

Mitochondrial DNA Depletion and Thymidine Phosphate Pool Dynamics in a Cellular Model of Mitochondrial Neurogastrointestinal Encephalomyopathy*

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Mitochondrial (mt) neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disease associated with depletion, deletions, and point mutations of mtDNA. Patients lack a functional thymidine phosphorylase and their plasma contains high concentrations of thymidine and deoxyuridine; elevation of the corresponding triphosphates probably impairs normal mtDNA replication and repair. To study metabolic events leading to MNGIE we used as model systems skin and lung fibroblasts cultured in the presence of thymidine and/or deoxyuridine at concentrations close to those in the plasma of the patients, a more than 100-fold excess relative to controls. The two deoxynucleosides increased the mt and cytosolic dTTP pools of skin fibroblasts almost 2-fold in cycling cells and 8-fold in quiescent cells. During up to a two-month incubation of quiescent fibroblasts with thymidine (but not with deoxyuridine), mtDNA decreased to ~50% without showing deletions or point mutations. When we removed thymidine, but maintained the quiescent state, mtDNA recovered rapidly. With thymidine in the medium, the dTTP pool of quiescent cells turned over rapidly at a rate depending on the concentration of thymidine, due to increased degradation and resynthesis of dTMP in a substrate (=futile) cycle between thymidine kinase and 5'-deoxyribonucleotidase. The cycle limited the expansion of the dTTP pool at the expense of ATP hydrolysis. We propose that the substrate cycle represents a regulatory mechanism to protect cells from harmful increases of dTTP. Thus MNGIE patients may increase their consumption of ATP to counteract an unlimited expansion of the dTTP pool caused by circulating thymidine.

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)² is an autosomal recessive human mitochondrial

(mt) disease, caused by the defective function of the cytosolic enzyme thymidine phosphorylase (1) and is associated with depletion (2), multiple deletions (2, 3), and point mutations of mtDNA (4). Thymidine phosphorylase converts the pyrimidine deoxynucleosides thymidine and deoxyuridine to free bases and deoxyribose 1-phosphate. Human blood from healthy individuals contains <0.05 μM of the two deoxynucleosides, whereas both thymidine and deoxyuridine are found at 10–20 μM concentrations in plasma from MNGIE patients (5, 6). It has been proposed that this large increase in circulating deoxynucleosides results in a similar increase in the intracellular concentration of their triphosphates disrupting the normal balance of the 4 dNTPs and thereby interfering with the replication of mtDNA (4, 6). Experimental evidence supports this hypothesis. Addition of thymidine to the medium of cultured, rapidly growing cells lead to an increase in the dTTP pool and a rapid cessation of nuclear (and probably also mt) DNA replication caused by a depletion of the dCTP pool (7). Depletion of dCTP, as well as the concomitant increase of dGTP and dATP were secondary to the allosteric effects of the large dTTP pool on the enzyme ribonucleotide reductase. In that case (7) the concentration of thymidine in the medium was much higher (1 mM) than in the plasma of MNGIE patients. However, more recently HeLa cells growing at 50 μM thymidine in the medium showed small increases in dTTP and dGTP and a slight decrease in dCTP (8). After 8 months of growth in thymidine the mtDNA of the cells was reported to contain small amounts of multiple deletions.

Thymidine phosphorylase is a cytosolic enzyme that primarily affects the degradation and thereby indirectly also the phosphorylation of cytosolic thymidine. Why should the absence of this enzyme lead to mitochondrial dysfunction in MNGIE? We have for some time studied the relationships between cytosolic and mt dTTP synthesis in cultured cells. In isotope chase experiments with labeled thymidine we found that dTTP was synthesized independently in the two cellular compartments but that the two pools were in rapid communication (9–11). Two separate pathways are responsible for dTTP synthesis: (i) *de novo* synthesis from ribonucleotides in the cytosol; and (ii) phosphorylation of thymidine by TK1 in the cytosol and by TK2 in mitochondria. Both pathways are active in S-phase cells, with *de novo* synthesis being most important (9). In quiescent fibro-

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² The abbreviations used are: MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; mt, mitochondrial; TK1, cytosolic thymidine kinase; TK2, mitochondrial thymidine kinase; dNTPs, deoxynucleoside

5'-triphosphates; FCS, fetal calf serum; HPLC, high performance liquid chromatography.

blasts both *de novo* synthesis and TK1 activity are very low leaving the phosphorylation of thymidine by TK2 in mitochondria as the major pathway for the formation of dTTP (11). We obtained quiescent fibroblasts by maintaining contact inhibited cells for prolonged time periods in medium with 0.1% fetal calf serum. In those cells excess thymidine in the medium resulted in a rapid equilibration of extra- and intracellular nucleoside and a concomitant smaller increase in the intracellular dTTP pool, slightly more pronounced in mitochondria than in the cytosol. Addition of deoxyuridine to the medium produced a large increase in the dUMP pool, but a very small amount of dUTP.

Our experiments (11) characterized the metabolism of dTTP in quiescent cells but were also aimed at promoting our understanding of the relationship between thymidine metabolism and MNGIE. For this purpose we here prepared mtDNA from cells maintained for varying lengths of time in medium containing increased deoxynucleoside concentrations and analyzed their mtDNA. We also investigated the effect of increasing concentrations of thymidine on the stability and turnover of the dTTP pool. The present paper describes the results of such analyses.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Growth—Human skin fibroblasts between the 4th and 8th passage were donated by Dr. Roberta Tiozzo (University of Modena, Italy). An established line of lung fibroblasts (CCD 34Lu) was from the American Type Culture Collection. At intervals we checked the cells for mycoplasma contamination by the Venor GEM PCR-based method (Minerva Biolabs). We seeded cells routinely at 0.5 million/10-cm plate and grew them in Dulbecco's modified Eagle's medium with 4.5 g of glucose/liter + 10% FCS + non-essential amino acids + 20 mM Hepes buffer, pH 7.4, in a humidified incubator with 5% CO₂. Fresh medium was supplied twice a week. To obtain quiescent cells we transferred contact inhibited cells to medium with 0.1% FCS as described earlier (11). When required, thymidine and/or deoxyuridine were added at 3-day intervals to maintain their concentrations at 10–40 μM (11). We monitored the concentration of deoxynucleosides in the medium by HPLC (11). To test the viability of cells maintained for long periods in 0.1% FCS we trypsinized the cultures and reseeded 0.5 to 1 million cells/10-cm dish with fresh medium containing 10% FCS and 50 μg/ml of uridine. In all experiments we determined cell numbers with a Coulter counter and the percentage of S phase cells with a fluorescence-activated flow cytometer.

