the specific radioactivity of the precursor cytidine indicating that more than 95% of each nucleotide had been formed from small molecules. This was, however, not because of a deficient reduction of CDP, as the specific radioactivities of CTP and dCTP were similar and equally low (Table 2) suggesting that the dilution of isotope had occurred before the reduction of CDP, probably caused by poor phosphorylation of the radioactive cytidine relative to the rapid *de novo* synthesis of CMP from nonlabeled molecules (see Fig. 2). The specific radioactivities of dCTP and CTP did not differ

TABLE 2

Incorporation of [5-^{3}H]cytidine into CTP, dCTP, and DNA by cycling and resting lung fibroblasts

After incubation of the cells for the indicated time with 1 μM labeled cytidine (25,000 cpm/pmol), we determined the amounts and specific radioactivities of CTP and dCTP by HPLC and the incorporation of isotope into DNA (normalized to 1 million cells).

Fibroblasts	Time of incubation	CTP		dCTP		DNA
		Picomoles	Specific radioactivity	Picomoles	Specific radioactivity	
	min		*cpm/pmol*		*cpm/pmol*	*pmol dCMP incorporated/min*
Cycling	60	1,040	700	29	600	
	120	1,270	760	23	660	1.2
Resting	60	255	390	1.7	220	
	120	255	500	2.4	450	0.006

TABLE 3

Time-dependent incorporation of [6-^{3}H]deoxycytidine into dNTPs and DNA by cycling and quiescent lung fibroblasts

Cells were incubated with 1 μM labeled deoxycytidine (10,000 cpm/pmol) for the indicated time. The amount and the specific radioactivity of dNTPs were analyzed by HPLC, and the synthesis of DNA was calculated from isotope incorporation and the specific radioactivity of the dNTPs. All values are normalized to 1 million cells.

Fibroblasts	Incubation time	dCTP		dTTP		DNA, nucleotide incorporated
		Picomoles	Specific radioactivity	Picomoles	Specific radioactivity	
	min		*cpm/pmol*		*cpm/pmol*	*pmol/min*
Cycling	60	27	910	34	1,300	0.8 dCMP
	120	33	950	45	1,100	1.0 dTMP
Quiescent	60	4.1	2,260	2.1	1,100	0.03 dCMP
	120	4.0	3,010	2.1	1,600	0.04 dTMP

between cycling and quiescent cells, but cycling cells contained a more than 10 times larger dCTP pool and a 4 times larger CTP pool. This experiment shows that quiescent cells actively reduce CDP to dCDP but does not allow a comparison of the rate of reduction in quiescent and cycling cells. This question will be addressed below.

When we determined the rate of DNA synthesis from the incorporation of cytidine by the procedure described above for the incorporation of radioactive thymidine into DNA, we found an incorporation of 1.2 pmol/min of dCMP in cycling and 0.006 pmol/min in quiescent cells (Table 2), in reasonable agreement with the values found for the incorporation of dTMP in the parallel experiment of Table 1.

In an additional, slightly different experiment, we confirmed by another technique the reduction of CDP in quiescent lung fibroblasts and extended the results to skin fibroblasts. As described under "Experimental Procedures," we determined isotope incorporation not only into CTP and dCTP but into the total cytidine and deoxycytidine phosphate pools. The results fully confirmed an active CDP reduction not only in lung fibroblasts but also in skin fibroblasts with almost identical specific radioactivities of the cytidine and deoxycytidine phosphates and with a large dilution of the radioactivity by the *de novo* pathway (data not shown).

