⌘ *Author's Choice*

Deoxyribonucleotide Metabolism in Cycling and Resting Human Fibroblasts with a Missense Mutation in p53R2, a Subunit of Ribonucleotide Reductase*

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Ribonucleotide reduction provides deoxynucleotides for nuclear and mitochondrial (mt) DNA replication and DNA repair. In cycling mammalian cells the reaction is catalyzed by two proteins, R1 and R2. A third protein, p53R2, with the same function as R2, occurs in minute amounts. In quiescent cells, p53R2 replaces the absent R2. In humans, genetic inactivation of p53R2 causes early death with mtDNA depletion, especially in muscle. We found that cycling fibroblasts from a patient with a lethal mutation in p53R2 contained a normal amount of mtDNA and showed normal growth, ribonucleotide reduction, and deoxynucleoside triphosphate (dNTP) pools. However, when made quiescent by prolonged serum starvation the mutant cells strongly down-regulated ribonucleotide reduction, decreased their dCTP and dGTP pools, and virtually abolished the catabolism of dCTP in substrate cycles. mtDNA was not affected. Also, nuclear DNA synthesis and the cell cycle-regulated enzymes R2 and thymidine kinase 1 decreased strongly, but the mutant cell populations retained unexpectedly larger amounts of the two enzymes than the controls. This difference was probably due to their slightly larger fraction of S phase cells and therefore not induced by the absence of p53R2 activity. We conclude that loss of p53R2 affects ribonucleotide reduction only in resting cells and leads to a decrease of dNTP catabolism by substrate cycles that counterweigh the loss of anabolic activity. We speculate that this compensatory mechanism suffices to maintain mtDNA in fibroblasts but not in muscle cells with a larger content of mtDNA necessary for their high energy requirements.

Cells replicate their nuclear DNA during a defined period of the cell cycle, the S phase, which in most mammalian cells occupies <50% of the whole cycle. During this period a rapid synthesis of the four canonical dNTPs[2] occurs with a massive 10–30-fold expansion of pool sizes despite the high turnover of dNTPs during DNA replication (1). dNTPs are mainly produced *de novo* by reduction of ribonucleoside diphosphates, and their large increase depends on up-regulation of the two nonidentical subunits R1 and R2 of the enzyme ribonucleotide reductase (RNR). Transcription of the two genes is activated just before and during early S phase (2, 3).

Both subunits are required for catalytic activity. The larger protein R1 carries the catalytic site for the reduction of the ribose moiety, whereas the smaller R2 generates and harbors a stable tyrosyl free radical essential for the catalytic process (4). The reduction of ribonucleotides is tightly tuned to the requirements of the cells for dNTPs. Excessive or imbalanced dNTP pools cause genetic abnormalities and can lead to cell death (1, 5). An immediate defense mechanism against dNTP imbalance is provided by a highly complex system of allosteric effects that directs not only the overall activity of RNR but also the specificity of the enzyme for its substrates (1, 4). Moreover, nucleoside phosphorylases and 5′-deoxynucleotidases keep overproduction of dNTPs at bay in substrate cycles by their catabolic function (6). Once DNA replication is completed, the transcriptional activation of the R1 and R2 genes ceases, adapting ribonucleotide reduction to the greatly diminished consumption of dNTPs. Furthermore, after completion of DNA replication the R2 subunit is degraded rapidly (7). Its "KEN box," a specific amino acid sequence located in its N terminus, provides a signal for ubiquitylation of R2 during mitosis by anaphase-promoting complex Cdh1 leading to proteasomal degradation.

How do quiescent cells in the absence of R2 obtain the small amounts of dNTPs required for DNA repair and mtDNA replication? The discovery of p53R2, a third subunit of RNR coded by the *RRM2B* gene (8, 9), answered this question. On a protein basis, R2 and p53R2 are equally active in their function of generating the tyrosyl free radical used by R1 for catalysis (10). The primary structures of p53R2 and R2 are essentially identical, except for the absence in p53R2 of the N-terminal stretch of 33 amino acids that in R2 contains the KEN box. This difference turns p53R2 into a stable protein that persists during the whole cell cycle and is active also in nondividing cells. p53R2 is the subject of the present paper.

Actively dividing cells contain comparable amounts of R1 and R2, but only minute amounts of p53R2 (11, 12). After DNA damage, when p53R2 is induced by p53, S phase cells contain much less p53R2 than R2 (11). It is therefore difficult to believe that p53R2 plays a major role in dNTP synthesis during S phase. Under those circumstances it has a standby function, and R2

provides the tyrosyl radical for catalysis. The p53R2 subunit becomes relevant in quiescent and postmitotic cells where it together with R1 synthesizes the low levels of dNTPs necessary for nuclear DNA repair and mtDNA synthesis. *In situ* measurements of enzyme activity by isotope-flow technology demonstrated that in quiescent human fibroblasts ribonucleotide reduction amounted to ≈2–3% of that occurring in cycling cells (12).

The importance of p53R2 for the maintenance of mtDNA is demonstrated by the mitochondrial diseases caused by its genetic inactivation (13–18). Most patients with mutations of the *RRM2B* gene presented mtDNA depletion, especially severe in skeletal muscles, and died in early infancy (13–16). However, the clinical spectrum of p53R2 deficiency includes also milder phenotypes caused by mutations that interfere with assembly of the enzyme quaternary structure and cause depletion (18) or multiple deletions (17) of mtDNA. Severe mtDNA depletion was also found in various tissues of p53R2 knock-out mice (13) that die of renal failure (19, 20).

Thus, the phenotypic consequences of p53R2 deficiency are well documented *in vivo* and indicate that the function of p53R2 is essential for the stability of the mt genome in differentiated tissues. In cellular systems the effects of p53R2 inactivation have been studied by siRNA silencing in transformed cell lines (21, 22) or in p53-deficient cancer cells, unable to induce p53R2 expression after DNA damage (23, 24). In both cases the main emphasis was on the connection between p53R2 and DNA repair. In the absence of a functioning p53 the role of p53R2 during the DNA damage response was suggested to be taken up by R2 (23, 24) that became limiting for repair.