Isotope Experiments—We described the general procedure for these experiments earlier (9, 11). Briefly, we labeled cells with [³H]thymidine and chased the isotope by substitution of the labeled medium with medium with non-labeled thymidine or no thymidine. We manipulated cells in a 37 °C thermostated room and incubated them during the course of the experiment in a 37 °C incubator. We stopped the metabolism of the cells by transferring them on ice to a cold room and, after careful washing with phosphate-buffered saline, extracted dNTP pools with 60% ice-cold methanol (9). After boiling for 3 min we evaporated the methanolic solutions in a flash evaporator, dissolved

the residue in a small amount of water, and used portions of the solution for HPLC (11) and pool analyses by the DNA polymerase assay (11, 12).

Determination of mtDNA level by Southern Blotting—We grew lung fibroblasts to confluence, then shifted them to 0.1% FCS as described above and kept them in the presence or absence of nucleosides for up to 2 months. At the different time points shown in Fig. 5, starting from the day when FCS was reduced to 0.1% (day 0), we harvested the cells by trypsinization, washed them twice with phosphate-buffered saline, resuspended the pellet corresponding to 2 million cells in phosphate-buffered saline in Eppendorf tubes, and stored them at –20 °C. We extracted total cellular DNA subsequently as described for the Wizard Genomic DNA Purification kit (Promega), dissolved the DNA in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and quantified the amount in a Hoefer DyNA Quant200 fluorimeter (Amersham Biosciences) with calf thymus DNA as a standard.

We digested 2 μg of DNA from each sample with PvuII (New England BioLabs) to linearize mtDNA and electrophoresed 0.25 and 0.5 μg of total DNA on 0.8% agarose. After denaturation we blotted the gel onto Hybond N⁺ membrane (Amersham Biosciences) and hybridized the blot under standard conditions (13) with two separate probes, one at a time. The first probe detected a fragment of the gene encoding 18 S rRNA (14) and served as a loading control, the second was a probe for a mt fragment corresponding to nucleotide 2578–4122 kindly donated by Dr. N. G. Larsson (15). We stripped the filter before hybridization with the mt probe. We labeled both probes with [α-³²P]dCTP using the Rediprime Random Prime Labeling System (Amersham Biosciences). After washing, we exposed the membrane on a PhosphorScreen (PerkinElmer Life Sciences) and developed it in a Packard Cyclone Storage PhosphorSystem. We quantified signals with OptiQuant software. We normalized the signals for mtDNA relative to the 18 S rRNA signals to correct for loading variations. Results were proportional to the amount of total DNA loaded on the gel. All hybridizations were done at least twice at two DNA concentrations. Control cells maintained in low serum for up to two months did not change their mtDNA content. We always determined the amount of mtDNA in the experimental cells simultaneously with appropriate controls and express data as percentage of mtDNA relative to the mtDNA in the appropriate control.

Real-time PCR Quantitation of mtDNA—To further quantitate mtDNA relative to nDNA in cultured fibroblasts, real-time quantitative PCR assays were performed using the TaqMan® probe system and an ABI 7000 Sequence Detection system (Applied Biosystems Inc.) as described (3).

Restriction Fragment Length Polymorphism Analyses—Restriction fragment length polymorphism analyses were performed on DNA from cultured fibroblasts. For the T5814C mtDNA mutation (A5814G in the coding heavy strand), a 238-base pair (bp) mtDNA fragment spanning nucleotides 5784 and 6022 was amplified from total DNA using a forward mismatch primer (5'-CTTCTTCGAATTGCAATTCAATATGAGTA-3') (nucleotides 5714–5813; mismatched nucleotides underlined) and a reverse primer (5'-TCGGCTCGAATAAGGAGGCT-3') (nucleotides 6022–6003). The A5814G mutation introduces a RsaI site; the enzyme digests the 238-bp PCR prod-

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uct in the presence of the mutation into two bands of 28 and 210 bp. For the T16172C transition, a 228-bp mtDNA fragment spanning nucleotides 15975 to 16203 was amplified using a forward primer (5'-CTCCACCATTAGCAC-CCAAA-3') (nucleotides 15975–15994) and a reverse mismatch primer (5'-TTGTAAGCATGGGGAGGGGGTT-TTGATGCCG-3') (nucleotides 16203–16173; mismatched nucleotides underlined). The T16172C transition generates a HpaII site; the enzyme digests the 228-bp PCR product in the presence of the mutation into two bands of 198 and 30 bp. For both analyses, PCR cycle conditions were: one cycle at 94 °C for 5 min; 30 cycles 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and an extension cycle 72 °C for 7 min.

MtDNA Sequencing—The entire mtDNA was sequenced using overlapping PCR amplified mtDNA fragments as described (3).

Western Blotting—Cells were extracted with phosphate-buffered saline containing 1.5% SDS and a mixture of mammalian cell protease inhibitors for mammalian cell extracts (Sigma). After heating with mercaptoethanol, portions containing equal amounts of protein were separated by gel electrophoresis on 12% polyacrylamide gels, blotted to polyvinylidene difluoride membranes (Millipore), and probed with antibodies against cytochrome *c* oxidase subunit II (Molecular Probes) and porin (Calbiochem-Novabiochem). Immunoreactive material was detected by chemiluminescence (Amersham Biosciences) after incubation with anti-mouse IgG horseradish peroxidase conjugate.

Analytical Procedures—We determined the size and specific activities of dNTP pools by an enzymatic assay with DNA po-

lymerase (12) as modified and appended for the determination of specific activity (11). We separated dTTP, dTDP, and dTMP by thin layer chromatography on cellulose PEI sheets (J. T. Baker, Phillipsburgh, NJ) (11).

We monitored the incorporation of thymidine into DNA present in the cell residue remaining on the dishes after extraction with methanol. We dissolved the cells during 24 h at 37 °C in 2 ml of 0.3 M NaOH, precipitated portions of the alkaline lysate containing carrier salmon sperm DNA with 2 ml of 10% HClO₄, filtered them onto GFC glass microfiber filters (Whatman), and determined the radioactivity by liquid scintillation counting.