Experiments with [6-^{3}H]Deoxycytidine—In addition to ribonucleotide reduction, the *de novo* pathway for dNTPs requires the activity of dCMP deaminase and dTMP synthase for the synthesis of dTTP (see Fig. 2). Tritium in dCMP formed by phosphorylation of [6-^{3}H]deoxycytidine is retained during methylation of dUMP and appears in dTMP. The specific radioactivity of dTTP relative to that of dCTP then monitors the combined activities of the deaminase and the synthase. The experiment with labeled deoxycytidine in Table 3 was carried out in parallel to the thymidine and cytidine experiments of Tables 1 and 2. In both quiescent and cycling cells, the dCTP and dTTP pools were labeled, and the specific radioactivity of dTTP was close to that of dCTP, demonstrating an efficient methylation of dUMP. In quiescent cells the specific radioactivity of dCTP was almost 30% that of the precursor deoxycytidine, whereas in cycling cells the dilution of isotope was larger, indicating a more efficient competition by ribonucleotide reduction of nonlabeled CDP. From the amount of radioactivity present in DNA (Table 3), we can calculate values for the incorporation of dCMP and dTMP assuming a T/C ratio of 1.3 in DNA. The higher values found here in quiescent cells compared with those found in the parallel experiments with thymidine (Table 1) and cytidine (Table 2) are unexplained. Importantly, the present experiment shows that quiescent cells in addition to ribonucleotide reduction carry out an efficient *de novo* synthesis of dTMP. We will present further evidence on this point below.

Effects of Hydroxyurea in Quiescent Cells—This drug is a specific inhibitor of ribonucleotide reduction. Extensive experiments in several laboratories have shown that addition of the drug to growing cells primarily decreases the pool sizes of purine deoxynucleotides with a smaller effect on the dCTP pool. The dTTP pool remains largely unchanged and in some cases even increased. We employed hydroxyurea in several experiments to compare in cycling and quiescent fibroblasts the effect of the drug on the size of dNTP pools and the metabolism of labeled nucleosides. In a first experiment we added 2 mM hydroxyurea to cultures of cycling or quiescent lung fibroblasts without addition of nucleosides, and after 2 h we determined the size of all four dNTP pools (Fig. 3). In both types of cells hydroxyurea had the largest effect on the dATP pool. In cycling cells the pool diminished to 10%; in quiescent cells the pool became too small to be measured. The dGTP pool decreased to ~50% in both types of cells. Also dCTP showed some decrease, mostly in quiescent cells. dTTP remained unchanged in quiescent cells but showed a minor decrease in cycling cells. The main conclusion from this experiment is that the effects of hydroxyurea on dNTP pools in quiescent cells are similar to those in cycling cells, confirming the existence of ribonucleotide reductase also in quiescent cells. The pro-

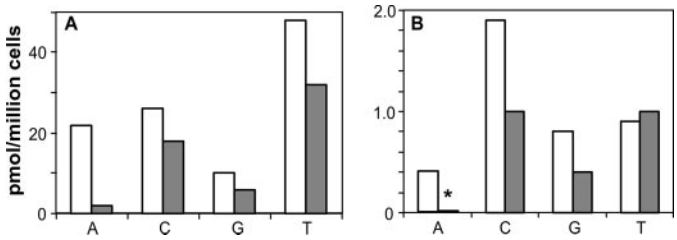

FIGURE 3. **Influence of hydroxyurea on the size of dNTP pools in cycling (*A*) and quiescent (*B*) human lung fibroblasts.** Hydroxyurea (2 mM) was present in the cultures for 3 h before extraction and analyses of dNTP pools. Pool sizes were analyzed by HPLC and the polymerase assay with similar results. The cycling cells (35% in S-phase) were analyzed after 3 days of growth in 10% dialyzed FCS. Quiescent cells had been maintained with 0.1% dialyzed FCS for 29 days after reaching confluence. *Open bars,* controls; *shaded bars,* samples with HU. *Asterisk* indicates that dATP was not found. *A,* dATP; *C,* dCTP; *G,* dGTP; *T,* dTTP.