We were interested in examining the consequences of p53R2 inactivation for the metabolism of dNTPs in normal noncycling cells not exposed to DNA-damaging agents, a model closer to the postmitotic cells affected *in vivo* by p53R2 deficiency. Here, we study deoxynucleotide metabolism in human fibroblasts from a patient with a homozygous missense mutation substituting a glycine by a valine residue in the diferric iron center of p53R2, thereby impeding the generation of the tyrosyl radical. The patient died in early infancy with severe depletion of mtDNA in muscle (15). We obtained quiescent fibroblasts by maintaining confluent cultures for up to 27 days in medium with 0.1% fetal calf serum and investigated the effects of the mutation on parameters related to the synthesis and metabolism of deoxynucleotides. In cycling cells and at different times during prolonged quiescence we determined (i) the distribution of cells in the cell cycle, (ii) the size of the four dNTP pools, (iii) the expression of enzymes relevant for dNTP synthesis, (iv) quantified *in situ* ribonucleotide reduction during incubation of cells with [³H]cytidine, and (v) measured the level of mtDNA. In cycling cultures, all these parameters did not differ between mutant and age-matched normal fibroblasts. Instead, in quiescent cultures the activity of RNR and related parameters decreased more in the mutant cells, but we found no depletion of mtDNA. Surprisingly, the overall down-regulation of cell cycle-related proteins, such as R2, thymidine kinase 1, and cyclin B1, was less marked in the mutant cell population, probably caused by a slightly higher frequency of S phase cells. Our experiments show that p53R2 function is dispensable in cycling cells during normal growth and that nonproliferating fibroblasts by repressing dNTP catabolism can cope with a strongly reduced ribonucleotide reduction without apparent destabilization of the mt genome.

EXPERIMENTAL PROCEDURES

Materials—[5-*methyl*-³H]Thymidine (20,000 cpm/pmol) was from PerkinElmer Life Sciences. [5-³H]Cytidine (30,000–40,000 cpm/pmol) was from Moravek (Brea, CA). It contained also [6-³H]cytidine as revealed by radioactivity found in dTTP after *in situ* labeling of cells. Antibodies used were anti-human R2 goat polyclonal (sc-10844; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-human p53R2 rabbit polyclonal (ab8105; Abcam), anti-human R1 mouse monoclonal mAb 3033 (Millipore), anti-β-actin mouse monoclonal (clone AC-74; Sigma), and anti-BrdU mouse monoclonal (clone BMG 6H8; Roche Applied Science).

Cell Lines and Cell Growth—p53R2-mutated human skin fibroblasts were isolated from a patient carrying a homozygous missense mutation in the *RRMB2* gene (15) and transferred from Gothenburg to Padova at the fourth passage in culture together with an age-matched control. Samples of both lines were frozen in liquid nitrogen and after thawing were used in parallel for experiments. In one case we also used an additional control line of human skin fibroblasts from a different healthy donor (25). All lines were cultured in 5- or 10-cm dishes in Eagle's minimal essential medium (MEM) with 10% fetal calf serum (FCS, New Zealand, Invitrogen) and nonessential amino acids (Invitrogen) in a humidified incubator with 5% CO_2. At intervals we checked the cells for mycoplasma contamination by the Venor GEM PCR-based method (Minerva Biolabs). To obtain quiescent fibroblasts we seeded 0.35 million cells/10-cm dish and grew them in MEM with 10% FCS. After 7–10 days, when cells were contact-inhibited, they were transferred to medium with 0.1% dialyzed FCS and maintained in culture for up to 4 weeks. Fresh medium containing 0.1% serum was supplied twice a week. During serum starvation we monitored the cultures for percent S phase cells by flow cytometry. During growth in 0.1% serum the percentage of cycling cells in the population decreased to below 1%. To check cell vitality during starvation we trypsinized the cells at the end of the starvation period, replated them in fresh medium with 10% serum, and followed their renewed growth and ability to incorporate BrdU during a further 7 days. We conducted in all three complete starvation experiments ending on the 21st or 27th day, making our analyses at selected days, as outlined under "Results." We made some additional experiments to confirm data at the end of the starvation period.

Isotope Experiments—We labeled cells by incubation with either 1 μM [³H]cytidine or 0.3 μM [³H]thymidine to determine the *in situ* metabolism of dNTPs by procedures described earlier (12, 26). Briefly, in the cytidine experiment we labeled the cells for 4 h and then chased the isotope with nonlabeled medium for 2 more h. We used the difference of isotope incorporation between the two time points to calculate the rate of ribonucleotide reduction from the sum of isotope incorporated into DNA and the products of dCTP catabolism (deoxyuridine + deoxycytidine). In the [³H]thymidine experiments we intro-

duced the isotope for either 1 or 4 h without chase and determined the rate of DNA synthesis from the difference of isotope incorporation between the two time points. In all experiments we determined the specific radioactivities of the relevant dNTP to calculate rates as pmol/min.

Analytical Procedures—At the end of incubation we transferred the plates on ice to a cold room, collected the medium in the chase experiments, washed the monolayers three times with PBS, extracted nucleotide pools with 60% ice-cold methanol, and processed them as described earlier (27, 28). We analyzed dNTP pools and determined the specific radioactivities of dTTP and dCTP by the DNA polymerase assay (29) as recently modified to avoid interference by rNTPs (30). We analyzed the concentration of rNTPs and the specific radioactivity of CTP by HPLC (28). To measure the excretion of nucleosides, we deproteinized the chase medium with perchloric acid (31) and determined the amount of radioactive deoxycytidine and deoxyuridine by HPLC on a LUNA C18 (Phenomenex, Torrance CA) column (250 × 4.4 mm) (32). We measured DNA synthesis by incorporation of isotope from [³H]cytidine or [³H]thymidine. In both cases we calculated rates as pmol/min from the isotope incorporated between the two time points and the determined average specific radioactivities of [³H]dCTP or [³H]dTTP. In the calculations of the cytidine experiments we assume incorporation of both dCTP and dTTP and use a T/C ratio of 1.3 in DNA (12).