RESULTS

Thymidine Increases the dTTP Pool—We studied how the presence of thymidine plus deoxyuridine in the medium affected cell growth and the size of pyrimidine dNTP pools of cultured human fibroblasts. As both nucleosides were degraded by thymidine phosphorylase in cells, we supplemented them in the culture medium daily during cell growth. By monitoring the level of thymidine and deoxyuridine in the medium by HPLC we maintained their concentrations between 9 and 22 μM (Fig. 1B). The presence of the two nucleosides did not affect the growth rate of the cells, which reached confluence on the 6th day (Fig. 1A). At the second and 7th day we prepared cytosolic and mt fractions and determined the size of pyrimidine dNTP pools (Table 1). The first sample was from cycling cells (15% S phase), the second was from quiescent cells (0.7% S phase). Skin fibroblasts grow relatively slowly and cycling cells contain a smaller percentage of S phase cells than lung fibroblasts or cancer cells used in our previous pool determinations (10, 11), nevertheless, the present pool analyses gave comparable values. We found striking increases in the size of the dTTP pool in the presence of deoxynucleosides, both in the cytosol and mitochondria. In cycling cells the dTTP pool increased in both compartments almost 2-fold, whereas in quiescent cells it increased 8.5-fold in mitochondria and 5.5-fold in the cytosol (Table 1). Thus the relative increase was much larger in quiescent cells. The absolute increase was, however, larger in cycling cells given their larger pool size. The dCTP pool in mitochondria was not affected by the nucleosides, but was somewhat decreased in the cytosol of quiescent cells. In general, the results for both pools resemble earlier data from lung fibroblasts (11).

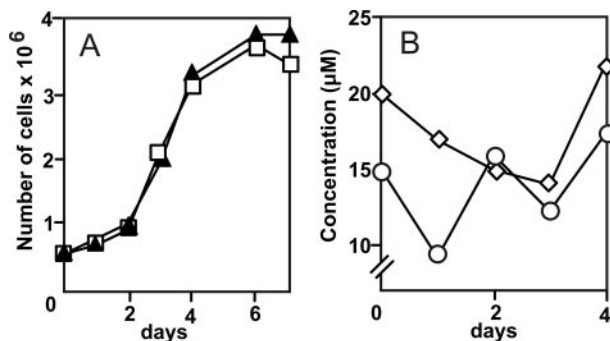


FIGURE 1. Effects of deoxynucleosides during growth of skin fibroblasts. A, time course for cell growth with (□) or without (▲) deoxynucleosides in the medium. B, variations in thymidine (◇) and deoxyuridine (○) concentrations in the medium during growth. The deoxynucleosides were added to the medium each day to maintain their concentration.

TABLE 1

Size of pyrimidine dNTP pools of skin fibroblasts growing in the presence of thymidine + deoxyuridine

We analyzed cells on the second (cycling cells) and seventh day (quiescent cells) from the treated and control cultures shown in Fig. 1.

	dTTP			dCTP		
	Control	TdR + UdR	-Fold increase	Control	TdR + UdR	-Fold increase
	pmol/million cells			pmol/million cells		
Mitochondria						
Cycling cells ^a	1.9	3.3	1.7	2.0	1.9	0.95
Quiescent cells ^b	0.22	1.9	8.6	0.33	0.39	1.20
Cytosol						
Cycling cells ^a	84	148	1.8	64	67	1.05
Quiescent cells ^b	1	5.4	5.4	3.2	1.7	0.53

^a 15% cells in S phase.

^b 0.7% cells in S phase.

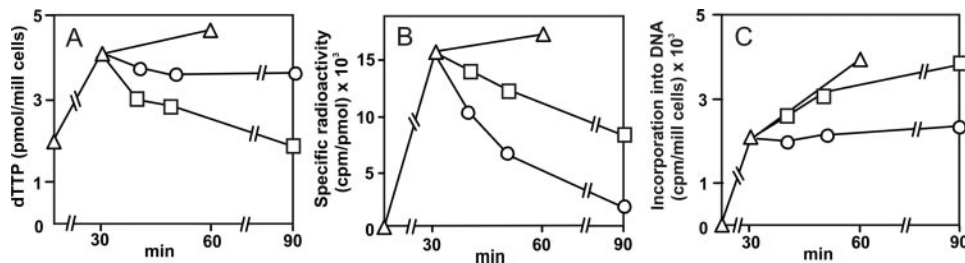


FIGURE 2. **Pulse-chase experiment.** Lung fibroblasts were incubated with $1 \mu\text{M}$ [^3H]thymidine, specific radioactivity 20,000 cpm/pmol (Δ). After 30 min the radioactive medium was in some cultures replaced with fresh medium containing either no thymidine (\square) or with medium containing $1 \mu\text{M}$ cold thymidine (\circ) and incubation was continued for 1 h. A, size of dTTP pool; B, specific radioactivity of dTTP pool; C, incorporation of radioactivity into total (nuclear + mitochondrial) DNA.

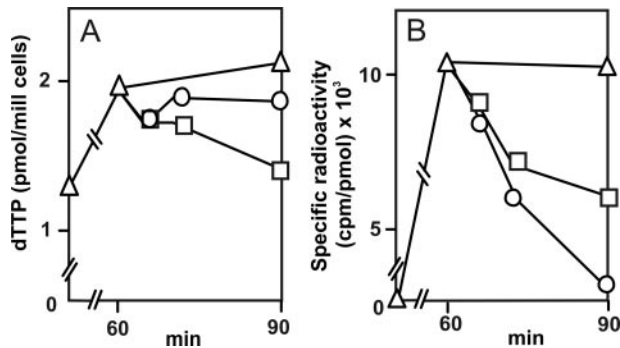


FIGURE 3. **Pulse-chase experiment with $0.1 \mu\text{M}$ [^3H]thymidine.** We carried out this experiment and the one described in Fig. 4 similar to the experiment described in the legend to Fig. 2 but with different concentrations of [^3H]thymidine and in a different time frame, with the chase starting at 60 min. Δ , continuous presence of radioactive thymidine; \circ , chase in the presence of $0.1 \mu\text{M}$ cold thymidine; \square , the chase without cold thymidine. A, size of the dTTP pool; B, specific radioactivity of the dTTP pool.

The dTTP Pool Turns Over Rapidly—We next tested how the stability and turnover of the total dTTP pool was affected by the amount of thymidine in the medium. Initially, we labeled quiescent lung fibroblast cultures with $1 \mu\text{M}$ [^3H]thymidine for 30 min, removed the labeled thymidine, and chased the isotope in the dTTP pool during 1 h in two different ways: half of the cultures were incubated with fresh medium containing $1 \mu\text{M}$ non-labeled thymidine, the other half was incubated without thymidine. From the changes in the size and the specific radioactivity of the dTTP pool during the chase, we calculated how thymidine in the medium affected the turnover of the dTTP pool.