TABLE 4

Influence of hydroxyurea on the incorporation of labeled nucleosides into dNTPs in cycling and quiescent lung fibroblasts

Cells were incubated for 1 h with 1 μM thymidine (20,000 cpm/pmol), cytidine (25,000 cpm/pmol), or deoxycytidine (10,000 cpm/pmol). Where indicated, 2 mM hydroxyurea (HU) was added 30 min before the isotope and was present during incubation. After 1 h the specific radioactivity of the dTTP pool was determined by the polymerase assay, and CTP and dCTP were determined by HPLC. Quiescent cells had been maintained for 29 days with 0.1% FCS.

Labeled nucleoside	Specific radioactivity					
	Cycling cells			Quiescent cells		
	CTP	dCTP	dTTP	CTP	dCTP	dTTP
	cpm/pmol					
[^{3}H]Thymidine						
Control			5,500			13,000
HU			11,000			14,000
[5-^{3}H]Cytidine						
Control	730	630		350	350	
HU	620	140		370	40	
[6-^{3}H]Deoxycytidine						
Control		930	1,200			
HU		3,750	3,500			

nounced inhibition of DNA synthesis in cycling cells found in the next experiment appears to be primarily caused from lack of dATP.

How does hydroxyurea influence the incorporation of the labeled nucleosides into DNA and dNTPs? In both cycling and quiescent cells the amount of isotope incorporated into DNA decreased to a few percent demonstrating a severe inhibition of DNA synthesis (data not shown). The influence of the drug on the labeling of dNTPs depended on the nature of the nucleoside (Table 4). With [^{3}H]thymidine, hydroxyurea increased the specific radioactivity of dTTP in cycling cells 2-fold but had only a small effect in quiescent cells, agreeing with the presence of a highly active hydroxyurea-sensitive ribonucleotide reductase in cycling cells that provides nonlabeled dNTPs in the absence of the drug. With [5-^{3}H]cytidine, hydroxyurea in both cycling and quiescent cells almost wiped out the radioactivity from dCTP without affecting CTP. In quiescent cells the radioactivity of dCTP dropped to 10% demonstrating a severe but not complete inhibition of ribonucleotide reduction. From [6-^{3}H]deoxycytidine isotope is incorporated into nucleotides without the intervention of ribonucleotide reduction. With this nucleoside, hydroxyurea increased the specific radioactivity of both dCTP and dTTP 3–4-fold by decreasing the dilution of radioactivity from nonlabeled dCTP formed via ribonucleotide reduction. Taken together the hydroxyurea experiments strongly support the existence of ribonucleotide reduction in quiescent cells.

Comparison of the Rate of CDP Reduction in Quiescent and Cycling Cells—During steady state conditions the rate of dCTP synthesis equals the rate of its consumption. A comparison of the rate of dCTP synthesis between quiescent and cycling cells can therefore be made from the total consumption of dCTP during the two growth stages. dCDP, the product of the reduction of CDP, is incorporated as dCMP and dTMP into DNA and, after catabolism, is excreted as deoxycytidine, deoxyuridine, and thymidine into the medium. Both processes occur in cycling cells. In quiescent cells incorporation into DNA is minimal (see Table 2), and the amount of catabolism is unknown. We now present data comparing the catabolism of dCTP during the two growth stages.

We first labeled quiescent or cycling cells with [5-^{3}H]cytidine. After 4 h we replaced the labeled medium with medium either containing 1 μM nonlabeled cytidine or no cytidine at all for a chase experiment and continued the incubation. We then determined at intervals in the cells the specific radioactivity of the dCTP pool (Fig. 4*C*) as well as the incorporation of radioactivity into DNA. The specific radioactivity did not change in quiescent cells, but it decreased in cycling cells by 35% during 1 h. During the chase fresh labeled dCTP is continuously provided by reduction of prelabeled CDP, and the decay of radioactivity of dCTP is therefore small and cannot be used to measure its consumption. From the labeling of DNA (data not shown) we calculated that cycling cells incorporated 2 pmol/min of dCMP. The incorporation by quiescent cells was difficult to determine but amounted to at most 0.006 pmol/min.