BrdU Incorporation—Cycling and quiescent fibroblasts were grown on coverslips. After either 16- or 30-h incubation with 30 μM BrdU the cells were fixed with 50 mM glycine, pH 2, and 70% ethanol for 20 min at −20 °C. Then the coverslips were incubated with the primary anti-BrdU antibody following the manufacturer's instructions (Roche Applied Science) and washed three times with PBS. The slides were incubated with anti-mouse Ig-fluorescein for 30 min at 37 °C. After further washing, they were mounted with mounting medium containing 0.2 μg/ml DAPI.

Real-time RT-PCR—We used the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) to quantify by real-time RT-PCR the mRNA expression of the three RNR subunits, thymidine kinase 1, and cyclin B1. Total RNA was extracted from cycling and quiescent cultures with TRIzol reagent (Invitrogen). We prepared cDNAs by reverse transcription and performed real-time PCR assays in 96-wells optical plates as described in Ref. 25. Primers for R1, R2, and succinate dehydrogenase complex subunit A were reported earlier (25). New primers used here were the p53R2 primers (5′-GAGGCTCGCTGTTTCTATGG-3′ and 5′-ATCTGCTA-TCCATCGCAAGG-3′), thymidine kinase 1 primers (5′-CCC-TGACATCGTGGAGTTCT-3′ and 5′-TGCCGAGCCTCTT-GGTATAG-3′), cyclin B1 primers (5′-TCCAGTTATGCAG-CACCTGGCTA-3′ and 5′-TGCCACAGCCTTGGCTAAAT-CTT-3′), and an additional set of primers for R2 (5′-TGGCA-GACAGACTTATGCTGGA-3′ and 5′-GGACTTGACATCA-CTCCCATCC-3′). To normalize the expression of individual target genes in control and mutant cells we chose succinate dehydrogenase complex subunit A as reference because it is expressed equally during cell proliferation and quiescence.

Each cDNA preparation was analyzed at least six times. We employed the comparative Ct method (33) for data processing.

Quantification of mtDNA—We determined human mtDNA copy number with the TaqMan probe system and Applied Biosystems 7500 Real Time PCR as described in Ref. 34. Briefly, total DNA was extracted from control and mutant fibroblasts with Puregene Core Kit B (Qiagen). The mt 12 S ribosomal RNA TaqMan probe and primers (PerkinElmer Life Sciences) were used to quantify mtDNA whereas for nuclear DNA we used the RNase P primers and probes contained in TaqMan predeveloped assay (PerkinElmer Life Sciences). mtDNA and nuclear DNA copy numbers were determined using calibration curves generated by serial dilution of a mixture of plasmids carrying the two PCR amplicons as described (34).

Western Blotting—We collected pellets of 1–2 million cells, washed them with 0.9% NaCl, and lysed them with lysis buffer (10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100). After addition of NaCl to a final concentration of 0.2 M and additional vortexing, we centrifuged the extract at 19,000 × g for 20 min and used the supernatant solutions for assays (35). We determined protein concentration by the BCA protein assay (Pierce) and loaded equal amounts of protein for electrophoresis on 9% polyacrylamide gels. We used separate gels for each subunit of RNR. When the number of samples to be analyzed was too high to be run in a single gel, we loaded aliquots of the same reference sample on both gels to be compared. We transferred proteins to a Hybond-C extra membrane (GE Healthcare), blocked the unspecific sites with 2% ECL blocking agent (GE Healthcare), and incubated the filter overnight at 4 °C with the primary antibody. After washing the membrane three times for 5 min each, we incubated it with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After further washing we developed the filter in an ECL-advanced system (GE Healthcare). The signal was detected and quantified with a Kodak one-dimensional Imaging station 440CF.

RESULTS

Growth of Mutant and Control Fibroblasts—Parallel cultures of a human fibroblast line with mutated p53R2 and a matched normal control, both from the fifth passage, showed similar growth behavior in medium containing 10% FCS, including the distribution of cells in the cell cycle at different days of growth (Fig. 1, *A* and *B*). After 10 days, when the cells had reached confluence, we changed the medium to contain dialyzed 0.1% FCS (day 0) and maintained the cultures for up to 27 days to deplete the cells of protein R2 further and arrest nuclear DNA replication. Both mutant and control cells remained attached to the plates during serum starvation, but cell cycle analyses demonstrated a small progressive increase in the subG₁ fraction indicating some cell death, that amounted to close to 10% after 21 days, slightly higher in the mutant cell population (Fig. 1*C*). When trypsinized at the end of the starvation period and replated in medium plus 10% FCS, mutant and control cells after a lag period started to grow at similar rates and eventually became inhibited at the same cell density (data not shown).
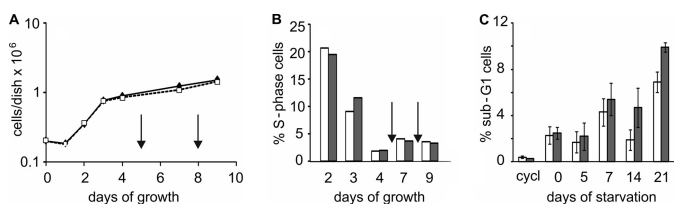
FIGURE 1. **Mutant and normal human fibroblasts in culture have similar growth characteristics.** *A*, time-dependent growth in culture. Mutant (*broken lines*) and control (*continuous lines*) cells from the fifth passage were grown on 5-cm dishes in MEM with 10% FCS, with medium changes after 4 and 7 days (*arrows*). *B*, percentage of S phase cells during growth. *Arrows* indicate medium changes. *C*, percentage of subG$_1$ cells during maintenance in MEM with 0.1% dialyzed FCS after reaching confluence (day 0) in complete medium. *Error bars* show individual values from two separate experiments. *Shaded columns* are mutant cells, *open columns* are matched controls. *Cycl*, cycling cell culture.
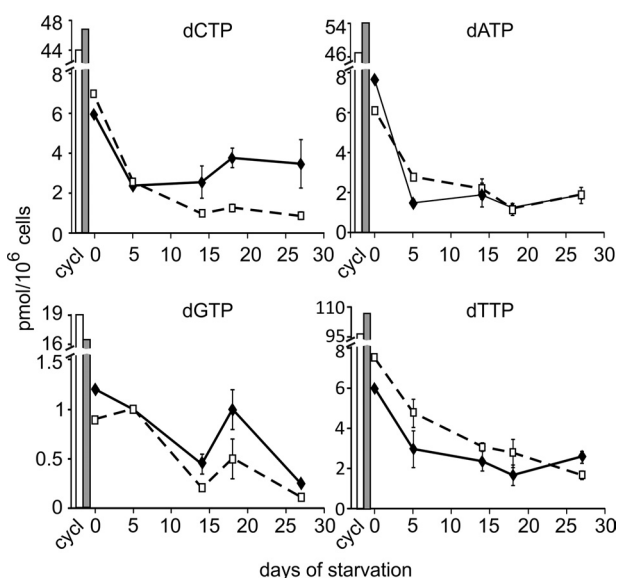