With thymidine present during the chase, the specific radioactivity of dTTP decreased rapidly, with a half-life of ~ 15 min (Fig. 2B). The loss of radioactivity was not caused by incorporation into nuclear plus mtDNA (Fig. 2C) or by a decrease in the size of the dTTP pool because the pool maintained its pre-chase size during the chase (Fig. 2A). Instead, the data indicate a rapid turnover of the dTTP pool where degradation and resynthesis balanced each other. Based on a half-life of 15 min (Fig. 2B) and a pool size of 4 pmol/million cells (Fig. 2A) we calculate that the turnover amounted to 0.13 pmol of dTTP/min per million cells.

In the absence of cold thymidine during the chase, the radioactivity disappeared more slowly (Fig. 2B) and the pool size decreased (Fig. 2A). Thus, in this case, resynthesis of dTTP did not compensate for its disappearance. During the first 20 min of the chase the dTTP pool decreased from 4 to 2.8 pmol

(=0.06 pmol/min) and lost a total of 28,000 cpm. Of these, 1,100 cpm were incorporated into DNA (Fig. 2C). The remaining $\sim 27,000$ cpm were lost by degradation of radioactive dTTP. To convert counts/min to picomoles, we divide 27,000 cpm by the average specific radioactivity of dTTP (14,000 cpm/pmol) arriving at a total of 1.9 pmol/20 min or 0.095 pmol/min total degradation of dTTP. Of these 0.095 pmol, 0.06 pmol are accounted

for by the decrease in the size of the pool. The difference between 0.095 and 0.06 indicates that ~ 0.035 pmol of dTTP were resynthesized each minute during the chase. Thus, there was a small but significant turnover of dTTP also in the absence of supplemental cold thymidine.

To evaluate how variations in the concentration of thymidine affect dTTP turnover we carried out two additional chase experiments: one at $0.1 \mu\text{M}$ thymidine, the other at $10 \mu\text{M}$ (the thymidine concentration found in the plasma of MNGIE patients). We labeled the quiescent cells with [^3H]thymidine for 60 min before the chase to ascertain that they had reached isotope equilibrium. In both experiments the chase medium contained either no thymidine or the same concentration of cold thymidine as that used for labeling. Fig. 3 depicts the results for $0.1 \mu\text{M}$, Fig. 4 the results for $10 \mu\text{M}$ thymidine, with *panel B* in each figure showing the specific radioactivity and *panel A* the size of the dTTP pool.

In both experiments the results of the chase were qualitatively similar to those in Fig. 2. During the labeling period, the size of the dTTP pool increased rapidly, 4-fold with $10 \mu\text{M}$ [^3H]thymidine and 1.4-fold with $0.1 \mu\text{M}$. At $10 \mu\text{M}$ the specific radioactivity of the pool was identical to that of the isotopic thymidine indicating that all dTTP was derived from thymidine in the medium. At $0.1 \mu\text{M}$ thymidine the specific radioactivity of dTTP was instead only 50% indicating dilution by non-labeled intracellular thymidine. During the chase with non-radioactive thymidine the specific radioactivity of the dTTP pool decayed with a half-life of 5 min at $10 \mu\text{M}$ and with a half-life of 12 min at $0.1 \mu\text{M}$. From these values, we calculated the turnovers of the dTTP pools as described for the previous experiment. They are summarized in Table 2, together with the earlier calculations.

The column of Table 2 entitled "Resynthesis" shows the actual turnover of the dTTP pool, *i.e.* the amount of the nucleotide that at each minute was resynthesized after having been degraded. This value was calculated from the disappearance of radioactivity from dTTP. Resynthesis of dTTP from thymidine involves the intermediate formation of dTMP and dTDP and it was important to ascertain that the turnover of each of these two nucleotides paralleled that of dTTP. For this purpose we determined the percentage of the total radioactivity of each of the three nucleotides after different times of the chase (Fig. 4C). As shown in the *inset* to the figure the total amount of radioactivity of the 3 pools decreased to 10% during the 30 min of the chase. About 80% of the radioactivity was present in dTTP, 15% in dTDP, and 5% in dTMP at all time points,

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demonstrating that the turnover simultaneously involved all three nucleotides, including the dTMP pool. The final degradation of dTMP presumably occurred by dephosphorylation by a 5'-deoxynucleotidase.

The amount of turnover increased with increasing concentrations of thymidine in the medium from 0.08 pmol/min at 0.1 μM thymidine to 0.57 pmol/min at 10 μM thymidine. MNGIE patients with large pools of intracellular deoxynucleosides therefore may have a high turnover of dTTP, and by analogy also of dUTP. The resynthesis of the two dNTPs from deoxynucleosides requires ATP indicating an increased consumption of ATP in all cells of the body. We will return to this point under "Discussion."

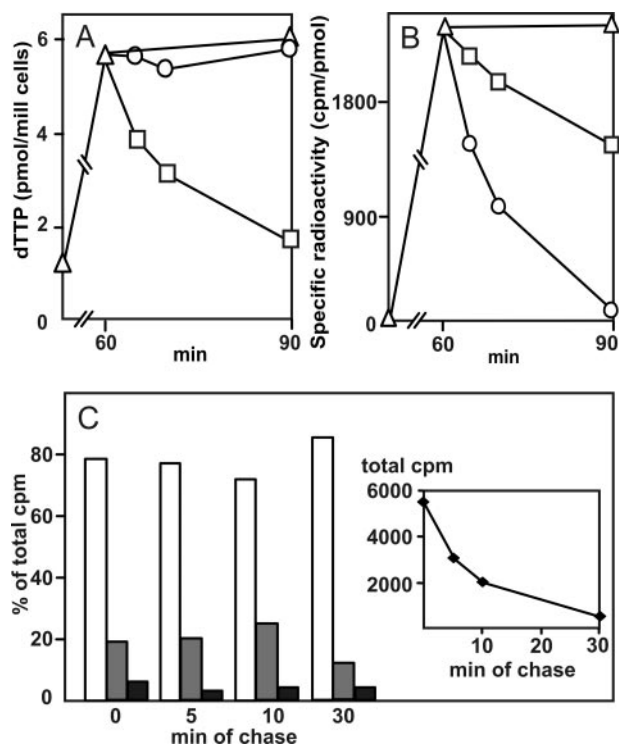


FIGURE 4. Pulse-chase experiment with 10 μM [^3H]thymidine. *A* (size of the dTTP pool) and *B* (specific radioactivity of the dTTP pool) show an experiment done under identical conditions as that in Fig. 3 except for the concentration and a decreased specific radioactivity of [^3H]thymidine (2,400 cpm/pmol). Δ , continuous presence of radioactive thymidine; \circ , chase in the presence of 10 μM cold thymidine; \square , chase without cold thymidine. *C* gives the % distribution of the total radioactivity in dTTP (open bars), dTDP (shaded bars), and dTMP (black bars) at different times during the chase in the presence of 10 μM cold thymidine. The inset shows the loss of total radioactivity from the sum of the three nucleotides during the chase.