At the different time points we also determined in the medium the excretion of deoxyuridine and deoxycytidine from their radioactivity. Cycling cells (Fig. 4*B*) excreted slightly more deoxycytidine than deoxyuridine, whereas the opposite occurred during the quiescent stage (Fig. 4*A*). In the former case the excretion of deoxycytidine during 60 min amounted to 20 pmol and that of deoxyuridine to16 pmol, giving a total excretion of deoxynucleosides of 0.60 pmol/min. A similar calculation for quiescent cells shows the excretion of 0.15 pmol/min. The excretion of the combined two deoxynucleosides thus was four times larger in cycling than in quiescent cells.

In this experiment a portion of the radioactive dCDP formed by reduction of CDP was after deamination transformed to dUMP and further methylated to dTMP. During the methylation step tritium from the labeled 5-position of cytosine is released as radioactive water to the medium. We could therefore measure the total methylation of dUMP (providing both dTTP for incorporation into DNA and thymidine for excretion) by determining the amount of ^3H_2O in the medium. We assumed that the dUMP pool had the same specific radioactivity as the dCTP pool, and from this value and the determined radioactivity in water we calculated the picomoles of dTMP formed from dUMP in cycling and quiescent cells. Time curves from the 1-h chase (Fig. 5) show an almost 100-fold larger release of tritium (and therefore dTMP synthesis) in cycling than in quiescent cells. Quiescent cells incorporated only 0.01 pmol/min dTTP into DNA (Table 1), and the bulk of dTMP is

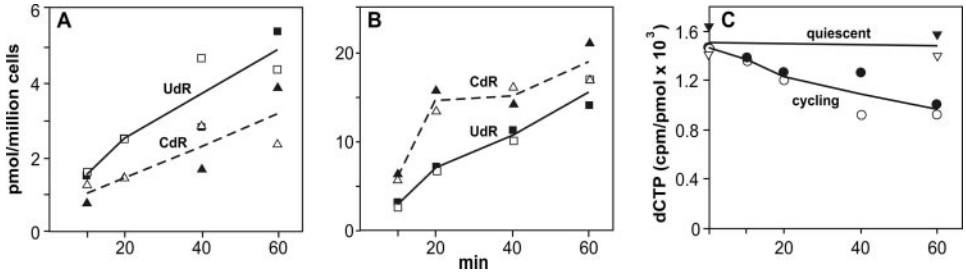

FIGURE 4. **Time curves for the excretion of deoxynucleosides (*A* and *B*) and for the specific radioactivity of the dCTP pool (*C*) during chase of the dCTP pool prelabeled from [5-^{3}H]cytidine.** We labeled cultured cells 22 days after confluence (*A*) or cycling cells at the 3rd day of growth (*B*) with 1 μM [5-^{3}H]cytidine (25,000 cpm/pmol) for 4 h. We then removed the labeled medium and replaced it by medium with dialyzed FCS (0.1% in *A*, 10% in *B*), either with 1 μM nonradioactive cytidine (*closed symbols*) or without cytidine (*open symbols*). At the indicated times we removed the medium and determined the excretion of deoxyuridine (□, ■) and deoxycytidine (△, ▲). We also measured the specific radioactivity of the dCTP pool (*C*) in quiescent (▼, ▽) and cycling (● ○) cells. Duplicates were analyzed for each time point, one from the chase without cytidine and the other from the chase with cold cytidine. We found no systematic differences between the two conditions and have drawn the curves using the average values from the two incubations.