FIGURE 2. **Decrease in the size of the four dNTP pools during prolonged serum starvation.** Mutant (*broken lines*) and control (*continuous lines*) cultures were grown in MEM + 10% FCS for 10 days to reach confluence (day 0). Incubation continued in MEM + 0.1% dialyzed FCS for 27 days with medium changes twice a week. Pool sizes were determined at different days. *Error bars* indicate extreme values from three separate experiments. *Cycl*, cycling cell culture; *shaded columns*, mutant cells; *open columns*, control cells.

Changes in the Size of dNTP and rNTP Pools during Starvation—Cycling cells contained relatively large pools of each of the four dNTPs, of equal size in mutant and control cultures. Each pool decreased 15–30-fold during attainment of quiescence with dATP and dTTP showing similar changes in both cell lines whereas dCTP and probably also dGTP decreased more in the mutant line (Fig. 2). Thus, analyses of four separate pairs of cultures after 27 days of starvation showed that the mutant cultures contained 0.9 ± 0.2 pmol/million cells of dCTP whereas the controls contained 3.5 ± 0.7 pmol. The very low values for the dGTP pool did not permit a reliable comparison of the two lines. The size of each of the 4 rNTP pools did not differ between mutant and control cells and decreased by ~50% during quiescence, with the ATP pool decreasing from 15,000 to 8000, UTP from 4000 to 2000, GTP from 2500 to 1500, and CTP from 1500 to 1000 pmol/million cells.

mtDNA in Quiescent Cells—In two independent experiments, we determined in mutant and control cells from the

10th and 12th passages the number of mtDNA copies/cell at days 0 and 27 of serum starvation. We found for the control cells an average copy number of 708 ± 70 at day 0 and 620 ± 66 at day 27; for the mutant cells we found 716 ± 52 at day 0 and 587 ± 158 at day 27. Thus, there were no significant differences. A parallel determination on total DNA from human skeletal muscle gave ~30,000 mtDNA copies/nuclear genome.

In Situ Ribonucleotide Reduction in Quiescent Cells—To compare the rate of ribonucleotide reduction in mutant and control cells maintained in 0.1% FCS we labeled the cells with [³H]cytidine in pulse-chase experiments and calculated total ribonucleotide reduction by a method described earlier in detail (12). Briefly, we determined the total amount of CDP reduction during the chase from the accumulation of isotope in the end products of [³H]dCTP metabolism, *i.e.* from the sum of radioactivity incorporated into DNA in the cell nucleus and into products of dCTP catabolism (deoxycytidine and deoxyuridine in the culture medium). Fig. 3*A* shows schematically in an abbreviated manner the flow of isotope from cytidine to the end products used to calculate the total rate of CDP reduction.

Fig. 3, *B–D*, shows the results of the relevant analyses at different times of starvation. The bases for all calculations were the specific radioactivities of dCTP (Fig. 3*B*). From these values we could transform radioactivity to pmol assuming in all labeled metabolic products a specific radioactivity identical to that of dCTP (see Ref. 12). After 5 days, dCTP and CTP had very low specific radioactivities compared with the precursor cytidine both in mutant and control cells (Fig. 3*B*), indicating a high dilution with nonradioactive material derived from the *de novo* synthesis of CTP. The values did not differ significantly before and after the chase and are therefore given as averages. They increased almost 10-fold after prolonged serum starvation, but now were consistently lower in the mutant cells than in the controls. The specific radioactivities of dCTP and CTP covaried closely (Fig. 3*B*) and thus in both cell lines the progressive increase of dCTP specific radioactivity during prolonged starvation reflected changes in CTP and not increased ribonucleotide reduction. The reason was either more effective phosphorylation of labeled cytidine or, more likely, smaller dilution by nonradioactive CTP. In fact, the *de novo* synthesis of pyrimidine ribonucleotides is down-regulated in nondividing cells (36). We have no explanation for the differences in CTP-specific radioactivity between mutant and control cells.