TABLE 2

Effects of thymidine on the turnover of the dTTP pool of quiescent fibroblasts

In the experiments shown in Figs. 2–4 we pulsed quiescent cells with 3 different concentrations of [^3H]thymidine and then chased either with cold thymidine at the labeling concentration or without thymidine. From the determined pool size and specific radioactivity of dTTP we calculated the resynthesis of dTTP (=turnover) as described in the text. All data refer to one million cells.

[^3H]TdR	dTTP turnover						
	No TdR in chase			+TdR in chase			
	Total ^a	Pool decrease ^b	Resynthesis ^c	Total ^a	Pool decrease ^b	Resynthesis ^c	
		<i>pmol/min</i>				<i>pmol/min</i>	
0.1 μM	0.095	0.035	0.06	0.08	0	0.08	
1.0 μM	0.095	0.06	0.035	0.13	0	0.13	
10 μM	0.44	0.25	0.19	0.57	0	0.57	

^a Total degradation calculated from the half-life of radioactivity.

^b Loss of pmol of dTTP from pool.

^c Difference between footnotes a and b.

mtDNA Is Depleted during Treatment with Thymidine—In previous experiments (11) we maintained quiescent skin or lung fibroblasts with 10–40 μM thymidine and/or deoxyuridine in the medium for up to 43 days and found that the deoxynucleosides increased the intracellular dTTP and/or dUTP pools during the entire time period. We now report that in these and in further experiments the presence of thymidine (but not deoxyuridine) resulted in a depletion of mtDNA. When thymidine was removed from the depleted cells they rapidly recovered their normal level of mtDNA.

Altogether we carried out 4 experiments with quiescent lung fibroblasts and two experiments with quiescent skin fibroblasts. In each, we measured first by Southern blotting the amount of mtDNA. In control cultures, the prolonged quiescent state did not result in a depletion of mtDNA. However, in the presence of thymidine mtDNA declined. Fig. 5A summarizes combined results with lung fibroblast incubated for various times with deoxyuridine and/or thymidine. The figure shows the amount of mtDNA relative to control cells in the same experiment. Despite considerable scatter, it is evident that mtDNA was depleted in cultures containing thymidine alone or thymidine plus deoxyuridine but not in those with only deoxyuridine. In the latter case, there may even have been a slight increase. Fig. 5B demonstrates that for the first of the four experiments, we measured mtDNA (relative to nDNA) by real-time PCR and confirmed depletion of mtDNA in cells cultured in thymidine \pm deoxyuridine. Fig. 6 shows the time curve for one such experiment with both thymidine and deoxyuridine in the medium during a period of two months. Cells lost mtDNA continuously and after 64 days contained 56% of the original amount. At this point, we removed the deoxynucleosides from some cultures and continued incubation with 0.1% FCS in their absence. Already after 6 days, the mtDNA had increased and after 2 weeks was back to normal. The cells maintained contact inhibition and did not divide. This part of the experiment shows that depletion of mtDNA was fully reversible in quiescent cells.

In an additional experiment involving long term treatment of quiescent cultures with thymidine, deoxyuridine, or both, we removed the nucleosides, trypsinized the cells, and seeded them at low density in medium with 10% FCS to determine their growth capacity. Parallel control cells kept without deoxynucleosides were treated similarly. All cultures started to divide after a lag period of about 3 days and attained final den-

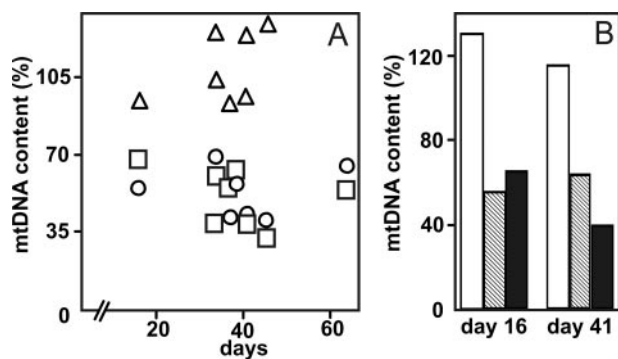


FIGURE 5. Time-dependent change of mtDNA. Quiescent lung fibroblasts incubated with thymidine (○), deoxyuridine (△), or thymidine + deoxyuridine (□). *A* gives results from four separate experiments where we incubated cells with deoxynucleosides for the indicated time periods and quantized their mtDNA by Southern blotting. Control cultures incubated for an identical time period but without deoxynucleosides were analyzed similarly. We express the results as the percentage of mtDNA of the experimental cells relative to their controls. *B* shows one of the experiments where we determined the decrease of mtDNA by real-time PCR instead of Southern blotting. *Open columns* represent incubation of the cells with deoxyuridine; *striped columns*, incubation with thymidine; and *black columns*, incubation with deoxyuridine + thymidine.

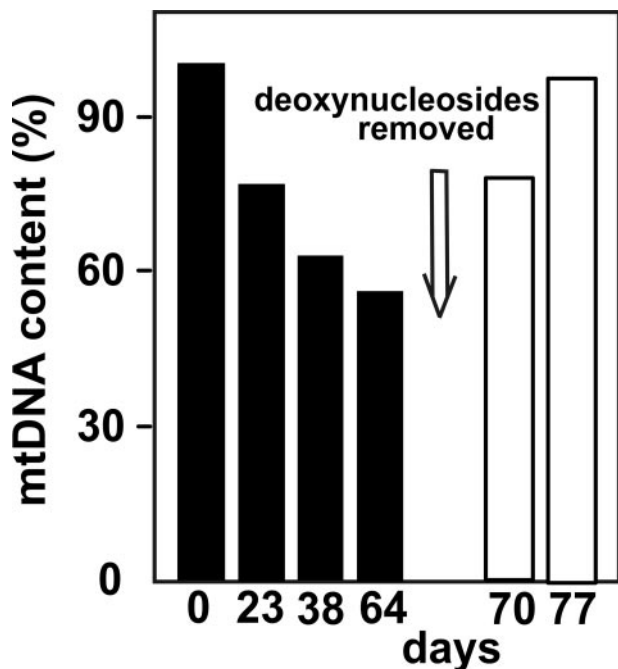


FIGURE 6. Time-dependent loss and recovery of mtDNA. We incubated lung fibroblasts in the presence of thymidine + deoxyuridine and analyzed the cells at time intervals as described in the legend to Fig. 5*A*. After 64 days we removed the deoxynucleosides from the medium and continued incubation for another 2 weeks. The *black columns* show the decrease of mtDNA at different times of incubation with deoxynucleosides, the *open columns* show the recovery of mtDNA after their removal.