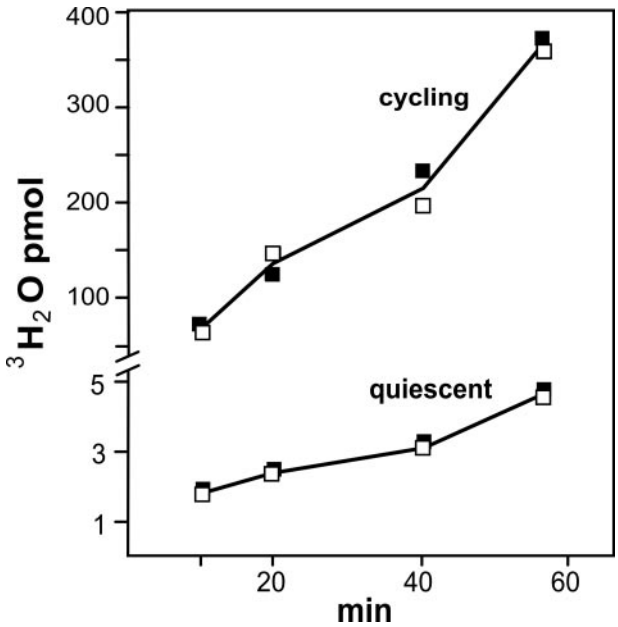

FIGURE 5. **Time curve for the methylation of dUMP during the chase of dCTP labeled from [5-^{3}H]cytidine.** In the chase experiment of Fig. 4 tritium was released as ^3H_2O to the medium from the 5-position of the pyrimidine ring during the methylation of dUMP to dTMP. We determined the time curve of this release by cycling and quiescent cells and expressed it in picomoles of ^3H_2O by dividing the accumulated radioactivity by the average specific radioactivity of dCTP. The values represent the total amount of dTMP formed up to each time point. *Open symbols* are from the chase without cytidine and *closed symbols* from the chase with nonradioactive cytidine. Note the 100-fold difference between quiescent and cycling cells indicated by the different scales for the two experiments.

therefore consumed by excretion of thymidine into the medium. The data in Fig. 5 suggest an excretion of 4.1 (4.7–0.6) pmol of thymidine during the 1-h chase, a value that closely matches the excretion of deoxycytidine (3.2 pmol) and deoxyuridine (4.9 pmol) shown in Fig. 4. In cycling cells as much as 360 pmol of ^3H_2O were released during 1 h. In this case dTMP synthesis represents the sum of the incorporation of dTTP into DNA and the excretion of thymidine into the medium.

We can now calculate the rate of ribonucleotide reduction from the consumption of the dCTP pool in cycling and quiescent cells assuming that incorporation of dCMP into DNA and catabolism by excretion of deoxycytidine and deoxyuridine together with methylation of dUMP account for the total drain on the pool. In cycling cells this amounted to 2 (dCMP into DNA) + 0.6 (deoxycytidine + deoxyuridine excretion) + 6 (dUMP methylation) = 8.6 pmol/min; in quiescent cells the values were 0.02 (dCMP + dTMP into DNA) + 0.135 (deoxycytidine + deoxyuridine excretion) + 0.07 (thymidine excretion) = 0.225 pmol/min, suggesting an almost 40-fold faster rate of CDP reduction in the cycling cell population. In quiescent cells at least 90% of the newly synthesized dCDP is excreted into the medium, whereas cycling cells incorporate deoxynucleotides into DNA.

DISCUSSION

The discovery of p53R2 as a component of ribonucleotide reductase added a new chapter to our understanding of how dNTPs for DNA replication and repair are supplied in mammalian cells (15, 16). p53R2 can functionally substitute for R2 (17) the canonical radical-providing subunit of ribonucleotide reductase (1). As implied from its name, p53R2 was from the beginning linked to the tumor suppressor p53, a protein with many reported functions in the regulation of cell damage control, apoptosis, and cancer (30). One of its many functions is to increase the transcription of p53R2 mRNA after DNA damage and this is how the p53R2 gene was originally identified (15). For this reason p53R2 has been assumed to facilitate the repair of damaged DNA by providing an increased supply of dNTPs. Mammalian cells show a low constitutive expression of p53R2 protein (14–16), and the original cDNA cloning was from a mouse skeletal muscle cDNA library supporting its expression in nondamaged cells (15). The exact physiological function of p53R2 is not known, but it is essential for cell survival because mice lacking functional p53R2 apparently grow normally up to 6 weeks but then die from kidney failure (31, 32).