The labeled cytidine used here, unlike the material used earlier, contained ³H in the 6-position, and the isotope was therefore transferred also to dTTP, which reached the same specific radioactivity as dCTP. Thus, both radioactive dCTP and dTTP were incorporated into DNA. We calculated rates of DNA synthesis (Fig. 3*C*) from the incorporation of both nucleotides as described earlier (12) assuming a T/C ratio of 1.3. Already after 5 days of serum starvation the rates of DNA synthesis corresponded to only 1% of that found earlier for cycling fibroblast (12). After 27 days starvation rates had decreased a further 30-fold with the mutant cells maintaining 2–3-fold higher rates than the controls. This progressive decline of residual DNA synthesis during serum starvation was confirmed in C63 human fibroblasts, an independent normal line (25) available in the laboratory (data not shown).
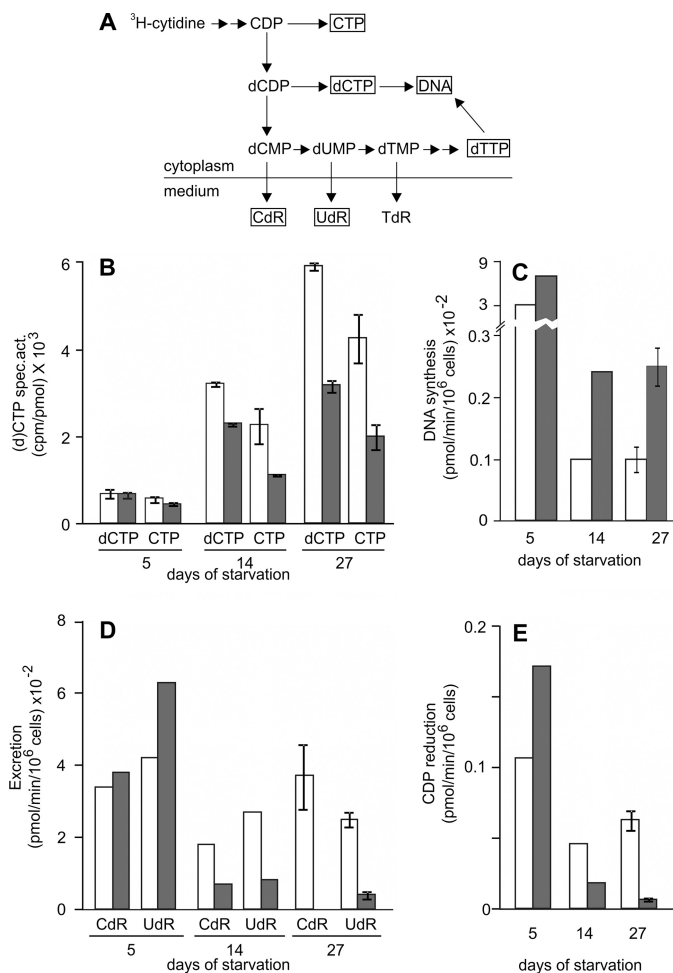
FIGURE 3. *In situ* determination of ribonucleotide reduction. *A*, pathways for the incorporation of cytidine into dNTPs and DNA. [³H]Cytidine is phosphorylated to CDP, reduced to dCDP by ribonucleotide reductase and after phosphorylation to dCTP incorporated into DNA. dCDP is also transformed to dCMP, deaminated to dUMP and further methylated to dTMP that after two phosphorylations is converted to dTTP and incorporated into DNA. The monophosphates are dephosphorylated by 5′-deoxynucleotidases, and the deoxynucleosides (deoxycytidine, deoxyuridine, and thymidine) are excreted into the medium. No thymidine excretion was detected in the present experiments. *Boxes* highlight the analyzed radioactive compounds reported in *B–D*. *B–E*, at the indicated days of maintenance in low serum, mutant (*shaded columns*) and control cells (*open columns*) were incubated with 1 μM [³H]cytidine (40,000 cpm/pmol) for 4 h followed by a 2-h chase without cytidine. At both times we determined the specific radioactivity of the CTP and dCTP pools, the incorporation of radioactivity into DNA, and the radioactivity incorporated into the deoxynucleosides excreted into the medium. From these values we calculated the rate of DNA synthesis and the excretion of radioactive deoxycytidine and deoxyuridine in pmol/min as described under "Experimental Procedures." *B*, average specific radioactivity of the dCTP and CTP pools. *C*, rate of incorporation of radioactive nucleotides into DNA. *D*, excretion of deoxycytidine (*CdR*) and deoxyuridine (*UdR*) into the medium. *E*, total ribonucleotide reduction was calculated from data in *B–D*. *Error bars* were from two separate experiments.

Fig. 3*D* shows the excretion of [³H]deoxyuridine and [³H]deoxycytidine into the medium after 5, 14, and 27 days of culture in 0.1% FCS. Results from a second independent experiment after 27 days are included in the figure. There was no corresponding excretion of labeled thymidine. In the control cells the length of serum starvation had no effect on excretion. The mutant cells excreted at day 5 amounts similar to the controls, but already after 14 days the excretion of both deoxynucleosides decreased strongly, and after 27 days we found no deoxy-

TABLE 1

BrdU incorporation into cell nuclei during serum starvation

Cycling and quiescent mutant and control cells were incubated during serum starvation with 30 μM BrdU for either 30 h (Experiment 1) or 16 h (Experiment 2), and the percentage of BrdU-labeled cell nuclei was determined by immunofluorescence. After 21 days in MEM + 0.1% dialyzed FCS the cells were trypsinized, replated in fresh MEM + 10% FCS, and labeled with BrdU after 5 days. For each time point 2500 −3000 cells were scored. M/C, ratio between labeled nuclei in mutant and control cells.

	% BrdU-labeled nuclei					
	Exp 1			Exp 2		
Cells	Control	Mutant	M/C	Control	Mutant	M/C
Cycling	49	57	1.2			
Days of starvation						
0	18	24	1.3			
3	2.7	5.7	2.1	3.9	4.8	1.7
7	1.5	3.9	2.6	0.9	2.2	2.5
21	0.5	1.1	2.2			
10% serum						
5	46	55	1.2			

cytidine and only traces of deoxyuridine in the medium of the mutant cultures.

In the control cells the total turnover of dCTP after 5 days was distributed equally between incorporation into DNA and catabolism (Fig. 3, *C* and *D*). Thereafter, catabolism accounted for >95% because the incorporation into DNA decreased precipitously whereas catabolism changed only marginally. Mutant cells behaved differently. They also decreased incorporation into DNA, but in addition lost almost all catabolism between the 5th and 27th days.

We can now calculate the total reduction of CDP from the turnover of dCTP, *i.e.* from the sum of DNA synthesis and catabolism (Fig. 3*E*). In the controls, CDP reduction changed little during the entire period of serum starvation whereas in the mutant cells it decreased, reaching 10% of the original value after 27 days. Clearly the absence of a functional p53R2 resulted in a large drop in CDP reduction but not in its complete abolition.

DNA Synthesis in Quiescent Cells—We used two additional methods to determine the progressive demise of DNA replication from mutant and control cultures during the starvation period: (i) BrdU incorporation into individual cell nuclei detected by immunostaining and (ii) incorporation of [³H]thymidine into total cellular DNA detected by scintillation counting. The first method measures the number of cells passing through S phase during a defined time period, and the second method provides the average DNA synthesis of the whole cell population without considering cell heterogeneity. In this case we determined thymidine incorporation into DNA and in addition the size and specific radioactivity of the dTTP pool, the proximal precursor of DNA, to correct for differences between mutant and control cells in the phosphorylation of labeled thymidine. With highly radioactive thymidine this method becomes more sensitive and accurate than the first method.

