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Synthesis of Mitochondrial DNA Precursors during Myogenesis, an Analysis in Purified C2C12 Myotubes*[S]

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Background: In developing muscle, stimulation of mitochondrial biogenesis and mtDNA expansion occur with down-regulation of deoxynucleotide synthesis.
Results: siRNA silencing of mitochondrial thymidine or deoxyguanosine kinase impacts myotube differentiation causing depletion of mtDNA and of all four deoxynucleotides.
Conclusion: Shortage of even a single deoxynucleotide may upset the regulation of all DNA precursors.
Significance: Deoxynucleotide analysis in myotubes unveils unexpected outcomes of synthetic enzyme deficiencies.

During myogenesis, myoblasts fuse into multinucleated myotubes that acquire the contractile fibrils and accessory structures typical of striated skeletal muscle fibers. To support the high energy requirements of muscle contraction, myogenesis entails an increase in mitochondrial (mt) mass with stimulation of mtDNA synthesis and consumption of DNA precursors (dNTPs). Myotubes are quiescent cells and as such down-regulate dNTP production despite a high demand for dNTPs. Although myogenesis has been studied extensively, changes in dNTP metabolism have not been examined specifically. In differentiating cultures of C2C12 myoblasts and purified myotubes, we analyzed expression and activities of enzymes of dNTP biosynthesis, dNTP pools, and the expansion of mtDNA. Myotubes exibited pronounced post-mitotic modifications of dNTP synthesis with a particularly marked down-regulation of *de novo* thymidylate synthesis. Expression profiling revealed the same pattern of enzyme down-regulation in adult murine muscles. The mtDNA increased steadily after myoblast fusion, turning over rapidly, as revealed after treatment with ethidium bromide. We individually down-regulated p53R2 ribonucleotide reductase, thymidine kinase 2, and deoxyguanosine kinase by siRNA transfection to examine how a further reduction of these synthetic enzymes impacted myotube development. Silencing of p53R2 had little effect, but silencing of either mt kinase caused 50% mtDNA depletion and an unexpected decrease of all four dNTP pools independently of the kinase specificity. We suggest that during development of myotubes the shortage of even a single dNTP may affect all four pools through dysregulation of ribonucleotide reduction and/or dissipation of the non-limiting dNTPs during unproductive elongation of new DNA chains.

Myogenesis includes stimulation of mitochondrial (mt)[3] biogenesis to accommodate the high energy demands of muscle contraction (1, 2). Myogenesis and mt biogenesis are interconnected and defects in the latter affect the development of fully functional muscle fibers. The expansion of mtDNA copy number contextual to the increase of mt mass and its upholding during the whole life of the organism depend on the availability of dNTPs, the precursors for DNA synthesis. In a post-mitotic cellular environment, the pathways of dNTP synthesis have undergone profound changes, resulting in a reduced rate of dNTP production compared with that in proliferating cells. Mitochondrial diseases identified over the last decade demonstrate that skeletal muscle is exquisitely sensitive to defects in the synthesis and regulation of the dNTPs (3, 4). Two partially overlapping pathways produce dNTPs: (i) cytosolic *de novo* synthesis with ribonucleotide reduction as a rate-limiting step and (ii) salvage of deoxynucleosides by kinases distributed between the cytosol and mitochondria (Fig. 1) (5).

This enzyme network is more active when the need for dNTPs is highest, *i.e.* during nuclear DNA replication (6). Genes specifically induced at the onset of S-phase are those coding for the two subunits of ribonucleotide reductase, R1 and R2, and for cytosolic thymidine kinase (TK1) (7–9). Both R2 and TK1 are only transiently present in the cells, because a signal in their primary sequence leads to their degradation by regulated proteolysis during mitosis (10, 11). The basic structure of a functional ribonucleotide reductase consists of a homodimeric large subunit (R1) plus a homodimeric small subunit (R2), both of which are required for catalysis (12). Outside

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[S] This article contains supplemental Table 1.
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[3] The abbreviations used are: mt, mitochondrial; dNTP, deoxynucleoside triphosphate; dTMP, thymidine monophosphate; CdR, deoxycytidine; DC, differentiated culture; dGK, deoxyguanosine kinase; DM, differentiation medium; mtDNA, mitochondrial DNA; Myh3, embryonic myosin heavy chain 3; p53R2, p53-inducible R2 subunit of ribonucleotide reductase; PM, proliferation medium; R1, ribonucleotide reductase subunit 1; R2 ribonucleotide reductase subunit 2; TdR, thymidine; TK1, thymidine kinase 1; TS, thymidylate synthase.

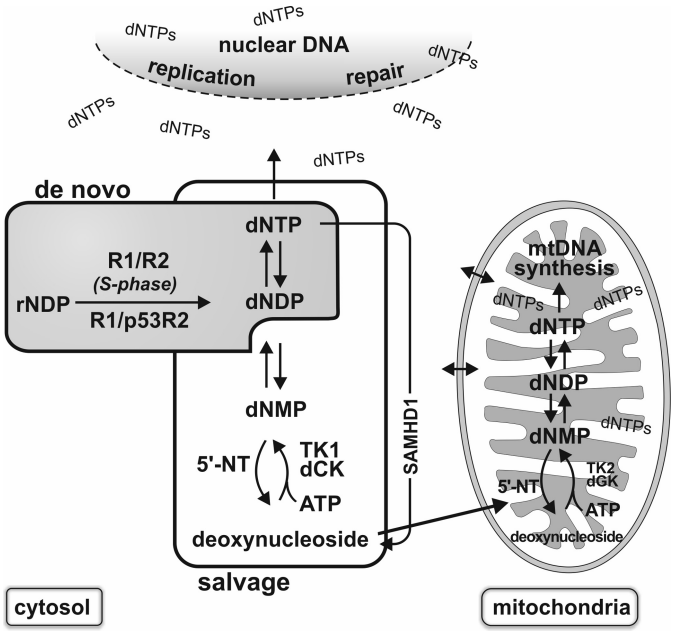


FIGURE 1. **Synthesis of DNA precursors in the cytosol and mitochondria.** The synthesis of dNTPs occurs by two pathways, ribonucleotide reductase-dependent *de novo* synthesis in the cytosol and salvage of deoxynucleosides, performed by two parallel sets of kinases in the cytosol and in mitochondria. Ribonucleotide reductase changes its subunit composition during S-phase (R1/R2) and outside S-phase or in non-dividing/differentiated cells (R1/p53R2). The first step of the salvage pathway is catalyzed by deoxynucleoside kinases TK1 and deoxycytidine kinase in the cytosol and TK2 and dGK in mitochondria. The R2 subunit of ribonucleotide reductase and TK1 appear at the beginning of S-phase and are degraded before completion of mitosis. Several catabolic activities participate in the regulation of the pools. The catabolic activity of 5′-nucleotidases (*5′-NT*) counteracts the irreversible reaction catalyzed by deoxynucleoside kinases creating regulatory "substrate cycles" both in the cytosol and mitochondria. The newly discovered deoxynucleotide triphosphohydrolase SAMHD1 converts dNTPs to deoxynucleosides (58). dNTPs move freely across the nuclear envelope creating a single pool of cytosolic and nuclear dNTPs, separated from mt dNTP pools by the impermeable mt inner membrane. Protein carriers in this membrane (*double-headed arrows*) establish a bidirectional communication between the two pools. The dNTPs are used in the nucleus and in mitochondria for DNA replication and repair.

S-phase p53R2, an alternative form of R2 devoid of degradation signal, replaces R2 permitting ribonucleotide reduction to continue, although at a substantially lower rate, during the whole cell cycle and in non-dividing cells (13, 14). As indicated by its name, p53R2 is p53-inducible, and its level increases after DNA damage (15, 16). Its active participation to UV-induced excision repair in quiescent human fibroblasts where R2 is absent has been recently demonstrated in our laboratory (17, 18). However, a major physiological function of p53R2 is the production, together with R1, of precursors for mtDNA maintenance. Genetic inactivation of p53R2 in humans and mice leads to severe depletion and structural instability of mtDNA in skeletal muscle and other differentiated tissues (19, 20).