p53R2 is 80–90% identical to R2 but lacks its 33 amino-terminal residues, including the KEN box required for degradation during mitosis by anaphase-promoting complex Cdh1-mediated proteolysis (33). Unlike R2, p53R2 is therefore present also in G_0/G_1 cells, and it was proposed that together with low levels of the R1 protein, its major role may be to provide dNTPs for DNA repair and mtDNA synthesis in such cells (14). We showed previously that mitochondria from cycling cultured cells obtain dNTPs for their DNA synthesis by import from the cytoplasm (13). In quiescent cells the intra-mt salvage pathways involving phosphorylation of deoxynucleosides were dominant. The importance of mt thymidine kinase 2 and mt deoxyguanosine kinase for the supply of dNTPs is also apparent from the existence of mt diseases arising from deficiencies of the two deoxynucleoside kinases (11, 12). These diseases preferentially affect terminally differentiated cells.

Here we determined the *in situ* activity of the *de novo* pathway in quiescent human fibroblasts in culture to compare ribonucleotide reduction by the R1-R2 plus R1-p53R2 complexes in cycling cells with that of the R1-p53R2 complex in quiescent cells. We employ isotope experiments with labeled nucleosides similar to those used previously (18–20) to determine the interrelation between salvage and *de novo* synthesis of pyrimidine dNTPs in cycling mouse 3T6 cells. One important difference between this study and our earlier experiments is that we now provide fresh medium containing dialyzed serum to the cells immediately before the labeling period. In previous experiments we provided conditioned medium with nondialyzed serum. During growth, cells continuously excrete deoxynucleosides, and conditioned medium therefore contains the substrates for the salvage pathway of dNTP synthesis. Although the emphasis of the earlier experiments was on cycling cells with the major purpose to understand the interplay between salvage and *de novo* synthesis, in the present experiments we wished to characterize the *de novo* pathway of dNTP synthesis in quiescent cells with minimal interference from the salvage pathway.

The labeled nucleosides provided in the medium rapidly transfer isotope to intracellular deoxynucleotides and DNA as illustrated in Fig. 2. Two of them, [C^3H_3]thymidine and [6-^{3}H]deoxycytidine, are incorporated into labeled dTTP and dCTP + dTTP, respectively, by phosphorylation, bypassing ribonucleotide reduction. The third, [5-^{3}H]cytidine, gives via ribonucleotide reduction rise to labeled dCTP and nonlabeled dTTP + tritiated water. In the latter case isotope from the 5-position of the pyrimidine ring is released into the medium and quantifies the methylation of dUMP to dTMP.

These experiments conclusively show that quiescent human skin and lung fibroblasts have the ability to produce dCTP by reduction of CDP confirming conclusions drawn previously from experiments with quiescent mouse 3T3 fibroblasts (14). In human lung fibroblasts the transition from cycling to quiescence resulted in a 2-fold increase in the level of p53R2 protein (Fig. 1). Previously work (34) had demonstrated an up to 5-fold increase of p53 protein in quiescent WI-38 human fibroblasts after serum deprivation of proliferating cells. A similar increase of p53 in the lung fibroblasts may be responsible for the increase in p53R2 during quiescence. Protein R2 was instead degraded during the transition to the quiescent state (33) and was no longer detectable on Western blots after some time (Fig. 1). These data provide overwhelming evidence that the reduction of ribonucleotides found in our metabolic experiments was catalyzed by a reductase containing p53R2 and not R2.