To determine the amount of cycling cells we incubated mutant and control cells for either 16 or 30 h with BrdU in two separate experiments (Table 1). A larger proportion of nuclei was labeled after 30 h but in both instances the number of S phase cells decreased during prolonged starvation. At 21 days, 1% or less of the cells were BrdU-positive compared with 50−60% in the cycling cultures. Unexpectedly, the mutant cul-
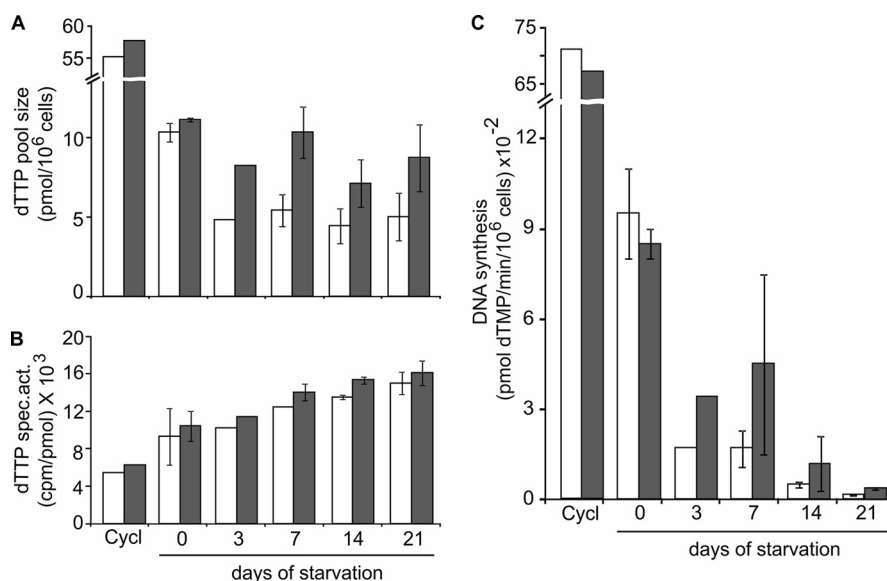
FIGURE 4. **Synthesis of DNA measured by incorporation of [³H]thymidine.** Contact-inhibited cells were maintained for up to 21 days in MEM + 0.1% dialyzed FCS. At the indicated days mutant (*shaded columns*) and control (*open columns*) cultures were incubated with 0.3 μM [³H]thymidine (20,000 cpm/pmol) for 1 or 4 h. Parallel cultures of cycling cells (*cycl*) were incubated similarly. The size of the dTTP pool (*A*), the specific radioactivity of the dTTP pool (*B*), and the rate of DNA synthesis (*C*) were determined as described under "Experimental Procedures." We calculated the rate of DNA synthesis as pmol/min from the difference between the radioactivity incorporated into DNA after a 1- and 4-h incubation by division with the specific radioactivity of the dTTP pool. *Error bars* are from two separate experiments.

tures contained more labeled nuclei than the controls. When after 21 days we transferred the cells to fresh medium containing 10% serum, both mutant and control cells recovered rapidly, and after 5 days in complete growth medium the percentage of labeled nuclei in both cultures was similar to that of cycling cells (Table 1). These data demonstrate that the long starvation period did not specifically damage the mutant cells and that both lines behaved similarly to other normal human fibroblasts studied earlier (31).

In parallel to the BrdU experiments of Table 1, we determined the rate of DNA synthesis from the incorporation of [³H]thymidine into DNA and dTTP. We found a decrease of incorporation similar to that occurring for the labeling of cell nuclei from BrdU. After prolonged starvation, both mutant and control cells incorporated <1% dTMP into DNA compared with cycling cells (Fig. 4C). At all time points in 0.1% serum mutant cells incorporated approximately twice as much radioactivity as the controls. For our calculations it was necessary to know the size (Fig. 4A) and the specific radioactivity (Fig. 4B) of the dTTP pool. Before serum starvation, mutant and control cells had identical pool sizes, but starting already at the 3rd day the mutant pool was almost 2-fold larger than the control pool with the difference remaining the same at all time intervals (Fig. 4A). We present evidence below (Fig. 5) for a higher thymidine kinase 1 activity in the mutant cell population, which probably explains these results. Even though in mutant and control cells the dTTP pools differed in size, their specific radioactivities differed only marginally (Fig. 4B). They increased progressively 3-fold during the 21-day period in both cell lines and eventually reached almost the same value as the labeled thymidine. These changes are likely caused by a diminished *de novo* synthesis of nonradioactive dTTP during prolonged starvation.
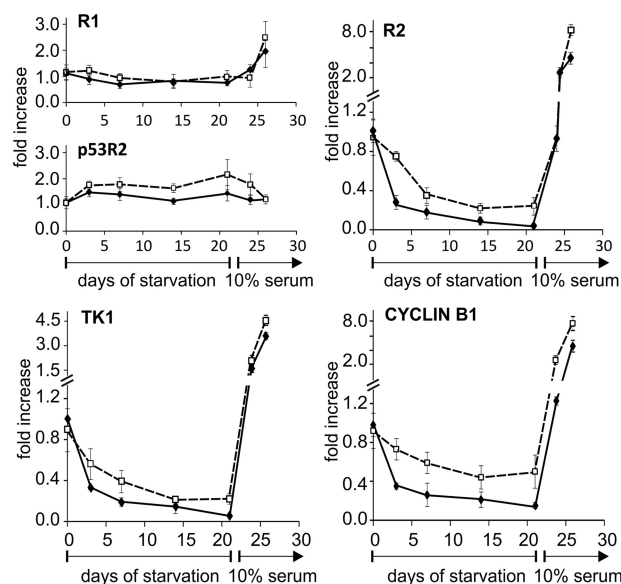

FIGURE 5. **Levels of mRNAs for R1, R2, p53R2, thymidine kinase 1 (*TK1*), and cyclin B1.** The mRNAs of mutant (□) and control (♦) cells during maintenance in MEM + 0.1% FCS were measured by real-time RT-PCR as described under "Experimental Procedures." At the 21st day of serum starvation we trypsinized the remaining cultures, replated them in MEM + 10% FCS, and redetermined mRNA levels after 3 and 5 days. mRNA levels are expressed as -fold increase relative to day 0. The data with *error bars* are means ± S.D. from three independent experiments. We used two different sets of primers for R2.