The salvage pathways also experience important modifications in post-mitotic cells. Cytosolic deoxycytidine kinase and mitochondrial TK2 and deoxyguanosine kinase (dGK) are constitutively expressed (21), but cytosolic TK1 undergoes regulated degradation during mitosis (11), removing from the cells the main kinase activity responsible for thymidine and deoxyuridine phosphorylation. The expression of the other three kinases presents varying degrees of tissue-specificity and is not totally independent of the proliferation state. Differentiated tissues may therefore differ markedly in their ability to produce dNTPs. The role of the three "constitutive" kinases is important in non-cycling cells, as indicated by the pathological phenotypes arising from their genetic deficiency (22–24), particularly for TK2 and dGK. The phenotype of TK2 deficiency is specifically myopathic, that of dGK deficiency although mostly hepatopathic may also present myopathic traits (25).

A number of parameters of mt biogenesis have been monitored during muscle differentiation (1, 2, 26). However, the changes taking place in dNTP biosynthesis and pool sizes during early steps of muscle differentiation have not been examined in detail. Few of the genes coding for the relevant enzymes vary above the threshold of microarray expression analyses and the low amounts of the individual proteins escape detection in proteomic surveys (27–29). Enzyme assays in protein extracts from animal tissues (30) may be biased by the presence in the tissues of multiple cell types. Thus, the features underlying the distinctive sensitivity of skeletal muscle to genetic defects of enzymes of dNTP metabolism are only incompletely understood, and the consequences of the enzyme deficiencies for the availability of precursors during mt biogenesis are largely unknown. Determination of the dNTP pool sizes of muscle fibers *in vivo* is technically challenging and impracticable in the case of patients with severe myopathies (31).

We have chosen cultures of C2C12 murine myoblasts to study how the enzyme network regulating dNTP synthesis changes during the initial phases of myogenesis and how a down-regulation of enzymes involved in mtDNA depletion syndromes affects the differentiation of myotubes and the expansion of mtDNA. Differentiated cultures of C2C12 cells contain a significant residual fraction of mononucleated cells whose dNTP pool sizes and enzymatic setup differ from those of differentiated myotubes. To avoid the confounding influence of non-fused cells, we devised a protocol for the purification of differentiated myotubes. In proliferating myoblasts, differentiated cultures, and isolated myotubes, we compared the expression and activities of key enzymes of dNTP synthesis and the size of the four dNTP pools, demonstrating a low abundance of dNTPs in myotubes and a low expression of their biosynthetic pathways. By siRNA transfection, we individually down-regulated TK2, dGK, or p53R2 to examine how a further reduction of synthetic activities impacts the development of multinucleated myotubes.

Unexpectedly, knockdown of TK2 or dGK produced a depletion of all four dNTPs in the myotubes. We suggest that shortage of either pyrimidine (TK2 silencing) or purine (dGK silencing) dNTPs interferes with the allosteric regulation of ribonucleotide reduction and/or leads to repeated initiation of abortive DNA molecules, with dissipation of the non-limiting dNTPs.

EXPERIMENTAL PROCEDURES

C2C12 Cell Culture and Differentiation

Mouse C2C12 myoblasts were purchased from ATCC (Manassas, VA) and grown at 37 °C in a controlled humidified 5% CO_2 atmosphere. They were maintained in gelatin-coated

plates in proliferation medium (PM) (Dulbecco's modified Eagle's medium supplemented with 10% FCS) up to 60% confluence. To initiate differentiation, cells were allowed to reach 90% confluence, and the medium was changed to differentiation medium (DM, the same as PM but with 2% horse serum). DM was renewed daily. The time of shift to DM was considered day 0 of differentiation. Myotube fusion and spontaneous twitching appeared after 4–8 days.

Evaluation of Myogenic Differentiation

Myogenic differentiation was assessed either by monitoring the expression of the mRNAs for myogenin and embryonic myosin (Myh3) as described below, or by determining the fusion index, *i.e.* the percentage of nuclei inside myotubes containing ≥3 nuclei in a sample of at least 2000 nuclei. To score fusion indexes, cells grown in DM on μ-Dish 35-mm plates (Ibidi GmbH, Germany) were washed four times with phosphate-buffered saline (PBS) and fixed for 15 min in methanol at −20 °C. After three washes in PBS, cells were permeabilized 10 min in Triton X 0.2% and stained with mouse anti-myosin heavy chain monoclonal antibody (1:30; MF20, Developmental Studies Hybridoma Bank) and donkey anti-mouse Alexa Fluor 488 secondary antibody (1:20; Invitrogen) using the MAXpack immunostaining media kit (Active Motif). Coverslips were mounted with Vectashield (Vector Laboratories) containing 0.02 μg/ml DAPI. Images were acquired and analyzed on an epifluorescent microscope (Leica 5000).

BrdU Incorporation

Differentiated C2C12 cultures grown in μ-Dishes were incubated for 30 min with 30 μM BrdU and fixed with 50 mM glycine, pH 2, and 70% ethanol for 20 min at −20 °C. The plates were incubated with primary anti-BrdU antibody and fluorescein-conjugated anti-mouse Ig following the manufacturer's instructions (5-bromo-2′-deoxy-uridine) labeling and detection kit 1 (Roche Applied Science) and stained with DAPI as above.

Purification of Myotubes

Myotubes were separated from the mononucleated cells present in fully differentiated cultures (DC) on the basis of their different adhesion properties. Cultures were trypsinized gently (0.05% trypsin-EDTA) for 5 min at 37 °C. The detached cells were resuspended in fresh PM and plated in a new 5-cm Petri dish. After 30 min at 37 °C, the material still floating consisted mostly of myotubes that were transferred to a second Petri dish to allow the mononucleated cells to attach to the plate during a second 30-min incubation at 37 °C. The floating myotubes were collected and used for further analyses.

Gene Silencing by siRNA Transfection

Stealth (si)RNA oligonucleotides targeting mouse TK2 (MSS250034, MSS250036), dGK (MSS248717, MSS248718), and p53R2 (MSS283984, MSS283986), and control oligonucleotides (Stealth RNAi siRNA negative control) were from Invitrogen. Transfections were carried out using RNAiMax reagent (Invitrogen) according to the manufacturer's instructions. 0.1×10^6 cells were seeded in 10-cm Petri dishes in PM and transfected after 24 h with 30 nM TK2 or dGK siRNA or 20 nM p53R2 siRNA or with 30 nM negative control siRNA. After 48 h, when the cells reached 90% confluence, the medium was changed to DM, and the cells were retransfected with the above dose of siRNA (day 0 of differentiation). 50% medium was replaced after further 48 h and completely renewed daily for the remaining days.

RNA Extraction

Total RNA was extracted from proliferating and differentiated myoblasts or mouse tissue with TRIzol reagent (Invitrogen). CD1 mice (Charles River) were sacrificed by cervical dislocation at three months of age. The extensor digitorum longus (fast glycolytic) and soleus (slow oxidative) muscles from both hind limbs were immediately removed by microdissection and dispersed in 1 ml of TRIzol. The tissue was homogenized with an ultra-turrax-T8 blender (IKA-Werke), and total RNA was isolated according to a standard protocol. The integrity of the RNA was confirmed by analyzing a small aliquot in an Agilent 2100 Bioanalyzer using the RNA 6000 LabChip kit (Agilent Technologies). Mouse procedures were approved by the competent authority of the University of Padova and authorized by the Italian Ministry of Health.

mRNA Expression Analysis by Real-time RT-PCR

We used the Applied Biosystem 7500 real-time PCR system to quantify by real-time RT-PCR the mRNA expression of the three ribonucleotide reductase subunits (R1, R2, and p53R2), TK1, TK2, dGK, thymidylate synthase, Myh3, and myogenin. We prepared cDNAs by reverse transcription and performed real-time PCR assays in 96-well optical plates as described previously (32). To normalize the expression of individual target genes, we used Rplp0 (ribosomal protein large P0) as suggested by Stern-Straeter *et al.* (33). Rplp0, Myh3, and dGK mRNAs were analyzed by Quantitect Primer Assay kit (Qiagen). Other primers used were as follows: for R1 (5′-CCCAATGAGTGTCCTGGTCT-3′ and 5′-GTTCTGCTGGTTGCTCTTCC-3′), R2 (5′-CCTACTAACCCCAGCGTTGA-3′ and 5′-GTTTCAGAGCTTCCCAGTGC-3′), p53R2 (5′-ACACACGCACACACCACCTGTA-3′ and 5′-TGACAAATGGGAAGCACAGAGC-3′), TK1 (5′-ACAAGTGCCTGGTCATCAAGTA-3′ and 5′-ACAATTACTGTCTTGCCCTCGT-3′), TK2 (5′-TGTCCTTGTGTGAGAGAGCAGT-3′ and 5′-CTCCAGGGTATACGGTCATCAT-3′), thymidylate synthase (5′-ATGTGGTGAATGGGGAACTGT-3′ and 5′-GAGCTTTGGGAAAGGTCTTGG-3′), and myogenin (5′-CCAACCCAGGAGATCATTTG-3′ and 5′-ACCCAGCCTGACAGACAATC-3′). Each cDNA preparation was analyzed in triplicate. Data were processed by the comparative C_t method (34).