sity after 9 days, without any growth difference between controls and cells treated with deoxynucleosides (data not shown). In a second experiment, we compared the growth of cells after 64 days quiescence with that of normal cycling cells (Fig. 7). The cells that had been quiescent had a longer lag phase and a slightly slower growth rate but both cultures reached the same final density. Taken together these results show that quiescent cells that had been kept up to 2 months in medium containing 0.1% dialyzed calf serum, with or without deoxynucleosides,

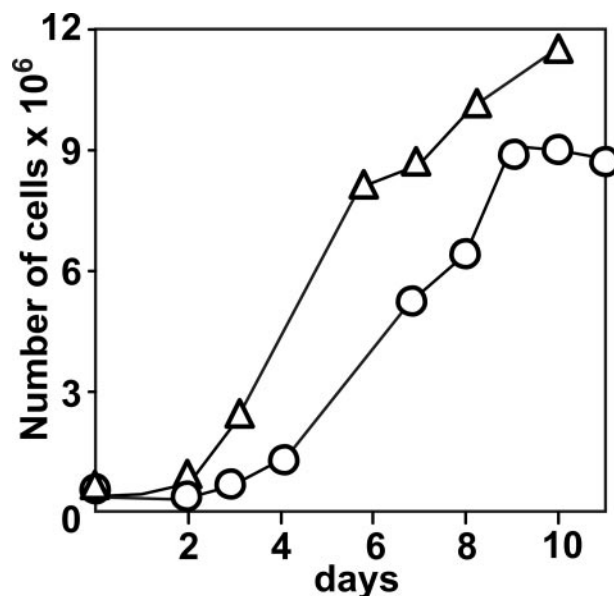


FIGURE 7. Growth curve of lung fibroblasts after quiescence. After a period of 64 days in 0.1% FCS medium we replated quiescent cells in fresh medium containing 10% FCS and determined their growth (○). A normal growth curve is given for comparison (△).

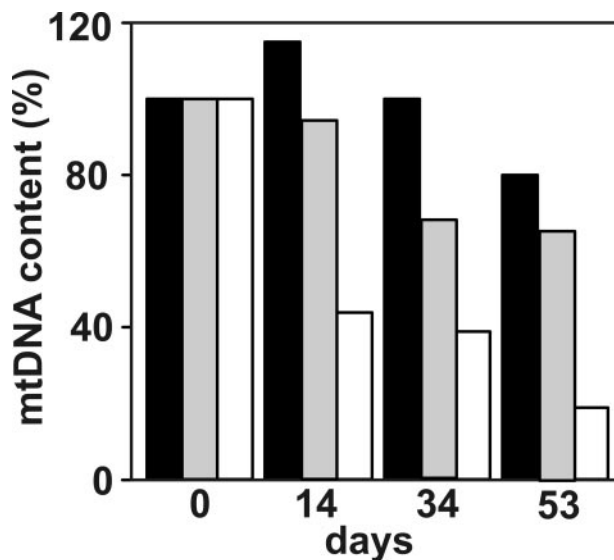


FIGURE 8. Effect of fetal calf serum on the loss of mtDNA. We maintained skin fibroblasts in culture with thymidine + deoxyuridine in media containing 3% FCS (*black bars*), 1% FCS (*shaded bars*), or 0.1% FCS (*open bars*) and determined the amount of their mtDNA at different times by Southern blotting.

retained viability with a growth capacity comparable with that of normal cells.

The aforementioned results described lung fibroblasts, but slower growing skin fibroblasts also developed depletion of mtDNA in similar experiments. We maintained quiescent cells with thymidine + deoxyuridine in media with 0.1, 1, or 3% FCS and, at fixed time points, measured their mtDNA. Appropriate controls of quiescent cells without deoxynucleosides were analyzed in parallel. Fig. 8 shows mtDNA of the deoxynucleoside-treated fibroblasts as percentage of the non-treated cells. We found no change with time with 3% FCS, a possibly slight decrease with 1% FCS, and a clear decrease with 0.1% FCS. After

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53 days the treated cells in 0.1% FCS contained only 20% of the control mtDNA suggesting a more profound depletion than in lung fibroblasts. The depletion of mtDNA in cells grown in 0.1% serum for 14 and 34 days was confirmed by real-time PCR (data not shown). In a second experiment with skin fibroblasts the mtDNA depletion was only 50% (data not shown) and more in line with the value from lung fibroblasts.

In addition we measured by Western blotting the levels of the mtDNA-encoded cytochrome *c* oxidase subunit II in extracts from quiescent lung fibroblasts treated for up to 46 days with thymidine and the corresponding signals from control cells kept for the same time in 0.1% FCS medium. All signals were normalized to that of porin, a mt protein encoded in nuclear DNA. We found no significant decrease in the signals from the mtDNA-depleted cells (data not shown).

mtDNA Shows No Deletions or Point Mutations—Samples of mtDNA extracted from cells treated and untreated with deoxynucleosides were screened by PCR for two species of deletions (5-kb “common” mtDNA deletion and an 8.1-kb deletion) and two point mutations (T5814C and T16172C) that were relatively abundant in tissues from MNGIE patients (4). We did not detect these mtDNA deletions or point mutations in quiescent skin fibroblasts cultured without nucleosides or with both deoxyuridine and thymidine for up to 53 days (data not shown). Similarly, we did not detect the deletions or point mutations in quiescent lung fibroblasts cultured without nucleosides or with both deoxyuridine and thymidine for 16 and 41 days (data not shown). The lower limit for detecting mtDNA point mutations is 2% heteroplasmy so very low levels of these mtDNA mutations cannot be completely excluded (4).

In addition, to screen for mtDNA point mutation in quiescent lung fibroblasts treated with thymidine plus or minus deoxyuridine, we sequenced both strands of the entire mtDNA. No differences of mtDNA were detected between cells cultured for 41 days with thymidine alone, with thymidine and deoxyuridine, or without supplemental nucleosides (data not shown). By direct sequencing, the detection limit for mtDNA point mutations is ~20% heteroplasmy; therefore, low levels of mtDNA mutations may have escaped detection.