Expression of Cell Cycle-regulated Proteins during Serum Starvation—We analyzed the expression of the three subunits of RNR and of thymidine kinase 1, an enzyme of dNTP metabolism characterized by a cell cycle dependence very similar to that of R2 (37). In addition, we also examined the expression of cyclin B1 (38), a cell cycle-regulated protein not directly related to dNTP synthesis that we used as an additional marker for the presence of cycling cells in the cultures.

dNTP Metabolism in p53R2-deficient Fibroblasts

Fig. 5 shows results from real-time RT-PCR analyses of mRNA levels. We relate the values at the different time points to the relative amount of mRNA at the start of the starvation period (time 0). As shown in Table 1 and Fig. 4C, starvation in 0.1% serum greatly decreased the rate of DNA synthesis in both mutant and control cells, reflecting a corresponding decrease in the number of S phase cells. The mRNAs for protein R2, the S phase-specific subunit of RNR, for thymidine kinase 1 and for cyclin B1 decreased strongly, whereas the mRNAs for protein R1 and protein p53R2, the two stable subunits of RNR, were less affected (Fig. 5). Interestingly, the mutant cells had higher mRNA levels of all five proteins than the controls during the whole time period. For R2 we confirmed this result in additional independent experiments.

The Western blots in Fig. 6 show that proteins R1 and R2, but not protein p53R2, strongly decreased with time in both mutant and control cells. For R2 the decrease was more pronounced in the controls with an average residual level in three separate experiments of 10% after 21 days compared with 25% in the mutants, paralleling the mRNA variations (Fig. 5). For R1, both control and mutant cells decreased to 25%. The stability of the p53R2 signal in both kinds of cells indicates that the missense mutation in *RRM2B* did not affect the transcription and translation of the inactive protein. After stimulation with 10% serum, both mutant and control cells rapidly recovered growth and regained high concentrations of R1 and R2 without any change in p53R2.

Fig. 7 shows that the ratio between mutant and control cells for both R2 and thymidine incorporation into DNA increased ~2-fold early during starvation and remained at this level during the whole experiment. The number of BrdU-labeled nuclei increased similarly, suggesting that the larger concentration of R2 in the mutant cells depended on a larger proportion of cycling cells in the culture.

DISCUSSION

Cycling fibroblasts contain at least 10 times more R2 than p53R2 and an excess of R2 over R1 (11). Accordingly, in our experiments the absence of p53R2 activity did not measurably affect the growth and survival of cycling mutant fibroblasts that employ R2 and not p53R2 for deoxynucleotide production. A defect of p53R2 became apparent only when the cells became quiescent and had lost R2. We forced both mutant and control cells into a quiescent state by prolonged serum starvation that accumulated them in G_0/G_1. Also during the attainment of quiescence and after recovery of growth in high serum the two cell lines behaved similarly, but with two noticeable differences: (i) the number of $subG_1$ cells increased slightly more in the mutant population on prolonged starvation, and (ii) the number of S phase cells declined precipitously to <1% but, surprisingly, the decline in the mutant was less pronounced. We then compared the two cell lines for different parameters related to ribonucleotide reduction to understand how the quiescent mutant cells coped with the loss of an active p53R2 protein, hoping also to gain some understanding of the mtDNA depletion syndrome manifested by the donor patient.

In earlier experiments we found that during quiescence normal human fibroblasts maintained a low level of ribonucleotide
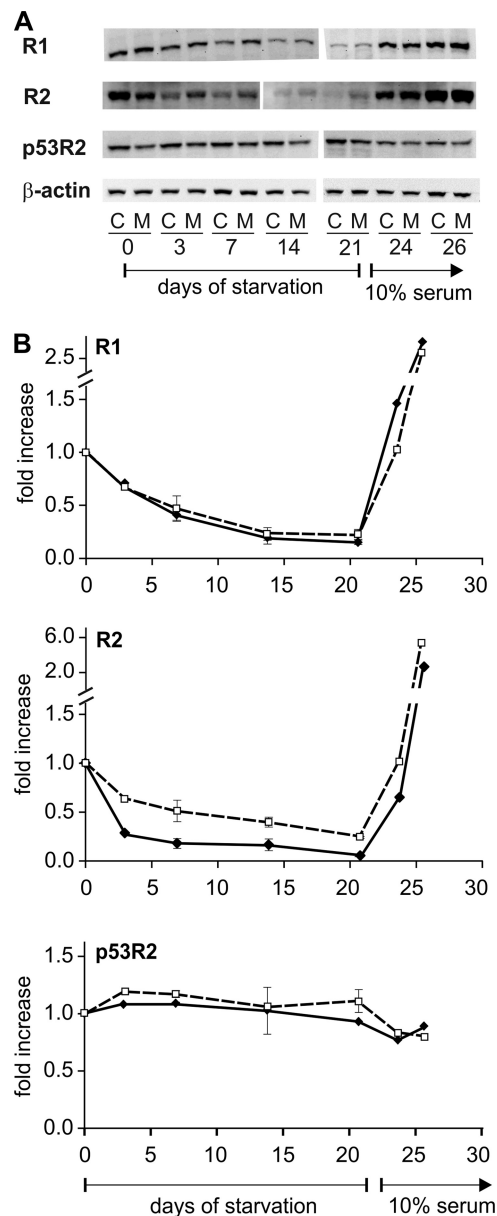

FIGURE 6. **Expression of RNR subunits.** *A*, Western blots of cell extracts from mutant (*M*) and control (*C*) cells during maintenance in MEM + 0.1% FCS made in parallel to the determinations of mRNAs shown in Fig. 5. At the 21st day the cells were replated in MEM + 10% FCS as in Fig. 5. We blotted cell extracts from the indicated days, containing 10 μg of total protein for R1 and R2 or 2 μg of total protein for p53R2 and β-actin, used as control, and detected each protein by immunoblotting as described under "Experimental Procedures." The results are from a single typical experiment. *B*, quantification of the average signal intensities of the immunoblots from three independent experiments with mutant (□) and control (♦) cells. For each protein and cell line the value at day 0 is set as 1. *Error bars* show extreme values.

reduction, as determined *in situ* from the formation of [³H]dCTP from [³H]cytidine, with the p53R2 protein substituting for R2 degraded during the last mitosis. Only a small part of the [³H]dCTP produced was used for DNA synthesis. More than 90% was instead catabolized and excreted as deoxynucleosides into the medium (12).