Microarray Analysis of Gene Expression

Sample Labeling—Cy3-labeled experimental cRNA samples were generated using the Quick Amp labeling kit, One-Color (Agilent Technologies), in the presence of RNA Spike-In controls, following the manufacturer's instructions. Briefly, 1 μg of total RNA was used to generate first-strand cDNA (2 h at 40 °C), and enzyme was then inactivated (15 min at 65 °C). Synthesis of the labeled cRNA was performed by run-off *in vitro*

transcription (2 h at 40 °C), and the linearly amplified cRNA was purified on Qiagen RNeasy mini columns.

Hybridization—1.65 μg of labeled cRNA was fragmented (30 min at 60 °C) before hybridization on the 44,000 mouse oligonucleotide arrays (Agilent Technologies, G4122F), according to the manufacturer's instructions. Fragmented cRNA was mixed with an equal volume of 2× GEx hybridization buffer HI-RPM (Agilent Technologies), and 100 μl were loaded onto the subarray. Incubation proceeded for 17 h at 65 °C at 10 rpm in a microarray hybridization oven (Agilent Technologies). Finally, slides were washed for 1 min at 22 °C in wash solution 1 and for 1 min in wash solution 2, prewarmed to 37 °C.

Microarray Data Processing—Microarray slides were inserted into a GenPix 4000B scanner (Agilent Technologies) for fluorescence detection at 5-micron resolution. Information was extracted from the scanned image using the Feature Extraction Software (Agilent Technologies) that also performed intra-array data normalization. The Expander software was applied to quantile normalize the complete series of experiments (35). The heat map was constructed with MultiExperiment Viewer (MeV, version 4.7.4), a part of the TM4 Microarray software suite (36). All microarray data are MIAME (Minimum Information About a Microarray Experiment) compliant. Raw and normalized data are available in the GEO database (accession no. GSE41877).

mtDNA Quantification

We determined the mouse mtDNA copy number with the TaqMan probe system and Applied Biosystem 7500 real-time PCR as described in Andreu *et al.* (37). Genomic DNA was extracted by Puregene Core kit B (Qiagen) from samples of myoblasts, differentiated cultures, and purified myotubes. The mitochondrial cytochrome *c* oxidase subunit I TaqMan probe 6FAM-5′-TACTACTAACAGACC-3′-MGB (Applied Biosystems) and primers cytochrome *c* oxidase subunit I (forward) (5′-TGCTAGCCGCAGGCATTACT-3′) and cytochrome *c* oxidase subunit I (reverse) (5′-CGGGATCAAAGAAAGTTGTGTTT-3′) were used to quantify mtDNA. For nuclear DNA, we used the actin β (ACTB) primers and probe contained in TaqMan predeveloped assay (Applied Biosystems). mtDNA and nuclear DNA were determined using calibration curves generated by serial dilution of a mixture of plasmids carrying the two PCR amplicons. Each DNA sample has been analyzed in triplicate.

Enzymatic Assays

Total cell extracts were prepared as described previously (38) by adding a protease inhibitor mixture (Roche Applied Science) to the lysis buffer. Protein concentration was measured by the colorimetric procedure of Bradford (39) with BSA as standard. All enzymatic assays were run with two different aliquots of extracts to check for proportionality. TK1 and TK2 activities were assayed using 1 μM [^{3}H]TdR or 0.2 μM [^{3}H]bromovinyl deoxyuridine, respectively, as described previously (40). In extracts of TK2-silenced myotubes, TK2 activity was measured using 1 μM [^{3}H]TdR instead of [^{3}H]bromovinyl deoxyuridine to increase the sensitivity of the assay, in the presence or absence of the TK2 inhibitor KIN109 (100 μM). TK2 activity corresponded to the difference between the activities measured under these two assay conditions. dGK and deoxycytidine kinase were tested as described in Leanza *et al.* (41), with some modifications. Namely, dGK activity was measured with 1 μM [^{3}H]deoxyguanosine in the presence of 0.5 mM deoxycytidine (CdR) to inhibit deoxycytidine kinase. The activity of deoxycytidine kinase was determined with 1 μM [^{3}H]CdR in the presence of 15 μM tetrahydrouridine to inhibit CdR deaminase (42). We express enzyme activities as pmol product min^{-1} mg^{-1} protein extract.

dNTP Pool Analysis

dNTP pools were extracted from cell cultures with 60% ice-cold methanol as described earlier (43). The cells remaining after extraction were dissolved in 2 ml of 0.3 M NaOH. The $A_{260\,nm}$ of the NaOH fraction containing the cellular macromolecules left after pool extraction was used as an index of cell mass (44). The sizes of the dNTP pools were determined with a DNA polymerase-based assay (45) with the modifications reported in Ferraro *et al.* (46) Two different aliquots of each pool extract were analyzed, and pool sizes were normalized by the $A_{260\,nm}$ of the NaOH lysates.

Isotope Experiments

The procedures for the isotope experiments and determination of the specific radioactivity of dNTP pools were described earlier (14, 43). Before adding either 0.03–0.3 μM [6-^{3}H]deoxycytidine (5,000 cpm/pmol) or 0.025–1 μM [5-methyl-^{3}H]thymidine (22,000 cpm/pmol) for the indicated times, we substituted the medium of DC cultures with fresh DM with 2% dialyzed horse serum and left the culture to equilibrate for 2 h. Instead, myotube samples were labeled with the radioactive precursors during the purification step in PM with 10% dialyzed FCS. At the end of the incubation, we extracted the dNTP pools as described above and determined the size and the specific radioactivity of the relevant dNTP by the DNA polymerase assay (46).

Immunoblotting of Ribonucleotide Reductase Subunits

Protein lysates were prepared from proliferating or differentiated C2C12 cultures and quantified as detailed in Pontarin *et al.* (14). We detected p53R2 and R1 in 25 μg of extracts with the antibodies and the conditions detailed previously (14). To detect R2 in 5 μg of proliferating cell extracts or 50 μg of DC or purified myotube extracts, we used a rat monoclonal antibody at 1/500 dilution (47). The internal calibrator GAPDH was measured in 1 μg of extract with a monoclonal antibody (MAB374, Millipore) at 1/1000 dilution. We quantitated the signals with Kodak one-dimensional image analysis software.

Statistical Analysis

Most experiments were performed at least three times, unless otherwise indicated. Data are presented as means ± S.D. Group means were compared using parametric *t* test or nonparametric Mann-Whitney test when the sample size was small.