DISCUSSION

To satisfy the requirements for building blocks for DNA replication and repair, the concentration of dNTPs is strictly regulated in eukaryotic cells by genetic controls and allosteric mechanisms. In mammalian cells (16, 17) as well as in yeast (18), deviations from the normal pool sizes of all 4 dNTPs or their relative proportions are mutagenic. In humans they can lead to genetic disease. The intracellular concentrations of dNTPs depend on the balance between their synthesis and degradation and their utilization for DNA replication, which occurs during a limited period during the life cycle of cells (S phase). In S phase the sizes of dNTP pools are 100 times or more larger than in quiescent cells (16), but a small amount of each dNTP is present also in quiescent cells, presumably to satisfy requirements for DNA repair and mtDNA synthesis. *De novo* synthesis from ribonucleotides via their reduction to deoxyribonucleotides (19) is the dominating pathway that provides and regulates dNTP pools for DNA replication in S phase. Quies-

cent cells contain minute amounts of two of the subunits (R1 and p53R2) of ribonucleotide reductase (20) but deoxyribonucleotides are largely produced by a second pathway (9), the phosphorylation of deoxynucleosides by appropriate kinases (21, 22). Regulation of pool sizes then occurs by catabolic 5'-nucleotidases (10, 23) and phosphorylases (24, 25). The second synthetic pathway also exists in S phase cells but there it is largely overshadowed by ribonucleotide reduction (9).

Malfunction or loss of enzymes of the second pathway lead to genetic diseases affecting the function of mtDNA. Considering the above outline of dNTP metabolism we hypothesized that the enzyme defects primarily have consequences for mtDNA synthesis in quiescent cells. Support for this concept comes from model experiments (26) with cells from a patient with a dysfunctional deoxyguanosine kinase, a synthetic enzyme of the second pathway.

In MNGIE thymidine phosphorylase, a catabolic enzyme of the second pathway is non-functional resulting in the accumulation of intracellular dTTP. We demonstrated that quiescent fibroblasts permanently exposed in culture to an excess of thymidine in the medium maintained a considerably enlarged intracellular dTTP pool (11). In similar experiments with deoxyuridine, the cells, due to the presence of dUTPase, contained a large dUMP pool and a very small dUTP pool. We hypothesized (11) that such fibroblasts may be considered as a model system for MNGIE. Here, we investigated the consequences of the enlarged pools on the maintenance and integrity of mtDNA and on the dynamics of the dTTP pool.

In both skin and lung quiescent fibroblasts the enlarged dTTP pool caused a depletion of mtDNA without leading to noticeable deletions or point mutations. The absence of mtDNA deletions in cultured fibroblasts is not surprising because these alterations of mtDNA have never been identified in cultured cells of patients with MNGIE or other autosomal diseases with multiple deletions of mtDNA (27). Instead, in these mendelian disorders, multiple deletions of mtDNA accumulate over many years in post-mitotic tissues such as skeletal muscle (4). In contrast, site-specific point mutations of mtDNA have been detected in replicating and quiescent cells of MNGIE patients (4). The levels of heteroplasmy of the point mutations is generally low (usually <10% for each change); however, the T5814C transition has been detected at levels up to 81% in cultured fibroblasts and the T16172C substitution at 63% in ovarian tissue from MNGIE patients (4). Most of the somatic mtDNA mutations detected in MNGIE patients have been 5'-AAT to 5'-AAC changes, which have been attributed to misincorporation of dGMP opposite template thymine bases followed by the “next nucleotide effect” (increased dTTP levels causing acceleration of mitochondrial DNA polymerase γ and impaired exonuclease proofreading function) (4). Presumably, these site-specific mtDNA mutations accumulate over many years in MNGIE because most patients begin to manifest symptoms in their late teenage years; therefore, cultured cells may need to be treated with thymidine plus or minus deoxyuridine for many months or years before developing somatic mtDNA mutations. Furthermore, endogenous thymidine phospho-

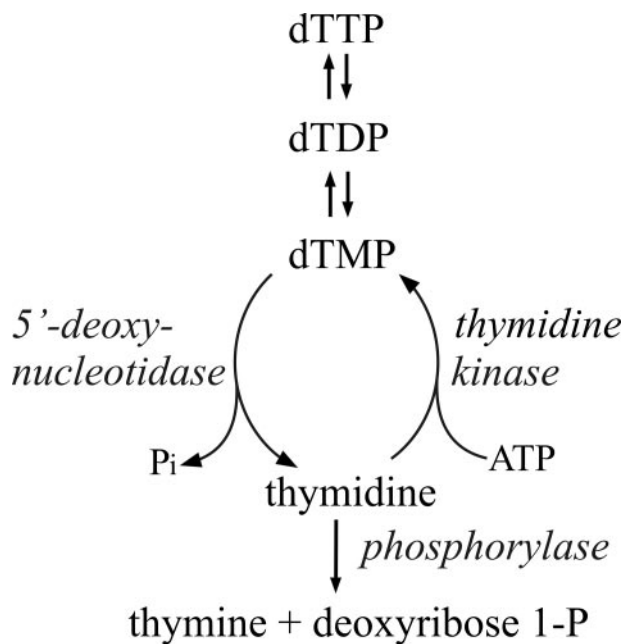


FIGURE 9. Scheme for the turnover of thymidine phosphates in quiescent cells. dTMP is formed by phosphorylation of thymidine (*thymidine kinase*) with ATP as the phosphate donor, followed by two transphosphorylation steps to give dTTP. Degradation of dTTP to dTMP occurs by transphosphorylations in the opposite direction and is followed by dephosphorylation of dTMP to thymidine (*5'-deoxynucleotidase*). The kinase and the deoxynucleotidase form a substrate (= futile) cycle that affects the size of the dTMP pool and, via the two transphosphorylation steps, the size of the dTTP pool. Normally, thymidine is degraded by *thymidine phosphorylase* and continuously removed from the cycle. MNGIE patients lack a functional phosphorylase. Thymidine accumulates, leading to a different dynamic equilibrium of the substrate cycle with a higher concentration of dTMP. Now, both the synthesis and the degradation of dTMP are accelerated. As a consequence the cycle consumes larger amounts of ATP.

rylase in cultured fibroblasts may be ameliorating the toxic effects of exogenous nucleosides.