In similar experiments with p53R2 mutant fibroblasts we now find a large decrease in ribonucleotide reduction with the difference between mutant and control cells increasing during prolonged starvation. The mutant cells adapted to the smaller
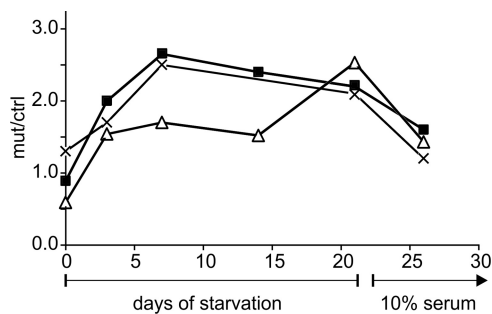
FIGURE 7. **Quiescent mutant cells contain more R2, incorporate more BrdU into cell nuclei, and synthesize more DNA from dTMP than matched controls.** Ratios between mutant and control cells (*mut/ctrl*) for content of R2 (△), BrdU incorporation into cell nuclei (×) and dTMP incorporation into DNA (■) at various days during maintenance in MEM + 0.1% FCS. At day 21 the cells were replated in MEM + 10% FCS.

supply of deoxynucleotides by decreasing the size of the dCTP pool to one third. Also the dGTP pool apparently decreased, whereas dATP and dTTP remained unchanged. The most striking difference concerned, however, the catabolism of dCTP that all but disappeared in the mutant. In the control cells with an active p53R2 the catabolism of dCTP served as a brake to prevent accumulation of the nucleotide that might lead to genetic instability. In the mutant cells that had a smaller dCTP pool due to impaired ribonucleotide reduction the brake was released.

In the absence of a functional p53R2, ribonucleotide reduction depended on R2 in the mutant cells. Their higher level of R2 might be thought to be an emergency measure to compensate for p53R2 deficiency. Such a compensatory mechanism with R2 substituting for p53R2 was suggested earlier to occur after DNA damage in human HCT-116 p53$^{-/-}$ colon cancer cells that cannot induce p53R2 by p53. In that case DNA repair appeared to depend on R2 (23). Those were, however, cycling cells where p53R2 probably has no major physiological function and not, as in our case, a resting cell population.

We consider a compensatory mechanism of R2 for p53R2 unlikely both in our experiments and during DNA repair in cycling p53$^{-/-}$ cells. In both instances a serious artifact arises from the heterogeneity of the cell populations. Cell cycle-regulated proteins, including R2 and thymidine kinase 1, abound in S phase cells and occur at very low concentrations or not at all in resting cells. In cycling cells the block in S phase after DNA damage leads to accumulation of S phase-specific proteins. This was reported for thymidine kinase 1 whose cell cycle regulation is identical to that for protein R2 (39). Conversely, even a small number of S phase cells present in a quiescent cell population has a major impact on the total amount of such proteins in the culture. In our case, the BrdU results (Table 1) showed indeed a larger number of cycling cells in the mutant cultures, and the surplus of R2 and thymidine kinase 1 probably depended on the larger number of S phase cells. The presence of higher levels of cyclin B1 mRNA (Fig. 5) supports this view. A possible explanation for the higher number of cycling cells in the mutant cultures comes from our finding of a slightly larger frequency of dead cells (Fig. 1C). The loss of these cells from the plate may make room for the division of neighboring cells. Our results serve also as a general caveat concerning the interpreta-

tion of experiments where parameters related to dNTP and DNA synthesis are compared between different cell populations without strict control of the distribution in the cell cycle.

Although the higher number of cycling cells may explain the permanence of some R2 in the mutant, it does not account for the decrease of ribonucleotide reduction, the smaller dCTP and dGTP pools, and the almost complete arrest of dCTP catabolism. On the contrary, the higher frequency of S phase cells may in these instances lead to an underestimation. The block of dCTP turnover beautifully illustrates regulation of dNTP pools by catabolism via substrate cycles (6, 32). The key regulators are 5′-deoxynucleotidases, whose activity, due to their high K_m values for deoxynucleoside monophosphates, strongly depends on substrate concentration (40, 41). In the present case, the lowered concentration of dCTP (and dCMP) resulted in lower nucleotidase activity. These changes demonstrate how in quiescent cells the lack of p53R2 activity affects the interplay between dNTP anabolism and catabolism. Besides the dCTP pool also the steady-state level of the dGTP pool appeared decreased, but it is reasonable to assume that the strong reduction of catabolism concerned all four dNTPs.

Given the severity of the mtDNA depletion in the patient from whom the fibroblasts were derived, the relatively mild effects of p53R2 inactivity in our experiments may be surprising. The alterations of dNTP metabolism emerged slowly after the confluent cells were shifted to low serum medium and mtDNA copy number remained unchanged during almost a month of serum starvation.

We were unable to find an effect of the decreased dNTP pools on the content of mtDNA in the mutant fibroblasts during prolonged quiescence in culture. Nor did the mutant fibroblasts obtained directly from the patient show any depletion of mtDNA. In previous experiments (31), we found a depletion of mtDNA in a cultured fibroblast model of MNGIE, a mtDNA disease caused by the genetic deficiency of thymidine phosphorylase (42). In our model, as well as in the disease, overproduction of dTTP by salvage of increased extracellular thymidine consumes large amounts of ATP that must be regenerated by mitochondria. We then suggested that the continuous large ATP consumption might stress the mitochondria and contribute to the disease. In the present model the p53R2-mutant fibroblasts were kept in low serum with limited requirements for ATP and no additional metabolic stress. In comparison with skeletal muscle fibers, fibroblasts contain few mitochondria and <5% of mtDNA molecules per nuclear genome, requiring considerably less dNTPs for their maintenance.

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