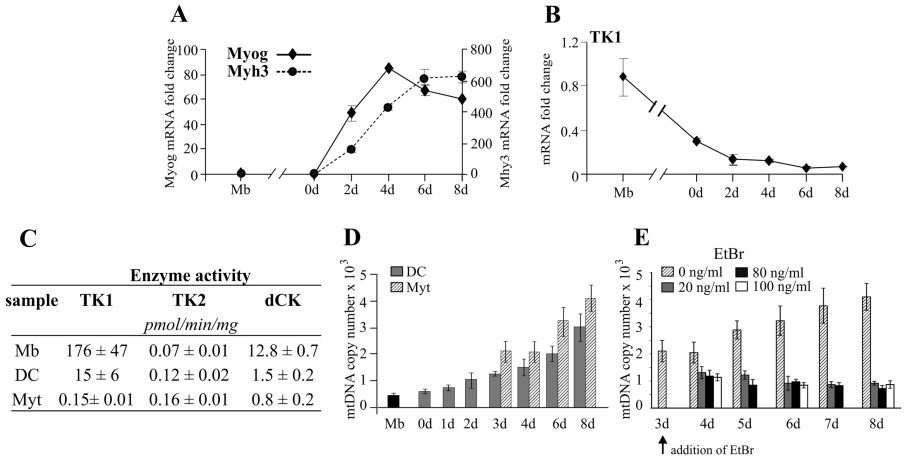


FIGURE 2. **Induction of myogenin and myosin heavy chain, down-regulation of cytosolic thymidine kinase, and mt biogenesis during differentiation of C2C12 myoblasts.** *A*, increase of myogenin (*Myog*) and myosin heavy chain (*Mhy3*) mRNAs during 8 days of differentiation (*0d–8d*) in DM relative to their expression in myoblasts (*Mb*), determined by real-time PCR. *B*, decrease of cytosolic thymidine kinase (TK1) mRNA during differentiation determined as described in *A*. *C*, enzyme activities of TK1, TK2, and deoxycytidine kinase (*dCK*) measured as described under "Experimental Procedures" in protein extracts from cycling myoblasts, 8 days differentiated cultures (*DC*) and myotubes (*Myt*) purified from DC at 8 days of differentiation. *D*, number of mtDNA copies per nuclear genome in myoblasts, cultures incubated in DM for 0–8 days (*DC*), and purified myotubes (*Myt*). mtDNA/nDNA ratio was measured by quantitative PCR using cytochrome *c* oxidase subunit I as a probe for mtDNA and ACTB as nuclear gene. *E*, decrease of mtDNA copy number in myotubes purified from C2C12 cultures incubated in DM for 3–8 days and treated with the indicated doses of ethidium bromide (*EtBr*) from day 3. mtDNA was quantified as described in *D*. All data are means ± S.D. from two experiments, from four experiments in *C*.

RESULTS

Mouse C2C12 cells are a well characterized untransformed muscle cell line, widely used to study muscle differentiation *in vitro*. When shifted to DM deprived of growth factors and mitogens, C2C12 myoblasts rapidly stop proliferating and initiate a differentiation program leading to their fusion into myotubes that after 4 to 8 days manifest spontaneous twitching. In our study, the differentiated cultures after 8 days in DM reached a fusion index of 72%. Incorporation of BrdU showed that only 0.4% of the nuclei of mononucleated cells were in S-phase. Although at this stage the cultures were confluent, the mononucleated cells might still express cell cycle-regulated enzymes of dNTP metabolism such as the R1/R2 isoform of ribonucleotide reductase and TK1 and contain relatively large dNTP pools. To reduce this potentially disturbing contamination, we developed a method for the separation of myotubes from unfused myoblasts. The procedure, described in the experimental part, exploits the different adhesion properties of the two cell types when replated in fresh medium + 10% FCS after gentle trypsinization.

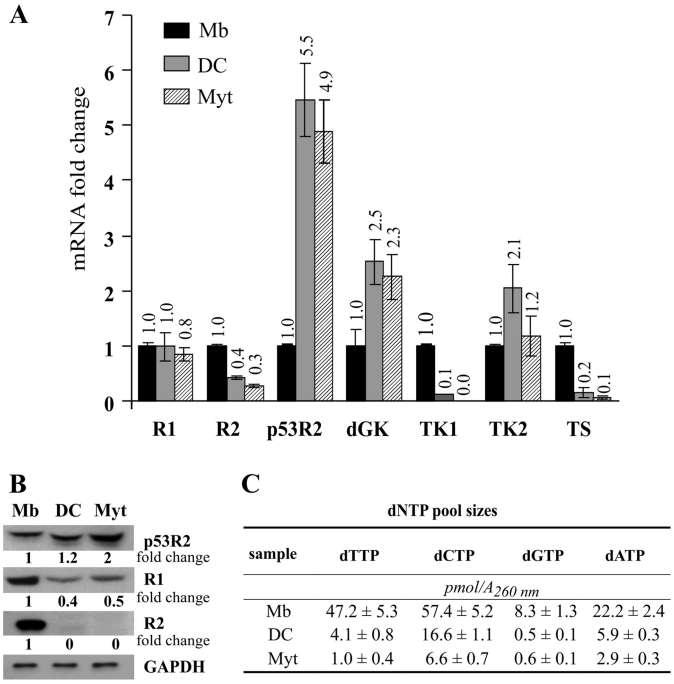
Below, we first present data demonstrating the usefulness of the myotube purification procedure to determine changes occurring in the enzymatic pathways for dNTP synthesis and in mtDNA during the differentiation of myoblasts. Thereafter, we report effects of siRNA silencing of TK2, p53R2, or dGK on these parameters.

dNTP Metabolism in Myotubes Isolated from Differentiated Cultures of C2C12 Cells—To induce the differentiation of myoblasts, we shifted confluent C2C12 cultures to DM and monitored the expression of myogenin and embryonic myosin heavy chain (Myh3) mRNAs during the following 8 days. In parallel, we measured the expression of three kinases involved in the salvage of deoxynucleosides.

Expression of myogenin and Myh3 increased strongly from day 2 in DM (Fig. 2*A*). The progressive decrease of the mRNA of TK1, the S-phase-specific cytosolic thymidine kinase, indicated the exit of the cells from the cell cycle during differentiation (Fig. 2*B*). To monitor the purity of the myotube fraction, we analyzed by specific assays described in the experimental part the activities of TK1 and the constitutively expressed mt TK2 in protein extracts from proliferating myoblasts (50% confluent), DC and purified myotubes harvested at 8 days of differentiation. TK1 activity was high in myoblasts but almost undetectable in myotube preparations (Fig. 2*C*). Its residual activity in DC is probably explained by the presence of non-fused cells. Conversely, TK2 activity was exceedingly low in myoblasts and in DC, as was previously observed in skeletal muscle (30). The marginal TK2 increase when muscle cells stop dividing distinguishes them from fibroblasts (32). The activity of deoxycytidine kinase decreased 15-fold in myotubes compared with myoblasts (Fig. 2*C*).

To correlate myogenesis with mitochondrial biogenesis, we measured the mtDNA copy number in myoblasts, DC (from d0 to d8), and purified myotube samples (d3-d8) (Fig. 2*D*). Mitochondrial biogenesis was induced immediately on addition of DM with an almost 6-fold increase of mtDNA within 3 days, after which time the expansion of mtDNA continued at a slower rate (Fig. 2*D*). The progressive increase of the mtDNA/nuclear DNA ratio in the differentiating myotubes reflected a stimulation of mtDNA replication and implied an increased consumption of DNA precursors. To demonstrate a turnover of mtDNA in the myotubes, we treated differentiating cultures with increasing concentrations of ethidium bromide starting at day 3 in DM (Fig. 2*E*). mtDNA decreased immediately and amounted after 5 days to only 25% of the control value suggesting a continuous turnover of mtDNA.