In our experiments mtDNA was depleted only in fully quiescent fibroblasts maintained in 0.1% FCS supporting the idea that an increased dTTP pool affects mtDNA in resting but not in cycling cells and suggesting that this also applies to the situation in MNGIE. The mechanism for this effect remains unknown. Our results are somewhat reminiscent of earlier experiments (26) in which quiescent deoxyguanosine kinase-deficient fibroblasts from a patient with hepatocerebral mtDNA depletion syndrome during prolonged culture depleted their mtDNA. Depletion of mtDNA did not occur in cycling cells. Quiescent cells also incorporated less bromodeoxyuridine into mtDNA than cells in S phase. Presumably the *de novo* synthesis of dGTP in resting cells did not suffice to sustain mtDNA synthesis in the absence of mt deoxyguanosine kinase. In that case it was the lack of a dNTP that caused depletion of mtDNA, whereas in our experiments it was the excess of a dNTP. In both instances the depletion occurred only in resting cells and affected only mtDNA. Only when the residual level of mtDNA in patient cells fell to about 30% was cytochrome *c* oxidase significantly reduced (26). In our wt fibroblasts the 50% depletion of mtDNA induced by thymidine (Fig. 5) was clearly not sufficient to lead to depletion of cytochrome *c* oxidase.

In our pulse experiment with 0.1 μM thymidine the final specific radioactivity of dTTP was half that of the administered

thymidine. From this dilution we estimate that quiescent cells maintain a concentration of $\sim 0.1 \mu\text{M}$ endogenous thymidine. Addition of 10 μM thymidine to the medium thus increased the thymidine pool 100-fold but led to only a 5-fold increase in the size of the dTTP pool (Fig. 4A). An explanation for this comes from the chase experiments that demonstrated that expansion of the dTTP pool was limited by degradation (Figs. 2–4). dTTP is in equilibrium with dTMP and the degradation occurred at the level of the monophosphate, probably by the two specific nucleotidases cdN and mdN (23) that function in substrate cycles (9, 10) with thymidine kinases (Fig. 9). Both deoxynucleotidases have high K_m values for dTMP (28) and their activity therefore increases directly in proportion to the concentration of dTMP. When thymidine phosphorylase is present this enzyme removes thymidine from the system and affects the balance between dTMP and thymidine, the two substrates of the substrate cycle. In the absence of a functional enzyme the concentration of thymidine increases enormously with repercussions for the dTTP pool. A similar situation can be envisaged for a dUMP/deoxyuridine cycle. In MNGIE the deoxyribonucleotidases of the substrate cycles provide barriers against extensive expansions of the dUMP and dTMP pools. As a consequence it can be expected that increased amounts of ATP (Fig. 9) are continuously consumed in the body of patients.

REFERENCES

- Nishino, I., Spinazzola, A., and Hirano, M. (1999) *Science* **283**, 689–692
- Papadimitriou, A., Comi, G. P., Hadjigeorgiou, G. M., Bordoni, A., Sciacco, M., Napoli, L., Prella, A., Moggio, M., Fagiolarì, G., Bresolin, N., Salani, S., Anastasopoulos, I., Giassakis, G., Divari, R., and Scarlato, G. (1998) *Neurology* **51**, 1086–1092
- Nishigaki, Y., Marti, R., and Hirano, M. (2004) *Hum. Mol. Genet.* **13**, 91–101
- Nishigaki, Y., Marti, R., Copeland, W. C., and Hirano, M. (2003) *J. Clin. Invest.* **111**, 1913–1921
- Spinazzola, A., Marti, R., Nishino, I., Andreu, A., Naini, A., Tadesse, S., Pela, I., Zammarchi, E., Donati, M. A., Oliver, J. A., and Hirano, M. (2002) *J. Biol. Chem.* **277**, 4128–4133
- Marti, R., Nishigaki, Y., and Hirano, M. (2003) *Biochem. Biophys. Res. Commun.* **303**, 14–18
- Bjursell, G., and Reichard, P. (1973) *J. Biol. Chem.* **248**, 3904–3909
- Song, S., Wheeler, L. J., and Mathews, C. K. (2003) *J. Biol. Chem.* **278**, 43893–43896
- Pontarin, G., Gallinaro, L., Ferraro, P., Reichard, P., and Bianchi, V. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 12159–12164
- Rampazzo, C., Ferraro, P., Pontarin, G., Fabris, S., Reichard, P., and Bianchi, V. (2004) *J. Biol. Chem.* **279**, 17019–17026
- Ferraro, P., Pontarin, G., Crocco, L., Fabris, S., Reichard, P., and Bianchi, V. (2005) *J. Biol. Chem.* **280**, 24472–24486
- Sherman, P. A., and Fyfe, J. A. (1989) *Anal. Biochem.* **180**, 222–226
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Chabi, B., Mousson de Camaret, B., Duborjal, H., Issartel, J. P., and Stepien, G. (2003) *Clin. Chem.* **49**, 1309–1327
- Larsson, N. G., Holme, E., Kristiansson, B., Oldfors, A., and Tulinius, M. (1990) *Pediatr. Res.* **28**, 131–136
- Reichard, P. (1988) *Annu. Rev. Biochem.* **57**, 349–374
- Kunz, B. A., and Kohalmi, S. E. (1991) *Annu. Rev. Genet.* **25**, 339–359
- Chabes, A., and Thelander, L. (2003) *Cell Cycle* **2**, 171–173
- Jordan, J., and Reichard, P. (1998) *Annu. Rev. Biochem.* **67**, 71–98
- Håkansson, P., Hofer, A., and Thelander, L. (2006) *J. Biol. Chem.* **281**, 7834–7841

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21. Berk, A. J., and Clayton, D. A. (1976) *J. Biol. Chem.* **248**, 2722–2729
22. Arnèr, E. S., and Eriksson, S. (1995) *Pharmacol. Ther.* **67**, 155–186
23. Bianchi, V., and Sychala, J. (2003) *J. Biol. Chem.* **278**, 46195–46198
24. Friedkin, M. R. (1954) *J. Biol. Chem.* **207**, 245–256
25. Cohen, A., and Barankiewicz, J. (1985) *Proc. Soc. Exp. Biol. Med.* **179**, 437–441
26. Taanman, J.-M., Muddle, J. R., and Muntain, A. C. (2003) *Hum. Mol. Genet.* **12**, 1839–1845
27. Carozzo, R., Hirano, M., Fromenty, B., Casali, C., Santorelli, F. M., Bonilla, E., DiMauro, S., Schon, E. A., and Miranda, A. F. (1998) *Neurology* **50**, 99–106
28. Mazzon, C., Rampazzo, C., Scaini, M. C., Gallinaro, L., Karlsson, A., Meier, C., Balzarini, J., Reichard, P., and Bianchi, V. (2003) *Biochem. Pharmacol.* **66**, 471–479