Moreover, we now find that the quiescent cells also had the capacity to form dTTP *de novo* by a process involving ribonucleotide reduction. We can visualize two pathways as follows: one involving the reduction of UDP to dUDP followed by methylation of dUMP; the other involving deamination of dCMP to dUMP + methylation. In both cases tritium attached to the 5-position of the ring appears in water. dTTP formation from [6-^{3}H]deoxycytidine does not involve ribonucleotide reduction and is initiated by deamination of dCMP. Also the dTTP formation from [5-^{3}H]cytidine proceeded via dCMP deamination because UTP (and therefore UDP) received very little isotope from cytidine during the prelabeling period. At the beginning of the chase the specific radioactivity of UTP was only 0.8, compared with 1,500 in dCTP. Our results thus document the combined activities of dCMP deaminase and dTMP synthase in quiescent cells.

Surprisingly, quiescent cells exported sizeable amounts of labeled deoxycytidine and deoxyuridine. The incorporation of nucleotides into DNA was very low, and the cells contained very small dNTP pools in comparison with the cycling cells (see Fig. 3) and in agreement with many earlier results. Nevertheless, their export of deoxynucleosides in a chase experiment after labeling with cytidine amounted to almost 25% that of cycling cells. We have shown previously that cycling cells in culture export large amounts of deoxynucleosides into the medium via substrate cycles that couple the activity of kinases from a salvage pathway with the catabolic activity of 5′-nucleotidases and thereby regulate the intracellular concentration of dNTPs (18–20, 24). In cycling cells, however, the export is only a fraction of the total dNTP synthesis. The new data suggest that similar substrate cycles actively degrading dCMP, dUMP, and dTMP also exist in quiescent cells and that the export of the corresponding deoxynucleosides accounts for the major turnover of the dCTP pool. In view of the low requirements for dNTPs for synthetic purposes during the quiescent stage, this was unexpected but may be the price the cells must pay to maintain the dNTP pools required for DNA repair and mtDNA synthesis.

The rate of the *de novo* synthesis of deoxynucleotides is much smaller in quiescent than in cycling cells. Attempts to measure the rate from the disappearance of tritium from dCTP during a chase after labeling with cytidine did not succeed as the cells contained a very large pool of labeled CTP with a low turnover rate that continuously fed fresh isotope into dCDP during the chase. Also attempts to use hydroxyurea to block reduction of CDP were unsuccessful because the block was incomplete (data not shown). We therefore determined dCTP turnover from the appearance of isotope in the end products of dCTP metabolism. However, we could not include pyrimidine-containing liponucleotides in the equation. As far as known, they show a slow turnover (35), and we do not believe that our omission influences the results very much. With this caveat, we calculated that the turnover of dCTP in quiescent cells amounted to between 2 and 3% that in cycling cells.

This may appear to be more than enough to satisfy requirements for DNA repair and mtDNA replication. There are, however, aspects of dNTP pool sizes that are not understood. In S-phase cells, pool sizes are 20-fold larger than in quiescent cells. Does this reflect the K_m values of different polymerases? On the other hand in S-phase cells hydroxyurea blocks incorporation of dTTP into DNA completely within a few minutes when the size of all four pools has barely halved and remains much above the size of pools in resting cells (20). One solution to this problem may be that the dNTPs are concentrated close to the site of DNA damage or within the mitochondria in resting cells.

There are now two independent demonstrations using different techniques showing that mammalian cells in G_0/G_1 indeed have an ongoing ribonucleotide reduction. This reduction is catalyzed by low levels of an R1-p53R2 complex, and we postulate that the activity is essential for basal level DNA repair

and mtDNA synthesis. The physiological importance of the increase in p53R2 protein and dNTP pools after DNA damage and p53 induction is unclear especially because maximal induction is reached no earlier than 24 h after DNA damage when most DNA repair should be completed. Our data underscore the importance for mtDNA replication of the low ribonucleotide reduction dependent on the constitutive levels of p53R2 in resting mammalian cells.

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