Induction of a post-mitotic state in differentiated muscle cultures was accompanied by a strong decline in the mRNAs for R2, TK1, and thymidylate synthase, most evident in the myotube fraction (Fig. 3*A*). Instead, p53R2 and dGK mRNAs were



sample	dTTP	dCTP	dGTP	dATP
	pmol/$A_{260\ nm}$			
Mb	47.2 ± 5.3	57.4 ± 5.2	8.3 ± 1.3	22.2 ± 2.4
DC	4.1 ± 0.8	16.6 ± 1.1	0.5 ± 0.1	5.9 ± 0.3
Myt	1.0 ± 0.4	6.6 ± 0.7	0.6 ± 0.1	2.9 ± 0.3

FIGURE 3. **Changes in the expression of enzymes of dNTP metabolism and dNTP pool sizes in C2C12 muscle cells during differentiation.** *A*, levels of the mRNAs for ribonucleotide reductase subunits R1, R2, and p53R2, deoxynucleoside kinases dGK, TK1, and TK2, and thymidylate synthase (*TS*) in myoblasts (*Mb*), differentiated cultures (*DC*), and purified myotubes (*Myt*) determined by real time RT-PCR after 8 days in DM. The level of each mRNA is compared with that in myoblasts. Data are means ± S.D. from four experiments. *B*, immunoblot indicating the abundance of ribonucleotide reductase subunits in extracts from myoblasts, DC, and myotubes after 8 days in DM. Fold changes relative to expression in myoblasts. GAPDH was used as a loading control. *C*, sizes of the four dNTP pools in extracts from myoblasts, DC, and myotubes after 8 days in DM. Picomoles of dNTPs normalized by the $A_{260\ nm}$ of the alkali lysate obtained after nucleotide pool extraction. Data are means ± S.D. from four experiments.

induced, whereas TK2 expression remained unchanged. The expression of ribonucleotide reductase subunits was measured also at the protein level. With differentiation, R2 disappeared in both DC and myotube extracts, R1 decreased by 50%, and p53R2 increased 2-fold compared with proliferating myoblasts (Fig. 3*B*).

The observed changes in the expression of synthetic enzymes affected the dNTP content of myotubes (Fig. 3*C*). In myoblasts, dTTP and dCTP were the largest pools, and dGTP was the smallest. In myotubes, all pools decreased to ≈10% with dCTP remaining the largest. Correct analyses of the concentrations of dNTPs in myotubes clearly require the extensive removal of contaminating myoblasts.

Similar Expression of dNTP-synthesizing Enzymes in C2C12 Myotubes and Mature Skeletal Muscle—To assess how the *in vitro* C2C12 cellular model compares with muscle *in vivo* from the point of view of dNTP metabolism, we performed a DNA microarray analysis of gene expression in purified C2C12 myotubes and in two mouse muscles with different metabolism, the fast glycolytic extensor digitorum longus and the slow-oxidative soleus (Fig. 4). In agreement with the above myotube data (Fig. 3*A*), the expression of most cytosolic enzymes of dNTP metabolism was much lower in muscle fibers than in proliferating myoblasts. Interestingly, the most significantly down-regulated genes were those coding for R2 and R4 enzymes of dTMP *de novo* synthesis, whereas only three genes in this group coded for catabolic enzymes (Fig. 4*C*). The trend of gene expression in myotubes and in the two muscles was basically the same, despite the different energy metabolism of the two muscles. More marked changes in mature muscle likely reflect the earlier differentiation stage of the *in vitro* myotubes. We conclude that isolated myotubes are a suitable model to investigate dNTP synthesis in muscle fibers.

In Situ Measurements of dNTP Synthesis in Isolated Myotubes—Do the changes in the pattern of dNTP synthesizing enzymes occurring during differentiation affect the metabolism of dNTPs? We investigated this question by isotope experiments with differentiated cultures and myotube fractions, measuring the incorporation of different concentrations of ^{3}H-labeled thymidine (TdR) or CdR into the dTTP and dCTP pools (Fig. 5*A*). In all instances, we determined both the size and the specific radioactivity of the two pools after 30- and 60-min incubation. The size of both pools was 2- to 3-fold larger in the DC than in the myotube extracts. With 1 μM [^{3}H]TdR in the medium, but not at lower concentrations, the dTTP pool increased ~3-fold (Fig. 5*D*), whereas [^{3}H]CdR had no effect on either the dTTP or the dCTP pool (Fig. 5, *B* and *D*). The specific radioactivities of the two pools indicate the efficiency with which the deoxynucleosides were transformed to the triphosphates and signals the potential of the salvage pathways including TK1, TK2, and deoxycytidine kinase. For both deoxynucleosides, the incorporation depended strongly on the concentration of the deoxynucleoside in the medium. At the highest concentrations (1 μM TdR and 0.3 μM CdR), they approached the specific radioactivities of the precursors (20,000 cpm/pmol for TdR and 5,000 for CdR), particularly in the purified myotube fraction (Fig. 5, *C* and *E*).

These results indicate a highly efficient synthesis of dNTPs both from TdR and CdR provided that the deoxynucleoside is present in the medium at a sufficiently high concentration. It is surprising to find such an efficient salvage of TdR, although TK2 and TK1 activities are so low (Fig. 2*C*). The salvage of CdR appeared to be still more efficient, with the specific radioactivities of dCTP and dTTP being identical to that of the precursor CdR already when the deoxynucleoside was provided at the low concentration of 0.3 μM. Incorporation of isotope from CdR into dTTP involves a reaction sequence of 5 steps (Fig. 5*A*). The results in Fig. 5, *C* and *E*, show that in the presence of [^{3}H]CdR, all steps were saturated with the isotope. Clearly the salvage enzymes compete efficiently with the ribonucleotide reductase synthetic pathway for the synthesis of pyrimidine dNTPs despite their low expression (Figs. 2*C* and 3*A*).

Silencing of TK2, p53R2, and dGK in Differentiated C2C12 Myotubes—Mutations in TK2, p53R2, and in some cases also dGK give rise to myopathic mtDNA depletion syndromes *in vivo*. We individually down-regulated each of the three enzymes during C2C12 differentiation by repeated siRNA transfections with two different siRNAs (named a and b) applied separately in independent cultures. After 5 days in DM, most myotubes in the TK2- or dGK-silenced cultures detached from the dishes, whereas the non-fused mononuclear cells remained adherent. This was not the case in cultures transfected with control siRNAs or p53R2 siRNAs that reached 8

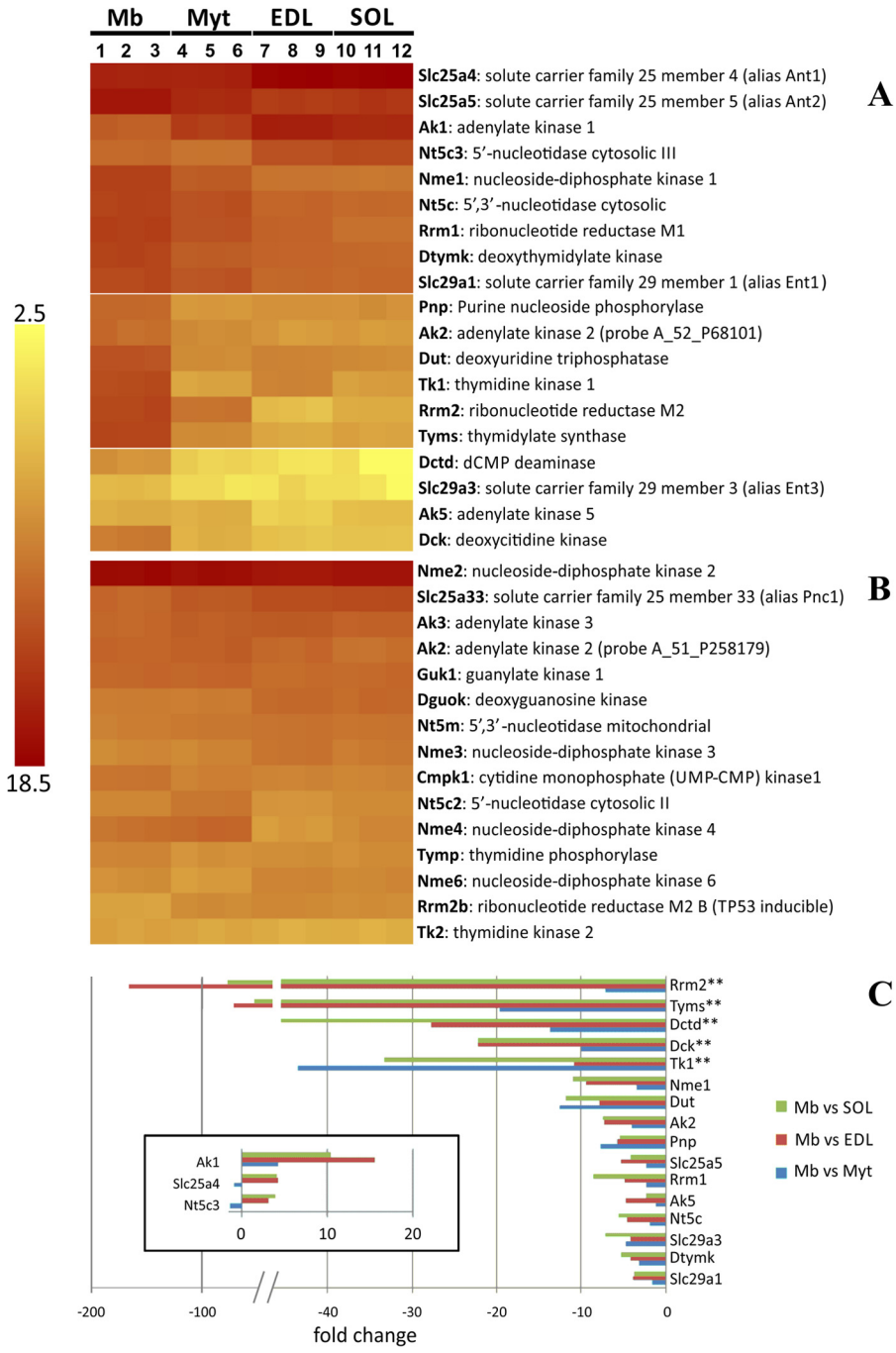


FIGURE 4. **Microarray analysis of genes regulating the dNTP pools in muscle cells.** Samples were as follows: myoblasts (*Mb*), proliferating C2C12 myoblasts at 50% confluence; *Myt*, myotubes purified at 8 days of differentiation; *EDL*, extensor digitorum longus; and soleus (*SOL*), skeletal muscles dissected from adult CD1 mice. To measure expression levels, Cy3-labeled target from myoblasts, extensor digitorum longus and soleus preparations were hybridized in triplicate experiments on whole mouse genome oligonucleotide microarrays (4 × 44,000, Agilent Technologies). Normalized values were converted to log2 (scale on the *left*), and heat maps were constructed with MultiExperiment Viewer to visualize gene expression levels. All genes involved in dNTP metabolism were annotated on the basis of their cytosolic or mitochondrial localization (supplemental Table 1). To evaluate the significance of the expression changes, pairwise Significance Analysis of Microarrays two class analyses were carried out between myoblasts and each of the other samples. *A*, genes differentially expressed in at least one test using a false discovery rate of 1%. *B*, genes that showed no significant changes in muscle fibers compared with myoblasts. *C*, genes from *A*, sorted according to fold-change values to underline the predominant down-regulation observed in comparisons between cycling and differentiated muscle cells. A *double asterisk* marks genes with the most significant changes (false discovery rate, 0% in at least two tests). The *inset* shows the few genes with positive fold-changes.

days of differentiation without problems. Therefore, we had to analyze the results of TK2 or dGK down-regulation after 4 days, when the silenced cultures were well adherent (Fig. 6*C*), but could use 8 days for the effect of p53R2 knockdown. The two separate siRNA sequences gave a similar degree of silencing for each target gene and below, where not specified, average data are presented. The mRNA for TK2 decreased to ~35% of the control, those for dGK and p53R2 even more (Fig. 6, *A* and *B*). A similar down-regulation occurred in the enzyme activities of TK2 and dGK (Fig. 6*A*) and in the amount of p53R2 detected in

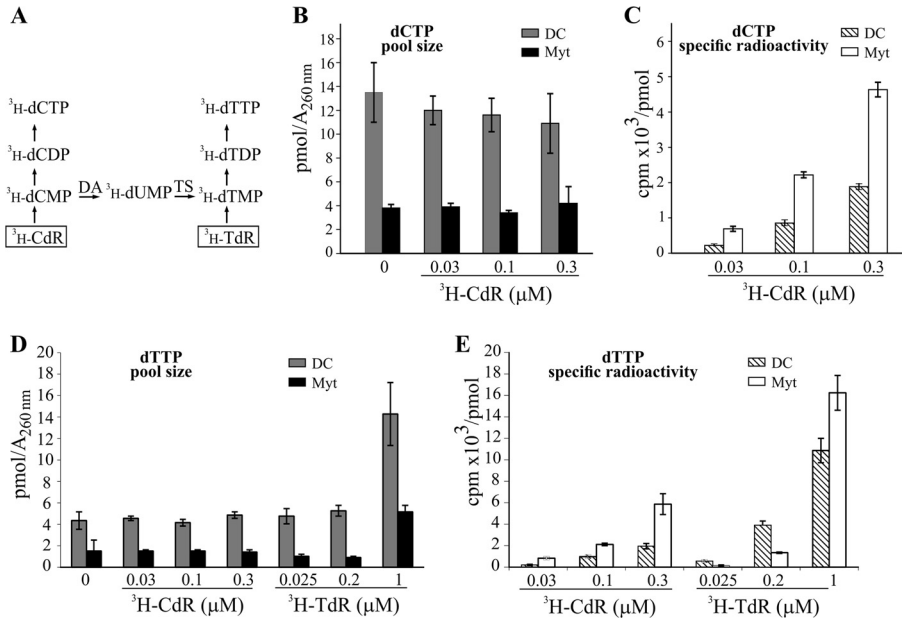


FIGURE 5. ***In situ* measurements of dNTP synthesis in differentiated C2C12 cultures and purified myotubes.** Differentiated cultures and isolated myotubes at 8 days of differentiation were incubated with the indicated concentrations of [^{3}H]CdR (5,000 cpm/pmol) or [^{3}H]TdR (20,000 cpm/pmol) in DM containing dialyzed horse serum or dialyzed FCS, respectively. Pool sizes and specific radioactivities were measured by an enzymatic assay as described under "Experimental Procedures." *A*, the schematic indicates the reactions through which radioactive CdR and TdR are incorporated into dCTP and dTTP. *DA*, dCMP deaminase; *TS*, thymidylate synthase. *B*, dCTP pool sizes expressed as pmoles/$A_{260\ nm}$ of the alkali lysate of each DC, and each myotube sample was incubated with 0–0.3 μM [^{3}H]CdR. *C*, specific radioactivities (cpm/pmol) of dCTP extracted from the DC and myotube samples analyzed in *B*. *D*, dTTP pool sizes expressed as pmoles/$A_{260\ nm}$ of the alkali lysate of each DC, and myotube samples were incubated with 0.03- 0.3 μM [^{3}H]CdR or 0.025–1 μM [^{3}H]TdR or in the absence of deoxynucleosides. *E*, specific radioactivities (cpm/pmol) of dTTP extracted from the DC and myotube samples analyzed in *D*. In all panels, values are means ± S.D. of results of the 60-min incubations from two experiments. *Myt*, myotubes.

A

siRNA (4d)	C	TK2	dGK
TK2 activity *pmol/min/mg*	0.5 ± 0.2	0.05 ± 0.04	nd
dGK activity *pmol/min/mg*	0.6 ± 0.2	nd	0.14 ± 0.02
residual mRNA %	100	33 ± 11	23 ± 4

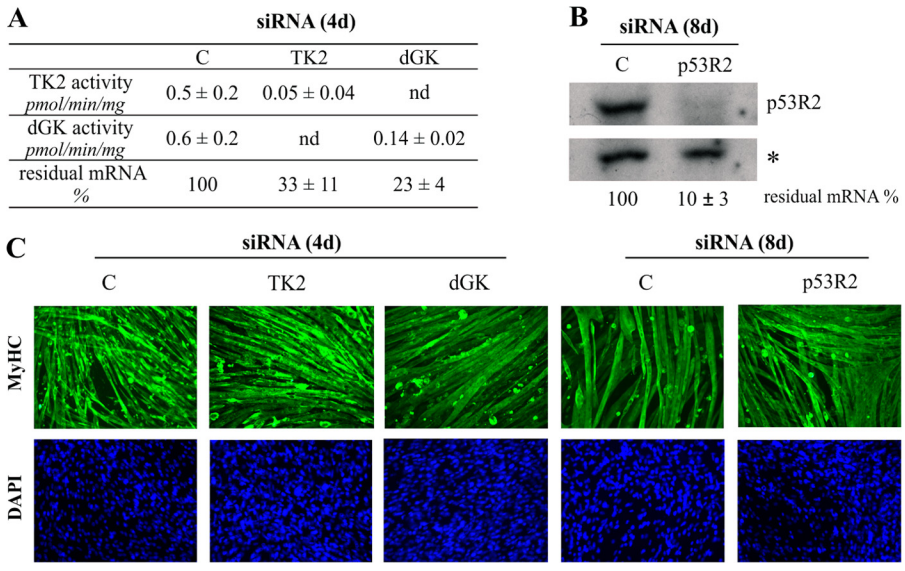


FIGURE 6. **Silencing of TK2, dGK, or p53R2 in differentiated C2C12 cultures.** *A*, residual TK2 and dGK activities in extracts from control and TK2- or dGK-silenced purified myotubes after 4 days (*4d*) of differentiation in the presence of siRNAs. Two different control or anti-TK2 or -dGK siRNA sequences were used with similar results, averaged in the table. Enzyme activities were measured by the radioactive assays described under "Experimental Procedures." The mRNA levels remaining in the silenced myotubes are indicated as percent of the controls transfected with non-targeting siRNAs. Data are means ± S.D. from four experiments. nd, not determined. *B*, immunodetection of p53R2 in protein extracts from control and p53R2-silenced myotubes after 8 days of differentiation. An unspecific band (*asterisk*) was used as loading control. Residual mRNA in silenced myotubes is indicated as percent of the control transfected myotubes ± S.D. Results were obtained from four experiments. *C*, immunofluorescence analysis of myosin heavy chain expression (*MyHC*) in C2C12 cultures transfected with control or anti TK2, dGK, or p53R2 siRNAs for 4 or 8 days. DAPI staining indicates that the large majority of nuclei were present inside myotubes in all cases.

immunoblots (Fig. 6*B*). At 4 and 8 days of differentiation, the controls and the corresponding siRNA-transfected cultures were well differentiated (Fig. 6*C*), showing similarly high fusion indexes (88–91%) and expression of myosin heavy chain. All transfected cultures contained a low number of S-phase cells determined by BrdU labeling (1% after 4 days and 0.5% after 8 days).

After 4 days, the mtDNA copy number determined by quantitative PCR was ~50% after TK2 and dGK silencing compared with the control (Fig. 7*A*). The silencing of p53R2 had no clear

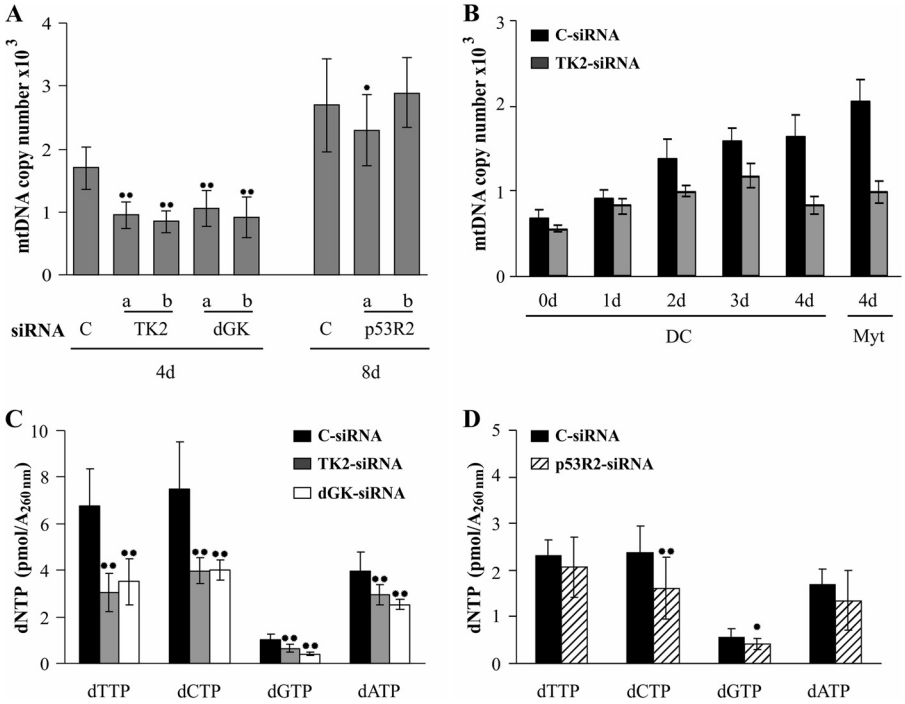


FIGURE 7. **Effect of TK2, dGK, or p53R2 silencing on the content of mtDNA and dNTPs of isolated myotubes.** Cultures of C2C12 cells were separately transfected with two different sequences of siRNAs for each target gene according to the protocol described under "Experimental Procedures." Controls were transfected with either of two non-targeting siRNAs. TK2 and dGK-silenced cultures could not be maintained longer than 4 days during transfection in DM, whereas p53R2-silenced myotubes remained adherent to the plates for 8 days. *A*, reduction of mtDNA level in myotube samples silenced with two different sequences (a and b) for each target gene. mtDNA/nuclear DNA ratio was measured by quantitative PCR as in Fig. 2. Values are means ± S.D. from at least three experiments for each siRNA sequence, from eight experiments in the case of p53R2-siRNA sequence a. *B*, variations of mtDNA copy number, measured as described in *A*, occurring during differentiation in DM in control and TK2 siRNA-transfected DC. At day 4 (*4d*), mtDNA was measured also in purified myotubes (*Myt*). Data are means ± S.D. from two experiments. *C*, sizes of the four dNTP pools in myotubes purified after 4 days of transfection in DM with anti TK2 or dGK siRNAs or control siRNA. Pool sizes are expressed as pmol/$A_{260\ nm}$ of the alkali lysates obtained after pool extraction. Values are means ± S.D. from seven experiments for TK2-siRNA and four experiments for dGK-siRNA. *D*, sizes of the four dNTP pools in myotubes purified after 8 days of transfection in DM with anti p53R2 siRNAs or control siRNA. Values are means ± S.D. from seven experiments. *Asterisks* indicate *t* test significant differences of the means in the silenced samples relative to controls. **, $p < 0.001$; *, $p < 0.05$. Note that the *bars* indicate S.D. of the whole data set and overlapping does not contradict the results of the *t* test.

effect, giving at most a 15% decrease after 8 days. During TK2 silencing, the increase in mtDNA started at a lower pace than in the controls and stopped completely between day 3 and 4 (Fig. 7*B*).

Multiple deletions were recently reported in the mtDNA of patients carrying p53R2 mutations (48). We analyzed the mtDNA of p53R2-silenced myotubes by long template PCR and found no evidence of truncated mtDNA fragments.

Silencing of TK2 and dGK affected the size of the four dNTP pools in myotubes (Fig. 7*C*). Surprisingly, all four dNTP pools became significantly smaller. The specificity of the involved kinases suggests that silencing of TK2 would affect the dTTP and possibly the dCTP pool and silencing of dGK the size of the dGTP and possibly the dATP pool (49, 50). Instead, the silencing of either enzyme activity decreased all four pool sizes with roughly similar efficiency (Fig. 7*C*). The effect of p53R2 silencing was smaller (Fig. 7*D*), in agreement with the marginal effect on mtDNA but consisted in a significant reduction of the dCTP and dGTP pools, as was observed in p53R2-mutated quiescent human fibroblasts (51). The generalized decrease of dNTP pool sizes in differentiating myotubes silenced for TK2 or dGK was puzzling. We hypothesized that the depletion of the unexpected dNTPs, *e.g.* dGTP and dATP in the TK2-silenced myotubes, may be a secondary effect of the depletion of the expected dNTPs, in the case of TK2 silencing primarily dTTP. To test our hypothesis, we incubated the TK2-silenced cultures with CdR during 4 days of differentiation in DM. The labeling experiments in Fig. 5 had shown that CdR is incorporated into both the dCTP and the dTTP pool, without altering their pool sizes. To obtain an increase of the dTTP and dCTP pool, we had to use 500 μM CdR, because concentrations between 5 and 100 μM were ineffective. The activity of catabolic enzymes kept the dCTP pool under control. With 500 μM CdR, we observed an expansion of the pyrimidine pools in both control and TK2-silenced myotubes (Fig. 8*A*), lower in the latter that could only phosphorylate CdR by deoxycytidine kinase, due to TK2 silencing. The increase in pyrimidine dNTPs was accompanied in the TK2-silenced myotubes by a normalization of the dATP pool and a partial recovery of the dGTP pool (Fig. 8*B*) and the mtDNA depletion (Fig. 8*C*).

DISCUSSION

Skeletal muscle is a major target of mitochondrial diseases owing to its high energy requirements, first during myogenesis and later after differentiation for its contractile functions. Muscle fibers have the problematic task of producing and maintaining a large array of mtDNA molecules with a feeble enzymatic toolbox for the synthesis of DNA building blocks, in particular dTTP. In differentiating myotubes, and especially in adult muscle, the expression of the enzymes dedicated to convert dCMP

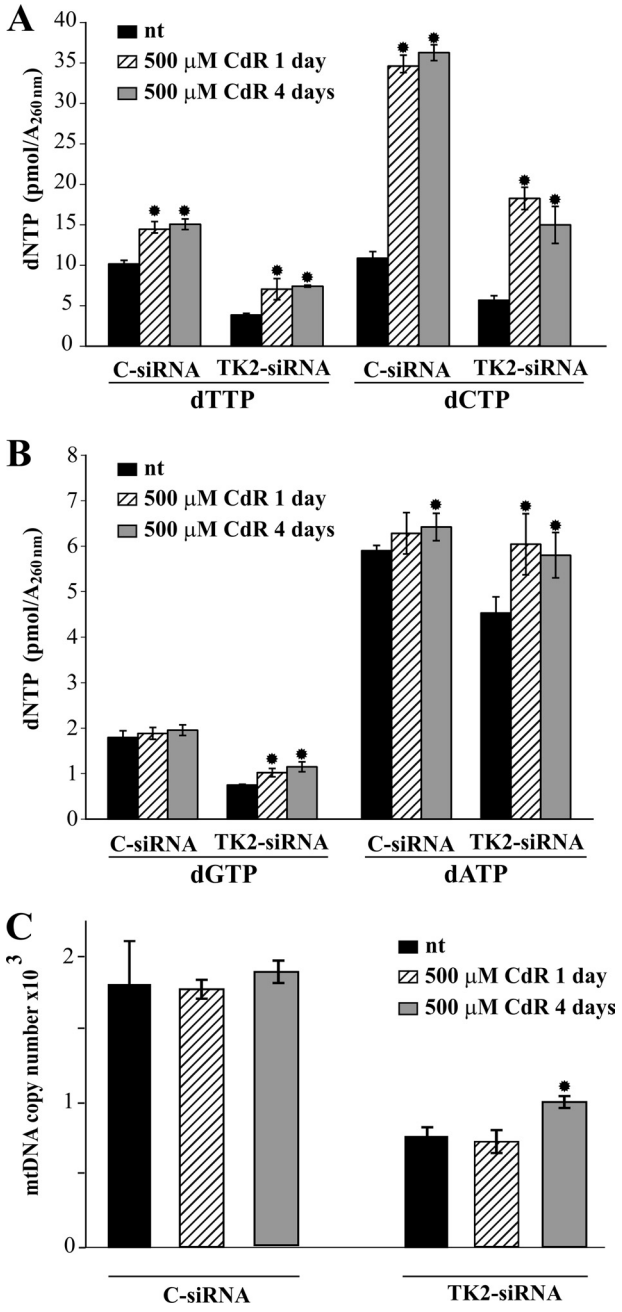


FIGURE 8. **Incubation with deoxycytidine during TK2 silencing partially prevents depletion of purine dNTPs and attenuates mtDNA depletion in differentiating myotubes.** Cultures of C2C12 cells transfected with control or TK2 siRNA were incubated in DM with 0 or 500 μM CdR either from day 0 to day 4 or during the last 24 h of silencing. At the end of treatment, myotubes were purified and analyzed. Pyrimidine (*A*) and purine dNTP (*B*) pool sizes (pmol/$A_{260\ nm}$ of the alkali lysates) are shown. Values are means ± S.D. from two experiments. Comparisons were made between CdR-incubated and not incubated samples by the Mann-Whitney's test. *, $p < 0.05$. *C*, mtDNA copy number measured as in Fig. 2. Values are means ± S.D. from three experiments. CdR-incubated samples were compared with not incubated samples by *t* test. *, $p < 0.01$.

to dTMP (dCMP deaminase and dTMP synthase), *i.e.* the main pathway of dTMP *de novo* synthesis in mammalian cells (52), is very low (Figs. 3*A* and 4*C*). With the disappearance of TK1 (Fig. 2, *B* and *C*), TK2 becomes the only enzyme capable to phosphorylate thymidine inside muscle fibers, but its expression is lower than in other differentiated tissues (42, 53) and remains unchanged, in contrast to the induction observed in cultured quiescent fibroblasts (32). A similar lack of TK2 induction was observed in murine brain and heart (54), suggesting that TK2 activity is not up-regulated during differentiation. The importance of TK2 in muscle is demonstrated by the acute myopathy associated with its genetic inactivation in humans and mice (22, 30, 55). It is confirmed here by the inability of TK2-silenced C2C12 cells to increase their mtDNA content early during differentiation and the drop in mtDNA copy number between day 3 and 4 (Fig. 7*B*).

In situ determinations of dNTP synthesis in isolated myotubes by incubation with labeled deoxynucleosides (Fig. 5) suggest that the salvage of pyrimidine deoxynucleosides and the reactions converting dCMP to dTMP remain active in myotubes although the enzymes involved are expressed much less than in myoblasts (Fig. 3*A* and Fig. 4, *A* and *C*). However, *in vivo*, the extracellular concentrations of deoxynucleosides do not suffice to provide sufficient precursors for these pathways. In human blood the concentration of TdR was estimated to be below 50 nM and that of CdR possibly lower (56, 57). The enzymes in myotubes probably recycle deoxynucleosides produced intracellularly by catabolic enzymes rather than contributing to the uptake of the minute amounts of extracellular free deoxynucleosides.

Genetic defects in enzymes of dNTP metabolism result in myopathic mtDNA depletion syndromes that are attributed to alterations of dNTP pool composition in muscle fibers. However, the sizes of dNTP pools in human or murine muscle have never been measured directly. This we have done here in purified myotubes, with the unexpected finding that down-regulation of either TK2 or dGK resulted in a generalized lowering of all 4 dNTP pools (Fig. 7*C*), despite the different substrate specificities of the two kinases (49, 50). Earlier determinations of dNTP levels in organs of young $TK2^{-/-}$ mice had revealed a decrease of the dTTP or dCTP pool, in agreement with the pyrimidine-specificity of TK2 (30). The global pool decrease observed here in newly formed myotubes may be due to the peak of mtDNA synthesis occurring in the early phase of myogenesis. We tried to "cure" the TK2-silenced myotubes by adding to the medium CdR that is a precursor of both dCTP and dTTP (Fig. 5*A*). Incubation with a high concentration of CdR during differentiation attenuated the effects of TK2 silencing on all dNTP pools and the depletion of mtDNA (Fig. 8). We speculate that the increase in the purine dNTPs in the presence of CdR may depend on two separate effects: (i) dTTP stimulates the reduction of purine ribonucleotides by ribonucleotide reductase and/or (ii) a compensation of pyrimidine pool shortage avoids dissipation of purine dNTPs through abortive DNA replication.

p53R2 silencing reduced the dCTP and dGTP content of myotubes, similar to the dNTP pool changes observed earlier in quiescent fibroblasts lacking p53R2 activity (51) but, in contrast to the rapid mtDNA depletion after TK2 silencing, had little or no effect on mtDNA after 8 days of differentiation (Fig. 7*A*), in agreement with the earlier onset of pathological phenotypes in mouse models of TK2 deficiency (20, 30, 55). Our *in vitro* analysis of dNTP metabolism during myogenesis highlights how the mtDNA expansion typical of skeletal muscle rests on a vulner-

able enzyme network where the synthetic but not the catabolic activities are down-regulated during differentiation.

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